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Educational handout

Title

Bioengineering and industrial microbiology

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Master II in Microbial biotechnology

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Preface

This educational document is designed for second-year Master's students in microbial biotechnology to acquire and provide fundamental notions in bioengineering and industrial microbiology. The objective is to enhance students' mastery of key concepts and principles applied in this field. This handout is divided into two parts:

The first part deals with the elements of biochemical engineering, it will give students operational skills in bioengineering and handling bioreactors with the study of the variability of culture processes, control of kinetics, it includes two chapters, the first gives the definition of fermentation, and the different fermentation processes and the second chapter discusses fermenters with basic notions on their hydrodynamics.

The second part is reserved for industrial microbiology which is divided into several chapters.

The third chapter provides a general overview of industrial microbiology, focusing on the main players involved: industrial microorganisms and the characteristics of strains used in industrial production, the ways of obtaining microorganisms and the strategy followed for the search for new industrial strains, starting with isolation, then selection and the identification of new industrial strains, the methods used for the improvement of industrial microorganisms such as random mutagenesis and site-directed mutagenesis, and ending with the different techniques used for the conservation of industrial strains.

The fourth chapter describes the industrial production of primary metabolites, such as organic acids (citric acid), microbial cells as a source of single-cell proteins (SCPs), methanogenic bacteria, biogas, and ethanol.

The next chapter illustrates the industrial production of secondary metabolites, including antibiotics (penicillins), enzymes (enzyme production and immobilization techniques), and toxins. The last chapter of this handout covers the fundamental notions on bioconversion and enzyme engineering.

The chosen teaching approach is predicated on clear, intelligible language that is backed up by examples. To help students better grasp the material, it is enhanced with a wealth of illustrations in the form of simplified diagrams, figures, and summary tables.

There might be some errors in my first version, therefore I would appreciate any feedback and comments from anyone who could offer suggestions. I express my gratitude to the colleagues who graciously reviewed the manuscript and offered helpful criticism to help me improve it.

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List of abbreviations

ATP: Adenosine triphosphate

C : Concentration of dissolved gas (mol/L)

C_L : Oxygen concentration in the liquid phase (mol/L)

C* : Oxygen concentration at saturation (mol/L)

D :Diameter of the agitator (m)

G: Generation time (h)

Fl: Flow Number

Fr: Froude Number

H : Henry's constant (L.atm/mol).

k_d : Specific death rate (h^{-1})

K_{La} : Volumetric transfer coefficient (s^{-1})

K_s: Half-saturation constant

N : Rotational speed of the agitator (rev/s)

Na : Aeration number

NAD: Nicotinamide Adenine Dinucleotide

Np : Power number (blade)

OTR : Oxygen Transfer Rate (moles of O_2/L.h)

OUR : Oxygen Uptake Rate (moles of O_2/L.h)

P : Power consumed in (Watt)

p: Partial pressure of the gas phase above the liquid (atm)

P_0 : Initial product concentration or Product concentration at time t_0 (g/L)

P_f : Final product concentration (g/L)

P_X : Biomass productivity at time t (g/L/h)

P_{xtot}: Total biomass productivity (g/L/h)

P_{xmax}: Maximum biomass productivity (g/L/h)

P_p: Product productivity at time t (g/L/h)

P_t: Product concentration at time t (g/L)

ρ: Density of the medium expressed (kg/m^3)

Qg : volumetric flow rate of the gas (m^3/s)

Q_p : Specific production rate of the product (h^{-1})

Re : Reynolds number

r_p : Product formation rates (g/L.h)

r_S : Substrate consumption rate (g/L.h)

r_x : Biomass growth rate (g/L.h)

S : Substrate concentration (g/L)

S_0 : Initial substrate concentration or Substrate concentration at time t_0 (g/L)

S_f : Final substrate concentration (g/L)

SSF: Solid-State Fermentation, or Solid Substrate Fermentation

t: Time (h)

T_{exp}: Exponential growth period

t_m : Time at which this maximum concentration is reached (h)

$\boldsymbol{\tau}$: Residence time (h)

μ : Dynamic viscosity of the fluid (Pa.s)

μ_{max}: Maximum growth rate (h^{-1})

V : Culture volume (L)

X : Biomass concentration (g/L)

X_0 : Initial biomass concentration or Biomass concentration at time t_0 (g/L)

X_f : Final biomass concentration (g/L)

X_m: Maximum biomass concentration (g/L)

X_t: Biomass concentration at time t (g/L)

$Y_{P/S}$: Product yield relative to the substrate (g/g)

$Y_{X/S}$:Biomass yield relative to the substrate (g/g)

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Bioengineering and industrial microbiology are two interdisciplinary fields that play a vital role in modern biotechnology, offering innovative solutions for numerous sectors, including healthcare, agriculture, and environmental sustainability.

The objective of bioengineering is to solve complicated problems by applying biological principles, chemistry, physics, engineering methods, and cutting-edge technology, frequently with an emphasis on creating bio-based materials and products. In order to create systems and methods that can scale from laboratory research to massive industrial production, it combines expertise from the fields of biology, chemical engineering, genetics, and materials science. ranging from developing life-saving medical devices to harnessing the power of microorganisms for industrial and environmental applications [1].

The roots of bioengineering can be traced back to the 20th century, when advancements in both biology and engineering began to intersect. Early pioneers in this field were instrumental in the creation of medical instruments, artificial organs, and devices that saved and improved lives. Over time, with advancements in biotechnology and genetic engineering, bioengineering has become one of the most important fields in modern healthcare. For example, the development of artificial organs, such as the heart-lung machine in the 1950s, marked the beginning of bioengineering's significant role in medical applications. The subsequent development of pacemakers, prosthetics, and more sophisticated medical imaging techniques has made a profound impact on both preventive and corrective medical care [1].

The future of bioengineering holds great promise, with the potential to tackle some of the world's most pressing healthcare challenges. With advances in personalized medicine, bioengineering is evolving toward treatments that are tailored to individuals' genetic makeup and specific medical needs. In the future, bioengineering could enable the creation of artificial organs, advanced neuroprosthetics, and highly specific drug therapies that target individual diseases at the molecular level.

Industrial microbiology, on the other hand, involves the study and application of microorganisms in industrial processes. It is central to the development of products like antibiotics, vaccines, enzymes, biofuels, and fermented foods. By harnessing the natural capabilities of bacteria, fungi, and yeast, industrial microbiology has revolutionized industries ranging from pharmaceuticals to food processing and waste management. The manipulation of microbes, their systems, and processes for the benefit of industry, society, and the environment is what is meant by the many definitions of industrial microbiology, often known as microbial biotechnology. The potential for sustainability, using and producing renewable resources, and the ability to substitute biocompatible materials for

fossil fuels, non-biodegradable plastics, and highly polluting chemical processes are two of industrial microbiology's most notable features [1, 2].

Industrial microbiology covers all bioconversion or biosynthesis processes carried out by microorganisms. It is a branch of applied microbiology and biotechnology that focuses on the use of microorganisms (bacteria, yeasts, fungi, etc.) in industrial processes to produce or transform large quantities of products for commercial purposes. It aims to exploit the metabolic and enzymatic capabilities of microorganisms to produce a variety of high-value products, such as drugs, bioenergies, chemicals, enzymes, foods and beverages, while optimizing culture conditions and fermentation processes. Industrial microbiology also involves techniques such as the improvement of microbial strains, the management of large-scale fermentations and the biotransformation of molecules [2-3].

The first applications of industrial microbiology for human health date back to antiquity, with the empirical use of microorganisms for fermentation in the production of bread, beer, wine, cheese, vinegar and food preservation. Ancient civilizations, such as the Sumerians, Egyptians and Greeks, discovered these processes without understanding the underlying biological mechanisms, but by observing their beneficial effects for the preservation of food and the production of fermented beverages [4].

Numerous and abundant in bioactive compounds, microorganisms have long been studied for potential use in human applications. Through the production of curd, cheese, wine, alcohol, and other products, humans have learnt how to use microbes. The emergence of contemporary industrial microbiology can be attributed to the manufacturing of penicillium and insulin. Industrial microbiology involves the study and application of microorganisms in industrial processes. It is central to the development of products like antibiotics, vaccines, enzymes, biofuels, and fermented foods. By harnessing the natural capabilities of bacteria, fungi, and yeast, industrial microbiology has revolutionized industries ranging from pharmaceuticals to food processing and waste management [5].

Together, bioengineering and industrial microbiology are transforming the production of bio-based products, improving efficiency, reducing environmental impact, and contributing to a more sustainable future. This synergy is key to the development of green technologies, renewable energy solutions, and advanced therapeutics, positioning these fields at the forefront of the biotechnology industry.

I. Fermentation processes

I.1. Fermentation definition

Fermentation is a very old technology for processing and preserving food. The term fermentation comes from the Latin *fervere*, which means to boil. Louis Pasteur became interested in fermentation between 1862 and 1877. He studied ferments, the formation of vinegar and the transformation of alcohol into acetic acid by *Mycoderma aceti* [4].

Industrial fermentation developed between 1900 and 1940 with the production of acetone, butanol, glycerol, citric acid and lactic acid. From 1940 onwards, using strain selection and mutation programmes, various productions such as antibiotics, amino acids, nucleotides and enzymes were carried out. From the 1980s onwards, genetic engineering made it possible to improve microbial strains with high industrial potential and to explore new biochemical pathways. Industrial fermentations thus concern a large number of sectors: food, fine chemicals, pharmaceuticals, agro-industry and cosmetics [6].

Fermentation in biochemistry and microbiology refers to a metabolic process in which cells convert sugars (like glucose) into acids, gases, or alcohols in the absence of oxygen (anaerobic conditions). This process is typically carried out by microorganisms like bacteria, yeast, and some fungi, but it can also occur in muscle cells under certain conditions [7].

The word fermentation has two different meanings for biochemists and industrial microbiologists.

In Biochemistry: In biochemistry, fermentation is a catabolic process that generates energy (ATP) from organic molecules without the involvement of oxygen. It is anaerobic catabolic pathways during which organic compounds serve as both electron donors and acceptors, with ATP synthesis being achieved by phosphorylation at the substrate level. The primary pathway involves the breakdown of glucose through glycolysis, leading to the production of pyruvate. Under anaerobic conditions, pyruvate is converted into other metabolites such as lactate (in animals) or ethanol and carbon dioxide (in yeast), rather than being fully oxidized to carbon dioxide and water through aerobic respiration. This process allows cells to regenerate NAD+ (important for glycolysis to continue) when oxygen is not available [7].

***In microbiology*:** the term fermentation refers to the unit operation that produces biomass or bioconversion products by culturing microorganisms. It is often studied as a method by which microorganisms obtain energy. For example, *Saccharomyces cerevisiae* (baker's yeast) ferments glucose to produce ethanol and carbon dioxide, which is fundamental in baking and alcohol production. In lactic acid bacteria (such as *Lactobacillus*), fermentation produces lactic acid as the

main end product. These microorganisms use fermentation to survive in environments where oxygen is limited [7]. The term industrial fermentation applies in industry to aerobic and anaerobic metabolisms.

Fermentation is a biological uses many forms of microorganisms and cells to convert complex molecules into simpler compounds called metabolites [8]. Microbiologists use the term "fermentation" to refer to any procedure that uses the mass cultivation of microorganisms to produce a product.

I.2. Different fermentation processes

Three major fermentation systems commonly used in industrial biotechnology: submerged fermentation (SmF), solid-state fermentation (SSF), and immobilized-cell fermentation (IMF). Depending on the type of culture, cells can be cultured in suspension or immobilized. In cell culture and fermentation, different methods of feeding nutrient medium can be used. There are three types of fermentation processes (Figure I.1) based on how nutrients are provided to the cells:

- Batch process or fermentation is interrupted;

- Fed-batch process or fed discontinuous fermentation;

- Continuous culture process.

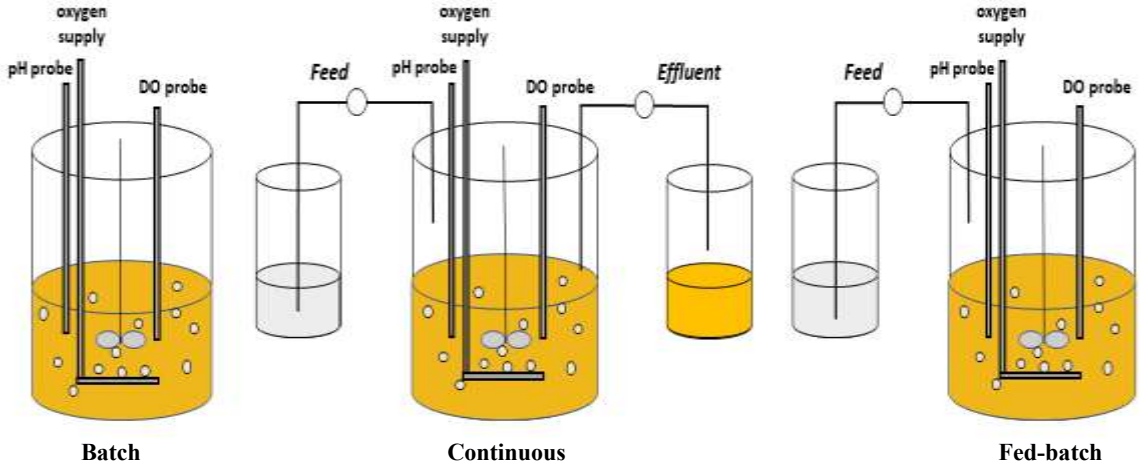

Figure I.1. Three general modes of fermentation operation: batch, continuous, and fed-batch processes [9].

I.2.1. Submerged fermentation

I.2.1.1. Batch process

In a batch process or discontinuous fermentation (figure I.1), all nutrients (sugars, salts, growth factors, etc.) are added to the fermenter at the beginning of the fermentation, and nothing is added or removed until fermentation is complete, except oxygen (in case of aerobic microorganisms), an antifoam agent, and acid or base to control the pH. The process runs for a fixed period until the cells reach a certain growth phase or the desired product is produced. The cells grow exponentially in the beginning, followed by a stationary phase, and then a decline phase as nutrients deplete and waste products accumulate which can impact the growth of cells and the quality of the product. The culture is allowed to grow until the nutrients are exhausted or the desired product is produced. This is the simplest and most commonly used method in laboratory-scale for studying fermentation processes [10].

In a batch process, a fixed volume of a medium is introduced to a microbial culture (Figure I.2). At the same time the substrate is consumed by the microorganism and the desired product appears. As the microorganisms multiply, they use up the nutrients in the medium and generate by-products, which gradually slows their growth and causes them to enter the stationary growth phase [11].

a. Modeling of batch fermentation processes

A mathematical model for any biochemical process should have three main parts: (1) a description of the metabolic pathway for the microorganism involved in the biochemical conversion; (2) development of rate equations for biomass production, substrate consumption, and product formation; and (3) the use of rate equations to balance the mass flux of all metabolites over the reactor. The metabolic pathway's enzymatic reactions require a variety of kinetic parameters, which can be found by minimising the squares of the error of the difference between simulation and experimental data [12].

Michaelis-Menten kinetics and Monod kinetics are the two most widely used models in the literature for kinetic modelling of biomass increase. The biomass production rate in the Monod kinetic model is expressed in terms of the specific growth rate, which includes an inhibition component that can be either substrate or byproduct inhibition, depending on the situation. One crucial component of the mathematical model is the impact of product inhibition on a particular growth rate [12].

The performance of the bioprocess can be verified by modelling and optimising culture yields and productivity using data on metabolite production and microbial growth kinetics. Let's look at the process of growing a bacterium in a batch to produce biomass (Figure I.2). To facilitate the study, we will make the following assumptions: the only limiting substrate is the carbon source (energy source); there is no cell death; no product is produced; the fermenter is infinitely mixed (homogeneous culture); and there is an excess of oxygen provided.

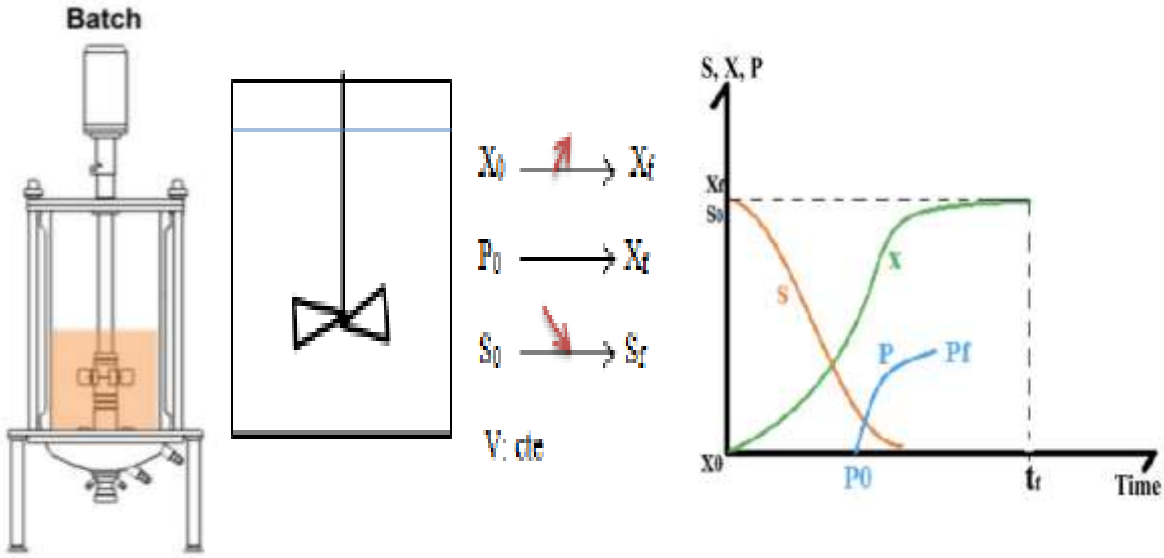

Figure I.2. Batch mode illustration [11].

V = culture volume (or useful volume) (m^3), S = limiting substrate concentration at time "t" (g/L), X = biomass concentration (g/l) (with a change from X_0 to X_f), The index "0" for the initial state and "f" for the final state.

The microbial strain must have known kinetic behavior during fermentation. No culture medium is introduced except for the neutralization reagents, or for the antifoam introduced in very small quantities. Similarly, no culture is removed until it is finished. The volume in the fermentation tank is therefore constant. The biomass concentration (X) increases inversely with respect to that of the substrate (S) which is consumed. The desired product (P) appears and its concentration increases.

The reactor has no inlet or outlet. $Q_E = Q_S = 0$, and the volume is constant.

Substrate balance: $-\boldsymbol{r_s} = \frac{\boldsymbol{dS}}{\boldsymbol{dt}}$

Biomass balance: $+\boldsymbol{r_X} = \frac{\boldsymbol{dX}}{\boldsymbol{dt}}$

Product balance: $+\boldsymbol{r_P} = \frac{\boldsymbol{dP}}{\boldsymbol{dt}}$

r_S is the substrate consumption rate often modelled by the Monod equation:

$$\mu = \mu_{max} \frac{S}{S+K_s} \text{.....(1)}$$

r_x and r_p are the biomass growth and product formation rates (g/L.h), respectively.

It is also given by the following relationship:

$$\frac{dX}{dt} = r_X = \mu X = \mu_{max} \frac{S}{S+K_s} X \text{.....(2)}$$

Where, X: cell concentration; S: limiting substrate concentration; μ_{max}: maximum growth rate; K_s: is the half-saturation constant, which is the substrate concentration at which the growth rate is half of its maximum value.

b. Specific growth rate

The specific growth rate (μ) is a measure of how fast a microorganism or cell population grows, typically expressed as the change in biomass (or cell concentration) per unit of time, per unit of biomass. It reflects the rate at which the population increases in terms of its size or number of cells. It represents the growth rate under optimal conditions [13].

The growth rate at time t is:

$$r_X = \frac{dX}{dt} = \mu X \text{.....(3)}$$

Using differential mathematical function), r_X is expressed in "biomass concentration. time^{-1}", for example, in g.L^{-1}.h^{-1}.

In mathematical terms, the specific growth rate is given by:

$$\mu = \frac{r_X}{X} = \frac{dX}{dt}\frac{1}{X} \text{.....(4)}$$

μ is expressed in time^{-1}, for example in h^{-1} or in min^{-1}).

Rearrange: $$\mu dt = \frac{dX}{X} \text{.....(5)}$$

During the exponential growth phase, the specific growth rate (μ) is maximum and constant, it is called maximum specific growth rate or exponential growth rate. It is generally referred to as μ_{max} or μ_{expo}. So, it was written: $$\mu_{max} dt = \frac{dX}{X} \text{.....(6)}$$

After integration we obtain: $\mathbf{Ln([X]_{t+\Delta t}) = Ln([X]_t) + \mu_{max}.\Delta t}$ ……*(7)*

or in exponential function we obtain: $[X]_{t+\Delta t} = [X]_t e^{\mu_{max} dt}$ ……*(8)*

c. Generation time

The Generation time (G) is the time interval it takes for a cell division to double to occur, it is also called the doubling time. It is expressed in hours or minutes [13].

Let us consider that $\Delta t = (t_2 - t_1) = G$; in this case, the biomass will have doubled when the generation time has elapsed: $X_2 = 2X_1$, or, in logarithmic conversion:

$LnX_2 = Ln(2X_1)$ this gives:

$\mathbf{Ln(2.X_1) = LnX_1 + \mu_{max}.G}$, Therefore: $\mu_{max} = \frac{Ln(2X_1) - LnX_1}{G}$ or $G = \frac{Ln2 + LnX_1 - LnX_1}{\mu_{max}}$

which therefore simplifies to $\mu_{max} = \frac{Ln\ 2}{G}$. All that remains is to isolate the generation time:

$$G = \frac{Ln\ 2}{\mu_{max}} \quad \text{……*(9)*}$$

d. Yields of a fermentation

A growth curve can be created by measuring the rise in bacterial mass or numbers over time after inoculating microbial cells in a liquid fresh media, provided that the medium has all the nutrients needed for growth and that the ambient conditions are ideal. A growth curve exhibits multiple discrete growth phases (Figure I.3).

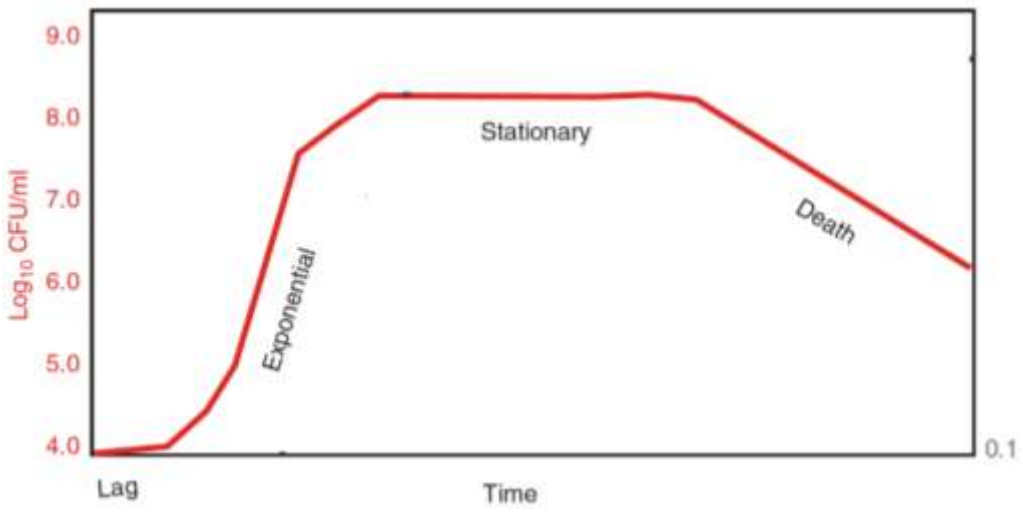

Figure I.3. Typical growth curve for a bacterial population [13].

These include [12] :

The lag phase, where the growth rate is practically zero, is one of these. After the starting population has doubled, it is considered to enter the exponential phase. It is supposed that the lag phase results from the physiological adaptation of the cell to the culture conditions.

The exponential or log phase, is defined by a period of exponential growth, which is the fastest growth achievable in the batch system's current conditions. The quantity of cells in the culture at any given time determines the rate at which the cells develop during exponential growth. The following formula can be used to mathematically characterise cell growth during the exponential phase:

$$\frac{dX}{dt} = \mu X \quad \text{.....(10)}$$

where t is time, μ is the specific growth rate constant ($time^{-1}$), and X is the number or mass of cells (mass/volume).

***The stationary phase*,** is the third phase of growth. It is defined as a state of no net growth in a batch culture, and it is represented by the following equation:

$$\frac{dX}{dt} = 0 \quad \text{.....(11)}$$

The death phase, is the last phase of the growth curve, is defined by a net loss of culturable cells. Individual cells may continue to divide and metabolise even during the death phase, but there is a net loss of viable cells because more viable cells are lost than are gained. Although the rate of cell death is typically slower than the rate of growth during the exponential phase, the death phase is frequently exponential. The following formula can be used to characterise the death phase:

$$\frac{dX}{dt} = -k_d X \quad \text{.....(12)}$$

where k_d is the specific death rate.

Each of these phases denotes a unique growth phase linked to typical physiological changes in the cell culture. There are significant differences in the growth rates linked to each phase [13].

Either over a time interval dt and d[S] (kg/m^3) of substrates are consumed to form d[X] (kg/m^3) of biomass and d[P] (kg/m^3) of product. $Y_{X/S}$ and $Y_{P/S}$ are the instantaneous yields of growth and product, respectively [14].

Biomass yield

Biomass yield ($Y_{X/S}$) is defined as the dry mass of cells produced per unit mass of substrate consumed (g/g) in a given culture volume. For an entire batch fermentation, the biomass yield relative to the substrate is therefore given by the following equation:

$$Y_{X/S} = -\frac{d[X]}{d[S]} \quad \text{.....(13)}$$

The overall conversion yield of S into biomass over an overall period is calculated by this formula [14]:

$$Y_{X/S} = -\frac{\Delta[X]}{\Delta[S]} = -\frac{X_f - X_0}{S_f - S_0} = \frac{X_f - X_0}{S_0 - S_f} \quad \text{.....(14)}$$

Where X_f is a final biomass concentration;X_0 is a initial biomass concentration; S_f is a final substrate concentration and S_0 is a initial substrate concentration.

Product yield

Product yield ($Y_{P/S}$) is defined as the mass of product formed per unit mass of substrate consumed (g/g) in a given volume of culture. For an entire batch fermentation, the product yield relative to the substrate is given by the following equation:

$$Y_{P/S} = -\frac{d[P]}{d[S]} \quad \text{.....(15)}$$

The overall conversion yield of S into product over an overall period is calculated by this formula:

$$Y_{P/S} = -\frac{\Delta[P]}{\Delta[S]} = -\frac{P_f - P_0}{S_f - S_0} = \frac{P_f - P_0}{S_0 - S_f} \quad \text{.....(16)}$$

Where P_f is a final product concentration; P_0 is a initial product concentration; S_f is a final substrate concentration and S_0 is a initial substrate concentration.

The substrate chosen to calculate product yield is often the source of carbon and energy, particularly if the product is growth-dependent. However, it may be justified to choose a different substrate for certain fermentations, particularly in the case of bioconversions.

For some products with relatively simple metabolic synthesis pathways (primary metabolites in particular), it is possible to predict how much product will be formed from a given amount of substrate using stoichiometry: this is the maximum theoretical yield (Yp/s). The maximum theoretical yield in bacterial fermentation, typically represented as Yp/s is a measure of how

efficiently a microorganism can convert substrate (glucose) into product (biomass, ethanol, or another metabolic product). It is defined as the ratio of product formed (P) to substrate consumed (S), and it is based on stoichiometry and thermodynamic principles [15].

For bacterial fermentation, this theoretical yield can be calculated under ideal conditions, where all the substrate is converted into the desired product without any loss or side reactions. The general formula for the maximum theoretical yield is:

$$Y_{P/S} = \frac{\textbf{moles of product (P)}}{\textbf{moles of substrate (S)}} \quad \text{.....(17)}$$

In practice, this stoichiometric yield is rarely achieved, as many factors can reduce production efficiency; however, it can be useful to calculate it to compare it with the actual product yield obtained (Yp/s). Indeed, the closer the practical yield is to the maximum theoretical yield, the more efficiently the substrate is invested in product synthesis during fermentation [15].

For example, the overall equation for alcoholic fermentation can be used to lower the maximum theoretical output yield when *S. cerevisiae* produces ethanol from maize sugar (glucose):

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

According to the equation, the fermentation would provide two moles of ethanol (2 moles.46g/mole = 92g) for every mole of glucose spent (1 mole x 180g/mole = 180g) if all of the glucose consumed were converted to ethanol. This would result in a maximum theoretical yield of:

$$Y_{P/S_{max}} = \frac{[Ethanol]}{[Glucose]} = \frac{92\ (g)}{180\ (g)} = 0,511\ g/g = 51,1\%$$

$Y_{X/S}$ and $Y_{P/S}$ are expressed as "quantity of X or P formed per quantity of S consumed" (for example in g of biomass (or product) per g of S). They depend a priori on the physiological conditions at a given time t of the culture. When the volume is constant, these yields can be assumed to be constant throughout a culture phase. The substrate consumption rate r_S ($kg.m^{-3}.s^{-1}$) and the biomass formation rates r_X and product r_P follow the relationships [14]:

$$Y_{X/S} = -\frac{d[X]}{dt}.\frac{dt}{d[S]} = -\frac{r_X}{r_S} \quad \text{.....(18)}$$

$$Y_{P/S} = -\frac{d[P]}{dt}.\frac{dt}{d[S]} = -\frac{r_P}{r_S} \quad \text{.....(19)}$$

$$r_S = -\frac{r_X}{Y_{X/S}} = -\frac{r_P}{Y_{P/S}} \quad \text{.....(20)}$$

Product yield relative to biomass and specific production rate

The dynamics of production and growth are intertwined in the case of growth-dependent products so that production is always proportionate to growth. Therefore, if the ratio between the two is known, production can be indirectly evaluated by measuring growth at any point throughout fermentation. The product yield to biomass (Y P/X) is represented by this ratio, which is defined as the mass of product made per unit dry mass of cells produced (g/g). It can be readily evaluated using the data needed to compute Y P/X and YX/S using the following equation :

$$Y_{P/X} = \frac{P - P_0}{X - X_0} \quad \text{.....(21)}$$

since $Y_{P/X}$ and $Y_{X/S}$ are determined for equal substrate consumption:

$$Y_{P/X} = \frac{(P - P_0)/(S_0 - S)}{(X - X_0)/((S_0 - S)} = \frac{Y_{P/S}}{Y_{X/S}} \quad \text{.....(22)}$$

In the event of a growth-dependent product, this yield also offers valuable and useful information for the examination of production kinetics. These kinetics are in fact comparable to growth kinetics, and as a result, the following differential equation can be used to analytically quantify the rate of synthesis of such a product at any point during growth [16].

$$\frac{dP}{dt} = Q_P X \quad \text{.....(23)}$$

Where: P is the product concentration (g/l), t is the time (h), Q_p is the specific production rate of the product (h^{-1}), X is the biomass concentration (g/l), which represents the living organism (bacteria, yeast).

This demonstrates that the amount of biomass present X and the specific production rate Q_p determine the rate of product production at any given time.

A relationship between the product production rate Q_p, the product yield $Y_{P/X}$, and the growth rate μ may be obtained by combining the biomass growth equation ($dX/dt=\mu X$) and the product production equation ($dP/dt=Q_P X$). The following equation represents this relationship:

$$Q_P = Y_{P/X} \cdot \mu \quad \text{.....(24)}$$

The specific production rate (Q_P) for a growth-dependent product is therefore always proportional to the specific growth rate (μ). Because of this, it has a maximum value at the exponential phase's

maximum growth phase (Q_{pmax}), which is easily computed from the equation if we know μ_{max} and $Y_{P/X}$.

For products that are partially growth-dependent or growth-independent, since the relationship between growth and production is not constant during fermentation, the specific production rate (Q_p) is not related to the specific growth rates (μ) by a constant relationship involving $Y_{P/X}$. $\boldsymbol{Q_p}$ is then determined from the experimental analysis of the unique production kinetics of the particular fermentation in question [16].

e. Productivity

Productivity in bacterial fermentation is an essential parameter that measures the amount of product formed per unit of time and volume. It depends on various factors, including the bacterial strain used, the culture conditions, and the operating parameters of the fermenter [17].

Biomass productivity

Biomass productivity in fermentation is a key measure of the efficiency of biomass production (the cells of the growing organism) during the fermentation process. It expresses the amount of biomass produced per unit volume and per unit time. Biomass productivity (P_X) can be defined by the following equation [17] :

$$\boldsymbol{P_X = \frac{\Delta X}{\Delta t} = \frac{X_t - X_0}{t - t_0}} \quad \ldots\ldots\ldots\ldots (25)$$

Where: P_X is the biomass productivity at time t (g/L/h); X_t: Biomass concentration at time t (g/L); X_0: Biomass concentration at time t_0 (g/L); t: time (h).

Generally, times t0 and t represent the beginning and conclusion of fermentation, respectively, and biomass productivity is computed for the duration of the fermentation. Additionally, total productivity (P_{xtot}) is computed. However, in certain fermentations, production continues after growth stops, in the idiophase, which lowers the productivity of biomass as a whole. To evaluate growth performance in these situations, it would be pertinent to compute maximum biomass productivity (P_{xmax}).

Maximum biomass productivity

Maximum biomass productivity (P_{xmax}) is a more accurate measure for assessing a microorganism's growth performance because it focuses only on the period when biomass growth is fastest, i.e., during the exponential growth phase. Maximum productivity is calculated by analyzing the biomass growth curve and determining the maximum slope of the exponential phase. It gives the rate at which

biomass grows at its maximum. This provides a more accurate assessment of growth efficiency, unaffected by periods when biomass is no longer growing, such as during the idiophase [17].

The equation for maximum productivity can be expressed as:

$$P_{Xmax} = \frac{X_m - X_0}{t_m - t_0} \quad \text{.....(26)}$$

Where: X_m is the maximum biomass concentration obtained during the exponential phase (g/L), t_m is the time at which this maximum concentration is reached (h), X_0 and t_0 are the concentration and time at the beginning of the exponential growth phase, respectively.

This productivity is generally used to optimize culture conditions and to better understand the performance of an organism during its active growth phase.

Total Biomass Productivity

Generally speaking, total biomass production is computed throughout the duration of the fermentation process, that is, from start to finish. It gives a general indication of how well biomass is produced during fermentation [17]. The equation for total productivity is as follows:

$$P_{Xmax} = \frac{X_t - X_0}{t - t_0} \quad \text{.....(27)}$$

Where: X_t is the final biomass concentration at the end of fermentation (g/L); X_0 is the initial biomass concentration (g/L); t is the total fermentation time (h); t_0 is the initial time (h).

Thus, total productivity reflects the total increase in biomass over the entire fermentation period.

Product productivity

The quantity of product (metabolite, biomass, or other product of interest) produced per unit volume and per unit time is referred to as product productivity in fermentation. It is a measure of how efficiently a microorganism produces a specific product during fermentation [16]. Productivity in product (P_P) can be expressed as follows:

$$P_p = \frac{\Delta P}{\Delta t} = \frac{P_t - P_0}{t - t_0} \quad \text{.....(28)}$$

Where: P_p is the product productivity at time t (g/L/h); P_t: Product concentration at time t (g/L); P_0: Product concentration at time t_0 (g/L); t: time (h).

Since productivity in batch mode is computed over the duration of the fermentation, times t0 and t, respectively, represent the beginning and ending of the fermentation. Since the final moment of

fermentation, when productivity is at its peak, will be carefully selected to stop the reactor and retrieve the product, the total productivity thus estimated for the product will always be equal to the maximum productivity. In fact, continuing a fermentation whose overall productivity is declining would be economically ridiculous because, on average, less product would be produced with each hour of fermentation.

Productivity is so important in the actual world of industry that it is frequently required to include reactor downtime in calculations to quantify productivity and evaluate the profitability of a fermentation process. In fact, a number of critical functions hinder the reactor's continuous operation, resulting in periods of inefficiency that nonetheless demand financial and human resources. Harvest time (t_h), cleaning time (t_c), filling time (t_f), sterilisation time (t_s), and emptying time (t_e) are these times. Productivity is obviously greatly decreased when these processing durations are added to the actual fermentation period, and this can be computed as follows: Indeed,

$$\mathbf{Ln}\frac{X_f}{X_0} = \mu_m T_{exp} \quad \text{.....(29)}$$

where T_{exp}: exponential growth period; t_0: called the growth delay time with: $\mathbf{t_0 = t_h + t_c + t_f + t_S + t_e}$.

A production cycle's total duration can be found using: $T_t = T_{exp} + t_0 = \frac{1}{\mu_m}\mathbf{Ln}\frac{X_f}{X_0} + t_0$

We can also calculate the total amount of biomass produced: $\mathbf{X_f - X_0 = Y_{X/S}\, S_0}$ (g/L)

The total productivity for a discontinuous culture is:

$$\textbf{Productivity} = \frac{X_f - X_0}{T_{Total}} = \frac{Y_{X/S}\, S_0}{\frac{1}{\mu_m}\mathbf{Ln}\frac{X_f}{X_0} + t_0} \quad \text{.....(30)}$$

Multiplying this equation by μ_m gives (g/L.h):

$$\textbf{Productivity} = \frac{\mu_m\, Y_{X/S}\, S_0}{\mathbf{Ln}\frac{X_f}{X_0} + \mu_m\, t_0} \quad \text{.....(31)}$$

I.2.1.2. Fed-batch fermentation

Similar to the batch technique, fed-batch fermentation extends the active growth period by adding more substrate and nutrients to the bioreactor (Figure I.1) once the microbes have consumed all of the substrate and nutrients in the cultivation medium. Both the pace of product synthesis and the proliferation of microorganisms can be controlled by varying the feed rate (Figure I.4). The maximal specific substrate utilisation rate for the used microbial strain must be taken into account when creating a feed plan [11].

More substrate will eventually begin to build up in the bioreactor if more is supplied than the cells can use. When the concentrations are high, it may directly or indirectly obstruct biomass development by encouraging the production of inhibitory metabolites. A same circumstance could occur if the product inhibits the cells, hence diminishing development over time as the product builds up within the reactor.

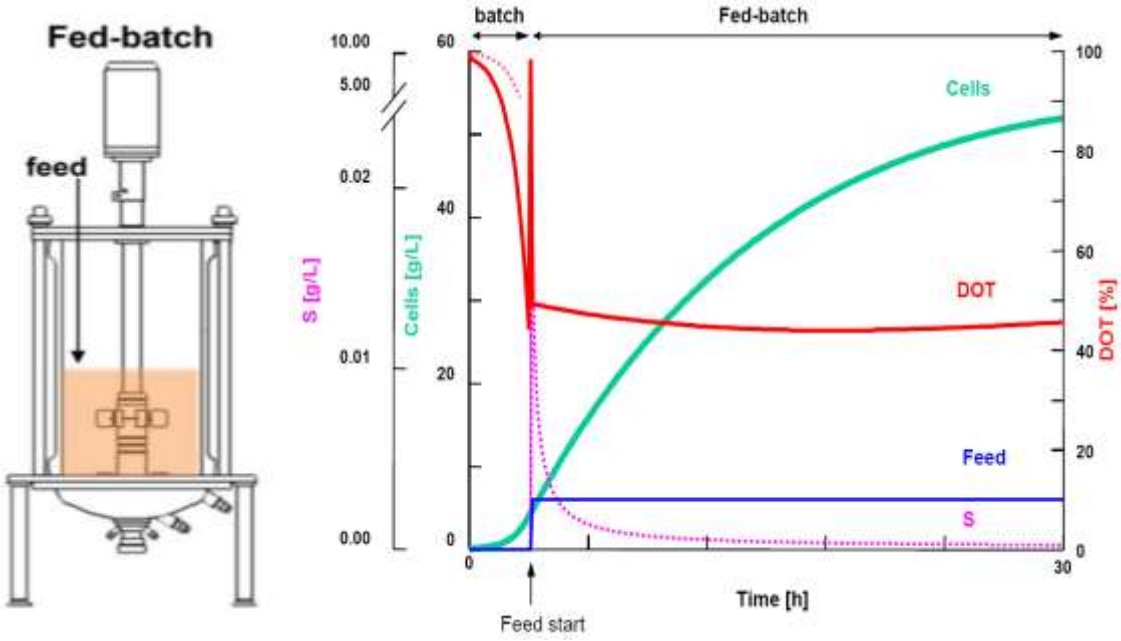

Figure I.4. Fed-batch mode illustration [11].

