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Faculty of Natural Sciences, Life Sciences, Earth and the Universe.

Departement of Biology

Course Handout

Biochemical Engineering and Industrial Microbiology

Specialization: Applied biochemistry

Master 2 level

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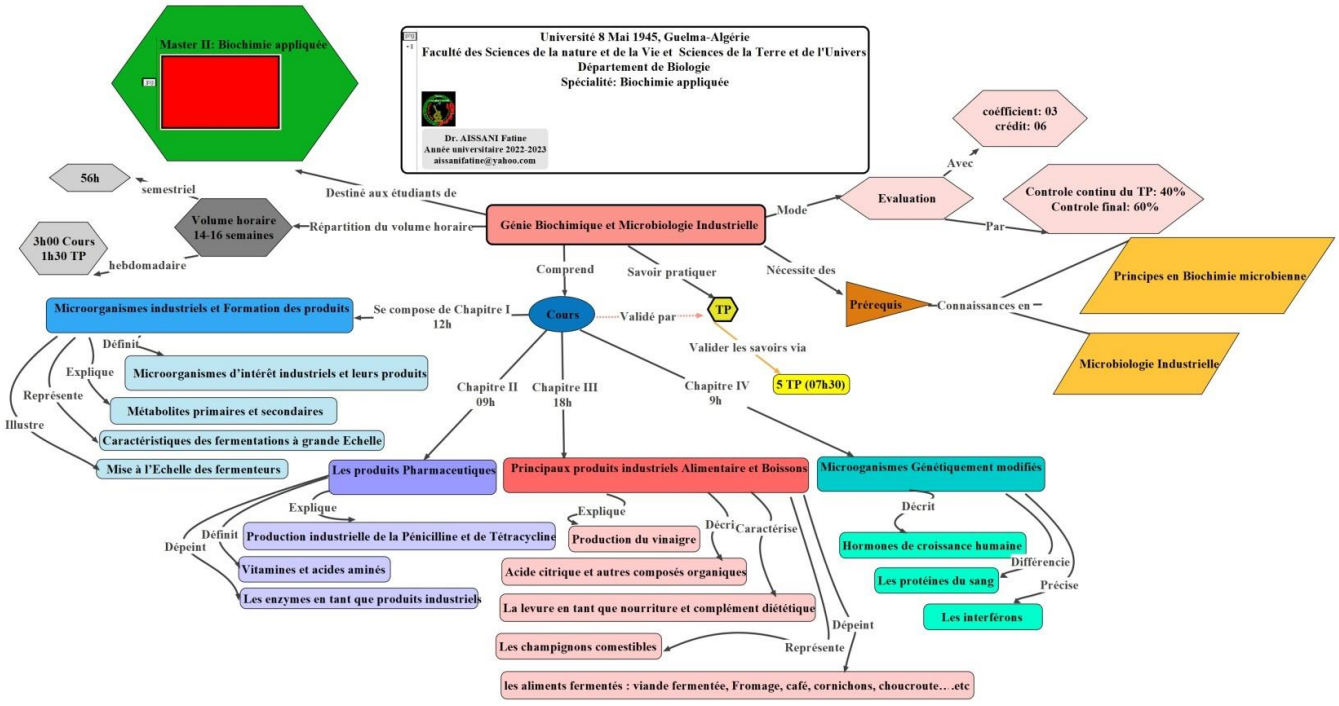
Introduction

This course is intended for Master II students in Applied Biochemistry. It is designed for learners with knowledge in microbial biochemistry and industrial microbiology. Indeed, microbes (bacteria, fungi, molds, etc.) are ubiquitous and form the basis of high-yield production of molecules of industrial interest. It is thanks to microbial metabolic reactions that scientists have been able to produce interesting pharmaceutical products (vitamins, enzymes, amino acids, gases, etc.) in unlimited quantities.

* In line with this objective, in addition to the twice-weekly biochemistry and industrial microbiology courses taken by students at the University of 8 May 1945, "Guelma," practical work is distributed to allow students to learn the different microbial culture techniques, mastery of microbial metabolism kinetics, and industrial production at the level of bioreactors in a practical learning setting.



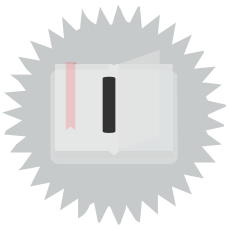
Conceptual Map.

Objectives

The objectives of this course are to:

- Identify the elements of biochemical engineering and industrial microbiology.
- Gain an understanding of the basic concepts of microbial biochemistry at an industrial scale.
- Acquire operational skills in bioreactor engineering and handling.
- Instill the study of the variability of microbial culture processes and industrial production of pharmaceutical products.
- Develop proficiency in applying various industrial microbiology techniques.

Pre-requisites



To fully benefit from this course, students should have a basic understanding and scientific background in the following areas :

- Fundamentals of Metabolic Biochemistry and Microbiology,
- Microbial Culture Concepts and Microbial Needs,
- Familiarity with Microbial Fermenters,
- Laboratory Safety and Aseptic Techniques,
- General Microbiology Concepts.

Course Content

The content of this course is organized into four chapters:

✓ The first Chapter delves intro the world of Industrial Microorganisms and Product Formation.

✓ The second chapter explain the industrial production steps of pharmaceutical products.

✓ The thrid chapter explores the production of major industrial food and beverage products using microorganisms.

✓ The forth and last chapter is est dedicated to geneticaaly modified microorganisms (GMOs).

Each chapter will end with solved exercises and additional unsolved ones.

Practical work

There will be five practical work on the various unit operations.

Evaluation

Final examination : 60%.

Practical work (lecture, micro-interrogation, other): 40%.

Table of contents

Chapter I : Industrial microorganisms and product formation

Objectives

At the end of this chapter, the student or learner will be able to:

- Identify microorganisms of industrial interest and their products.
- Define primary and secondary metabolites and show their examples.
- Know the characteristics of large-scale fermentations and differentiate between fermenter types.
- Demonstrate fermenter scaling.

History

- Born nearly three centuries ago from the discovery of the microscope, microbiology remained isolated from other biological disciplines for a long time.
- In 1676, the renowned Dutch microscopist Anthony Van Leeuwenhoek, who had developed the first compound microscope, revealed to the world the existence of microbes.
- Approximately 190 years after Van Leeuwenhoek's discovery of microbes, it was Louis Pasteur who, despite extraordinary opposition, established a connection between the presence of microbes and the production of wine (1856), beer, vinegar manufacturing, the disease of silkworms (Nosema bombycis), anthrax affecting certain animals (*Bacillus anthracis*: anthrax disease), and rabies in dogs.
- Pasteur demonstrated that the spoilage of foodstuffs was due to the action of microbes originating from the air, soil, water, or the human body, rather than some mysterious form of spontaneous generation.
- By the end of the 19th century, the cell theory had brought a unifying factor to biology by showing that all organisms, no matter how complex, are composed of elementary units, the cells, in which the same organization is always found.
- In 1881, Robert Koch isolated pure cultures (*tuberculosis bacillus*) of what we now call bacteria. (The term "microbe" includes bacteria, viruses, and forms of algae, fungi, and protozoa that are so small that the human eye requires a microscope to see them).
- Pasteur discovered through microscopic observations that there is a connection between a microbe and fermentation, i.e., between a form of life and a chemical process. His work laid the foundation for subsequent advances in biochemistry.
- Under the influence of Pasteur's work, Lister introduced asepsis in surgery (1867) and was the first to use a disinfectant, phenolic acid (phenol), to destroy contamination agents.
- Penicillin is the first significant antibiotic that was successfully produced from a microbe, which is a fungus of the genus Penicillium. For example, penicillin inhibits the growth of microbes that cause scarlet fever and abscesses, but it has no effect on the typhoid bacillus.

- The second half of the twentieth century witnessed significant changes in the fermentation industry. Most of these changes stemmed from the discovery of antibiotics as effective weapons against diseases.
- Research and production of new antibacterial, antifungal, antitumor, and antiviral molecules became major industries in Japan, the United States, and Western Europe.
- The technology of fermentation originated with the earliest civilizations that harnessed the ability of microorganisms to produce alcoholic beverages, bread, and cheese.
- The first Sumerian city-states (four millennia before B.C.) produced beer and wine, respectively, from fermented barley and grapes.
- Ancient civilizations also made cheese and salted and fermented meat to preserve their food for longer periods.
- Modern innovations in food preservation began with the Frenchman François Appert, who developed canning methods in 1809.
- During the first half of the twentieth century, the production of wine and beer, vinegar, and baker's yeast transitioned from ancient artisanal methods to established scientific techniques.
- Large-scale microbial processes for the production of citric and lactic acids were developed.
- Acetone, butanol, and ethanol were produced in industrial quantities through fermentation, but they are now generally produced through chemical synthesis.
- Other products generated by microorganisms, including vitamins, sterols, organic acids, amino acids, aromatic agents, enzymes, and fermented beverages or foods, have gained significant commercial importance.
- During the First and Second World Wars, microbial fermentation and similar processes were employed to produce chemical compounds related to weaponry, such as glycerol (propane-1,2,3-triol) and acetone (propanone).
- Thus, in recent years, the use of genetically modified organisms has revolutionized industrial microbiology.

1. INTRODUCTION

1.1. Definition of industrial microbiology

Industrial microbiology is a field of biotechnology "according to the OECD." IM encompasses all processes of bioconversion or biosynthesis carried out by microorganisms for agricultural, medical, food, and other purposes.

It involves the large-scale cultivation of microorganisms, either to produce molecules or value-added substances or to perform delicate chemical steps through processes.

It concerns the use of microorganisms in the production of organic substances (ethanol, glycerol, acetate, propionate...), antibiotics, pharmaceutical compounds, and food additives...

Biotechnology = Bio-industry = Fermentation industry = Industrial microbiology

Industrial Microbiology

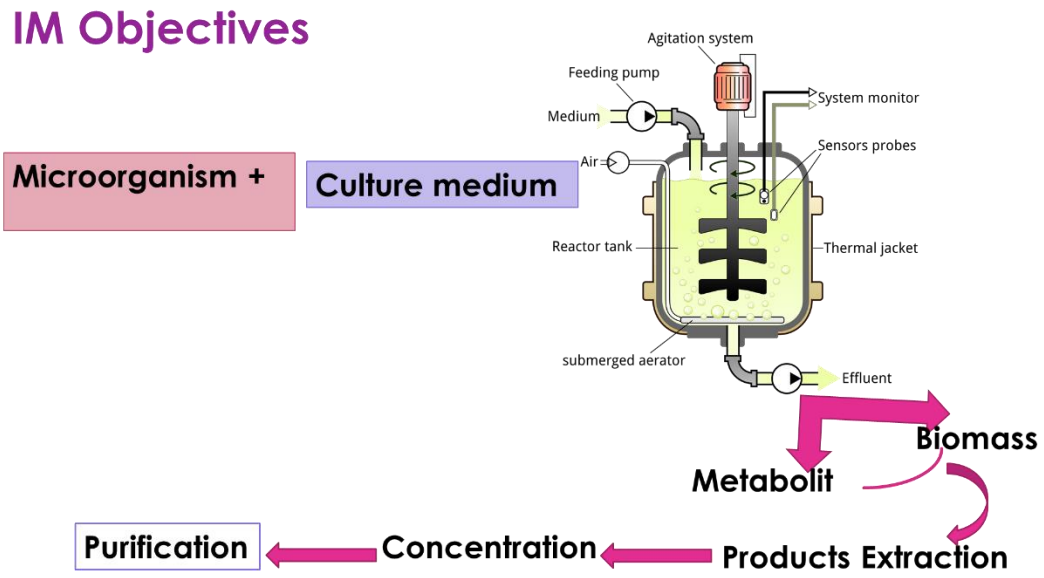
Microbiology		*Industry*
Bacteria, Viruses, Yeasts, Molds		Large-scale production (commercial)

- The use of microorganisms for the large-scale production of useful biological molecules.

1.2. The main objectives of industrial microbiology

The main objectives of this discipline are:

- Obtaining microorganisms in large ;
- Obtaining products of interest secreted by microorganisms (antibiotics, enzymes, etc.);
- Obtaining products metabolized by microorganisms.



1.3. Fields of activity of industrial microbiology

IM's main areas of activity are as follows :

I. **Agro-alimentation :** Foods, Flavor enhancers, dietary supplements, beverages; emulsifiers, alcoholic fermentations, lactic fermentations.

II. **Agriculture, horticulture :** A vehicle for the production of GMOs, biopesticides: herbicides, insecticides, plant hormones).

III. **Chemistry and Energy** : Biogases (ethanol, methane, hydrogen), organic acids, biodetergents, oxygenated compounds, solvent production (acetone, butanol...).

IV. **Environement** : Wastewater depollution and treatment (biological purification), pesticide biodegradation, bioremediation of polluted soils, mineral extraction, or bioleaching...

V. **Health (pharmaceutical industry)** : production of antibiotics, vaccines, vitamins, amino acids, insulin, growth hormone, hydrocortisone, interferon beta-1b, and many other products in the groups of antitumor agents, immunosuppressants, anti-inflammatories, etc.).

1.4. The benefits of using micro-organisms in industrial microbiology

Here are some advantages that make the use of microorganisms interesting:

- ✓ Lower Cost compared to chemical processes (Enzymatic catalysis, etc.): Microbial processes often have a lower cost compared to chemical methods.
- ✓ Synthesis and Biotransformation (e.g., prostaglandins, steroids) of certain molecules can only be achieved through microbial pathways - specificity of the reaction.
- ✓ Feasibility: Some molecules can only be synthesized or biotransformed using microbial pathways. Microbial processes are often more feasible for these compounds due to their specificity in reactions.
- ✓ Increased health safety: absence of viruses (e.g., HIV, hepatitis B) or prions; for example, cases of Creutzfeldt-Jakob disease transmission through growth hormones extracted from contaminated cadaveric pituitaries.
- ✓ Large-scale production in a short period (typically a few hours): Microorganisms can accomplish syntheses that are beyond the reach of traditional chemistry.

It is clear that the potential is vast and immense; it simply requires knowing the suitable microorganism, controlling its metabolism and growth, and being able to use it on a large scale."

1.5. Microbial products of industrial interest

Microorganisms are used in various agri-food and industrial sectors.

Major commercial products obtained from microorganisms include :

Foods, Flavor Enhancers, Dietary Supplements, and Beverages:

- ❖ Foods :
  - o Dairy products (yogurt, cheese)
  - Baker's yeast for baking, brewing, or food, and the substances produced by these cells.
  - Fermented meats (sausages, salamis, hams)
  - Edible mushrooms
  - Coffee
  - Pickles, olives, sauerkraut
  - Proteins from unicellular organisms.

- Flavor Enhancers and Dietary Supplements : Vinegar, Amino acids.
- Beverages : Wines, Beers.
- Vitamins : Cobolamin 'B12', Riboflavin B2.
- Organic acids : Citric acid, Itaconic acid.
- Enzymes and Microbial Transformations : Commercial enzymes, Sterol conversions.
- Inhibitors : Biocides, Antibiotics.
- Products from Genetically Modified Microorganisms (GMOs) : Insulin, Hormones.

2. Industrial Microorganisms

2.1. Definition

Microorganisms, also known as microbes, are living organisms too small to be seen with the naked eye. This group includes archaea, bacteria, fungi (yeasts and molds), protozoa, and microscopic algae. It also includes viruses, which are non-cellular entities located at the boundary between the living and non-living worlds.

2.2. Microorganisms used in industry:

Microorganisms are widely used in industry to accomplish tasks beneficial to humans.

They have proven particularly useful due to :

- The ease of their cultivation,
- The rapidity of their growth,
- Their ability to utilize inexpensive substrates (many of which are waste products from the agri-food industry),
- Their capacity to undergo genetic manipulations easily.

❖ These properties are originally present in wild microbial strains from the natural environment. These strains are isolated and then improved in the laboratory through mutation and the selection of the most efficient mutants.

2.2.1. Examples of useful microorganisms in industry

a. Archaea (archaebacteria)

Archaea are single-celled prokaryotic microorganisms. Often resembling bacteria in appearance, archaea have long been considered particular extremophilic bacteria (extreme thermophiles).

They are found in various environments, including oceanic hydrothermal vents, volcanic hot springs, salt lakes, soil, seawater, marshes, and the intestinal flora.

Description

The size and shape of archaea are generally similar to those of bacteria (1 to 4 µm). They can be both aerobic and anaerobic. Archaea consist of three main phenotypic groups as shown in figure 1 :

1- **Extreme Thermophilic Archaea:** These archaea thrive at temperatures exceeding 80°C, so they are restricted to environments where geothermal energy is available, such as hot springs, solfataras, and geothermally heated marine sediments.

2- **Extreme Halophilic Archaea (Haloarchaea):** These archaea only grow at NaCl concentrations greater than 1.8 M. They can be facultative or obligate aerobes. Most of them use amino acids, carbohydrates, or organic acids as an energy source.

3- **Methane-Producing Archaea (Methanoarchaea):**These archaea are strict anaerobes and inhabit the most anaerobic environments on Earth, including waterlogged soils, rice paddies, lake sediments, marine sediments, and the digestive tracts of animals. They can be psychrophilic, mesophilic, or thermophilic and play a role in the carbon cycle.

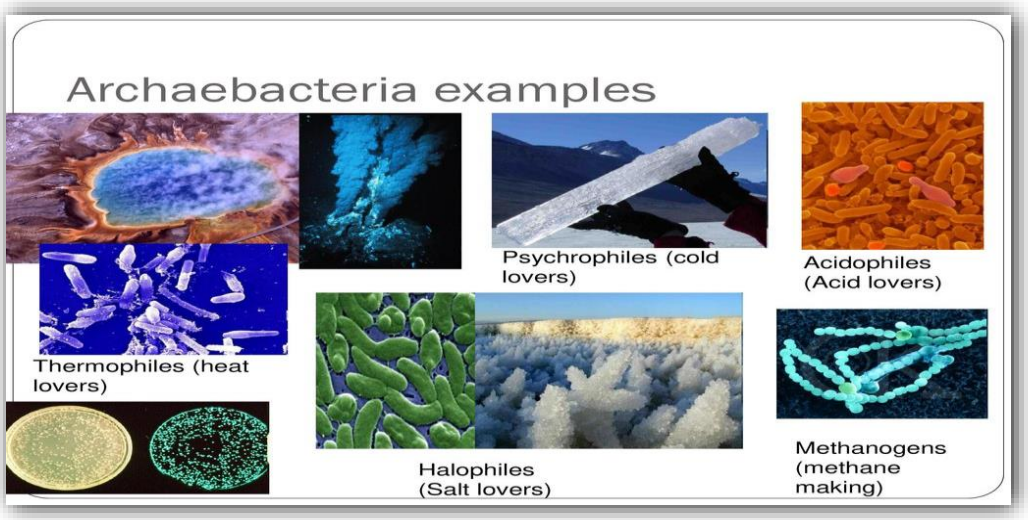


Figure 1 : Different type of Archaebacteria.

Industrial applications

The Archaea of particular industrial interest are mainly extreme thermophiles.

- Enzymes from extreme thermophilic Archaea can have significant commercial applications due to their high optimal temperature and thermostability.
- High-temperature activity is a critical characteristic because several industrial processes occur at temperatures between 50 and 100°C.
- Enzymes with a high optimal temperature are less costly than others (they provide the same activity with fewer enzymes).
- Heat resistance is correlated with higher resistance to moderate temperatures and denaturing chemicals.

Examples: Alkaline Proteases

These are thermophilic enzymes primarily used in laundry detergents.

- ✓ These enzymes have high activity at a temperature of 50°C (the temperature of washing water).
- ✓ temperature, which extends their shelf life in supermarkets.
- ✓ They are resistant to other components of detergents that can denature enzymes.

Examples: DNA Polymerase (Pyrococcus and Thermococcus)

These are thermophilic enzymes primarily used in laundry detergents. This is an enzyme commonly used in the biotechnology industry for Polymerase Chain Reaction (PCR) and DNA sequencing.

- ✓ In addition to their high activity and thermostability, these enzymes are valuable due to their proofreading activity and low error rate (compared to bacterial enzymes).
- ✓ This enzyme is widely used in research, diagnostics, and forensic medicine.
- ▪ They are primarily used for methane (CH4) production. All methanogenic bacteria are strict anaerobes and perform one of two reactions:

$CO2 + 2H2 \rightarrow CH4 + O2$ (ex: ***Methanobacterium***)

Acetic acid $\rightarrow$ $CH4 + O2$ (ex: ***Methanosarcina***)

- ▪ Methane is of great interest as it represents a non-polluting biofuel. Methanogenic bacteria are often used to produce methane from sludges. This process helps purify them by treating the polluted waters they contain. This is referred to as "sludge digestion."
- ▪ The main archaea encountered in methane production include *Methanobacterium, Methanosarcina, Methanobrevibacterium, Methanothermus, and Methanococcus*.

b. Bacteria

Bacteria are unicellular prokaryotic organisms (lacking a true nucleus).

• The nuclear apparatus of bacteria consists of a single chromosome (circular, closed loop, does not contain large repetitive regions) located in the cytoplasm.

• There may be extrachromosomal genetic material: plasmids (circular double-stranded DNA, transferable).

• Based on their source of carbon and energy, bacteria are categorized as: photoautotrophs, photoheterotrophs, chemoautotrophs, and chemoheterotrophs.

- ❖ Several bacteria can be used in the industrial field, such as actinomycetes, including the genus Streptomyces, which alone provides 70% of the antibiotics used.

Phylogenetic classification

- The Bacteria domain comprises several phyla (around 20 phyla), including Proteobacteria and Firmicutes.

The Proteobacteria form a very vast and heterogeneous group.

•Classification based on 16S rRNA reveals five main subgroups of Proteobacteria, referred to as subdivisions (subclasses): alpha, beta, gamma, delta, and epsilon-Proteobacteria.

•The majority of bacteria in this group are Gram-negative.

Examples of industrially important *Proteobacteria:* Vinegar Bacteria: *Acetobacter* and *Gluconobacter*

- Vinegar is used as a preservative and for seasoning. The two genera Acetobacter and Gluconobacter (acetic acid bacteria) are the primary microorganisms responsible for vinegar production.
- These two genera are natural inhabitants of flowers, fruits, honey, cider, and others. Acetobacter and Gluconobacter are Gram-negative bacteria capable of oxidizing ethanol into acetic acid (CH3COOH).
- They can be detrimental in industrial winemaking. Furthermore, they can cause viscosity, turbidity, and unpleasant tastes in beverages, including non-alcoholic drinks (sodas, fruit juices).
- The Acetobacter genus is also used industrially for the production of keto acids such as ascorbic acid (vitamin C).

Examples of industrially important Proteobacteria: Rhizobia Inoculants.

- ✓ Rhizobia are Gram- bacteria, often belonging to the α and β-Proteobacteria subclasses.
- ✓ Species of rhizobia, such as Rhizobium and Bradyrhizobium, that are commercially marketed as inoculants, are used to stimulate the formation of root nodules and nitrogen fixation in leguminous plants, thereby enhancing the production of these plants.

Examples of industrially important Proteobacteria: Bacteria for Amino Acid, Sugar, and Derivative Production

- ✓ Escherichia coli : E. coli is a Gram- bacterium belonging to the family Enterobacteriaceae, γ-Proteobacteria subclass.
- ✓ E. coli is used for the production of several amino acids, including L-tryptophan and L-threonine.
- ✓ It is also used for the production of certain sugars, such as the bioconversion of L-rhamnose into L-rhamnulose.
- ✓ The enzyme glucose isomerase from *E. intermedia* and *E. freundii* is used in the production of fructose (conversion of glucose to fructose).

Examples of industrially important Proteobacteria: Bacteria for Polysaccharide Production

- ✓ Xanthomonas belongs to the Xanthomonadaceae family of proteobacteria. This group includes the species Xanthomonas campestris, which is of industrial importance for the production of an exopolysaccharide called xanthan.
- ✓ Xanthan is soluble in cold water and is used as a food additive (E415) for its thickening and gelling properties to modify the consistency of foods.

Domain : bacteria ; Phylum: Firmicutes.

Firmicutes are Gram+ bacteria, heterotrophic in nature. Most of them are cocci or rods, and some produce endospores. They can be aerobic or anaerobic.

Lactic acid Bacteria

- They are found in many natural environments, ranging from soil and decomposing plants to animals. The gastrointestinal tract of mammals is colonized by lactic acid bacteria such as *Bifidobacterium, Lactobacillus* and *Leuconostoc.*
- Lactic acid bacteria can be aerotolerant anaerobes or microaerophiles and have demanding nutritional requirements since they are unable to synthesize certain amino acids.

Role of lactic acid bacteria in industry

- ➢ They are organisms capable of fermenting carbohydrates into lactic acid. This group includes genera like *Lactobacillus, Pediococcus, Aerococcus, Abiotrophia, Leuconostoc and Oenococcus*. They are used in the preparation of dairy products.
- ➢ Citrate Metabolism: In the dairy industry, citric acid present in milk is considered the primary precursor for the formation of appreciated aromatic compounds like acetate.
- ➢ Lactic acid bacteria such as *Lactobacillus bulgaricus and Streptococcus thermophilus* are used in yogurt production. Other species like *Corynebacterium glutamicum* and *Propionibacterium shermanii* are known for their ability to secrete glutamic acid and vitamin B12.
- ➢ The genera *Streptococcus*, *Lactococcus* and *Enterococcus* are widely used in lactic fermentation industries, including dairy, butter, cheese, as well as in brining and curing processes. They play crucial roles as lactic acid producers and coagulants in these industries.
- ❖ The *Bifidobacteriaceae* from the order Bifidobacteriales are also classified among lactic acid bacteria.
- ❖ They produce lactic acid, which helps to acidify the substrate and consequently inhibits the proliferation of pathogenic microorganisms or undesirable agents that can cause organoleptic changes.
- ❖ These bacteria are glycolytic and produce acetic and lactic acid, as well as small amounts of formic acid, ethanol, and succinic acid.

- ❖ Bifidobacterium, formerly known as Lactobacillus bifidus, is used in some "probiotic" yogurts.

C. Eubacteria : *Streptomyces*:

- They are Gram-positive filamentous Eubacteria and are present in the soil.
- They are mainly producers of antibiotics, antifungals, and antitumor agents.

d. Fungi

- Fungi are eukaryotes, with a nucleus, and they can be unicellular (yeasts) or multicellular (molds). They are found wherever there is a food source.

1. Yeasts

Yeast is a unicellular fungus capable of causing the fermentation of animal or plant organic matter. Yeasts are used in the production of wine, beer, industrial alcohols, leavened bread, and antibiotics.

Example : Baker's yeast is obtained from various strains of Saccharomyces cerevisiae, either alone or in combination.

Saccharomyces cerivisiae

The species Saccharomyces cerevisiae is the most widely used yeast in the industrial field, primarily in:

- Alcoholic fermentation, resulting in finished products like ethyl alcohol and carbon dioxide.
- This fermentation is used in baking and flavor production.
- The production of proteins, recombinant proteins, and various biopharmaceutical products.
- It possesses several intrinsic characteristics, such as expression system stability and ease of cultivation.
- As an alternative to S. cerevisiae, yeasts like *Pichia pastoris* and *Kluyveromyces lactis* can be used for protein production. Other yeasts like Candida and Torulopsis also have relevance in the production of unicellular proteins.

2. Molds

These are generally multicellular organisms. Most molds are heterotrophic, and some species have a mixed metabolism.

- Penicillium are filamentous fungi.
- They are very common fungi in the environment and can be responsible for many forms of degradation.
- Various species are cultivated on an industrial scale for the production of :

  Cheeses (Penicillium roqueforti, Penicillium camembertii), where they are involved in curd fermentation.

 Metabolites production such as :

• Antibiotics like penicillins (*Penicillium notatum, Penicillium chrysogenum*).

• Gluconic acid (by *Penicillium purpurogenum*).

Penicillium roqueforti

- ❖ is a species of saprophytic ascomycete fungi that is widespread in nature.
- ❖ Its main agricultural, artisanal, or industrial use is in dairy processing for blue cheese varieties such as Roquefort and Fourme d'Ambert…
- ❖ They are also used in the production of flavors and fragrances.

Penicillium notatum

- ❖ It is a microscopic fungus in the Penicillium genus, famous for being associated with the discovery of penicillin.
- ❖ Penicillium can be found on moldy bread, fruits, and certain types of cheeses.

Aspergillus niger

- It is a mold used for the industrial production of citric acid, which serves as an acidifier and antioxidant to enhance flavors and preserve fruit juices.
- Among the molds used in protein production, notable examples include *Rhizopus oligosporus, Zygorhynchus moelleri, Oospora lactis, Penicillium roqueforti*, and others.
- These molds are characterized by their production of proteins with nutritional value for animals and even humans. The proteins they produce complement cereal products.

Myciculture

- Myciculture, or fungi-culture, refers to the cultivation of edible mushrooms (culinary mushrooms).
- The largest share of the market is occupied by the common mushroom, Agaricus bisporus, commonly known as the button mushroom, as well as by Asian mushrooms such as shiitake (Lentinula edodes).

Common mushroom (*Agaricus bisporus*)

It is cultivated under the name "champignon de Paris" or "button mushroom." It is the most widely cultivated mushroom in mushroom farms, which can be located in cellars or dedicated buildings, because it is simple and quick to grow. Production of the common mushroom represents approximately three-quarters of the world's mushroom production.

e. Algae

They are organisms capable of photosynthesis and primarily live in aquatic environments, making them a type of plant. Algae can be unicellular and microscopic, forming the phytoplankton, while other species are multicellular and can reach impressive sizes.

Classification

- This group includes both prokaryotic organisms (cyanobacteria) and eukaryotes.

- Eukaryotic algae are divided into several taxa, with the most important being blue-green algae (cyanobacteria), brown algae, red algae, and green algae.

Algae application

Algae have various applications, including :

- Many algae species are edible for humans and animals.
- They are used as fertilizers.
- Several projects are exploring algae for biofuel production.
- Algae are a source of soda ash (sodium carbonate) and iodine.
- Algae are exploited for the production of gelling agents used in the food and chemical industries.
- Alginates, a type of gelling agent produced by algae (such as *Ascophyllum nodosum*), are used in the manufacture of numerous products in diverse fields like chemistry, food, cosmetics, environment, and healthcare.

f. Microalgae

are photosynthetic microorganisms, and they can be eukaryotic or prokaryotic, unicellular, or multicellular. Cyanobacteria of the Arthrospira genus, such as Spirulina, are used as dietary supplements to combat malnutrition.

Spirulina is known to produce a wide range of products through photosynthesis, which have pharmaceutical, cosmetic, and especially nutritional value. Spirulina is rich in proteins, and its essential amino acid content is better balanced than that found in plant sources (which often lack lysine).

g. Viruses

Viruses are infectious agents that require a host (obligate parasites). They are classified based on the nature of their genome's nucleic acid (DNA or RNA), the structure of the nucleic acid (single-stranded or double-stranded), and the shape of the nucleic acid (linear, circular, segmented, or non-segmented). Viruses are used as tools, for example, to enable a cell to produce a protein of interest or to study the effects of introducing a new gene into the genome.

Example:

- Baculoviruses are extensively studied insect viruses that serve as a means to control or replace chemical insecticides.

2.3. Sources of microorganisms used in biotechnology

There are several sources of microorganims that are used in biotechnology (Figure 2).

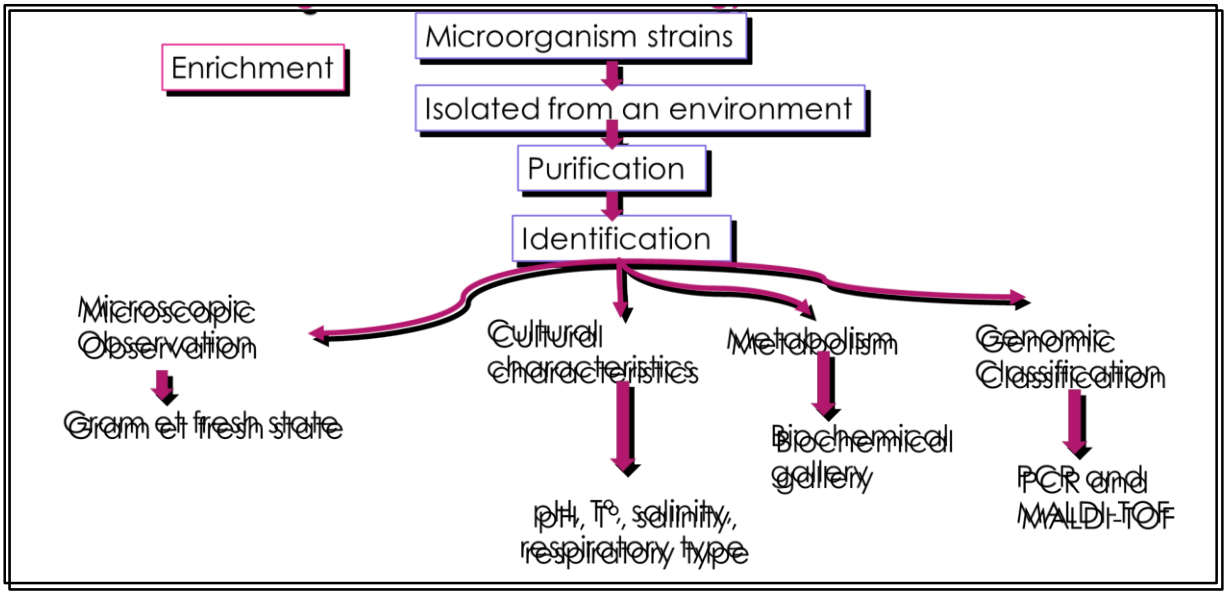


Figure 2 : microorganims extraction methods.

2.4. The strategy for finding new industrial strains

2.4.1. Isolation and selection of strains of industrial interest

There are essentially two ways to obtain microorganisms of industrial interest :

- Obtaining them already isolated by turning to large national collections of microorganisms, such as the American Type Culture Collection (ATCC) in the United States, the Biological Resource Center of the Pasteur Institute in France, or the Centraal Bureau voor Schimmelcultures (CBS) in the Netherlands. These collections provide strains for teaching, research, and industry.

1. The isolation step is random since it involves collecting microorganisms from natural environments (food, water, soil, organic matter...) related to the desired organism.
2. The sample is then inoculated onto an appropriate medium to isolate the target microbe.
3. The next step involves isolating and purifying the various obtained clones. Purification is achieved through streaking for isolation. This technique allows for the isolation of colonies and obtaining pure cultures.

2.4.2. Selection of industrial microorganisms

After isolation, the obtained microorganisms then undergo selection based on their biological and technological aptitude.