For bioprocesses that aim to produce high cell densities and product titers, a fed-batch operation is especially advantageous because it usually leads to a considerable accumulation of biomass, especially when the goal product is favourably correlated with microbial growth. Furthermore, both product and biomass yields per fed substrate are increased since the substrate is not overfed during the process and by-product accumulation is kept to a minimum [11].

Comparing fed-batch operations to batch procedures, it is often possible to get a greater product titer per fermentation, which effectively raises the product space time yields. For example, when comparing a 72-hour batch to a 72-hour fed-batch fermentation, Muri et al. increased the bioethanol productivity in *S. cerevisiae* fermentation by 1.8 times, boosting the overall process productivity [18].

a. Modeling of feed-batch fermentation processes

The following assumptions will be considered: no cell mortality; excess oxygen is provided; the limiting substrate is carbon source; adequate amounts of other nutrients are provided. The equations

for batch culture and the first period are similar. To make the model easier to use, a few conditions are selected during the fed-batch period (metabolite production time) [19]:

S≈0: during fed-batch, the substrate is completely consumed as it is added;

$V=V_0$: volume of culture medium in the tank at the start of the production phase;

$V<V_f$: where Vf is the maximum final volume (usable volume) of the fermenter;

X_p = cell concentration at the start of the production period.

Model of biomass in a Fed-Batch fermenter

The volume of the culture media in a fed-batch fermenter rises with the addition of substrate. The dilution rate, due to the addition of substrate, is therefore an important component in the change in biomass concentration *X* in the fermenter. The change in cell concentration is generally expressed as follows:

$$\frac{dX}{dt} = -\frac{dV}{dt}.\frac{X}{V} \quad \text{.....(32)}$$

Model of substrate in a Fed-Batch fermenter

The variation of substrate concentration in the culture medium of the fed-batch fermenter depends on: the rate of consumption of the medium by the cell ($-r_s$); the dilution of the medium in the fermenter by the addition ($-\frac{dV}{dt}.\frac{S}{V}$); the contribution of substrate by the addition ($\boldsymbol{Q}.\frac{S_a}{V}$).

$$\frac{dS}{dt} = -r_s - \frac{dV}{dt}.\frac{S}{V} + \frac{dV}{dt}.\frac{S_a}{V} \quad \text{.....(33)}$$

Under the conditions below, it is assumed that the substrate concentration remains constant and close to zero S=0 therefore:

$$\frac{dS}{dt} = -r_s + Q.\frac{S_a}{V} \quad \text{.....(34)}$$

Model of product in a Fed-Batch fermenter

The variation in the concentration of metabolic products in the culture medium depends on:

- The rate of production of the latter by the cells $+r_p$;

- The dilution of the culture medium by the addition ($-\frac{dV}{dt}.\frac{P}{V}$), therefore:

$$\frac{dS}{dt} = +r_P - \frac{dV}{dt}.\frac{P}{V} \quad \text{.....(35)}$$

The evolution of the parameters during the two periods of culture is shown schematically in Figure I.5. During the production period, we observe [18]:

- A linear increase in volume $\frac{dV}{dt} = Q = Cte$;

- A decrease in cell concentration following dilution $\frac{dX}{dt} = -\frac{dV}{dt}.\frac{X}{V}$ .....(36)

- An increase in product concentration $\frac{dP}{dt} = r_P - \frac{dV}{dt}.\frac{P}{V}$ .....(37)

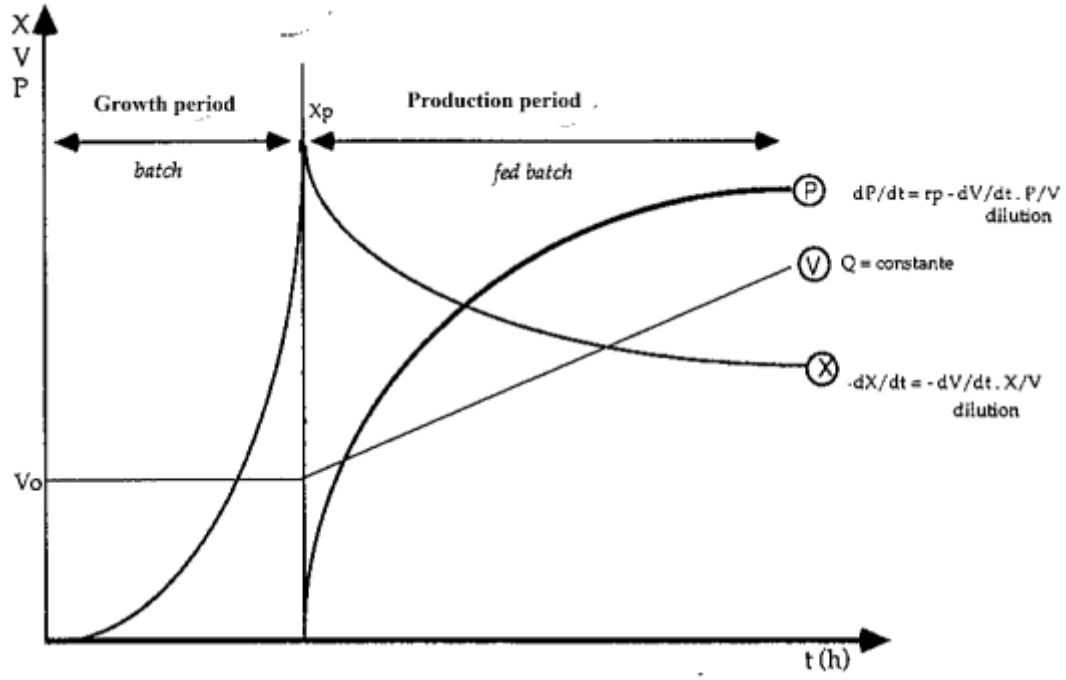

Figure I.5. Evolution of variables X, V and P during the two periods of a "fed-batch" fermentation[11].

Cells mortality

Although the working hypothesis has up till now ignored all cell mortality, cell mortality can have a substantial impact during the manufacturing phase. Therefore, the biomass balance expression needs to be modified in this situation: $\frac{dX}{dt} = r_X = -K_d.X - Q\frac{X}{v}$ .....(38)

where, K_d: the mortality rate (1/h).

Cell mortality affects the length of the production phase but does not change the final product concentration. Therefore, only the overall productivity of the process is affected. Productivity for fed-batch cultivation is calculated as follows:

$$\boldsymbol{Productivity_{Fed-batch}} = \frac{\boldsymbol{X_f - X_0}}{\boldsymbol{T_{Total}}} \quad \ldots\ldots\ldots\ldots(39)$$

with: $\mathbf{T_{Total} = t_{fb} + t_0}$

where, $\boldsymbol{t_{fb}}$ is a cultivation period with addition (Fedd-batch) and t_0 is delay time ($t_h + t_c + t_f + t_S$).

I.2.1.3. Continuous fermentation

On the other hand, continuous fermentation keeps the medium level in the bioreactor constant by continuously introducing fresh culture medium, usually at a steady pace, and collecting the cells and spent medium at the same time (Figure I.1). While maintaining a consistent culture volume, this procedure allows for the elimination of harmful metabolites and the replenishment of nutrients that have been eaten [11].

The fermentation volume is constant in continuous fermentations because sterile medium is continuously introduced into a fermenter and the fermented product is continuously removed (Figure I.6). Continuous fermentations are usually initiated as batch cultures, and feeding starts once the concentration of the microbial population is reached [20].

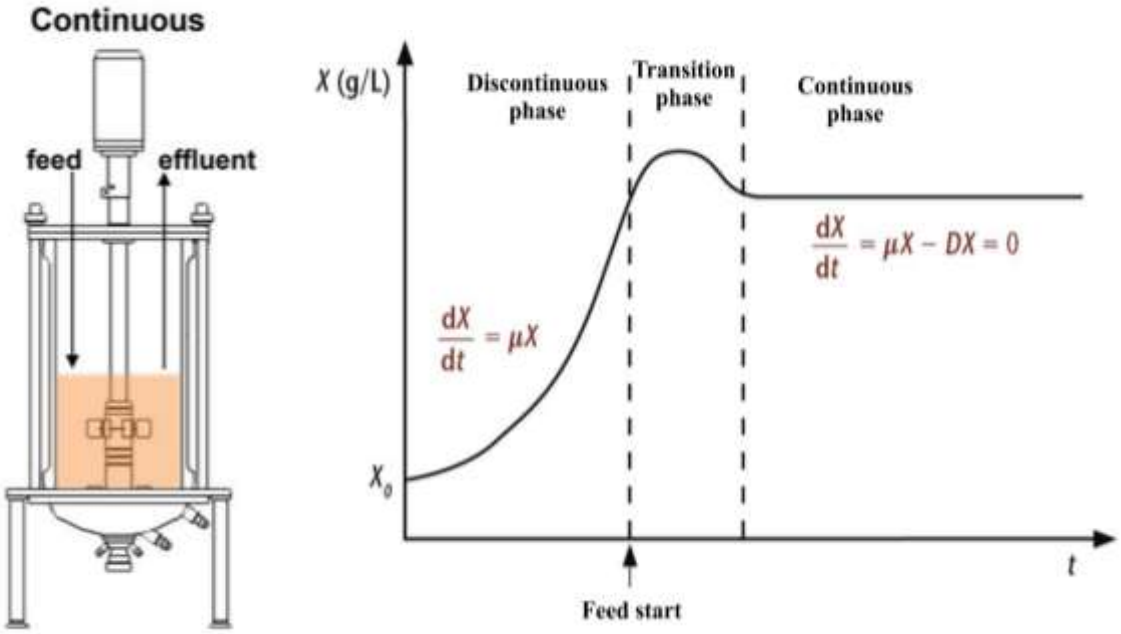

Figure I.6. Continuous mode illustration [11].

Continuous inoculation is required for "plug flow" fermentation methods (Figure I.7.a), such as lengthy tubes that prevent back mixing. A plug flow device's fluid moving components act like miniature batch fermenters. Therefore, plug flow fermenters make it very easy to convert true batch processes into continuous operations, particularly where aeration and pH control are not needed. Although the fermentation conditions (such as low pH, high alcohol, or salt content) may occasionally be chosen to favour the desired microbes above any possible contaminants, continuous cultures are especially vulnerable to microbial contamination.

The medium's feed rate in a "well-mixed" continuous fermenter (Figure I.7.b) should be such that the dilution rate, that is, the volumetric feed rate divided by the constant culture volume, remains below the microorganism's maximum specific growth rate in that medium and under those specific fermentation conditions. The microbe will be removed from the fermenter if the dilution rate is higher than the maximum specific growth rate [20].

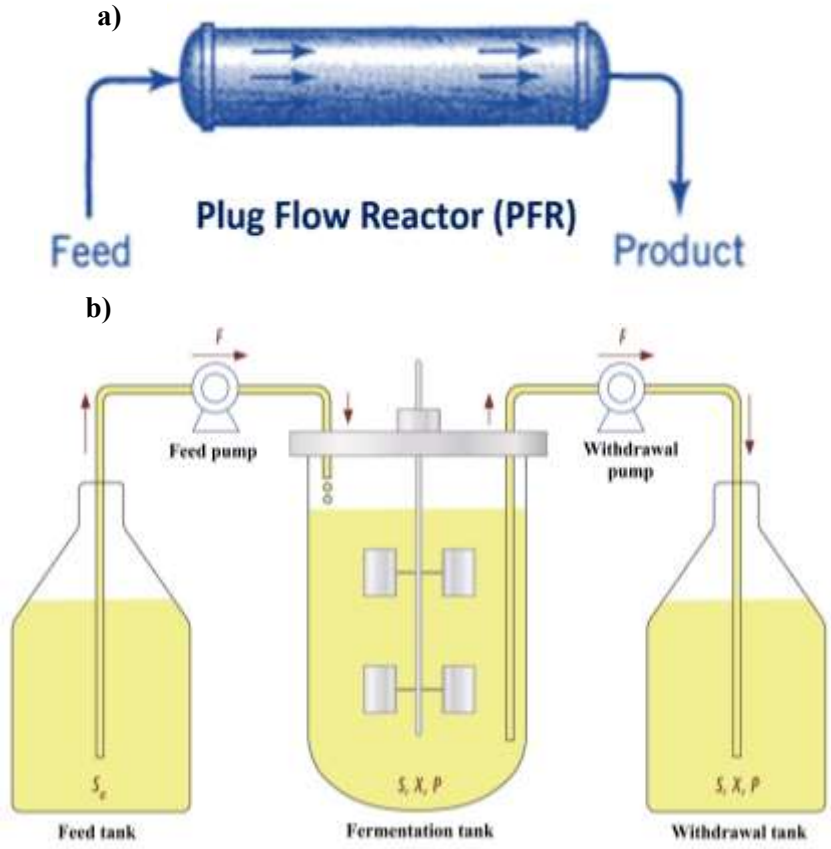

Figure I.7. Continuous fermentation methodologies without biomass recycle [20].
(a) continuous plug flow fermentation; ((b) well - mixed continuous fermentation

a. Modeling of continuous fermentation processes

It is first necessary to comprehend that a continuous culture is a system in equilibrium, at constant volume, perpetually diluted at a fixed rate; this dilution rate is related to the flow rate and the culture volume according to: In order to determine the exact flow rate to maintain the culture in exponential growth under constant conditions where productivity is maximum [20].

$$\boldsymbol{Dilution\ (D) = \frac{Flow\ rate}{Culture\ volume} = \frac{Q}{V}} \text{ ……..(40)}$$

The average residence time ($\boldsymbol{\tau}$) for the dilution rate to equal the growth rate is:

$$\boldsymbol{\tau = \frac{1}{D}} \text{ ……..(41)}$$

In practice, we begin to seed the tank containing a volume V of liquid. Feeding and withdrawal are carried out at the same flow rates $Q_f = Q_w = Q$

Mass balance relative to the substrate: $\boldsymbol{QS_0 - r_s.V = QS + \frac{d(V.S)}{dt}}$ ……..(42)

Mass balance relative to the biomass: $\boldsymbol{QX_0 + r_X.V = QX + \frac{d(V.X)}{dt}}$ ……..(43)

Mass balance relative to the product : $\boldsymbol{QP_0 + r_P.V = QP + \frac{d(V.P)}{dt}}$ ……..(44)

When the equilibrium state for biomass is established, the balance equation becomes:

$$\boldsymbol{QX_0 + r_X.V = QX}, \text{ either } \boldsymbol{D = \frac{Q}{V}}, \Rightarrow \boldsymbol{D(X - X_0) = r_X = \mu X}$$

With $X_0 = 0$, the evolution of the biomass is:

$$\boldsymbol{\frac{dX}{dt} = r_X - D.X = \mu X - DX = (\mu - D)X} \text{ …..(45)}$$

with: +μ.X: positive component of cell growth; −D.X: negative component resulting from dilution by the fresh medium.

For the substrate: $\boldsymbol{\frac{dS}{dt} = D.S_a - r_S - D.S}$ therefore:

$$\boldsymbol{\frac{dS}{dt} = D.(S_a - S) - r_S} \text{ ……..(46)}$$

+D.S_a: positive component resulting from the substrate provided by the fresh medium; -r_s: negative component resulting from cell consumption; -D.S: negative component resulting from the dilution effect. S_a: concentration of limiting substrate in the addition. For the product:

$$\frac{dP}{dt} = r_P - D.P \quad \text{.....(47)}$$

rp: positive component resulting from cellular synthesis; D.P: negative component resulting from the dilution effect.

Therefore, the choice of the dilution rate "D" throughout the continuous culture setup determines the evolution of the biomass, substrate, and product concentrations. The objectives of the continuous culture must be taken into consideration when selecting this parameter. There are two main types of continuous culture system that are generally used: Chemostat and Turbidostat.

Chemostat

Monod in France and Novick & Szilard in the US independently described the continuous culturing technique with a chemostat in 1950, it is a continuous fermentation without feedback control. In order to achieve steady-state growth, a chemostat works by continually supplying a growth-limiting nutrient and removing a portion of the fermentation broth at the same rate (Figure I.8). Carbon, nitrogen, phosphorus, or any other necessary nutrient that affects the particular development rate can be the growth-limiting nutrient. The ability to adjust the growth rate by varying the feed rate of the growth-limiting ingredient is a key benefit of chemostat mode over batch mode [21].

In actuality, two pumps and an effluent tube "plunged at the desired level" can be used in a laboratory fermenter to precisely equalise the feed and effluent flow rates. Another method, which is popular even for installations with relatively high volume, involves using gravity to take off the wastewater when a solenoid valve that is controlled by the bioreactor's weight, which must remain constant, is activated.

Because the availability of a single medium component (the limiting substrate) controls the growth rate, the fermenter is known as a chemostat. Two key characteristics are that the limiting substrate is fed to the culture by the constant addition of fresh medium, and the dilution rate determines the culture's particular growth rate [21].

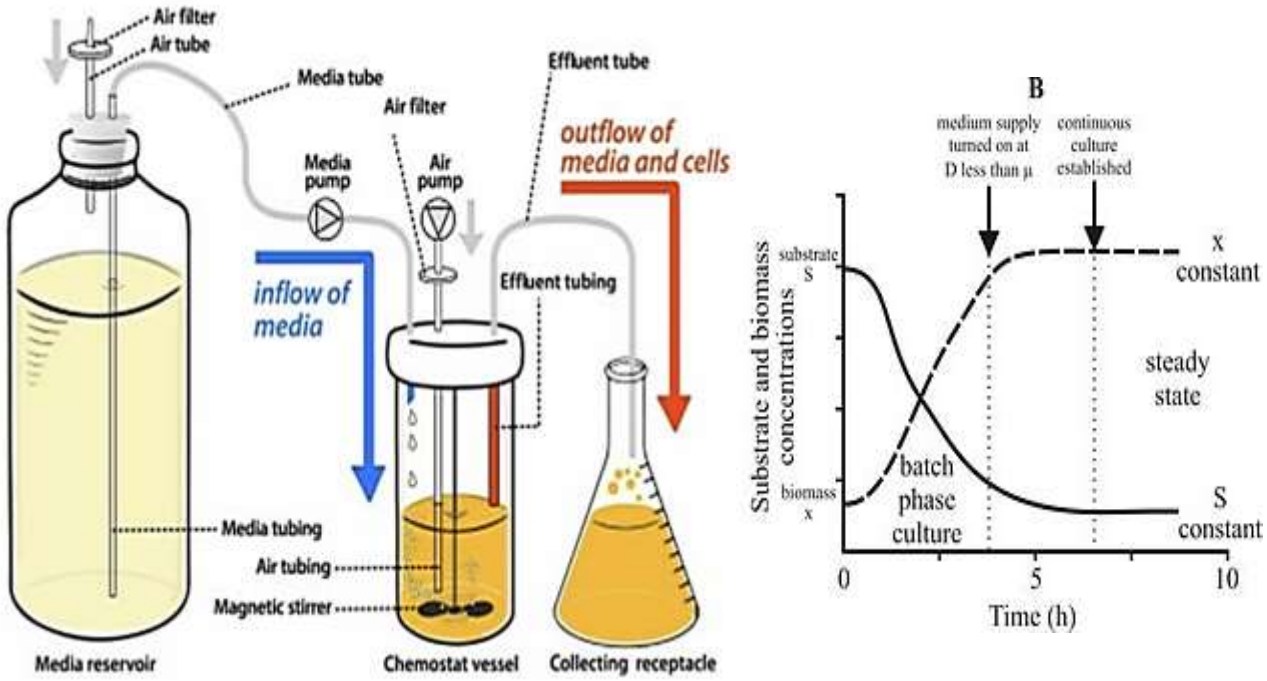

Figure I.8. Chemostat mode illustration [21].

The dilution rate D is fundamental for a chemostat which is defined as the frequency or rate of renewal of the reactor in new medium (Figure I.8). Chemostats inoculated with a pure strain exhibit a remarkable property with two possible situations [22]:

- **If D < μ_{max}** the chemostat will reach a stationary dynamic or steady state for which D=μ and d[X]/dt=0 and d[S_f]/dt=0. The stationary state obtained is independent of the initial inoculum load. It obviously depends on the composition of the feeding medium and the dilution ratio and of course on the strain used and the temperature.
- **If D>μ_{max},** the dynamics of the chemostat is its washing, i.e. it will be washed and little by little the biomass concentration ([X]) will tend towards zero and the composition of the bioreactor medium will become identical to that of the new medium (the biomass gradually tends towards 0).

b. Monod modeling of chemostat

The chemostat will be modeled using a series of simple differential equations. In this fundamentally simple model, cells multiply, and any cell mortality is ignored, and no production of any product in the medium by the cells is considered. Similarly, no inhibitors appear The growth kinetics of cells growing in steady state when restricted by a single growth limiting nutrient have been described by a variety of models in a chemostat. The Monod model is preferred due to the fact that the parameters in question specific growth rate μ, maximum specific growth rate μ_{max} of bacteria, rate limiting

substrate concentration S, and substrate affinity constant K_s have empirical measurements and meanings that can be obtained through experimentation [23].

Limitation of μ (Equation 1)

D is the dilution rate, where the specific growth rate is a function of time dependent concentration S(t), when considering a simple system like the chemostat, with a total biomass X, and an initial substrate concentration of S_0. We make the assumption that one of the nutrients, S_L, will be the one that sets a limit on the biomass's particular growth rate (μ). The concentrations of the other nutrients are sufficiently high to prevent this same limiting impact. The following equation of Monod controls the limits of μ:

$$\mu = \mu_{max} \cdot \frac{S_L}{S_L + K_{S_L}} \quad \text{…… (Equation 1)}$$

Monod constant K_{SL} is defined as the concentration of substrate, that allows cells to grow at ½ μmax [22].

Biomass concentration balance (Equation 2)

For biomass [X] in the bioreactor:

$$\frac{dX}{dt} = r_X - D.X = \mu X - DX = (\mu - D)X \quad \text{……..(Equation 2)}$$

Where μX is production rate (variation in biomass concentration per unit of time), and DX is the output of biomass through the effluent in variation of concentration per unit of time.

Concentration balance for substrates S, including S_L (Equation 3)

The final balance for the different S substrates (also valid for Sl, the limiting substrate of μ):

$$\frac{dS}{dt} = DS_{feed} - D.S - \frac{r_X}{Y_{X/S}} - m_S X \quad \text{……..(Equation 3)}$$

Where DS_{feed} is input flow rate of substrate S in concentration variation per unit time, DS is the output flow rate of substrate S in concentration variation per unit time and $\frac{r_X}{Y_{X/S}} - m_S S$ is rate of consumption of substrate S by cells in variation of concentration per unit of time.

by replacing rX by μ[X] in this equation we obtain:

$$\frac{dS}{dt} = DS_{feed} - D.S - \frac{\mu X}{Y_{X/S}} - m_S X = D\left(S_{feed} - S\right) - \left(\frac{\mu}{Y_{X/S}} + m_S\right)X \quad \text{……..(48)}$$

mS, maintenance coefficient is often (not always) very low compared to $\frac{\mu}{Y_{X/S}}$ and therefore, the maintenance term can often be neglected in this equation.

The system that combines the three equations regulates the chemostat's operation:

$$\mu = \mu_{max} \cdot \frac{S_L}{S_L + K_{S_L}} \dots\dots\dots\dots\dots Equation\ 1$$

$$\frac{dX}{dt} = (\mu - D)X \dots\dots\dots\dots\dots Equation\ 2$$

$$\frac{dS}{dt} = D\left(S_{feed} - S\right) - \left(\frac{\mu}{Y_{X/S}} + m_S\right)X \dots\dots\dots\dots\dots\dots\dots Equation\ 3$$

A well "balanced" system since we have for example 3 unknowns: μ, X and S_L (limiting substrate of μ) for 3 equations. The other elements are, in fact, physiological parameters of the strain in culture (μ_{max}, K_{SL}, $Y_{X/Sl}$, m_S) or "construction" parameters of the chemostat (S_{feed}, and D).

Turbidostat

Turbidostat is another continuous culture system first introduced by Bryson and Szybalski (1952). Turbidostats are continuous fermentations with feedback control in which the medium feeding rate is regulated to maintain the cell concentration at a constant level [24]. Turbidistats dynamically adjust the flow rate (and therefore the dilution ratio) to maintain constant turbidity. They use a spectrophotometer/turbidimeter to measure optical density for control purposes (Figure I.9).

This device ensures continuous culture where the specific growth rate is maximum (μmax) and a constant biomass X, which will be chosen in the exponential phase and will remain constant thanks to a regulation of D, i.e.: μ = D. The apparatus is very simple, comprising a photoelectric cell set to a chosen value of X and connected to the fresh medium inlet valve. This is a self-regulation of D based on the biomass measured at the outlet [25].

Turbidostat operating principle:

• If the turbidity increases too much, the supply of fresh medium is increased, which dilutes it and returns it to its initial value.

• If the turbidity decreases too much, the supply of fresh medium is decreased until growth has allowed it to return to its initial value.

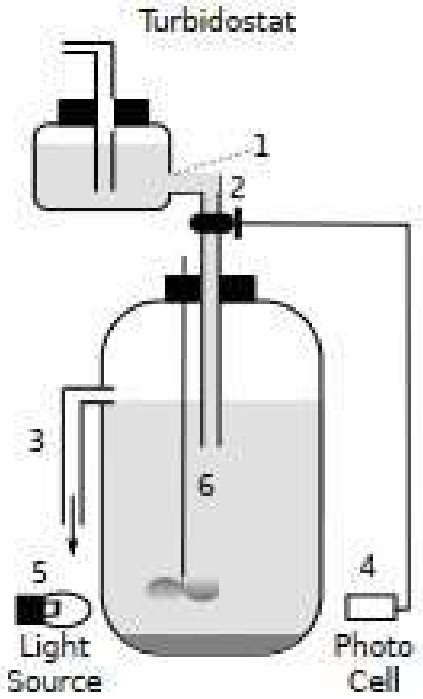

Figure 1.9. Turbidostat.

Through steps 1 and 2, new resources enter the system. Mature species and certain resources are pumped out through 3 after species are cultivated in 6. The system's dilution process is managed by the feedback options (4 and 5), maintaining a constant species density [25].

The turbidostat differs from the chemostat in the following ways:

- The dilution ratio D in a chemostat varies rather than remaining constant in the turbidostat.

- The culture medium does not contain a limiting factor in the turbidostat.

Continuous fermentation with biomass recycling

There are various techniques to recycle biomass [26]:

**** Centrifugation :*** Centrifugation is an effective method that involves subjecting the effluent from the fermentation tank to centrifugation. The microorganisms are collected (pellet) and then reinserted into the fermenter. This fermentation method is frequently used, particularly for the production of ethanol via alcoholic fermentation.

**** Static decantation:*** The effluent passes through a container in which the biomass is concentrated by sedimentation. This solution produces relatively good results with microorganisms that tend to flocculate (coagulation = clumping). This method is used particularly in biological wastewater treatment to recycle activated sludge. This method is rarely used on an industrial scale.

**** Micro- or ultrafiltration:*** the application of membrane modules for micro- or ultrafiltration in order to attain a high concentration of cells. Indeed, the connecting of the fermenter and the membrane modules needs the employment of circulation pumps. Installing a heat exchanger fixes the heating phenomena that arise from this. Shear phenomena caused by pumps and modules may have an impact on the microorganisms' condition. Consideration must also be given to filter clogging.

I.2.2. Solid state fermentation

The growth of microorganisms without a free-flowing aqueous phase is known as solid-state fermentation, or solid substrate fermentation (SSF). is employed in systems that cultivate microorganisms on the surface of a concentrated water-insoluble substrate with a low concentration of free water. This substrate often contains polysaccharides as a source of carbon and energy. For the generation of value-added products such as antibiotics, single-cell proteins, polyunsaturated fatty acids, enzymes, organic acids, biopesticides, biofuel, and fragrances, the SSF is an alternative to submerged fermentation [27, 28].

I.2.2.1. Solid-state sermentation process and applications

In order to transform a starting substrate into products with added value, SSF is a three-phase heterogeneous process that involves solid, liquid, and gaseous phases. Due to its capacity to produce more products with a lower risk of contamination while maintaining economic and environmental sustainability, SSF has attracted a lot of attention in the last 20 years for the development of industrial bioprocesses. SSF is influenced by a number of factors that are crucial to the technical and economical feasibility of process development [29].

Finding the ideal physical, chemical, and biological process factors (such as pH, aeration, temperature, humidity, and solid material properties) is one of these parameters, along with choosing the appropriate microbe/consortium and substrate. Another factor that affects the viability of producing SSF is the product's purification.

Two of the primary SSF issues to be resolved in scale-up are heat accumulation during the fermentation process and the substrate's heterogeneous character (a complex gas-liquid-solid multiphase system). SSF proves to be a viable approach in a number of domains beyond the manufacture of biopesticides and biostimulants.

SSF is frequently used in the manufacturing of biofuels, metabolites (such as antibiotics, aromas, biosurfactants, enzymes, and organic acids), and environmental applications, such as bioremediation. This method not only lowers process costs but also lowers product prices for customers. Different types of bioreactors are used to carry out these productions [28, 29].

I.2.2.2. Typical features of solid substrate fermentation

The primary characteristics of this system differ significantly from those of classical submerged cultivation, as indicated in Table I.1, because solid substrate fermentation is characterized by extremely low water activity when the relative humidity of the gaseous phase in equilibrium with the moist solid is significantly below 1. SSF has a number advantages over traditional submerged technology, including a) the use of a concentrated medium, which reduces the reactor volume and capital investment costs; b) the low moisture levels and substrate complexity, which reduce the risk of bacterial and yeast contamination; c) the ease of use of the technology and the minimal amount of effluent water produced during the process; d) the increased product yield and simpler product recovery; and e) the use of agricultural wastes as substrates for specific applications, such as feed supplements and cellulolytic enzymes [27].

Table I.1. Main Differences between Submerged fermentation and Solid Substrate Fermentation[27].

Solid substrate fermentation	Submerged fermentation
Nonhomogeneous system, gradient of nutrients	Homogeneous system
Complex substrate insoluble in water, high local concentration of nutrients	Nutrients are dissolved in water, concentration of nutrients is lower
Low water content of the cultivation medium (40–80%)	Liquid cultivation medium (~95% water content)
Three-phase system: gas–liquid–solid	Two-phase system: gas–liquid
Limitations in heat, oxygen, and nutrient transfer	Transport processes are usually not limited (exception can be oxygen transfer)
Gas–liquid and liquid–solid oxygen transfer	Gas–liquid oxygen transfer
Microorganisms are grown on the surface of the solid substrate	Microorganisms are grown in the liquid medium
High concentration of the product	Product is dissolved in the liquid phase
Process monitoring and control are difficult	Online monitoring and control of the process are common
Heat is removed by using a stream of air or by placing the bioreactor into the temperature-controlled chamber	Cooling is achieved by bioreactor jacket cooling system

I.2.2.3. Substrates and microorganisms used in SSF Processes

The efficiency of Solid-State Fermentation (SSF) depends heavily on the selection of the solid substrate. Ideal substrates should have a porous structure with a large surface area, support gentle compression and mixing, contain biodegradable carbohydrates, absorb water relative to their dry weight, maintain high water activity for microbial growth, and absorb added nutrients like nitrogen sources and mineral salts. The best materials are small granular or fibrous particles that remain intact and don't clump together. Common substrates include wheat, wheat bran, soybean, rice, barley, oats, and other cereals [27].

Filamentous fungi are preferred for SSF processes because they can (a) grow on substrates with low water activity, (b) penetrate their hyphae into the solid substrate, and (c) produce exoenzymes (amylolytic and cellulolytic enzymes) that break down polysaccharides, the main carbon source in solid substrates. When using yeast, a material pretreatment step (such as steam explosion, acid or alkali treatment, followed by enzymatic digestion, or a combination) is required. Alternatively, a mixed culture can be used, where complex cellulosic or starchy materials are initially degraded by molds that produce extracellular enzymes, and the released glucose is then consumed by yeast (or less often by bacteria) to yield the desired product [27].

I.2.2.4. Challenges and limitations of solid substrate fermentation

Even though SSF has many advantages over liquid cultivation, its primary challenges are its inability to precisely control the microbial environment due to poor heat and mass transfers within the substrate and its limited ability to monitor important cultivation parameters online, such as temperature, pH, dissolved oxygen, nutrient concentrations, or water content. One of the main obstacles to process scale-up is the heat produced by microbial metabolism; from 1 kg of digested substrate, up to 3000 kcal of heat can be released, creating a 5°C/cm radial gradient in the reactor's center.

The system's low water content and the SSF substrates' poor heat conductivity encourage the heat gradient, which is exacerbated by little movement (the molds are extremely sensitive to shear stress) and lessened primarily by evaporative cooling, which can, on the other hand, make more water loss worse. Another problem is the pH gradient brought on primarily by the synthesis of organic acids and the use of proteins; pH monitoring and control are challenging due to the fact that no pH electrode currently in use can function without free water. Because urea can counteract acidification, it should be added, or the substrate buffering capacity should be increased (by adding $CaCO_3$) to prevent pH fluctuations. By aerating with moist air, which also aids in the desorption of carbon dioxide and controls temperature and moisture content, oxygen gradients can be reduced [27].

I.2.2.5. Solid-state fermentation bioreactors

A few grams of matrix can be processed using inert supports (such as Petri dishes, flasks, and bottles) in laboratory-scale SSF devices to do quick screenings (such as determining the ideal temperature or inoculum-to-matrix ratios). At this scale, forced aeration and agitation are typically not used; instead, the temperature is the only controllable parameter. Pilot and industrial scale processes are examined and optimized in bioreactors with advanced control systems after the ideal lab scale conditions (such as the inoculum rate, matrix amounts, and ideal temperature) are achieved. The presence or lack of forced aeration and agitation is the primary factor that distinguishes the various types of bioreactors [29].

The simplest type is the tray bioreactor (Figure I.10.A) in which the solid material is laid on trays constructed with inert material (metal, wood, plastic). Trays are placed in a tray chamber with a suitable gap among them in which a circulating air controls temperature and humidity. In tray bioreactors, the air is not forced, and agitation might occur occasionally based on the process carried out.

Packed-bed bioreactors, which are glass or plastic column reactors with solid material packed inside, were discovered in the presence of occasional agitation and forced aeration (Figure I.10.B). Fluxing air from the bottom ensures aeration, and external equipment, such as heat exchangers or cooling/heating jackets, regulate the temperature. Packed-bed bioreactors can also be utilized with forced aeration and occasional mixing, as long as airflow or a mechanical stirrer is used to agitate the mixture.

Two varieties of stirred drum bioreactors are available for SSF that require gradual, continuous agitation without the need for forced aeration (Figure I.10. C). These bioreactors work by filling the drum with solid material and blowing air into it. Paddles inside the drum unit in the stirred-drum bioreactor (below) and a spinning drum in the rotating-drum bioreactor (above) ensure agitation [29-30].

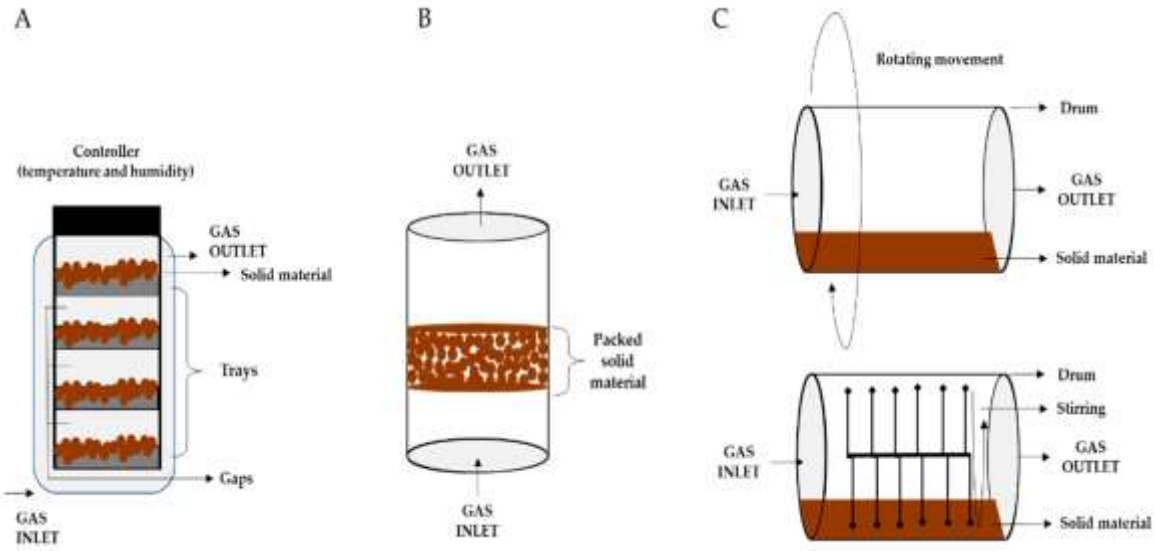

Figure I.10. SSF bioreactors without forced aeration and with occasional agitation.

A; Tray bioreactor; with forced aeration and sporadic stirring; B. Packed-bed bioreactor); and without forced aeration and with gradual, continuous agitation; C. Two types of stirring drums [29].

Three different types of bioreactors can be utilized in SSF with forced aeration and slow continuous agitation: stirred-aerated bioreactors (Figure I.11. A), gas-solid fluidized beds bioreactors (Figure I.11.B), and rocking drums bioreactors (Figure I.11. C).

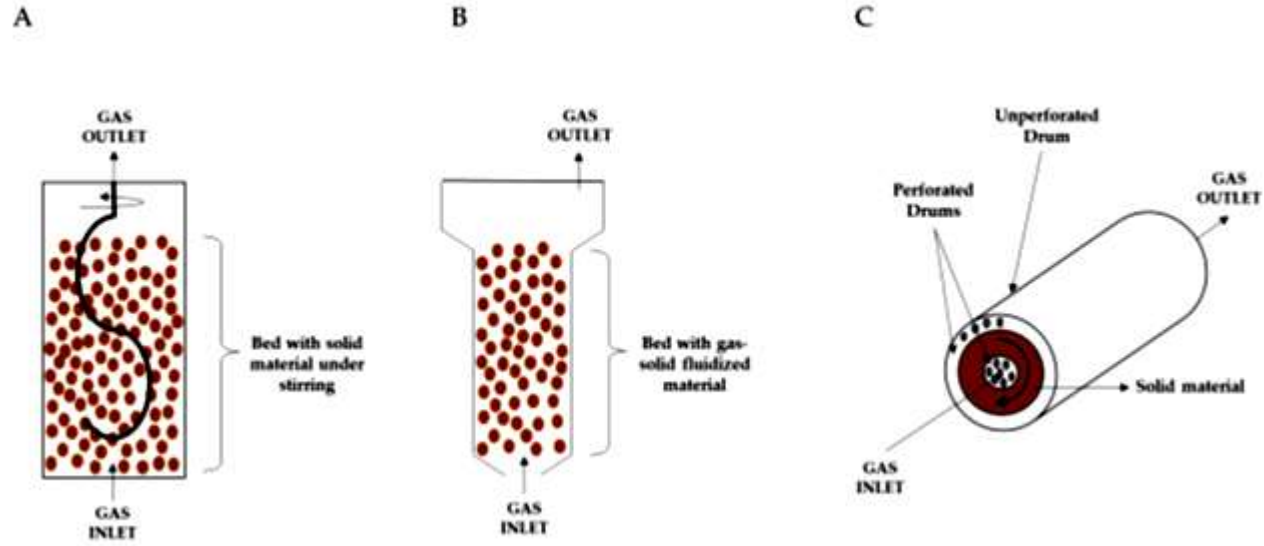

Figure I.11. SSF bioreactors with forced aeration and continuous agitation.

(*A). Stirred aired bioreactor; (B). Gas-solid fluidized bed bioreactor; (C). Rocking drum bioreactor* [29].

These reactors agitate the bed by vigorously blowing air through it. Such a bioreactor can typically be operated in one of two modes, depending on the type of mixing: intermittently mixed bioreactors or continuously mixed bioreactors. The mixing system lowers the cooling requirement since water is added to the bed. Whether to use continuous or intermittent mixing will depend on the mechanical and adhesive properties of the substrate particles, as well as the microbes' sensitivity to shear effects from mixing. Despite the fact that numerous research has employed and established a wide range of substitutes for these fermenters, the tray- or drum-type bioreactor continues to be the initial model for their design [29-30].

I.2.3. Immobilized cell systems

The physical confinement or localisation of intact cells to a certain region of space; without loss of desired biological activity is one definition of immobilisation of whole cells. The terms "bioencapsulation" or "microencapsulation" are used to describe the encapsulation of cells in an immobilised cell system, while the latter word is used to describe the immobilisation of cells in microcapsules, which are systems that are micrometres in size and encased in a barrier membrane. Proteins and other biological molecules have recently been successfully nanoencapsulated [31].

The producing microorganisms in this type of process are immobilized on a substrate or matrix rather than developing freely in the media. A fermented medium containing the desired product is introduced at the output, while fresh medium is introduced at the input to feed the biomass. Immobilized cells have been used to produce bacitracin, cephalosporin, and chlortetracycline [27].

Immobilized cell systems can be divided into four groups (Figure I.12) based on their physical location and the type of microenvironment.

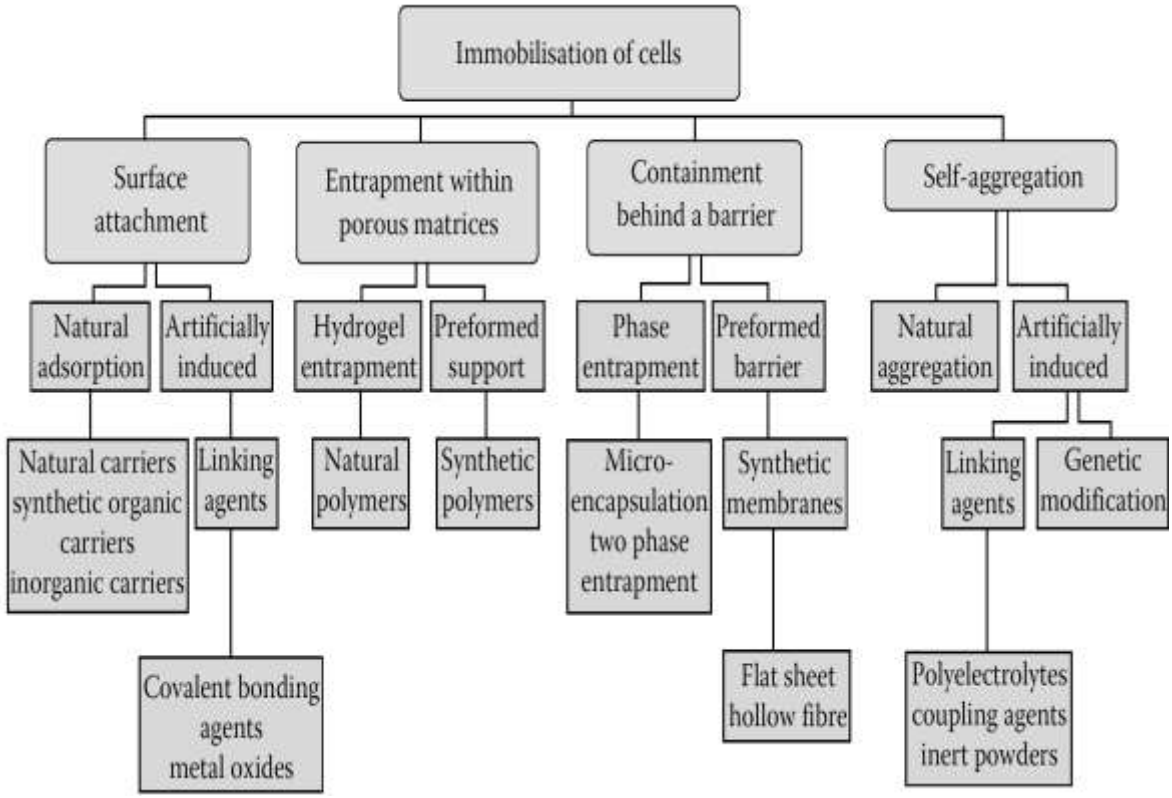

Figure I.12. Classification of immobilized cell systems according to the physical localization and the nature of the microenvironment [31].

The development of these systems presents several advantages:

-At the end of culture, biological material is preserved so that it can be utilized again for subsequent cycles;

-Because the strain is not dispersed throughout the reaction medium, product recovery is simpler;

-Reaction rates and cell density have increased.

I.2.3.1. Immobilized cell methods

All cell types can, in theory, be immobilized by a variety of methods that are based on the methods for enzyme immobilization. There are four possible forms of immobility [27-31]:

-Adsorption: which is an old process used in the manufacture of vinegar, in which *Acetobacter* are adsorbed onto beech shavings. Now, PVC beads, DEAE cellulose (Diethylaminoethyl cellulose) are used (*Nocardia erythropolis*: conversion of steroids).

-Inclusion: with incorporation of microorganisms into a matrix of a rigid polymer such as sodium alginate (*Sc. cerevisiae*: production of alcohol, *Methanosarcina barkeri*: production of methane, polyacrylamide (*Arthrobacter simplex*: production of hydrocortisone, *B. subtilis*: production of

alpha-amylase, *Aspergillus niger*: production of citric acid). Several embodiments are possible: the gel can be placed in a column, in which circulates the fresh medium which comes out with the biosynthesis products. The gels can be used in batch or continuous culture in conventional bioreactors.

-Covalent bonding: which is less used for microorganisms. This is an irreversible chemical covalent bond that involves the groups of the side chains of amino acids and a ligand fixed to the support. In addition to irreversibility, this system has the disadvantage of using reagents as ligands that are often toxic to microorganisms;

-Flocculation: a system by which the aggregation of cells is caused, for example by the addition of). It involves hydrogen bonds and Wan der Waals bonds. It is notably used in polyelectrolytes (chitosan) for the treatment of wastewater in activated sludge.

I.2.3.2. Immobilized cell bioreactors

Three categories can be distinguished: bioreactors filled with (1) mixed, suspended particles; (2) fixed particles or large surfaces; or (3) moving surfaces. Most bioreactors (Figure I.13) contain three phases: solid (the carrier or cell aggregate), bulk liquid, and gas (air/oxygen or gas feed, gaseous products) [31-32]:

****Bubble column bioreactor (BCRs):*** The chemical, biochemical, and petrochemical industries heavily rely on bubble columns as multiphase contactors and reactors. They offer a number of benefits during operation and upkeep, including low operating and maintenance costs, compactness, and high heat and mass transfer rates.

****Air lift bioreactor:*** An alternative to BCRs are airlift bioreactors (ARLs). The primary distinction is a central tube or additional parts (channels) that ensure effective fluid mixing and recirculation. Airlift reactors are pneumatically agitated bioreactors in which the reaction broth circulates between the riser and an associated downcomer compartment that contains a smaller (or insignificant) gas phase when a gas stream is injected into a particular reactor compartment, the riser.

****Fluidized-bed bioreactors and tapered fluidized bioreactor:*** Tall columns that have an aspect ratio (height to diameter) of more than 10:1 are used as fermenters for fluidized bed (FB) operations. For fluidized bed fermentations (FBFs), the fluidizing medium is typically broth; for biological fluidised beds (BFBs), it is wastewater; the fluid flows up through the bed; the fluidizing medium can be a gas, liquid, or a combination of the two; the flow is upward for particles that are denser than the fluid or downward for particles that are less dense, where clogging of the beads is avoided by adjusting their density and flow rate, allowing for better mass transfer.

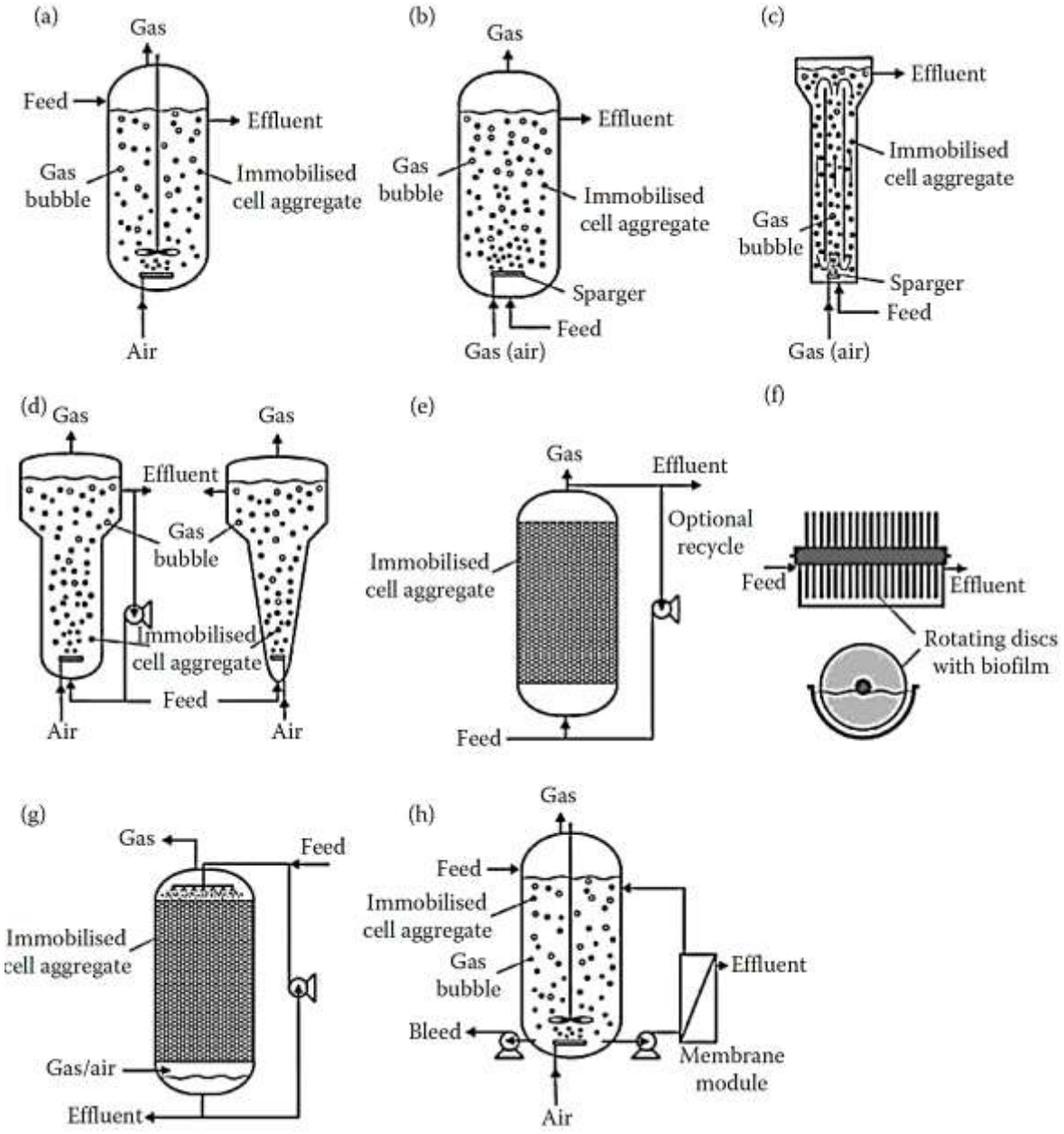

Figure I.13. Immobilized cell bioreactors.

(*a) stirred-tank bioreactor; (b) bubble column bioreactor; (c) air lift bio reactor; (d) fluidized-bed bioreactor (left), tapered fluidized bioreactor (right); (e) packed-bed bioreactor with optional recycle loop; (f) rotating drum bioreactor; (g) trickle bed bioreactor; and (h) membrane cell-recycle bioreactor* [31].

***Fixed-bed bioreactors (Packed-bed bioreactors):** The primary components of packed bed bioreactors (PBRs) are a reservoir that circulates the oxygenated nutritional medium across the bed and a packed bed that supports the cells on or within carriers. beads are packed in a column, with aeration and various controls performed by an external system.

Rotating drum bioreactors: There are two primary characteristics of rotating-drum bioreactors. First, a horizontal or inclined drum is used to hold the substrate bed, and the drum's rotation is used to mix the bed. Second, the headspace above the solid bed is blown with air. This indicates that it is not compelled to pass through the bed. Instead, the gas exchange between the headspace and the bed aerates the bed.

Trickle bed bioreactors: A column containing packing material that permits cell growth is called a trickle-bed reactor. Depending on the application, the gaseous substrate flows either downhill (cocurrent) or upward (countercurrent) while the microbial culture media constantly flows downward. In order to maximise gas-liquid contact and minimise resistance to mass transfer, fermentation media trickles down the bed as a thin film while microorganisms grow as biofilm on the bed. There is no need for agitation because the trickle-bed reactors are run at atmospheric pressure.

Membrane bioreactors: Specialised membranes having a certain molecular weight cutoff are used in membrane bioreactors for product separation, nutrient delivery, or in situ aeration. In a cell compartment, they are intended to preserve cell biomass and perhaps recombinant protein products. Despite the fact that membrane bioreactors reduce the shear stress on cultured cells, research has shown that mass transfer is crucial, even for plant cells that grow rather slowly [31-32].

II. Fermenters

The fermenter is an essential part of the fermentation process. A fermenter (bio-reactor) is a closed vessel that is set up for aeration, agitation, pH control, and temperature control. Additionally, it has a drain or overflow vent to remove the waste biomass of the farmed microbes and their products. It is a containment system that provides a controlled environment for the growth of bacteria. In order to create the appropriate product, the gadget must nurture favourable bacteria. Additionally, it stops contaminating microorganisms from entering and growing from the external environment. The fermenter serves as a containment mechanism for the growth of fungus and bacteria. Insect or mammalian cells can be cultivated in bio-reactors, which are fermenters. In commercial manufacturing, live cells use low-value substrates to produce high-value products in a fermenter. Fermenters are employed in waste treatment and food processing in addition to fermentation [31-32].

The primary purpose of a fermenter is to:

1. Provide a controlled and favourable environment, including pH, temperature, and pressure, for the biological agent (microorganisms).
2. To act as a defined microbial mixture prior to sufficient mixing for the transmission of nutrients and products.
3. Sufficient sparging to aerate and degas produced gases.
4. To achieve the intended result following fermentation.
5. To use mineral power input to create a uniform culture.
6. To make it possible to preserve ideal circumstances for the development of cells and products.
7. A sturdy vessel that can endure the several procedures needed, such as pressure sterilisation and high heat.
8. The sampling valve for taking a sample for various tests and the inoculation point for aseptic transfer of inoculums.

So, the fermenter ensures and enables:

- Homogenization of the culture (biomass and medium) as well as temperature homogenization and oxygenation of the culture.

- Monitoring and/or regulation of certain parameters: temperature, pH, dissolved oxygen, foam levels, various inputs, etc.

- Aseptic process control if necessary (by far the most common case).

Two things are achieved by stirring: (i) the gas bubbles are mixed through the liquid culture media, and (ii) the microbial cells are mixed through the liquid culture medium. Stirring in this manner guarantees that microbial cells have consistent access to the nutrients. Depending on the stirring method, there are two types of fermenters [32-33]:

II.1. Mechanically stirred fermenter

Mechanically agitated bioreactors (impellers propeller). They are very popular and most studied. Stirred Tank Reactors (STRs) are reactors that are mechanically stirred. Due of its simplicity, this model is the most popular. Volumes ranging from one litre to 500,000 litres are available for mechanically stirred fermenters. Batch, fed-batch, continuous fed with chemostat withdrawal, perfused (biomass recycling), and other key culture techniques can all be utilised with STR reactors.

Impellers are classified according to the flow patterns they produce (Figure II.1); they frequently display multiple dominating flow patterns.

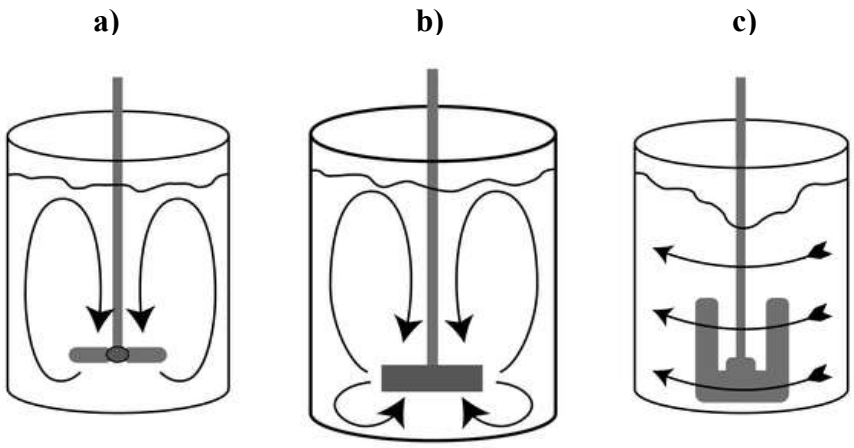

Figure II.1. Axial (a), radial (b) and tangential (c) flow pattern(Online Source 1).

II.1.1. Axial agitator

Propeller with large, thin blades, marine-style. Axial agitators are commonly used in fermenters that require large-scale, continuous mixing or where the process benefits from pumping liquid in a vertical direction. They are especially effective in processes that require high flow rates and gentle mixing. The blades of an axial agitator are mounted on a vertical shaft, and they push the liquid along the axis of rotation (upward or downward). The blades are typically angled to generate axial flow, meaning the liquid moves in the same direction as the shaft rotation.

When axial flow impellers are used, the media flows parallel to the impeller's axis of rotation. These impellers' blades are tilted less than 90 degrees to the plane of rotation, which creates a "top-to-bottom cyclic" flow pattern inside the tank. Fluid near the top is pulled downward by the impeller

until it reaches the bottom, where it distributes across the tank floor and then flows upward along the wall before being dragged back into the impeller. This movement prevents sediments and solutes from settling to the bottom of the tank by effectively spreading the fluid throughout it. Solid suspensions and fluids with low to medium viscosities can be used with axial flow impellers. They are perfect for shear-sensitive media, such as non-Newtonian fluids or those impacted by stress-induced changes in viscosity, because of their low shear properties. In order to create significant vertical currents, they are usually placed in tanks with high liquid levels and are also used in heat transfer applications [32-33].

II.1.2. Radial agitator

Propeller with straight or curved blades. Radial agitators have blades that rotate perpendicular to the axis of the agitator shaft, and they push the liquid in a radial direction (outward from the center). Fluid is moved sideways and then either up or down, before moving back to the center. Radial flow is suitable for high-shear applications like emulsification or liquid-gas dispersion. While shear tends to be higher, there's less flow compared to axial flow. This causes the fluid to move outward from the center of the fermenter to the walls and then back toward the center. Radial agitators are often used in applications that involve slurry or systems where gas dispersion is important. They are particularly effective for the agitation of viscoelastic materials or systems where foam is a concern.

A "side-to-side flow pattern" is created within the tank by radial flow impellers, which force the media to flow perpendicular to the impeller's axis of rotation. The fluid that is ejected from the impeller travels in the direction of the tank walls, then either upward or downhill before coming back to the centre of the impeller. The contents of the tank are completely mixed by this constant action. Baffles are required to reduce swirling and vortex formation because radial flow impellers, in contrast to axial flow impellers, lack inclined blades that guide fluid downward. Because of their sideways fluid movement, radial flow impellers cause significant shear and reduced overall flow. They work well for gas-liquid and liquid-liquid dispersions as well as for mixing viscous liquids. These impellers are frequently utilised in low-level mixing applications in extended tanks [32-33].

II.1.3. Tangential agitator

Fluid is moved horizontally around the vessel as with a paddle. This is most suitable for mixing high-viscosity materials. Shear is low and there is little vertical flow. Tangential flow impellers cause the fluid to revolve around the vessel in tandem with the impeller blades by directing the media to flow in a circular pattern around the shaft. When the fluid strikes the tank wall, this circular motion produces very little vertical flow. Low shear forces are a

characteristic of tangential flow impellers. Usually, these impellers are used to mix extremely viscous materials and encourage stratification inside the tank [32-33].

II.2. Pneumatically stirred fermenters

These bioreactors are combined non-mechanically (pneumatically), either by air bubbling or pneumatically, without the use of mechanical agitators. These consist of split airlift, airlift with internal draft tube, and external loop airlift bioreactors, and also bubble columns. Compared to mechanical, they have many advantages [33]:

1. Less expensive, more straightforward, and simpler to build.
2. Less energy is needed for mixing.
3. Typically, there is very little hydrodynamic stress.

II.2.1. Bubble column fermenters

The bubble column is a reactor also used for the aerobic production of microorganisms. Air bubbles injected at the base of the column provide oxygen to the microorganisms and mix the liquid phase during their upward movement. A sparger is employed to supply air into the continuous liquid phase at the reactor's bottom, and density-driven fluid movements are used to agitate the bioreactor. When convective flows turn turbulent, the fluid intensely mixed at high gas flow rates. The diagram of Bubble column fermenters shown in figure II.2. Bubble column fermenters have several benefits, including low maintenance, easy operation, and good heat and mass transfer. Bubble column fermenters' drawbacks include one significant disadvantage that has a negative impact on product conversion is back mixing [33].

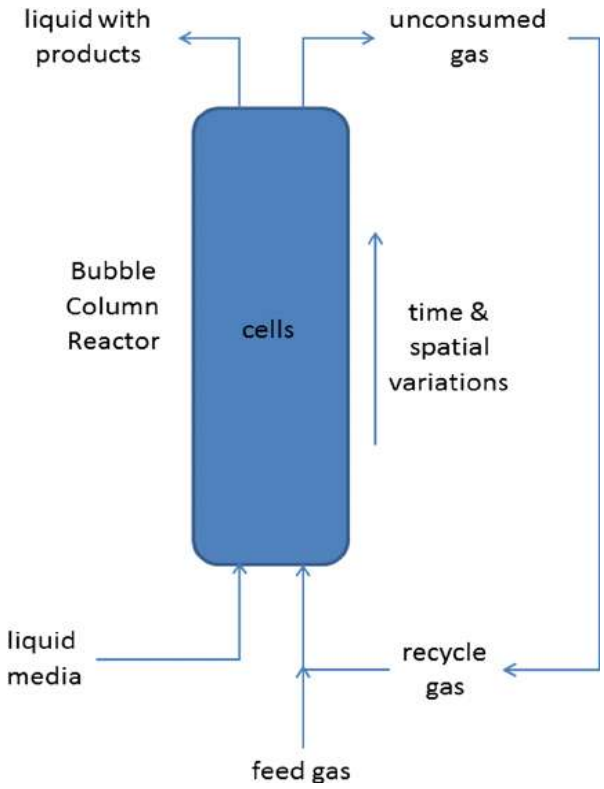

Figure II.2. Bubble column fermenter [33].

II.2.2. Airlift fermenters

This device is derived from the bubble column and is used exclusively for aerobic cultures. An airlift reactor is a bubble column comprising either an internal concentric tube placed above the gas distributor or an external recirculation tube. The addition of these devices significantly modifies the circulation of the liquid phase. The movement of the liquid phase is induced by the difference between the apparent densities of the gas-liquid dispersion located in the central part of the reactor and the liquid phase located in the other part.

Air is pumped through a baffle or draft tube that serves as the vessel's medium. This might cause a bubble to form in the medium, which would eventually aid in the fluid's ascent through the baffle tube and pull the surrounding fluid along with it. Figure II.3 shows the diagram. In the process, the air stirs up the contents [33].

Two varieties of airlift fermenters exist [33-34]:

1-Internal-loop airlift fermenter has a single container with a draft carrying out the fermentation process.

2- External loop airlift fermenter has an external loop to separate samples or liquids in different channels carrying out fermentation.

Benefits of Airlift fermenters: it creates minimal friction and very little shear stress, the fermenter may be built with less effort, it is reasonably priced and there is less need for energy.

Cons of Airlift fermenters is this method requires high pressure, and its main disadvantage is that it lacks a shaft that would act as a foam breaker [33].

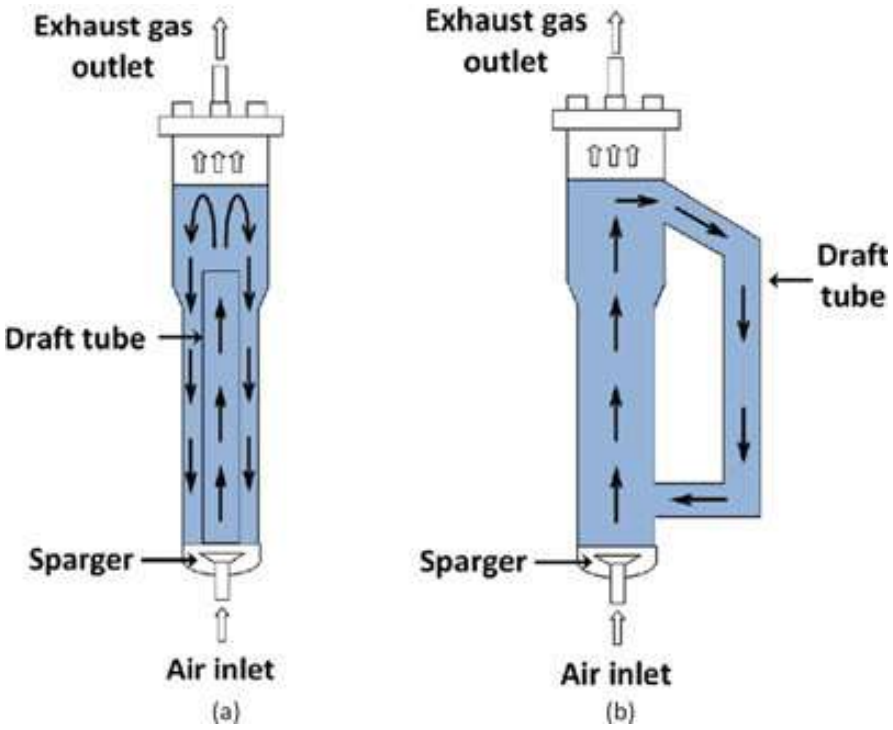

Figure II.3. Airlift fermenter with (a) internal and with (b) external circulation loop [34].

II.3. Hydrodynamics of fermenters

II.3.1. Power consumed

The economics of the mixing process are significantly impacted by power usage. The incorrect equipment selection can result in significantly higher power consumption than is required to provide the intended outcome. However, in order to guarantee that the endpoint of the mixing process may be reached in a reasonable amount of time, enough power must be provided to the fluid [34-35]. One of the most important factors to take into account when it comes to mixing tanks is their power consumption. Before building begins, it is helpful to compare different mixing tank configurations to see which is best. A dimensionless number known as the power number is used to do this. The mechanical power P (Watt) consumed is calculated using the power number Np, also called the drag coefficient of the agitator in the fluid, representing the ratio between the external forces and the inertial forces [35]:

$$P = N_P N^3 D^5 \rho \text{.....(49)}$$

Where ρ: density of the medium expressed in kg/m^3, D is the diameter of the agitator in m, N rotational speed of the agitator in rev/s , Np is the power number (blade) its value is determined from the Rushton chart representing Np = f(Re) and P is the power consumed in watts.

We can analyse the power draw of two distinct mixing tanks and, consequently, determine which is most effective for any given purpose by comparing the power numbers of various impeller diameter configurations [35].

II.3.2. Reynolds number

The Reynolds number (Re) represents the ratio between the inertial and viscosity forces, it is dimensionless and it characterizes the type of flow. It can be used to identify whether a flow is laminar, transient, or turbulent by measuring the level of turbulence created by the impeller in a particular mixing system. This can be found using following equation:

$$Re = \frac{N D^2 \rho}{\mu} \text{.....(50)}$$

Where D (in m) is the diameter of the agitator, N (in rev/s) is the rotation speed, ρ (in kg/m^3) is the density of the fluid and μ (in Pa.s) is the dynamic viscosity of the fluid [35].

The flow is considered laminar flow, if Re <2000, between 2000 and 4000 it is a transitional flow (between laminar and turbulent), and if 4000<Re the flow is turbulent (Figure II.4).

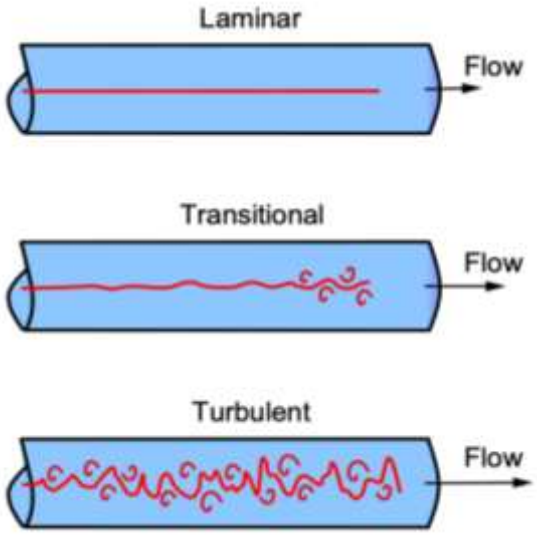

Figure II.4. Fluid flow regimes (Online Source 2).

II.3.3. Hydrodynamic regimes

In general, the degree of interfacial contact has a significant impact on the process efficiency of gas-liquid liquid-stirred tank reactors. Other crucial operating characteristics like mass transfer coefficients and volumetric heat also change as the gas–liquid interface varies per unit liquid volume (a). Two dimensionless numbers have been used to report and describe three regimes (Figure II.5) in the literature [36].

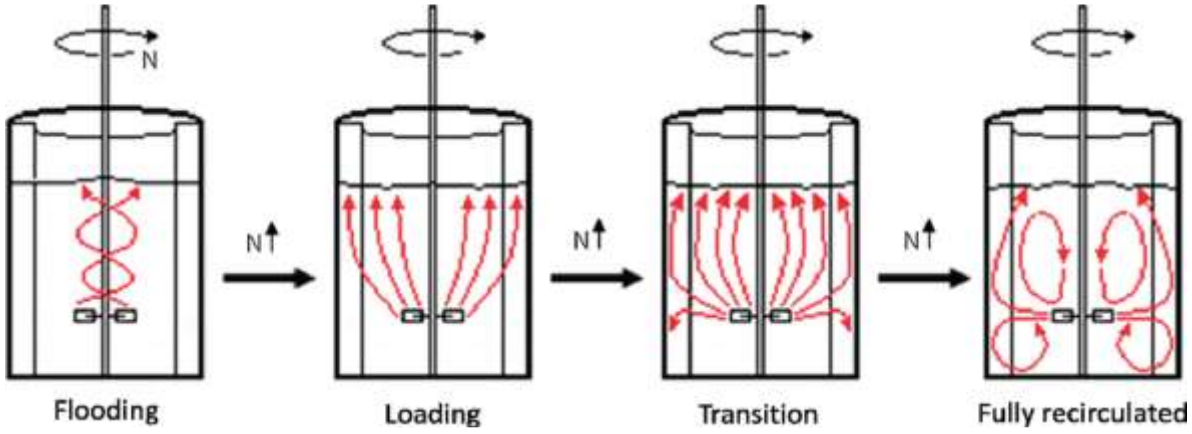

Figure II.5. Flow regime transition from flooding to loading to the fully recirculated regime.

More areas of the tank are filled with gas bubbles when N (impeller rotational speed) rises [36].

The two dimensionless numbers are the Flow Number (Fl) and the Froude Number (Fr), and these three regimes are flooding, loading, and fully recirculated. The ratio of the impeller-driven acceleration to gravity is the Fr number, and the ratio of the gas flow rate to the impeller-driven flow rate is the Fl number. In equation form,

$$\boldsymbol{Fl} = \frac{\boldsymbol{Q_g}}{\boldsymbol{ND^3}} \text{ ……..(51)}$$

$$\boldsymbol{Fr} = \frac{\boldsymbol{N^2\, D}}{\boldsymbol{g}} \text{ ……..(52)}$$

Where g is the gravitational constant, D is the turbine diameter, N is the impeller rotational rate, and Qg is the gas flow rate from the sparger [36].

The value of dimensionless numbers such as Na (aeration number) plays a crucial role in characterizing the transitions between different flow regimes in a stirred reactor or fermenter, particularly when dealing with systems that involve gas-liquid interactions, such as in fermentation processes. The aeration number (Na) is defined as:

$$Na = \frac{Q_g}{N\,D^3} \ldots\ldots\ldots\ldots (53)$$

Where Qg is the volumetric flow rate of the gas in m^3/s.

II.3.4. Solubility of gaz in liquide phase

At the gas-liquid interface, the concentration of the soluble species in the liquid phase is generally described by an equilibrium relationship. In the case of poorly soluble gases, such as oxygen, the equilibrium relationship is described by Henry's law:

$$P = H * C \ldots\ldots\ldots\ldots (54)$$

Where P = Partial pressure of the gas phase above the liquid in atm, C = concentration of dissolved gas in mol/L, H = Henry's constant L.atm/mol.

Henry's law is valid at the infinite dilution limit for solute i in solvent j. Hence, it is an appropriate choice for approximating poor solubilities in a solvent [37-38].

For aerobic germs, the dissolved O_2 concentration can be a critical limiting element in a process. To illustrate simply, we can recall and apply Monod's model of the limitation of the specific growth rate to O_2:

$$\mu = \mu_{max} \cdot \frac{[O_2]}{[O_2]+K_{50}} \ldots\ldots\ldots\ldots (55)$$

$K_{50} = K_{O2}$: this is the monod constant: the O_2 concentration which leads to half of μ_{max}.

II.4. Oxygen gas exchange

The objective of oxygen gas exchange is to satisfy the gas exchange needs of crops. Microorganisms use dissolved oxygen and release CO_2 in equilibrium with the gas phase. Two essential concepts emerge [37-38]:

II.4.1. Oxygen transfer rate

Transport phenomena between the liquid and gaseous phases are essential to the bioreactor's operation because it is a multiphasic system. The primary examples of the necessity for effective mass transfer include oxygen molecules, nutrients, and poisonous compounds that must be taken up or expelled from cells. The movement of oxygen in the gas-liquid phase is a limiting step since it is poorly soluble in aqueous systems. The mass transfer coefficient (K_{La}) and the concentration gradient across the gas-liquid interface are what define the oxygen transfer rate (OTR) in the development of

a novel bioreactor and systems for suspension cell culture in biopharmaceutical production. The gradient close to the gas-liquid interface is a complicated phenomenon that can range from relatively sluggish flow close to the interface to turbulent flow in the bulk phases [38].

Oxygen transfer rate is defined by the following relationship, which expresses the amount of oxygen transferred per unit of time (OTR) or transfer rate (rO_2) expressed in moles of O_2/L.h Oxygen transfer rate [37-38]:

$$\boldsymbol{OTR = K_{La}\,(C^{*} - C_{L})} \quad \text{……..(56)}$$

Where C_L is the oxygen concentration in the liquid phase in mol/L, C* is the oxygen concentration at saturation in mol/L and K_{La} is the volumetric transfer coefficient in s^{-1}.

Note that (C* - C_L) does not depend on agitation, but on the nature of the medium, the temperature, and the partial pressure of the constituent to be dissolved.

The transfer capacity of the reactors will depend on several variables:

- The agitation power and the efficiency of the agitation system (dispersion),

- The solubility of the gas depends on the pressure, flow rate, composition, temperature, and composition of the liquid medium.

II.4.2. Oxygen uptake rate

The Oxygen Uptake Rate (OUR) is a key parameter used to quantify the rate at which oxygen is consumed by microorganisms, cells, or any biological system. It is particularly important in processes such as aerobic fermentation, fermenter design, and cell culture. It measures the oxygen consumption requirements of the culture and is expressed in mole/l [37-38]:

$$\boldsymbol{OUR = \frac{r_X}{Y_{X/O_2}} = \frac{X\,\mu}{Y_{X/O_2}} = \frac{X}{Y_{X/O_2}} \cdot \frac{\mu_{max}\cdot[O_2]}{K_{O_2} + [O_2]}} \quad \text{……..(57)}$$

Where:

μ (s^{-1}) is the growth rate,

μ_{max} (s^{-1}) is the maximum growth rate,

r_X is the biomass formation rate ($kg/m^3.s^{-1}$),

$[O_2]$ is the dissolved oxygen concentration, (kg/m^3),

$Y_{X/O2}$: biomass yield relative to oxygen,

K_{O2} (kg/m^3): Monod constant for oxygen.

II.4.3. Balance between biological reaction and gas-liquid mass transfer

For a quasi-steady state, the oxygen transfer rate is equal to its consumption rate by bioreaction. Equilibrium between biological reaction and gas-liquid mass transfer is found during aerobic cultures of microorganisms. Oxygen passes from the gas phase, dispersed in the form of bubbles, to the pseudo-homogeneous liquid phase containing the microorganisms, where it is consumed. The two phenomena, gas-liquid mass transfer and bioreaction (Figure II.6), are successive and the dissolved oxygen concentration in the liquid phase results from a rapidly established quasi-steady state, for which the oxygen transfer rate is equal to its consumption rate by bioreaction [39].

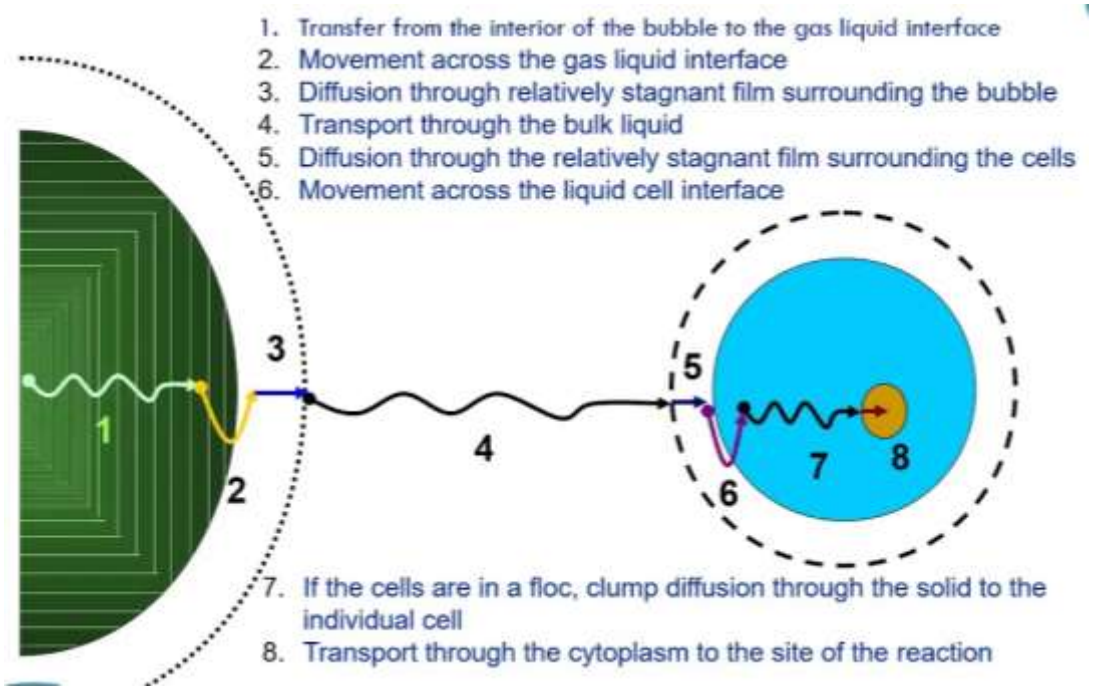

Figure II.6. Steps for transfer of oxygen from gas bubble to cell [39].

Given its very low solubility, the limiting substrate is generally oxygen; assuming that the influence of dissolved oxygen concentration on growth rate obeys Monod's law, we can then write in quasi-steady state: **OTR = OUR,** therefore :

$$\boldsymbol{OTR = K_{La}\,(C^{*} - C_{L}) = \frac{r_X}{Y_{X/O_2}} = \frac{X\,\mu}{Y_{X/O_2}} = \frac{X}{Y_{X/O_2}} . \frac{\mu_{max}.[O_2]}{K_{O_2} + [O_2]}} \quad \text{……..(58)}$$

III. Industrial microorganisms

For centuries, microbes have greatly benefited humanity and are present everywhere. Additionally, the diversity of bacteria suggests that their metabolic pathways and enzyme composition would be more diversified and functionally vital. For thousands of years, people have used traditional methods to preserve milk and vegetables and produce goods like bread, wine, beer, distilled spirits, vinegar, cheese, pickles, and other fermented materials. Jars containing leftovers of rice wine were found in China around 7000 BCE, providing the oldest indication of alcoholic beverages. Even though milk products have been fermenting since 10,000 BCE, Louis Pasteur didn't discover that microorganisms are the cause of lactic acid fermentation until 1857 [40].