2.4.3. Properties of microorganims of industrial interest :

They should have the following properties :

- Grow rapidly (short generation time) in an inexpensive and available culture medium (agri-food waste).
- Not pathogenic for humans or the nevironment and can be genetically manipulated.

- The product must not include toxic or undesirable products, especially consumables.
- Physiological stability and ease of extraction.
- Produce products that are easy to extrract and separate, and in abundant quantities.
- Produce the substance of industrial interest in a short period of time.

2.5 Improvement of selected strains

A- Objectives

The improvement strategies aim to :

a) Increase the yield of the desired product concentration or biomass.

b) Enhance substrate specificity and production rate.

c) Regulate the activity of constitutive or inducible enzymes (present at all times as part of the basic metabolism or induced by the presence of substrate).

d) Improve resistance to unfavorable conditions (temperature, pH, toxins, bacteriophages).

e) Reduce the production of undesirable metabolites.

B- Strain improvement Procedures :

- More Efficient Extraction System.
- More Productive Culture Medium (in composition).
- Ensuring the most favorable conditions for microorganism activity (temperature, pH).
- Better control of the fermentation process.
- Genetic Improvement of Strains (the best approach).

➢ Regulation of the activity of enzymes secreted by the microorganism.

➢ Increasing the permeability of the cell wall if the product is secreted outside.

➢ Selection of high-production strains from the natural population.

➢ Manipulation of genetic material of the strain:

1. Manipulation of genetic material not involving foreign DNA.
2. Manipulation of genetic material involving foreign DNA.

a. Manipulation of genetic material involving foreign DNA

Methods used include (figure 3) :

1. Transduction

2. Conjugation

3. Transformation

4. Recombination

5. Protoplast fusion

6. Directed Mutation

7. Genetic engineering

8. Metabolic ingineering

- **Protoplast Fusion :** Allows for the fusion of two different species to form a new hybrid that inherits genetic properties from both parent species.
- **Directed mutation at a site :** Involves inducing one or more mutations in a genome in a precise and deliberate manner. This method requires a group of specialized enzymes that recognize target sites.

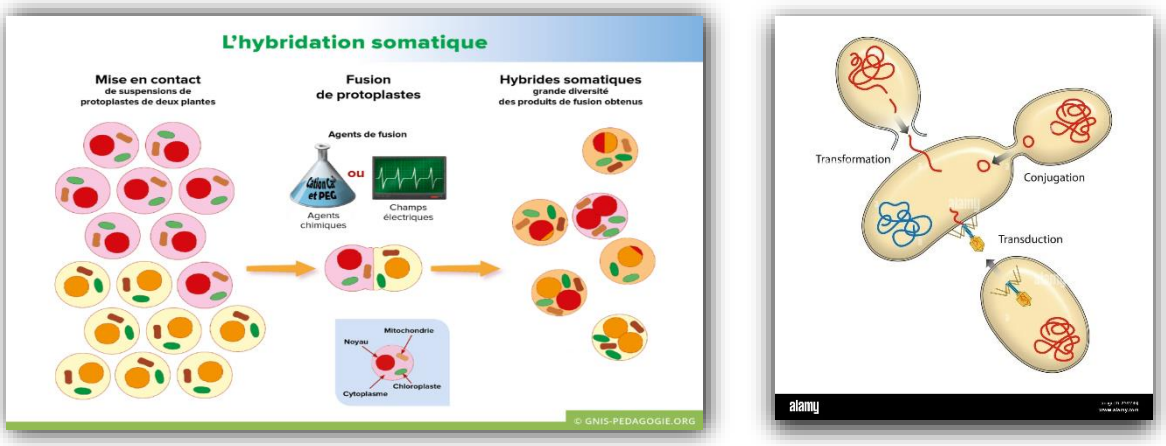


Figure 3 : different type of genetic manipulation.

- **Metabolic engineering :** Metabolic engineering involves creating or modifying metabolic pathways by manipulating the genes within the pathway. The goal is to improve the production of a metabolite, eliminate or reduce an undesirable metabolite, or shift production towards a new metabolite.
- **Genetic Engineering (Recombinant DNA Technology, Molecular Cloning, Gene Cloning) :**
  - Involves the excision of a specific portion of donor DNA,
  - Insertion of this portion into a replicative DNA (vector, e.g., plasmid),
  - Transfer of the recombinant DNA into the host cell,
  - Isolation of host cells that have effectively received the recombinant DNA.

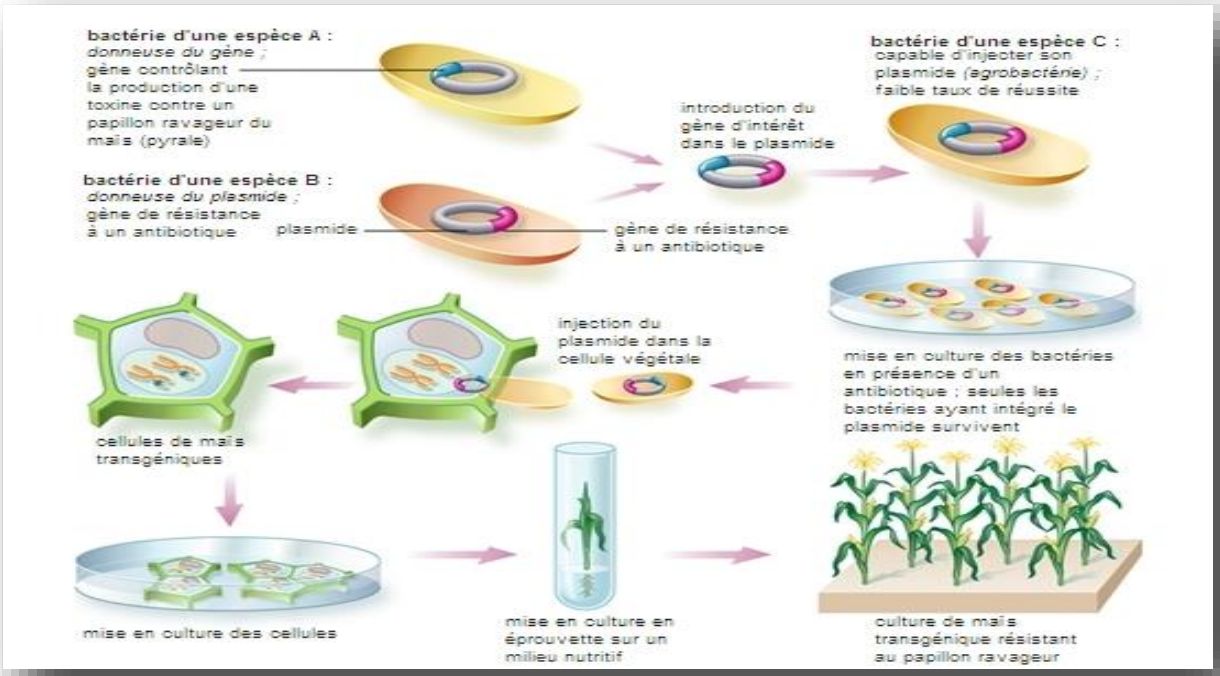


Figure 4 : Steps in the genetic improvement of industrial strains (Chang *et al*., 2017).

2.6 Conservation of strains

Culture collections : Each bioindustry maintains a collection of microorganisms in storage. The types of collections can be divided into

• Collection of various microorganisms :

- **American Type Culture Collection (ATCC)** : A biological resource center whose mission focuses on acquiring, producing, conserving, developing, and distributing microorganisms and other materials for research in life sciences.

• Specific collections :

Pathogens : National Collection of Type Cultures (NCTC) in London.

Industrial strains : National Collection of Industrial Bacteria (NCIB) in Scotland.

Specific types (fungi) : Center of Braun Schweitzer (CBS) in Holland.

2.6.1. Methods of preservation

The choice depends on the microorganism and the intended purpose :

Principle : Reduce the rate of metabolism of the organism.

Methods of preservation (conservation) involve one or more of the following techniques:

(a) Reduction of the growth temperature.

(b) Desiccation or dehydration.

(c) Limitation of available nutrients for the microorganism.

a) Methods Based on reduction of growth temperature

1. **Preservation on agar with regular refrigeration (4-10°C)**

- Aerobic microorganisms : slant agar.
- Anaerobic microorganisms : deep agar + paraffin or oil.
- Storage for 3 to 12 months.

Advantages

Cost-effective method as it does not require special equipment.

Disadvantages

- Refrigeration temperature does not completely inhibit the growth of microorganisms, requiring repeated subculturing of the strain.

- Risk of contamination and mutation due to repeated subculturing.

- Requires a significant amount of storage space.

- Subculturing can be labor-intensive.

2. **Preservation in freezers (from -20°C to -80°C)**

• Solid agar chunks + cryoprotectant (glycerol, raffinose, lactose, or trehalose).

• Glass beads in a culture broth + cryoprotectant.

Advantages

- The method is simple and requires minimal equipment.
- Storage for up to 3 years.
- Suitable for various types of organisms.
- Tubes require little space.
- Beads thaw quickly, saving time.
- Different colors of beads can be used, facilitating microorganism recognition.
- Suitable for both aerobic and anaerobic microorganisms.

3. **Preservation in Liquid or Vapor Phase Nitrogen (-156, -196°C):**

Culture broth + 10 to 20% glycerol or 5-10% dimethyl sulfoxide (DMSO) in polypropylene straws.

• This method is desirable for microorganisms that do not tolerate freeze-drying.

• The method of choice for preserving precious organisms.

Disadvantages

- Regular replacement of liquid nitrogen (it evaporates).
- Risk of straw explosion during thawing.
- The equipment is expensive.
- Not suitable for transport.

b) Dehydration-based methods

Principle : Removal of the water necessary for metabolism.

b. Drying on Sterile Silica Gel :

• A screw-cap tube is half-filled with sterile silica gel and sterilized in an oven.

• After cooling, the microbial suspension is poured into the tube, which is dried at 25°C and then stored in a container containing a desiccant.

f) Drying on Sterile Filter Paper (for spores).

g) Lyophilization (Freeze-Sublimation-Drying) :

Involves removing free water from the sample through freezing and then evaporating the ice under vacuum (using a lyophilizer).

Advantages

- Suitable for preserving large quantities of microorganisms in the industry.
- Relatively inexpensive equipment.
- Ampoules take up little space.
- Longevity of organisms (up to 10 years).

c) Methods based on nutrient reduction

Methods

Preservation in Sterile Distilled Water (with or without refrigeration).

• This method is suitable for preserving only a few organisms.

Choice of method

- Based on literature.
- Through experimentation.
- Preserving the industrial properties of the strain.

3. Industrial culture media

Introduction

Once a strain capable of producing the desired product with high yield has been obtained, it is necessary to develop a culture medium that can be used on an industrial scale.

3.1. Definition

The culture medium is the matrix that allows the growth of microorganisms.

• It is an aqueous solution in which all the essential nutrients for the growth of microorganisms are dissolved.

• The best carbon substrates for microorganisms are simple sugars, which is why sugar is the major ingredient used in the preparation of culture media.

• For industrial production, it is necessary to carry out cultures in very large quantities in tanks with volumes that can reach several cubic meters.

3.1.1. Factors determining the choice of industrial culture media.

The main factors that affect the choice of industrial culture media are as follows :

- Inexpensive substrate and year-round availability.
- Consistent physico-chemical quality even after sterilization.
- Low transportation and storage costs, especially regarding temperature.
- Easy to handle in both solid and liquid forms.
- Easy to sterilize.
- Viscosity that does not hinder the agitation or aeration of the industrial culture medium during the fermentation process.

3.2. Basic Components of industrial culture media

- A culture medium must be complete, whether it is synthetic or complex (table 1).
- Since the majority of industrial microorganisms are hetero-chemotrophic, it is necessary to provide the culture medium with :
- A source of carbon, which is often also a source of energy; a source of nitrogen; Inorganic salts (such as K_2HPO_4, $CaCl_2$, $MgSO_4$); Trace elements (like Zn, I, Co, Cu, Mn, etc.).
- Some demanding microorganisms require the presence of specific growth factors (vitamins, amino acids, etc.) and water.
- The absence of growth-inhibiting substances.

Table 1 : Main constituents of culture media used in industrial processes.

Source	Raw material
Carbon and energy	Molasses, whey, agricultural waste
Nitrogen	Soybean flour, Slaughterhouse waste, NH3 and nitrate, Vinasse
Vitamins	Plant and animal products
Iron, trace-elements	Inorganic chemical derivatives
Anti-foam	Alcohols, Silicones, Vegetable oils.

a) Carbone source (carbohydrates)

Carbon is the most abundant element in microbial cells. It represents the largest portion of the culture medium.

The carbon nutrients in the medium can vary widely and include : Potato starch, Molasses from sugar cane or sugar beets, Starch hydrolysates, Glucose syrups, Saccharified starch, Sucrose.

a) Major industrial carbon substartes

- **Molasses**: Molasses is a byproduct of sugar refinement from sugar beets or sugar cane.

Sugar cane molasses typically contains sucrose (32%), glucose (14%), and fructose (16%), while sugar beet molasses contains saccharose (48-50%), raffinose (1%), glucose+fructose 1%, vitamins, and amino acids.

- **Starch,**
- **Lactose (lactosérum)**
- **Starch and dextrin :**

Starch is typically derived from corn but can also be obtained from other cereals. To be used as a carbon and energy source, starch undergoes hydrolysis into a sugar syrup containing mainly glucose and dextrin, typically using diluted acids or amylolytic enzymes.

b) Nitrogen source

Most industrial microorganisms can use nitrogen sources in both organic and inorganic forms.

- Inorganic nitrogen can be provided in the form of ammonium salts, such as ammonium sulfate ((NH4)2SO4), potassium nitrate (KNO3), and diammonium phosphate ((NH4)2HPO4),
- Organic nitrogen sources include amino acids, proteins, urea, glutamate…etc, and complex sources like peptone, soybean flour, and more.

Nitrogen sources are often provided in a raw form, typically derived from agri-food industry byproducts, such as: Corn steep liquor, yeast extracts, peptones, and soybean flour. Pure amino acids are only used in specific cases.

a) **«Corn steep»**

Corn Steep Liquor is a byproduct of corn starch, specifically the concentrated soaking water from corn seeds (containing 8% nitrogen and vitamins).

- Corn is soaked in water with sulfuric acid to make the corn seeds easier to grind.
- The resulting supernatant is the corn steep liquor.
- This material is used in the production of certain antibiotics like penicillin and rifampicin.

b) Soybean flour

Soybean flour is the remainder of the seeds after extracting soybean oil.

- It is desirable for actinomycetes.
- Used in the production of certain antibiotics and plant hormones.

c) Mineral elements

Play a crucial role in the composition and structure of certain enzymes (P, S, Mg, I). For example, corn steep liquor contains a sufficient quantity of mineral salts to meet the fermentation requirements.

d) Growth Factor

Such as vitamins, amino acids, fatty acids, sterols, and nucleotides must be added to cultures if the microorganism has specific nutritional requirements.

e) O_2 supply

The supply of oxygen is essential, and it depends on the microorganism's oxygen needs and respiratory type. Oxygen can be injected into the fermenter in the form of air containing approximately 21% (v/v) oxygen, or it can be provided as pure oxygen when the microorganism's oxygen requirements are particularly high. The air or oxygen injected into the fermenter must be sterilized through filtration.

f) Adding antifoams

The addition of antifoaming agents is necessary to reduce foam formation during fermentation, which is primarily caused by proteins in the culture medium.

- If foam formation is not controlled, it can block air filters, leading to a loss of asepsis.
- There are three approaches to control foam formation :

I. Modifying the composition of the culture medium ;

II. Using mechanical foam breakers ;

III. Adding chemical antifoaming agents, which are surfactant molecules, such as vegetable oils (soybean oil, sunflower oil, and rapeseed oil), and fish oil.

4. Products of industrial fermentations

4.1. Primary metabolites obtained through microbial fermentation

There are two main types of microbial metabolites : primary and secondary.

- ❖ A primary metabolite is formed during the exponential growth phase (trophophase), whereas a secondary metabolite is typically produced towards the end of the exponential phase or during the stationary phase (idiophase).

Primary metabolites obtained through microbial fermentation include amino acids, organic acids, biogases (H2, CH4,…).

a. Amino acids

- The most produced amino acid is glutamic acid, which is used as a flavor enhancer (sodium monoglutamate SMG, E621).
- Two other important amino acids, aspartic acid and phenylalanine, are the basis for the artificial sweetener aspartame, a non-caloric sweetening agent used in sugar-free beverages.
- Lysine, an essential amino acid for humans and some livestock, is used as a food additive.

b.Organic acids

Most organic acids have applications in the agri-food industry and are primarily used as :

Some of the main organic acids useful to humans include :

- ✓ Acetic acid (produced by *Clostridium thermoaceticum*),
- ✓ Citric acid (produced by *Aspergillus niger*),
- ✓ Lactic acid
- ✓ And Gluconic acid.

c.Alcohol and solvents, biofuels, and biogas

Alcohol, a typical primary metabolite, is a product of anaerobic metabolism in certain yeasts and bacteria and is part of the energy metabolism.

- Acetone, dihydroxyacetone, polyols, butanol, and ethanol…
- Ethanol, of which 80% of the global production is produced by fermentation from cellulose, glucose, starch, etc.
- Hydrogen is considered a new energy source intended to replace petroleum.
- Methane is produced through the anaerobic decomposition of organic matter and other hydrocarbons.
- Photosynthetic bacteria (purple bacteria and cyanobacteria) and algae produce H2 by carrying out the following reaction : $2H_2O \rightarrow 2H_2 + O_2$

- Fermentative bacteria tend to produce hydrogen gas through a different reaction : Glucose + 2H2O → 4H2+ 2 acetic acids + 2CO2.

Note: Methanogenic bacteria require specific bioreactors (anaerobic bioreactors). When used for sludge digestion, they are inoculated in depth to ensure anaerobic conditions.

4.2.Secondary metabolites obtained through microbial fermentation

- Antibiotics (penicillin, streptomycin, tetracycline).
- Polysaccharides.
- Vitamins.
- Enzymes.

- Secondary metabolites are all produced during the idiophase.
- They are not essential for growth or reproduction.
- They provide microorganisms with secondary functions such as protection, toxin production, and degradation of macromolecules…etc

a.Antibiotics

Antibiotics are produced by microorganisms to kill or inhibit the growth of other microorganisms. They are organic molecules derived from the secondary metabolism of filamentous fungi and bacteria, especially actinomycetes.

b.Polysaccharides

Polysaccharides of industrial interest come in two types :

- Reserve or secondary polysaccharides, which are produced during the idiophase.
- Structural polysaccharides (e.g., from the cell wall), which are produced during the trophophase. For example, Xanthomonas campestris is used to produce xanthan gum.

- Algae and molds also play a significant role, with many species utilized in the production of cellulose, alginates, agars, and more.

c.Vitamins

- ❖ Vitamins are organic substances essential for the functioning of the human and animal organism.
- ❖ Vitamin B12 (cobalamin) is exclusively synthesized by microorganisms in nature. For industrial production of vitamin B12, strains of Pseudomonas, Bacillus, and Streptomyces are used.

d.Enzymes

- ❖ Enzymes are peptides or proteins that act as catalysts for the metabolic reactions of microorganisms.
- Lactase: *Kluyveromyces fragilis.*
- Invertase: *Saccharomyces cerevisiae* and *Aspergillus usamii*…

5. Characteristics of large-scale fermentations

The formation of products : fermentation technology

5.1. Definition of industrial fermentation

A biological process using the mass culture of aerobic or anaerobic microorganisms. Fermentation involves cultivating microorganisms with the aim of having them produce biologically relevant molecules for commercial purposes at a reasonable cost. It is a biotechnological process that involves transforming an organic substance under the influence of a ferment (microorganisms, enzymes...) in order to collect the microorganisms themselves (such as baker's yeast) or a byproduct of the reaction (alcohol, antibiotics...).

5.2. Fermenter

For industrial production, it is necessary to carry out cultures of microorganisms in very large quantities in vessels that can reach several cubic meters in volume. These installations are called "bioreactors or fermenters".

5.2.1. Definition of a fermenter or bioreactor

A vessel used for the culture of a microorganism, providing optimal conditions for the production of biomass, the production of a metabolite, or the bioconversion of a target molecule.

• Bioreactors are classified based on their maximum volume and come in various sizes :

- 1-20 liters in the laboratory.
- Pilot-sclae bioreactors (300-3000 L).
- 100,000-500,000 (500 m3) liters in industry.

The equation inside a bioreactor is as follow :

Substrate + Biomass = (Biomass) n + Metabolites + substrate residues

5.3. Components of a bioreactor : A bioreactor consists of (figure 5) :

- **A culture vessel or chamber**, made of glass or stainless steel, with a variable volume ranging from a few liters to several cubic meters in the case of industrial units, where all biochemical reactions take place. The vessel is tightly sealed and does not allow the exchange of air between the interior and exterior environments.

- **The cover :** It is a large plug that covers and protects the interior environment from external contamination by preventing the entry and exit of air.
- **A syringe :** It is used for the sampling and injection of various solutions during the culture.
- **An agitation system** is used to ensure the mixing and aeration of the culture. It consists of an external motor and one or more internal turbines (depending on the bioreactor's volume).
- **Sensors :** These sensors are responsible for measuring temperature (thermometer), pH (pH meter), and dissolved oxygen concentration (oxygen probe) to ensure the right conditions inside the bioreactor.
- **A control unit** managed by a computer records and controls all the operating parameters of the bioreactor.

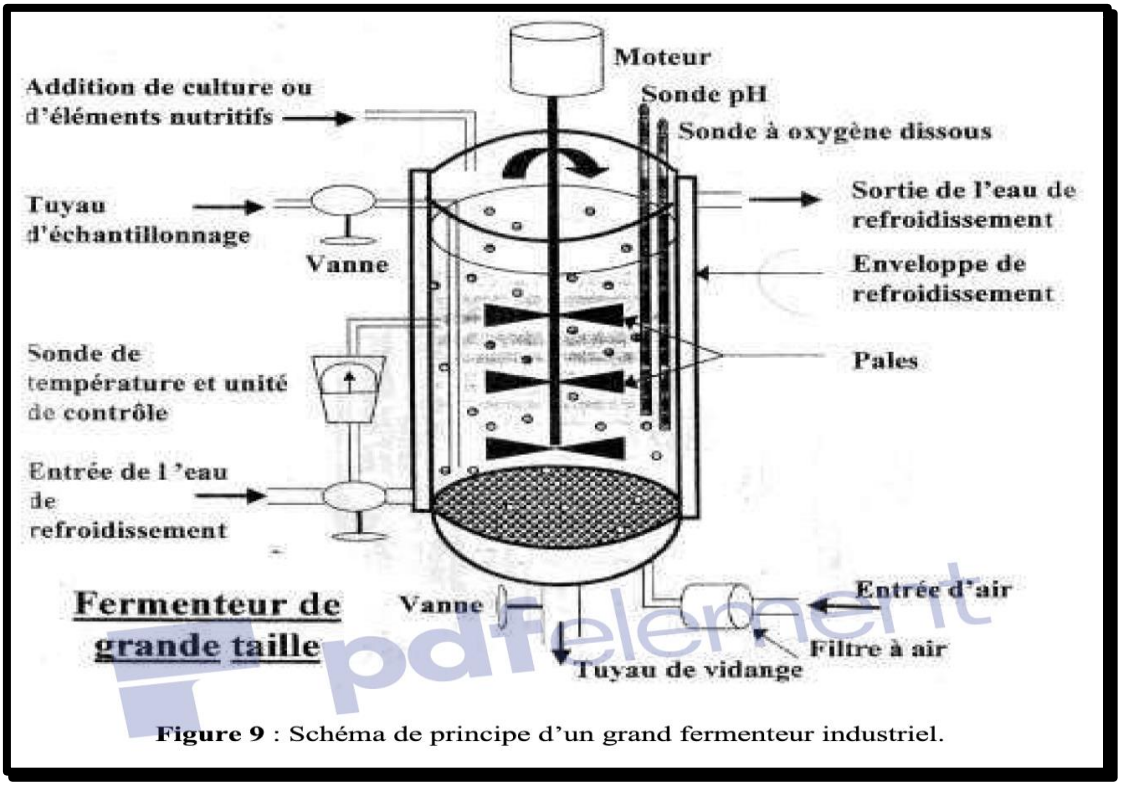


Figure 5 : Components of a bioreactor.

5.3.1. Bioreactor design

Choice of Installation Location

Bioreactors are located in isolated facilities away from the laboratory.

To choose the installation location of a bioreactor, two specific conditions must be considered:

- Preliminary assessment of all risks of microbial pollution of the air or water.
- The ability to have a sufficient quantity of water for bioreactor cooling.

a.Choice of a bioreactor

The choice of a bioreactor is governed by several criteria :

- ✓ Type of microorganisms used, distinguishing between aerobic and anaerobic bioreactors.
- ✓ Perfect contact between the liquid phase (the medium) and the solid phase (biomass).
- ✓ Good mass transfer between the cell and the culture medium.
- ✓ Adequate oxygen transfer.
- ✓ Easy heat transfer to and from the cells, as well as to the exterior.
- ✓ Easy collection of products.

b. **Bioreactor preparation :** The startup of a bioreactor is controlled by the following parameters :

c. **Sterilization :** Sterilization is carried out through heat treatment using two methods :

1. In-situ Sterilization

- Injection of steam at 121°C for 20 to 30 minutes.
- Injection of superheated water (120°C to 140°C), with the duration varying depending on the bioreactor's size.
- Heating the chamber using resistors.

2. Ex-situ Sterilization : This method is for small-volume glass bioreactors and is done through autoclaving.

Gaz injection

Some microorganisms require (CO2) or (N2) for their growth. These gases are stored in pressurized bottles and introduced into the bioreactor after passing through a sterilizing filter.

Temperature

Cells generate heat energy during culture, leading to temperature variations. Therefore, a sensor is used to detect these variations and control the appropriate temperature for growth. Warming or cooling of the chamber is achieved through an isolated water circuit (cooling system) that does not come into contact with the microorganisms.

Pressure

Some productions require pressurizing the bioreactor. For this purpose, pressure gauges (pressure measuring devices) are placed to continuously monitor the pressure level in all parts of the installation.

Agitation

This parameter is crucial because it helps homogenize the culture medium, temperature, and pH, preventing cell aggregation.

d- Cultivation Modes in a Bioreactor :

To produce a substance of interest using a microorganism on an industrial scale, it's important to first understand its requirements and growth kinetics. This knowledge helps in choosing the most suitable fermentation mode for its cultivation.

There are three main modes :

- Batch Fermentation (Closed Reactor).
- Fed-Batch Fermentation (Intermittent Feeding).
- Continuous Fermentation (Open Reactor).
-

1. Batch fermentation

- The bioreactor is filled with sterile culture medium and then inoculated with the industrial strain.
- During this fermentation, no additional culture medium is added to the bioreactor, except for neutralization reagents or a very small amount of antifoam agent.
- Similarly, no culture is withdrawn until it is complete.
- Over time, the biomass concentration increases inversely to the substrate concentration, which decreases until it is exhausted.
- The volume in the fermentation vessel remains constant.
- At the end of the culture, the bioreactor is emptied, and the desired product is extracted.

This is the most classic mode of fermentation and, despite its low productivity, it is widely used.

This is due to the following advantages :

- Better control of the fermentation process and its short duration.
- Low risk of microbial contamination because the system is closed.
- Low risk of mutation because the number of cell divisions is limited.

Reminder of microbial growth : consists of 5 phases :

- **Lag phase :** The growth rate is zero. It is the time needed for the bacteria to adapt to the new substrate (enzyme synthesis).
- **Exponential growth phase :** The growth rate reaches its maximum, and the growth rate is constant.
- **Slowdown Phase :** The growth rate decreases. There is depletion of the culture medium, accumulation of waste products, and the beginning of autolysis of the bacteria.
- **Stationary Phase :** The growth rate becomes zero. Bacteria begin to synthesize metabolites to better resist damage.

- **Decline Phase :** The growth rate is negative. All nutrient resources are exhausted. There is an accumulation of toxic metabolites. There is a decrease in viable organisms and cell lysis due to the action of endogenous proteolytic enzymes.

2. The fed-batch fermentation mode

- This mode starts in a small volume of sterile culture medium, and the biomass increases rapidly (batch fermentation).
- When the microorganisms are in the exponential phase, sterile culture medium is added to the fermenter at a determined rate.
- The volume in the tank increases over time.
- The growth rate in this mode is therefore constant, which ensures the constancy of nutrient and biomass concentrations throughout the culture.
- The increase in biomass is mainly due to the growth of microorganisms.
- Feeding is stopped when the tank is filled to its useful volume.

Advantages

- ❑ The desired product can be collected at any time.
- ❑ This mode helps to avoid inhibition problems, such as substrate toxicity.
- ❑ It is widely used in practice, saving time and improving reactor productivity.

Disadvantages

- ❑ The challenge with this mode is to adjust the feed rate so that the substrate concentration remains constant in the culture.
- ❑ It's important to note that the risk of contamination is higher in this type of fermenter.

3. Continuous fermentation

In this mode, the bioreactor is constantly supplied with fresh medium while an equivalent volume of spent medium is withdrawn to maintain concentrations in equilibrium in the culture (constant reactor volume).

Unlike batch fermentation, continuous fermentation allows for the maintenance of :

- Cellular concentration
- Biomass growth rate
- Nutrient balance in the medium
- Production of the desired metabolites.

❖ The total emptying of the bioreactor is only done in case of mutations or contaminations.

Advantages

- Intensive use of the bioreactor.
- Time savings by eliminating washing, sterilization, cooling, and substrate replenishment.
- Continuous processes are easily automated.
- Very high productivity compared to the previous two modes.

Disadvantages

- Risk of microbial contamination as the system is open and operates over long periods.
- Contaminants with slow growth that are not a problem in batch fermentation (4 to 10 days) can be a significant issue in continuous fermentation (3 to 9 months).
- The need to use highly genetically stable strains to minimize the risk of mutation due to the long duration of the culture and the high number of divisions.
- High costs of peripherals adapted to this mode of culture.

5.4.The process of scaling up (or extrapolation)

- Regardless of the application field of industrial microbiology, transitioning from laboratory Erlenmeyer flasks to industrial bioreactors remains a challenge (figure 6).
- Hence the idea of the scale-up process, which involves transferring the microbial culture prepared in laboratory Erlenmeyer flasks to small-volume laboratory bioreactors, then to pilot-scale bioreactors. Subsequently, the culture is introduced into an industrial fermenter (Figure).
- The goal is to achieve the same yield despite the increase in the culture volume.
- We move on to the laboratory-scale fermenter, which is a glass bioreactor with a capacity of 1 to 10 liters for the initial scale-up steps.
- At this stage, when the composition variation tests show promise, we proceed to the pilot-scale fermentation stage using bioreactors ranging from 300 to 3000 liters, which are closer to commercial size but still reasonably cost-effective.
- Finally, we reach the industrial-scale fermenter, which has a capacity ranging from 10,000 to 500,000 liters. Throughout all these stages, close monitoring of aeration and how volume affects the evolution of oxygen concentration in the culture is crucial.

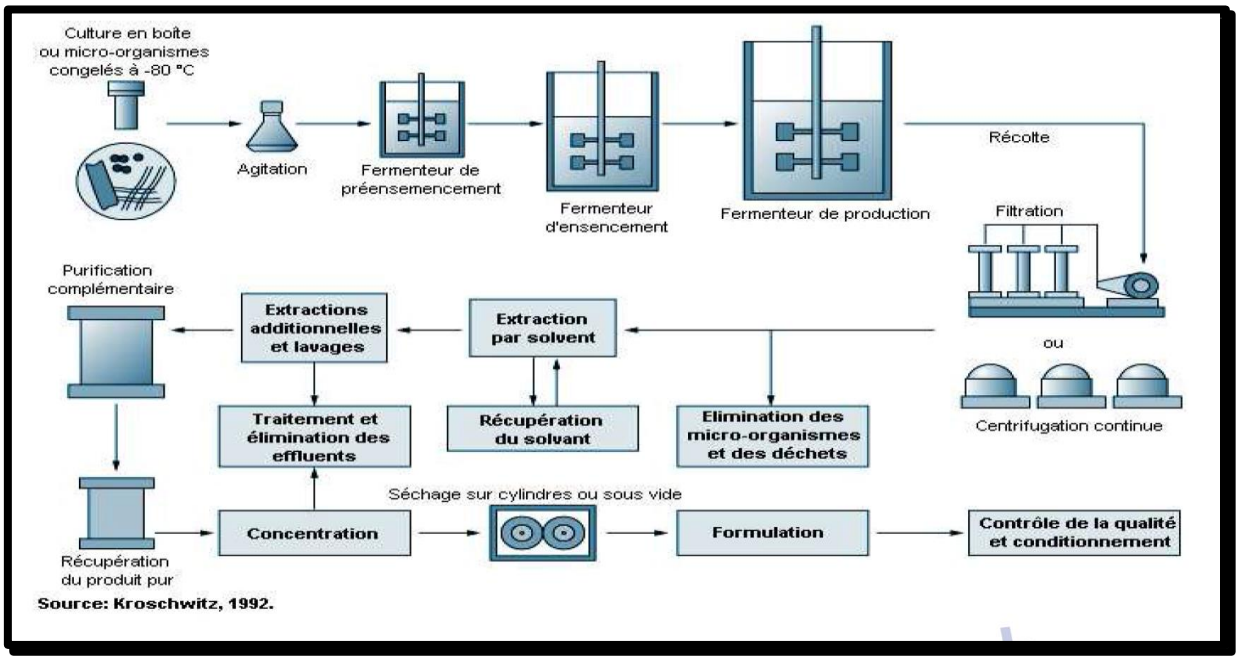


Figure.6 : General scaling diagram followed by product extraction and purification steps.

5.4.1. Inoculation and Seeding

Definition

The inoculum is any living material (spores, cells, hyphae) used to introduce into a propagation or production medium.

Preparation of the inoculum

- A spore suspension is prepared, diluted, and streaked onto the surface of an inclined agar tube.
- Next, a pre-inoculum is prepared in a liquid medium (flask, Erlenmeyer flask) incubated on rotary shakers. It is essential that the chosen medium yields a large quantity of spores.
- The process then proceeds to a small fermenter of 300 to 500 liters
- and subsequently to a larger fermenter (germinator) of 3000 to 5000 liters, which serves as a seed tank. Using a mobile metal container (Bazooka), the fermenter is aseptically inoculated with sterile compressed air, and the pressure drives the contents of the container into the fermenter.
- All piping is sterilized with steam.

Inoculum properties

At each stage, rigorous controls are in place to detect three ever-present threats : contamination, bacteriophages and mutations.

Purity

The presence of contaminants is detected through sterility tests using microscopic examinations and subcultures. These tests should be conducted from the inoculum to the main culture.