The first large-scale fermentations used to produce solvents, organic acids, vitamins, enzymes, and other chemicals marked the beginning of modern industrial microbiology in the early 20th century. The production of antibiotics like streptomycin and penicillin, as well as other value-added products like enzymes and secondary metabolites like steroids and hormones, brought industrial microbiology to the forefront after the success of large-scale penicillin manufacturing contributed to notable economic growth. Additional significant goods with increasing market significance include organic acids, amino acids, and enzymes with novel uses. Microbes' ability to produce metabolites has been used in biotechnological companies to produce vaccines, antibiotics, and enzymes in large quantities [40].

III.1. Application fields of industrial microbiology

The application of microbes in diverse industrial processes to generate valuable goods or services is the main focus of industrial microbiology. Industrial microbiology has a wide range of application areas, and have a significant impact on several sectors:

III.1.1. Food and beverage industry

Food production relies heavily on industrial microbiology, particularly when it comes to fermentation and the creation of food additives [41]:

Emulsifiers and flavouring agents: Compounds that improve food products' flavour and texture are produced by microorganisms such as bacteria and yeasts (monosodium glutamate creation by *Corynebacterium glutamicum* fermentation).

Lactic and Alcoholic Fermentation: Lactic acid fermentation in dairy products (yoghurt, cheese) and alcoholic fermentation (wine, beer) are both accomplished by yeasts.

III.1.2. Agriculture

Microorganisms are essential to agriculture, especially in the creation of genetically modified organisms (GMOs), biopesticides, and biostimulants [42]:

Biopesticides: Toxins produced by bacteria such as *Bacillus thuringiensis* function as biological insecticides.

GMOs: Agrobacterium tumefaciens facilitates the production of GMOs by introducing genes into plants.

Herbicides and Insecticides: By producing natural substances that function as herbicides and insecticides, some microorganisms lessen the need for artificial chemicals.

Plant hormones: Auxins and cytokinins, which are produced by microbes, can encourage plant resistance and growth.

III.1.3. Organic substance production and energy generation

The production of organic acids, biofuels, and other useful chemicals strongly relies on industrial microbiology [43]:

Bioethanol: An alternative fuel made by using yeast to ferment sugars.

Biogaz and methane: Methanogenic bacteria break down organic matter to create biogas, which is also known as methane.

Organic Acids: Used in the food, pharmaceutical, and chemical sectors, organic acids including citric, lactic, and acetic acids are produced by microorganisms.

III.1.4. Solvent Production

Chemical manufacture, pharmaceuticals, food processing, and biofuels are among the many of the industries that use industrial microbiology to produce solvents. A crucial process for creating solvents like ethanol, acetone, butanol, and other alcohols needed for industrial uses is microbial fermentation.

Anaerobic bacteria, specifically *Clostridium acetobutylicum*, ferment sugars and other organic substrates to create a combination of solvents, mostly acetone, butanol, and ethanol. This process is known as "Acetone-Butanol-Ethanol Fermentation." Known by another name, Clostridial Solvent manufacturing, this fermentation technique played a vital role in the industrial manufacturing of acetone during World War I, which was essential for the production of explosives [44].

III.1.5. Molecular biology

Industrial microbiology plays a crucial role in molecular biology, bridging the gap between microbial processes and the molecular techniques that allow for innovation in various industries, particularly in the production of restriction enzymes and other tools for DNA manipulation [45]:

Restriction enzymes: These enzymes, produced by bacteria, cut DNA at specific sites and are essential for genetic engineering, cloning, and DNA mapping.

Microbial hosts for gene cloning: Recombinant DNA technology frequently uses microorganisms such as *Escherichia coli*. These bacteria are used by industrial microbiology to express, clone, and purify desired proteins for use in food production, medicine, and other sectors. The synthesis of goods like insulin, growth hormones, and enzymes used in detergents and other industrial operations is based on this.

Gene editing: To modify genes for scientific purposes or to create genetically modified organisms (GMOs) for commercial use, methods like CRISPR/Cas9 depend on an understanding of microbial systems. This has important ramifications for the manufacturing of biofuel, medicines, and agriculture.

III.1.6. Environmental applications

Microorganisms are essential to environmental management because they provide long-term solutions for pollution control, wastewater treatment, and environmental cleanup. The use of industrial microbiology in environmental management makes use of microorganisms' special capacity to degrade toxic materials and cleanse pollutants in ways that are frequently more environmentally benign than traditional chemical techniques. The following provides more thorough explanations of the roles that microorganisms play in biological water treatment, bioremediation, and biolixiviation.

Bioremediation: Utilising microorganisms, bioremediation breaks down or detoxifies environmental pollutants, especially heavy metals, organic toxins, and hydrocarbons (such oils and insecticides). These microbes, which are frequently naturally occurring bacteria, fungi, or algae, have the ability to metabolise poisonous substances, changing them into less toxic forms or destroying them entirely [46].

Biolixiviation (Bioleaching) **:** The technique of employing microorganisms to extract precious metals from ores—known as biolixiviation or bioleaching—instead of environmentally hazardous chemical processes (such sulphuric acid) is known as biolixiviation. This approach is more economical, energy-efficient, and environmentally friendly. In bioleaching, acidophilic bacteria like *Thiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* are frequently employed. These bacteria

liberate metal ions into solution, where they are readily recoverable, by oxidising metal sulphides, such as copper, gold, and nickel. *Aspergillus niger* and other fungi are also used in bioleaching procedures, namely to break down sulphide ores and extract gold and other valuable metals [47].

***Biological Water Treatment*:** By using microorganisms to decompose organic contaminants in wastewater, biological water treatment renders the water suitable for discharge, reuse, or additional treatment. Wastewater treatment facilities and the control of municipal, agricultural, and industrial effluents depend heavily on this procedure. In wastewater, organic molecules including oils, lipids, carbohydrates, and proteins are broken down by microorganisms, especially bacteria and fungi. Among the common bacteria that are engaged in this process are *Nitrosomonas*, *Azotobacter*, and *Pseudomonas* [48].

III.1.7. Pharmaceutical industry

Industrial microbiology plays a crucial role in the pharmaceutical industry, where microorganisms are utilized in the production of a wide range of drugs, vaccines, and therapeutic products. The application of microorganisms in this industry extends beyond traditional fermentation to include the production of antibiotics, hormones, vaccines, biopharmaceuticals, enzymes, and diagnostic agents. The contributions of industrial microbiology have revolutionized the way medicines are produced, ensuring higher efficiency, safety, and scalability [49].

***Antibiotic production* :** The fermentation processes used to produce antibiotics are among the most important contributions of industrial microbiology. Antibiotics, which are essential for treating bacterial infections, are produced by microorganisms like bacteria and fungus. Microorganisms like *Penicillium* produce penicillin and other antibiotics, which are essential in treating bacterial infections.

***Vaccine production* :** The creation of vaccinations, which stimulate the body's immune system to identify and combat viruses, depends on microorganisms (Live attenuated vaccinations, inactivated vaccines, and recombinant vaccines). *Saccharomyces cerevisiae*, or baker's yeast, is genetically modified to produce the hepatitis B surface antigen, which is subsequently employed as a vaccine component in recombinant vaccines, which are vaccinations against hepatitis B.

***Biopharmaceuticals and monoclonal antibodies* :** Drugs made from live organisms are referred to as biopharmaceuticals, and monoclonal antibodies (mAbs) are a crucial component of contemporary therapeutic medicine. Numerous illnesses, such as cancer, autoimmune conditions, and infectious infections, are treated with monoclonal antibodies. Microorganisms (like *Escherichia coli*) can be modified to manufacture complicated proteins like monoclonal antibodies through the use of recombinant DNA technology. For instance, insulin and other proteins required to treat diabetes and

other illnesses are frequently produced by the E. coli bacteria. Gene therapy is an additional biopharmaceutical application in which therapeutic genes are delivered into human cells using modified viruses, typically lentiviruses or adenoviruses. The treatment of genetic abnormalities is the focus of this quickly evolving field of medicine [49].

Hormone production **:** A key component of modern pharmaceutical manufacture is the employment of microbes to produce hormones. Hormones were obtained from animal sources before to the development of microbial fermentation, which created problems with supply and purity. Traditionally, animal pancreases were used to extract insulin, which is used to treat diabetes. These days, it is made using recombinant DNA technology, which genetically modifies *Saccharomyces* cerevisiae or *E. coli* to create human insulin. *E. coli* has also undergone genetic modification to produce Human Growth Hormone (HGH), which is used to treat some genetic abnormalities and growth issues.

Enzyme production **:** In the pharmaceutical sector, enzymes are employed for a number of tasks, such as the manufacture of medications, the development of medical diagnostics, and the management of specific illnesses. L-Asparaginase produced by *Erwinia chrysanthemi* or *E. coli*, is used to treat leukaemia by depleting asparagine, an amino acid necessary for the proliferation of cancer cells. A medicinal enzyme, chymotrypsin is made by fermentation methods using microorganisms and is used to treat digestive and inflammatory conditions. Lipase and amylase are two examples of fungus-derived enzymes that are utilised in therapeutic formulations and for catalysis throughout the drug production process.

Anticancer agents and immunosuppressive drugs **:** Numerous microorganisms are employed in the manufacturing of immunosuppressive medications and anticancer medicines. Cyclosporine is a common immunosuppressive medication used to stop organ rejection in transplant recipients, it is produced by the fungus *Tolypocladium inflatum*. Taxol (paclitaxel), originally derived from the Pacific yew tree, is currently made via microbial fermentation, namely with the help of strains of the yew tree-associated bacterium *Taxomyces andreanae* [49].

III.2. Industrial fermentation

The industrial production of microbial products typically relies on fermentation. Industrial fermentation is the cultivation of large quantities of microbes or other isolated cells to produce a substance of commercial interest. The tanks used for industrial fermentation are called bioreactors. The main factors considered in the design of these tanks are aeration, pH, and temperature control. There are several types of bioreactors, but the most well-known are continuous-mixing units [50].

III.2.1. Type of fermenters

The types of fermenters or bioreactors vary depending on their capacity, design, and use in different phases of industrial production. Each type of fermenter is designed to meet specific needs at each stage of the fermentation process, from laboratory to industrial production. Laboratory and pilot fermenters allow for small-scale testing and optimization of processes before scaling up to large-scale production in industrial fermenters [50]:

III.2.1.1. Autoclavable laboratory fermenters

Autoclavable laboratory fermenters, with capacities up to 18 L, are used in laboratories for experimental testing and small-scale production. They are generally designed to be autoclaved, which ensures the aseptic culture conditions required for reliable experiments. They have many applications, such as microorganism culture tests and optimizing fermentation parameters (pH, temperature, aeration, etc.) before scaling up.

III.2.1.2. In situ sterilizable laboratory fermenters

Unlike autoclavable fermenters, these bioreactors, with capacities up to 30 L, are designed to be sterilized directly in situ (in situ), without the need for disassembly for autoclave sterilization. They are used for larger-scale experiments than small laboratory bioreactors, but always for process development prior to large-scale production.

III.1.2.1.3. Pilot-scale fermenters

These fermenters, up to 300 L, are used for the pilot phase of a fermentation process. They allow fermentation conditions to be tested on a larger scale than laboratory bioreactors while remaining manageable for trials and adjustments before scaling up to industrial scale. Their main applications are large-scale testing of fermentation conditions, evaluating process viability, and preparing for industrial production.

III.2.1.4. Industrial fermenters

These fermenters (up to 500,000 L, or 500 m^3) are used in large-scale industrial production. They are designed to produce large quantities of fermented products, such as antibiotics, enzymes, bioethanols, or other commercial bioproducts. They are used for continuous or batch industrial production of various fermented products on a very large scale.

III.2.2. Fermentation steps

Fermentation is a critical process in the production of a wide range of bioproducts, including pharmaceuticals, biofuels, food, and beverages. The entire fermentation process can be broken down

into several key stages. Each stage is essential to ensure the successful growth of microorganisms and the efficient production of the desired product [51].

III.2.2.1. Cultural medium formulation

The culture medium supplies the vital nutrients needed for microorganisms to flourish during fermentation. It usually includes nitrogen sources (like ammonium salts or peptones), carbon sources (like sucrose or glucose), vitamins, minerals, and occasionally growth hormones. The culture medium's composition is customised to meet the unique needs of the microorganism being utilised in the fermentation process. The microorganisms' rate of growth and the product's yield are greatly influenced by the culture medium's quality and composition. To ensure maximum microbial activity, the medium must be optimised to offer a balance of nutrients.

III.2.2.2. Fermenter and its equipment sterilisation

In order to preserve aseptic conditions, sterilisation is an essential step. Contamination can raise expenses, lower product yield, and jeopardise the process. It guarantees that the fermentation process is not contaminated by undesirable bacteria. Sterilisation is required for the fermentation vessel, or bioreactor, as well as its pipes, valves, and other components. To get rid of any pre-existing microorganisms, the culture media is also sterilised. Usually, filtration or autoclaving (steam sterilisation) are used to accomplish this. To minimise time and reduce the chance of contamination, sterilisation is sometimes done in situ, or inside the bioreactor.

III.2.2.3. Preparation of the inoculum

To start fermentation, a small amount of microbial culture is added to the bioreactor as the inoculum. In order to ensure that the culture is healthy and at the proper stage of growth, it is typically created in a small-scale vessel under controlled conditions. Usually, a seed culture or starter culture is scaled up for the inoculum preparation process. To guarantee that fermentation progresses at the best possible rate, the inoculum needs to be of the highest calibre and include a sufficient number of live cells. The time it takes for the fermentation to achieve the required productivity might also be influenced by the inoculum preparation.

III.2.2.4. Production in the fermenter

At this step, the microorganisms multiply and generate the desired product in the fermenter during the actual fermentation process. To create the perfect conditions for microbial development, important variables including temperature, pH, dissolved oxygen, and agitation are carefully regulated. The microbes stay in their ideal growth phase, increasing product yield, thanks to constant monitoring and modification of these parameters. The majority of the product is synthesised at this

point. Environmental parameters must be carefully managed because even slight departures from ideal circumstances might have an impact on microbial activity and the final product yield.

III.2.2.5. Extraction of the product and its purification.

The product is removed from the fermentation broth once the fermentation process has reached the intended endpoint. Usually, this entails removing the microbial biomass from the liquid phase and purifying the final product through downstream processing. Depending on whether the product is intracellular or extracellular, extraction can be accomplished via techniques including centrifugation, filtering, or cell lysis. To achieve the criteria needed for its intended application (such as in food, medicine, or biofuels), the product must be purified. To eliminate contaminants and increase product purity, purification entails a number of processes, such as concentration, filtration, chromatography, and even crystallisation.

III.2.3. Scaling-up of fermentation process

The process of moving the fermentation process from a small-volume laboratory bioreactor to a large-scale industrial bioreactor is known as "scaling-up." This is a crucial stage in the development of biotechnology since circumstances that are effective in the laboratory might not be directly relevant in an industrial setting. There are multiple steps (Figure III.1) involved in scaling up [51]:

III.2.3.1. From the erlenmeyer flask to the benchtop bioreactor

Transferring the fermentation from a small flask, typically an Erlenmeyer flask, to a tabletop bioreactor is the first stage in scaling up. Better control over variables like temperature, pH, and aeration is made possible by this shift. The process is refined in a benchtop bioreactor, which usually holds 1–10 litres, before being transferred to larger tanks.

III.2.3.2. From the benchtop bioreactor to the laboratory pilot bioreactor

The procedure is then moved to a pilot bioreactor at the laboratory scale, which usually has a capacity of 50–300 litres. This step helps to evaluate the process's scalability, allowing for the testing and optimisation of more substantial modifications to the bioreactor's size and operating parameters for large-scale manufacturing.

III.2.3.3. From the laboratory pilot bioreactor to the industrial bioreactor

The final step is transferring the process to an industrial-scale bioreactor, which can range from several hundred to several thousand liters in capacity (up to 500,000 liters for some industrial applications). This phase involves fine-tuning the process parameters for maximum yield and efficiency at an industrial scale. Challenges such as oxygen transfer, mixing efficiency, and heat management become more pronounced, requiring careful optimization.

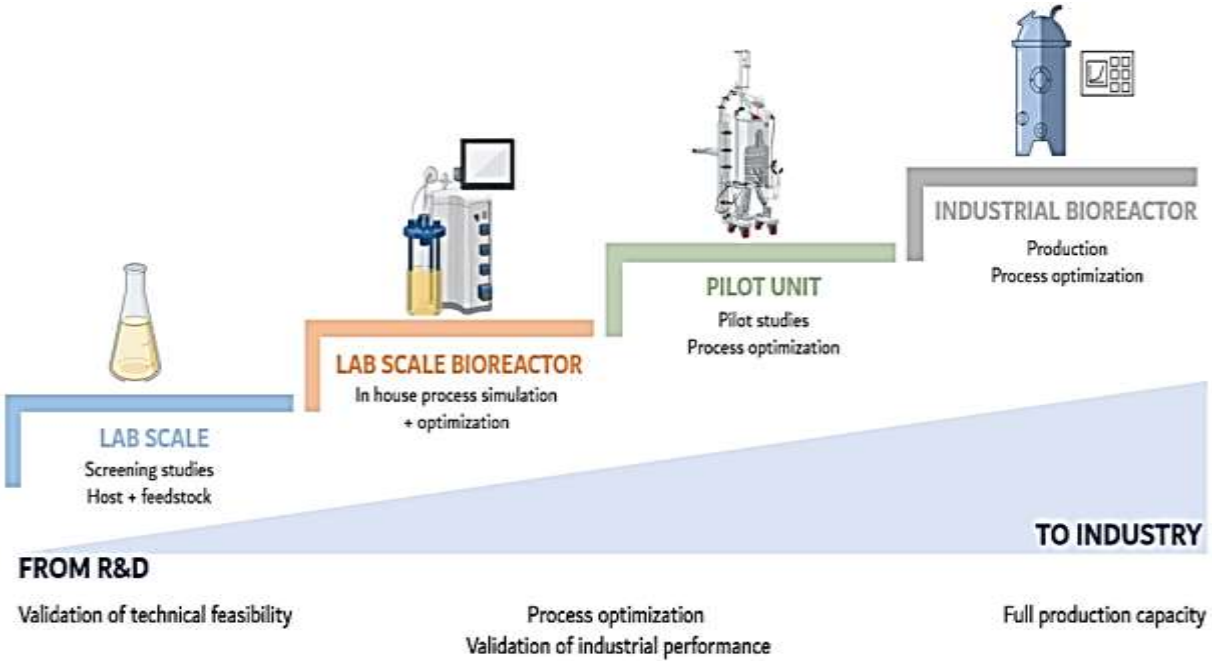

Figure III.1. Steps of scaling up of fermentation (Online Source 3).

III.3. Microorganisms of industrial importance

Microorganisms including yeast, moulds, and bacteria that are employed in extensive industrial processes to generate a variety of products are known as industrial microorganisms. These organisms are essential to agriculture, food production, biotechnology, pharmaceuticals, and environmental management. They are employed because of their capacity to inexpensively and efficiently carry out particular biological activities. These microbes are perfect for the mass manufacture of valuable compounds because of their rapid growth, simplicity of genetic modification, and capacity to thrive in controlled environments. Some common products derived from industrial microorganisms include [52]:

-Enzymes (used in detergents, food processing, and textiles)

-Antibiotics (like penicillin from *Penicillium* species)

-Alcohols and organic acids (such as ethanol and citric acid)

-Vitamins and amino acids

-Biofuels and bioplastics

Microorganisms used in industry can be either naturally occurring strains or genetically modified for higher yield or better performance. They are often grown in fermenters, large bioreactors that provide optimal conditions for growth and product formation.

III.3.1. Yeasts

Yeasts can be defined as unicellular fungi and are much larger than bacteria, eukaryotic microorganisms that reproduce by budding or fission. Their presence depends primarily on the availability of organic carbon, then on temperature, pH, and the presence of water. Since ancient times, yeasts have been utilised to produce fermented foods and drinks like bread, wine, beer, and sake. However, historical considerations rather than scientific ones frequently influence the selection of a specific yeast strain or species for a given industrial use. Furthermore, yeast must contend with conditions and difficulties that are different from those found in conventional food fermentations in novel biotechnological yeast applications, such as the synthesis of second-generation biofuels [52].

Yeasts have been used by humans for millennia without their knowledge, particularly in the production of alcoholic beverages and bread. It was not until the work of Pasteur in the years 1866-1876 that the role of yeasts in alcoholic fermentation was highlighted. Their ease of cultivation and the safety of a large number of species have made them the most widely used microorganisms for the production of alcoholic beverages and bakery products. They are also used as a source of protein and vitamins in human and animal nutrition [53]:

- For breadmaking: *Saccharomyces cerevisiae* (baker's yeast),

- Yeast-food rich in proteins and vitamins (dietary supplement: (Single-Cell Organism Proteins (SCPs)): *Kluyveromyces, Candida, Schwaniomyces, Lipomyces, Pichia, Rhodotorula, Sacharomycopsis*

- For winemaking or oenology, science of wine: *S. ellipsoideus* and *S. oviformis* (they are the most alcohol-producing species)

- For beermaking: *S. cerevisiae* and *S. carlsbergensis*.

- For cheesemaking: *S. cerevisiae* and *Candida versatilis.*

- Production of industrial alcohol like industrial ethanol: *Saccharomyces*, *Torulopsis*, and *Candida* species can produce large quantities of alcohol.

- Industrial production of riboflavin (B2) : *Ashbya gossypii*

III.3.2. Moulds

Moulds, or filamentous fungi, are of great economic importance due to both their harmfulness and their usefulness. Their harmful activities are numerous: spoilage of food products and deterioration in many other areas, production of mycotoxins, and parasitic life at the expense of humans, animals, and plants. The harmful effects of these microorganisms are largely offset by their beneficial activities. Responsible for the destruction of a large portion of the Earth's organic matter, molds contribute significantly to the completion of major natural biological cycles [54].

They have long been used by humans for food preparation, notably as fermentation agents in cheese making. They synthesize a large number of economically important complex substances: enzymes, organic acids, antibiotics. The exploitation of these microorganisms for the industrial production of interesting metabolites only began at the beginning of the 20th century. Understanding the primary and secondary metabolites and the genetics of these microorganisms allows for increasingly better control of their biosynthetic capabilities and their use in humankind [54]:

- Cheese production: *Penicillium camemberti* and *Penicillium roqueforti* (Flavor production)

- Antibiotic production: *Penicillium (chrysogenum=natanum*) and *Cephalosporum genera*.

- Organic acid production (citric acid and gluconic acid): *Aspergillus niger*

III.3.3. Bacteria

Bacteria are tiny living beings made of a single cell, present almost everywhere: air, soil, water, skin. Some cause illnesses (colds, listeriosis), others are useful to humans: present in the intestine, they aid digestion and are used to make foods like yogurt, ….etc. [40]:

- Dairy product production: lactic acid bacteria: *Leuconostoc, Lactobacillus bulgaricus*.

- Cheese production: *Leuconostoc*, *Lactococcus*, *Propionibacterium*.

- Probiotics: *Lactobacillus casei*, *Lb acidophilus*, *Lb lactis*, *Bifidobacterium*.

- Vinegar production: *Acetobacter*, *Gluconobacter*.

- Lactic acid production: *Lactobacillus thermobacterium*, *Streptococcus*, *Pediococcus*, used as a preservative in drinks and fruit juices, and in the plastics industry as a monomer.

- Antibiotic production: Actinomycetes genus *Strypromyces.*

- Solvent production: *Clostridium acetobutylicum*, *Fusobacterium*

- Food gelling agent production (dextran, xanthan, or alginate): *Leuconostoc*, *Xanthomonas*

- Ethanol production : *Zymomonas mobilis.*

III.3.4. Microalgae

Microalgae are microscopic, photosynthetic organisms found in freshwater and marine environments. They have a wide variety of industrial applications thanks to their richness in bioactive compounds (lipids, proteins, pigments, etc.), their applications include [55]:

-Food industry : *Spirulina* and *Chlorella* as superfoods and natural colorants.

-Cosmetics : used in anti-aging and skin-repair products.

-Pharmaceuticals : source of antioxidants, antivirals, and anti-inflammatory compounds.

-Biofuels : production of biodiesel from lipid-rich species.

-Wastewater treatment : removing nutrients and pollutants from water.

-Agriculture : used as biofertilizers and soil conditioners.

III.4. Interest in industrial microorganism use

Large-scale microorganisms are used in industrial microbiology to either create goods with added value or carry out complex chemical reactions by methods that are essentially enhancements of metabolic reactions currently carried out by bacteria. The following benefits make using microorganisms appealing [56]:

*Less expensive than chemical procedures (catalysis by enzymes, ……etc.).

*Only microbes can synthesise and biotransform certain chemicals, such as steroids, prostaglandins,….etc.

*Production in large quantities.

*Production without regard to geographical or seasonal limitations, for example, industrial manufacturing has turned towards fermentation techniques due to the high cost of animal-derived enzymes.

*Low toxicity and biodegradability of some biomolecules.

III.5. Characteristics of strains used in industrial production

For industrial biotechnology to be successful and economical, the microbial strains (bacteria, yeasts, moulds, etc.) that are employed must have certain important characteristics. These characteristics guarantee the production of the desired molecules (enzymes, antibiotics, vitamins, etc.) in a stable,

quick, cost-effective, and secure manner. The criteria for choosing an industrial microorganism include [56]:

- *Good productivity (high yield):* It is the ability to produce the target molecule in large quantities and the strain must be highly productive. It is measured by its ability to convert the substrate into a product and to obtain a high yield of product per unit of time. Example: *Aspergillus niger* is a mold used to produce citric acid on an industrial scale.

- *Rapid growth* : To produce molecules of interest or biomass in a short period of time in large quantities. Example*: Escherichia coli* multiplies every 20 minutes, enabling rapid protein production.

- *Safety (non-pathogenic):* It must not be hazardous to humans or the environment, and do not produce undesirable metabolites such as toxins. Example: *Bacillus subtilis* is a GRAS (Generally Recognized As Safe) bacterium often used in industry.

- *Biological criteria specific to microorganisms and their application:* For example, lactic acid bacteria are selected based on their acidifying and flavoring properties, as well as their bacteriocin production.

- *Genetic stability during fermentation processes:* they do not lose their characteristics after numerous multiplications in a bioreactor and during storage, especially after freezing or freeze-drying. It must retain its characteristics for several generations (avoid unwanted mutations).

- *Easy to manipulate genetically:* Improvability and susceptibility to genetic manipulation (ideally, the strain should be able to evolve under pressure from industry, with the aim of improving or adapting production).

- *Adaptability to the industrial environment:* The microorganism's ability to adapt to the type of process implemented and be able to withstand to various technological processes. It must withstand production conditions (pH, temperature, pressure, etc.).

-*Ease of cultivation:* It must be able to grow easily on simple and inexpensive media and do not have specific requirements regarding growth factors. Example: Some strains can be cultivated on agricultural (by-product recovery) and food industry waste.

- *Optimal growth temperature:* Using a microorganism with an optimal temperature under 40°C significantly reduces cooling costs for large-scale fermentation;

- *Ease of product recovery from the culture:* Extracting the desired product after microbial growth must be simple. If the product is secreted into the medium (extracellular), its recovery is easy and

inexpensive. If it is intracellular, it requires cell lysis, which complicates and accelerates the process. Easy recovery reduces production costs and simplifies purification.

- Bacteriophage resistance: This criterion involves restriction enzymes and is linked to the number and shape of surface proteins, which act as binding points for bacteriophages [56].

III.6. Methods of obtaining industrial microorganisms

There are essentially two ways of obtaining microorganisms of industrial interest:

III.6.1. Microorganism collections

Microorganisms are already isolated by contacting major international microorganism collections, such as the American Type Culture Collection (ATCC) in the United States, the Biological Resource Center of the Pasteur Institute in France, or the CentraalBureau voor Schimmelcultures (CBS) (Central Bureau for Fungal Biodiversity) in the Netherlands. Table III.1 provides a selection of the major collections, these collections provide strains for teaching, research, and industry [56].

Table III.1. Major collections of cultures [56].

Culture Collection	Website
National Collection of Type Cultures (NCTC)	http://www.phe-culturecollections.org.uk
National Collection of Industrial Food and Marine Bacteria (NCIMB Ltd)	http://www.ncimb.com
National Collection of Yeast Cultures (NCYC)	http://www.ncyc.co.uk
UK National Collection of Fungus Cultures	http://www.cabi.org
The British Antarctic Survey Culture Collection	http://www.cabi.org
National Collection of Plant Pathogenic Bacteria	http://ncppb.fera.defra.gov.uk
American Type Culture Collection (ATCC)	https://www.atcc.org/
Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ)	https://www.dsmz.de
Centraalbureau voor Schimmelcultures (CBS)	http://www.cbs.knaw.nl
Czech Collection of Microorganisms (CCM)	http://www.sci.muni.cz/ccm/index.html
Collection Nationale de Cultures de Microorganismes (CNCM)	https://www.pasteur.fr/fr/recherche/unites/%20Cncm/index-en.html
Japan Collection of Microorganisms (JCM)	http://jcm.brc.riken.jp/en/

While the environment contains a wide variety of species, only a small percentage of them may be adequate, such collections may offer organisms with known characteristics but may not include those

with the most desired traits. Purchasing a culture is undoubtedly less expensive than isolating oneself from the natural world. Nonetheless, it is always beneficial to buy cultures that exhibit the necessary traits, even if they are poor, since they can be used as model systems to create assay and culture methods that can then be used to evaluate natural isolates [56].

III.6.2. Isolation from natural environments

In order to determine which microorganisms, carry out the desired reaction or produce the desired product, the ideal isolation procedure starts with an environmental source, usually soil, that is extremely probable to be rich in the desired types. It is then designed to favour the growth of those organisms possessing the industrially important types.

In certain situations, the isolation process can be planned so that the growth of possible producers is promoted or that they can be identified during the isolation phase. In other situations, however, organisms must be isolated and producers identified at a later time. It is important to keep in mind that the isolate must ultimately complete the procedure economically, therefore choosing the appropriate culture involves striking a balance between the organism's productivity and the process's financial limitations [56].

Conventional isolation and cultivation methods can be used to separate microorganisms from a variety of natural habitats, such as water, soil, and organic debris. Modern techniques have been developed to increase the effectiveness of microbial discovery in addition to traditional microbiological procedures. These methods enable the identification of microorganisms with particular and desirable traits by combining isolation and selection into a single, quick, and efficient procedure. This method, also known as "technical screening", makes it easier to find strains that may be useful in biotechnology, industry, agriculture, and environmental management.

III.6.3. Strategy for searching new industrial strains

A rigorous strategy is followed in the search for new industrial microorganisms in order to find strains that are highly productive, safe, stable and cost-effective for industrial applications (like enzyme production, antibiotics, biofuels, ……..etc.).

III.6.3.1. Natural environments exploration

Researchers begin by studying various environments likely to host unusual or extremophilic bacteria, the goal is to find microorganisms with specific metabolic capabilities or resilience to harsh environments. These include [57-58]:

-Soil (rich in diverse microorganisms)

-Thermophilic creatures, or hot springs

-Deep-sea habitats (barophiles)

-Decaying organic matter and compost are sources of decomposers

-Industrial waste (organisms adapted to pollutants)

III.6.3.2. Microorganism isolation

To isolate particular microbes and to getting pure, culturable strains for testing, collected samples are processed in the laboratory [56-58]:

-Targeting particular types (such as bacteria or fungi) involves the use of selective substrate and media.

-Using pure culture methods to isolate and cultivate a single strain.

-Depending on the type of organism, either aerobic or anaerobic conditions are used.

In summary, the sample is inoculated onto an appropriate medium in order to isolate the desired germ. The next step is to isolate and purify the different clones obtained. Purification is carried out by depletion streaking. This technique allows the isolation of colonies and the obtaining of pure cultures.

III.6.3.3. Enrichment liquid culture

In essence, enrichment culture types are isolation techniques that depend on the usage of desirable characteristics as selecting factors. Through the use of enrichment culture, the quantity of a particular organism in the original inoculum is increased in comparison to the quantity of other types. The procedure entails taking an environmental source, typically soil, that has a mixed population and creating conditions that are either favourable for the growth of the desired type or unfavourable for the growth of the other organisms. For instance, this can be done by adding specific inhibitors or by providing specific substrates. It is frequently beneficial to expose the sample to conditions that promote the organisms' survival before the culture stage. For instance, actinomycetes will have a better chance of surviving if the soil is allowed to air dry [56].

In liquid enrichment cultures, growth is typically done in shake flasks. Over time, the medium changes, potentially allowing other organisms to grow. To maintain selectivity, subculturing into fresh medium is repeated until the desired organism dominates. Isolation is done when this organism becomes dominant, which depends on its growth rate and affinity for nutrients. The fastest-growing organism usually dominates, but sometimes the goal is to isolate one with higher substrate affinity, not just speed [56].

III.6.3.4. Screening and selection of microorganism

After isolation, the microorganisms obtained undergo selection based on their biological and technological suitability. Several techniques and methods can be implemented to select strains of interest. The technique is adapted according to the desired biological activity. Here are some examples of techniques for isolating and selecting strains that produce products (substances) of industrial interest:

III.6.3.4.1. Selection of enzyme-producing strains

Enzymes are most often induced by the presence of the substrate; therefore, enzyme-producing microorganisms are sought where the substances to be hydrolyzed are abundant (cellulase-producing microorganisms are sought in cellulose-rich forest soils; lactase producers in dairy products, etc.).

If certain properties are also sought in the enzyme (such as thermostability), appropriate environments are targeted (hot springs, etc.). The principle of selection is based on the use of selective culture media (depending on the targeted producing microorganism: fungi, actinobacteria, etc.). The selection medium must contain the enzyme substrate as the sole carbon source [59].

Research and confirmation of strain enzymatic activity

This involves investigating enzyme synthesis and quantifying it. Principle is to demonstrate the enzymatic activity of strains isolated on agar media containing specific enzyme substrates. The methods described for investigating and confirming microbial enzymatic activity, such as demonstrating enzyme synthesis, quantifying activity, and utilizing selective culture media, are well-established in microbiological research. These techniques are detailed in various studies and protocols.

To demonstrate enzymatic activity, the isolated strain or the enzyme extract obtained after culture on liquid media can be used. In the case of extracellular enzyme where this later secreted into the culture medium, the supernatant is often collected after centrifugation or filtration. For intracellular enzymes, the cells can be recovered after their destruction and lyse by physical means (mechanical grinding, sonication, etc.), chemical means (detergents, etc.), or enzymatic mans (enzymatic digestion), and release the enzymes [60-61].

The formation of halos around colonies on agar plates is a widely used method to visualize enzymatic activity. The size of the halo often correlates with the level of enzyme activity, providing a semi-quantitative measure. This method is utilized in various studies to assess the enzymatic potential of microbial strains [62].

Example of cellulase activity detection

On an agar medium (15 g of agar per 1000 ml of distilled water) containing CMC (carboxymethyl cellulose with 5 g/L), the isolated strain of interest is inoculated onto the surface. After incubation at the ideal temperature and time, the enzymatic activity is visualized by covering the entire surface of the plate with a Congo red solution (1%) (Figure III.2). The appearance of an orange halo on a red background indicates the presence of cellulase (cellulase production by the strain). When using an enzyme extract, the well technique is performed (4-6 mm diameter wells are dug on the agar medium and a quantity (approximately 50-100 µL) of the enzyme extract is placed inside [60-61].

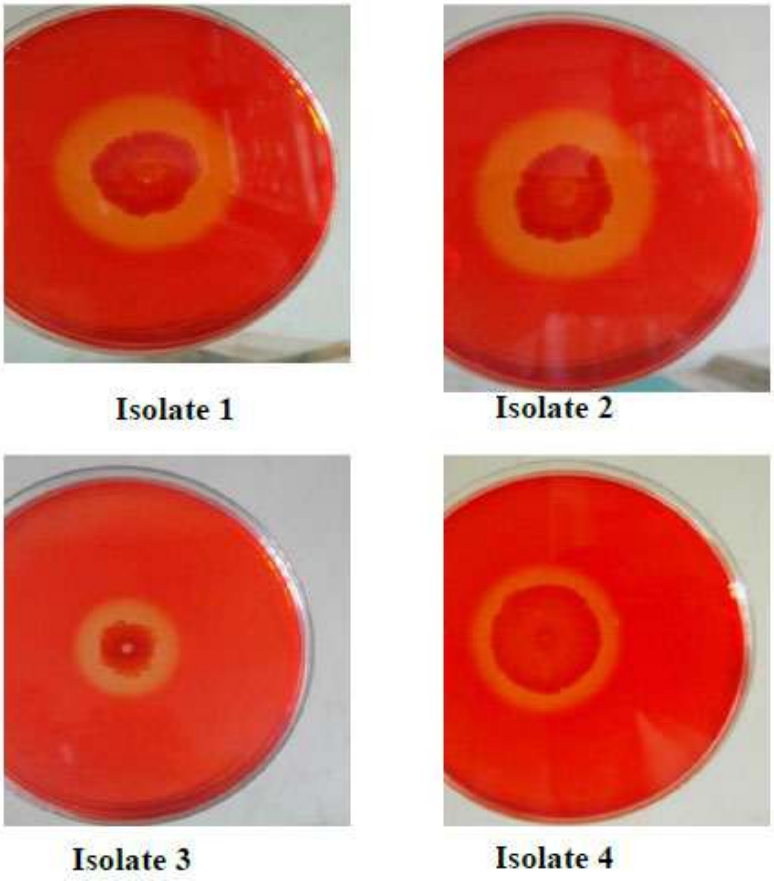

Figure III.2. Screening for cellulolytic bacteria after revelation with Congo red (1%).

Applying Congo red dye to the petri plates when secreted CMCase hydrolyses carboxymethylcellulose (CMC), a zone of clearing forms around the colonies [62].

III.6.3.4.2. Selection of antibiotic-producing strains

Traditionally, screening has been used for discovering antibiotics. This method isolates a significant number of strains in pure culture that may naturally produce antibiotics. They are then tested for their production of inhibitory compounds against control microorganisms (target germs) chosen to be representative of or very close to pathogenic germs (antagonism tests), using several methods. The

appropriate technique depends on whether the strain is used directly or the extract after production in a liquid medium [63-64].

When the strain (isolate) is used directly, there are two widely used techniques: the agar cylinder technique and the cross-streak technique. Whereas when the extract (culture supernatant or filtrate) is used, there are three more suitable techniques (Figure III.3): the well technique, the impregnated disk techniques, and spot technique [65-66].

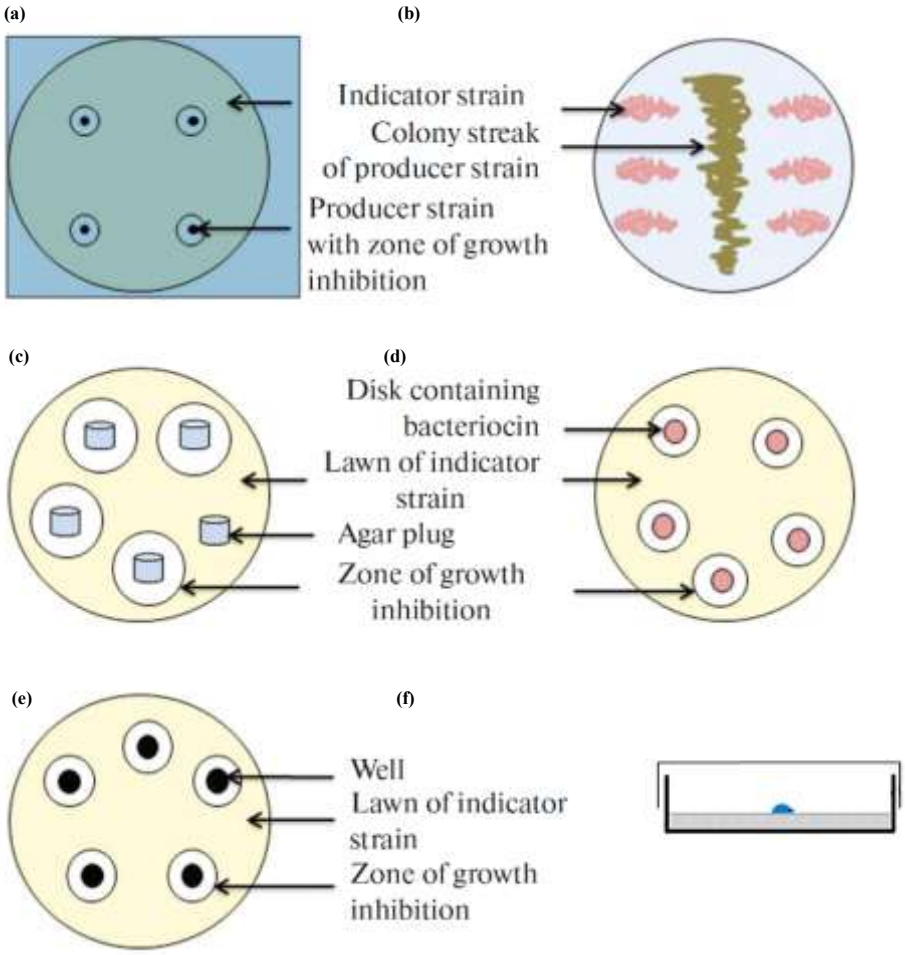

Figure III.3. Diagrammatic representation of the many agar-based techniques for assessing antibacterial activity.