5.4.2. Products of Industrial Fermentation.

Single Cell Proteins (SCP)

1. Definition

Single Cell Proteins (SCP), also known as "Protéines d'Origine Unicellulaire" (P.O.U) in French, refer to any microbial biomass rich in proteins intended for human or animal consumption. SCP is not pure proteins but also contains : Carbohydrates, Lipids, Nucleic acids, Mineral salts and Vitamins.

2. Microorganisms used for SCP production

Generally, four types of microorganisms are used. These include :

- ✓ **Yeasts**

Saccharomyces cerevisiae : primarily used as a food additive.

Candida utilis: after heat inactivation, it is used as a nutritious food source since it is rich in proteins and free amino acids and has a mild meat-like flavor.

- ✓ **Filamentous fungi**

The mold, *Fusarium venenatum* : used for the production of Quorn, a brand of mycoprotein-based meat substitute with a taste resembling chicken meat.

- ✓ **Microalgae, Bacteria**

Cyanobacteria can be used as dietary supplements, as seen with spirulina, mainly produced from the species Arthrospira platensis.

Spirulina is rich in proteins (60% protein content), vitamins, mineral salts, and trace elements.

3.Criteria for selecting microorganisms for SCP production

Before being used as a source of SCP, the microorganism must meet certain criteria. It should be : Non-pathogenic ; Have a high protein content ; Have a high growth rate ; Be easy to harvest ; Exhibit good resistance to variations in production conditions.

4. Industrial production of SCP

Culture conditions

The ideal ratio for the various sources of carbon, nitrogen, and phosphorus in the culture medium for SCP production should be approximately 100 / 5 / 1.

The incubation temperature is typically between 30 and 35°C, depending on the microorganism ; while the pH should be maintained between 4.0 and 5.5.

A critical parameter is the dissolved oxygen concentration. For aerobic fermentations, the medium should be saturated with 40% oxygen.

5.Fermentation and purification of SCP

After sterilizing the culture medium, biomass production is carried out in a fermenter. The SCP is then recovered through multiple centrifugations or dried to obtain a powder free of any living cells.

Advantages of SCP

The use of microorganisms as a source of single-cell proteins (SCP) has several advantages compared to animal or plant-based protein sources :

- Rapid growth rates compared to livestock.
- High protein content (30–80% of dry weight).
- The ability to use a wide range of inexpensive substrates, including organic waste, for their growth.
- Requires less space and water compared to traditional livestock farming.
- Helps address environmental issues.
- SCP production is independent of climate variations.

However, there are some potential drawbacks to using SCP as a protein source :

They may produce toxins or other harmful metabolites.The nucleic acid content of SCP limits their use in human food.

Chapter II : Pharmaceutical products

Industrial production of Penicillin and Tetracycline

Objectives

At the end of this chapter, the student or learner will be able to :

- Explain the steps involved in the industrial production of Penicillin and Tetracyclin.
- Describe and outline the microorganisms that produce vitamins and amino acids. Describe the stages involved in the industrial production of pharmaceutical products.

Introduction

The role of microbiology in the advancements of the pharmaceutical and medical industries has led to significant discoveries. The biochemical properties of microorganisms are indeed exploited in numerous pharmaceutical processes, including the synthesis of antibiotics, vaccines, and other medically relevant molecules.

1. Secondary metabolites obtained through microbial fermentation.

1.1. Antibiotics

Antibiotics are natural or synthetic substances that possess the property of inhibiting growth (bacteriostatic activity) or even destroy target bacteria (bactericidal activity). Some antibiotics are bacteriostatic at low doses and bactericidal at high doses.

- The spectrum of action is wide or narrow.
- Antibiotics are naturally produced by microorganisms during their growth to survive in a competitive environment. They are produced by bacteria and Fungus ‘filamentous fungi’: actinomycetes
- Although 4,000 antimicrobial molecules have been isolated from various microorganisms, only about fifty are used as antibiotics for the treatment of various infections.
- The immense therapeutic potential of antibiotics has required the development of industrial techniques for the production of these molecules.
- The manufacturing of antibiotics is most often carried out through microbiological methods, harnessing the metabolic activity of certain bacteria and fungi on an industrial scale.

1.1.1. Target of bacterial inhibition

Antibiotics act on bacteria by primarily targeting bacterial wall synthesis, synthesis of membrane proteins, intracellular proteins or enzymes and DNA synthesis (figure 7).

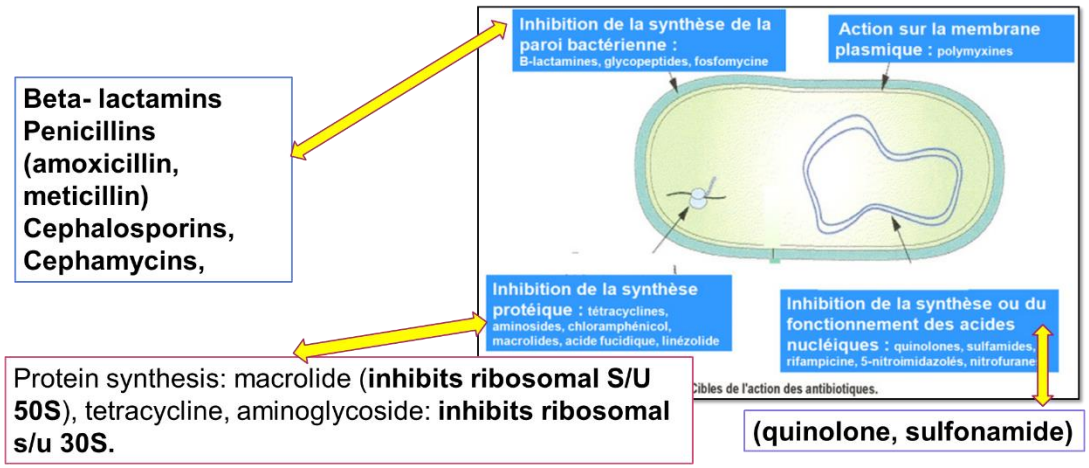


Figure 7 : Target of bacterial inhibition.

1.2. Antibiotics classification

These natural molecules are classified based on their origin, chemical nature, mechanism of action, and spectrum of activity. There are several categories:

1. Beta-lactams: These are low-toxicity bactericidal antibiotics (e.g., Penicillin, Cephalosporins). They are generally used orally for infections in the ENT (ear, nose, and throat) area, bronchial infections, and in the urinary and digestive systems.
2. Compounds with cyclic structures: For example, Tetracyclines, which are bacteriostatic and have some liver toxicity.
3. Macrolides: These are low-toxicity bacteriostatic antibiotics (e.g., Erythromycin). They are used as an alternative to penicillins for infections in the ENT and pulmonary systems.

1.3. Global Procedure for the industrial production of antibiotics

The production and commercialization of a new antibiotic must go through a series of studies at multiple levels:

- ✓ Preliminary studies to isolate and preserve strains of interest.
- ✓ Laboratory-scale production studies through fermentation.
- ✓ Analytical studies of physical and chemical properties.
- ✓ Pharmacological studies of antimicrobial activities, as well as toxicity and therapeutic effects in animals.
- ✓ Industrial-scale production studies to establish manufacturing and quality control methods.
- ✓ Economic and commercial studies.

1.4. Qualities sought in a new antibiotic

For suitable medical use, a new antibiotic must possess a number of qualities:

- Absence of toxicity.
- Solubility in water at an acceptable pH.
- Stability in relation to various physicochemical factors.
- Absence of pyrogens and histaminic compounds.
- Good tolerance.
- Absence of side effects on cells, serum, red blood cells, and white blood cells.
- Low production costs.

• Examples of antibiotics production

1.Penicillin :

The most commonly used antibiotic families in medicine are the β-lactams, such as penicillins, which are obtained from the mold *Penicillium*.

The discovery was accidental in 1928 when A. Fleming observed the inhibitory activity of Staphylococcus by a "fluid" produced by *Penicillium notatum.* Despite promising results, and due to numerous challenges related to the production of large quantities of penicillin, the product was forgotten (figure 8).

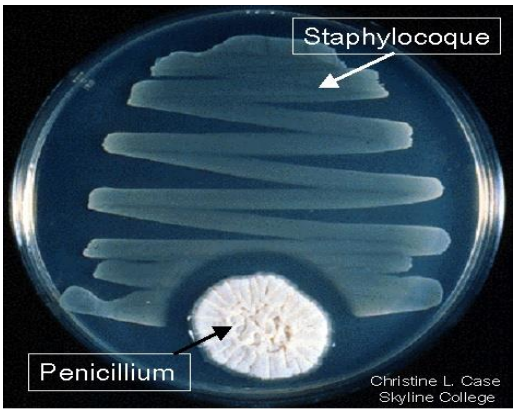


Figure 8 : *Staphylococci* cannot grow near *Penicillium notatum.*

- Penicillin was only industrially produced starting in 1941. Since then, numerous research efforts have led to such improvements that this production process is currently a highly efficient microbiological process.
- Fleming, Florey, and Chain were awarded the Nobel Prize in Physiology or Medicine in 1945 for "the discovery of penicillin and its curative effect in various infectious diseases."

• This antibiotic can be produced by many species of *Penicillium and Aspergillus*.

• However, the first strain isolated by Fleming, *Penicillium notatum*, produced only a small amount of antibiotic. In 1946, the NRRL (Northern Regional Research Laboratory) in the United States isolated a more productive species, *Penicillium chrysogenum*, which was then improved through a series of mutations and selections.

- As a result, the variants used today produce 55 times more penicillin than the strain isolated by NRRL, and thousands of times more than the one isolated by Fleming.
- Penicillin is produced through fermentation in aerated and agitated tanks. Penicillium is capable of producing five types of penicillin (Penicillin G, X, F, K, and dihydropenicillin F). However, penicillin G was favored due to its ease of production.

The class of β-lactams or penicillins

- This class of molecules contains antibiotics that have a β-lactam ring formed by three carbon atoms and one nitrogen in their molecular structure (figure 9) :

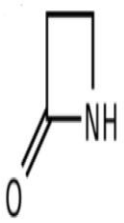


Figure 9 : Beta-lactam core structure.

- Penicillin is characterized by a cycle of 4 fused β-lactam atoms with a five-atom heterocycle (thiazolidine) (figure 10).
- On this five-membered ring, two methyl groups and one carboxyl group are attached.
- The β-lactam nucleus carries an amine function to which various side chains are attached by an amide bond.

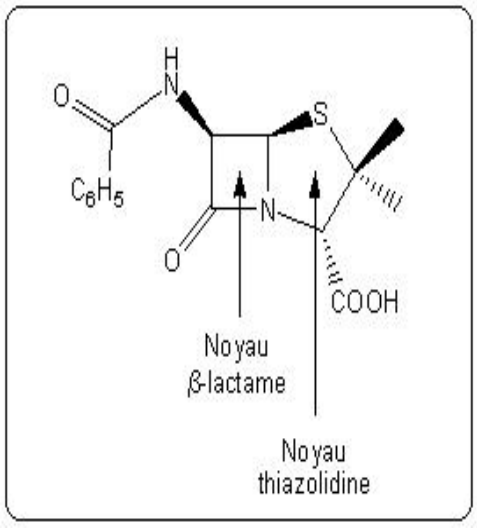


Figure 10 : Structure of benzylpenicillin or "penicillin G".

- More than 100 β-lactams, mainly penicillins and cephalosporins, have been approved for human use, and they represent more than half of the antibiotics produced worldwide.

- This group is particularly useful due to its broad spectrum of activity against various Gram-positive bacteria.
- They are time-dependent bactericidal antibiotics that inhibit the peptidoglycan in the bacterial cell wall after binding to target enzymes located at the cytoplasmic membrane: penicillin-binding proteins (PBPs). This prevents the final step of peptidoglycan synthesis (an essential component of the cell wall), leading to bacterial lysis (figure 11).

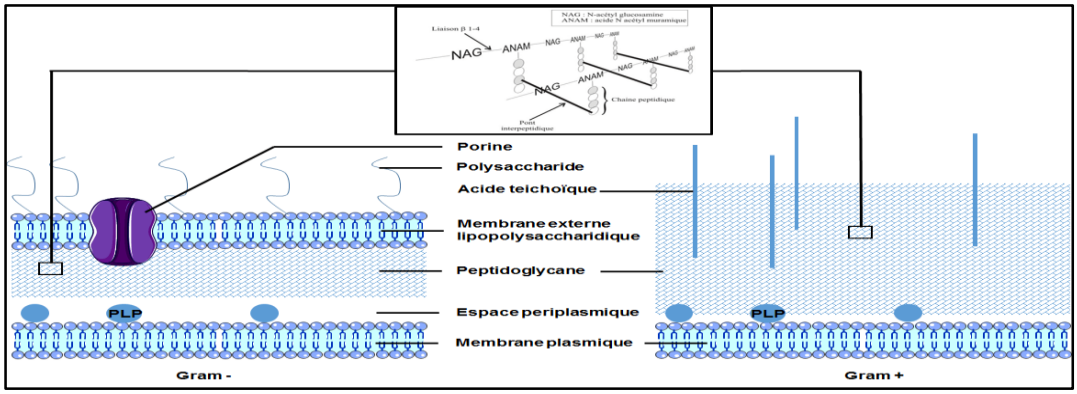


Figure 11 : Penicillin target.

• β-lactams are divided into two major subfamilies:

♪ Penicillins

♪ Cephalosporins

- The majority of penicillins are now semi-synthetic, obtained through chemical modification of natural penicillin from the species *P. chrysogenum*. This modification is achieved by substituting the natural acyl group attached to 6-aminopenicillanic acid (6-APA) with other groups, giving the molecule new properties (figure 12, 13).

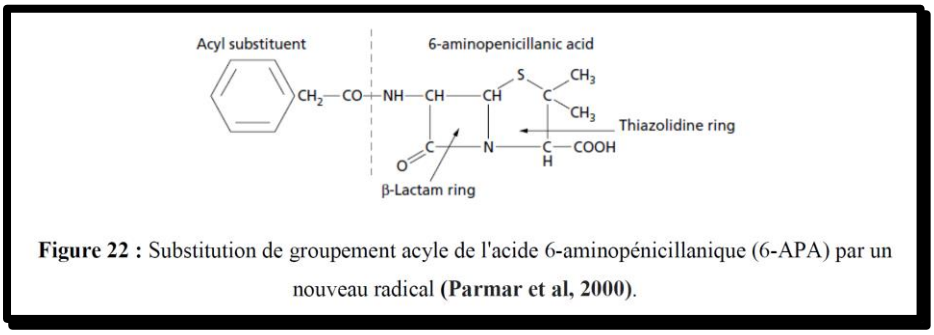


Figure 22 : Substitution de groupement acyle de l'acide 6-aminopénicillanique (6-APA) par un nouveau radical **(Parmar et al, 2000)**.

Figure 12 : substitution of the acyl group of 6-aminopenicillanic acid (6-APA) by a new radical (Parmar *et al.*, 2000).

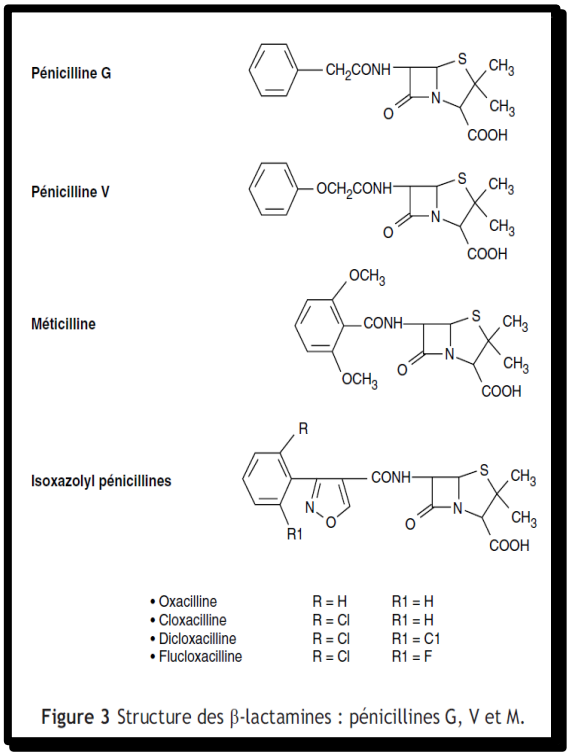


Figure 13 : Structure of β-lactammines: penicillins G, V and M.

Industrial production of penicillin

Stages involved in the production of Penicillin

1. Selection of microorganisms,
2. Selection of raw materials,
3. Preparation of the inoculum,
4. Fermentation process,
5. Recovery process.

Selection of microorganisms

- Mainly *Penicillium notatum* and *Penicillium chrysogenum* are used for the fermentative production of penicillin.
- *Penicillium notatum:* has high efficiency for antibiotic production.
- *Penicillium chrysogenum:* has a long mycelium that can utilize more nutrients and grow faster.
- So, the high-efficiency gene is produced in the laboratory and transferred to *P. chrysogenum.*

Thus, the genetically modified organism *P. chrysogenum* X 1612 is produced.

Selection of raw materials

- The production of penicillin from the culture of the *P. chrysogenum* strain is summarized as follows. The culture medium used for the industrial production of penicillin is formed by:

➢ A carbon source: which is typically glucose, lactose, and sucrose. These carbon sources are used either alone or in combination, such as the combination of glucose (1%) /lactose (3.5%). Initially, the fungus uses glucose as a carbon source for good growth, and once glucose is depleted, *P. chrysogenum* utilizes lactose to initiate penicillin biosynthesis.

➢ A nitrogen source: the most commonly used nitrogen source is Corn steep liquor (3.5%).

➢ Other ingredients may also be added, such as ammonia, mineral salts (calcium carbonate (1%) and monopotassium phosphate (0.4%)) can also be added.

- Ammonium is the best nitrogen source to ensure rapid growth:

✓ If one chooses to continue feeding with ammonium salts, they will be added in a semi-continuous mode. Generally, nitrogen for production (3rd phase) will be provided in the form of complex sources; for example, soybean or peanut flours rich in proteins. The addition of these elements is accompanied by oxygenation control.

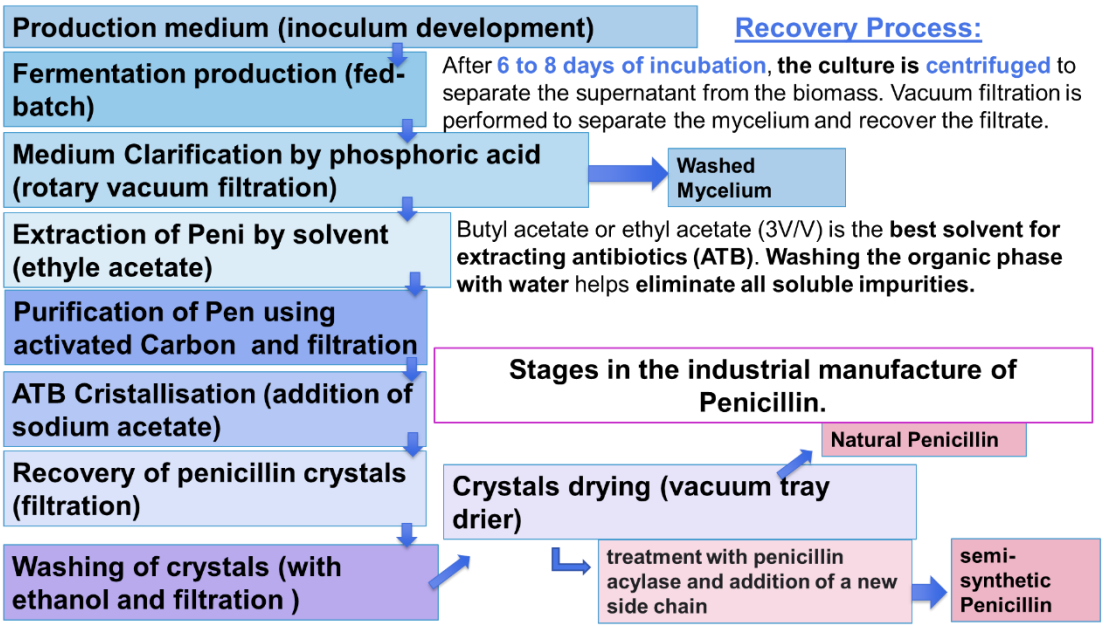
- Note that complex sources serve multiple functions. For example, if soybean flours serve as a nitrogen source, they also provide nucleic acids, vitamins, trace elements, lipids, sulfur, and phosphorus.

- Fatty acids and their derivatives are often supplied by oils in the form of triglycerides. The most commonly used oils include soybean, peanut, corn, and rapeseed oils. Besides their role as an energy source and potential precursors, fatty acids exert appropriate physicochemical actions such as emulsion formation, foam reduction, and modification of membrane permeability.

Fermentation process

➢ After preparing and sterilizing the culture medium, it is introduced into Fed-Batch type fermenters with a capacity of (20-40) x 10^3 liters.

➢ The oxygen level must be maintained between 25-60 mmol/L/h, and the culture should be incubated at a temperature of 25-27 °C with a pH range of 6.5-7.7.



- **Packaging of the antibiotic**

Packaging (figure 14), after the addition of various excipients, can take the form of :

- Powder for pediatric preparations,
- Capsules, Animal feed additives, Solutions for ready-to-use injectable preparations and Tablets.

Figure 14 : different type of packaging of antibiotic.

2. Tetracycline

- First discovered in 1948 by Duggar, tetracyclines constitute the most economically significant group of antibiotics due to their broad spectrum of activity.
- Their synthesis has primarily been attributed to the species *Streptomyces aureofaciens*, whose production capabilities have been significantly enhanced through selective isolations and induced mutations.

Definition of Tetracyclines

• Bacteriostatic antibiotics with intra and extracellular diffusion (except in the central nervous system, cerebrospinal fluid, and joints).

• Inhibit bacterial protein synthesis: They disrupt protein synthesis at the 30S ribosome subunit, leading to bacterial inhibition.

• Broad spectrum of action: Effective against both Gram+ and Gram- bacteria. The primary indications for the use of tetracyclines are infections caused by *Escherichia coli* and *Staphylococcus aureus.*

• Limited use due to side effects and resistance.

Structure of Tetracyclines

As their name suggests, tetracyclines (TC) have a common chemical structure composed of four fused hexacyclic rings in a linear arrangement (figure 15).

- Members of this class have four (4) hydrocarbon rings and are known by their name with the suffix "-cycline".
- The first member of this class was chlorotetracycline (Aureomycin).

Tetracyclines are divided into natural and semi-synthetic cyclines.

- Natural cyclines include: Chlorotetracycline and Tetracycline
- Semi-synthetic cyclines include: • Oxytetracycline • Doxycycline • Minocycline

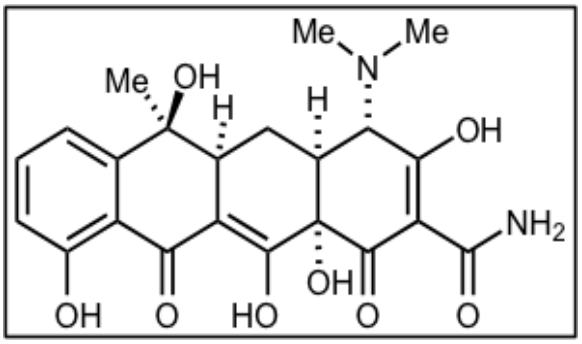


Figure 15 : Tetracycline structure.

- Tetracycline is produced through the fermentation process in aerated and agitated stainless steel or aluminum fermenters.
- The manufacturing process consists of two stages: a growth stage and a production stage.
- Fermentation typically lasts for about two days.

Search for Antibiotic-Producing Species

- Species that produce antibiotics are mainly sought in soil; indeed, the soil has a highly diverse microflora, increasing the likelihood of finding antibiotic-producing microorganisms there.
- For the selection of antibiotic-producing species, the process represented in the following diagram is carried out.

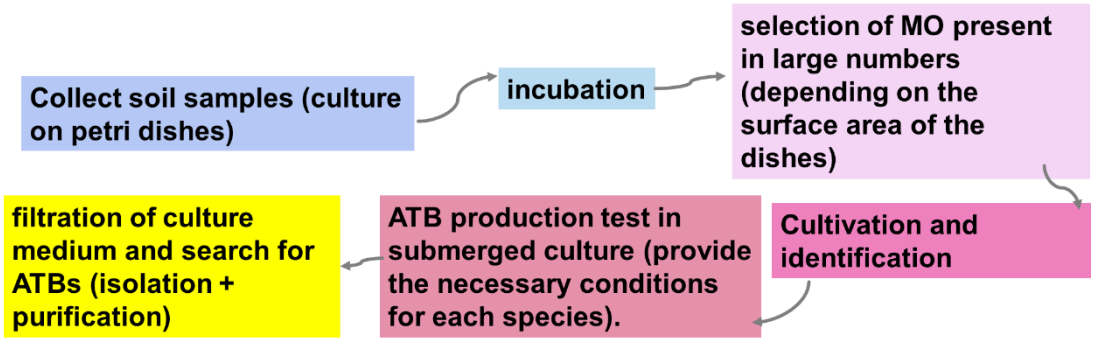


Diagram 1: Methodology for searching and isolating antibiotic-producing species from soil.

2. Vitamins

- Vitamins are active organic substances essential in small quantities for the metabolism of living organisms (growth factors).
- Humans and higher animals cannot synthesize vitamins themselves, so they must obtain them from their food.
- In humans, three vitamins are synthesized by intestinal bacteria: vitamins K, B8, and B12.
- However, the absence or insufficiency of vitamins in the diet can lead to the development of severe deficiency diseases.
    - Inadequate intake or a lack of vitamins can respectively cause hypovitaminosis or avitaminosis, which are the underlying causes of various diseases such as scurvy, beriberi, rickets, etc (figure 16).
    - Excessive intake of fat-soluble vitamins (primarily A and D) can result in hypervitaminosis, which can be highly toxic to the body.

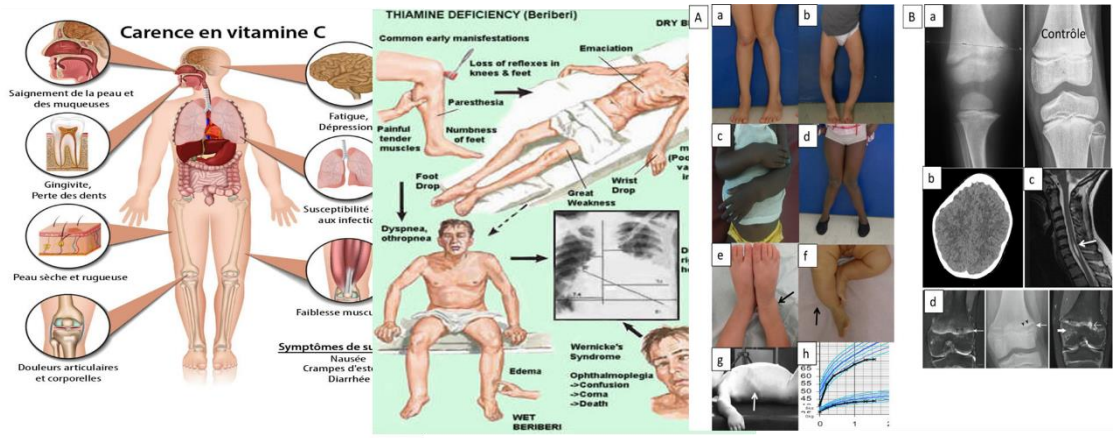


Figure 16 : The effects of insufficiency of vitamins in the diet.

2.1.Classification of vitamins

There are 13 different vitamins, which are classified into two groups based on their solubility: water-soluble vitamins and fat-soluble vitamins.

Hydrosoluble Vitamins

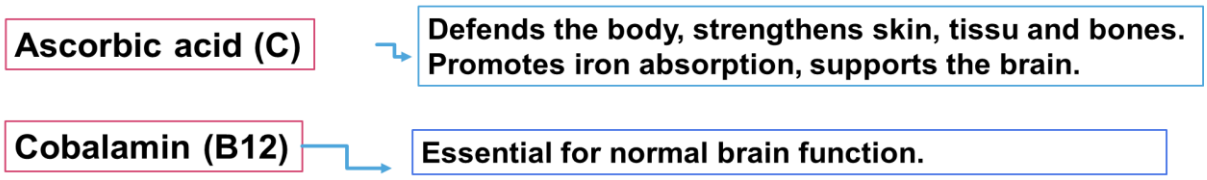
These include **vitamin C and the B-vitamins (B1, B2, B3 or PP, B5, B6, B8, B9, and B12).**

They are **soluble in water** and, consequently, **disperse in the body's fluids without being stored.** This factor makes them very non-toxic because even in case of **overconsumption, they are excreted in urine**.

Their **maximum effect in the body occurs 8 to 14 hours after ingestion**. In general, water-soluble vitamins are primarily obtained **from fruits and vegetables**.

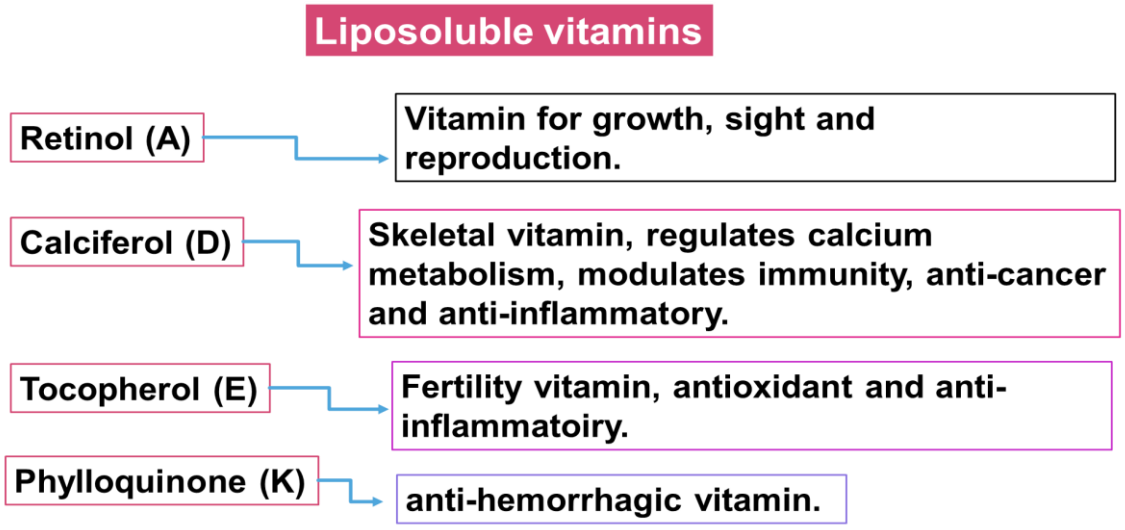
Hydrosoluble Vitamins

Thiamine (B1)	**Anti-berberic, nervous system vitamin.**
Riboflavin (B2)	**vitamin for energy and muscle crumps.**
Niacin (B3 or PP)	**(nicotinamide) anti-dandruff.**
Pantothenic acid (B5) **Biotin (B8)**	**Skin and hair vitamin, energy production.**
Pyridoxine (B6)	**Amino acid and protein metabolism regulator.**
Folic acid (B9)	**anti-anemic.**



Liposoluble vitamins

- These are **vitamins A, D, E, and K**.
- They are **dissolved and stored in fatty tissues,** which can make them **toxic in high doses.**
- This property also means that they can be **consumed less regularly than water-soluble vitamins**.
- In general, liposoluble vitamins are obtained from **dietary fats** (**oils, fatty fish, egg yolks, organ meats, liver, etc.),**
- Except for **vitamin D,** for which the **most significant source remains sunlight.**



2.2. Biosynthesis of vitamins by microorganisms

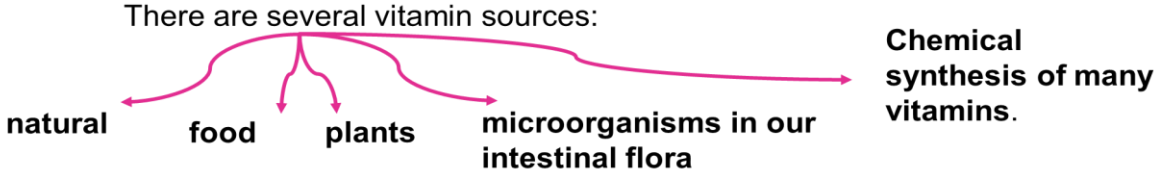
- Prototrophic microorganisms are capable of synthesizing all growth factors, including all the vitamins they need, and some of them release significant amounts of these vitamins into the environment.
- It is possible, through metabolic disruption, to have microorganisms produce most vitamins or provitamins (pyridoxine, biotin, thiamine, folic acid, nicotinamide, riboflavin, cyanocobalamin, precursors of vitamins A, C, D, K, etc.).
- Some of these productions are of great industrial interest, such as vitamin B2 or riboflavin, and especially vitamin B12 (cyanocobalamin), which is only sourced from microorganisms (table 2).

Table 2 : Examples of vitamins produced by micro-organisms.

Vitamins	Producing Microorganisms
Thiamine (vitamine B1)	*Torula utilis, Sacharomyces cerevisae*
Riboflavin (vitamine B2)	*Clostridium acetobutylium ; Pichia miso (yeast)*
Cobalamin (vitamine B12)	*Bacillus megaterium ; Streptomyces olivaceus ; Pseudomonas denitrificans ;*
Retinol (vitamine A)	*(fungus) Blakeslea trispora ; Rhodotorula gracilis (yeast)*
Calciferol (vitamine D)	*Saccharomyces cerevisiae ; Aspergillus niger*
Tocopherol (vitamine E)	*Euglena gracilis*

Source and production

There are several vitamin sources:



- Almost all microorganisms are **capable of synthesizing water-soluble or fat-soluble vitamins**.
- However, their **production is very low**, and these factors do not accumulate in the culture medium. Therefore, the **chemical synthesis industry takes over.**

Furthermore, it can be observed that yeasts such as **Torula utilis, baker's yeast, and brewer's yeast** constitute :

- ❑ an important source of a mixture of **water-soluble vitamins (B1, B2, B3, B5, B8, B9)** known as **"B complex."**
- ❑ This B complex is used by the **pharmaceutical industry** for both **human and veterinary purposes**.

- ❑ In addition to yeasts, other microorganisms also have the ability to produce mixtures of various B-group vitamins, including *Bacillus polymyxa*, *Bacillus megatherium*, and *Aspergillus aerogenes*.
- ❑ For most vitamins, microbial production involves the addition of precursors to the culture medium.

Cobolamin (Vitamin B12)

➢ Vitamin B12, is a **hydrosoluble vitamin essential for :**

Normal brain function (involved in the **synthesis of neuromediators** and the **nervous system).**

Blood formation.

cofactor in cellular metabolism, particularly in **DNA synthesis** and **regulation .**

Fatty acid synthesis and **energy production.**

One of the eight B vitamins sources: meat, shellfish, fish and dairy products.

- Cobalamins have a chemical structure similar to heme, but the central iron atom is replaced by a cobalt atom, which is why they are named as such.
- No animals, and generally no eukaryotes, can synthesize vitamin B12.
- All animals, therefore, rely on other organisms, such as bacteria (vitamin B12 is produced exclusively by bacteria and archaea).
- Microorganisms producing vitamin B12 can also be found in the digestive system of animals. This allows these animals to acquire the necessary vitamin B12 for their growth.
- Subsequently, the food chain transfers this nutrient from herbivores to carnivores, so that all animal-derived products contain it, but not plants.
- In humans, the production of vitamin B12 by intestinal bacteria (intestinal microbiome) is not sufficient to meet daily needs. Vitamin B12 is absorbed in the small intestine (ileum), while bacterial production occurs further down in the large intestine (colon), downstream from the absorption area.
- Moreover, the bacteria in the intestine also use this molecule for their growth.
- In doing so, they modify vitamin B12 to form analogs that are adapted to their needs. The analogs of vitamin B12 produced in this way are not assimilable by humans.
- Therefore, the primary source of vitamin B12 in humans comes from food, whether it be from meat or fermented foods.

Vitamin B12 and human health

- A deficiency in vitamin B12 leads to a form of anemia, one of the characteristics of which is the presence of greatly enlarged red blood cells (macrocytosis).
- People with a vegetarian diet are not the only ones at risk of vitamin B12 deficiency. In addition to dietary deficiency, the deficiency can be caused by poor absorption, bacterial

infection with *Helicobacter pylori*, the use of antacid medications for the stomach, or genetic factors. Pregnant women and the elderly are also more susceptible to vitamin B12 deficiency.

- Therefore, vitamin B12 can be chemically synthesized in a laboratory, but this method is time-consuming and expensive.
- The production of vitamin B12 by bacteria in a fermenter is more cost-effective.
- To achieve this, a Brazilian research team uses an industrial waste product to support bacterial growth.

1- A carbon source (Glucose, beet or citrus molasses).

2- A source of nitrogen (fish flour, corn steep (soluble, concentrated protein fraction of corn grain) , casein hydrolysate (rich in amino acids and mineral salts).

3- A buffer (CaCO3).

Culture medium for Vit B12 production

4-A source of cobalt (CoCl2).

5-A precursor: 5',6-dimethylbenzimidazol from riboflavin.

- For the industrial production of vitamin B12, strains of the bacterial genera ***Propionibacterium*** and ***Pseudomonas*** are used.
- There is a **cobalt atom at the center of vitamin B12**, and yields of vitamin B12 are **enhanced by adding a small amount of cobalt** to the culture medium.

The process is carried out by **adding cobalt in two phases**.

✓ **Anaerobic Phase:** This is a **preliminary** phase that can last from **2 to 4 days.**
✓ In the **anaerobic phase**, **5'-deoxyadenosylcobinamide** is **primarily produced**.

✓ **Aerobic Phase**: In this phase, **5,6-dimethylbenzimidazole** is produced from riboflavin, which incorporates to **ultimately form the coenzyme of vitamin B12, namely 5'-deoxyadenosylcobalamin.**

❑ The **fermentation** typically takes place at **27°C for 3 days** with **agitation and aeration.**
❑ Subsequently, the **mycelium is separated** by **centrifugation at 12,000 rpm for 10 minutes at 4°C** and then **resuspended**.
❑ The vitamin is extracted either by **thermal shock (80-120°C) or by acidification (sodium sulfite).**
❑ Under these conditions, **vitamin B12 is released**.
❑ A **concentrate of this vitamin** is prepared after **filtration and drying (75 mg of B12 is obtained per kilogram of medium used).**

Riboflavin (vitamin B2)

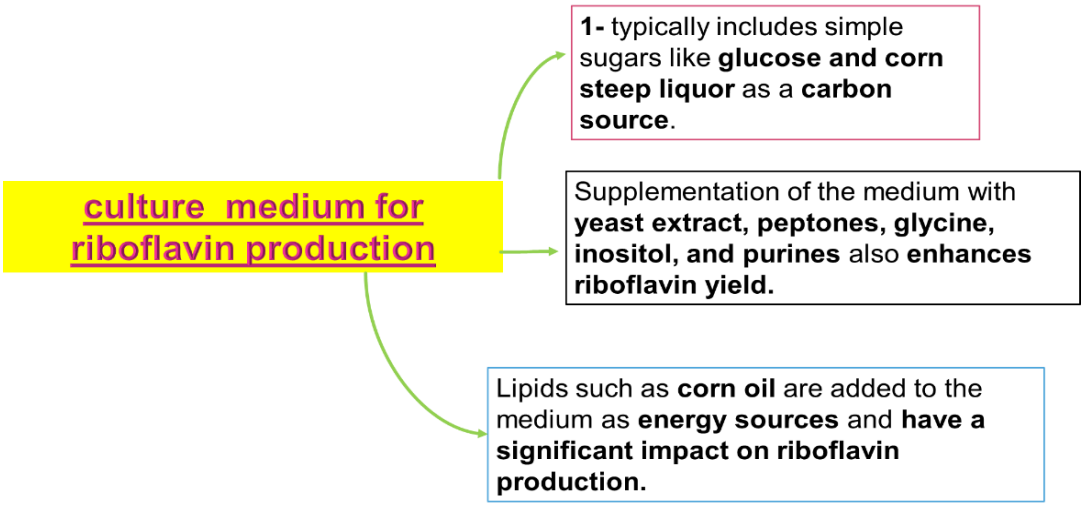
Riboflavin is a water-soluble vitamin that is essential for growth and reproduction in humans and animals.

- A deficiency of riboflavin in rats causes growth retardation, dermatitis, and eye lesions.
- In humans, a deficiency of vitamin B2 results in cheilosis (cracks at the corners of the mouth), glossitis (a purplish tongue), dermatitis, and hair loss.
- Riboflavin carries out its biochemical functions through coenzymes, namely, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).

The production of riboflavin (vitamin B2)

Plays a fundamental role in ATP formation.

- It is found in microorganisms bound to certain nucleotides to form FMN or FAD.
- Many microorganisms are capable of synthesizing this vitamin in very large quantities exceeding their needs, which accumulate in the culture medium.
- In fact, microorganisms overproduce riboflavin in cases of abnormal formation of toxic purines in the environment to eliminate this compound.
- Riboflavin is synthesized by numerous bacteria, yeasts, and molds. The fungus *Ashbya gossypii* naturally produces very large quantities of it (up to 7g/L) and is used in most production processes.
- Iron, which inhibits the production of vitamin B2 in Clostridium and yeasts, has no effect on *A. gossypii*.



a) Phased fermentation: It is now recognized that fermentation occurs in three phases.

Phase I:
This phase is characterized by **rapid organism growth** using **glucose**. **Pyruvic acid accumulates**, leading to an **acidic pH**. Growth of the organism **stops** when **glucose is depleted**. In Phase I, **there is no riboflavin production**.

Phase II:
The **concentration of pyruvate decreases**. Simultaneously, there is **an accumulation of ammonia** (due to enhanced deaminase activity) that **makes the medium alkaline**. This phase is **characterized by maximum riboflavin production**, mainly in the form of **FAD, with a small portion in the form of FMN.**

Phase III:
In this final phase, **cells undergo autolysis**, leading to the **release** of **FAD, FMN, and free riboflavin into the medium.**

b) Recovery

- Riboflavin is found in a fermentation broth and in a form bound to cells.
- It can be released through heat treatment (120°C) for one hour.
- The cells can be discarded after filtration or centrifugation.
- The filtrate can be further purified and dried as needed.

3. Amino acids

Amino acids

- Amino acids are the **primary building blocks of proteins**.
- They are **vital molecules**, and their **contribution to the functioning of the organism is crucial.**
- **Humans are unable to synthesize amino acids** and **must therefore obtain them from their diet**.
- However, **food products of plant origin** contain only very **low levels of essential amino acids such as lysine, methionine, or tryptophan**.
- In contrast, **amino acids are synthesized by animals, microorganisms, and plants.**

AA role

- **Structural: protein monomers, involved in protein synthesis,**
- **Energetic: energy substrate: their oxidation contributes to 20% of the energy produced by the body,**
- **Metabolic: precursors of molecules of biological interest or metabolic intermediates (hormone precursors, purine and pyrimidine bases: aspartate, glycine and glutamate).**
- **They are used as food additives, participating for example in:**
  - **Prevention of cardiovascular disease and hypertension.**
  - **Combatting stress.**

Amino acids applications:

They are **used in food products** to enhance flavor. For example, **glycine and alanine** are **added to certain food products to improve their taste.**
Glutamic acid is used, in the form of its **salt** (**monosodium glutamate or MSG**), as a **flavor enhancer**, often referred to as **"umami"** or the **"meatiness" of a dish**.

Used as **sweeteners due to their "sweet taste"** in human nutrition. exp, **aspartame = aspartate and phenylalanine**.

Lysine and methionine are added to **plant-based products** to increase their **nutritional value, especially in terms of essential amino acids.**

Glutamate is also used by **pharmaceutical and cosmetic industries** for various applications.

Amino acid production by microorganisms:

Many microorganisms have the ability to **produce the amino acids** they **need for protein synthesis and growth** from **carbohydrates and mineral salts** (Table 2).

Table 3 : Examples of amino acids produced by microorganisms.

Amino acids	Producing Microorganisms
Aspartic acid	*Bacillus megaterium ; Pseudomonas*
Alanine	*Pseudomonas ; Corynebacterium ; Brevibacterium*
Arginine	*Corynebacterium glutamicum ; Brevibacterium flavum*
Glutamic acid	*Corynebacterium glutamicum ; Brevibacterium ; Micrococcus*
Lysine	*Brevibacterium lactofermentum ; Saccharomyces Candida*
Methionine	*Corynebacterium glutamicum ; Rhodotorula ; Candida*
Phenylalanine	*Brevibacterium lactofermentum ; Flavobacterium spp ; Micrococcus*

There are several methods of amino acid production:

➢ They can be obtained through chemical synthesis.

➢ Through enzymatic catalysis.

➢ Through extraction from a raw material rich in the desired amino acid.

➢ Through microbial culture (which is the focus of our course).

Metabolic pathways for amino acid synthesis:

- For the synthesis of an amino acid by a microorganism, the presence of intermediates is required to form the carbon chain and others for the amine function (NH2).
- The synthesis of the carbon chain of amino acids is carried out from intermediate products of carbohydrate metabolism (glycolysis, pentose phosphate pathway, and Krebs cycle).
- These intermediates include phosphoglycerate, phosphoenolpyruvate, pyruvate, acetyl-CoA, oxaloacetate, and alpha-ketoglutarate (Figure).
- The amine group comes from another amino acid through a transamination reaction (ALAT, ASAT), or it can come from an inorganic molecule (NH4+, N2..) through the amination process (oxidative deamination by glutamate dehydrogenase).
- In fact, glutamate serves as the donor of the amine group to many other amino acids through transamination reactions (figure 17).

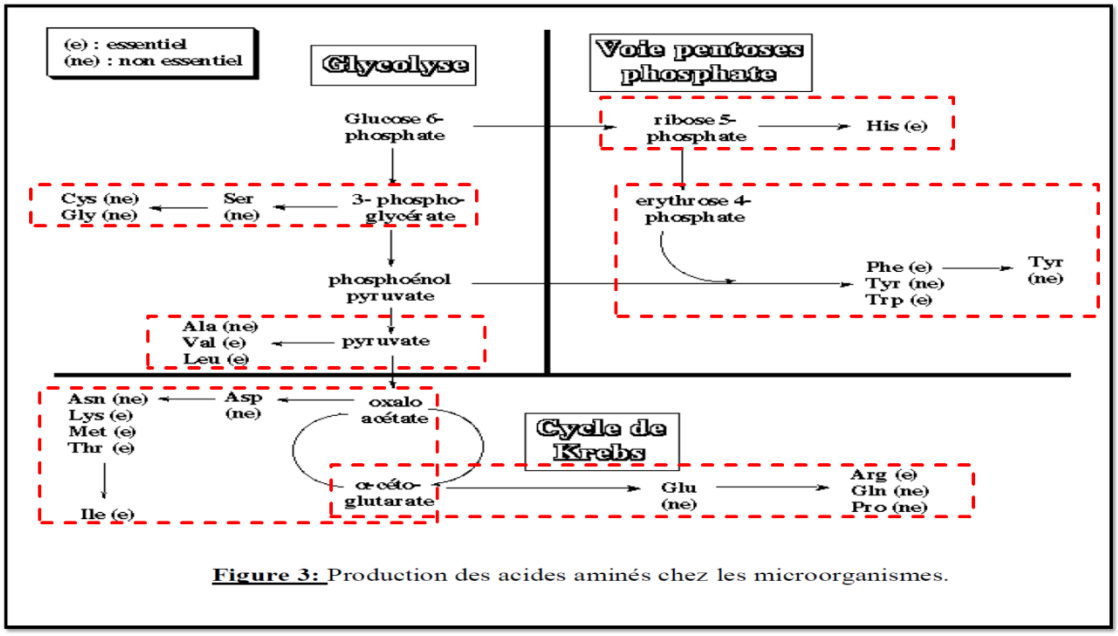


Figure 3: Production des acides aminés chez les microorganismes.

Figure 17 : Amino acids productions by microorganisms.

- Industrial production of amino acids through microbiological processes is expanding and currently holds significant social and economic importance. These methods have become competitive with classical chemical synthesis techniques.
- Furthermore, the microbiological approach allows for the direct production of the L forms of amino acids, which are usable by mammals, unlike chemical methods that can only provide racemic compounds.

As an example, let's consider the industrial production of glutamic acid:

- Glutamic acid (glutamate) is not an essential amino acid for humans or animals but is industrially produced in the form of monosodium glutamate (MSG, E621).
- Nonetheless, it is widely used in the food industry as a flavor enhancer or taste modifier.
- This molecule is primarily used as a food additive to enhance the taste of various food products, as it is responsible for the umami taste.
- Glutamate is also one of the most active neurotransmitters in the brain.
- It can be extracted from natural sources such as beet molasses, soy flour, or gluten, but in insufficient quantities to meet demand.

The main microbial strains used

- Glutamic acid production belongs to the Brevibacterium, Corynebacterium, and Micrococcus genera. These are Gram+, non-motile, strict aerobic bacteria, auxotrophic for biotin.
- The industrial strains used for glutamic acid production are mutants of *Corynebacterium glutamicum* and *Brevibacterium flavum* species.

To overcome challenges encountered during industrial glutamic acid production, several steps are followed:

· Genetic modification of industrial strains, primarily aimed at blocking metabolic pathways that lead to the undesirable byproducts.

· Inhibition of the regulatory mechanism for glutamic acid production through retro-inhibition: In wild strains of Corynebacterium glutamicum, when the final product accumulates to a desirable final concentration, cytoplasmic glutamic acid production is inhibited by the retro-inhibition mechanism.

- Reducing the activity of the enzymatic complex α-ketoglutarate dehydrogenase, also known as the oxoglutarate dehydrogenase complex (OGDC), which are enzymes in the Krebs cycle, catalyzes the conversion of α-ketoglutarate to succinyl-CoA (figure 18).

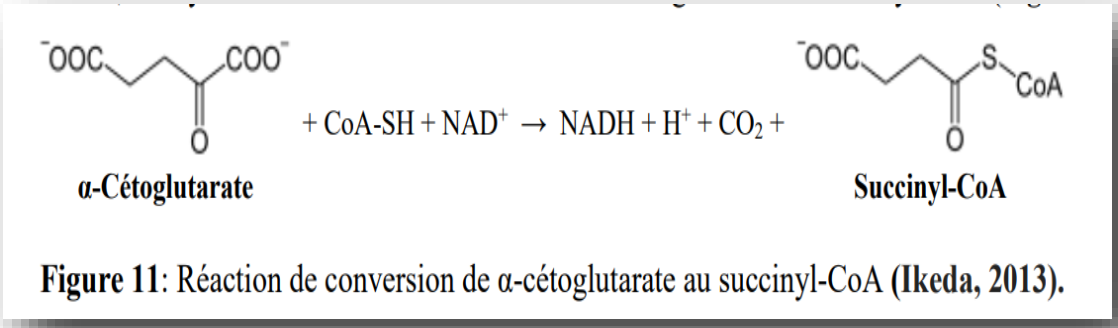


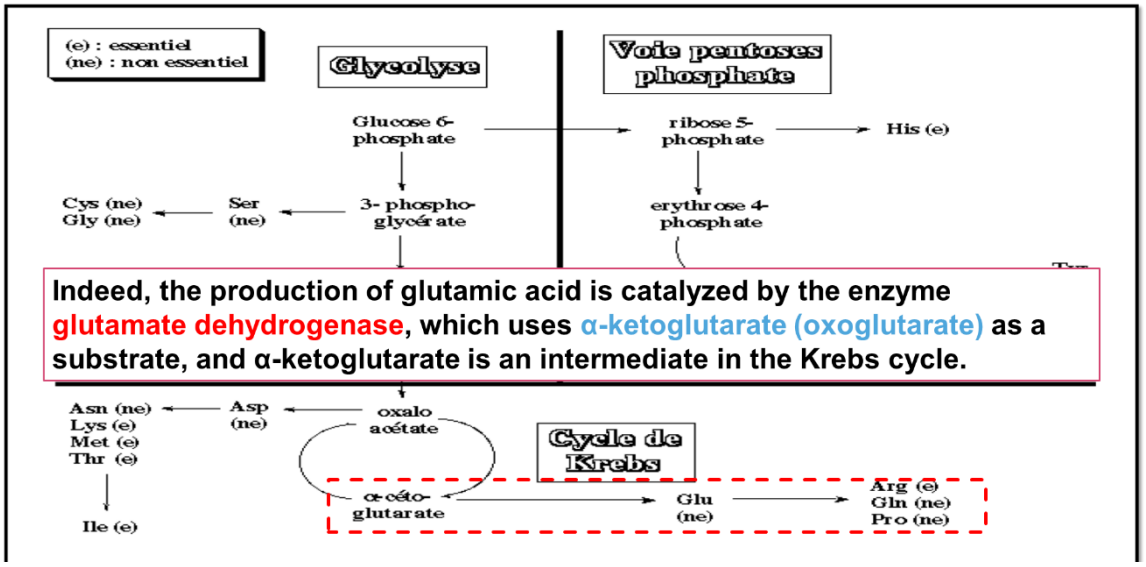
Figure 11: Réaction de conversion de α-cétoglutarate au succinyl-CoA (Ikeda, 2013).

Figure18: Conversion reaction of α-ketoglutarate to succinyl-CoA.

Industrial production of glutamic acid: can be carried out using two main processes:

- **Single-stage process:**

- In this process, the amino acid is directly produced by a single microorganism.
- The synthesis of the amino acid's carbon chain occurs from intermediate products in carbohydrate metabolism (glycolysis, pentose phosphate pathway, and the Krebs cycle). Glucose is oxidized to form citrate, which is then further oxidized to α-ketoglutaric acid. Glutamic acid is subsequently produced from α-ketoglutarate through the action of glutamate dehydrogenase (figure 19).
- Strains used in this process are *Corynebacterium glutamicum* and *Brevibacterium flavum*.



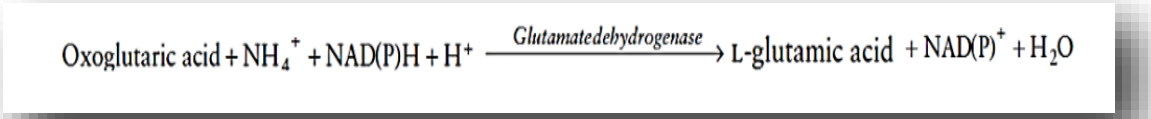


Figure 19 : Production and reaction of the formation of L-glutamate (Ertan, 1992).

The two-stage process :

- Involves first preparing α-ketoglutaric acid through microbiological means, which is then converted to glutamic acid either using a second microorganism or enzymatically.
- This process is of significant industrial importance because the intermediate product, α-ketoglutaric acid, can be readily obtained through well-established microbiological techniques. (figure 19).
- It can be produced by various genera of microorganisms using glucose, a source of mineral or organic nitrogen, and mineral salts.

- To direct the **metabolism of mutant strains** towards **overproduction of glutamic acid**, the activity of the enzyme **α-ketoglutarate dehydrogenase (OGDC:2) decreases under biotin-limiting conditions**. This allows the **accumulation of α-ketoglutarate** and consequently **improves the yield of glutamic acid production**.
- It is important to note that the optimal **biotin concentration for glutamate production** falls **between 2 and 5 µg/L**.

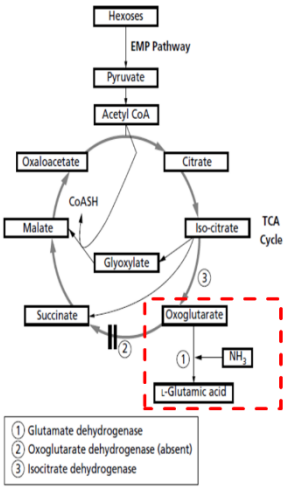


Figure 13 : Biosynthèse de l'acide l-glutamique chez les souches mutantes (Sonenshein, 2021).

Figure 19 : L-glutamic acid biosynthesis in mutant strains (Sonenshein, 2021).

1- A carbon source (glucose, fructose, sucrose, lactate, pyruvate, ethanol, serine).

2-A source of nitrogen (ammonium sulfate, ammonia).

Culture medium for Glutamate production

3- Growth factor: Biotin.

Fermentation conditions:
Corynebacterium glutamicum is mainly used for the industrial **production of L-glutamate and L-lysine. pH= 7-8, 30°C.**

Recovery
- Product recovery is achieved by **separating the cells from the culture medium.**
- L-glutamate is **then crystallized by lowering the pH to 3.2 with hydrochloric acid**.
- The L-glu crystals are **filtered and washed**.

4. Enzymes as industrial products

Enzymes

Enzymes are **complex proteinaceous biological catalysts** that have the ability to **accelerate chemical reactions in living cells** without being **permanently transformed themselves.**

- **Each chemical reaction** in a cell has its **own catalyst**. Therefore, there are a large number of enzymes in a single living cell.

❖ In the case of ***E. coli***, there are **4,485 genes**, of which **33.5% code for distinct enzymes**.

- Out of the hundreds of enzymes used industrially:

❖ In the absence of enzymes, the vast majority of reactions are not possible, even if one waits for years, and life as we know it would not exist.

4.2. Enzymes properties

The key properties of enzymes compared to other types of catalysts include:

1. Very high catalytic power ; Specificity; Regulation of catalytic activity by natural substances.

4.3. Origin of Industrial Enzymes

- Industrial enzymes can come from plant, animal, or microbial sources.
- Extraction from plants and animals, however, is limited by challenging-to-control parameters.
- The main advantages of enzymes produced industrially over extracted enzymes are:

✓ Independent production from seasonal and geographical constraints,

✓ The possibility of using inexpensive raw materials,

✓ Increased production yields through the improvement of microbial strains and the optimization of production conditions.

4.3. Protease Sources

- Proteases can be extracted from plants, animals, or microorganisms, and they are purified from various biological raw materials.
- However, only microbial enzymes produced through fermentation have experienced significant expansion and industrial preparation. This is because:
- microorganisms offer several advantages as a source of enzymes, such as exponential growth and availability.
- The choice of the appropriate strain is crucial for the production of an industrial enzyme, especially since most cases involve significant developments in applications for food purposes.

4.4. Enzymes of microbial origin

Microbial enzymes originate from various microorganisms, including approximately 50 bacteria and fungi (GRAS*).

*GRAS: are generally recognized as safe, meaning they pose no health hazards when used.

- Bacteria are primarily represented by *Bacillus subtilis*, *Bacillus licheniformis*, and various *Streptomyces* species.
- Fungi are typically represented by *Aspergillus*, *Mucor*, *Rhizopus*, *Kluyveromyces*, and *Saccharomyces* (table 4).
- In non-food sectors such as chemistry, diagnostics, various analyses, the selection of strains is not subject to the same constraints.
- In general, microorganisms are chosen based on the following main criteria:

Ø Providing good enzyme production.

Ø Achieving this in a minimum amount of time.

Ø Preferably producing extracellular enzymes (usually hydrolases) over intracellular ones (which are difficult to extract).

Ø The strain should be capable of growing on cost-effective substrates.

Table 4: microorganisms producing enzymes.

Enzymes	Micro-organismes producteurs
Invertase	*Saccharomyces cerevisae,Kluyveromyces lactis ; Aspergillus usamii*
Lipase	*Mucor javanicus; Lucor mihei Rhizopus arrhizus; Aspergillus effusus*
Pectinestérase, Pectine lyase, Polygalacturonase	*Aspergillus usamii; Aspergillus wentii Aspergillus niger; Penicillium funiculosum*
Alpha amylase	*Bacillus licheniformis; Bacillus subtilis Aspergillus niger; Aspergillus oryzae*
Glucose isomérase	*Streptomyces albus Actinoplanes missouriensis Bacillus coagulans*
Lactase	*Kluyveromyces fragilis Kluyveromyces lactis*

4.5. Production medium

Can be either synthetic or complex and contain raw materials that provide essential nutrients such as energy, carbon,nitrogen,phosphorus, sulfure, vitamins, etc.

Sources of carbon and energy.	**Cereal flour, soybean flour, cornstarch, potato starch, by-products like whey and molasses.**
Sources of organic nitrogen.	**Fish flour, gelatin, casein, soybean flour, corn, and peanuts.**
Mineral salts and growth-promoting substances	**Yeast extract, vegetable oils, and flour from oilseeds.**

4.6. Fermentation processes

Fermenters with volumes ranging from 100 to 200 m3. Depending on the enzymes and processes involved, fermentation can last from 30 to 150 hours.

4.6.1. During fermentation

- A rich medium is used, and various physico-chemical parameters are continuously regulated, including oxygen levels, pH, temperature, and foam formation (which is reduced by adding anti-foaming agents).
- Enzyme activity is also measured at regular intervals.

4.6.2. Induction

Ø Inducers must be present in the production media. For example:

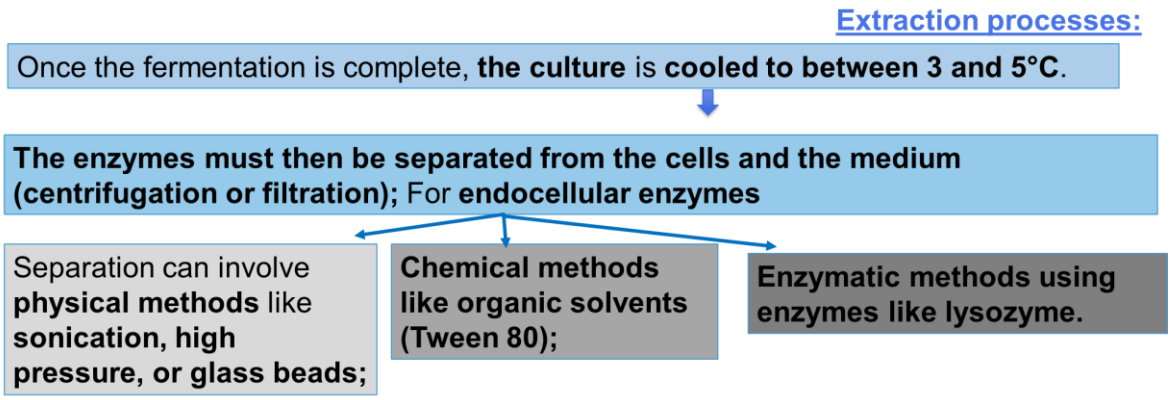
- Starch serves as an inducer for amylase production,
- Urea induces urease production,
- Xylose is used as an inducer for xylose isomerase.

Ø Some molecules can act as inducers at low concentrations and repressors at high doses, such as cellobiose for cellulases.

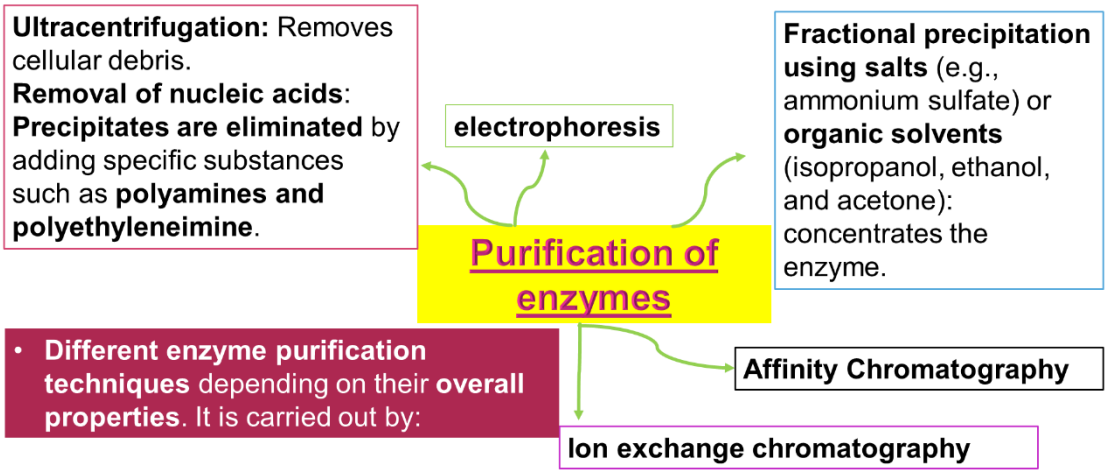
Ø Inductive effects are often demonstrated using substrate analogs. For example, isopropyl-beta-D-thiogalactoside serves as an analog of lactose for beta-galactosidase induction.

Ø Coenzymes can also have an inductive effect to optimize production. For instance, thiamine increases the production of pyruvate carboxylase.

Note: It's important to note that enzymatic activity (the rate of production) is regularly measured during production to ensure that the conditions are being maintained effectively.



4.7. Purification of enzymes



Separation by chromatography techniques including : ion exchange, size exclusion, affinity and hydrophobic interaction chromatography. Among these methods, **ion exchange chromatography is commonly used for enzyme purification**.

4.8. Conservation

- The concentrated form of the enzyme can be obtained through drying. This can be done using evaporators or freeze dryers. The dried enzyme can then be packaged and commercialized.
- Finally, enzymes are processed to achieve a commercial preparation that meets the desired criteria for purity and stability.

The benefits of enzymes: Enzymes produced industrially are used in various fields, as examples:

In the agri-food industry:
- **Amylases** are used in the production of **sugars, syrups, and bread (they enhance yeast activity)**.
- **Cellulases and pectinases** are widely used for **clarifying fruit juices (removing particles and making the juices clearer).**
- **Papain tenderizes meat before cooking.**
- Several enzymes are used in **cheese production and maturation (e.g., chymosin, pepsin**).

In laundry:
- Enzymes are experiencing a renewed interest in the **household cleaning products industry** because they **break down organic matter and stubborn dirt** (dishes, floors, walls, ovens, appliances, clothing, etc.).
- The main enzymes used in laundry are **lipases, pectinases, amylases, and cellulases.**

In therapy:
- Enzymes are used for **diagnosing certain diseases**, often in **immobilized form**. Examples: **aldolase is used to diagnose muscular disorders.**
- **Alkaline phosphatase** is used to **analyze body fatty acids.**

In Molecular Biology :
- **DNA ligases, DNA polymerases, restriction enzymes**, and all others that act on **DNA or RNA.**
- For PCR, these enzymes help **identify microorganisms (diagnosis of infectious diseases**) and **search for specific substrates in a sample.**

Chapter III : Key industrial food and beverage products

Objectives

At the end of this chapter, the student will be able to :

- Know the production stages of vinegar, citric acid and other organic compounds.
- Know the nutritional composition of yeast.
- Define edible fungi and their production stages.
- Unveil the synthesis of fermented foods: fermented meat, coffee, pickles, sauerkraut....etc.

1. Vinegar production

History

- Vinegar has been known to most ancient civilizations. It is used as a condiment, as a preservative, or diluted in water as a beverage. The Babylonians produced it 5,000 years before Christ from palm wine.
- In fact, when wine is exposed to air for a certain period, it naturally transforms into a liquid with an acidic taste, giving birth to vinegar or “vin-aigre."
- About 2,000 years ago, vinegar was the centerpiece of the world's most expensive meal, during a bet between Mark Antony and Cleopatra. Cleopatra won the bet by dissolving a massive pearl in her vinegar before drinking it.
- In 1822, the botanist Persoon attributed vinegar production to the "vinegar mother," which forms on the surface of wine left exposed to air. Believing it to be a fungus, he named it *Mycoderma Acéti.*
- In 1864, Louis Pasteur identified the responsible bacterium, of the *Acetobacter* genus, and defined acetic fermentation with the chemical equation:

- **CH_3-CH_2OH + O_2 → CH_3-COOH + H_2O + 348 kJ**
- **Louis Pasteur** scientifically identified the three essential criteria for vinegar production:

1-Presence of Alcohol: It comes from wine, cider, or other alcoholic beverages.

2-Presence of Oxygen: The oxygen in the air is perfectly suitable.

3-Presence of a Ferment: *Mycoderma acéti,* which is actually a bacterium later renamed *Acetobacter aceti.*

- While vinegar is commonly used for seasoning various salads, it has other functions, especially in the medical and even aesthetic fields.

1.1.Definition of vinegar and regulations

The term "vinegar" etymologically derives from "vin" (wine) and "aigre" (sour). It refers to wine that has become sour due to the development of acetic bacteria.

- ❖ By extension, any product obtained through the acetic fermentation of beverages (such as wine) or alcoholic dilutions has been called vinegar. Vinegar is used as a condiment or preservative because of its acidity (pH = 3).
- ✓ According to the FAO (1987), vinegar is a liquid suitable for human consumption, produced from suitable agricultural materials.
- ✓ The production of vinegar is attributed to acetic bacteria known as “Acetobacter”.
- ✓ Similarly, the Codex Alimentarius and Algerian legislation require a minimum content of acetic acid of 6% for wine vinegar and 5% for other vinegars (JORA, 1998).
- ✓

1.2.What is fermentation?

It is a very ancient technology for processing and preserving food. During certain fermentations, there is indeed a release of gas, often CO2, with the formation of foam, similar to what happens when a liquid boils. There are several types of fermentation.