(a, f) Point inoculation method (spot diffusion), (b) cross streak method, (c) agar plug diffusion method, (d) disk diffusion method, (e) agar well diffusion assay [65-66].

a. Agar plug (cylinder) technique

Using this technique, test strain-containing agar plugs are placed onto an agar plate that has been inoculated with the indicator microorganism (Figure III.4). An inhibitory zone is produced by the diffusion of antimicrobial compounds from the plug, signifying antimicrobial activity [64-67].

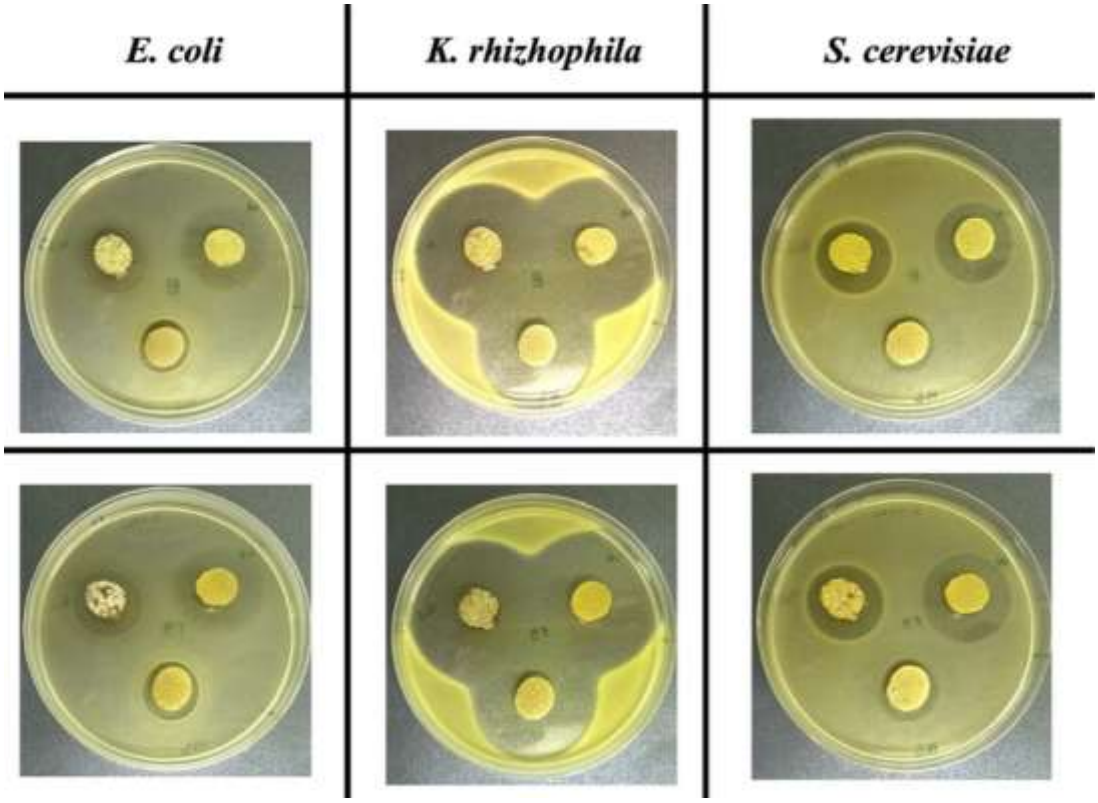

Figure III.4. Antimicrobial activity assay of *Streptomyces* sp. AV8 and *Streptomyces* sp. AV19 using the agar plug method [67].

The test strain is inoculated in tight streaks on the agar medium and incubated at the ideal temperature and time for the strain. Agar plugs (cylinders) of approximately 6mm diameter are then removed with a cookie cutter and placed on the surface of a medium (Muller Hinton) previously inoculated with the target germs. The inoculated dishes are kept at 4°C for 2 hours before being incubated to allow the diffusion of the active substances while temporarily preventing the growth of the target microorganisms. The inhibition zones are measured with a "caliper" after an incubation time appropriate to the target germ (24 hours at 37°C for bacteria / 48 to 72 hours at 28°C for fungi).

b. Cross-streak technique

This method involves streaking the test microorganism over the middle of an agar plate. Indicator strains are streaked perpendicular to the original stripe following incubation (Figure III.3 and Figure III.5). The intersections will show distinct zones of inhibition if the test strain generates antimicrobial chemicals [65, 66, 68].

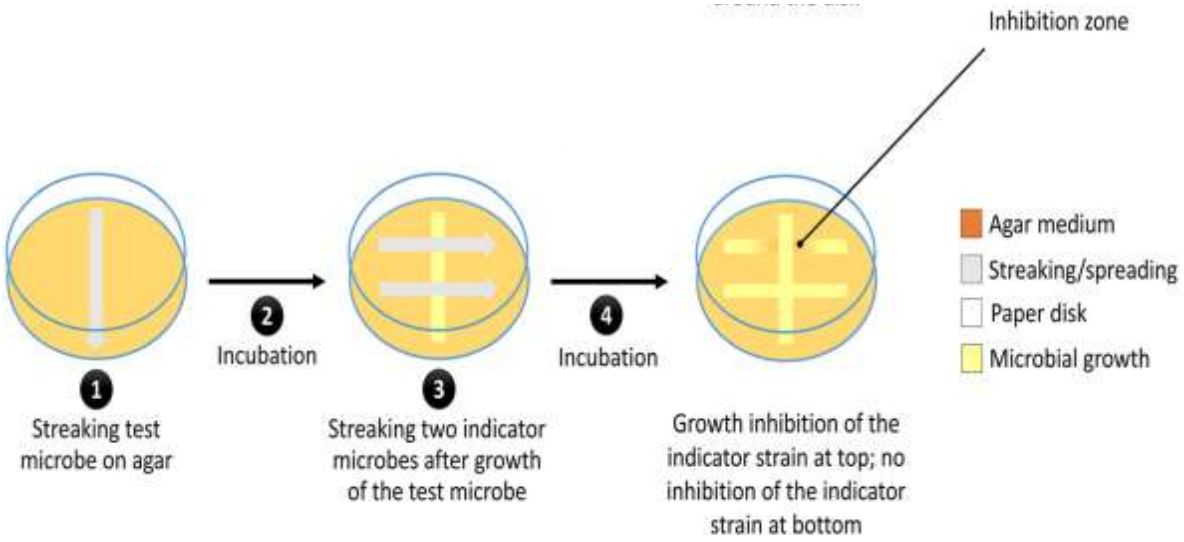

Figure III.5. Diagrammatic illustration of the cross-streak assay procedures used to measure antibiotic activity.

The test organism is first streaked over the agar surface in a straight line (1) in the cross-streak method. The indicator strains are streaked perpendicular to the test microorganism (3) after an incubation time (2). The plate is inspected to determine whether clean zones are present at the intersections following a second round of incubation (4) [68].

This test consists of inoculating the test germ (the isolate) in a single line onto a suitable agar medium. After incubation at the ideal temperature and time for the test germ, the germs are inoculated in streaks perpendicular to that of the test germ. The plates are incubated (24 hours at 37°C for bacteria / 48 to 72 hours at 28°C for fungi). The inhibition zones between the edge of the test germ streak and that of the target germs are measured with a caliper.

c. Disk diffusion assay

This method involves impregnating filter paper discs with the antimicrobial extract and setting them on an agar plate that has been infected with the indicator bacterium (Figure III.3 and Figure III.6). An inhibitory zone is created when the drug diffuses from the disc [65, 66, 68].

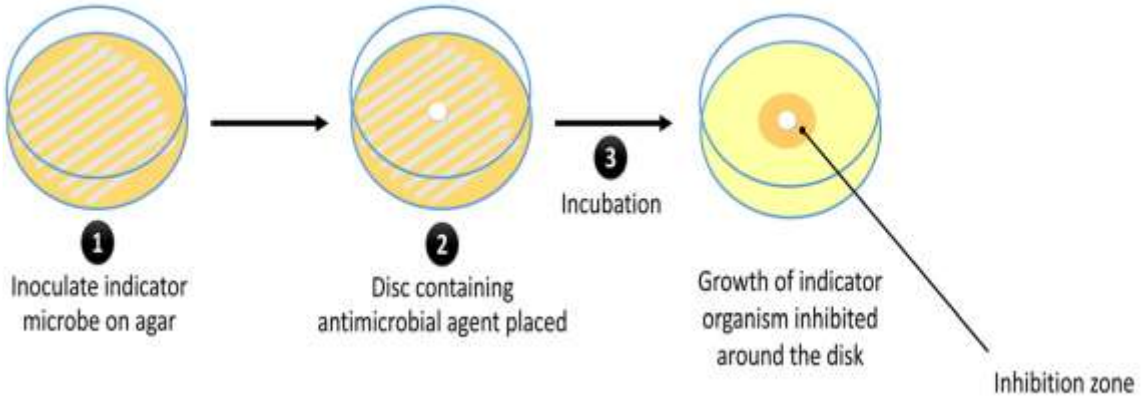

Figure III.6. Diagrammatic illustration of the disc diffusion assay procedures used to measure antibiotic activity.

The indicator organism is uniformly dispersed throughout the agar surface in the disc diffusion test (1). (2) involves placing a disc in the middle of the plate that contains the antimicrobial agent at the proper concentration. The plate is checked for the existence of a distinct zone of inhibition following incubation (3) [68].

Watman paper discs (No. 1) (previously sterilized) are soaked with the extract using a micropipette, then dried with a dryer (remove the solvent in which the extract is diluted). Then place the loaded discs, aseptically, on the surface of the agar previously inoculated with the target germ. The inoculated plates are kept at 4°C for 2 hours before being incubated (24 hours at 37°C for bacteria and 48 to 72 hours at 28°C for fungi). The inhibition zones are then measured with a caliper.

d. Well diffusion assay

Using this technique, wells are made in an agar plate that has been inoculated with the indicator microbe. These wells are filled with the antimicrobial extract (Figure III.7). Following incubation, a clear zone is formed by the antimicrobial agent's diffusion from the well, signifying activity [65-66].

This is a diffusion method on a Petri dish (agar medium) probably seeded with the target germ and on the surface of which wells of about 6mm diameter are dug (using a punch). The wells are then filled with (50-100 µL) of the supernatant or culture filtrate. The seeded dishes are kept at 4°C for 2 hours before being incubated (24h at 37°C for bacteria / 48 to 72h at 28°C for fungi). The inhibition zones are then measured with a "caliper".

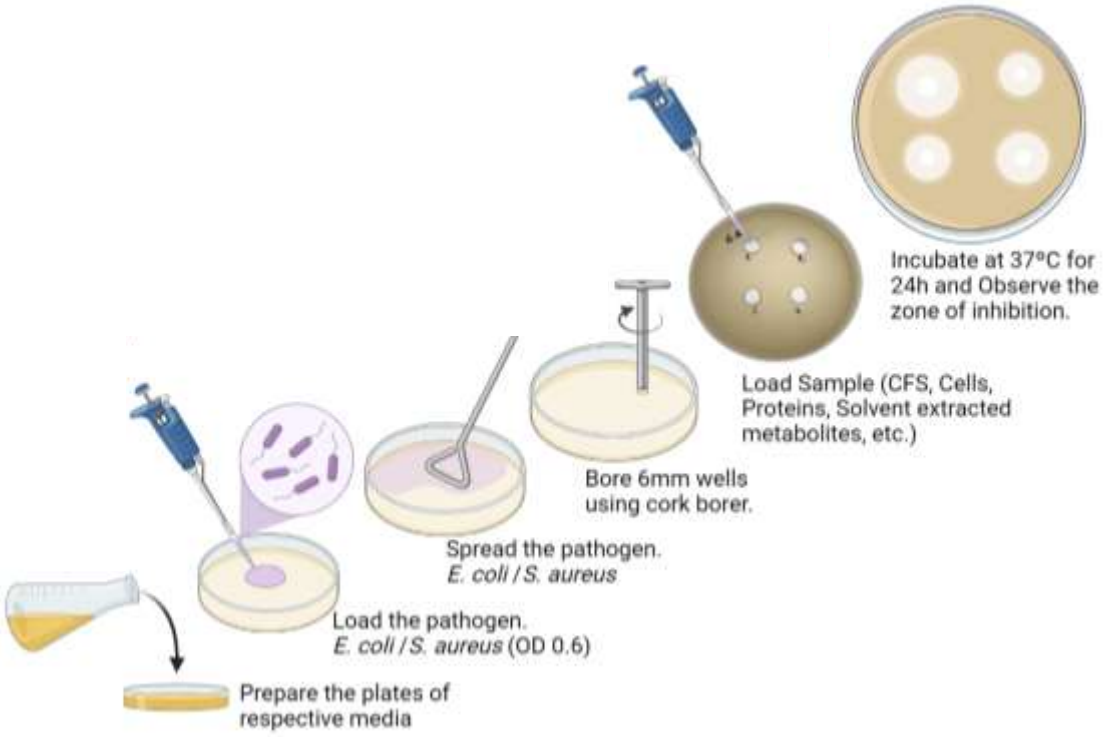

Figure III.7. Diagrammatic illustration of the well diffusion assay procedures used to measure antibiotic activity (Online Source 4).

e. Spot diffusion assay

The spot diffusion technique, also called Spot-on-Lawn Assay, is a simple and effective method used in microbiology to detect antimicrobial activity of microorganisms or their metabolites against a target (indicator) organism (Figure III.3). The technique is based on the diffusion of antimicrobial compounds from a spot-inoculated microbial culture or extract into an agar medium pre-inoculated with a test microorganism (usually a pathogen or indicator strain). If the spotted sample produces antimicrobial substances, they diffuse and inhibit the growth of the indicator organism, creating a clear zone (zone of inhibition) around the spot [65, 66, 68, 69].

A volume of the extract is aseptically deposited, using a micropipette, on the surface of the agar previously inoculated with the target germ. The inoculated dishes are kept at 4°C for 2 hours before being incubated (24 hours at 37°C for bacteria / 48 to 72 hours at 28°C for fungi). The inhibition zones are then measured with a caliper.

III.6.4. Molecular identification of selected strains

When a strain is selected and meets the selection criteria, and after phenotypic identification, it is subject to molecular identification based on sequencing of 16s rDNA for bacterial identification and

Internal Transcribed Spacer (ITS) region of ribosomal DNA for fungal identification, due to its high variability among species and conservation within species.

The phenotypic approach is a classification that groups organisms based on observable characteristics and according to the similarity of their phenotypic characteristics such as morphological, physiological, metabolic (biochemical), ecological, immunological response, physicochemical properties. This method is foundational in microbiology, particularly for identifying and characterizing bacteria and archaea [70].

Phenotypic classification offers useful and observable standards for classifying organisms, although it does not always correctly represent evolutionary relationships. For this reason, it is frequently combined with genotypic techniques to offer a more thorough comprehension of organismal classification.

By analysing molecular indicators like DNA or RNA sequences, molecular or phylogenetic approaches classify species according to their evolutionary relationships. These markers are dependable indicators of phylogenetic relationships because of their low variability and strong evolutionary stability. To determine the genetic relatedness of species, methods including DNA-DNA hybridisation, DNA fingerprinting, and DNA sequencing are used. Depending on the level of precision needed, the study can focus on individual bacterial chromosome or plasmid segments or complete genomes. Plasmid typing techniques, such as degenerate primer MOB typing (DPMT) and PCR-based replicon typing (PBRT), for example, use plasmid sequences to ascertain phylogenetic relationships. Numerous genotypic techniques, like as PCR-based methods, restriction fragment length polymorphism (RFLP) analysis, and sequencing of conserved genes like 16S rRNA, have been developed to circumvent the constraints of phenotypic identification. These techniques improve our knowledge of microbial taxonomy and evolution by providing more precise and reproducible outcomes [71].

III.7. Strain improvement of industrial microorganisms

During fermentation, the chemical modifications caused by microbial activity in various organic substrates transform the substrates into a more valuable product. Different genetic varieties of a microorganism that is appropriate for a specific fermentation method can be called strains. The wild-type strain is the naturally occurring, well-characterized strain that is used as a standard reference. Although this wild-type strain may be able to convert substrates into products, the process may not be optimal. For a fermentation process to be commercially successful, it must be as productive and high-quality as possible. Often, strain improvement is necessary to fulfil these criteria. Therefore, the insertion of specific desirable genetic modifications by mutations, selection, or genetic recombination is referred to as strain improvement [72].

III.7.1. Interest in strain improvement

Strain improvement from the original culture is necessary before profitable commercial exploitation of the synthesized product. Essential molecules are produced by natural microorganisms, although wild-type strains usually generate yield in low quantities. Strain homogeneity and purity are first checked before proceeding with improvment. strain improvement can add traits such as [72]:

- Over time, strain improvement raises product yield per vessel and enhances productivity.

- Lower production costs and reduce the production of undesirable metabolites.

- Use cheaper substrates.

- Improve fermenter utilization for other products.

- Stress tolerance (abiotic, toxic products, solvents, etc.)

- Resistance to nutritional and anti-metabolite repression.

- Reduced inhibitor by-product formation benefits downstream processing.

Other advantages include genetic stability, bacteriophage resistance, better enzyme regulation, increased cell permeability, non-toxicity, and Elimination of undesirable characteristics [72].

Classical strain improvement" describes techniques like dominant selection, directed evolution, and random mutagenesis. These can result in additional genetic background variations when combined with some natural gene transfer processes like transformation and conjugation.

III.7.2. Random mutagenesis

A classic and still widely used technique in industrial microbiology for improving microbial strains to increase bio-product production (such as antibiotics, enzymes, biofuels, amino acids, etc.) is random mutagenesis.

A quicker and more economical way to get rid of particular unwanted traits is through random mutagenesis. The accumulation of numerous accidental, undesired, and desirable mutations is a major drawback of this approach. This technique does not require prior knowledge of the genome or physiology of the strains being treated. Random mutagenesis induces genetic changes randomly within the genome; the location and nature of the mutations are unpredictable and cannot be controlled. Mutations are introduced into the genome of microorganisms through the action of physical or chemical mutagenic agents, each with a different effect on the genome. The process of random mutagenesis involves a number of important considerations. Following mutagenesis, strains are chosen based on the desired phenotype. Working with these mutagenic agents is dangerous

though, therefore care must be taken and contact should be avoided. For instance, a fume chamber should be used while handling chemically dangerous chemicals [72].

The selection of the mutagen (Table III.2) is a crucial element with many implications. Chemical mutagens such nitrosoguanidine (NTG), 4-nitroquinoline-1-oxide, mustards (IR170), methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and hydroxylamine (HA) and physical mutagens like UV, gamma, and X-ray radiations are categorised in Table III.2 [72].

Table III.2. The damage caused by different mutagens [72].

Mutagen	**Damage**
Nitrous acid	Deamination of adenine, cytosine, guanosine
Ethyl methanesulfonate	Alkylation of cytosine and adenine
N-methyl-N0-nitro-N-nitrosoguanidine	Methylation at high pH
Nitrosamine	Alkylation
Hydroxylamine	Deamination of cytosine
Far-UV	Hydroxylation of bases
UV	Pyrimidine dimers

III.7.2.1. Physical agents

In physical mutagenesis, heavy-ion, laser, ultraviolet (UV), X-ray, and gamma radiations were applied to cells using specific radiation doses. Ionising radiation and ultraviolet light (254 nm) cause errors and, more significantly (Figure III.8), substitutions during DNA repair (SOS function). UV rays are rather safe and simple to utilise. In a glass Petri plate, cells are suspended in TES buffer (N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid) at pH 7. Stirring helps keep the suspension uniform. The bactericidal lamp is positioned 20 to 30 centimetres away. It is not advised to employ ionising radiation, X-rays, or gamma rays in a strain enhancement program since they harm and significantly alter the genetic material [72-75].

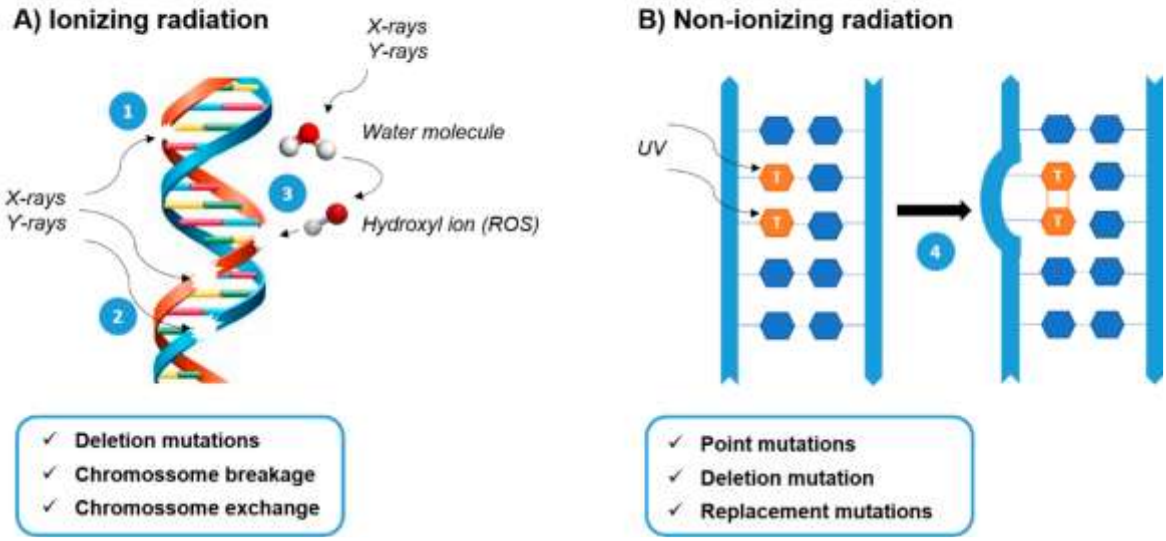

Figure III.8. DNA mutation mechanisms by physical mutagenesis. *(**A**) Ionizing radiation, (**B**) Non-ionizing radiation* [75].

III.7.2.2. Chemical agents

Chemical mutagens have also been widely used, and both their mechanisms of action and mutagenic potentials are well characterized. Alkylating agents, or compounds with an active alkyl group that replaces an alkyl group on a DNA base, typically guanine, with a hydrogen ion, are the most often employed chemical mutagens. Nucleotide substitutions, insertions, or deletions are added to the DNA sequence during DNA replication, frequently as a result of the DNA polymerase misinterpreting the nucleotides on the chemically modified template strand [72, 75].

Nitrosoguanidine (NTG, MNNG), ethyl-nitrosourea (ENU), methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and N-methyl-N-nitrosourea (MNU) are typical alkylating agents (Figure III.9). Among them, EMS and NTG/MNNG are most commonly utilized in microalgal strain improvement. Similar chemical mutagenesis mechanisms in DNA are triggered by these substances, allowing for high-frequency point mutation and the creation of new phenotypes. MNNG causes a wider spectrum of mutations, whereas EMS alkylation is exclusive to guanine and causes G/C to A/T transitions [72, 75].

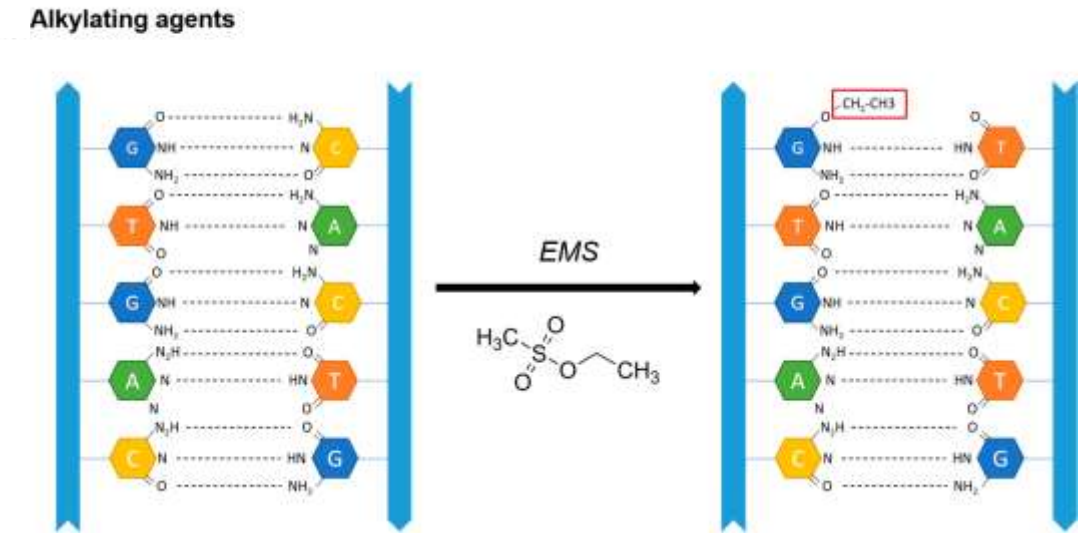

Figure III.9. DNA mutation mechanisms by chemical mutagenesis using alkylating agents as EMS [75].

Other agents used in chemical mutagenesis include:

* Non-alkylating agents: hydroxylamine and 4-nitroquinoline oxide (NQO) or nitrous acid.

* Base analogs: 5-bromouracil (thymine analog) and 2-aminopurine (adenine analog).

* Intercalating agents: acridine orange and ethidium bromide. Intercalating agents and nucleobases are not effective in inducing stable mutations in actinomycetes and fungi. All of these mutagenic agents are carcinogenic and must be handled with extreme caution.

III.7.2.3. Combination of treatments

A combination of chemical and physical mutagens can be used to increase the efficiency of mutagenesis. Moreover, the presence of certain substances can enhance the mutation rate. Caffeine, for example, inhibits DNA repair mechanisms, and nalidixic acid increases the rate of erroneous repairs. ARTP (Atmospheric and Room Temperature Plasma) is a modern mutagenesis method used to create mutations in the genetic material of microorganisms (bacteria, yeast, fungi, etc.) in order to improve certain properties (enzyme production, stress tolerance, metabolic efficiency, etc.). It is a physicochemical method of mutagenesis. It is physical, because it uses an electric field and energetic particles from the plasma. It is chemical, because the plasma also generates reactive molecules (O•, OH•, NO•, etc.) which chemically interact with the DNA [75].

III.7.3. Site-directed mutagenesis

Another important method for strain improvement is site-directed mutagenesis. The main concept behind site-directed mutagenesis is the use of synthetic oligonucleotides (primers) containing the

desired mutation to change a particular nucleotide sequence. During DNA replication or amplification, these primers act as templates, allowing a changed sequence to be substituted for the original.

Site-directed mutagenesis can be achieved using various gene-editing tools that allow the creation of tailor-made genotypes. Among these, techniques such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR-Cas9) and zinc finger nucleases (ZFNs) are commonly employed. In addition, RNA interference (RNAi), together with advances in the field of synthetic biology, has been applied to engineer specific genes for enhanced biomass production and increased yields of value-added compounds, ultimately contributing to reduced production costs [75].

Recombinant DNA technology involves the design of desirable genetic constructs through gene manipulation techniques such as gene knockout, gene knockin, and targeted mutagenesis, followed by their introduction into an appropriate host organism. This approach enables the combination of favorable traits while eliminating undesirable characteristics. In well-characterized organisms, the use of natural recombination systems through sexual or parasexual reproduction has also proven to be effective [72]. However, protoplast fusion is a more recent and adaptable method for producing recombinants when the biochemistry or genetics of a metabolite's production are poorly understood, or when a recently isolated strain is poorly defined [76].

III.7.3.1. Protoplast fusion

The term protoplast refers to a cell structure free of cell wall components. The production of viable protoplasts has revolutionized genetic techniques, allowing high-rate intraspecific fusions, some interspecific fusions, and transformations. Currently, protoplast fusion is widely used. Genome-wide recombination in bacteria can be accomplished through protoplast fusion. In this traditional genetic engineering approach, bacterial cells are first deprived of their cell walls and then chemically fused, enabling chromosomal exchange between the parent cells. Initially developed for standard genetic manipulation, protoplast fusion has since become a valuable strategy for creating microorganisms with enhanced phenotypes in biotechnology, by merging advantageous alleles from distinct strains or even different species. Protoplast fusion has been used to improve low spore-producing strains of certain antibiotics, such as *P. chrysogenum* and *Cephalosporium acremonium*. In some instances, *Streptomyces griseus* and *Streptomyces tenjimariensis* nonantibiotic-producing mutants fused protoplasts to create indolizomycin, a novel antibiotic [76-78].

For protoplast preparation, cells are cultured in isotonic solution while being treated with enzymes such as β-galacturonidase. The protoplasts are then regenerated using osmotic stabilizers such as sucrose. When hybrids are formed, the desired recombinants are identified by selective plating

techniques. After cell wall regeneration, the product of protoplast fusion can be used for further studies. Figure III.10. shows a flowchart that illustrates the fundamental stages of protoplast fusion [72].

The benefits of protoplast fusion include:

-Interspecies fusions: A major advantage of the protoplast fusion technique is the ability to fuse protoplasts from different microbial species, even if they are not very closely related. For example, the fusion of protoplasts from *Penicillium roqueforti* and *Penicillium chrysogenum*.

-High recombinant rate: This is generally high for all regions of the chromosome and higher than that obtained with conjugation (10^{-3} to 10^{-7});

-High multiple crossover rate: This can reach a frequency of 25%, compared to 1 to 2% for conjugation.

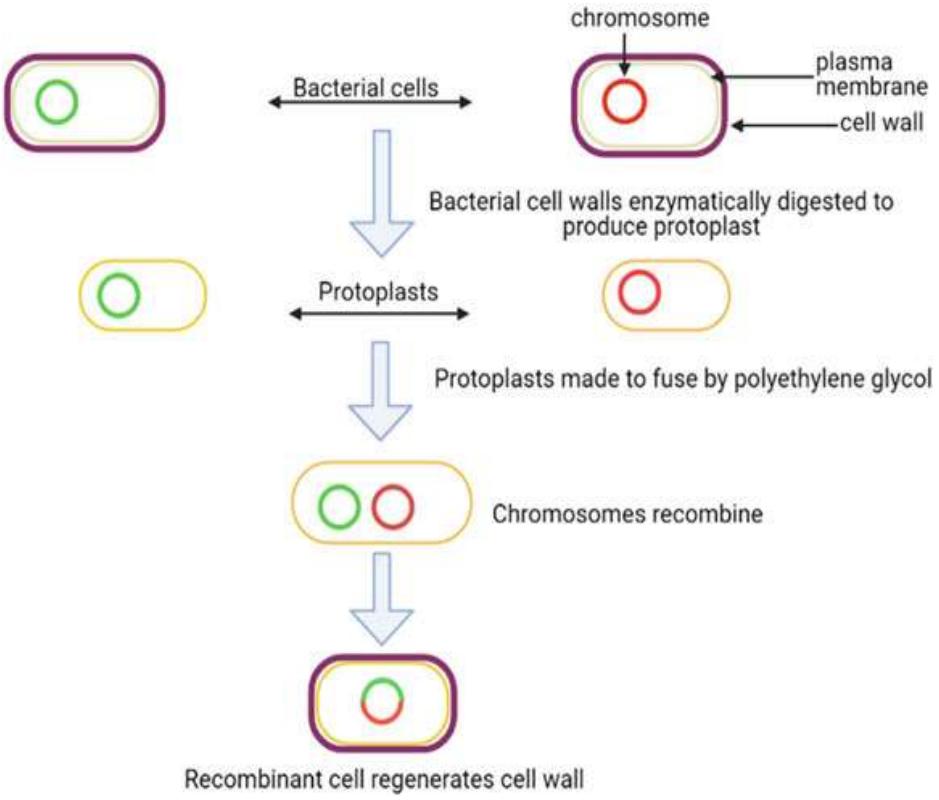

Figure III.10. Protoplast fusion [72].

III.7.3.2. Insertion of short DNA sequences

The insertion of short DNA sequences through site-directed mutagenesis (SDM) is the process of purposefully adding a few nucleotides, usually ranging from one to a few hundred base pairs, to a specific location inside a gene or regulatory area. With this method, bacterial DNA can be precisely

modified to add new functional elements, change the structure of proteins, or change how genes are regulated [79]. This technique is widely used in bacterial biotechnology to:

-Introduce epitope tags, restriction sites, or signal peptides;

-Insert short regulatory sequences (promoters, ribosome-binding sites, or transcriptional terminators);

-Modify enzyme active sites or protein domains for enhanced function.

The same concepts as point mutation apply to insertion mutagenesis; however, the oligonucleotide primer is made to include an extra brief DNA fragment in between sections that are complementary to the target sequence. This inserted fragment is integrated into the freshly created DNA strand during PCR amplification. Only the altered plasmid or chromosomal copy remains after the original (wild-type) DNA template is later eliminated (often by DpnI digestion).

a. Insertion of short DNA sequences methods

PCR-Based insertion mutagenesis: The insertion sequence is embedded within the forward or reverse primer. High-fidelity DNA polymerase amplifies the plasmid containing the mutation. The mutated plasmid is transformed into competent *E. coli* for replication and expression.

OE-PCR or Overlap Extension PCR : Two fragments of the target gene are amplified separately, each containing part of the insertion. These fragments are fused in a second PCR step through complementary overlapping regions that include the inserted DNA.

CRISPR/Cas9-Assisted insertion: A single guide RNA (sgRNA) directs Cas9 to the target site. A short donor DNA sequence (repair template) containing the insertion is provided. Homology-directed repair (HDR) integrates the inserted sequence precisely into the bacterial chromosome [75].

b. Applications in bacterial improvement

Site-directed mutagenesis is widely employed in industrial strain improvement to enhance certain functional characteristics, such as[80]:

Protein engineering: The insertion of short peptide tags, such as His-tags or FLAG-tags, is widely used to facilitate protein purification, detection, and characterization in bacterial systems. Additionally, the introduction of linker sequences between protein domains can enhance enzyme flexibility, improve substrate accessibility, and increase overall protein stability, thereby contributing to the optimization of enzyme performance and functionality in biotechnological applications.

Gene expression optimization: The insertion of short promoter sequences or ribosome-binding sites (RBS) can significantly enhance gene transcription and translation efficiency in bacterial systems. Moreover, the incorporation of synthetic regulatory elements enables precise modulation of gene expression, allowing fine-tuning of metabolic enzyme activity. Such optimization strategies are particularly valuable for improving metabolic flux and increasing the yield of biotechnological products, including bioplastics and biofuels.

Functional studies: Short DNA insertions can be strategically employed as molecular markers to investigate protein folding, subcellular localization, and protein–protein interactions within bacterial cells. Insertional mutagenesis also serves as a valuable tool for identifying functional domains, regulatory elements, and other critical regions within bacterial genes, thereby contributing to a deeper understanding of gene function and cellular mechanisms.

Synthetic biology and metabolic engineering*:* The insertion of regulatory motifs or genetic switches enables precise control over cellular metabolic fluxes. This approach allows engineered bacterial strains to optimize the synthesis of target compounds and to modulate their metabolic responses according to specific environmental or physiological conditions[80].

c. Considerations and challenges

Although site-directed mutagenesis is a potent and accurate method of genetic modification, its effectiveness can be influenced by a number of biological and technical factors. To guarantee the precision, stability, and functioning of the induced alterations, it is essential to comprehend these limitations.

-The insertion of short DNA sequences through site-directed mutagenesis presents several important considerations.
-Improperly designed insertions may disrupt the reading frame or affect protein folding, potentially leading to loss of function or instability. The efficiency of insertion largely depends on factors such as primer design, polymerase fidelity, and the cellular DNA repair mechanism involved.

- In the case of chromosomal insertions, the length of homologous regions used for recombination and the selection strategy employed are critical determinants of successful integration and overall experimental efficiency [79].

III.7.3.3. Modification of gene expression

In addition to inserting new genes into microorganisms, one can also modify gene regulation by altering transcriptional control, fusing proteins, or removing feedback inhibition. These strategies enable overproduction of many valuable compounds. This approach may also involve intentionally

rewiring metabolic pathways by inactivating or deregulating specific genes. Such strategies fall under the field of metabolic pathway engineering. For instance, penicillin production has been enhanced via metabolic engineering of Penicillium and bacterial host strains. Similarly, variants of erythromycin have been generated by blocking certain biosynthetic steps in its precursor pathway, thereby producing modified end products [80].

III.7.4. Selection of mutants

The isolation of mutant strains with desired phenotypes is a cornerstone technique in microbial genetics, metabolic engineering, and synthetic biology. In a doctoral research context, one aims not only to apply selection methods, but also to justify them, discuss their limitations, and position them relative to the literature. Below is a refined and expanded draft.

III.7.4.1. Antibiotic-resistance selection

In this strategy, one co-cultivates the wild-type (prototrophic) strain and mutagenized populations in a medium containing a specific antibiotic. Only those mutants harboring a resistance-conferring alteration (in the antibiotic target, an efflux pump, or via acquisition of a resistance gene) survive; wild-type (sensitive) cells are eliminated. This is a classical method in molecular biology for plasmid maintenance and selection of transformants, as well as for enrichment of mutants with altered antibiotic targets. However, its use in strain engineering carries caveats, such as the risk of horizontal gene transfer of resistance markers, regulatory constraints in industrial or therapeutic use, and metabolic load of resistance determinants (see review: *Antibiotic-Free Selection in Biotherapeutics*).

When implementing antibiotic selection, one must optimize antibiotic concentration to maximize the selectivity index (the ratio between killing of sensitive cells and survival of resistant cells), avoid “leaky” survival of wild-types, and account for stress responses that might increase mutation rates [81].

III.7.4.2. Antimetabolite-resistance selection

Antimetabolites are structural analogs of essential metabolites that competitively inhibit enzymes or block metabolic pathways, thereby causing growth inhibition or lethality. By applying such analogs in culture medium, wild-type cells are suppressed, while mutants with reduced sensitivity (via target modifications, bypass pathways, or increased efflux) can survive. This approach has been widely used in plant cell culture and microbial systems. Protocols were provided for selecting antimetabolite-resistant mutants, including amino acid analogs, base analogs, metabolic inhibitors, and herbicide-like compounds.

Its advantages include a potentially more subtle selection pressure than outright antibiotic killing, and a capacity to select for altered metabolic regulation rather than simple resistance genes. But it

demands careful calibration of analog concentration, as overly harsh conditions may eliminate rare beneficial mutants, while too mild concentrations permit wild-type survival [82].

III.7.4.3. Auxotrophic mutant selection

An auxotroph is a mutant unable to synthesize a critical metabolite (amino acid, nucleotide, vitamin, etc.), and therefore incapable of growth in minimal (unsupplemented) medium. By contrast, prototrophs grow without supplementation. The method of auxotrophic selection exploits this: one uses a host strain already auxotrophic, or induces auxotrophy via mutagenesis, and then complements that defect (via plasmid-borne gene) to permit growth only of recombinants. This approach avoids antibiotic selection and is especially prized in systems where antibiotic resistance is undesirable (in industrial bioprocessing or regulatory-constrained applications) [83].

In yeast biotechnology, multiple auxotrophic markers can be stacked; for example, a quadruple auxotrophic *Saccharomyces cerevisiae* strain was engineered for stable plasmid maintenance without antibiotic selection. One must take care that the auxotrophic mutation is stable (non-reverting), that background revertants are rare, and that metabolic burden or unintended cross-feeding do not cloud the selection [84].