1.3.Chemical principle of vinegar production

1.3.1. Traditional vinegar production technique through double spontaneous fermentation (Orleans Method)

- The Orleans method is the most well-known traditional technique.
- The transformation of wine into vinegar occurs through natural surface fermentation.
- Vinegar results from two successive chemical reactions: alcoholic fermentation and acetic fermentation.
- Vinegar is obtained through contact of the wine's surface with the air. This is due to the presence of acetic acid-producing bacteria (*Acetobacter*) in the air.

Alcoholic Fermentation

- Occurs in anaerobic conditions.
- It is carried out by yeast (of the genus *Saccharomyces*) at 30°C for a few days, converting glucose into ethanol and carbon dioxide.
- The production of alcohol plays a significant organoleptic role in the quality of the product.
- The cessation of gas bubbles (CO2) is often considered an indicator of the end of alcoholic fermentation.

Acetic Fermentation

- Under the influence of Acetobacter bacteria, it transforms (oxidizes) ethanol into acetic acid in the presence of oxygen.
- The optimal temperature for aeration is between 30 and 32°C; beyond 33°C, there is overoxidation of acetic acid into carbon dioxide and water.
- It is acetic acid that gives vinegar its characteristic acidity and makes it an effective preservative.
- The alcohol content is typically between 7° and 12°, as beyond 12°, ethanol is transformed into carbon dioxide and water.

Yeast

$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2CO_2$

$CH_3CH_2OH + O_2 \rightarrow CH_3COOH + H_2O$

(acetic acid)

Vinegar can be considered as such when all the alcohol has been transformed into acetic acid (figure 20).

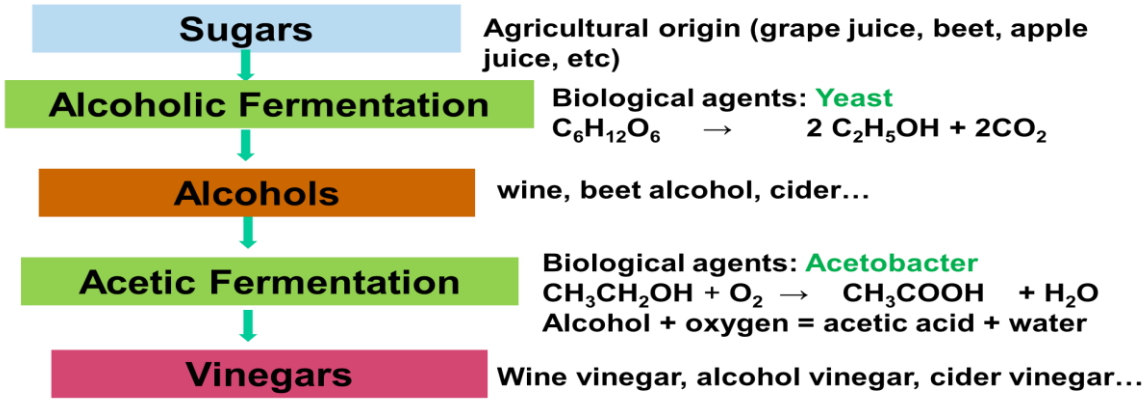


Figure 20 : Vinegar chemistry steps.

Essential conditions for acetic fermentation :

1 – Presence of oxygen : Acetobacter is a major consumer of O2

2 – Temperature regulation between 28 and 32° C.

3 – Sufficient nutrients in the medium.

Acetification processes

- The first process developed by humans involved the spontaneous conversion of wine into vinegar due to the action of bacteria found on fruits, in containers, or in the air, by letting the wine come into contact with the air.
- The organisms used for industrial vinegar production are *Acetobacter aceti and Gluconobacter oxydans*.
- From a technological perspective, there are two vinegar production processes (acetification): the trickle system and the submerged system.
-

1.3.2. The "slow" traditional processes: These are static methods in which acetic acid bacteria are placed in the liquid-air interface, in direct contact with oxygen from the air. There are two notable methods:

1. The Orléans Method or Pasteur Method

- Wine is oxidized in barrels exposed to the air. A film of acetic acid bacteria forms.
- Vinegar is drawn off, and wine is added through the bottom of the container.
- Large open containers with a capacity of 200 to 400 liters are used, filled halfway. Every eight days, 10 to 15 liters of wine are added, while an equal amount of vinegar is removed.
- The most favorable temperature ranges from 25 to 30°C.
- This method produces vinegar with a pleasant bouquet and excellent quality but is slow, has a relatively low yield (the operation is discontinuous), and can only produce small quantities.
- As a result, it is mainly used in small vinegar factories and households.

2. The Trickle System (German Method: Schuzenbach)

- One of the industrial systems for vinegar production is the Frings trickle generator.
- The generator, which is constructed from cypress or mahogany wood, is filled with beechwood shavings, which are a porous wood on which acetic acid bacteria develop in greater quantities.
- The collection chamber at the base has a capacity of approximately 13,250 liters.
- A pump circulates the acetic acid-water-ethanol mixture from the collection chamber and sprays it onto the surface of the central chamber.
- The temperature is controlled by the liquid flow, ranging from 29°C at the surface to 35°C at the bottom.
- The Frings system is aerated, producing bubbles at the bottom of the tank, and the aeration rate and temperature are meticulously controlled to prevent any evaporation of acetic acid.

- Ethanol must be continuously supplied to maintain the bacterial population.
- The maximum acetic acid concentration in the solution is between 13% and 14%.
- The lifespan of such a generator is approximately 20 years. This generator is an example of continuous fermentation.
- The Frings model is the most commonly used in the vinegar industry.
- An estimate is that a single bacterium could acetify 10,000 times its own weight of alcohol or wine in 48 hours, provided it had plenty of oxygen or a hydrogen acceptor.
- Rationalized cultivation of this bacterium has accelerated the natural fermentation process: what once took at least three weeks has been optimized by the industry.
- Nowadays, a vinegar maker can produce enormous quantities of vinegar in less than 24 hours.

Modern 'fast' processes:

3. Batch Fermentation (Industrial Method) or Submerged Culture

- This method is decidedly the most modern and takes less than 2 days, thanks to the injection of a large number of microbubbles of air into very large tanks, capable of holding thousands of liters, at a temperature of 30°C (figure 21, 22).
- This process is carried out in fermenters equipped with a forced aeration system called an “acetator”. It involves a submerged batch fermentation process.
- The Frings fermentation consists of a stainless steel tank with an agitator at its base, which constantly mixes microorganisms, air, ethanol, and nutrients to provide a favorable environment to bacterial growth.
- Small air bubbles are introduced at the base of the tank to provide aeration. Any foam that may form on the surface due to aeration is eliminated by a foam destroyer located at the top of the device.
- The temperature is maintained at 30°C by circulating cold water through a cooling tube. Ethanol, bacterial inoculum, and selected nutrients for bacterial growth are added to the tank all at once.

- An electronic system allows monitoring of the ethanol concentration.
- When the alcohol level drops below 0.2% (v/v), roughly one-third of the finished product is removed from the tank, and nutrients and ethanol are added.
- It takes about 35 hours to produce vinegar at 12%.
- The submerged Frings fermenter (acetator) is more efficient than the continuous trickle fermenter.

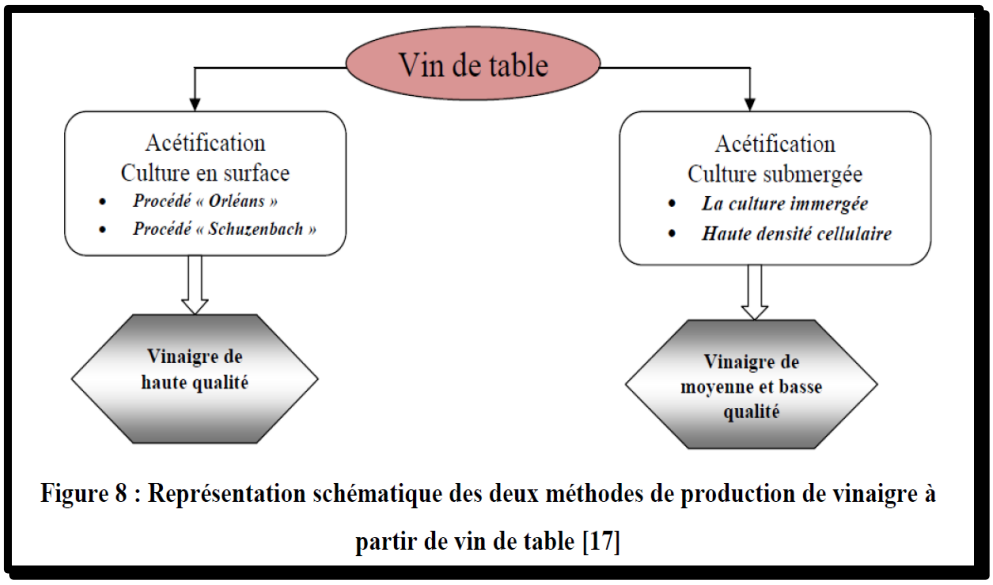


Figure 8 : Représentation schématique des deux méthodes de production de vinaigre à partir de vin de table [17]

Figure 21: Schematic representation of the two methods for producing vinegar from table wine.

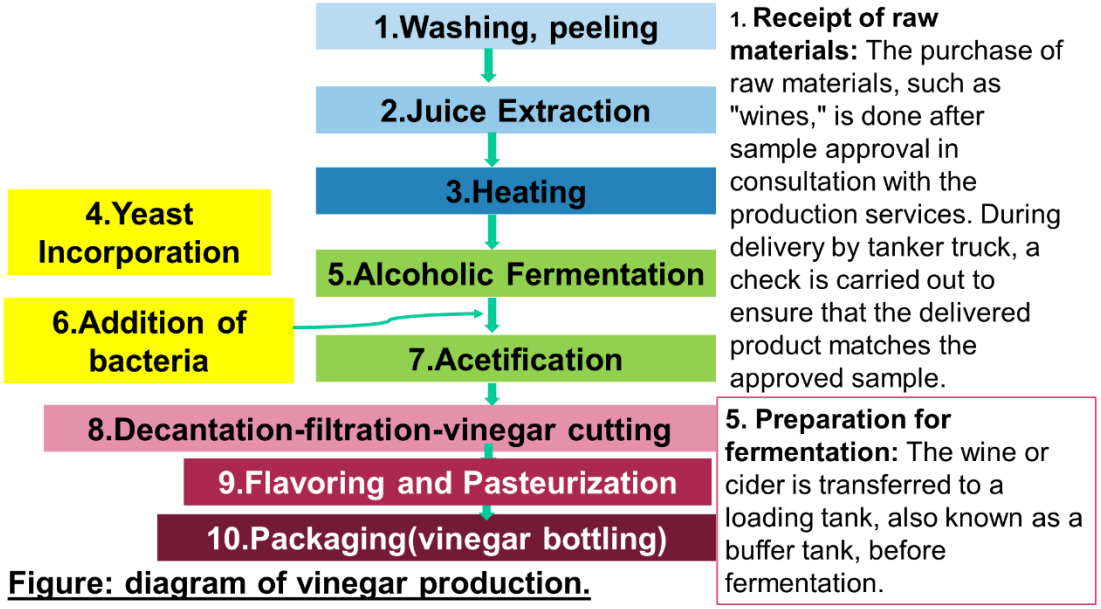


Figure: diagram of vinegar production.

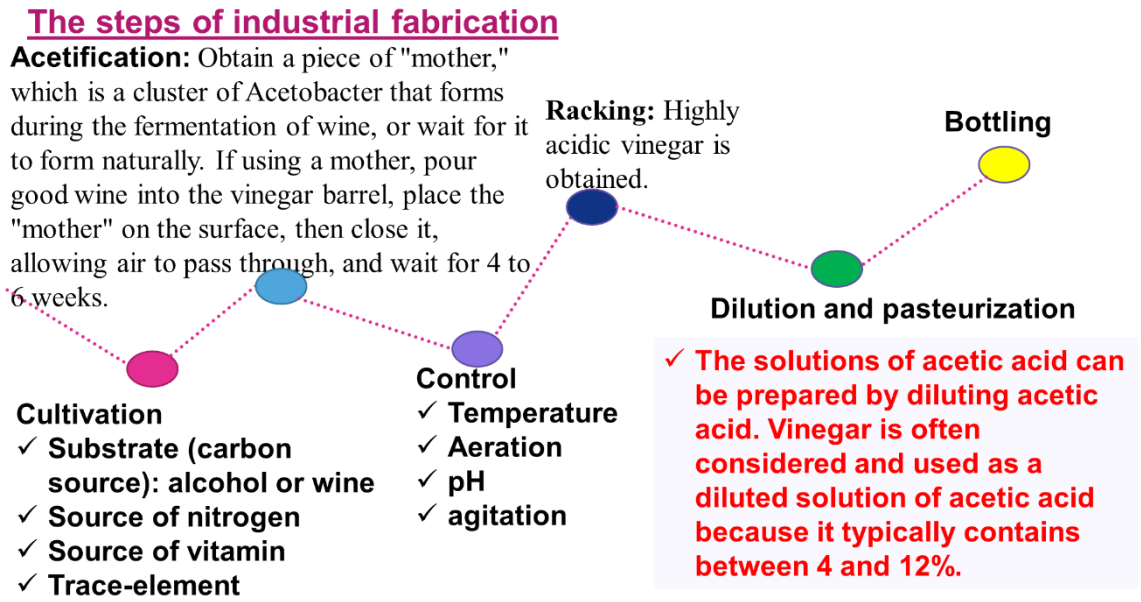
8. Removal of high acetic acid vinegar
Apart from losses due to alcohol and acid vapors, the reaction is said to be "one-to-one": **one degree of alcohol yields one degree of acetic acid**. Therefore, a **10° alcoholic wine** will produce a **vinegar with 10° acetic acid** (high acetic acid vinegar).

8. Filtration:
is a general technique for **separating two phases**: a **solid phase (the impurities causing cloudiness)** and a **liquid phase (the vinegar),** by passing them through a **porous barrier** that acts as **a filter, retaining the solid phase**. As the liquid passes through **the filters, it becomes clearer**.

10.Packaging: There are 2 glass-bottle packaging lines, with virtually identical equipment: **a turner/blow-moulder, a volumetric filler, a corker** (plastic, cork or screw caps), a marking system, and cardboard or bundling.

Figure 21 : Diagram of vinegar production.

1.4.The steps of industrial fabrication



1.5.Vinegar production

Vinegar Composition :

The main constituent is **acetic acid.**

- **Tartric acid**
- **Succinic acid**
- **Nitrogenous materials**
- **Nutrients** added to the reaction medium
- **Dilution water**.

However, other compounds are formed during **acetic fermentation (fermentation products)** or result from the interaction of components with each other, such as **ethyl acetate, which contributes to the flavor of vinegar.**

Vinegar Composition (continued):

The **differentiation criteria** between **types of vinegars** are the levels of **non-sugar extract, sorbitol, acetoin, lactic acid, tartaric acid, or lactose.**

- **Wine vinegar contains Tartric acid.**
- **Apple vinegar contains L-malic acid.**
- **Whey vinegar contains D- and L-lactic acid.**
- **Lemon vinegar contains citric acid.**

1.6.The main types of vinegar

The Main Types of Vinegar

Many types of vinegar are produced and are classified **based on the origin of the ethanol** that bacteria transform into acetic acid. The **differences between these vinegar varieties** are primarily related to the **starting raw material**. The main types of vinegar are as follows:

- **Alcohol vinegar (white vinegar):** It is produced in **larger quantities,** using **pure alcohol as the raw material,** which is diluted and previously denatured by alcohol vinegar. This vinegar is commonly **used in the condiment industry.**
- **Wine vinegar:** Made from **low-quality wine**, subject to oxidation by air. Both **white and red wines can be converted into vinegar, depending on the type of wine used.**
- **Cider vinegar:** Made from **fermented apple juice.** This vinegar is **rich in pectic substances.**
- **Glucose vinegar:** Obtained by **acetic acid fermentation of alcoholic liquid** from the fermentation of a **commercial glucose** solution.
- **Beet vinegar:** Obtained by subjecting **beet juice to acetic acid fermentation**. It is usually **mixed with an equal volume of alcohol vinegar**.

- **Fruit juice vinegar:** In each fruit-producing country, vinegar corresponding to that fruit is generally produced. Examples include:· kiwi vinegar · Lemon vinegar · Cider vinegar · Date vinegar, etc.
- **Starchy product vinegar:** A saccharification step of starch is essential before alcoholic fermentation. Among them, we have:

·**Beer vinegar or malt vinegar (from barley):** Made from alcohol obtained from the fermentation of corn or barley starches treated with enzymes that release sugars for fermentation.

· **Rice vinegar:** Derived from glutinous rice fermentation. This vinegar, characterized by its low acetic acid content, is used in Asia.

· Wheat vinegar, balsamic vinegar, tea vinegar, and so on.

❖ The color and aroma of any vinegar depend significantly on the initial substrate.

1.7.The use of vinegar

a) Vinegar in the Kitchen

❖ Vinegar has played a very important role in cooking since ancient times because, beyond making vinaigrettes and marinades and its condimental use, its antiseptic qualities were very useful before the advent of refrigerators for food preservation, especially for meat.

❖ Some uses include: • Tenderizing meat • Turning natural vegetables into marinades • Marinating • Food and meat preservation • Cooking.

b) Non-food uses

- Vinegar, like hydrogen peroxide, is used in the livestock industry to kill bacteria and viruses before refrigeration.
- Since vinegar is a readily available source of diluted acetic acid, it is used for descaling (in faucets, toilets, coffee makers), cleaning certain stains (removing ink, coffee, rust, wine stains, etc.).
- In the past, vinegar was primarily used for its antiseptic properties.
- In the household, it can disinfect and deodorize; make mirrors, windows, brass, copper, and leather shine; replace fabric softener for machine laundry.
- With its distinctive odor, vinegar repels mosquitoes.
- Vinegar can be used for jellyfish stings, as long as the species is identified.
- Le vinaigre peut être utilisé pour les piqûres de certaines méduses, dans la mesure où l'espèce est identifiée.

1.8.Therapeutic Effects of Apple Cider Vinegar (ACV)

❑ Digestive Disorders: ACV is an excellent gastric acid buffer. When taken during meals, it combats food poisoning, flatulence, and spasms. When taken before meals, it stimulates digestion.

❑ Before bedtime, it fights constipation and regenerates intestinal flora.

a. **Antibacterial effect of ACV**

❑ Apple cider vinegar has an antibacterial effect on several types of bacteria, including *E. coli* and *Salmonella*…

❑ This effect is primarily due to acetic acid, which is considered the active substance in ACV. Acetic acid plays a role in controlling and preventing the growth of a wide range of bacteria by modifying essential components responsible for their growth, such as DNA polymerase and pH.

b. **Side effects**

- ❑ The main ingredient in apple cider vinegar is acetic acid. Apple cider vinegar should always be diluted with water or juice; otherwise, it can damage tooth enamel and the oral and esophageal mucosa.
- ❑ Vinegar is known to cause skin irritation upon contact.
- ❑ Long-term use of apple cider vinegar may lead to decreased bone density.
- ❑ Apple cider vinegar could theoretically interact with diuretics, laxatives, and heart medications.

2. Citric acid and other organic compounds

History

- Citric acid was first isolated in crystalline form from lemon juice in 1784 by the Swedish chemist Carl Scheele.
- Its structure was established by Liebig in 1838.
- In 1869, in England, a physician obtained it in crystalline form.
- The chemical synthesis of citric acid from glycerin dates back to 1880.
- As early as 1893, Wehmer had the idea that filamentous fungi produced citric acid through the fermentation of substrates containing sugar.
- In 1923, citrate was isolated from a fungal fermented culture. While this method was initially conducted on a small scale in the laboratory, more than 90% of the world's citric acid production was carried out in Italy.
- At that time, the industrial fermentation process using *Aspergillus niger* as the producing microorganism and sugar as the raw material began to develop in Europe and the United States.
- In the early 1930s, 80% of the world's citric acid production was achieved through fermentation.

- Today, citric acid is produced using both "surface" and "submerged" fermentation techniques.
- Currently, 99% of the production is achieved through microbiological processes, with the remaining 1% extracted from citrus fruits.

2.1.Definition

Citric acid, or citrate, is a tricarboxylic acid with the chemical formula C6H8O7. It is abundantly found in lemons, which is the source of its name, and is widely distributed in nature. Citric acid is a solid, white compound with no odor and an extremely sour taste. It plays a crucial role in the metabolism of many animals and plants. This weak acid is significant in biochemistry as a metabolite in the Krebs cycle, a major metabolic pathway in all aerobic organisms.

2.2.Principle of citric acid production

It is produced in the first step of the Krebs cycle by condensation of acetyl-CoA (2C) with oxaloacetate (4C) to form citrate (6C), releasing CoA in the process, catalyzed by citrate synthase (figure 23, 24).

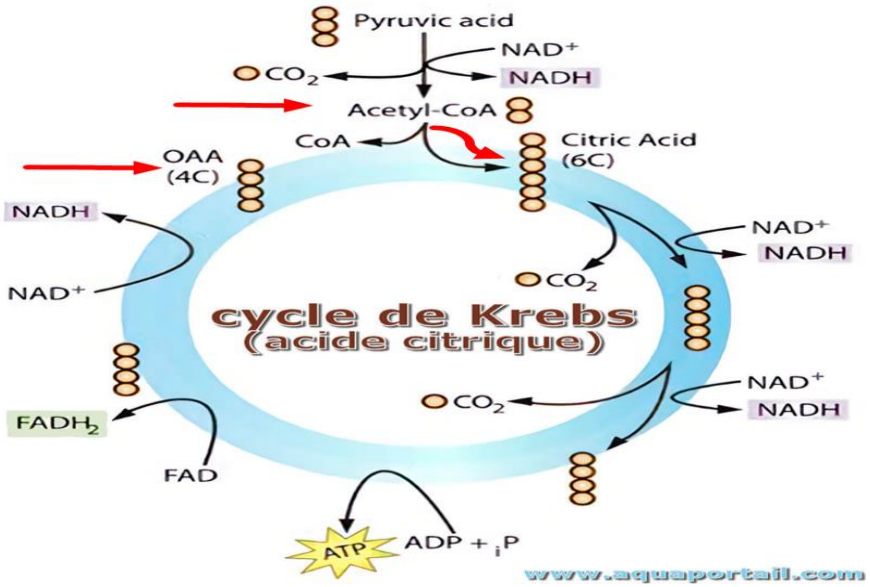


Figure 23 : Krebs cycle and citric acid production.

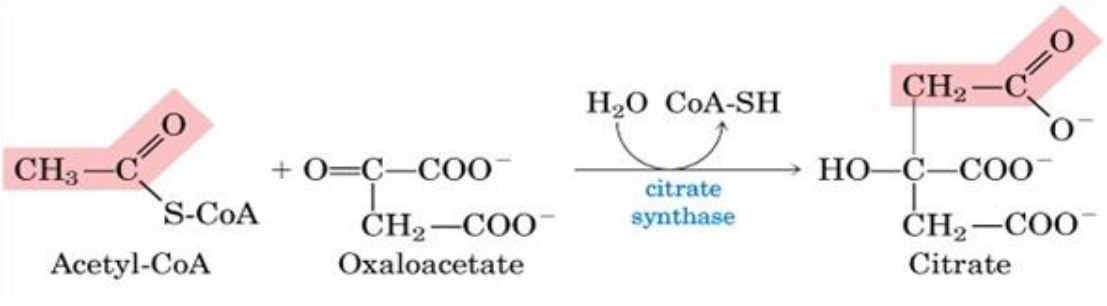


Figure 24 : citrate reaction production.

2.3.Citric acid production

Citric acid Production :

In terms of tons, citric acid is **the most significant commercial product** derived from fungi. **Global production exceeds 90,000 tons per year** from the **fungus *Aspergillus niger.***

Solid-state Fermentation (Koji fermentation) :

This relatively simple process involves cultivating a strain of ***A. niger*** on **fibrous starchy residues (e.g., potato residues).**

- **The temperature within the mass should not exceed 28°C.**
- The **pH drops to 1.8-2.0** during the **accumulation of citric acid.**
- After **5 to 8 days, the fermented mass** is extracted with water in percolators.
- Citric acid **purification is carried out** using conventional methods.

Many **Saccharomyces yeasts and molds** can produce citric acid, **but nearly all of them have sensitivity to metal ions and very low yields**.

Choice of substrate

The choice of substrate must meet certain criteria to achieve high yield with lower costs:

1. **Presence of a substrate rich in fermentable sugars (starchy).**
2. **Low content of trace toxins for the microorganism.**
3. **Cost-effectiveness of the substrate.**
4. **Ease of treatment and handling.**

Choice of microorganisms :

Mutant strains of *Aspergillus niger* are the microorganisms of choice on an industrial scale due to:

- **Insensitivity to trace metals.**
- **Ease of cultivation.**
- **Genetic stability.**
- **High yields.**
- **The ability to utilize inexpensive materials and the absence of undesirable metabolites.**

The advantages of using molds

-They allow for significant cost savings compared to lemon cultivation.

They are not as polluting compared to the waste generated by citrus fruit, which is often dumped in large quantities into the environment, causing significant pollution.

2.4.Citric acid utilisations

- **Food Industry**

Ø It is used as an additive (in beverages, jams, etc.). In beverages, it is generally used for its refreshing or effervescent properties.

Ø It is used in the production of candies, fruit preservation, fish preservation, ice cream, various confections, sauces, fruit juices, and syrups.

Ø It can be used as a cleaning agent for stainless steel due to its sequestering power.

- **Phamaceutical Industry**

Ø Citric acid indirectly promotes bone growth by aiding in calcium absorption and regulating the size of calcium crystals in bones.

Ø It is used as a component of bladder irrigation solutions.

Ø Citric acid and its salts prevent blood coagulation in stored blood.

2.5.Citric acid synthesis

The synthesis of citric acid can be divided into three major steps from a biochemical and metabolic standpoint.

Sugar Degradation

Three main sugar degradation pathways can lead to the formation of pyruvic acid: glycolysis, the pentose phosphate pathway, and the Entner-Doudoroff pathway (figure 25).

1. Glycolysis, also known as the Embden-Meyeroff-Parnas pathway, breaks down glucose to produce ATP and pyruvate, which is further used in the citric acid synthesis pathway.

2. Glucose proceeds through the pentose phosphate pathway. This metabolic process is significant because it enables the generation of precursors required for the synthesis of aromatic amino acids and vitamins.

3. The Entner-Doudoroff pathway : leads to the formation of gluconic acid, one of the key byproducts produced during fermentation.

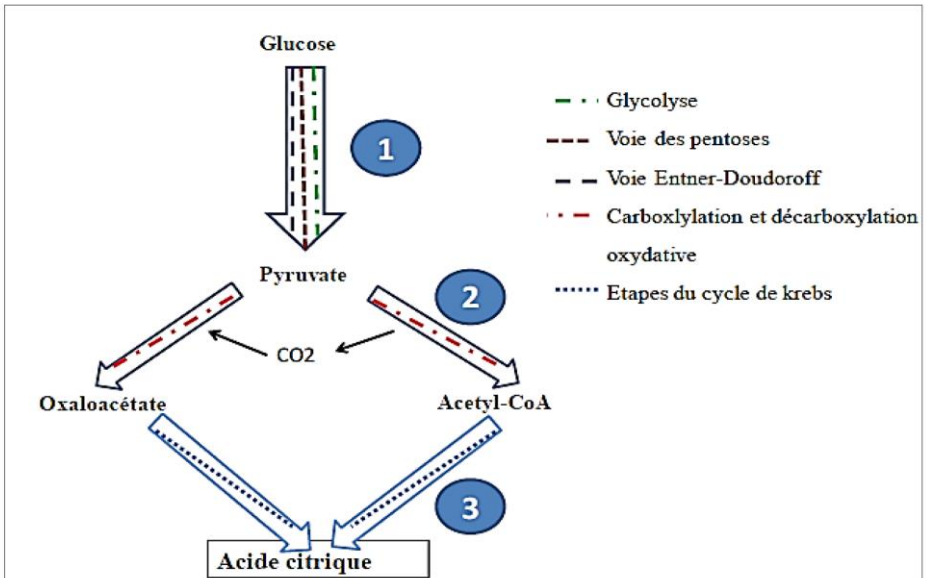


Figure 25: Citric acid synthesis from a biochemical and metabolic point of view.

Central hub

The central hub is where pyruvate is decarboxylated/oxidized and carboxylated. Two molecules of pyruvate are formed from one molecule of glucose. The first is decarboxylated to produce acetyl-CoA, and the second is carboxylated into oxaloacetate. Thus, acetyl-CoA and oxaloacetate play a role in the Krebs cycle.

Krebs cycle

Also known as the citric acid cycle or citrate cycle, the Krebs cycle consists of eight reactions described by Hans Krebs in 1940.

It is composed of two major enzymatic steps: the oxidation of acetyl-CoA and the regeneration of oxaloacetate.

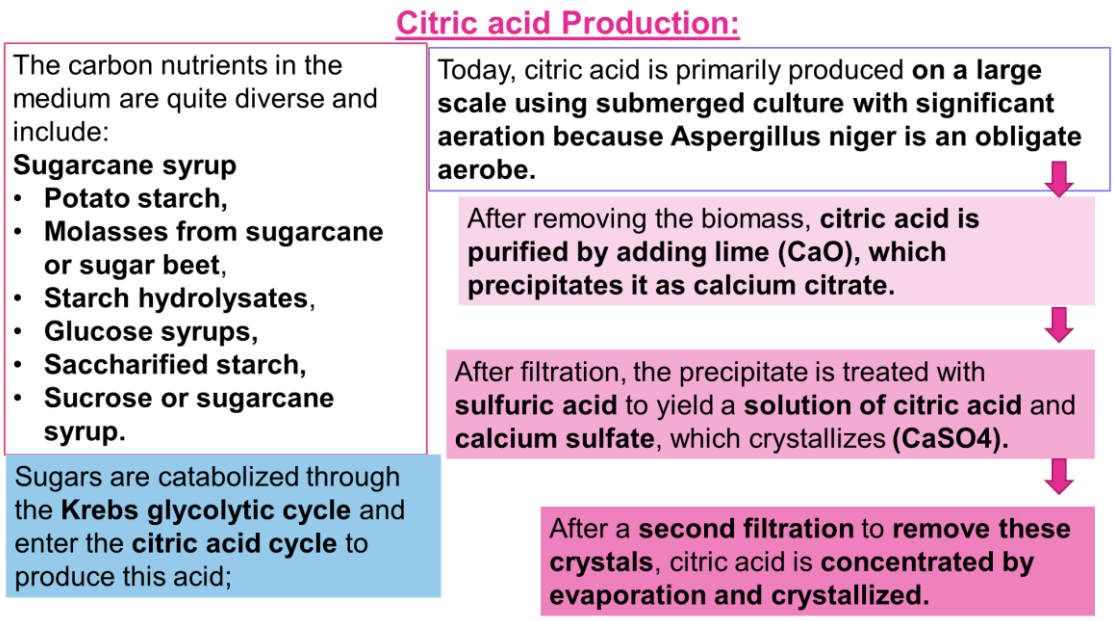
2.6.Microorganisms producing citric cid

- Throughout the history of citric acid fermentation, a large number of microorganisms (bacteria, fungi, and yeasts) have been reported to produce citric acid.
- These include numerous filamentous fungi (Aspergillus, Penicillium, Trichoderma, Mucor, etc.), yeasts (Candida, Saccharomycopsis), and a few bacteria (Arthrobacter, Corynebacterium) capable of excreting citric acid.
- With advancements in biotechnology, new genetically modified strains and the improvement of existing strains through mutagenesis and strain selection are used for industrial production. However, the effective use of these new strains presents some challenges, and there are various constraints in citric acid production.

2.6.1. Genus *Aspergillus*

- Fungi belonging to the genus *Aspergillus* were first described in 1729. These fungi are saprophytic, meaning they obtain their nutrition from decomposing organic substances.
- They have an aerobic metabolism.
- They are thermophilic (some species can survive temperatures close to 70°C), and do not require specific nutrients.
- Aspergillus exhibit rapid growth on standard culture media (such as malt agar, Sabouraud agar, Potato Dextrose Agar (PDA)) supplemented with antibiotics.
- After 96 hours of incubation, colonies develop characteristic colors, which can be brown, green, yellow, or black depending on the species (figure 03).
- Most *Aspergillus species* grow optimally between 22 and 25°C, but thermophilic species like *A. fumigatus* can thrive between 37 and 40°C and sometimes even up to 57°C.
- The colony color is a quick aid in species identification, such as gray-green for *A. fumigatus*, green-yellow for *A. flavus*, yellow turning to black for *A. niger*, and white for *A. candidus*.

2.6.2. Citric acid production



Bioproduction of citric acid

2.6.3. Bioproduction of citric acid

- Bioproduction of citric acid can be described in three major steps (inoculum preparation, fermentation, and extraction).
- Two types of fermentation are most commonly used, namely submerged fermentation or liquid-state fermentation (LSF) and solid-state fermentation (SSF).
- Each of the two techniques has its advantages and disadvantages; however, the most commonly used fermentation is liquid-state fermentation (LSF).

a. Liquid-state Fermentation (LSF)

- Large quantities of citric acid can be obtained by growing the fungus aerobically on an iron-deficient medium. Iron deficiency forces *Aspergillus niger* to overproduce citric acid to sequester the iron it needs in the medium by chelating it with the citric acid it produces.
- The production medium is treated to be iron-deficient, as are the stainless steel fermenters, which can be lined with an inner glass layer to prevent iron leaching from the walls at the acidic pH generated by citric acid accumulation during production.
- It is estimated that approximately 80% of the production is obtained by LSF
- Indeed, this technique reduces total investment costs and labor by 2.5 to 25%.

- It has several advantages, including high productivity and yield, as well as a lower risk of contamination.
- Typically, it is carried out between 5 and 12 days.

However, the disadvantages include:

- Higher energy costs and more sophisticated control requiring highly skilled personnel.
- Long reaction time and the need for a large volume.
- Susceptibility to contamination *by Penicillium* and other yeasts and lactic acid bacteria.
- Sensitivity to inhibition by trace metals.

c. **Solid-state Fermentation (SSF)**

(SSF) is generally defined as the growth of microorganisms on moist substrates in the absence of free water.

- ❖ The production of citric acid by SSF, using the Koji process, was developed in Japan.
- ❖ This fermentation can be carried out using various raw materials.
- ❖ In recent years, SSF has garnered significant interest because it offers numerous advantages for citric acid and enzyme production.
- SSF processes have low energy requirements, produce significantly fewer effluents, and, as a result, raise fewer environmental concerns.
- The ability to use agro-industrial residues as substrates for citric acid bioproduction also contributes to waste disposal solutions.
- Additionally, agricultural waste products like banana peels can be biotransformed in this manner.