III.8. Strain preservation

Strain preservation refers to the set of techniques used to maintain the viability, genetic stability, and phenotypic characteristics of microbial or cellular strains over long periods. It ensures that cultures used in research, biotechnology, pharmaceuticals, and agriculture remain unchanged and available for future study or industrial use. Without proper preservation, strains can undergo genetic drift, lose essential traits, or die, leading to the loss of valuable biological resources.

Microbial strains are vital in biotechnology, agriculture, medicine, and environmental research. Preserving them ensures that essential traits, such as enzyme activity or antibiotic production, are retained. It also supports long-term studies, quality control in pharmaceuticals, and biodiversity conservation in microbial culture collections.

Common preservation methods include cryopreservation, lyophilization (freeze-drying), and storage in protective media at low temperatures. These techniques maintain cellular viability and genetic integrity, allowing reliable recovery and consistent performance of engineered strains for research and industrial applications.

III.8.1. Short-term preservation

For short-term needs (weeks to months), strains can be stored on agar slants at 4 °C or covered with mineral oil to prevent drying. These methods are simple and inexpensive but not suitable for long-term storage, as frequent subculturing increases mutation risks and contamination.

III.8.2. Lyophilization (Freeze-drying)

Lyophilization removes water from microbial suspensions by freezing them and then applying a vacuum so that the ice sublimates. Freeze-drying (at -70°C to -196°C) allows for advanced dehydration compatible with very long storage times. The resulting dry material can be stored at room temperature or under refrigeration for many years. Protectants such as trehalose, sucrose, or skim milk are often used to stabilize cell membranes during dehydration [85].

Lyophilized cultures are easy to transport and store, making this method common in microbial culture collections. However, survival rates vary by species—spore-forming bacteria and fungi tend to survive better than non-spore-formers. The strain to be preserved is:

- Frozen and then dehydrated by sublimation of ice (evaporation of water directly from solid to vapor).

- Then the lyophilizate is sealed and stored in the dark, generally in a cool atmosphere (0-4°C).

Lyophilization is not compatible with all microorganisms. In some cases, it causes cellular and genetic alterations. Revival (rehydration) is achieved by adding culture medium to the lyophilizate vial. When the practical conditions for a given strain are perfect, lyophilization is an excellent long-term storage method that is easy to manage (no problem with liquid nitrogen or -80°C freezers). Examples: entire collections of strains in major international strain libraries.

III.8.3. Cryopreservation (Deep freezing)

Cryopreservation involves storing cells at ultra-low temperatures, usually −80 °C in mechanical freezers or −196 °C in liquid nitrogen. At these temperatures, all biological processes stop, preserving the genetic and metabolic state of the organism. Cryoprotectants such as glycerol or dimethyl sulfoxide (DMSO) are added to minimize ice crystal formation, which can damage cellular structures. This method is highly effective for most bacteria, yeasts, and fungi and can maintain viability for decades. However, it requires specialized equipment and careful control of cooling and thawing rates to avoid thermal shock [85].

At -20°C: the temperatures are not low enough, and the system evolves chemically quite slowly. Bacteria, yeasts, and moulds can be preserved for a few months to a few years, however.

At -80°C, chemical evolution is very, very slow. Most microorganisms can then be preserved with good viability if they have been frozen in the presence of cryoprotectants such as glycerol and DMSO, and if they are warmed quickly for reactivation. This makes them very useful for preservation over several years.

At -196°C, in liquid nitrogen, they can also be preserved for very long periods.

III.8.4. Simple desiccation method

Certain bacterial species exhibit a high tolerance to dehydration under natural dry conditions. This preservation technique can be enhanced through the application of a partial vacuum or the use of desiccating agents to accelerate moisture removal. During the dehydration phase, microorganisms are distributed onto an inert support material such as blotting paper, sand, or silica grains. Once desiccation is complete, the cultures must be stored in a completely dry state, typically at temperatures between 0 °C and 4 °C, within sealed containers containing a desiccant to maintain low humidity.

Revival of the preserved microorganisms is achieved by immersing the desiccated material directly into an appropriate culture medium, allowing for the rehydration and recovery of viable cells. This method is considered simple, cost-effective, and practical for strains that demonstrate resistance to drying. For instance, members of the *Enterobacteriaceae* family can be effectively preserved on blotting paper and remain viable for several months under dry storage conditions [86].

III.8.5. Preservation in spore form

Certain bacterial and fungal species possess the natural ability to differentiate into specialized, resistant structures known as spores, which serve as a survival mechanism under unfavorable environmental conditions. This intrinsic characteristic is effectively utilized for the long-term preservation of microbial strains.

Members of the genus *Bacillus* form endospores, which are highly resistant to desiccation, heat, and chemical agents, making them exceptionally stable during storage. Owing to this resilience, *Bacillus* strains are widely employed in industrial biotechnology, particularly for the large-scale production of enzymes and other bioactive compounds. Similarly, filamentous bacteria such as *Streptomyces* produce exospores, which, although less resistant to extreme conditions than *Bacillus* endospores, remain viable for extended periods when stored in dry, cool, and dark environments. These spores are valuable in the pharmaceutical industry, where *Streptomyces* species are a primary source of antibiotic compounds [85-87].

In laboratory practice, sporulation can be induced by cultivating the strains on suitable solid media that favor spore formation. The resulting spores, along with mycelial fragments, are gently removed from the agar surface using a sterile saline solution. The suspension is then filtered through sterile cotton wool to separate spores from mycelial debris. A cryoprotectant—such as glycerol—is subsequently added to enhance preservation during freezing. The final spore suspension is aliquoted into sterile vials or ampoules and stored under the same conditions used for vegetative cells, typically at −20 °C, −80 °C, or in liquid nitrogen. This approach is simple, reliable, and cost-effective for species capable of sporulation, providing long-term viability with minimal genetic alteration [85-87].

III.8.5. Factors affecting preservation

The success of microbial strain preservation is influenced by multiple factors, which can be broadly categorized into intrinsic factors, related to the inherent biological properties of the strain, and extrinsic factors, associated with environmental conditions and preservation procedures [85-87].

Physiological and biological factors*:* the survival of microorganisms depends on their growth phase, cell structure, and inherent resistance to stress. Cells in the late exponential or stationary phase and spore-forming species generally tolerate freezing or drying better.

Chemical and physical conditions*:* the type and concentration of protective agents (glycerol, DMSO, sugars), cooling and thawing rates, and storage temperature strongly influence cell viability. Controlled freezing and ultra-low temperatures (−80 °C to −196 °C) ensure better preservation.

Handling and storage procedures*:* proper preparation before preservation, stable low-temperature storage, and gentle rehydration or revival techniques are essential to maintain viability and prevent genetic or phenotypic changes

Some microorganisms, especially anaerobes and extremophiles, are difficult to preserve using conventional methods. Emerging techniques, such as vitrification (rapid freezing that prevents ice formation) and encapsulation in polymeric matrices, are being explored to overcome these limitations. The integration of genomic data into strain databases also ensures traceability and authenticity of preserved cultures [85-87].

III.9. Inoculum and inoculation

III.9.1. Inoculum

Inoculum refers to the microbial material—such as cells, spores, or tissue—introduced into a sterile medium to initiate microbial growth. The characteristics of the inoculum, including its viability, physiological state, and concentration, significantly influence the subsequent growth and metabolic activity of the culture. For instance, the physiological state of the inoculum can affect the lag phase

duration and overall growth kinetics. On an industrial scale, inoculation involves introducing the culture medium with a sufficiently large and active inoculum so that the desired strain can rapidly establish itself and outcompete potential contaminants. To achieve this, it is often necessary to prepare an inoculum capable of reaching volumes of 5,000 to 10,000 L, which can then be used to inoculate fermenters that are 10 to 20 times larger [88].

III.9.1.1. Inoculum properties

At each stage, rigorous controls detect the three ever-threatening causes of accidents: contamination, bacteriophages, and mutations[88].

a. Purity: Contaminant-free inoculum ensures experimental reliability and prevents unwanted microbial competition. The presence of contaminants is detected by performing sterility tests using microscopic examinations and subcultures. These tests must be performed from the inoculum to the main culture.

***b. Viability*:** Only living, metabolically active cells should be used.

c. Quality: Regarding mutations, control is much more difficult, and only yield reductions are observed, which are not always significant. Therefore, whenever possible, stable mutants should be chosen as the parent strain. Example: *Streptomyces griseus*, after 58 subcultures (subcultures), lost its ability to sporulate and produce streptomycin on agar medium, glucose-lacquered with yeast extract. Finally, the viability of the inoculum must be verified by subculture on solid medium.

d. Quantity: The cells (or spores) must be in sufficient quantity to allow for maximum inoculation. In some cases (in filamentous microorganisms), the quantity of inoculum influences the morphological characteristics of the strain during its growth.

e. Physiological state: Cells in the late exponential or early stationary phase often yield the best performance.

f. Size/Density: Adequate inoculum size ensures rapid and uniform growth, reduces lag phase, and improves process consistency.

III.9.1. 2. Preparing the inoculum

A spore suspension is prepared (*Actinomyces*), diluted, and subcultured onto the surface of an agar slant tube. Then, a pre-inoculum is created in a liquid medium (flasks, Erlenmeyer flasks) and incubated on rotating shakers. It is essential that the chosen medium yields a large quantity of spores by fragmenting the mycelium. The fermentation is then carried out in a small 300 to 500 L fermenter, then in a larger 3000 to 5000 L fermenter (germinator), which serves as a starter culture.

Using a mobile metal container (bazooka), the fermenter is aseptically inoculated using sterile compressed air, the pressure of which forces the contents of the container into the fermenter. All piping is steam sterilized.

III.9.2. Inoculation

Inoculation is the process of introducing the inoculum into a sterile growth medium, bioreactor, or culture system. Employing appropriate inoculation techniques is essential to prevent contamination, ensure uniform distribution of the inoculum, and maintain reproducibility across experiments or production batches. Various inoculation methods exist, such as aseptic transfer from liquid or solid cultures using sterile instruments, surface inoculation on agar plates (streaking or spreading), and submerged inoculation in liquid media or bioreactors, often utilizing a starter culture or seed culture[88].

Applications of inoculation are widespread in laboratory research, industrial fermentation, and biotechnological processes. In laboratory settings, inoculation is fundamental for pure culture studies, antimicrobial susceptibility testing, and microbial physiology investigations. In industrial fermentation, inoculation is critical for the production of enzymes, antibiotics, and probiotics. Biotechnological processes, such as biofuel production and wastewater treatment, also rely on effective inoculation techniques to ensure optimal microbial activity and process efficiency.

III.9.2.1. Inoculation success

The success of inoculation depends on a combination of biological, chemical, and environmental factors that collectively determine the growth, viability, and metabolic performance of the inoculated culture. Key factors include [88]:

a. Growth phase and physiological state of the inoculum: the physiological condition of the microbial cells at the time of inoculation is critical. Cells in the late exponential or early stationary phase often exhibit optimal metabolic activity and stress tolerance, which reduces the lag phase and enhances subsequent growth. In contrast, cells in the early exponential phase may be more sensitive to environmental stress, while aged cells may exhibit reduced viability.

b. Inoculum size and concentration: the volume and density of the inoculum directly influence the establishment of the culture. Insufficient inoculum can result in prolonged lag phases, slower growth rates, and increased susceptibility to contamination, whereas overly dense inocula may lead to nutrient depletion or oxygen limitation, affecting metabolic efficiency. Industrial processes often require carefully scaled inocula to match the volume of the fermenter for uniform culture establishment.

c. Medium composition and volume: the nutrient composition, osmolarity, and buffering capacity of the growth medium significantly affect inoculum performance. Media that provide balanced macronutrients, vitamins, and trace elements support robust growth, whereas suboptimal formulations can prolong lag phases or reduce yield. The total volume of the medium relative to the inoculum also affects nutrient availability and gas exchange, particularly in large-scale fermenters.

d. Environmental conditions: physical and chemical environmental parameters—including temperature, pH, oxygen availability, and agitation—affect microbial metabolism and growth kinetics. Optimal conditions promote rapid adaptation and metabolic activity, whereas deviations can lead to stress responses, slower growth, or metabolic shifts that reduce productivity. For aerobic cultures, sufficient oxygen transfer is essential to prevent hypoxic zones, while pH extremes can inhibit enzyme activity and cell division.

Overall, careful control and optimization of these factors are essential to ensure reproducible, high-yield inoculation, both in laboratory experiments and industrial fermentations. The interplay between inoculum quality, medium composition, and environmental conditions determines the lag phase duration, growth rate, and metabolic efficiency of the culture.

III.9.2.2. Inoculation methodologies

Inoculation is a fundamental step in microbiology and biotechnology, as it determines the initial establishment of a microbial culture. Several methodologies are widely employed depending on the type of microorganism, the growth medium, and the scale of the operation [88]:

a. Aseptic transfer from liquid or solid cultures: this method involves transferring microbial cells from a source culture to a fresh medium under sterile conditions using inoculating loops, needles, or pipettes. It is commonly used in laboratory settings to maintain pure cultures and prevent contamination. Proper aseptic technique ensures that only the desired microorganisms are introduced.

b. Surface inoculation on solid media: surface inoculation techniques are typically employed on agar plates to isolate or enumerate colonies:

c. Streaking*:* cells are spread across the agar surface in a pattern that dilutes the culture, leading to isolated colonies.

d. Spreading*:* a known volume of inoculum is evenly distributed over the agar surface using a sterile spreader, commonly used for colony counting or uniform culture establishment.

e. Submerged inoculation in liquid media or bioreactors: submerged inoculation is widely used in liquid cultures, including industrial fermentations. A starter or seed culture is introduced into a larger

volume of medium to achieve uniform distribution and rapid growth. This approach is critical for large-scale processes, where inoculum size and homogeneity directly influence productivity and process consistency.

f. Specialized methods: include aerosol inoculation, which is used for plant pathogens or environmental studies, and co-inoculation, employed for mixed cultures in bioprocesses that require synergistic microbial interactions.

These inoculation methodologies are applied across a wide range of contexts, including the study of pure cultures, antimicrobial susceptibility testing, enzyme production, industrial fermentation, and various biotechnological applications, such as biofuel synthesis and wastewater treatment. The selection of an appropriate inoculation method depends on the microbial species involved, their specific growth requirements, sterility considerations, and the intended scale of cultivation. Ensuring uniform distribution of the inoculum and minimizing the risk of contamination are fundamental objectives to achieve reproducible and efficient microbial growth[88].

IV. Primary metabolites

There are two categories of microbial metabolites: primary and secondary. Primary metabolites are produced by microorganisms while they are actively growing and include substances including proteins, carbohydrates, ethanol, acetone, and amino acids. Conversely, secondary metabolites are created after the microorganisms' active growth phase and serve as defence mechanisms against other microbes, plants, insects, and animals rather than directly affecting microbial development. The symbiosis between the bacterium and other living things, such as plants, nematodes, insects, and animals, can occasionally be facilitated by secondary metabolites [5].

Primary metabolites are essential compounds produced during the growth and development of organisms. They are directly involved in fundamental physiological processes such as growth, reproduction, and cellular maintenance. These metabolites are typically produced during the exponential (log) phase of microbial growth (trophophase) and are crucial for the survival and proliferation of the organism. Many primary metabolites are produced on an industrial scale for various applications.

The trophophase (Figure IV.1) is the active growth phase of a microbial culture, during which cells metabolize nutrients primarily for growth, maintenance, and reproduction. It corresponds to the exponential (log) phase of the microbial growth curve. During this phase:

-Cells exhibit high metabolic activity, consuming carbon, nitrogen, and other nutrients to build cellular components.

-Primary metabolites (such as amino acids, organic acids, ethanol, and nucleotides) are predominantly produced.

-The cell population increases rapidly, and the culture shows minimal stress responses.

The trophophase is contrasted with the idiophase, which is the phase where growth slows down, and secondary metabolites (antibiotics, pigments) are synthesized. Understanding the trophophase is crucial in industrial microbiology and biotechnology because maximizing this phase ensures high biomass yield and effective production of primary metabolites.

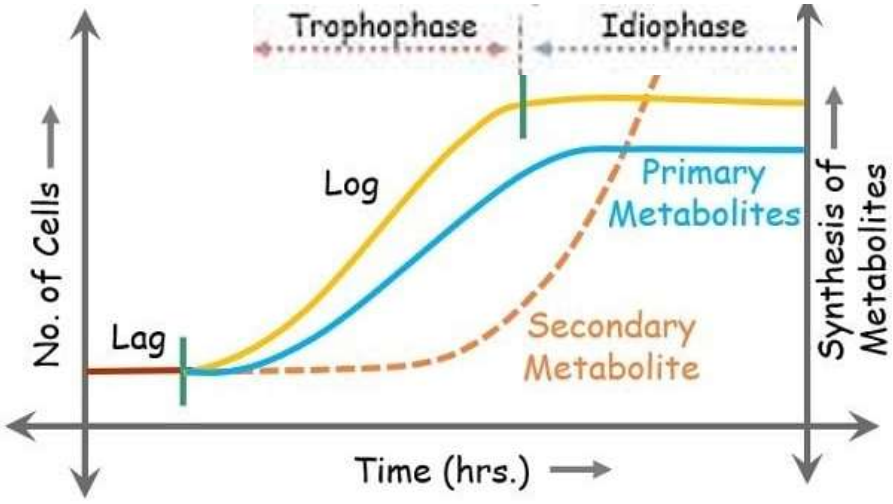

Figure IV.1. Phases of metabolite synthesis (Online source 5).

IV.1. Organic acid

Organic acids are a diverse group of low-molecular-weight compounds that contain one or more carboxyl functional groups (–COOH). They play essential roles in microbial metabolism and have broad industrial applications. Microorganisms such as bacteria, fungi, and yeasts are widely employed for the production of various organic acids through fermentation and metabolic processes. Microbial production of organic acids is attractive because it allows the use of renewable raw materials such as agricultural residues, lignocellulosic biomass, and industrial by-products. Furthermore, advances in metabolic engineering and synthetic biology have enabled the modification of microbial strains to increase yield and productivity.

Several organic acids are produced naturally or through engineered microbial pathways (Figure IV.2). The most significant examples include [89-91]:

Citric acid *:* produced mainly by *Aspergillus niger* via the TCA cycle. It is widely used in the food, pharmaceutical, and cosmetic industries as an acidulant and flavor enhancer.

Lactic acid : synthesized by *Lactobacillus* and *Streptococcus* species through homo- or heterofermentation of carbohydrates. It is an essential compound for the food industry and the production of biodegradable plastics such as polylactic acid (PLA).

Acetic acid: produced by acetic acid bacteria through the oxidation of ethanol or by anaerobic fermentation. It is primarily used in vinegar production and as a chemical reagent.

Succinic, fumaric, malic, and α-ketoglutaric acids : intermediates of the TCA cycle that can be overproduced through metabolic engineering. These compounds serve as building blocks for various polymers, solvents, and pharmaceuticals.

Itaconic, propionic, gluconic, and oxalic acids: produced by fungi and certain soil bacteria through specialized fermentation pathways. These acids have applications in pharmaceuticals, agriculture, and the chemical industry.

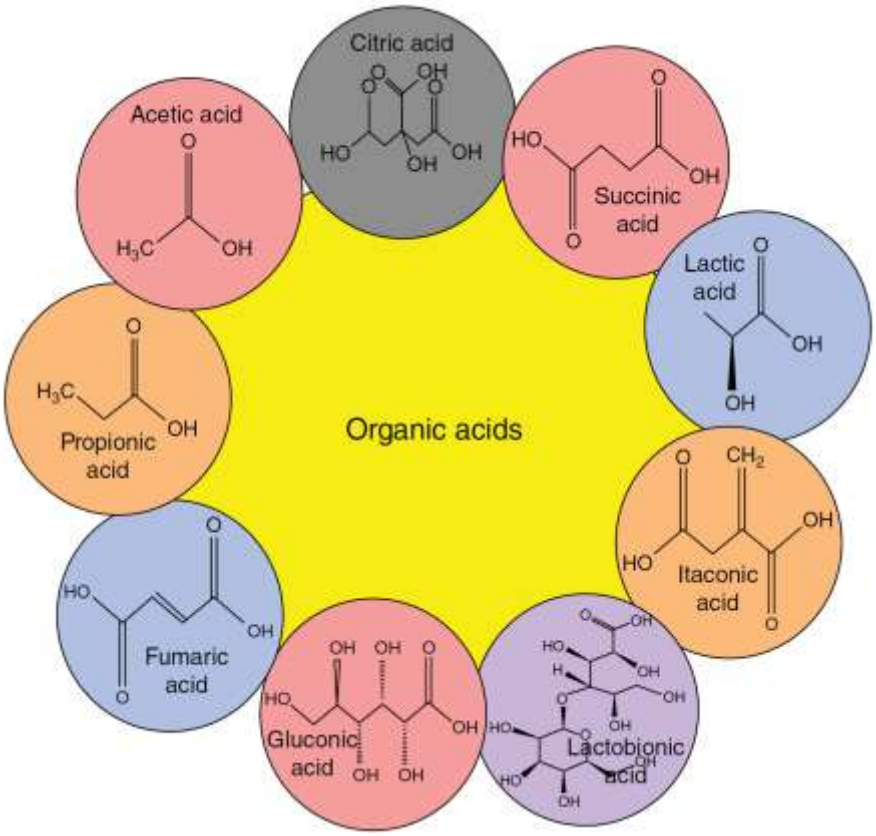

Figure IV.2. Different types and molecular structure of organic acids [91].

Microbial organic acids are used extensively in many different fields[91]:

Food industry: As flavorings, acidulants, and preservatives.

Pharmaceutical and cosmetic industries: As stabilizers, excipients, and active substances.

Polymer industry : it is involved in the manufacturing of biodegradable polymers like polylactic acid (PLA).

Agriculture: Organic acids enhance plant growth and nutrient solubilization.

Chemical industry: Act as "building blocks" or precursors for the production of bio-based compounds.

IV.1.1. Citric acid

One of the most common and adaptable organic acids is citric acid ($C_6H_8O_7$, 2-hydroxy-1, 2, 3-propane tricarboxylic acid). These are odorless, colorless, translucent crystals that taste strongly like acid. Citric acid was first isolated from lemon juice in 1784. However, in 1923, Wehmer discovered that *Penicillium glaucum* could produce citric acid as a byproduct of calcium oxalate formation. Earlier, in 1917, Currie reported the industrial production of citric acid using *Aspergillus niger* grown in a sugar-based medium. Due to its economic advantages and the efficiency of biological synthesis over chemical methods, this process eventually became the preferred technique for large-scale commercial production. It is now well established that filamentous fungi, yeasts, and certain bacteria are capable of producing citric acid[91].

Numerous bacterial species, including *Corynebacterium* sp., *Bacillus licheniformis*, and *Arthrobacter paraffinens*, have been studied for their ability to produce citric acid. These bacteria were found to grow effectively in media containing ammonium sulfate, calcium, urea, and glucose. Subsequently, other genera such as *Aerobacter*, *Pseudomonas*, *Micrococcus*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, and *Arthrobacter* were evaluated under conditions that included isocitric acid, which was later converted into citric acid (Table IV.1) [91].

Table IV.1. Citric acid production by major microbial classes [91].

Classe of microorganisms	Strains
Filamentous fungi	*Aspergillus niger; Aspergillus aculeatus ; A. awamori ; A. carbonarius ; A. foetidus ; A. phoenicis ; A. wentii; Penicillium janthinellum*
Yeast	*Saccharomycopsis; lipolytica; Yarrowia lipolytica; Candida tropicalis; C. oleophila; C. guilliermondii; C. citroformans; C. parapsilosis; C. lipolytica; Hansenula anomola; Brettanomyces; Debaromyces; Torulopsis; Kloeckers; Pichia*
Bacteria	*Arthrobacter paraffinens; Bacillus licheniformis; Corynebacterium* sp.; *Aerobacter; Pseudomonas; Micrococcus; Bacillus; Brevibacterium; Arthrobacter*

Since citric acid is the most adaptable and often used acidulant, its primary use (70%) is in the food and beverage industry. Its application in this industry has been confirmed by its pleasant taste, high water solubility, and flavor-enhancing ability, all of which have contributed significantly to the rise in demand for citric acid. Citric acid and certain of its salts are used as sequestering agents in the chemical processing and pharmaceutical industries due to their buffering properties and capability to

form complexes with heavy metals. The remaining demand is split as follows: 6% goes toward pharmaceuticals and cosmetics, 6% goes toward industrial and chemical processes, and 18% goes toward detergents and cleansers.

IV.1.1.1. Fermentation basics

Citric acid, a key metabolic intermediate of the Krebs cycle, is produced through the condensation of the acetyl group (two carbons) from acetyl-CoA with oxaloacetate (four carbons), forming citrate (six carbons) and releasing coenzyme A (CoA). This reaction, catalyzed by the mitochondrial enzyme citrate synthase, represents the first committed and irreversible step of the Krebs cycle. The high-energy thioester bond of acetyl-CoA drives the condensation reaction, ensuring its thermodynamic favorability. Citrate formation marks the entry point of acetyl units into the cycle, linking carbohydrate, lipid, and protein metabolism within the broader context of cellular energy production [91].

The production and accumulation of citric acid by microorganisms is a well-known example of a metabolically regulated process. Under normal physiological conditions, microorganisms do not excrete large amounts of citric acid, as it serves as an intermediate in the tricarboxylic acid (TCA) cycle, an essential pathway for cellular energy generation. However, when certain bacteria, yeasts, or filamentous fungi, notably *Aspergillus niger* and *Yarrowia lipolytica*, are cultivated under specific environmental or nutritional constraints, they can accumulate citric acid in significant quantities in the extracellular medium and citric acid concentration varies widely depending on the strain in use and the main carbon source [92].

The fundamental biochemical pathways involved in citrate metabolism are well understood, as shoun in figure IV.3.

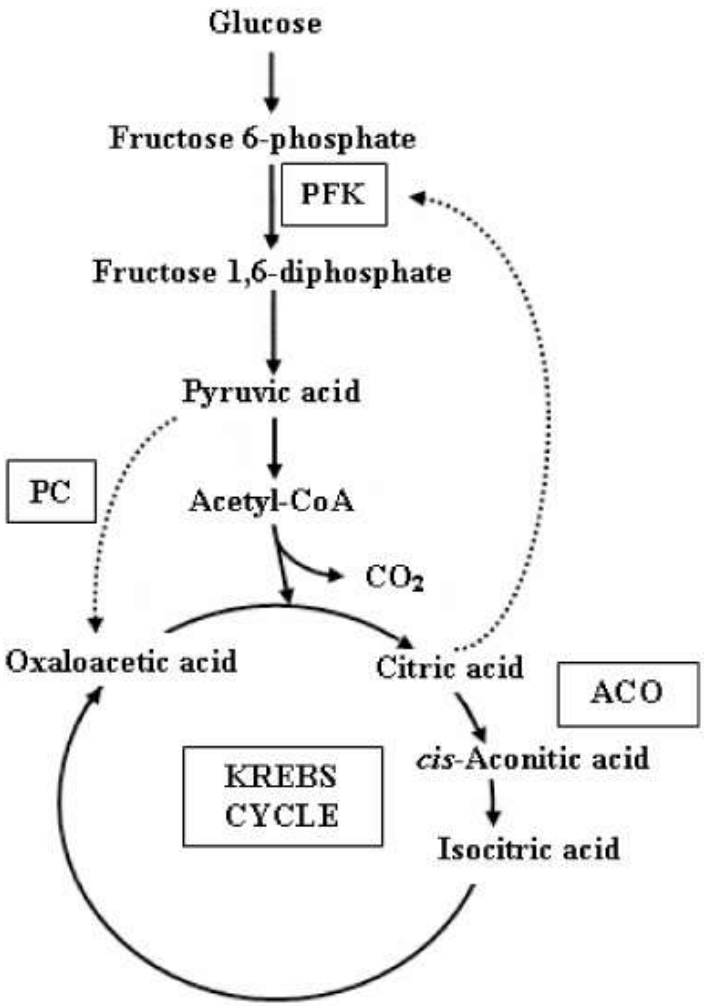

Figure IV.3. Main metabolic reactions involved in the citric acid production [93].

PFK: phosphofructokinase, PC: pyruvate carboxylase, ACO: aconitase

In *Aspergillus niger*, citric acid is synthesized from glucose through glycolysis, producing two molecules of pyruvate that are then converted to oxaloacetate. The enzyme citrate synthase catalyzes the condensation of oxaloacetate with acetyl-CoA to form citrate (Figure IV.4).

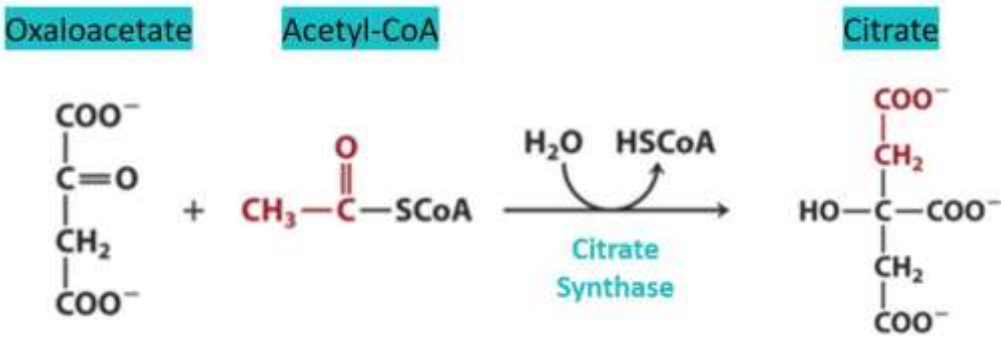

Figure IV.4. Citrate synthetase (Online source 6).

Citrate accumulation occurs when there is an imbalance between glycolysis and the TCA cycle, typically due to an increased glycolytic flux resulting from specific culture conditions that enhance glycolytic enzyme activity. A crucial step in this process is the formation of oxaloacetate via pyruvate carboxylase, an anaplerotic enzyme activated by high carbohydrate concentrations. The coordinated action of pyruvate carboxylase and citrate synthase leads to a buildup of intracellular citric. This unusual overproduction results from metabolic imbalances or genetic modifications that disrupt the normal flow of carbon through the TCA cycle acid [92-93]. For instance:

- Limitation of essential nutrients (such as iron, manganese, or phosphate) inhibits the activity of enzymes downstream of citric acid synthesis, particularly aconitase and isocitrate dehydrogenase, leading to citric acid accumulation.

- High carbon-to-nitrogen (C/N) ratios provide an excess of carbon substrate (usually glucose or sucrose) while restricting biomass formation, thus diverting metabolic flux toward product accumulation.

- Low pH and high aeration rates further enhance citric acid secretion by optimizing oxidative metabolism and minimizing by-product formation.

During citric acid production, isocitrate dehydrogenase (IDH), the enzyme that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate in the tricarboxylic acid (TCA) cycle, often acts as a metabolic bottleneck. Under normal growth conditions, IDH maintains the flow of carbon through the TCA cycle for energy production and biosynthesis. However, during citric acid fermentation, several factors can reduce IDH activity, causing citrate to accumulate.

In *Aspergillus niger*, IDH is sensitive to cellular energy and redox states. Low levels of manganese ions (Mn^{2+}), high ATP concentrations, and acidic pH conditions can all suppress IDH activity. When this enzyme is inhibited, the conversion of isocitrate to α-ketoglutarate slows down, while citrate and isocitrate continue to be produced upstream via citrate synthase. This leads to a metabolic imbalance, resulting in citrate accumulation and secretion into the culture medium. Therefore, isocitrate dehydrogenase acts as a regulatory checkpoint, and its partial inhibition is one of the mechanisms that enable *Aspergillus niger* to channel carbon flux toward citric acid overproduction rather than complete oxidation through the TCA cycle [92-93].

Therefore, the optimization of citric acid fermentation involves defining a delicate balance between nutrient limitation, aeration, pH, temperature, and substrate concentration. These parameters vary widely depending on the microbial strain, fermentation mode (batch, fed-batch, or continuous), and genetic background of the producer organism. In summary, citric acid accumulation is not a natural

physiological event but a controlled metabolic outcome resulting from carefully imposed constraints that redirect microbial metabolism from growth toward secondary metabolite production [92-93].

IV.1.1.2. Fermentation processes for citric acid production

A variety of fermentation processes have been developed to optimize citric acid production, depending on the microbial strain, carbon source, and process configuration. The two principal microorganisms used commercially are the filamentous fungus *Aspergillus niger* and the yeast *Yarrowia lipolytica*, each exhibiting distinct metabolic routes and substrate preferences [92-93].

For *Aspergillus niger*, both surface and submerged fermentation methods have been successfully applied. In surface fermentation, the fungus grows as a dense mycelial mat on the surface of the culture medium under relatively low aeration; this traditional method remains important for certain industrial applications due to its high product purity and simple downstream recovery. In contrast, submerged fermentation, where fungal pellets grow in aerated liquid media, is the predominant industrial process today, as it allows better oxygen transfer, pH control, and scalability in bioreactors. *Aspergillus niger* primarily utilizes carbohydrates such as glucose, sucrose, or molasses as carbon sources, converting them into citric acid through glycolysis and the tricarboxylic acid (TCA) cycle under conditions that favor high carbon flux and limit metal ions like manganese (Mn^{2+}) [92].

Meanwhile, *Yarrowia lipolytica*, an oleaginous yeast, has the unique ability to metabolize hydrophobic substrates such as n-alkanes, fatty acids, or vegetable oils, in addition to carbohydrates. Production with yeast is always performed in a submerged culture. When cultivated under nitrogen-limited and carbon-excess conditions, this yeast redirects acetyl-CoA derived from β-oxidation and glycolysis toward citric acid accumulation rather than lipid synthesis. Both microorganisms require finely tuned conditions, especially regarding carbon-to-nitrogen ratio, dissolved oxygen levels, trace metal concentrations, and pH, to maximize yield and minimize by-products. Continuous advances in metabolic engineering and process optimization are enhancing these fermentation systems, enabling more efficient and sustainable citric acid production from a variety of renewable substrates, including agricultural and industrial wastes [92].

IV.1.1.3. Production with *Aspergillus niger*

Culture conditions for citric acid overproduction

To achieve high citric acid excretion, the growth of the microorganism must be limited while maintaining active metabolism. Although high sugar concentrations stimulate glycolytic flux and promote precursor formation, nutrient limitation, particularly of nitrogen and phosphorus, is essential to shift metabolism from biomass synthesis toward citric acid accumulation. Additionally, trace metal deficiency, especially of iron (Fe^{2+}) and manganese (Mn^{2+}), is critical for optimal production,

as these ions influence key enzyme activities within the tricarboxylic acid (TCA) cycle and the mechanisms involved in citric acid transport and secretion[92].

Empirical studies have established general medium composition requirements for *Aspergillus niger* fermentation: a high carbon source concentration (typically 60–240 g L^{-1}), low nitrogen availability (2–3 g L^{-1}), limited phosphate levels (1.5–3.0 g L^{-1}), and extremely low concentrations of trace metals, manganese (<0.000001 g L^{-1}), iron (<0.0013 g L^{-1}), and zinc (<0.00025 g L^{-1}). The process also requires vigorous aeration to sustain aerobic metabolism and a low pH (2–3), which not only favors citric acid stability but also reduces contamination risks[92].

Carbon source: the carbon source could be starch, starch hydrolysate, sugarcane juice, glucose, sucrose, or molasses. In industry, molasses is the most commonly used carbon source. To achieve good citric acid production yields, the sugar concentration in the culture medium must be at least 140 g/L (14%). In industrial settings, beet and sugarcane molasses and glucose syrups are the primary carbon sources. However, since the composition of molasses varies widely depending on origin and processing, testing and pretreatment steps are necessary to adjust trace metal concentrations. Among these metals, manganese is particularly crucial; its excess can drastically suppress citric acid yield by stimulating undesired enzyme activities and diverting metabolic flux away from citrate accumulation.

To control trace metal availability, chelating agents such as sodium or potassium ferrocyanide are commonly added. These compounds bind excess metal ions, and when applied at slightly higher concentrations than the stoichiometric requirement, they can also contribute to partial growth inhibition, further enhancing product formation. Thus, successful citric acid production by *Aspergillus niger* depends on a delicate balance between nutrient limitation, metal ion control, and aeration, all of which collectively regulate metabolic flux distribution and promote efficient extracellular accumulation of citric acid [92-93].

Nitrogen source: Nitrogen is typically supplied in the form of ammonium salts, such as ammonium sulfate or ammonium nitrate, which are provided at concentrations ranging from 0.1 to 0.4 g/L. These compounds serve as readily assimilable nitrogen sources that support limited microbial growth while promoting citric acid accumulation. Excess nitrogen should be avoided, as it enhances biomass formation at the expense of product yield.

Mineral salts: Trace minerals, particularly ferrous ions (Fe^{2+}), must be carefully controlled or removed from the culture medium because concentrations above critical thresholds inhibit citric acid biosynthesis. The removal or reduction of inhibitory metal ions is typically achieved through ion-exchange chromatography or the use of chelating agents such as ferrocyanides. Maintaining ultra-

low levels of manganese, zinc, and iron is essential to direct metabolism toward citric acid accumulation.

pH control: During the initial stage of fermentation, the pH is adjusted between 5.0 and 7.0 to promote the germination and growth of *Aspergillus niger* spores. As fermentation progresses, the pH is gradually lowered below 2.0, which offers multiple benefits: it prevents contamination by other microorganisms and inhibits the formation of undesirable by-products such as oxalic acid and gluconic acid. Strict pH regulation is, therefore, a crucial parameter for achieving high citric acid yield and purity [92-93].

Factors affecting citric acid production

The regulation of trace metal concentrations is one of the most critical aspects of optimizing citric acid fermentation. In addition to chemical chelation using ferrocyanides, ion-exchange treatments can also be employed to remove excessive metal ions from the substrate. However, this technique is only effective for feedstocks with low salt content, such as corn syrups, since high ionic strength interferes with ion-exchange efficiency. Continuous efforts have been made to identify optimal mineral combinations and develop microbial strains less sensitive to metal ion regulation, allowing greater process flexibility and robustness[92].

Nutrient limitation remains a key driver for redirecting metabolism from growth to citric acid synthesis. Both nitrogen and phosphate restriction are effective in triggering this metabolic shift. In industrial fermentations with *Aspergillus niger*, ammonium salts such as ammonium sulfate and ammonium nitrate are the preferred nitrogen sources, typically added at concentrations between 1 and 3 g L^{-1}. The type and concentration of nitrogen strongly influence both biomass formation and citric acid yield; excessive nitrogen promotes cell growth at the expense of product accumulation, while too little may limit enzymatic activity. Phosphate limitation also enhances citric acid accumulation, particularly when trace metal levels are not tightly controlled, since phosphate can complex with metal ions and alter their bioavailability. However, moderate phosphate concentrations are still required for ATP generation and cellular maintenance[92].

Among the trace elements, magnesium (Mg^{2+}) plays a dual role, it is essential for cellular growth, serving as a cofactor for several glycolytic and TCA cycle enzymes, but it is also necessary in small amounts to sustain citric acid synthesis. Furthermore, the addition of lower alcohols such as methanol or ethanol, as well as lipid-based materials (vegetable oils), has been reported to enhance citric acid production. The stimulatory effect of these compounds is often linked to their influence on membrane permeability and trace metal chelation. However, when used in combination with high-purity substrates containing minimal metal ions, alcohols can exert an inhibitory effect, highlighting the delicate balance required in medium formulation. Overall, the interplay between nutrient limitation,

trace metal control, and specific stimulatory additives determines the metabolic state of the organism and governs the efficiency of citric acid accumulation during fermentation[92].

Regulation of citrate accumulation via aconitase inhibition

In industrial citric acid fermentation, maximizing citrate accumulation requires blocking its conversion to cis-aconitate, a reaction catalyzed by the enzyme aconitase. Therefore, inhibiting aconitase activity is a critical strategy for enhancing citric acid yields.

1. Removal of Iron Ions (Fe^{2+}): Aconitase is an iron sulfur enzyme that depends on Fe^{2+} ions as essential cofactors for its catalytic function. In the absence of iron, the enzyme becomes inactive, effectively halting the conversion of citrate to cis-aconitate and favoring citrate accumulation. Industrially, this is achieved by maintaining extremely low iron concentrations in the culture medium or by adding iron-chelating agents such as fluorocitrate, ferrocyanide, or ethylenediaminetetraacetic acid (EDTA). These agents sequester available iron, preventing its incorporation into the enzyme's active site.