3. Organic acid Production

3.1.Definition

- Many organic acids, resulting from either the anaerobic breakdown of sugars or their incomplete oxidation, are industrially produced using microorganisms such as bacteria and fungi.
- These microorganisms play a significant role in the production of organic acids, and their synthesis is linked to the Krebs cycle.
- An organic acid is a compound capable of releasing a positively charged ion (cation), H+, in aqueous environments.
- The most common organic acids are carboxylic acids, including citric acid, lactic acid, acetic acid, propionic acid, butyric acid, and so on.

3.2.Domain of use of organic acids

Organic acids are additives widely used in the food industry. They can serve as: Preservatives, Acidulants, Antioxidants and emulsifiers.

Note: Large quantities of organic acids can be extracted during the normal metabolism of certain microorganisms.

3.3.Microorganisms producing organic acids

The microbial specied involved in the production of organic acids vary depending on the type of industrial acid of interest (table 4).

Table 4 : Organic acids produced by microorganisms and their uses in the food industry.

Acides organiques	Micro-organismes producteur	Exemples d'utilisation
Acide acétique	Acetobacter aceti (bactérie)	Agent de conservation dans les mayonnaises, pâtisseries...
Acide lactique	Lactobacillus (bactérie) Aspergillus griseus (moisissure) Rhizopus (moisissure)	Agent de conservation et acidulant pour confitures, boissons gazeuses, olives, poissons...
Acide citrique	Aspergillus niger (moisissure)	Acidulant et antioxydant dans les boissons gazeuses, produits laitiers, fruits congelés..
Acide gluconique	Aspergillus niger (moisissure) Pénicillium chrysogenum (moisissure)	Acidulant pour viandes, renforce le goût dans la margarine...
Acide fumarique	Rhizopus (moisissure) Mucor (moisissure)	Acidulant dans les jus de fruits…
Acide malique	Pénicillium brevicompartum (moisissure) Leuconostoc (bactérie) Aspergillus oryzae (moisissure)	Acidulant dans les jus de fruits, crème glacée...
Acide tartrique	Pénicillium notatum (moisissure)	Acidulant pour boissons…
Acide propionique	Propionibacterium spp	Agent de conservation de nombreux aliments, dont les pâtisseries...

3.2. Organic acids

- Several other acids are produced by fungi, including itaconic acid, used in the production of acrylic resins, and gluconic acid, used in the production of calcium gluconate for the treatment of calcium deficiencies in humans and as a softening washing agent.
- Among other important chemicals produced by bacteria are sorbose, produced through the oxidation of sorbitol by Acetobacter and used in the manufacture of ascorbic acid (vitamin C), and lactic acid, produced by many bacteria and used as a food acidulant.
-

3.4.Lactic acid

- 2-hydroxypropanoic acid, or lactic acid, is the primary component of all acidified dairy products, giving them their fundamental characteristics.
- Lactic acid is produced in large quantities from sugars by homofermentative lactic acid bacteria; various species of *Lactobacillus and Lactococcus* are used for the production of this organic acid.
- However, other microorganisms can also be used, such as *Bacillus coagulans* and certain molds.
- Depending on the mode of fermentation, there are homofermentative and heterofermentative bacteria (Figure 26).
- The choice of microorganism type varies depending on the available substrate (whey, molasses, corn, cassava,etc.).

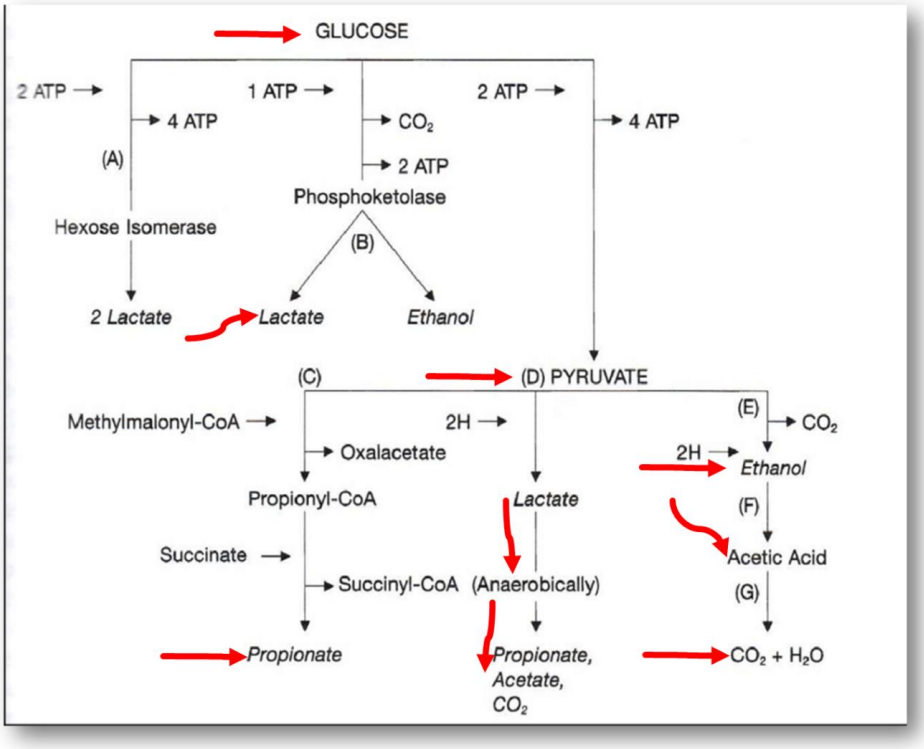


Figure 26 :lactic acid pathway production.

3.2.1. Usage

- ❑ Lactic acid is used to acidify jams, jellies, sweets, carbonated beverages, and other products.
- ❑ It can be added to brine during the production of pickles and olives to prevent fermentation or preservation defects.
- ❑ It serves as a preservative for fish and other foods.

4. Yeast as a food and dietary supplement

History

- Historically, yeasts are microorganisms that have been used for millennia in the making of various bakery products and alcoholic beverages.
- The relationship between yeast and humans probably dates back over 7,000 years when people tasted an alcoholic liquid derived from damaged and stored grapes. They didn't know it yet, but they were witnessing the phenomenon of spontaneous fermentation.

- Several significant discoveries have revolutionized the science of yeast, such as the first microscopic observations of yeasts made by Antoine van Leeuwenhoek in 1680,
- The identification of yeasts as the agents responsible for the conversion of sugar into carbon dioxide by Louis Pasteur in 1870,
- and the publication of the first complete genomic sequence of wine yeast (*Saccharomyces cerevisiae* AWRI1631) in 2008.

4.1.Definition and Classification

- The term "levure" (from Latin "levare," meaning "to raise") reflects its ability to "raise" bread dough by producing CO2 during fermentation.
- Yeasts are chemoheterotrophic eukaryotes, which means they can derive their energy from redox reactions or the fermentation of chemical compounds such as sugars. They belong to the group of fungi, but they are distinguished by their unicellular nature and the absence of a true mycelium.
- Yeasts refer to all microscopic unicellular fungi that have a spherical or ovoid shape and reproduce through budding.
- Among them, the yeast *Saccharomyces cerevisiae* was the first eukaryote whose genome was completely sequenced, in 1996.
- It is considered one of the most widely used model organisms in genetics due to:its rapid development, large populations, and low propagation cost.

Fungi, also known as mushrooms or mycetes, make up a highly diverse group estimated to include around one million species (figure 27).

However, they share several fundamental characteristics:

- They are eukaryotic organisms.

- They exhibit a heterotrophic mode of life.

  - They possess a branching, diffuse, and tubular vegetative structure.
  - Reproduction is achieved through spores.
  - They have a chitinous cell wall.

- Eumycetes represent a monophyletic group consisting of five taxa: Chytridiomycetes, Zygomycetes, Glomeromycetes, Ascomycetes, and Basidiomycetes.

Basidiomycetes and Ascomycetes are the best-characterized fungi.

The mycelium of Basidiomycetes and Ascomycetes is septate: they are septate fungi.

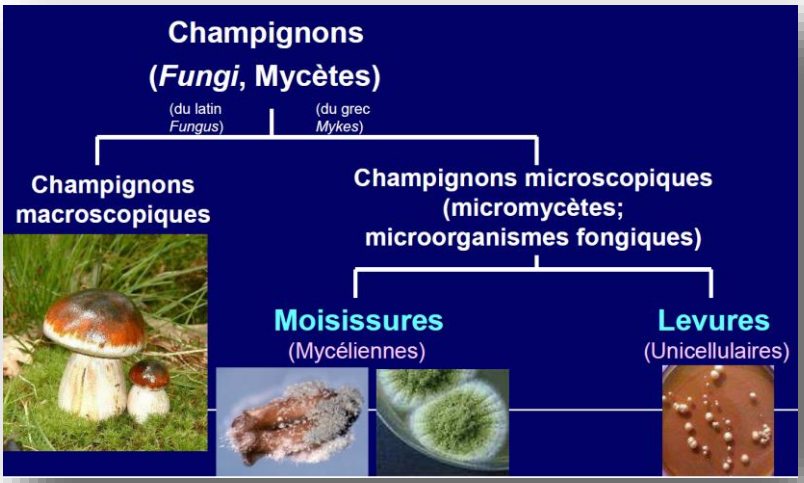


Figure 27 : Position of fungi in the tree of life.

The Saccharomycetes, of which 160 species are known, are mainly limited to the Saccharomycetaceae family.

Therefore, brewer's yeast, *Saccharomyces cerevisiae*, is an Ascomycete belonging to the Saccharomycetes class and the Saccharomycetaceae family.

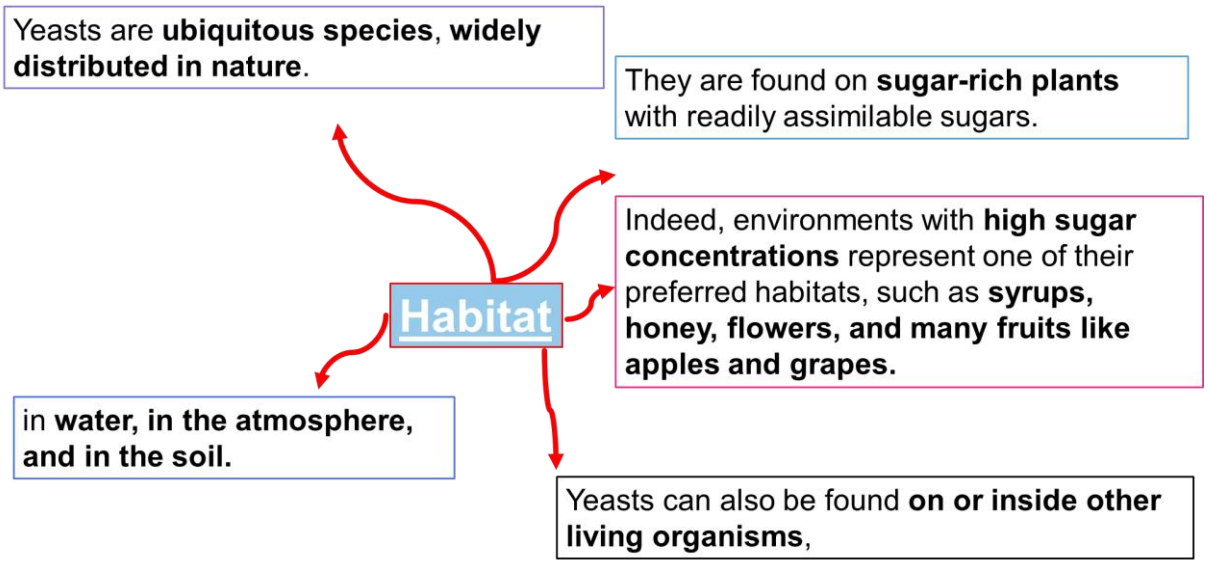
4.2.Description of brewer's yeast cell

4.2.1. Organization

- Yeast cells possess all the characteristics of eukaryotes but differ from plant or animal cells due to their small size.
- Indeed, yeast is the smallest among eukaryotic cells.
- Brewer's yeast, when observed under a microscope, appears as spherical or ovoid cells with an average size of 7 µm [Figure 3]. Typically, yeast colonies are white and regular.

- Like all eukaryotic organisms, a brewer's yeast cell has cell envelopes, cytoplasm, and organelles.

4.2.2. Habitat



4.2.3. Definition of Saccharomyces cerevisiae

❑ *Saccharomyces cerevisiae* (Sc) yeast is probably the **most studied and used species in the world**, commonly known as **"baker's yeast."**
❑ Sc yeast is a versatile microorganism with **GRAS status**.

Requirements

- Yeasts synthesize organic molecules from other organic molecules taken from the environment; they are **heterotrophic organisms**.
- Their environment must provide a **source of oxygen, a source of energy, and a source of food**.

4.2.4. Metabolism

Metabolism

- **Yeasts obtain the energy** required for their development through the **degradation of organic molecules**.
- To produce energy, they use **two different systems** depending on **the absence or presence of oxygen** in the environment:

- Alcoholic fermentation (under anaerobic conditions) ;
- Respiration (under aerobic conditions).

➢ The **amounts of energy released** are different in these two processes. It's worth noting that the enzymes for **fermentation are located in the cytoplasm,** while those for **respiration are located in the mitochondria.**

➢ **Alcoholic fermentation** is used in **making bread and alcoholic beverages.**

L'équation globale de la respiration est la suivante :

$C_6H_{12}O_6 + 6\ O_2 \Rightarrow 6\ CO_2 + 6\ H_2O + 36\ ATP$ [20]

L'équation peut également s'écrire :

$C_6H_{12}O_6 + 6\ O_2 \Rightarrow 6\ CO_2 + 6\ H_2O + 675\ Kcal$ [20]

$C_6H_{12}O_6 \Rightarrow 2\ CO_2 + 2\ C_2H_5OH + 2\ ATP$ [20]

L'équation peut également s'écrire :

$C_6H_{12}O_6 \Rightarrow 2\ CO_2 + 2\ C_2H_5OH + 25\ Kcal$ [20]

4.2.5. Objectives of yeast production

The industrial production of yeast cells holds significant importance in achieving a dual objective (figure 28):

1.The first objective is qualitative, aiming to select a strain that meets the following criteria:

- ❑ Enhanced fermentative capacity
- ❑ Greater stability
- ❑ Final product suitable for the specific client's needs (baking, winemaking, compressed yeast, dry yeast, etc.).

2.The second objective is economic; the manufacturer aims to attain the desired quality at the best possible cost.

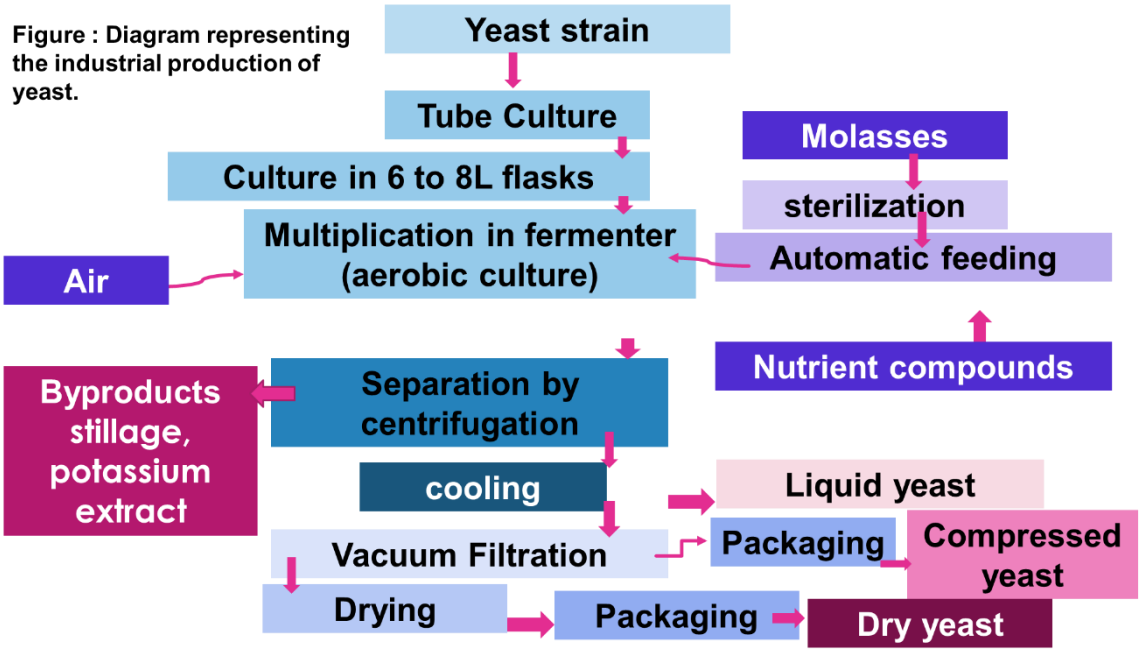


Figure 28 : Diagram representing the industrial production of yeast.

4.2.6. Applications of brewer's yeast

There are two types of brewer's yeast:

Active brewer's yeast : also known as live or revivable, has been slowly dried at a temperature not exceeding 40°C.

- In addition to its nutritional qualities, it retains its fermenting power and probiotic properties.

Inactive brewer's yeast or dead yeast, is the most common and least expensive. It has been subjected to high-temperature drying (over 40°C), resulting in cell death.

- Therefore, it loses its fermenting power and probiotic properties. However, it remains rich in proteins, vitamins, minerals, and trace elements, retaining its nutritional properties.

4.3. Applications in food : some examples

Baking use

Baker's yeast, used in the production of **bread, pizza dough, brioches**, etc., consists of **live *Saccharomyces cerevisiae*** cells. It primarily exists in **two forms:**

- **Fresh yeast in small cubes:** It has a **cream color** and is packaged in **small cubes of about 42 grams**.
- While **less expensive than the dry form** and capable of **being frozen for several months**, it only keeps for a **few days in the refrigerator** (**maximum of ten days**).

- **Dry yeast in sachets:** This form can be **stored for a longer period (up to a year)** at **room temperature**, away from **light, moisture, and air**.
- It can be used **directly without rehydration.**

- The baker's yeast should not be confused with baking powder. The latter is composed of a mixture of baking soda with an acidic agent (such as tartaric acid) and a stabilizing agent (cornstarch).
- The dough's rise is caused by the release of CO2 due to an acid-base chemical reaction that occurs during baking, unlike the action of baker's yeast, which starts before baking.
- As a result, baking powder is suitable for cakes but not suitable for making bread, pizzas, or brioches.
- Bread is a food made from flour, water, and salt in the process of baking.
- During this process, the flour undergoes fermentation facilitated by the microorganisms present in baker's yeast or sourdough starter, leading to the release of carbon dioxide in the dough, creating air pockets.

4.3.1. The steps involved in bread-making :

Bread-making consists of 6 stages (figure 29) :

1. The ingredients for the dough are first mixed during the "frasage" at low speed until a relatively homogeneous dough is obtained.
2. This is followed by a kneading phase where the dough is worked at high speed by mechanical force. This step allows the rearrangement of the protein network in the flour (gluten) in three dimensions, conferring specific viscoelastic properties to the dough.

3. "Pointage" constitutes the first fermentation during which the flavors of the bread develop before the dough is divided.
4. After shaping, the dough undergoes a second fermentation called "apprêt" that allows the dough to rise.
5. The bread-making process concludes by baking the dough at 250°C in the presence of steam to form the crust. Yeasts are killed during this step, and the ethanol produced during their metabolism is evaporated.

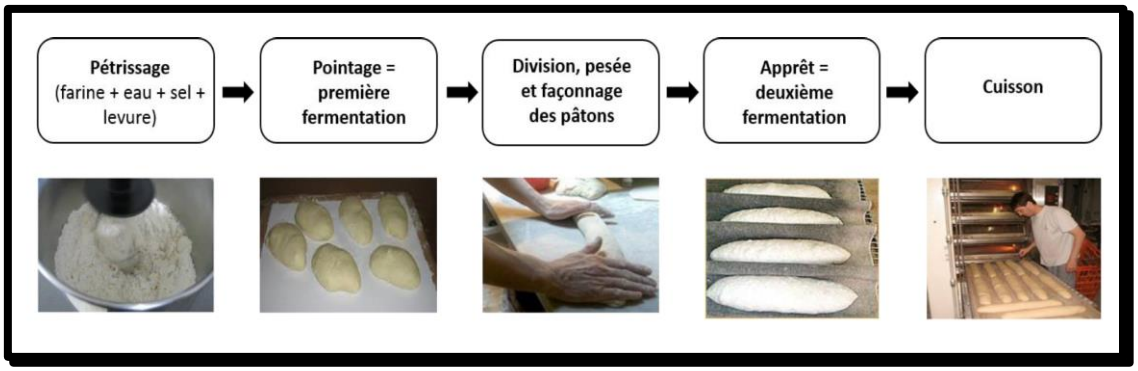


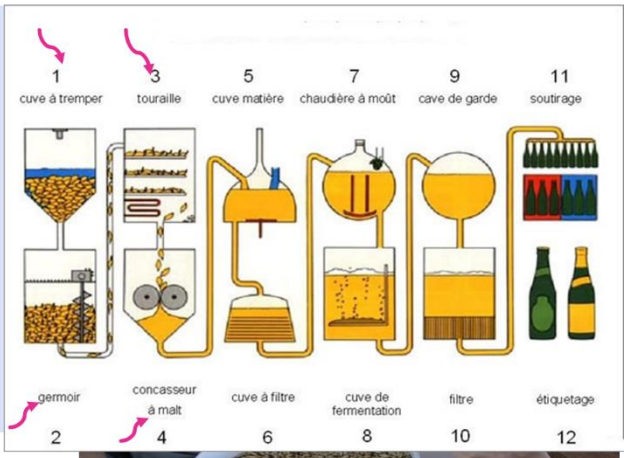
Figure 29 : Bread fabrication steps.

4.3.2. Usage in brewing

The yeast *S. cerevisiae* is essential in the beer-making process through alcoholic fermentation.

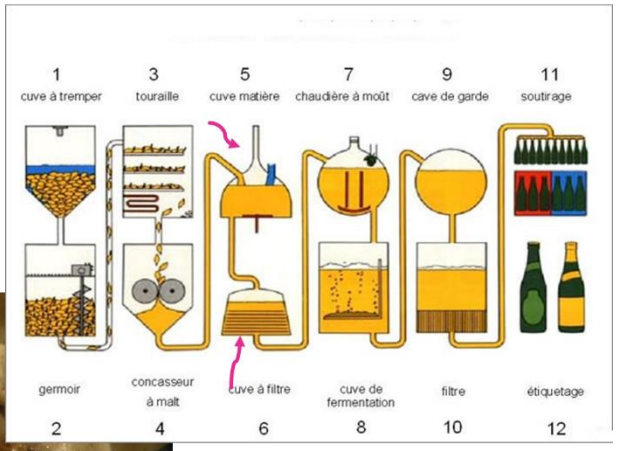
- The yeast transforms the sugars found in the wort (derived from germinated and roasted barley grains, also known as malt) into alcohol and CO2.
- Beer is the product of yeast fermentation in a brewing mix resulting from the partial breakdown of malted starch. It is made up of water, hops, and barley malt.

- **Malting** (steps 1,2 and 3) is typically managed by **malt houses**, less commonly by **brewers** nowadays.
- During malting, **barley is made fermentable**.
- The malting process occurs in three steps: **steeping, germination, and kilning** (**heating the barley**).
- This results in **malt**, which represents **barley grains** that have **sprouted and then been roasted.**



- **Milling the malt (concassage du malt):**
- In this step, the **grain's contents are expelled from its husk** to ease the **extraction of enzymes and sugars.**

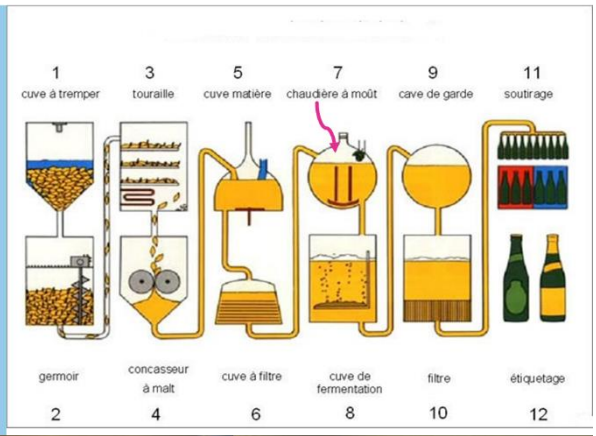
- **Mashing (Empatâge) :**
- Mashing involves **soaking the crushed malt in water** at a certain temperature to obtain a **sweet liquid called wort**. The **malt-water mixture** is known as the **mash**.



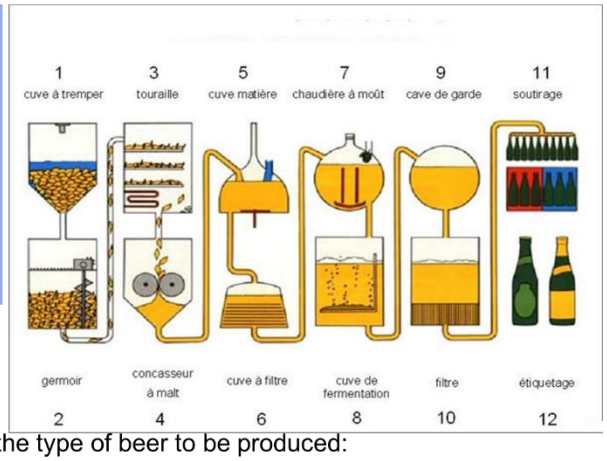
- **Filtration and rinsing :**
- At this stage, the **wort is separated from the soaked malt (draff). Rinsing is the process of extracting the maximum amount of sugar from the draff.** After filtration, the **wort is pumped to the wort kettle for boiling**.

- **Boiling and hopping :**
- The wort is then placed in a **kettle and brought to a boil.**
- The boiling process serves two purposes:

- **-Sterilization of the wort**
- **-Addition of bitter and aromatic hops**
- Depending on the type of beer being brewed, this is also the stage where certain **spices (such as coriander, orange peel, ginger, cinnamon**, etc.) are added. These steps are carried out at **different temperatures.**



- **Fermentation :**
- The **sterilized and flavored wort** is **quickly cooled and transferred to a fermentation tank**.
- **Yeast is added to transform the sugar into alcohol**. This stage can last between **4 weeks and several months**, **depending on the type of beer**.

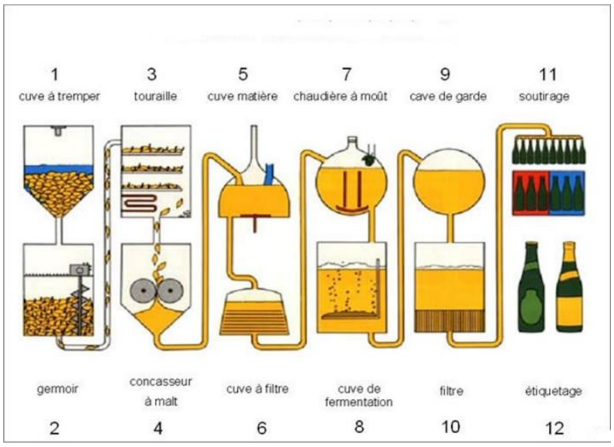


There are **two types of fermentation**, depending on the type of beer to be produced:

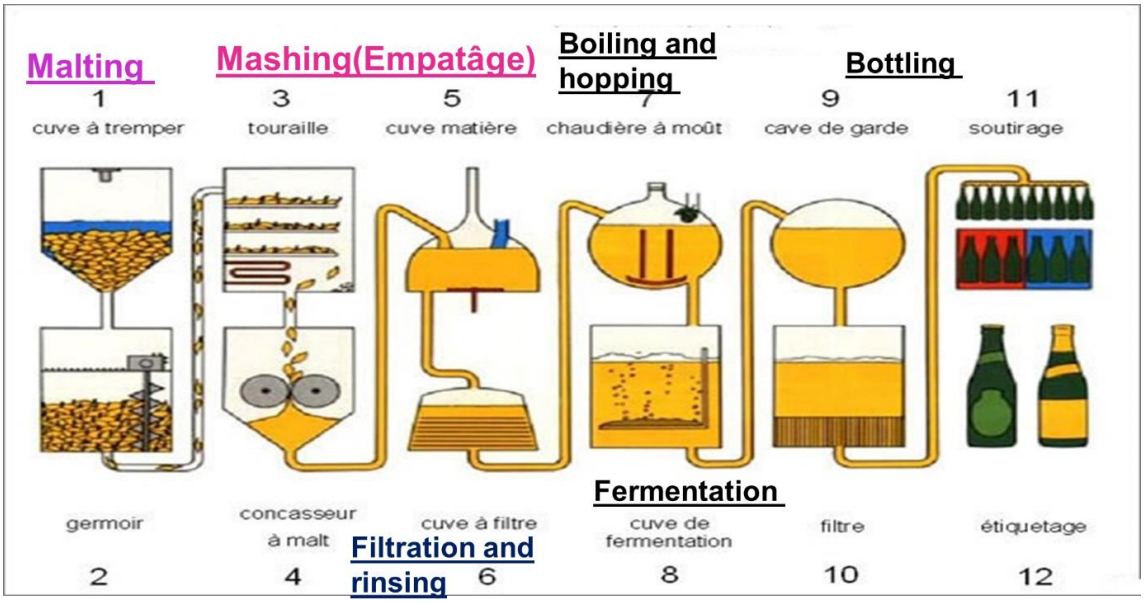
- **"Top fermentation"** where yeast is **seeded for 3 to 8 days at 22°C**; this is the **oldest and most common method**, resulting in **darker beers.**

- **"Bottom fermentation"** where yeast is **seeded for 7 to 10 days at 12°C**; this is a **newer process**, initially used for **pilsner type beers (pale lagers).**

- **Bottling:**
- Our beer is **finally alcoholic but still flat.**
- To give it its **fizz**, it is then sent to the **bottling line to be bottled, canned, or kegged**.
- **Sugar is added during the bottling process**. This sugar, **consumed by the yeast, transforms into CO2**.
- This **refermentation process lasts about a month, or even longer.**



Thus, yeasts not only produce alcohol and CO2 resulting from fermentation, but also other components such as **higher alcohols, organic acids, esters, aldehydes, ketones**, which directly contribute to the **sensory profile of beer (aromatic properties).**



4.3.3. Use in the production of other fermented beverages

a- Use in winemaking

Yeast, commonly used in baking and brewing, is also essential in wine production. The mature grape juice contains around 15 to 25% of fermentable sugars. *Saccharomyces.c* naturally occurs on the grape skins" (table 5).

Table 5 : Source of fermentation of major alcoholic beverages.

Major alcoholic beverages	Source for fermentation
Wine	**Fruit sugars (grapes)**
Beer	**Barley (sometimes rice or corn)**
Whisky	**Rye, corn, barley**
Tequila	**Blue Agave (*Agave tequilana*)**
Rum	**Cane sugar molasses**
Mead	**Honey**
sake	**Rice**

b- Use in the food industry

- In the food industries, yeast autolysates are incorporated at a rate of 1 to 3% in food products to enhance their nutritional qualities.
- These autolysates are obtained by the partial self-digestion of yeast cells using their own enzymes and have the advantage of being low in salt content.

Brewer's yeast is used in the food industries for various functions:

- It acts as a flavor enhancer and taste booster due to its richness in glutamic acid.
- In the form of autolysates, it's used in the production of soups, bouillon cubes, poultry stocks, and sauces to enhance flavors, giving a taste reminiscent of poultry and cheese.
- Brewer's yeast can replace monosodium glutamate, an industrially produced flavor enhancer added to many food products, particularly in Asian cuisine, to intensify taste.

- It serves as an adjunct to prevent thickening of textures in cheese and sausage production, and stabilizes the viscosity of liquid or semi-liquid dough (crepes, waffles, etc.)."

4.4.Therapeutic use

In addition to its nutritional benefits and its effects on skin and appendages, brewer's yeast has other beneficial effects on health that we will explore.

- Brewer's yeast, used for its probiotic properties, is active.
- Sc yeast is also used for the production of highly pure proteins of various hantaviruses used in vaccine development.
- Furthermore, as a eukaryotic cell easily manipulated genetically, it is considered the model microorganism for studying cancer biology and aging."

5. Edible mushrooms

5.1.Description

Basidiomycetes constitute a vast branch of fungi that include most species commonly referred to as mushrooms in everyday language. Edible mushrooms are mushrooms that can be eaten because, unlike toxic mushrooms, their consumption does not pose a health risk. However, not all edible mushrooms are necessarily palatable, meaning that some non-toxic mushrooms are not good for consumption based on taste criteria. According to the FAO, we consume approximately a thousand different species of mushrooms.

5.1.1. Biology of edible mushrooms

- There are two components in the edible mushroom: the vegetative part called the "Mycelium" and the reproductive part called the "Carpophore" (Figure 30).