2. Genetic Modification of Aspergillus niger Strains: An alternative approach involves genetic manipulation of *A. niger* to create strains with reduced or inactive aconitase expression. This can be achieved through mutagenesis or targeted gene disruption of the aconitase biosynthetic gene, resulting in transgenic strains incapable of converting citrate into subsequent intermediates of the tricarboxylic acid (TCA) cycle. Such strains maintain high intracellular citrate concentrations, leading to enhanced extracellular excretion of citric acid.

Together, these strategies iron deprivation and genetic inhibition of aconitase constitute fundamental biotechnological approaches for optimizing citric acid overproduction in industrial fermentations.

Fermentation processes in citric acid production by aspergillus niger

Two main industrial fermentation methods are used for citric acid production: the surface process and the submerged process.

The surface fermentation method was the first developed and remains in limited use due to its simplicity, lower energy requirements, and reduced sensitivity to trace metal variations, although it is highly labor-intensive. In this system, *Aspergillus niger* mycelium grows as a surface mat in shallow trays containing a diluted carbohydrate medium (≈15%) pretreated and adjusted to pH 5–7 to promote spore germination. After sterilization and inoculation with spores, the trays are incubated at 30°C under controlled humidity (40–60%) and continuous aeration, which ensures oxygen supply and heat removal. During fermentation, the pH gradually decreases to around 2.0, and the process is

completed within 7–15 days, achieving yields of approximately 70%. The resulting fermented broth is then separated from the mycelium for downstream recovery of citric acid [92].

In contrast, ***the submerged process***, now the predominant industrial method, is conducted in stirred-tank or tower fermenters under batch conditions, with aeration systems designed to maintain high dissolved oxygen levels, a key factor for optimal citric acid synthesis. The medium typically contains 15–27% carbon source, pretreated to eliminate manganese and reduce other trace metals. Optimal pH (2.5–3.0) and temperature (28–35°C) ranges are maintained, and inoculation is performed either with spores or mycelial precultures at a 1:10 ratio. Both the inoculum and medium composition strongly influence culture morphology, which in turn affects oxygen transfer and productivity. Formation of small, smooth, and compact pellets leads to superior mass transfer and reduced energy demand, enhancing overall efficiency. The fermentation duration generally ranges from 5 to 10 days, depending on strain, medium, and operating parameters[92].

Although ***fed-batch and continuous fermentation*** strategies have been explored for citric acid production, the batch process remains the industrial standard. Since citric acid accumulation is only partially associated with cell growth, continuous systems tend to be less efficient, as productivity declines once the growth phase is decoupled from metabolite synthesis. Similarly, fed-batch processes—despite allowing better control of nutrient supply and metal ion concentration—have not demonstrated significant productivity advantages to justify their large-scale adoption. To overcome this limitation and maintain high productivity over longer operational periods, researchers have investigated the use of immobilized mycelia of *Aspergillus niger*. Immobilization offers potential benefits such as increased cell density, stability, and reusability of the biocatalyst; however, results have been variable, and no industrial-scale process based on immobilized cells has yet proven economically viable [92].

Aspergillus niger mycelium has been successfully immobilized in various matrices such as alginate beads, collagen, and polyurethane foam to enhance process stability and reusability. However, despite these advances, citric acid productivity remains too low, and the complexity of the immobilization process makes it industrially unattractive. In contrast, recent progress in understanding fermentation physiology, along with the development of mathematical and kinetic models for process optimization, has yielded more substantial improvements in yield and control than these alternative configurations.

The simplest method for citric acid production is the solid-state fermentation (SSF) technique, commonly known as the Koji process. Originally designed for solid substrates such as sweet potato residues, rice bran, and wheat bran, this process has been modernized for sustainable production using agro-industrial wastes like fruit pomace and sugarcane bagasse. In the Koji process, the

carbohydrate substrate is moistened to approximately 70%, sterilized, and inoculated with conidia of *Aspergillus niger*. During fermentation, amylase enzymes secreted by the fungus hydrolyze starch into fermentable sugars, which are then converted into citric acid within 4–5 days. Although this technique is economically suitable for small-scale or decentralized production, it remains less competitive for large industrial operations due to lower productivity and more challenging process control[92].

IV.1.1.4. Industrial recovery of acid citric

In contemporary biotechnological manufacturing, citric acid is predominantly produced using the submerged fermentation method with *Aspergillus niger* as the production organism. This fungus is a strict aerobe, meaning it requires high levels of dissolved oxygen to achieve optimal productivity. Industrial fermentations are therefore conducted in large stainless-steel bioreactors equipped with powerful aeration and agitation systems that maintain high oxygen transfer rates and homogenous mixing [92].

Biomass separation: When fermentation is complete, the fungal mycelium (biomass) must be separated from the citric acid broth. This is achieved by filtration or centrifugation. The filtrate, which is rich in citric acid, proceeds to the recovery and purification stages, while the solid mycelial residue can be used as animal feed or composted (Figure IV.5).

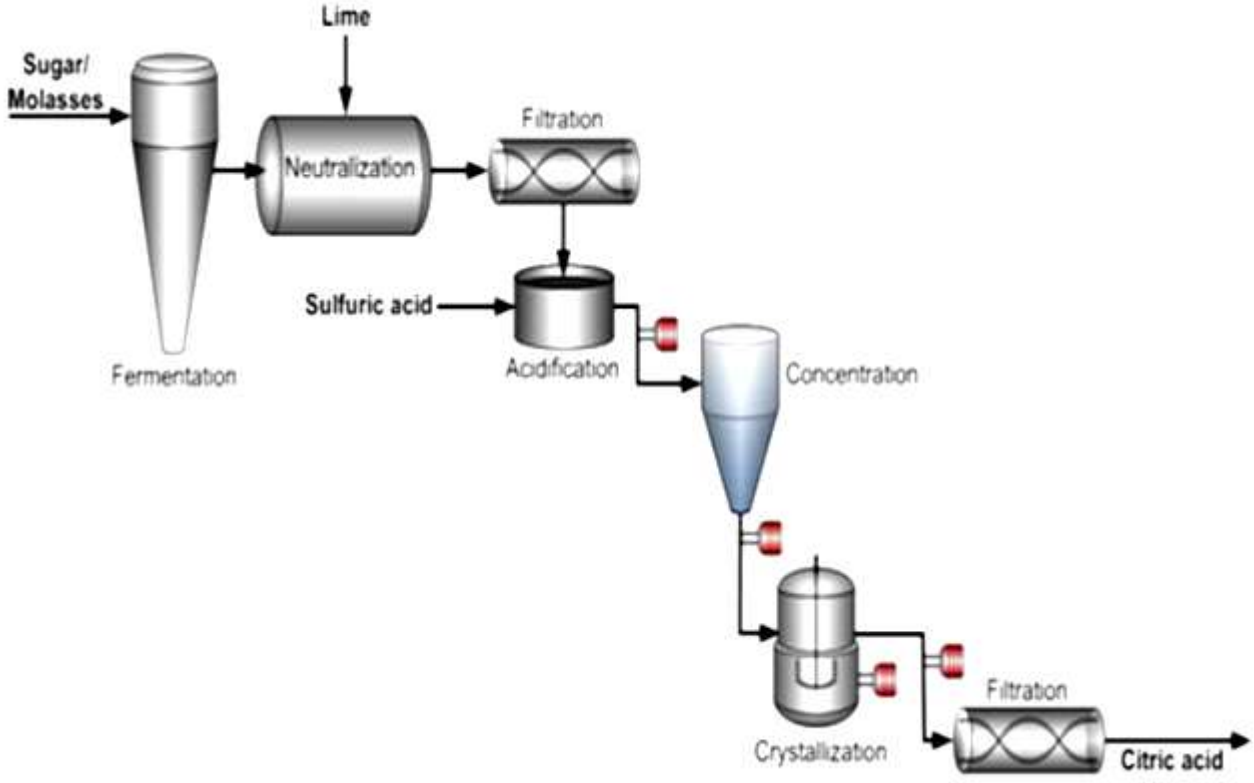

Figure IV.5. Industrial recovery of citric acid (Online source 7).

Precipitation of calcium citrate: The clear filtrate is heated to around 70–80 °C to inactivate enzymes and then neutralized by adding lime (CaO) or calcium hydroxide ($Ca(OH)_2$). This causes the citric acid to react with calcium, forming an insoluble calcium citrate precipitate according to the following reaction:

$$3Ca(OH)_2 + 2C_6H_8O_7 \rightarrow Ca_3(C_6H_5O_7)_2 + 6H_2O$$

The resulting calcium citrate is separated by filtration and washed to remove impurities.

Conversion to citric acid: The calcium citrate is then treated with dilute sulfuric acid (H_2SO_4) to regenerate citric acid and produce a calcium sulfate (gypsum) by-product:

$$Ca_3(C_6H_5O_7)_2 + 3H_2SO_4 \rightarrow 2C_6H_8O_7 + 3CaSO_4 \downarrow$$

The gypsum precipitate is removed by filtration, leaving a solution of citric acid.

Purification and crystallization: The resulting citric acid solution is often slightly colored due to residual organic compounds. It is therefore treated with activated carbon to remove pigments and then filtered again. Next, the solution is concentrated by evaporation under reduced pressure to avoid thermal degradation. Upon cooling, citric acid crystallizes from the concentrated solution. These crystals are separated by centrifugation, then washed, dried, and finally packaged as either citric acid monohydrate or anhydrous citric acid, depending on the drying conditions[92].

In traditional citric acid production, recovery involves precipitation with lime (CaO) and subsequent acidification with sulfuric acid, which leads to the formation of large amounts of calcium sulfate (gypsum). This by-product creates significant environmental and disposal challenges. Therefore, modern processes have focused on developing alternative recovery techniques that eliminate gypsum formation while maintaining product purity and process efficiency.

One of the most promising methods is solvent extraction (liquid–liquid extraction). In this process, citric acid is transferred from the aqueous fermentation broth into an organic solvent phase. The extraction is usually performed at low temperatures (10–25 °C) to maintain acid stability. A mixture of *n*-octyl alcohol and tridodecylamine (TDA) is commonly used because it forms a reversible complex with citric acid. The acid is then recovered by stripping the solvent with hot water (60–80 °C), yielding pure citric acid. This method completely avoids gypsum formation, reduces chemical consumption, and produces food-grade quality citric acid suitable for pharmaceutical applications [92-93].

Another sustainable approach involves membrane-based recovery systems such as microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. These techniques are used sequentially to remove

biomass, concentrate the acid, and achieve purification. When combined with adsorption resins, membranes can selectively retain citric acid and release it upon elution, providing an efficient, chemical-free process.

Ion exchange and adsorption methods are also applied, particularly using strong-base anionic exchange resins. The clarified fermentation broth is passed through these resins, which selectively bind citric acid. The acid is later desorbed with dilute acid or salt solutions, resulting in a highly purified product. This method offers continuous operation and high selectivity without generating solid waste. Another innovative method is electrodialysis, which uses ion-exchange membranes under an electric field to separate citric acid ions from impurities. This process is clean, continuous, and does not require chemical reagents, though membrane fouling remains a technical challenge. Overall, these advanced recovery methods aim to improve environmental sustainability, reduce waste, and lower operational costs. They also facilitate integration with continuous fermentation systems and support the transition toward greener biotechnological production of citric acid[92, 93].

IV.2. Microbial cells (Microbial biomass)

The industrial production of microbial cells, often called biomass production, is a fundamental process in biotechnology. It involves cultivating microorganisms such as bacteria, yeasts, molds, or algae at large scale to obtain their cells for use as food, feed, enzymes, pharmaceuticals, or industrial catalysts. This process is carried out under strictly controlled conditions to maximize cell yield, productivity, and desired characteristics. The production process begins with the selection of an appropriate microorganism, depending on the desired product. For example, *Saccharomyces cerevisiae* is used for single-cell protein and baking yeast; *Lactobacillus* species for probiotics; *Aspergillus niger* for enzyme production; and *Chlorella* or *Spirulina* for algal biomass. Selected strains are usually improved through mutation or genetic engineering to enhance productivity, substrate utilization, or stress tolerance. Industrial microbial biomass serves multiple purposes[95]:

Food and feed supplements (*Spirulina*, *Candida utilis*, *Saccharomyces cerevisiae*), known as single-cell protein (SCP).

Probiotics in functional foods.

Starter cultures for dairy or fermentation industries.

Biofertilizers and biocontrol agents in agriculture.

Biosorbents or bioadsorbents in environmental biotechnology.

The overall efficiency of microbial cell production depends on strain selection, substrate cost, aeration efficiency, and downstream processing. Advances in bioprocess engineering, metabolic modeling, and bioreactor design continue to improve yield, scalability, and sustainability.

Microorganisms can be exploited either for their cellular biomass or for the metabolites they produce. When used directly for their beneficial effects on health, they are known as probiotics. The term *probiotic* derives from the Greek words *pro* ("for") and *bios* ("life"), meaning "for life." Probiotics are living microorganisms, mainly bacteria and yeasts, that confer health benefits to the host when administered in adequate amounts. They help maintain a balanced intestinal microbiota, improve digestion, and support the immune system. The most common probiotic microorganisms include [94]:

Yeasts, particularly *Saccharomyces cerevisiae* (brewer's yeast), widely used as a dietary supplement for improving intestinal health and preventing diarrhea.

Lactic acid bacteria, such as *Lactobacillus casei*, *L. acidophilus*, *L. lactis*, and species of the genus *Bifidobacterium*, which are naturally present in fermented dairy products like yogurt.

When consumed regularly, probiotics help restore the natural balance of gut flora disturbed by stress, antibiotics, or infection. They can reduce the duration and intensity of digestive disorders (diarrhea, constipation, irritable bowel syndrome) and improve nutrient absorption and immune response. Probiotics are available as fermented foods (yogurt, kefir, sauerkraut), pharmaceutical formulations, or dietary supplements. Their effectiveness depends on the strain used, dosage, and individual health condition[94].

IV.2.1. Baker's yeast production

Baker's yeast (*Saccharomyces cerevisiae*) is widely used in the baking industry to produce leavened bread and is also a source of single-cell proteins (SCPs), dietary supplements, and various commercial biochemicals such as enzymes, nucleotides, and vitamins. Yeast extract, derived from autolyzed yeast, provides a rich source of B vitamins, amino acids, and growth factors, and is commonly used in microbial culture media. Globally, baker's yeast production exceeds 100,000 tons annually in countries like the United States[95].

The production process begins with the selection and preparation of a pure yeast strain, which is first grown in laboratory-scale seed cultures. The inoculum is then gradually scaled up through a series of progressively larger fermenters, ultimately reaching industrial fermenters with capacities up to 500 m^3. The fermentation medium typically contains a carbon source such as sugar beet or cane molasses, glucose, or sucrose; a nitrogen source like ammonium salts, urea, or yeast extract; and

phosphates, trace elements, and vitamins to support optimal growth. Sterilization of both the medium and equipment is essential to avoid contamination [95].

Fermentation is carried out under aerobic conditions, as oxygen is required for high biomass yield. Agitation and aeration ensure efficient mixing and oxygen transfer, while pH, temperature, and dissolved oxygen are continuously monitored and adjusted. Baker's yeast grows optimally at 30–35 °C and pH 5–6. Depending on the process, industrial fermentation may be conducted in batch, fed-batch, or continuous modes, with controlled sugar feeding to maximize biomass production and minimize ethanol formation. During fermentation, yeast converts sugars into carbon dioxide and ethanol:

$$\text{Sugar} \xrightarrow{\text{Yeast}} CO_2 + \text{Ethanol}$$

In baking, CO_2 becomes trapped in the gluten network of dough, causing it to rise, while ethanol and CO_2 escape during baking, leaving the characteristic porous structure of bread.

After fermentation, yeast cells are harvested by centrifugation or filtration (Figure IV.6) and concentrated into a dense paste (~60–70% moisture). The product can be formulated as moist yeast blocks, which must be refrigerated, or active dry yeast, produced via freeze-drying or fluidized bed drying to reduce moisture to 8–10% for a long shelf life. Quality control ensures cell viability, fermentation capacity, moisture content, and absence of contaminants before packaging and distribution. The dried product can be formulated as powder, granules, or pellets [95].

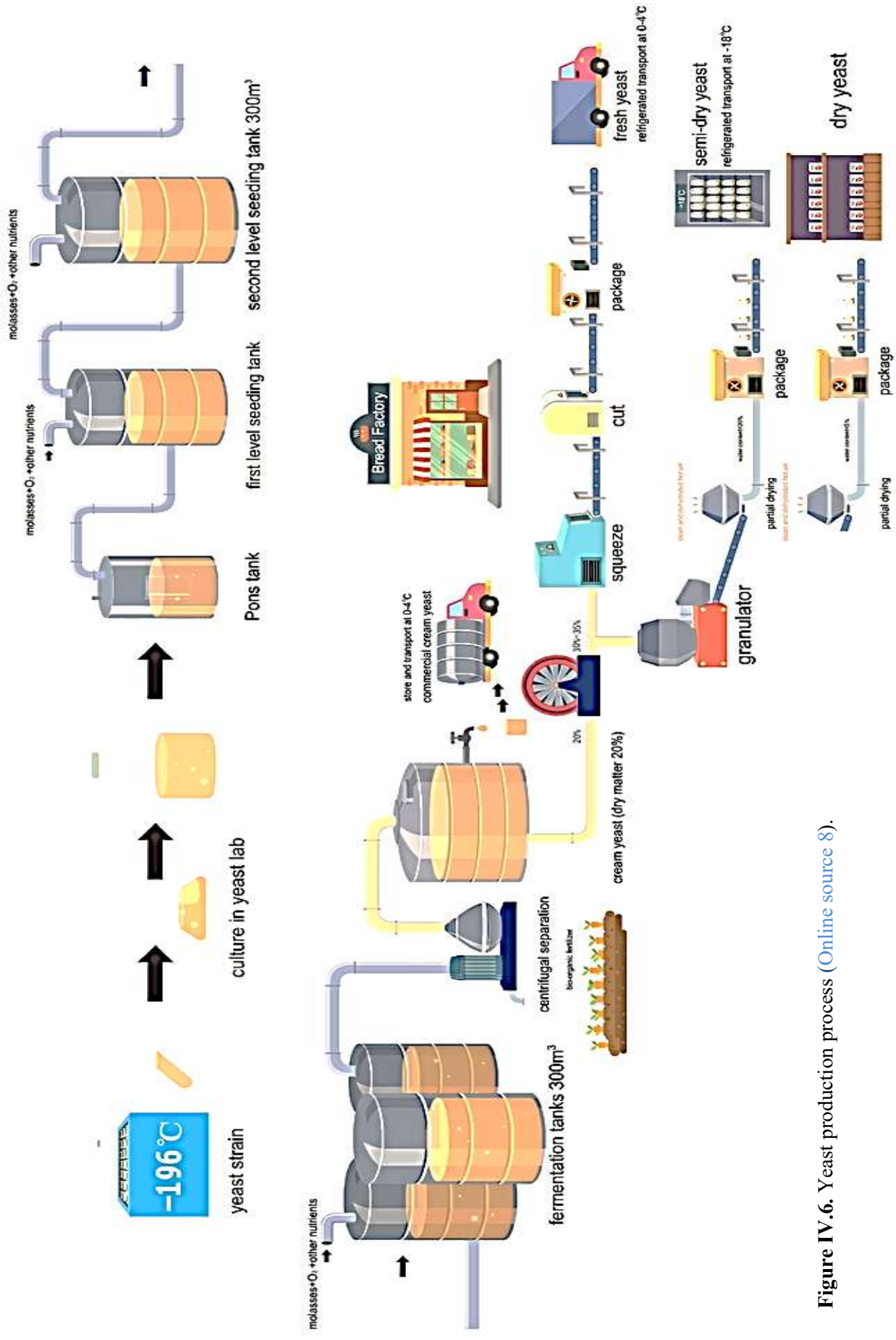

Figure IV.6. Yeast production process (Online source 8).

IV.3. Protein production by unicellular organisms

Unicellular organisms, including bacteria, yeasts, and microalgae, are widely used to produce proteins for food, feed, pharmaceuticals, and industrial applications. Proteins obtained this way are often referred to as single-cell proteins (SCPs). These organisms are advantageous because they can grow rapidly, utilize diverse substrates (including waste streams), and be cultivated at large scale in controlled environments. The concept of SCP production emerged as a response to global protein shortages, offering an alternative to traditional plant and animal protein sources[96].

IV.3.1. Microbial source in Single-Cell Protein (SCP) production

Microorganisms used for single-cell protein (SCP) production, such as bacteria, yeasts, fungi, and microalgae, are considered efficient and sustainable protein producers. Each group has distinct characteristics, nutrient profiles, and cultivation requirements that influence its suitability for food or feed applications. Their rapid growth rate, high protein content, and ability to use inexpensive substrates make them an excellent alternative to conventional protein sources from plants or animals [96].

IV.3.1.1. Bacteria

Bacterial species are especially efficient because of their rapid growth and high protein yields (up to 80% of dry weight). Strains such as *Methylophilus methylotrophus*, *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Ralstonia eutropha* can metabolize inexpensive carbon sources like methanol, methane, and food-derived organic wastes. They are particularly valuable for large-scale production of SCP for animal feed due to their high lysine and methionine content[97].

Methylophilus methylotrophus was used in the Pruteen process, one of the first industrial SCP projects. The process involved aerobic continuous fermentation at 30–37 °C and pH ≈ 7. Methanol was oxidized through the RuMP pathway to produce microbial biomass rich in protein. The final product contained about 70 % protein (dry weight) and all essential amino acids. The SCP was used mainly for animal feed (pigs, poultry). Its advantages include rapid growth, high yield, and efficient use of methanol. However, high production costs and energy demand limited its commercial success. It remains a classic example of methanol-based SCP biotechnology. Recent studies have revisited methylotrophic systems as a modern platform for SCP and recombinant protein production due to their high efficiency, low waste generation, and compatibility with renewable methanol derived from CO_2. Therefore, *Methylophilus methylotrophus* remains a model organism demonstrating the feasibility of methanol-based bacterial SCP.

Hydrogenobacter thermophilus is a thermophilic, chemolithoautotrophic bacterium that uses H_2, O_2, and CO_2 for growth. It converts CO_2 into biomass through the Calvin cycle, using hydrogen as an

energy source. Cultivation occurs in gas-fed bioreactors at ~70 °C and pH 6.5–7.0. The resulting biomass contains ≈ 75 % protein (dry weight) with high nutritional value. This SCP process requires no organic feedstock, offering a carbon-neutral solution. It can integrate renewable hydrogen to make sustainable microbial protein for aquafeed and human diets [97].

IV.3.1.2. Yeasts

Yeasts like *Candida utilis*, *Saccharomyces cerevisiae*, *Kluyveromyces fragilis* and *Yarrowia lipolytica* are highlighted for their robustness and ability to utilize waste materials such as molasses, whey, and other agro-industrial by-products. They produce protein-rich biomass (40–60% protein), vitamins (especially B-complex), and essential amino acids. Yeast-based SCP is widely used in food and feed industries because of its good digestibility and low nucleic acid conten [97].

One well-known example of SCP production using yeast is the cultivation of *Candida utilis* (also known as *Torula yeast*). This yeast is widely used for converting industrial and agricultural by-products, such as molasses, whey, and wood hydrolysates, into protein-rich biomass. During the fermentation process, *Candida utilis* grows rapidly under aerobic conditions and accumulates 40–60% protein in its dry weight. The resulting biomass is then harvested, dried, and processed into a high-protein supplement used in animal feed and human nutrition.

Another common yeast used for SCP production is *Saccharomyces cerevisiae*, the same species used in baking and brewing. It can be cultivated on spent molasses or other carbohydrate-rich wastes from food industries. The produced yeast biomass contains not only protein but also B-complex vitamins and essential amino acids, making it a valuable ingredient in functional foods and feed formulations.

These yeast-based SCP processes are cost-effective, sustainable, and scalable, as they transform organic waste into valuable nutritional products while reducing environmental pollution[97].

IV.3.1.3. Fungi (Molds)

Fungal species such as *Fusarium venenatum*, *Aspergillus oryzae*, and *Rhizopus oligosporus* are used for their ability to grow on lignocellulosic residues and complex organic wastes. They convert agricultural and food processing wastes into protein biomass with a fibrous texture, suitable for use in meat analogs and feed formulations.

Fusarium venenatum is a filamentous fungus widely known for producing the commercial mycoprotein Quorn™, one of the most successful SCP products for human consumption. It was first identified in 1967 during screening for high-protein microorganisms capable of growing on carbohydrates. This strain is cultivated under aerobic, continuous fermentation using glucose or starch hydrolysates as carbon sources. Optimal growth conditions are pH 6.0, 30 °C, with controlled aeration and agitation to maintain filamentous growth. The resulting biomass contains about 45–50%

protein (dry weight) and 25% fiber, with a favorable amino acid balance similar to meat. Before consumption, the mycelial biomass is heat-treated to reduce RNA content and improve digestibility. It is then dried, textured, and flavored to make meat-like products such as burgers, nuggets, and sausages. *F. venenatum* SCP offers a sustainable, low-fat, and cholesterol-free alternative protein source. Its industrial production demonstrates how fungal SCP can meet both nutritional and environmental goals [98].

Candida utilis (formerly *Torulopsis utilis*) is a unicellular yeast used for SCP production since the 1950s. It can grow on a variety of waste substrates such as molasses, whey, or wood hydrolysates, converting them into microbial protein. It is cultivated in aerated batch or continuous fermenters at 30–35 °C and pH 4.5–5.5. The biomass yield is high, with 45–55% protein (dry weight) and a good balance of essential amino acids. *Candida utilis* also produces vitamins (especially B-complex) and has low nucleic acid content, making it suitable for both feed and food. During World War II, it was mass-produced as "Torula yeast" for human consumption. Today, it is used in animal feed, food supplements, and flavor enhancers. Its ability to grow on low-cost industrial effluents supports circular bioeconomy models.This yeast remains a reference microorganism for SCP from agro-industrial waste.

IV.3.1.4. Microalgae and cyanobacteria

Photosynthetic microorganisms like *Chlorella vulgaris*, *Spirulina platensis*, and *Scenedesmus obliquus* are sustainable SCP producers because they fix atmospheric CO_2 and utilize sunlight as an energy source. They can also be cultivated in wastewater, contributing to nutrient recycling. Their biomass contains 50–70% protein, essential amino acids, and bioactive compounds such as carotenoids and polyunsaturated fatty acids [97].

Chlorella vulgaris is a unicellular green microalga widely used for SCP due to its high protein content (50–60% dry weight) and balanced amino acid composition. It grows photoautotrophically using CO_2, light, and mineral nutrients, or heterotrophically on organic carbon (glucose, acetate). Cultivation occurs in open ponds or photobioreactors at 25–30 °C and pH 6.5–7.5. Nutrients such as nitrates, phosphates, and trace metals are added to enhance growth. The protein is rich in essential amino acids, vitamins (B_{12}, E, β-carotene), and minerals. Biomass is harvested by centrifugation or filtration and then dried into a protein-rich powder. *Chlorella* SCP is used in human health supplements, animal feed, and aquaculture. It improves immune function, growth rate, and gut health in livestock and fish. Its ability to capture CO_2 makes it environmentally sustainable. Recent studies explore wastewater-based cultivation to reduce cost and recover nutrients[99].

Spirulina platensis (also known as *Arthrospira platensis*) is a filamentous cyanobacterium known as a "superfood" and one of the oldest life forms used for SCP. Cultivation in photobioreactors (PBRs) allows precise control of environmental conditions such as light, pH, and temperature, ensuring high productivity and minimal contamination. A typical process uses a tubular photobioreactor (Figure IV.7) made of transparent glass or plastic tubes through which culture medium circulates continuously. The system is aerated with air containing 1–2% CO_2, providing carbon for photosynthesis and maintaining mixing. The medium, rich in bicarbonates, nitrates, and minerals, is used to support growth. Optimal conditions include pH 9–10, temperature 30–35°C, and light intensity 150–200 µmol photons m^{-2} s^{-1}. Under these parameters, *Spirulina* grows rapidly, achieving biomass productivity up to 1.5 g/L/day and protein yield around 65% of dry weight with all essential amino acids except methionine. The biomass also contains vitamins (B_{12}, provitamin A), iron, and γ-linolenic acid, making it valuable for human and animal nutrition. After harvesting and drying, the product is sold as powder or tablets. It has antioxidant, antiviral, and immune-modulating properties. Industrial production is established in countries like the USA, India, and China. Spirulina SCP is approved by FAO and WHO as a safe, high-quality protein source. Its carbon-neutral growth makes it ideal for future food security under climate change.

Figure IV.7. Tubular photobioreactor (Online source 9).

In conclusion, microorganisms represent a promising and versatile source of single-cell proteins. Their rapid growth, ability to use diverse substrates, and high nutritional quality make them ideal for sustainable protein production. By integrating SCP processes into waste management and bioeconomy systems, it is possible to produce environmentally friendly and nutritionally valuable proteins to meet the growing global demand for food and feed.

IV.3.2. Advantages in Single-Cell Protein (SCP) production

SCP production has gained increasing attention as a sustainable alternative to conventional protein sources like meat and soy, due to its rapid growth rate, high protein yield, and ability to utilize low-

cost or waste-derived substrates. It represents a promising solution to global challenges related to food security, resource scarcity, and environmental sustainability[97].

Rapid growth and high productivity: Microorganisms such as bacteria, yeasts, fungi, and microalgae grow extremely fast, often doubling their biomass within a few hours. This rapid growth allows for continuous and large-scale protein production in a short period, making SCP more efficient than conventional plant or animal protein sources.

High nutritional value: Single-cell proteins are rich in essential amino acids, vitamins, and minerals. Their protein content ranges from 30% to 80% of dry weight, and they often contain all essential amino acids required for human and animal nutrition. Some microorganisms also produce valuable compounds such as lipids, pigments, and antioxidants, which enhance the nutritional quality of the biomass.

Use of low-cost and renewable substrates: A major advantage of SCP production is the ability of microorganisms to grow on inexpensive or waste-derived materials. Agricultural residues, food processing by-products, and industrial effluents can all serve as substrates. This not only reduces production costs but also helps in managing waste streams effectively.

Environmental sustainability: SCP production supports environmental protection and the circular bioeconomy. By converting organic waste and greenhouse gases like methane or CO_2 into protein, the process helps reduce pollution, minimize carbon emissions, and promote sustainable resource use.

Independence from climate and season: Unlike traditional agriculture and livestock farming, microbial protein production can be carried out in controlled bioreactors under any climatic condition. This ensures a stable, year-round supply of protein regardless of weather, soil quality, or regional limitations.

Efficient resource utilization: SCP production requires significantly less land and water compared to crop or livestock protein sources. This makes it an ideal solution for addressing food security challenges in regions with limited agricultural resources or arable land [100].

Despite these advantages, several challenges still exist. High production costs, the need for nucleic acid reduction, downstream processing difficulties, and consumer acceptance remain key barriers to large-scale SCP adoption. Continued progress in strain engineering, metabolic optimization, and low-cost feedstock utilization is helping to overcome these obstacles.

IV.3.3. Single-Cell Protein production processes

Several Single-Cell Protein (SCP) production processes have been developed and are now operational in many countries, including the United States, Eastern Europe, and Australia. The concept of using microorganisms as a protein source dates back to World War I, when food shortages encouraged scientists to explore microbial biomass as an alternative to conventional protein. Since then, advances in biotechnology and fermentation techniques have led to the establishment of several large-scale production systems based on various substrates and microbial strains[100].

IV.3.3.1. PRUTEEN

One of the most well-known examples of bacterial SCP production is the PRUTEEN process, developed by Imperial Chemical Industries (ICI) in the United Kingdom. This industrial process uses the bacterium *Methylophilus methylotrophus*, a methylotrophic species capable of utilizing methanol as its primary carbon and energy source. In the PRUTEEN process, *Methylophilus methylotrophus* is cultivated in large-scale aerobic fermenters, where methanol is continuously supplied along with essential nutrients such as nitrogen, phosphorus, and minerals. The bacteria grow rapidly, forming biomass with a protein content of around 70–80% (dry weight). After fermentation, the bacterial cells are harvested, dried, and processed into a protein-rich meal. The resulting SCP product, marketed as "Pruteen," was primarily developed for animal feed (particularly for pigs and poultry) [100].

This process demonstrated that methanol, a low-cost and abundant substrate, can be efficiently converted into high-quality microbial protein, making it one of the earliest large-scale industrial applications of SCP technology. The success of the PRUTEEN process inspired further research into bacterial protein production using other carbon sources such as methane, natural gas, and agricultural waste hydrolysates. Modern advances have built on this foundation, using genetically engineered bacteria to enhance protein yield and improve amino acid composition [100].

IV.3.3.2. WHEAST process

The WHEAST process utilizes whey, a by-product of the dairy industry, as a primary substrate for microbial growth. The yeast *Kluyveromyces fragilis* is used as the inoculum because of its ability to metabolize lactose, the main sugar in whey. During fermentation, the yeast converts lactose and other nutrients into high-protein biomass, which is then harvested, dried, and processed into SCP. This process not only provides a valuable protein source but also contributes to waste management by reducing the environmental impact of whey disposal[100].

IV.3.3.3. SYMBA process

The SYMBA process involves the cultivation of *Candida utilis* yeast strains on starch hydrolysates derived from agricultural raw materials such as grains or potatoes. This mixed-culture process allows

for efficient utilization of carbon sources and results in high yields of protein-rich biomass. The final product, containing around 45–55% protein, is used in animal feed formulations and as a potential food supplement. The SYMBA process demonstrates how fermentation technology can transform agricultural residues into a sustainable protein source.

These pioneering SCP production processes marked the foundation for modern biotechnological protein synthesis, paving the way for today's advanced methods that employ diverse microorganisms and waste valorization strategies for sustainable food and feed production[100].

IV.4. Methanogenic bacteria

Methanogenic bacteria are strictly anaerobic microorganisms that play a crucial role in biogas production through a process known as methanogenesis. These microorganisms belong mainly to the domain *Archaea* and are responsible for converting the end-products of organic matter degradation into methane (CH_4) and carbon dioxide (CO_2), the main components of biogas. During anaerobic digestion, complex organic materials such as carbohydrates, lipids, and proteins are broken down in four main stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In the first three steps, bacteria degrade polymers into simpler molecules like volatile fatty acids, alcohols, and hydrogen. The methanogenic bacteria act in the final stage, using these intermediates to generate methane [101].

The methane produced serves as a renewable bioenergy source, used for electricity, heat, or as upgraded biomethane fuel. Moreover, the process reduces organic waste, stabilizes sludge, and minimizes greenhouse gas emissions. Methanogenic bacteria are the biological heart of biogas systems. Their activity transforms organic waste into a valuable energy resource, linking microbiology with sustainable energy and waste management[101].

IV.4.1. The importance of methanisation

Methanisation, or anaerobic digestion, is of great environmental, energetic, and economic interest. It represents an innovative and sustainable technology that simultaneously addresses several global challenges related to waste management, energy production, and climate change mitigation[101-102].

From an environmental perspective, methanisation provides an effective solution for the treatment and recovery of organic waste. Instead of being landfilled or incinerated, biodegradable materials such as agricultural residues, food waste, and animal manure are biologically converted into biogas and digestate. This process significantly reduces greenhouse gas emissions, odors, and pathogens, while also limiting water and soil pollution.

From an energy perspective, the biogas produced during methanisation contains a high proportion of methane (CH_4), a renewable energy source that can replace fossil fuels. Biogas can be used directly

for heat and electricity generation, or purified into biomethane for injection into the natural gas grid or use as vehicle fuel. This contributes to energy diversification and the transition toward carbon-neutral energy systems.

Economically, methanisation promotes a circular economy by transforming waste into valuable resources. The digestate, a nutrient-rich residue from the process, can be used as a biofertilizer, reducing the need for chemical fertilizers and closing the nutrient loop in agricultural systems. Methanisation plants also create local employment opportunities and support rural development by providing farmers and communities with decentralized energy solutions.

In summary, methanisation offers a triple benefit:

- Environmental protection through waste reduction and emission control,
- Renewable energy production through biogas valorization,
- Economic and agricultural sustainability through nutrient recycling and job creation.

Thus, methanisation is a key component of the green energy transition and an essential tool for achieving sustainable development goals (SDGs) in the context of a bio-based and circular economy[101-102].

IV.4.2. The mechanism of biogas production in methanisation

Biogas production is a biological process that converts organic matter into a combustible gas mixture, mainly composed of methane (CH_4) and carbon dioxide (CO_2), through the action of microorganisms in the absence of oxygen. This process, known as anaerobic digestion (AD), has gained increasing importance as a sustainable method for managing organic waste and generating renewable energy. The substrates used for biogas production include agricultural residues, livestock manure, sewage sludge, food waste, and industrial effluents. By transforming these wastes into energy, biogas systems contribute significantly to circular economy and carbon-neutral strategies[102].

The methanisation process, also known as anaerobic digestion occurs in four main biological stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Each stage of methanisation is performed by distinct microbial communities, each possessing specialized metabolic functions. The biochemical pathways involved in the degradation of complex organic matter throughout the anaerobic digestion process are illustrated in Figure IV.8.

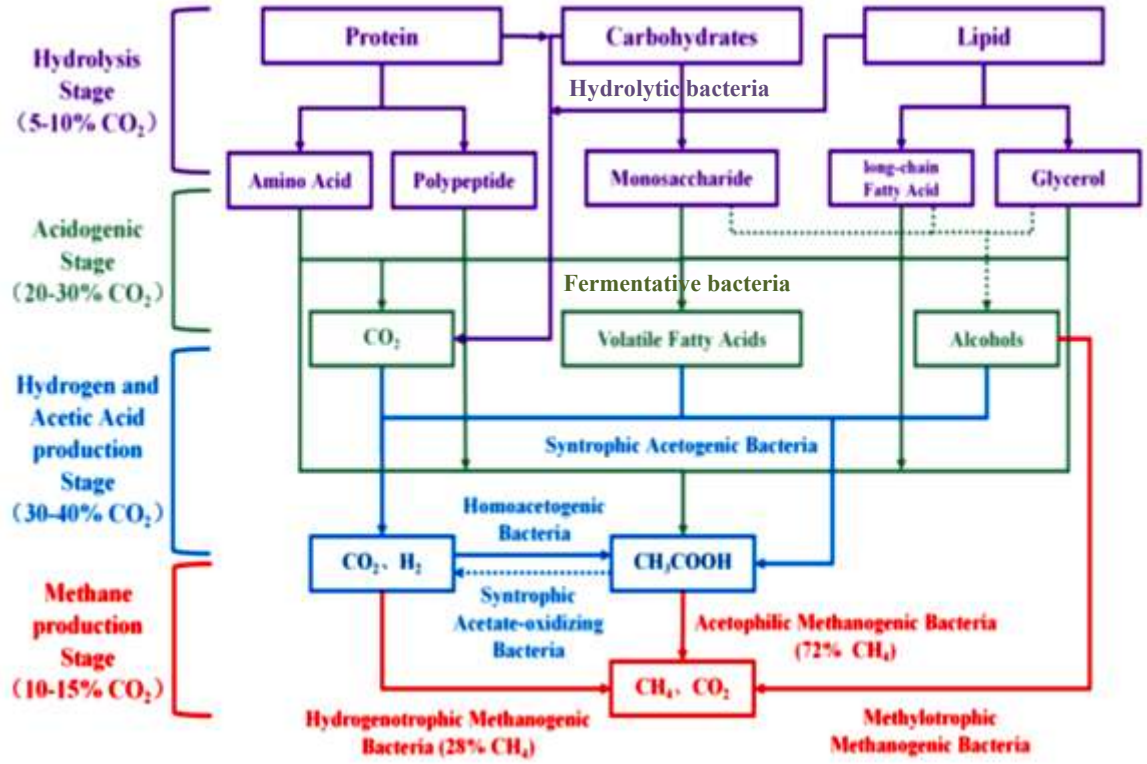

Figure IV.8. Metabolic pathways and microbial interactions during the methanisation process [102].

IV.4.2.1. Hydrolysis

During the hydrolysis stage, complex organic macromolecules such as carbohydrates, lipids, and proteins are enzymatically decomposed into simpler soluble compounds, primarily monosaccharides, long-chain fatty acids, and amino acids. This step is catalyzed by hydrolytic and fermentative bacteria, which secrete extracellular enzymes including cellulases, proteases, and lipases. The resulting small molecules can readily diffuse through cell membranes and serve as substrates for microorganisms in the subsequent stages of anaerobic digestion [102].