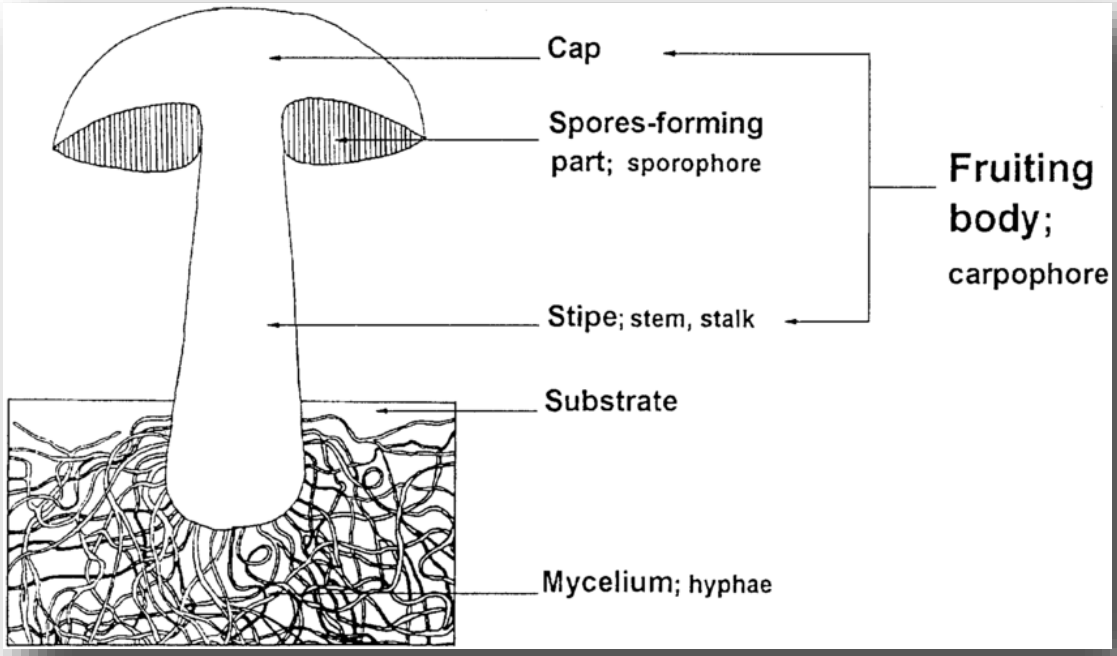
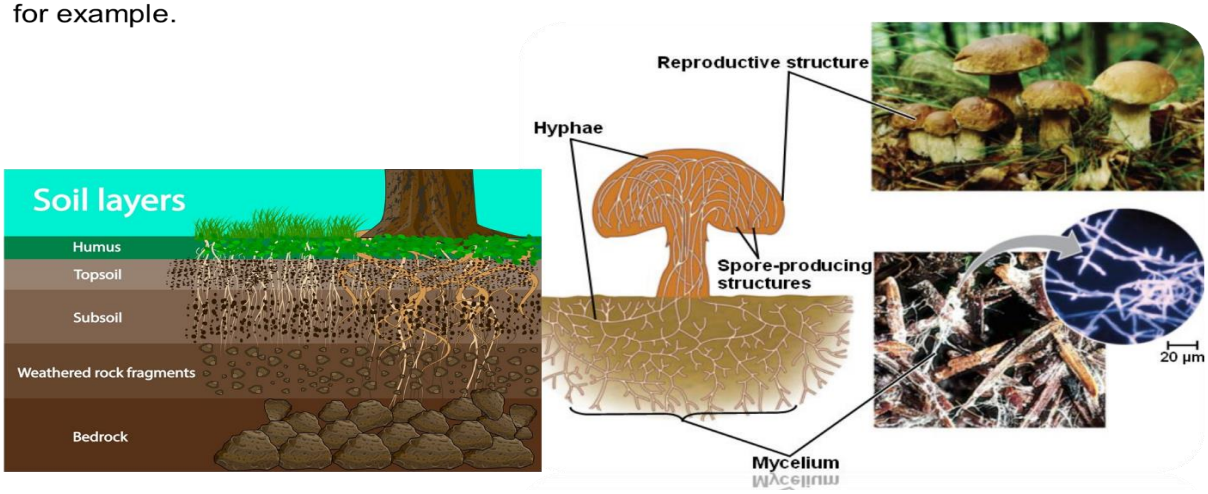


Figure 30: Anatomy of the Edible Mushroom.

The mycelium

The mycelium is composed of often **whitish filaments called "hyphae"** and is the **underground part** of the organism found in **humus, mineral soil, or decaying wood**, for example.



The carpophore

- It is the external part of the mushroom that ensures the organism's reproduction by releasing millions of spores.
- The carpophore consists of several components (hymenophore, cap, stalk or stipe, flesh).

- These mushrooms can be classified based on morphological criteria (shape of the stalk and cap, consistency of the flesh, color of the spores), organoleptic criteria (odor and flavor), and chemical criteria.

5.2.Nutritional modes of edible muchrooms

Mushrooms are heterotrophic organisms whose carbon nutrition depends on the presence of preformed organic matter, determining, depending on circumstances, their saprophytic, parasitic, or symbiotic life.

a- Saprophytic Nutrition

In this mode, mushrooms degrade dead or decomposing organic matter to extract essential mineral elements. They play a crucial role in recycling dead materials such as plant and animal debris.

b- Parasitic nutrition

Parasitic mushrooms feed on living animal or plant matter. They live on or within the body of a host. The parasite benefits from its host without giving anything in return, as the host provides it with nourishment, shelter, and reproduction opportunities.

c- Symbiotic nutrition

The last group of mushrooms prefers symbiosis or mutualism, representing an association with an autotrophic plant, with each organism benefiting from this association. Symbiosis sometimes leads to the creation of new entities, such as lichens or mycorrhizae.

5.3.Mode of reproduction of edible mushrooms

Mushrooms reproduce through spores, employing both asexual and/or sexual modes. The reproduction of Basidiomycetes is sexual, as illustrated in Figure 31.

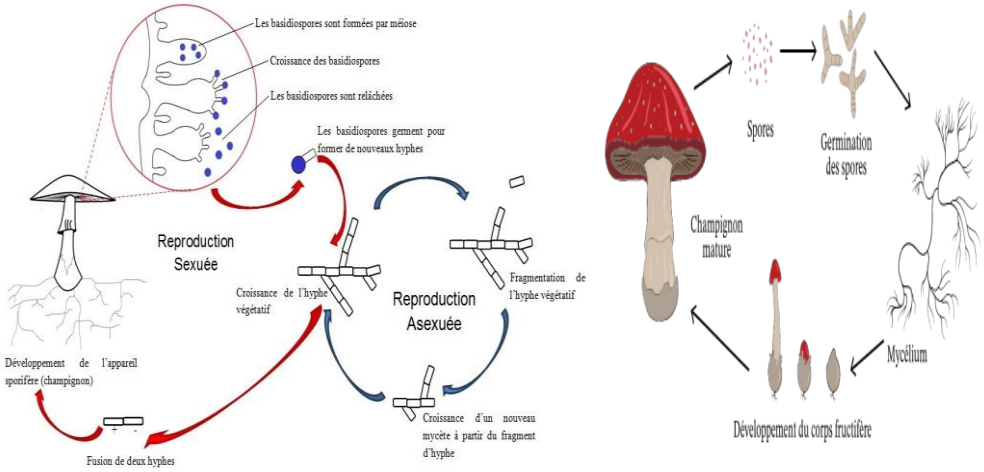


Figure 31: Mode of reproduction of Basidiomycetes.

5.4.Nutritional and Medicinal Importance of Edible Mushrooms

Edible mushrooms contain more protein than any vegetables and are rich in vitamin B.

Extractable products from medicinal mushrooms can be classified into the following categories:

- Category of Dietary Supplements/Nutraceuticals with Mushrooms:
- The increase in the consumption of unprocessed whole foods, such as mushrooms, appears to reduce the risk of obesity and overall mortality from diabetes and heart diseases. Mushrooms are abundant in antioxidants.
- Selenium Content:
- Selenium is a mineral not present in most fruits and vegetables but is found in mushrooms. It plays a role in liver enzyme function and contributes to the detoxification of certain carcinogenic compounds in the body. Additionally, selenium helps prevent inflammation and also decreases tumor growth rates.
- The vitamin D present in mushrooms also inhibits the growth of cancer cells by contributing to the regulation of the cell growth cycle.
- Folate in mushrooms plays a crucial role in the synthesis and repair of DNA, thus preventing the formation of cancer cells from mutations in the DNA.
- Edible mushrooms can be used for environmental protection and ecosystem cleaning.

- They analyze the wood residues from forest trees and play a key role in the life of forests, preserving the heat in the original forest.

5.5.Cultivation of edible mushrooms

- The number of mushrooms that can be cultivated is approximately 100 species. Most of them are saprophytes, such as the button mushroom (*Agaricus bisporus*) and oyster mushrooms (*Pleurotus ostreatus*).
- The main species are cultivated on a variety of organic substrates, including waste from cotton and coffee production.

5.5.1. Button Mushroom (*Agaricus bisporus*).

Agaricus bisporus is the wild form of the Button Mushroom. It is found in commerce in various forms, with the most common being small-sized and white. The Button Mushroom is cultivated on a large scale for consumption in natural caves dug into limestone hillsides, where temperature and humidity remain very constant (figure 32).

Figure 32 : Button Mushroom '*Agaricus biosporus*'.

a- Lifestyle

The Button Mushroom is a saprophyte; its carbon nutrition is derived from carbohydrate compounds.

Its nitrogen nutrition occurs either from organic nitrogen (amino acids and peptides) or from mineral nitrogen (ammonia or urea). On the other hand, an excessively high level of ammoniacal nitrogen is detrimental to it. The optimal growth of the mycelium occurs at 22-25°C, while that of the stalk and the mushroom is optimal at 15-10°C.

5.5.2. The yellow Oyster Mushroom (*Pleurotus citrinopileatus*).

- This variety is primarily known for its astonishing color.
- It prefers warmer temperatures ranging from 18°C to 30°C and grows in Asian tropical forests (China, Japan, Vietnam, etc.).
- It typically forms large clusters of fifty mushrooms or more, with a golden to yellow cap and a white stem.
- The mushroom cap is much smaller than that of other oyster mushrooms.
- It has a pronounced hazelnut flavor. This mushroom is very delicate and breaks easily if not handled with care, so a gentle touch is required during harvest.
- They are also highly perishable and should be consumed a day or two after harvest, as they tend to lose their yellow colors fairly quickly.

Nutritional value

- Oyster mushrooms are an excellent source of proteins, containing five times more than most vegetables. They are also rich in vitamin B and copper.

5.5.2.1.Production process of Oyster Mushrooms on an Industrial Scale:

There are 6 steps to follow: **1.Composting 2.Pasteurization 3. Inoculation 4.Incubation 5.Casing 6. Harvest.**

1. Composting

The preparation of the substrate.

- The **substrate** is the food for the mushrooms.
- In cultivated mushrooms, a distinction is generally made between **compost or casing mushrooms** (such as **Button Mushrooms**, Shaggy Ink Caps, etc.) and **lignivorous mushrooms that decompose wood and, more broadly, lignocellulosic materials** (**Oyster Mushrooms**, Shiitakes,...).
- **Oyster mushrooms** can be cultivated on **cereal straw or on wood (logs, wood chips, sawdust).**

The preparation of the substrate : involves possible **grinding, mixing with different materials, humidification**, and generally **pasteurization/sterilization.**



2. Pasteurization

This process allows for the elimination of some of the naturally occurring microorganisms in the substrate that would compete with the chosen mushroom.

- ✓ Pasteurization is generally done by heat, either by immersing the compost in water at 75°C for 1 hour, for example, or using steam.
- ✓ It can also be done by soaking for 12 hours in a bath of cold water and slaked lime. The abrupt change in pH (10-12) will eliminate some competitors and create a more favorable environment for the development of oyster mushroom mycelium.

3.Inoculation

- Inoculation, or seeding, simply means the seeding of the substrate.
- The seeds or "mushroom spawn" are typically composed of grains or sawdust colonized by mycelium.
- The production of spawn is the most delicate step in mushroom production as it requires very high levels of cleanliness and sterility.
- The more suitable and well-pasteurized the substrate is for the mushroom, the less mycelium needs to be added. Conversely, a poorly or unpasteurized substrate

significantly increases the risk of contamination. This means the appearance of other microorganisms (bacteria, molds), and therefore the seeding rate will be higher.

4- 5) incubation and Casing

This is the stage where the mycelium colonizes the substrate.

- It transitions from the grains distributed in the substrate to the straw or wood sawdust. To achieve this, it produces a multitude of enzymes specific to each variety and each substrate.
- At this stage, its metabolism generates heat and produces CO2. The higher the seeding rate, the faster the colonization, and the reduced risk of contamination.
- Indoors, the optimal incubation temperature is between 20 and 25°C. In nature, it can be cooler, and the incubation period will be extended.
- Along with fermentation, the addition of limestone to composts promotes above-ground growth at the expense of underground growth.

Exemple : Example: indoors at 22°C, oyster mushroom mycelium colonizes a straw substrate in approximately 2 weeks and a sawdust substrate in approximately 3 weeks.

4) Fruiting

- Oyster mushrooms **automatically fruit** as soon as they **have finished colonizing their substrate** (most **mycelium produce their fruits**, i.e., mushrooms).
- Fruiting requires a **very high humidity level for the mushrooms to develop properly**.

6) Harvesting

- The number of harvests on a substrate depends on the variety, the substrate, and the producer's decision to keep or discard the substrate for an extended period.
- Productivity decreases with each harvest, so producers typically harvest 2 to 3 times from one substrate.
- The quantity varies depending on the variety, substrate, and producer. However, it is estimated that oyster mushrooms generally yield between 15 and 25% fresh mushrooms compared to the initial moist substrate, over 2-3 harvests.

6. Fermented foods: Fermented meat, Cheese, Coffee, Pickles, Sauerkraut….etc

6.1.Definition

Scientists have defined fermented foods as those produced through selected microbial growth and the conversion of food components through enzymatic reactions.

These foods are not new; they have been part of our diet for thousands of years.

- ❖ Microorganisms are employed in food production because they enable the preservation of unprocessed products such as milk, meat, or vegetables, which have a limited shelf life.

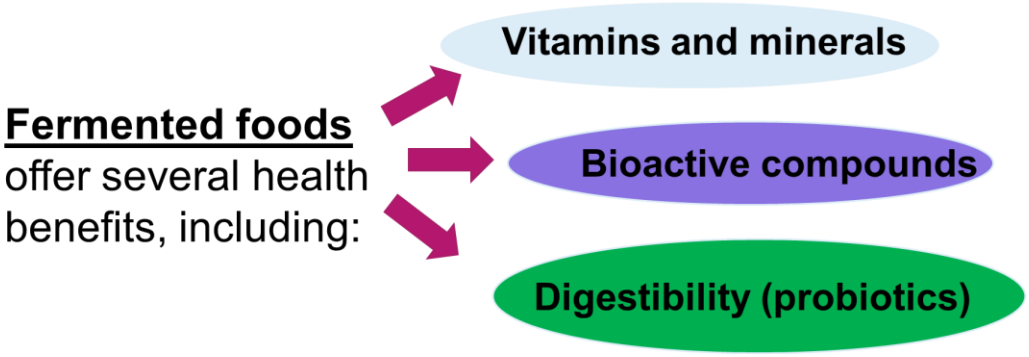
Fermented foods without live cultures (inactivated or removed during production):

- Bread, including sourdough (baked)
- Pickled foods and long-lasting fermented vegetables (thermally processed)
- Sausages (thermally processed)
- Soy sauce (thermally processed)
- Vinegar (thermally processed)
- Wine, most beers, distilled spirits (filtered)
- Coffee beans and cocoa beans (roasted)

Non-fermented foods (No use of live cultures in the production):

- Fresh sausages
- Vegetables in brine or vinegar
- Soy sauce made by a chemical process
- Non-fermented deli meats and fish
- Acidified cottage cheese

Beneficial effects of fermentation



A brief history of preservation :

- It is through observation that humans learn to preserve their food for longer periods, noting the effects of drying and cold storage.
- Between antiquity and the 18th century, various preservation methods such as drying, salting, smoking, cooking, coating, refrigeration, and fermentation were discovered and developed.
- Between 1800 and 1880, the industrial revolution brought about new discoveries, including sterilization.
- Lactic fermentation is one of the fermentations used in the food industry. It allows the production of foods such as sauerkraut and charcuterie.

- Various bacteria are responsible for lactic fermentation, such as *Lactobacillus plantarum* and *Lactobacillus cucumeris* for cabbage, which are naturally present on the leaves.

6.2.Fermented cabbage (Sauerkraut)

Fermented Cabbage (Sauerkraut):

Sauerkraut's origins trace back to **China**, where it was used to **feed workers during the construction of the Great Wall.**

- The **cabbage is finely sliced** into **thin julienne strips and ferments in a brine mixture of water and salt in a barrel** (**wooden or stoneware**). Each layer is about 20 cm thick, separated by a layer of salt (2.5-3% of the weight). A **wooden board seals the container to isolate it from air, and a stone compresses the mixture.**
- The **brine is more concentrated than seawater**.
- In traditional methods, production lasts between **6 and 8 weeks**. In the industry, the process is **expedited by agitation with white vinegar.**



6.3.Deli meats

It was mainly developed by the Romans, and there are various processes for making ham and sausage, including drying and salting.

Wet salt is used to prevent excessive and rapid water loss, followed by dry salt.

. The proportions are 2kg of salt for 10kg of meat.

- In the first few weeks, the meat is rubbed 4 to 5 times every 8 days.
- Depending on the aging process, fermentation lasts from 6 to 18 months.
- During this time, hams are moved to rooms with different temperatures and humidity levels.
- The ferments act for 6 months, creating an acidic environment. They need water to develop, and they use the water in the cellular environment. However, with salting, this water becomes a hypertonic solution that lyses the bacteria.

- In addition to aiding in the preservation of the meat, bacteria synthesize various aroma compounds through their lipolytic activity, contributing to the organoleptic qualities of the product.

6.4.Lacto-fermented vegetables

- Lactic fermentation is not only used to preserve dairy products but also for preserving mushrooms and various vegetables such as cabbage, beets, carrots, beans, onions, etc.
- This technique involves preserving vegetables by promoting the development of lactic acid bacteria, which acidify the environment and thus inhibit the growth of other undesirable organisms.
- Thus, vegetables must provide sugar, B-group vitamins, and minerals.
- As fermentation takes place in an anaerobic environment, vegetables are often covered with saltwater (the salt inhibits bacteria responsible for vegetable decomposition).

The fermentation occurs in 3 phases:

1. Pre-fermentation, lasting 2-3 days during which numerous species of microorganisms develop, leading to the decomposition and softening of vegetables.
2. Fermentation, which begins when lactic acid bacteria dominate other microorganisms.
3. Storage, when the pH drops below 4. Undesirable microorganisms can no longer develop, and new flavors emerge.

- The vegetables can then be stored for at least a year, even if the temperature rises above 10°C.
- This preservation method is not only economical as it requires no energy input but also beneficial for health, as lactic acid bacteria simultaneously produce numerous vitamins, and lactic acid has many digestive virtues.

6.5.Chocolate

An essential step in the development of chocolate flavors is the fermentation of cocoa beans, where aroma molecule precursors are synthesized. This process lasts for 4 to 5 days.

When the beans are removed from the pods, they are surrounded by a whitish envelope, the pulp, which contains yeasts, sugar, and bacteria (figure 33).

- First, the yeasts perform anaerobic alcoholic fermentation. This action leads to the degradation of the pulp.
- Next, the bacteria undergo acetic fermentation using the previously produced alcohol to produce aerobic acetic acid.
- The acetic acid penetrates the bean and activates enzymes that degrade sugar and proteins, which will be the precursors of chocolate aromas.
- To stop fermentation, the beans are dried to eliminate acetic acid and water.
- The chocolate flavors are revealed during roasting, where the precursors synthesized during fermentation combine and create new volatile molecules: the aromas, under the influence of heat.

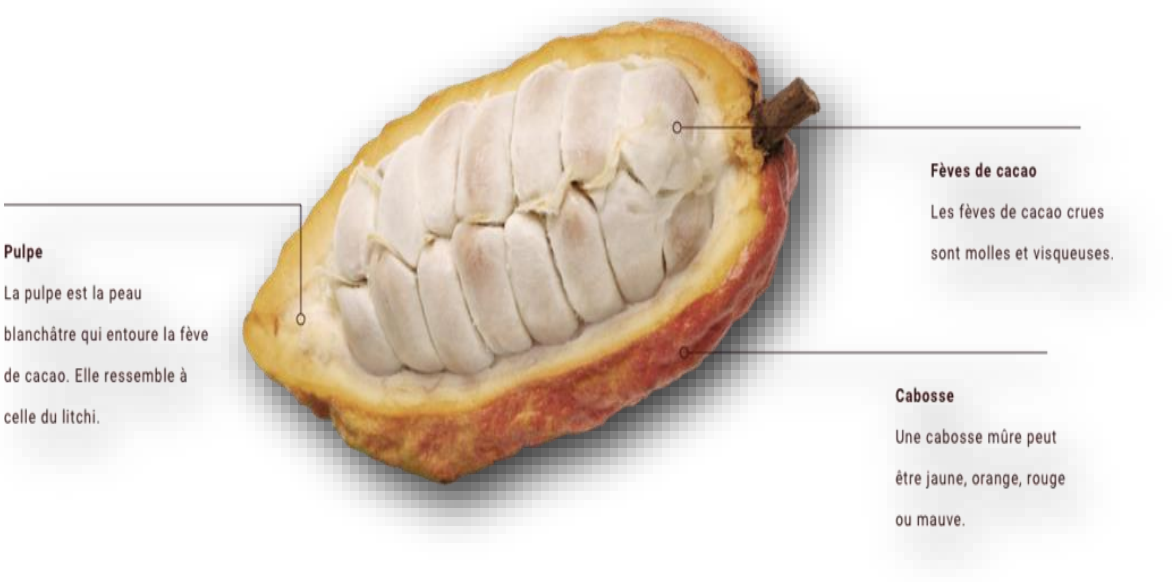


Figure 33 : Chocolate bean composition.

6.6.Coffee

The coffee bean is protected by the mucilage, pulp, and skin of the cherry.

- After harvesting, fermentation is the first transformation step that coffee undergoes. During this process, the mucilage is removed, leaving only the green coffee bean.
- Fermentation is primarily the process in which microorganisms (bacteria and yeasts) contribute to the degradation of sugars in the mucilage. While they play a significant role through the production of enzymes, alcohols, and acids.
- The fermentation process has three main variants: the washed, natural, and honey processes.

6.6.1. Washed Coffee (Wet Process) :

Washed coffee is the type of coffee that we are all familiar with, as it is the preferred fermentation method for producers in Central and South America and has been historically used by roasters for taste uniformity.

- This method involves completely removing the pulp and mucilage before drying the beans.
- Fermentation occurs as the depulped beans are placed in fermentation tanks. These are then usually rinced, dried, decorticated and roasted (at temperatures ranging from 180 to 220°C) and packaged for consumption.
- Washed coffees are known for their freshness and aromatic complexity, as well as a bright acidity and medium body (figure 34).

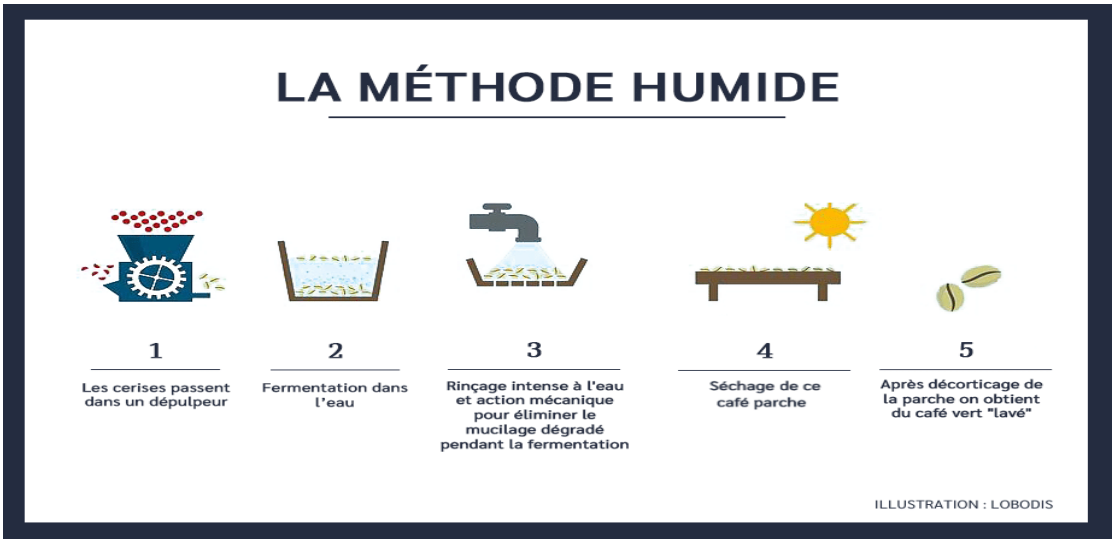


Figure 34 : Washed coffee preparation method

6.6.2. Natural Coffee (Dry or Natural Process) :

The coffee cherry is first dried before the fruit surrounding the bean is removed.

- This means that the coffee bean is in contact with the coffee cherry throughout the entire drying process and ferments directly within the coffee cherry.
- Traditionally, the cherries are dried on raised beds (African beds) or drying patios, and fermentation takes place until they are completely dry.
- This extended contact time allows for the transfer of organic compounds between different layers of the coffee cherry, causing the bean to absorb and acquire certain characteristics of the fruit.
- Naturally processed coffee tends to have a more fruity taste and a fuller body because the dried coffee bean has absorbed some of the sugars from the surrounding fruit.
- This method is faster and significantly reduces water usage, addressing issues associated with wastewater disposal (figure 35).

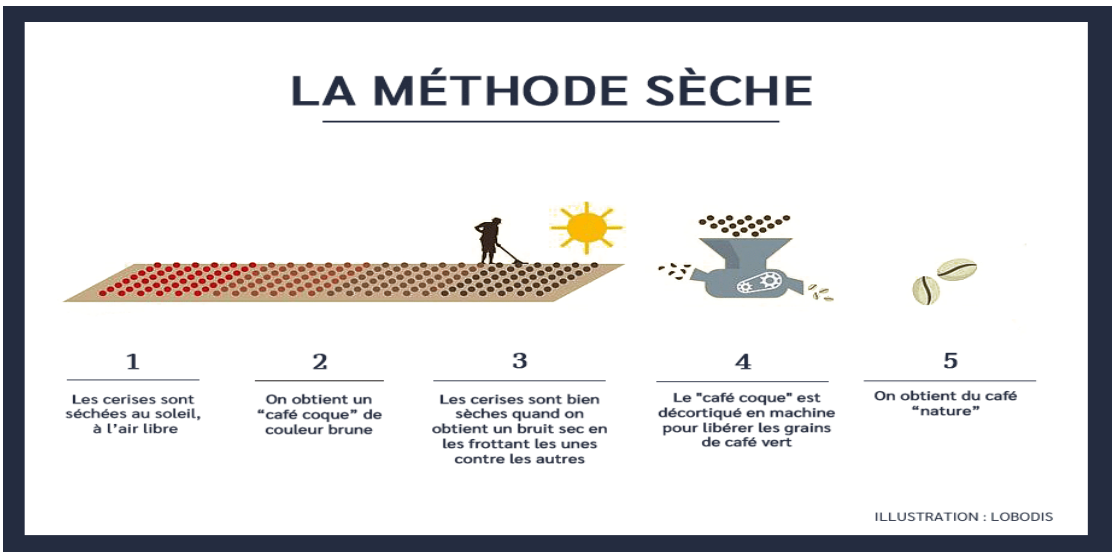


Figure 35 : Natural coffee preparation method

6.6.3. Honey Coffee (Honey process) :

The "honey" method is a process that owes much of its development to Costa Rica. The honey process gets its name from the mucilage, which has a texture similar to honey—viscous and sweet.

- The honey fermentation is a hybrid between the washed and natural processes. In this method, the coffee is stripped of its flesh (the pulp of the cherry), allowed to ferment in tanks, and then dried without rinsing off the mucilage from the bean.
- These coffees exhibit a medium to high acidity (similar to washed coffees) and have a more pronounced body and sweetness (like natural coffees).
- They strike a balance between flavor complexity and sweetness, offering a profile that is both complex, sweet, and has a gentle acidity.
- There are various variations such as white, yellow, gold, red, and black honey coffees, depending on the percentage of mucilage left on the beans during the drying process, which also coincides with the fermentation period (figure 36).

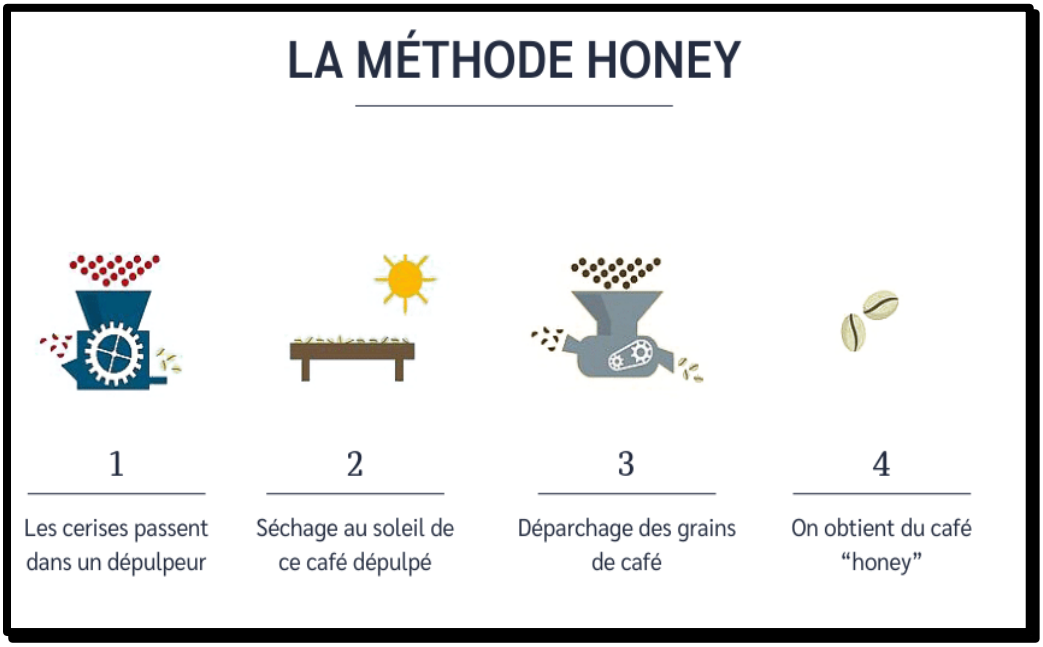


Figure 36 : Honey coffee preparation method

7. Dairy Products (Milk and its Derivatives)

7.1.Milk

Milk can be consumed in various forms and textures, such as fresh milk, semi-skimmed milk, or powdered milk. Its original flora includes *micrococcus sp., lactobacillus, streptococcus, lactococcus.*

7.1.1. Microbial activities in Milk

· Acidification : Microorganisms (lactic acid bacteria), through the action of β-galactosidase, hydrolyze the lactose in milk to produce glucose or galactose.

· Gas Production: Hetero-fermentative lactic acid bacteria from the soil can produce gases.

· Alcohol Production: Yeasts transform lactose into alcohol.

7.1.2. Milk Manufacturing Process

The milk manufacturing process begins with the milking of selected dairy cows chosen for the quality and quantity of their milk.

- The collected liters of milk are then refrigerated and analyzed before being transported in an insulated tanker truck from the farm to the dairy.
- At the dairy, the milk undergoes several processes:

❑ Pasteurization : This process eliminates undesirable microorganisms harmful to humans. The milk is heated for 15 seconds at 72°C.

❑ Skimming : It involves separating the cream and the milk using a cream separator that quickly mixes them. Cream (in varying amounts depending on the desired type of milk) is then added to a mixing tank.

❑ Sterilization : The milk is heated to 140°C using steam for 2 seconds, effectively destroying all microorganisms.

❑ Packaging : The milk is packaged in cartons or bottles to protect it from air and light, ensuring better preservation.

7.2. Yogurt (or Yoghurt)

Yogurt is a milk product fermented by two lactic acid bacteria (*Streptococcus thermophilus* living in symbiosis with *Lactobacillus bulgaricus*).

- When milk is subjected to the influence of lactic ferments, it can coagulate to form a gel. Lactic ferments break down the lactose present in the milk, transforming it into lactate. As a result, the acidity of the milk increases, forming aggregates of proteins in aqueous suspension and leading to the coagulation that results in this gel (curd).
- This gel can yield two types of products: fresh cheeses and yogurts.
- Yogurts differ from fresh cheeses by the absence of gel fragmentation followed by draining (removing the aqueous phase).

Quality control of Milk

- Pumped and filtered: elimination of residues.
- Analyses of its fat content and acidity.

Standardization

- 1.Standardization of fat content
- 2.Enrichment in milk proteins
- 3. Addition of sugar.

Lactic Fermentation or Inoculation: addition of 2 fundamental ferments:

- ***Lactobacillus bulgaricus*** for acidity
- ***Streptococcus thermophilus*** for aroma development.

Incubation:

- Incubation at 42°C to allow fermentation for 3 to 6h.

1. Yogurt is obtained by prolonged boiling of whole or semi-skimmed milk, which allows for sterilization and concentration.

2. The milk is then cooled to 45°C and inoculated with a pure culture or starter. The milk is maintained at this temperature in the incubator until the beginning of coagulation (between 3 and 6 hours), allowing the cultures to develop and transform the milk.
3. Finally, it is chilled to stabilize it.
4. The bacteria present must still be alive (10^7 bacteria/g of product) at the time of yogurt consumption, promoting better digestion and transit.
5. Additionally, there are different types of yogurt: firm, stirred, and drinkable, which differ, notably, in the time and temperature of incubation.

They are characterized by their lactic acid content: firm yogurts have a content of 0.75%, and stirred yogurts have a content of 1.2%.

7.3.Cheese

Cheese is a food product made from coagulated milk derived exclusively from dairy sources (whole milk, partially or fully skimmed milk, etc.), which may be mixed or not through the action of rennet.

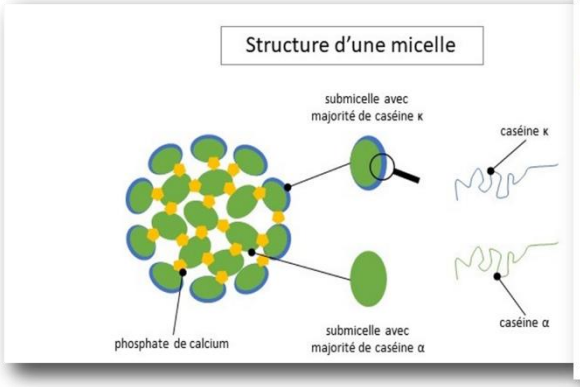
- Cheese is a fermented or non-fermented, aged or non-aged product.
- Depending on the milk's origin, we talk about cow cheese, sheep cheese, goat cheese, but also other mammals such as buffalo, camel, etc.
- We can distinguish at least four major families: hard cheeses, pressed cheeses, soft cheeses, and fresh cheeses.
- The texture will depend on the speed of acidification progression, and the flavor will depend on the metabolism of the used ferments.

- **Involvement of microorganisms in the cheese manufacturing process:**

The cheese-making process, whether industrial or artisanal, involves several manufacturing stages that can vary depending on the type of cheese being produced:

In the cheese manufacturing process, **after pasteurization of the milk**, similar to **the production of yogurt,** it is **coagulated (curdled) by adding rennet and lactic ferments** (such as ***streptococci, lactobacilli, leuconostocs, lactococci***...).

❖ Rennet is a solution composed of two **proteolytic enzymes: chymosin** (whose optimal activity occurs at **pH=3**, hence the importance of acidification before) and **pepsin**. These enzymes **facilitate the solidification** and **cohesion of the milk proteins**, **specifically the casein micelles**.



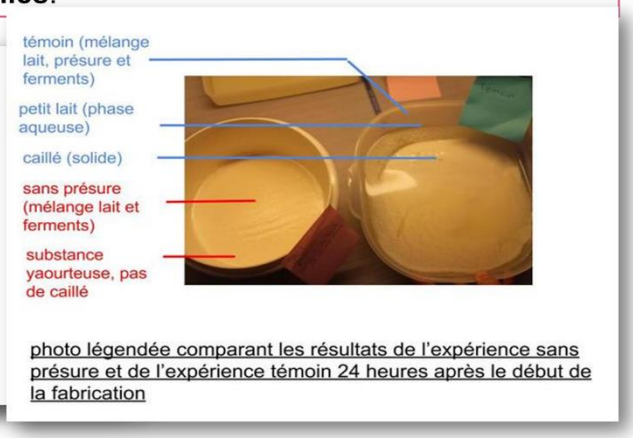


photo légendée comparant les résultats de l'expérience sans présure et de l'expérience témoin 24 heures après le début de la fabrication

- Draining: Once coagulated, the milk is **separated from the whey** (the **liquid part resulting from the coagulation of milk**), which helps **extend its shelf life** (an optional step performed depending on the type of cheese).

- Molding: It is **transferred into molds** that vary depending on the type of cheese. After this step, **fresh and white cheeses are consumed directly**.

- Pressing: The cheeses are **unmolded to remove excess water**. This step is not **mandatory and depends** on **the type of cheese you want to obtain.**

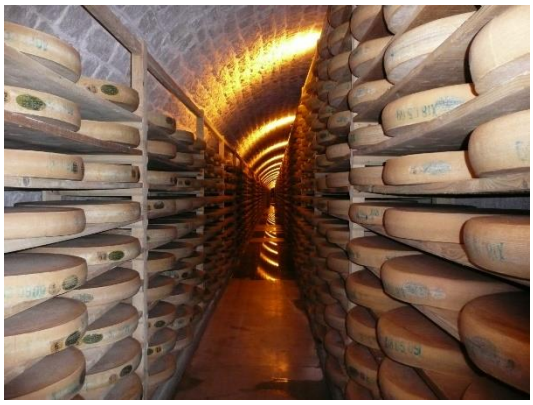
- Salting: the cheese is immersed in brine or salted directly through surface sprinkling.

The salt will:

- absorb the remaining moisture from the cheese and thus complete its draining.
- give the cheese its appearance and part of its aroma since it solidifies the crust.
- preserve the cheese and allow for a selection of the microflora in and on the cheese.

- inhibit the growth of certain microorganisms on the surface of the cheese, such as *Mucor*.

- The final step is maturation: which is a ripening period allowing for the physico-chemical evolution of cheeses and is characteristic of their tastes, flavors, appearances, and textures. The duration of maturation varies and can last from a few days to several months. Maturation takes place in a cellar.

Maturation

- Propionic bacteria come into play following lactic acid bacteria since they perform propionic fermentation, transforming lactic acid into propionic acid: 3 lactates → 2 propionates + 1 acetate + 1 CO_2 + 1 H_2O
- These bacteria, such as *Propionibacterium freudenreichii*, play a crucial role in the production of cooked-curd cheeses like Gruyère because they release carbon dioxide, responsible for the "holes" in Gruyère.
- The acetate and propionate produced contribute to the hazelnut flavor of Gruyère.
- Molds have similar roles to yeasts and play an essential role during the maturation of several cheeses as they enable the formation of the surface crust.
- *Penicillium candidum* develops first (from the 4th to the 7th day), forming a white mycelial layer, followed by *Penicillium album* and then *glaucum*, forming a blue mycelial layer…
- The work in the cheese cellar involves controlling and directing the development of these ripening agents.

This process varies based on the type of cheese one wants to make. For example:

- For bloomy-rind cheeses like Camembert, *Penicillium camembertii* is allowed to develop, giving the cheese a fluffy white appearance.
- For washed-rind cheeses like Munster, the cheese is washed with salted water and brushed to inhibit the growth of certain microorganisms (*Penicillium*) and promote the development of other surface bacteria (*Brevibacterium linens*, which imparts an orange color to the cheese).
- For blue-veined cheeses (blue cheese like Roquefort), the cheese is pierced to provide oxygen to the internal molds (*Penicillium roqueforti*) necessary for their development.
- Each type of cheese, for successful aging, requires a specific atmospheric environment (temperature, humidity). This makes it impossible to age different cheeses in the same cheese cellar (figure 37).

Figure : Steps in cheese making.

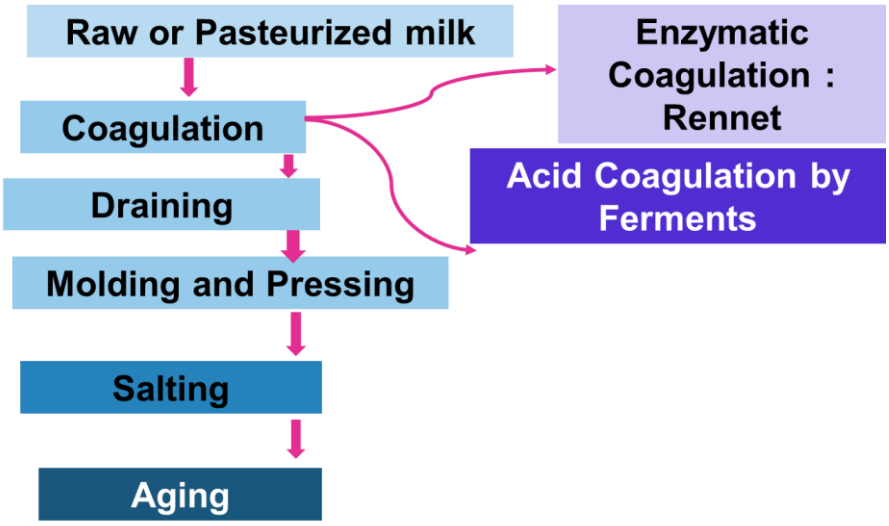


Figure 37 : Steps in cheese making.

7.4.Butters

Butters are obtained by churning cream or milk (removing the part of the milk where butterfat globules accumulate, leaving a product with the same composition as milk but with more fat).

- They contain lactic acid bacteria and saprophytic microorganisms (yeasts, molds).
- Butter is naturally yellow, but the color intensifies under the influence of heat.

- To qualify as butter, it must have a maximum water content of 16% in at least 82% dairy-origin fat.
- This fat consists of 500 different fatty acids associated with glycerol, forming triglycerides.
- Butter also contains vitamins A, D, and E (making it very energetic), and cholesterol (which can lead to cardiovascular diseases if consumed excessively).
- Due to its sensitivity to air oxidation, butter cannot be stored for a long time. To limit rancidity, it should be stored in the refrigerator in a sealed package, protected from air and light.

Chapter IV : Genetically modified microoganisms

Objectives

At the end of this chapter, the student will be able to:

- Explain genetically modified microorganisms.
- Describe human growth hormones and their compositions. Sketch blood proteins.

1. Human growth hormones

1.1.Introduction

Due to the limitations of chemical synthesis in producing certain macromolecules for therapeutic purposes, alternative strategies were developed to obtain large quantities of these molecules to meet a wide range of needs while ensuring satisfactory quality.

- This led to the emergence of therapeutic genetically modified organisms (GMOs) production.
- Currently, around 80 active principles are produced by culturing prokaryotic cells (e.g., *E. coli*) or eukaryotic cells (e.g., Chinese hamster) for therapeutic purposes. Most of these molecules couldn't be obtained through chemical synthesis, and some of them enable the treatment of incurable diseases.
- Today, it is possible to reprogram the genetic code of a microorganism by inserting a specific gene, allowing it to produce valuable recombinant proteins for medical purposes (e.g., interferon, insulin, growth hormone…) or industrial applications (e.g., enzymes…).

1.2.History

- The history of genetically modified organisms (GMOs) didn't happen overnight. It began with the discovery of the DNA molecule in 1944, followed by the revelation of its structure in 1953 by Crick and Watson and its crucial role in determining individual characteristics.

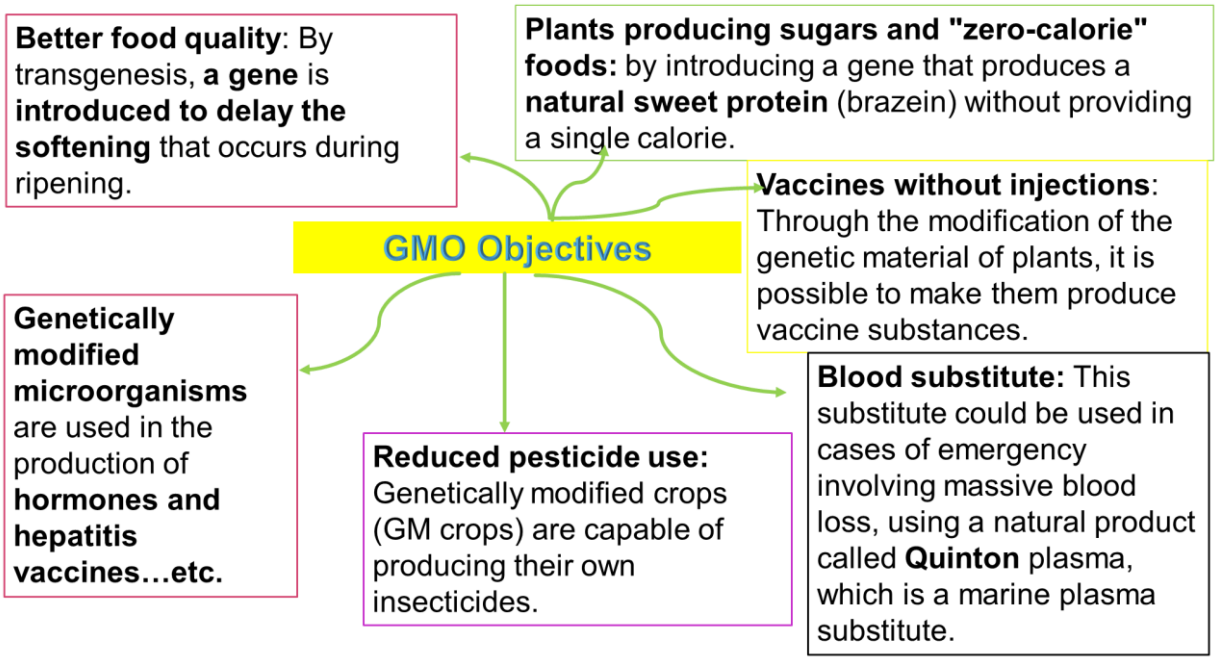
- What's novel about GMOs is the introduction of an additional foreign gene to enhance or modify an organism.
- The first significant step in this direction was taken in 1972 when an American team successfully hybridized simian DNA with that of a bacterium.
- In human applications, two human genes were introduced into bacteria to produce hormones: somatotropin in 1977 and insulin in 1978.
- It wasn't until the 1990s that transgenic products began to appear on the market, first in the United States and Canada, and then more cautiously in Europe (such as genetically modified canola, soy, and maize).

1.3.Definition

- The acronym GMO stands for "Genetically Modified Organism."
- In European regulation, a GMO is defined as "an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination" (Article 2 of Directive 2001/18).
- This involves genetic engineering techniques that allow for the addition of new genes, deletion, or modification of genes already present in the organism, typically to confer new characteristics.
- Genetic engineering techniques can be applied to animals, plants, or microorganisms. The introduced genes can come from any organism, including viruses, bacteria, yeast, fungi, plants, or animals, due to the universality of the genetic code.
- The applications of GMO techniques are broad, spanning from agriculture to industry and the medical field.
- Genetic engineering has been used to develop microorganisms that produce human proteins.
- Blood proteins and hormones are typically present in animals in small quantities, and purifying proteins from tissues, glands, or blood can be difficult and costly.

- Genetically modified bacteria grow rapidly in the presence of simple nutrients and serve as potential sources of blood proteins, hormones, and other proteins in unlimited quantities.
- The initial contribution of this technology to medicine was making therapeutic proteins such as insulin and human growth hormone (HGH) available in sufficient quantities.
- Medicine has gained many other benefits from recombinant DNA technology, including vaccines and therapeutic agents (gene therapies).
- Recombinant DNA technology has also impacted other products of interest in the agri-food and industrial sectors, including enzymes (rennet, proteases), amino acids (glutamate, succinate), and vitamins (B2, B12, C…etc).

1.4. GMO objectives



1.5.Examples of industrial hormone production

1.5.1. Human growth hormone (somatotropin)

- Growth hormone is a 191-amino acid protein produced by the pituitary gland that regulates growth and development. It acts on target cells that have receptors specifically binding to the hormone.

➨ Various pathologies are related to this hormone: dwarfism (in case of secretion deficiency), gigantism, and acromegaly (in case of excessive secretion).

* If this deficiency is diagnosed early enough, children can be given regular growth hormone injections that stimulate their growth so that they can eventually attain nearly normal heights (figure 38).

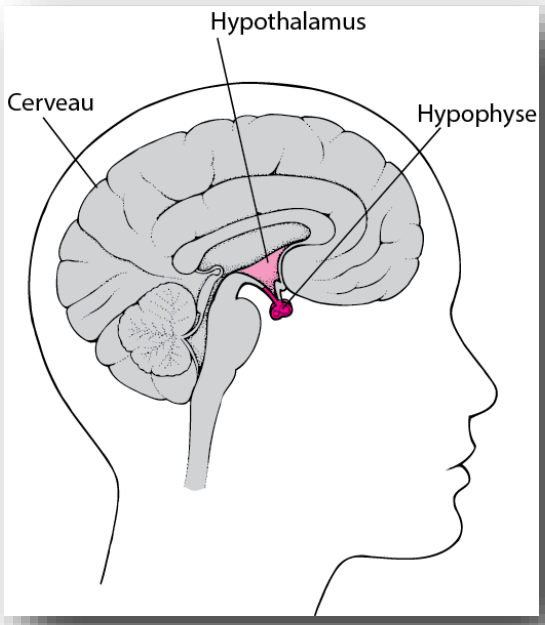


Figure 38 : Place of the human pituitary gland in relation to the encephalon.

http://www.gnis-pedagogie.org

1.5.2. Origine of Human Growth Hormone

Since animal growth hormones proved to be ineffective, human growth hormone (HGH) had to be used, extracted from the pituitary glands of deceased humans. Until 1985, it was manufactured through post-mortem brain extraction from human cadavers. This method had two drawbacks:

- The quantity of hormones obtained through this method was insufficient to treat all cases ;
- These human samples could potentially contain infectious agents such as viruses, bacteria, or prions (PROteinaceous INfectious particle) (e.g., Creutzfeldt-Jakob, HIV...).

- In 1987, the mass production of hormones was achieved using genetically modified bacteria, which means they contained the human growth gene.

Manufacturing Process of Growth Hormone

Step 1: Identification and isolation of the human DNA fragment containing the growth hormone gene (GH-1).

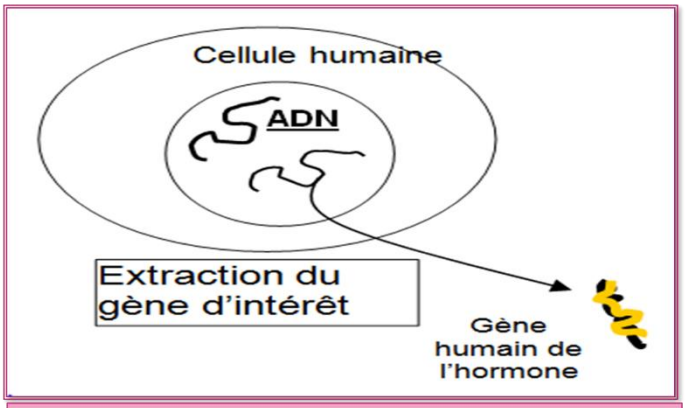


Figure: Gene Extraction.

Step 2 :

Insertion of the human DNA fragment into a plasmid (creation of a gene construct).

Introduction of the modified plasmid into the bacterium *Escherichia coli* (amplification of the gene construct).

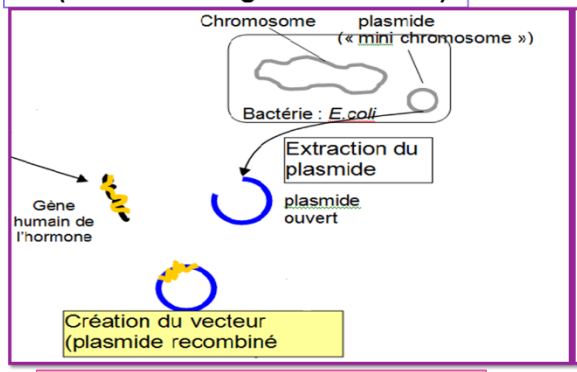


Figure : Creation of a recombinant vector.

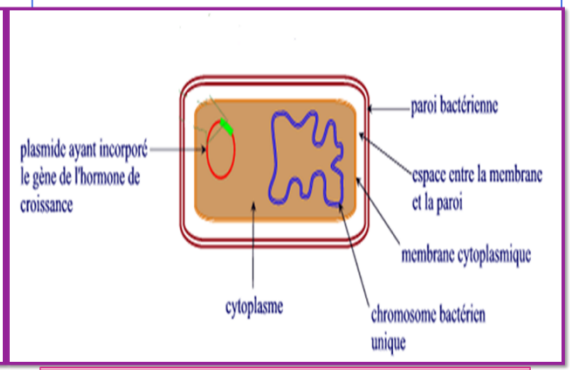


Figure : Transfer of the recombinant plasmid.

Step 3 : The culture (fermentation)

- Bacterial cells are initially multiplied in small bioreactors (fermenters) containing a nutrient solution. It is during this phase that the actual production of growth hormone takes place, after controlling temperature, pressure, oxygenation, and other parameters.
- The duration of multiplication depends on the cell growth cycle.
- *E. coli* cells divide every 20 minutes. In the span of 24 hours and under ideal conditions, a single cell can produce 4.7 x 10^{21} descendants.
- The hormone is released beyond the cytoplasmic membrane and appears to be identical to human growth hormone.

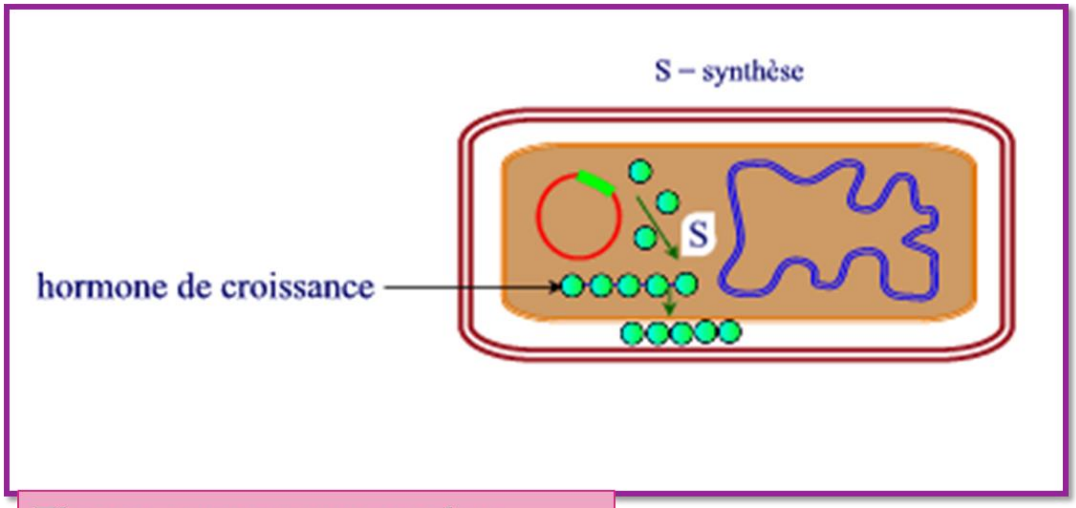


Figure : gene expression.

- A single tank containing 500 liters produces the same amount of growth hormone as 35,000 human pituitary glands.

Step 4 : Purification

- The aim of this purification process **is to extract the product and eliminate all so-called contaminants**, including those from the producing cells, the **raw materials used (reactants, culture medium...),** as well as **degradation products**.
- The hormone can be **recovered without destroying the bacteria**, which **avoids the risk of contamination by molecules or viruses** contained within the bacteria.
- In fact, through this process, the **hormone is not released into the external environment**; it **accumulates in a space located between the cytoplasmic membrane and the cell wall.** It is then sufficient to **dissolve this wall to recover the growth hormone**.

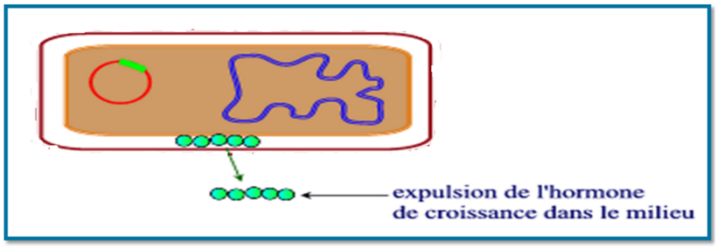
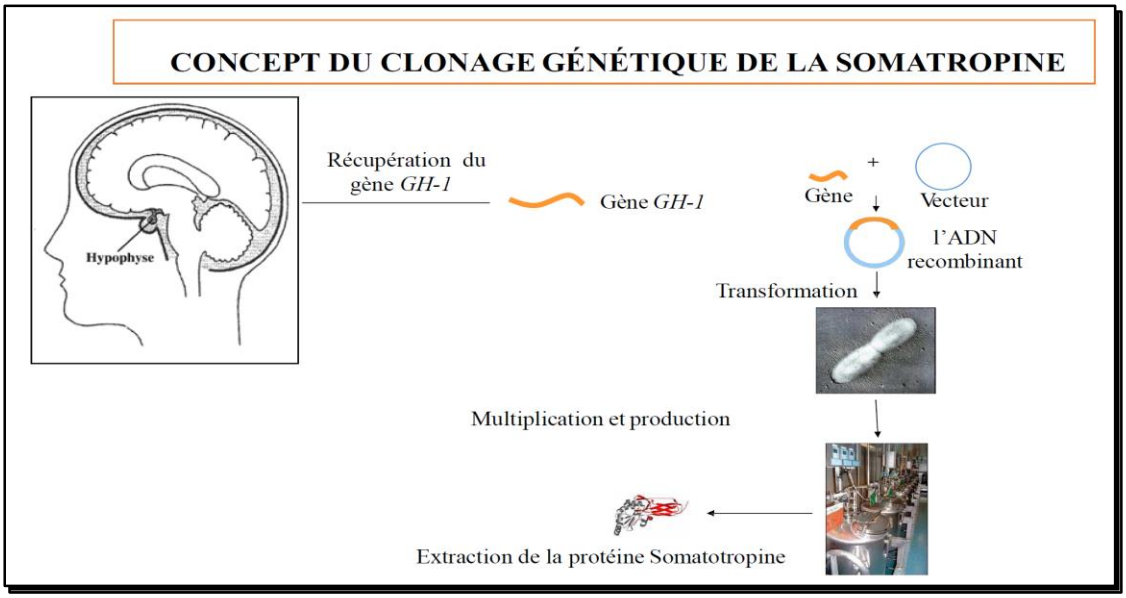


Figure : Growth hormone extraction.



1.5.2. The pharmaceutical formulation

- ✓ This step involves incorporating the therapeutic protein into a form that allows for its administration to the patient.
- ✓ Only a few large global pharmaceutical companies have the means to produce growth hormone.

- ✓ It is then available in injectable form, but obtaining a growth hormone treatment is expensive (approximately 1500 euros per month) and can only be obtained with a medical prescription.
- ✓ To do this, it is necessary to develop a formula with excipients whose role is to ensure the best possible solubilization of the protein and the maintenance of its physical and chemical integrity.

a- Interest of GH-1 gene cloning

Currently, biotechnology industries produce growth hormone through genetic engineering using the bacteria *Escherichia coli*.

Qualitative advantage

Production of a pure hormone, uncontaminated by viruses or prions.

Quantitative advantage

A 500-liter tank of bacteria produces the same quantity of growth hormone as 35,000 human pituitary glands.

2. Blood proteins and Interferons

2. 1.Blood and Plasma Proteins (Blood Proteins)

General information

Blood is the most extensively studied biological fluid. It is very rich in proteins, comprising 22% of its composition. Blood accounts for 6 to 8% of body weight (in a 70 kg adult, this is approximately 5 kg). Its volume is close to 5 liters.

2.1.1. Blood plasma

Plasma constitutes the liquid portion of blood, accounting for 55% of blood volume.

- ❖ It serves to transport blood cells, hormones, and numerous proteins essential for regulating physiological processes in the body, including growth, inflammation, and coagulation...etc.

  1.2. Albumin (≈40 g/l) and immunoglobulins (≈10 g/l) are the most abundant plasma proteins, representing approximately 80% of total plasma proteins .

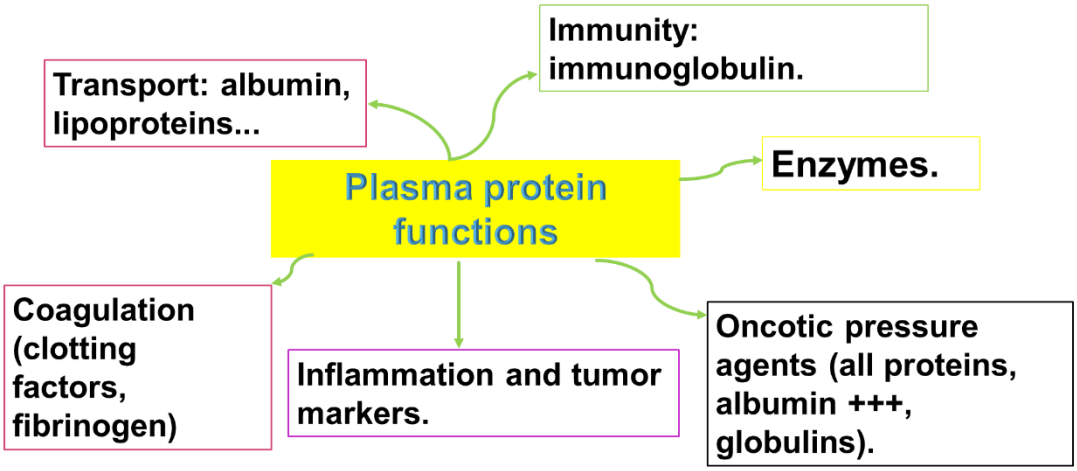
  Other proteins are present in lower quantities in plasma, such as α-1-antitrypsin (≈1 g/L) and antithrombin (<1 mg/L), which are protease inhibitors, or coagulation factors like factor VIII (a few ng/L).

Plasma serves as the raw material for the production of many therapeutic proteins used in the treatment of serious conditions such as:

- Coagulation disorders, Immune deficiencies,
- Autoimmune diseases,
- Critical patient care (e.g., hypovolemia, severe bleeding, thrombosis).

- ➢ This biological raw material is collected from donors and can be obtained either by centrifugation of whole blood (referred to as recovered plasma) or by apheresis (or plasmapheresis).
- ➢ Fractionated plasma for the production of therapeutic proteins is primarily obtained through plasmapheresis. This technique involves separating the various components of blood by centrifugation to isolate plasma, while blood cells (white blood cells, red blood cells, platelets) are returned to the donor.
- ➢ Additionally, this technique allows for the freezing of plasma just minutes after collection, which limits the risks of coagulation or fibrinolytic system activation.

2.2. Plasma protein functions



Oncotic pressure or colloido-osmotic pressure is the osmotic pressure that attracts water towards proteins.

2.3. Blood and Plasma proteins (blood proteins)

Currently, these proteins are produced by extraction from blood samples not intended for transfusion purposes. This method has certain limitations that affect the fractionation industry:

- ❑ The source is considered hazardous and variable, subject to recurrent regulatory additions that make its cost unpredictable;
- ❑ The possible contamination of sources by viruses and prions remains a major concern.
- ❖ Genetic engineering for the production of important blood proteins is currently in full development.

2.4. Fractionation and Purification of Plasma Proteins

- Ethanol fractionation remains the basic process in the modern plasma fractionation industry.
- The Cohn process (figure 39) is based on sequential ethanol precipitation of plasma proteins at low temperatures, combining the action of various parameters such as pH, ionic strength, temperature, and organic solvent concentration.
- The process flowchart describes the various steps and parameters of the fractionation process, resulting in the obtainment of five fractions enriched with different plasma proteins .

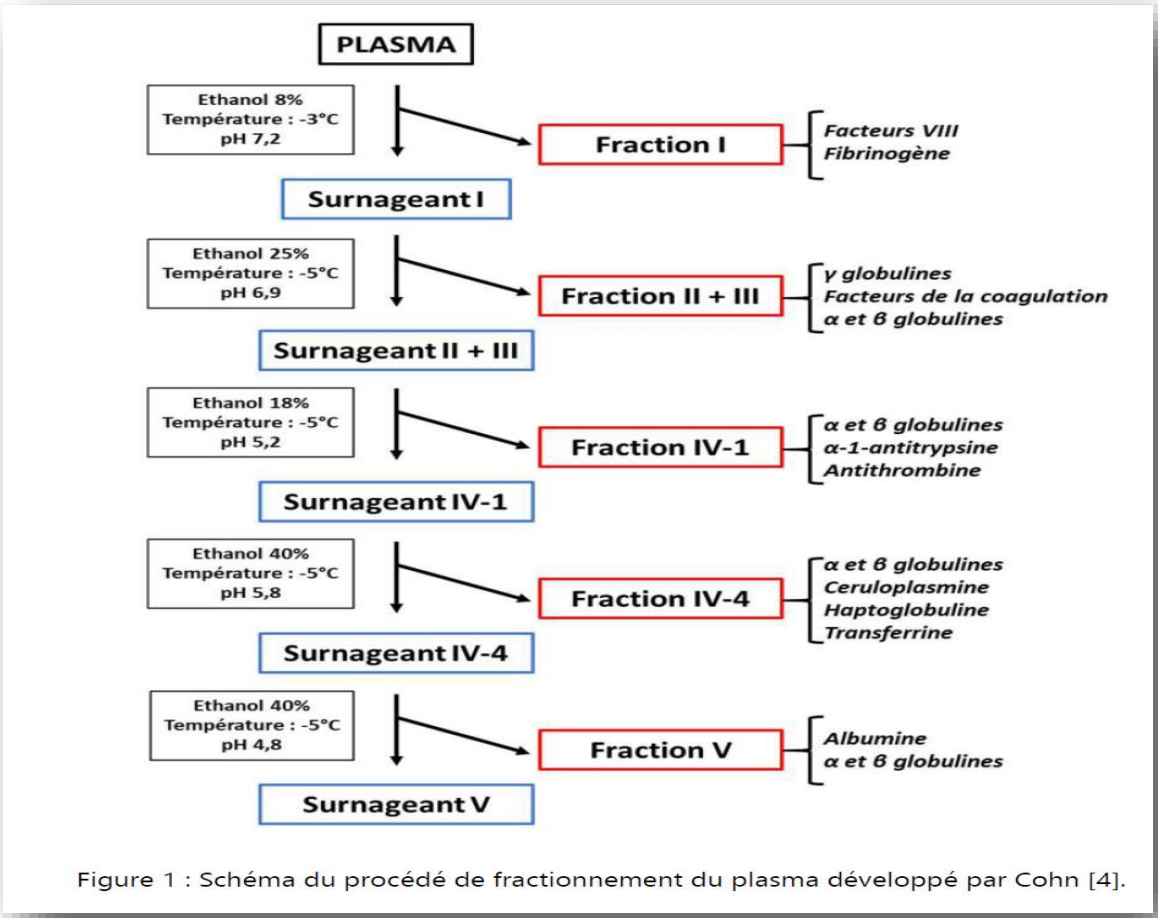


Figure 39: Diagram of the plasma fractionation process developed by Cohn.

Fraction V, obtained at the end of the process and enriched in albumin, is then the main fraction used for patient treatment.

However, the latest process improvements involve adding chromatography steps to enable the production of new proteins at high levels of purity.

- While most therapeutic plasma proteins are produced from plasma, they can also be produced using recombinant technologies in mammalian cells or transgenic animals.

- Factor VIII, Factor IX, activated Factor VII, and antithrombin are produced using these technologies and have been commercially available since the 1990s.
- The advantages of this type of production include:

✓ No risk of contamination by human viruses.

✓ Potentially lower production costs.

✓ Addressing challenges related to raw material supply (plasma procurement or low plasma concentration of the protein of interest), which can affect meeting product demand.

3. Human Albumin

- Albumin is a plasma protein used to manage hypovolemia and hypoalbuminemia that can occur during certain surgical procedures.
- It is also used as an excipient in various medications.
- It has been successfully produced in potatoes and tobacco plants, as well as in yeast *Saccharomyces cerevisiae* cultures in fermenters.

3.1. Coagulation factors

They are medications used in the treatment of hemophilia, a hereditary disease characterized by a deficiency of either factor VIII (hemophilia A) or factor IX (hemophilia B). This deficiency puts hemophiliacs at risk of severe bleeding episodes that can be life-threatening.

- More recently, synthetic coagulation factors have become available. These are factor VIII and factor IX produced using biotechnology techniques, often referred to as recombinant factors.
- Recombinant forms are gradually replacing plasma-derived concentrates.

4. Cytokines

- They are proteins naturally produced by the organs of the human immune system.
- They act on other immune system cells, modulating the body's response to disease and infection.

- Cytokines are molecules involved in intercellular communication, and there are several types of cytokines, including Interferons (IFN), Interleukins (IL-1…IL-34), and Tumor Necrosis Factor (TNF).

4.1. Interferons

Interferons constitute a complex family of natural glycoproteins induced by various stimuli in the host. These proteins are secreted by cells infected with inducing viruses and, in some cases, by other viruses as well. Some interferons are also potent anticancer agents.

- ❖ Interferons cause the inhibition of viral replication in virus-infected cells,
- ❖ the suppression of cell proliferation,
- ❖ and immunomodulatory activities such as enhancing macrophage phagocytic activity.

4.2. Interferons used in therapy

Are recombinant human proteins that possess the same properties as natural interferons, including antiviral, antimitotic, and immunomodulatory activity.

- There are two types: Interferon alfa 2a: Roferon-A; Interferon alfa 2b: Intron-A.
- They were among the first interferons registered in 1986 and are produced from a strain of *E. coli*.

They have several indications, including: Chronic Hepatitis B and C , Cutaneous T-cell lymphoma, Advanced-stage kidney cancer, Stage II malignant melanoma, and more.

5. Potential future developments

The future of genetically modified microorganisms capable of generating a wide range of molecules seems limitless.

- ✓ The purity of proteins, crucial for human health, is considerably higher than the same molecules extracted from animal tissues.
- ✓ Concerns about contamination by known or unknown viruses are also eliminated.
- ✓ Furthermore, production costs are minimized because microorganisms can multiply indefinitely in the presence of inexpensive nutrients.
- ✓ The future of this field of industrial microbiology indeed appears very promising.

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