The hydrolysis rate depends largely on the composition of the feedstock and the metabolic activity of the microbial community within the system. Carbon dioxide (CO_2) produced during this stage represents approximately 5–10% of the total CO_2 generated during the process, originating mainly from partial oxidation of organic matter and microbial respiration. Throughout hydrolysis, the chemical bonds within carbohydrates and lipids are cleaved, and some components undergo oxidation reactions, resulting in the formation of CO_2 and other low-molecular-weight intermediates [102].

The main bacterial genera involved in this step include *Clostridium spp*., *Bacteroides spp*., *Bacillus spp*., *Ruminococcus spp*., and *Cellulomonas spp*. These microorganisms play a crucial role in

initiating the degradation of complex substrates and rendering organic matter accessible to other microbial groups involved in later stages of methanisation.

IV.4.2.2. Acidogenesis

During the acidogenesis stage, the soluble monomers and oligomers produced from hydrolysis — such as sugars, amino acids, and long-chain fatty acids — are metabolized by acidogenic (fermentative) bacteria into a variety of intermediate products. These include volatile fatty acids (VFAs) such as acetic, propionic, butyric, and valeric acids, along with alcohols, lactic acid, carbon dioxide (CO_2), hydrogen (H_2), and ammonia (NH_3). This step represents a critical link between the breakdown of complex organic matter and the formation of substrates suitable for acetogenic and methanogenic microorganisms. The metabolic reactions in this stage are primarily fermentative and lead to a decrease in pH due to the accumulation of organic acids. Maintaining pH stability is therefore essential to prevent inhibition of downstream microbial activity. The composition of the microbial community and the nature of the substrate strongly influence the types and proportions of fermentation products formed [102].

Typical acidogenic bacteria involved in this phase include *Clostridium spp., Lactobacillus spp., Enterobacter spp., Escherichia col*i, and *Streptococcus spp.* These microorganisms play a vital role in transforming hydrolysis products into volatile intermediates, which will later be oxidized by acetogenic bacteria or directly utilized by methanogens in the subsequent stages of methanisation.

IV.4.2.3. Acetogenesis

In the acetogenesis stage, the intermediate products formed during acidogenesis, primarily volatile fatty acids (VFAs) such as propionic acid, butyric acid, and valeric acid (in the previous phase), are transformed into acetate, hydrogen (H_2), and carbon dioxide (CO_2). These three compounds serve as the main substrates for methanogenic archaea during the subsequent methanogenesis stage of the methanisation process. Acetogenesis is mainly catalyzed by two functional groups of bacteria: the homo-acetogenic bacteria, and the obligate hydrogen-producing acetogens (syntrophic acetogenic bacteria) [102].

a. Homo-acetogenic (Non-syntrophic) bacteria

Homo-acetogenic bacteria are microorganisms that produce acetate as their sole end product. Depending on the source of the acetate produced, they can be divided into two groups:

Group 1: These bacteria generate acetate through the fermentation of carbonaceous substrates. They are able to ferment simple sugars such as glucose or the products originating from the acidogenesis stage into acetate. Examples include *Butyribacterium spp.* and *Peptococcus glycinophilus*.

Group 2: These bacteria utilize hydrogen (H_2) and carbon dioxide (CO_2) to produce acetate according to the following reaction:

$$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COOH + 4H_2O$$

Representative species include *Acetoanaerobium noterae*, *Eubacterium limosum*, *Sporomusa spp.*, and *Thermoanaerobacter kivui*.

Through these pathways, homo-acetogenic bacteria play a major role in carbon dioxide fixation and acetate formation, serving as an alternative hydrogen sink under conditions where methanogenesis is partially inhibited.

b. Syntrophic acetogenic bacteria

Syntrophic acetogenic bacteria, also known as obligate hydrogen-producing acetogens (OHPA), are microorganisms that produce molecular hydrogen (H_2) as a by-product during the formation of acetate. Typical genera include *Syntrophobacter*, *Syntrophomonas*, *Syntrophus*, and *Syntrophococcus*.

The activity of these bacteria is highly sensitive to hydrogen accumulation, as an excess of H_2 inhibits their metabolism. Therefore, the hydrogen produced during this stage must be immediately consumed to maintain a low hydrogen partial pressure. This requirement creates a syntrophic relationship between acetogenic bacteria and hydrogen-consuming microorganisms, such as sulfate-reducing bacteria, homo-acetogens, and particularly methanogenic archaea. Such cooperative interactions are essential to ensure efficient metabolic balance and stable operation of the anaerobic digester, allowing the continuous conversion of organic intermediates into substrates for methane production [102].

IV.4.2.4. Methanogenesis

The methanogenesis stage represents the final and most critical phase of the methanisation (anaerobic digestion) process, during which the intermediate products from acetogenesis, smainly acetic acid, hydrogen (H_2), and carbon dioxide (CO_2), are converted into methane (CH_4) and CO_2. This step is carried out exclusively by methanogenic archaea, a specialized group of microorganisms distinct from true bacteria, belonging primarily to the *Euryarchaeota* phylum[102].

Methanogenesis proceeds through three principal biochemical pathways[102]:

1. Acetoclastic Methanogenesis, in this pathway, acetate is cleaved into methane (CH_4) and carbon dioxide (CO_2) according to the reaction:

$$CH_3COOH \rightarrow CH_4 + CO_2$$

This route accounts for 60–70% of total methane production in most anaerobic digesters. It is mainly carried out by the genera *Methanosaeta* (formerly *Methanothrix*) and *Methanosarcina*.

Methanosaeta concilii is specialized in environments with low acetate concentrations, showing high substrate affinity but slow growth rates.

Methanosarcina barkeri, by contrast, thrives in high-acetate environments, displaying rapid growth and metabolic flexibility.

2. Hydrogenotrophic methanogenesis, in this pathway, CO_2 serves as the terminal electron acceptor and is reduced by H_2 to form methane:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

This reaction is catalyzed by hydrogenotrophic methanogens such as *Methanobacterium formicicum*, *Methanospirillum hungatei*, and *Methanobrevibacter ruminantium*. These species are dominant under conditions with high hydrogen availability or low acetate concentration.

3. Methylotrophic methanogenesis, some methanogens can utilize methylated compounds (methanol, methylamines, methyl sulfides) as substrates for methane formation:

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$$

Representative methylotrophic species include *Methanomethylovorans hollandica*, *Methanosarcina barkeri,* and *Methanolobus spp*. Although less dominant, this pathway contributes significantly in environments rich in methylated substrates, such as marine sediments and digesters treating protein-rich wastes.

Methanogenesis is highly sensitive to environmental factors such as pH (optimal range: 6.8–7.5), temperature (mesophilic: 35–40 °C; thermophilic: 50–60 °C), and redox potential (below −300 mV). Disturbances in these parameters can lead to process instability and reduced methane yield. The methanogenic archaea form syntrophic associations with acetogenic bacteria, maintaining the balance of hydrogen and ensuring efficient carbon flow. As a result, methanogenesis determines the final composition and energy content of the produced biogas, which typically contains 50–70% CH_4 and 30–50% CO_2, with traces of H_2S and water vapor[102].

IV.4.3. Methanisation technology

The technology of methanisation, or anaerobic digestion technology, refers to the set of engineered systems and processes designed to convert organic matter into biogas and digestate under controlled anaerobic conditions. The process relies on the natural activity of microbial consortia operating in

oxygen-free environments, but modern technology allows this biological conversion to be optimized, stabilized, and scaled for industrial or agricultural use [103].

IV.4.3.1. Principle and types of methanisation systems

Methanisation technologies aim to reproduce and optimize the natural biodegradation of organic matter in controlled reactors called anaerobic digesters. Inside the digester, microorganisms successively carry out the biochemical reactions of hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The result is the production of biogas, rich in methane (CH_4), and a stabilized digestate that can be used as an organic fertilizer. Methanisation can be implemented using several technological configurations, depending on the nature of the substrate, the operating temperature, and the desired scale of production [101, 103]:

Wet digestion systems: Operate with substrates containing less than 15% dry matter, such as animal manure, sewage sludge, or food waste. These systems are the most common and often use continuous stirred-tank reactors (CSTRs).

Dry digestion systems: Designed for solid organic waste with high dry matter content (20–40%), such as municipal waste or crop residues. These systems typically use plug-flow reactors or batch reactors and require less water.

Mesophilic systems (35–40 °C) *vs.* ***Thermophilic systems (50–60 °C):*** Mesophilic digestion is more stable and less energy-intensive, while thermophilic digestion provides faster reaction rates and better pathogen reduction but requires tighter process control.

IV.4.3.2. Main components of a methanisation plant

A methanisation plant is composed of several interdependent units designed to ensure the efficient conversion of organic substrates into biogas and digestate under optimal anaerobic conditions. Each component plays a specific role in preparing, processing, and valorizing the feedstock and its by-products. A modern methanisation installation typically includes[103]:

Feedstock preparation and pre-treatment unit: this section is responsible for the collection, sorting, and homogenization of organic waste materials. Depending on the substrate type, it may include crushing, dilution, screening, or pasteurization stages to optimize particle size and ensure uniform feeding to the digester. Effective pre-treatment enhances biodegradability and ensures stable process performance.

Anaerobic digester (reactor): the digester is the core unit of the system, where microbial consortia perform the successive biochemical stages of hydrolysis, acidogenesis, acetogenesis, and methanogenesis. It operates under strictly anaerobic, temperature-controlled, and pH-regulated

conditions. Digesters may be continuous stirred-tank reactors (CSTRs), plug-flow, or batch systems, depending on substrate characteristics and operational scale.

Biogas collection and treatment system: the biogas generated in the digester is continuously collected through a gas-tight piping network. It is then subjected to purification and upgrading processes, such as desulfurization (H_2S removal), dehydration, and CO_2 separation, to enhance methane concentration. The treated gas can be stored, used for heat and power generation, or upgraded to biomethane for grid injection or vehicle fuel.

Digestate treatment and valorization unit: after digestion, the remaining solid-liquid mixture, known as digestate, is processed to facilitate its reuse. Common operations include solid-liquid separation, dewatering, and composting. The final product is a stabilized biofertilizer rich in nutrients (N, P, K), suitable for agricultural applications, thereby closing the nutrient cycle.

Instrumentation and control system: modern methanisation plants are equipped with automated monitoring and control systems to maintain optimal operating conditions. Sensors continuously measure parameters such as temperature, pH, biogas flow rate, methane content, and hydraulic retention time (HRT). These data are used to ensure process stability, energy efficiency, and operational safety.

IV.4.3.3. Biogas utilization technologies

The biogas produced through methanisation can be used in several ways[103]:

Cogeneration (CHP units): simultaneous production of heat and electricity.

Biomethane upgrading: purification of biogas to natural gas quality for grid injection.

Vehicle fuel: after upgrading and compression, biomethane can replace diesel or gasoline.

Industrial or domestic heat applications: direct combustion in boilers or furnaces.

Methanisation technology offers numerous benefits (Figure IV.9), including: renewable energy generation from waste streams, reduction of greenhouse gas emissions, stabilization of organic waste, and production of biofertilizers from digestate. For its utilization as an energy source, biogas undergoes a purification and upgrading process aimed at improving its quality and energy content. This treatment involves[103]:

-Removing solid particles and dust to prevent equipment damage,

-Dehydrating the gas to eliminate moisture and water vapor,

-Eliminating sulfur compounds, particularly hydrogen sulfide (H_2S), which are corrosive and toxic,

-Removing carbon dioxide (CO_2) to increase the methane concentration and calorific value of the final biogas.

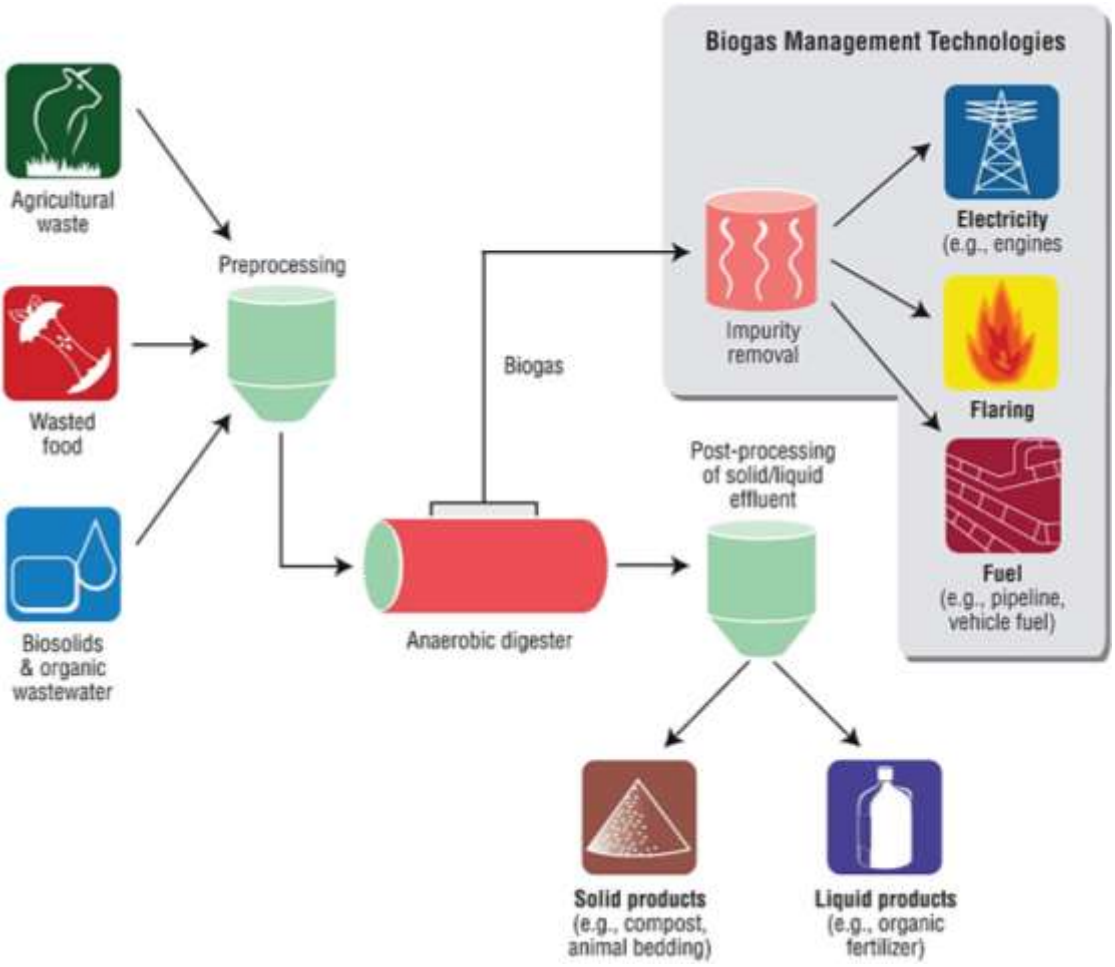

Figure IV.9. Biogas production and application (Online source 10).

Recent advances focus on improving process efficiency, energy recovery, and automation. Innovations include two-stage digestion, co-digestion of multiple substrates, high-rate reactors, and real-time process monitoring using sensor-based control systems[103].

IV.5. Alcohols and ethanol

Alcohols are organic compounds in which a carbon atom is bonded to a hydroxyl group (-OH). They have diverse industrial applications [104]:

Solvents: Ethanol is widely used in pharmaceuticals, perfumes, and personal care products.

Chemical reagents: Alcohols are precursors in esterification and other organic syntheses.

Antifreezes: Methanol and ethylene glycol prevent freezing in cooling systems due to their low melting points.

Alcohols can be primary, secondary, or tertiary, depending on the carbon atom attached to the hydroxyl group.

IV.5.1. Microorganisms producing alcohols

Alcohols are commonly primary metabolites produced by microorganisms during anaerobic energy metabolism. Their formation occurs simultaneously with microbial growth, as energy generation is required for cell maintenance and survival. Microbial alcohol production is of great industrial importance, particularly for the synthesis of ethanol and other valuable compounds[104].

IV.5.1. 1. Yeasts

Yeasts are the most widely used microorganisms for industrial alcohol production, especially ethanol. They efficiently convert sugars into alcohols under anaerobic conditions.

Saccharomyces cerevisiae: Industrially significant for ethanol fermentation, using glucose as the primary carbon source. Other yeasts, such as *Pichia stipitis* and *Candida* species, can ferment pentose sugars (xylose) into ethanol.

IV.5.1.2. Bacteria

Certain bacteria are also capable of producing alcohols, although their industrial use is more limited than yeasts. Bacterial fermentation can target a wider range of sugars, including pentoses.

Thermophilic bacteria: Species such as *Thermoanaerobacter ethanolicus*, *Clostridium thermohydrosulfuricum*, and genetically engineered *Bacillus stearothermophilus* convert xylose into ethanol at high temperatures.

Mesophilic bacteria: Examples include *Escherichia coli*, *Klebsiella oxytoca*, and *K. planticola*, which ferment pentose sugars under moderate temperatures.

In addition to ethanol, microbial fermentation can produce other valuable metabolites, including acetone (*Clostridium acetobutylicum*), dihydroxyacetone, polyols, and butanol[104].

IV.5.2. Industrial production of ethanol

Ethanol is a major biofuel, with approximately 80% of global production derived from fermentation of carbohydrates such as cellulose, glucose, and starch. In contrast, methane is primarily produced through the anaerobic decomposition of organic matter and other hydrocarbons. Within the context of alcoholic fermentation, ethanol serves as a primary metabolite and represents the end-product of energy metabolism, with carbon dioxide as a co-product. In this discussion, the focus will be exclusively on ethanol production for biofuel applications. Several industrial programs have been

established to produce ethanol biofuel (Figure IV.10) through the fermentation of sugars derived from agricultural biomass [104]:

Sugarcane molasses: The primary sugar is sucrose. Ethanol is produced via fermentation by *Saccharomyces cerevisiae*. This approach forms the basis of a large-scale industrial program in Brazil.

Hydrolyzed corn starch: The main sugars resulting from starch hydrolysis are glucose and maltose. Fermentation is also performed using *Saccharomyces cerevisiae*, and this process underpins a major industrial program in the United States.

Jerusalem artichoke molasses: Jerusalem artichoke (*Helianthus tuberosus*) is a tuberous plant belonging to the Asteraceae family. Cultivation is relatively simple, and the plant can thrive on marginal soils unsuitable for conventional crops. The tubers contain 8–10% carbohydrates, comprising approximately 50% inulin, along with glucose, fructose, and sucrose. Inulin is a polymer of β-fructose linked by β-1,2-glycosidic bonds. The yeast *Kluyveromyces marxianus* produces inulinase enzymes that hydrolyze inulin into fermentable fructose. Subsequently, fructose can be fermented into ethanol. Alternatively, industrial processes may involve a dedicated enzymatic hydrolysis of inulin, followed by fermentation using *Saccharomyces cerevisiae*. This approach is currently under industrial development in Switzerland, Canada, Japan, and France [105].

The ethanol production yield is optimized using a process that minimizes the use of carbohydrates as a carbon source for biomass growth. Ideally, most of the sugar substrate is directed toward ethanol fermentation for energy production, aligning with the objective of maximizing ethanol output. By recycling yeast cells from the bioreactor effluent, two key benefits are achieved [106]:

1. The yeast concentration in the bioreactor is maintained at a maximum.

2. Yeast growth (biomass accumulation) is minimized, which reduces glucose consumption for cell synthesis. Consequently, glucose is primarily used as an energy source for cellular maintenance, resulting in the production of ethanol as the primary metabolic by-product.

The overall chemical reaction for glucose fermentation to ethanol is:

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2$$

180 g of glucose → 2 × 46 g ethanol = 92 g

Maximum theoretical stoichiometric yield: 0.51 g ethanol per g of sugar. This yield is closely approached in industrial processes where biomass growth in the fermenter is kept very low.

Several critical factors must be considered in evaluating ethanol as a biofuel[106]:

Energy balance and cost: The net energy gain is not straightforward to determine. It depends on the petroleum equivalent produced versus petroleum consumed during agricultural production, transport, fermentation, and distillation. Additional concerns include irrigation water use, fertilizers, pesticides, and potential competition with food crops.

Lignocellulosic biomass as a feedstock: Plant biomass represents a promising alternative source of fermentable sugars, potentially with lower ecological impact than conventional agricultural feedstocks. These include: Glucose from cellulose hydrolysis and C5 sugars (xylose, arabinose) from hemicellulose.

Ethanol fermentation from lignocellulosic biomass faces two major technological challenges[106]:

Accessibility and hydrolysis of cellulose and hemicellulose: Efficient hydrolysis within the lignocellulosic matrix must achieve high conversion rates, rapid kinetics, and low cost. This remains an active area of research and development.

Microorganisms capable of fermenting C5 sugars: Successful conversion requires either genetically engineered yeasts or specialized bacteria such as *Zymomonas mobilis*. This aspect also remains under development.

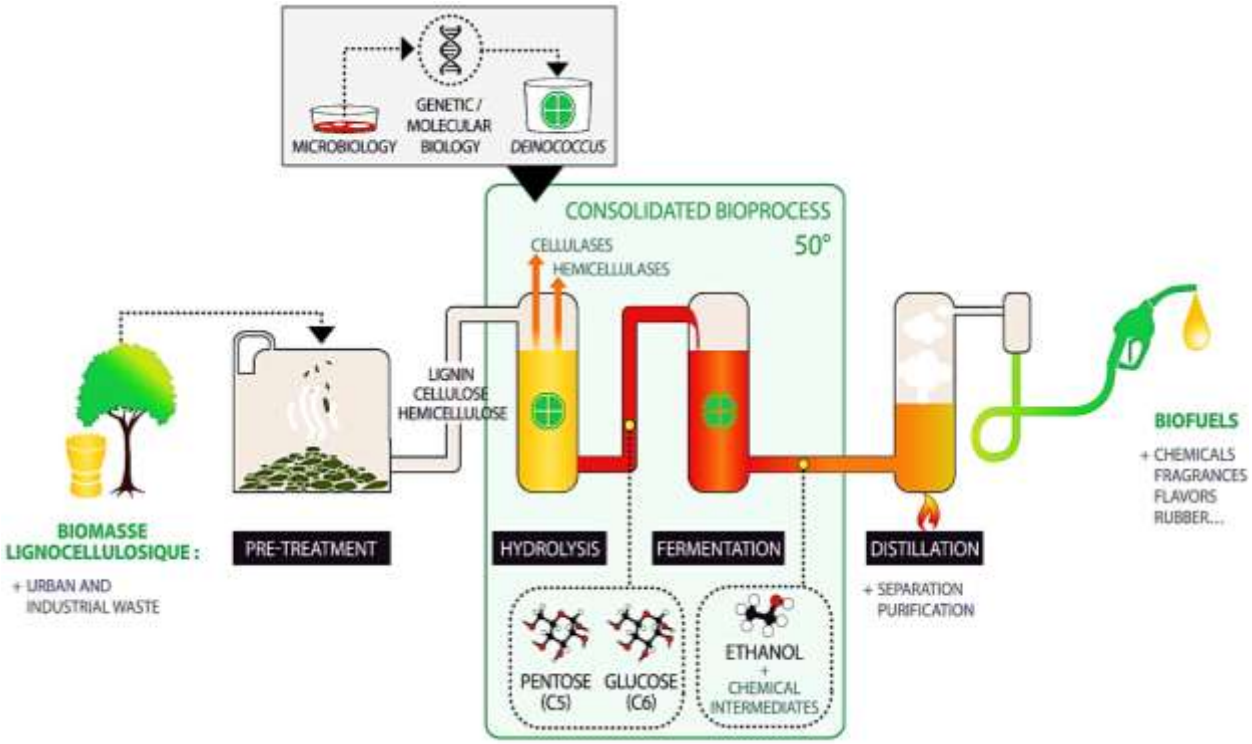

Figure IV.10. Bioethanol production process (Online source 11).

IV.6. Amino acids

Microbial production of amino acids constitutes a cornerstone of industrial biotechnology. Microorganisms are employed to convert simple carbon and nitrogen sources into amino acids at industrial scales, providing key compounds for human nutrition, animal feed, pharmaceuticals, and chemical synthesis. This biotechnological approach offers significant advantages over chemical synthesis, including high enantiomeric purity, reduced environmental impact, and cost-effectiveness. The global demand for amino acids is substantial. For example, L-glutamate and L-lysine are produced in volumes exceeding several million tons annually. Microbial fermentation has largely supplanted chemical synthesis for these and other amino acids due to improved efficiency and sustainability [106].

IV.6.1. Microorganisms producing amino acids

The choice of microbial host for amino acid production depends on the target amino acid and the specific requirements of the fermentation process. Key microorganisms employed in industrial production include [106]:

Corynebacterium glutamicum: A Gram-positive, non-pathogenic bacterium extensively used for the production of L-glutamate, L-lysine, L-threonine, and L-arginine. This organism is valued for its well-characterized metabolism, robustness under industrial conditions, and genetic tractability, which facilitate strain improvement and process optimization.

Escherichia coli: Commonly utilized for the production of amino acids and derivatives not naturally accumulated in high amounts by other strains. E. coli offers rapid growth, a wide array of genetic engineering tools, and the ability to metabolize diverse carbon sources, making it a versatile platform for metabolic engineering.

Yeasts and filamentous fungi: These organisms are employed for the production of specialty amino acids or when using alternative feedstocks. For example, *Saccharomyces cerevisiae* has been engineered for the industrial synthesis of L-tryptophan and L-phenylalanine.

IV.6.2. Metabolic pathways and production strategies

Amino acid biosynthesis in microorganisms is closely linked to central metabolic pathways, including glycolysis, the pentose phosphate pathway, and the tricarboxylic acid (TCA) cycle. Target amino acids are synthesized from key intermediates such as α-ketoglutarate, oxaloacetate, pyruvate, and phosphoenolpyruvate. Examples of pathways include [106]:

L-glutamate: Synthesized from α-ketoglutarate via glutamate dehydrogenase or transaminase-catalyzed reactions (Figure IV.11).

L-lysine: Produced via the diaminopimelate pathway from aspartate.

Branched-chain amino acids (valine, leucine, isoleucine): Derived from pyruvate through multi-step enzymatic reactions.

Industrial strategies aim to maximize flux toward the desired amino acid, including:

- Alleviating feedback inhibition of key enzymes to prevent metabolic bottlenecks.

- Enhancing precursor availability to increase substrate flux into the target pathway.

- Optimizing amino acid export systems to reduce intracellular accumulation and product inhibition.

- Deleting competing pathways to divert metabolic flux toward the desired amino acid.

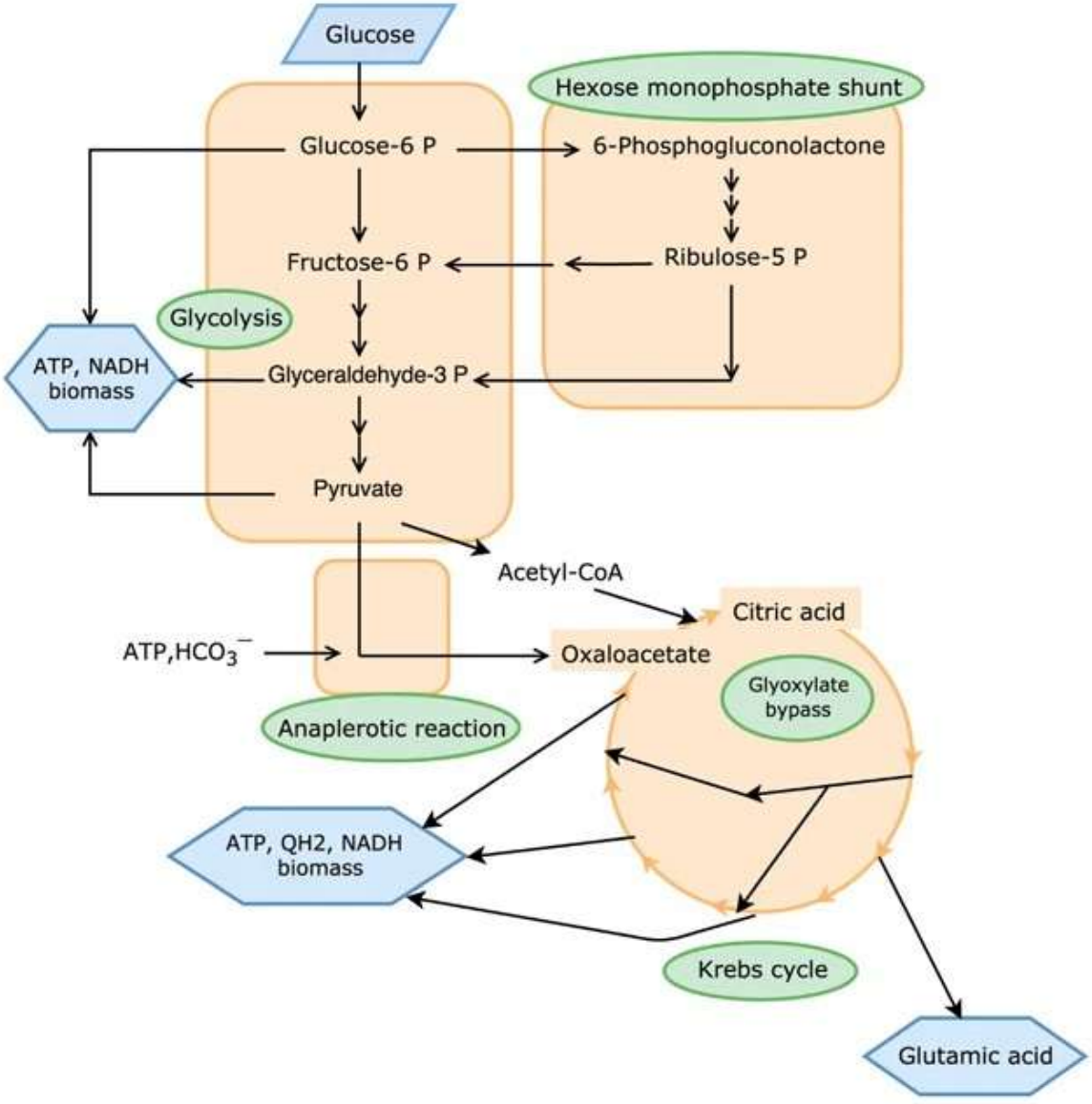

Figure IV.11. Biosynthesis of glutamic acid from glucose (Online source 12).

IV.6.3. Industrial process considerations for amino acid production

The efficiency and economic viability of microbial amino acid production rely on careful control of feedstocks, fermentation mode, environmental parameters, and productivity targets[106].

Feedstocks: Carbohydrate-rich substrates such as glucose, sucrose, and molasses serve as primary carbon sources, while nitrogen is typically supplied via ammonium salts, urea, or other suitable nitrogen-containing compounds. The choice of feedstock significantly affects yield, cost, and downstream processing requirements.

Fermentation mode: The fermentation strategy, batch, fed-batch, or continuous, is selected based on the specific amino acid and the metabolic characteristics of the microbial host. Fed-batch processes are often preferred industrially, as they allow controlled nutrient supply and reduce the accumulation of inhibitory by-products.

Environmental conditions: Parameters including oxygen availability, pH, temperature, and nutrient concentrations are tightly regulated to maximize amino acid production. Optimal control of these variables ensures both microbial growth and efficient conversion of substrates to the desired product.

Productivity targets: Industrial-scale production aims for high yield (percentage of carbon converted to product), high titer (concentration of amino acid in g/L), and high specific productivity (g/L/h). For example, L-glutamate fermentation can achieve yields of 70–80% of the theoretical maximum under optimized conditions. Consistent monitoring and process optimization are essential to approach these productivity benchmarks.

IV.6.4. Downstream processing (recovery and purification)

After fermentation, the amino acid product must be separated and purified from the microbial biomass and residual medium components. This stage is critical to achieving high purity and meeting regulatory standards. Downstream processing typically involves [106]:

Biomass separation: Removal of microbial cells by centrifugation, microfiltration, or sedimentation.

Concentration: The amino acid-rich supernatant is concentrated using techniques such as evaporation, ultrafiltration, or membrane filtration.

Purification: Further purification is achieved through crystallization, ion-exchange chromatography, or other chemical separation methods.

Formulation: Purified amino acids are converted into the desired final form, including powders, granules, or liquid solutions, suitable for feed, food, or pharmaceutical applications.

IV.6.5. Metabolic engineering and future directions

Advances in metabolic engineering, synthetic biology, and systems biology have enabled the development of strains with enhanced yield, productivity, and feedstock flexibility. Strategies include [106]:

* Overexpression of biosynthetic genes and deletion of competing pathways.

* Engineering of regulatory circuits to control enzyme levels dynamically.

* Adaptive laboratory evolution to enhance strain robustness.

* Utilization of alternative feedstocks, such as lignocellulosic hydrolysates, glycerol, or industrial by-products.

Future developments aim to address remaining challenges, including regulatory complexity, transport limitations, by-product formation, and strain stability, enabling sustainable production of both conventional and novel amino acids.

IV.7. Vitamins

Vitamins are low-molecular-weight organic compounds essential for normal physiological function in humans. Most vitamins cannot be synthesized endogenously in sufficient amounts, making dietary intake or supplementation necessary. Within cells, vitamins serve diverse roles, including acting as antioxidants, enzyme cofactors, and hormones (vitamin D). Vitamins are classified into thirteen types, grouped according to solubility [107]:

Fat-soluble vitamins: Vitamins A, D, E, and K.

Water-soluble vitamins: Including the eight B-complex vitamins, such as vitamin B1 (thiamine), whose deficiency causes beriberi.

Among water-soluble vitamins, vitamin B12 (cobalamin) is particularly important. It is essential for normal brain function, hematopoiesis, DNA synthesis and regulation, and the metabolism of amino acids and fatty acids. Clinically, vitamin B12 supplementation is used to treat pernicious anemia and other deficiency-related disorders.

Microorganisms, particularly prototrophic strains, are capable of synthesizing all required growth factors, including vitamins, and some secrete substantial amounts into the culture medium. This property has been exploited for industrial vitamin production. Examples include [107]:

Vitamin B2 (riboflavin): Produced by *Ashbya gossypii*, *Bacillus subtilis*, and *Candida famata*. These strains are optimized through classical mutagenesis or metabolic engineering to increase riboflavin yield.

Vitamin B12 (cobalamin): Industrially produced mainly by *Propionibacterium freudenreichii* and *Pseudomonas denitrificans*. These bacteria naturally synthesize cobalamin via the complex anaerobic pathway, and fermentation conditions are optimized to maximize extracellular accumulation.

β-Carotene (pro-vitamin A): Produced by fungi such as *Blakeslea trispora* and *Dunaliella salina*, which can convert carbon sources like glucose or glycerol into carotenoid pigments.

Other B vitamins, such as B1 (thiamine), B6 (pyridoxine), and B7 (biotin), are produced by various bacteria (*Escherichia coli*, *Bacillus subtilis*) and yeast strains (*Saccharomyces cerevisiae*), often through fermentation processes enhanced by genetic or metabolic engineering.

Microbial production of vitamins offers multiple advantages over chemical synthesis, including high specificity, sustainability, scalability, and lower production costs. This makes microbial biosynthesis the preferred method for producing several commercially significant vitamins [107].

V. Secondary metabolites

Microbial secondary metabolites are compounds not essential for growth, produced primarily during the idiophase (Figure IV.1), when cells shift from primary metabolism to secondary metabolism. They include antibiotics, toxins, pigments, and signaling molecules, often synthesized under specific environmental conditions such as nutrient limitation or stress. Their biosynthetic pathways are complex, involving multiple enzymatic steps and tightly regulated gene clusters. These metabolites are typically species- or even strain-specific, reflecting the genetic architecture of the producing organism.

Functionally, they confer ecological advantages, such as inhibiting competitors, deterring predators, or facilitating host interactions. Their structural diversity arises from modular enzyme systems like polyketide synthases and nonribosomal peptide synthetases. Beyond ecology, they have significant biotechnological and medical applications, including antibiotics, anticancer agents, and immunosuppressants. Production is often low under laboratory conditions, requiring metabolic engineering to enhance yields. Understanding their biosynthesis and regulation is key to exploiting their potential[108].

V.1. Antibiotics

Antibiotics are microbial secondary metabolites that inhibit the growth of or kill other microorganisms. They are not essential for the growth of the producing organism but provide a competitive advantage in natural environments. The biosynthetic pathways are often complex, involving multiple enzymatic steps and tightly regulated gene clusters that are highly specific to particular species or strains.

A bioactive molecule must meet several essential criteria to be considered an antibiotic: (i) it must exhibit selective activity against target microorganisms, (ii) it must be non-toxic to humans and animals, (iii) it should be cost-effective to produce, (iv) it should possess a broad spectrum of activity, (v) its mechanism of action must be well-defined, (vi) it should have high solubility, and (vii) it must remain stable under various physicochemical conditions. The number of microorganisms capable of producing antibiotics is very limited [109].

The majority of naturally occurring antibiotics are produced by microorganisms. Among these, actinobacteria remain one of the most extensively studied groups for the screening of new bioactive molecules, accounting for approximately 70% of all described antibiotics. Within this group, *Streptomyces* species are the most widespread and well-characterized in the environment. Fungi contribute roughly 20% of known antibiotics, bacteria about 10%, and algae around 0.8%.

The antibiotic families most commonly used in medicine include β-lactams, such as penicillins, obtained from the mold *Penicillium glaucum*, and cephalosporins, purified from the mold *Cephalosporium acremonium*. Aminoglycosides, such as streptomycin, are produced by *Streptomyces griseus*, while tetracyclines, broad-spectrum antibiotics, are synthesized by various *Streptomyces* species, including *Streptomyces aureofaciens* [109].

The production of these antibiotics is highly regulated and influenced by environmental factors, such as nutrient availability, pH, and oxygen levels. Advances in fermentation technology and metabolic engineering have enabled increased yields of these clinically important compounds. Beyond their therapeutic use, these antibiotics illustrate the structural diversity and biosynthetic sophistication of microbial secondary metabolites, reflecting the evolutionary adaptation of microorganisms to ecological competition[109].

V.1.1. Penicillins

Penicillin is a β-lactam antibiotic produced primarily by the fungus *Penicillium chrysogenum* (formerly *P. notatum*). It is one of the earliest antibiotics to be industrially produced by microbial fermentation. The antibiotic penicillin was discovered by Alexander Fleming in 1928, but large-scale industrial production required major advances in fermentation and strain development. The production organism used industrially is chiefly *Penicillium chrysogenum* (recently reclassified as *P. rubens*).

The chemical structure of penicillin (Figure V.1) is defined by a four-membered β-lactam ring fused to a five-membered heterocyclic thiazolidine ring. The thiazolidine ring is substituted with two methyl groups and a carboxyl functional group. The β-lactam ring contains an amide-linked amino group, which serves as the attachment point for variable side chains, conferring different penicillin derivatives with distinct pharmacological properties [110].

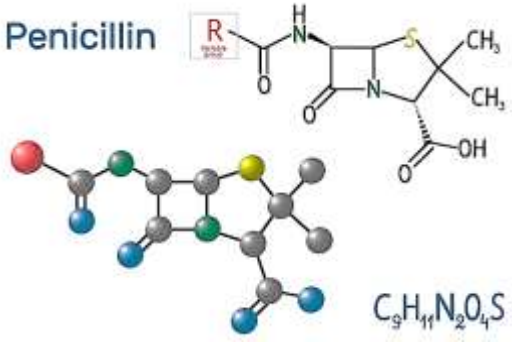

Figure V.1. Chemical structure of penicillin (Online source 13).

V.1.1.1. Biosynthesis of penicillin G

Penicillin G is synthesized through a sequence of enzymatically catalyzed reactions in *Penicillium chrysogenum* (Figure V.2):

1- The biosynthetic pathway begins with the condensation of three amino acids, L-α-aminoadipic acid, L-cysteine, and L-valine, by the non-ribosomal peptide synthetase δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) to form a linear tripeptide.

2- This tripeptide undergoes oxidative cyclization catalyzed by isopenicillin N synthase (IPNS), producing the bicyclic compound isopenicillin N, which contains both the β-lactam and thiazolidine rings.

3- Subsequently, the hydrophilic L-α-aminoadipyl side chain of isopenicillin N is replaced with a hydrophobic phenylacetyl group via the action of acyltransferase (AAT), yielding penicillin G, the bioactive antibiotic with its characteristic β-lactam structure.

This pathway illustrates the sequential enzymatic transformations that convert primary metabolites into a clinically important secondary metabolite[110].

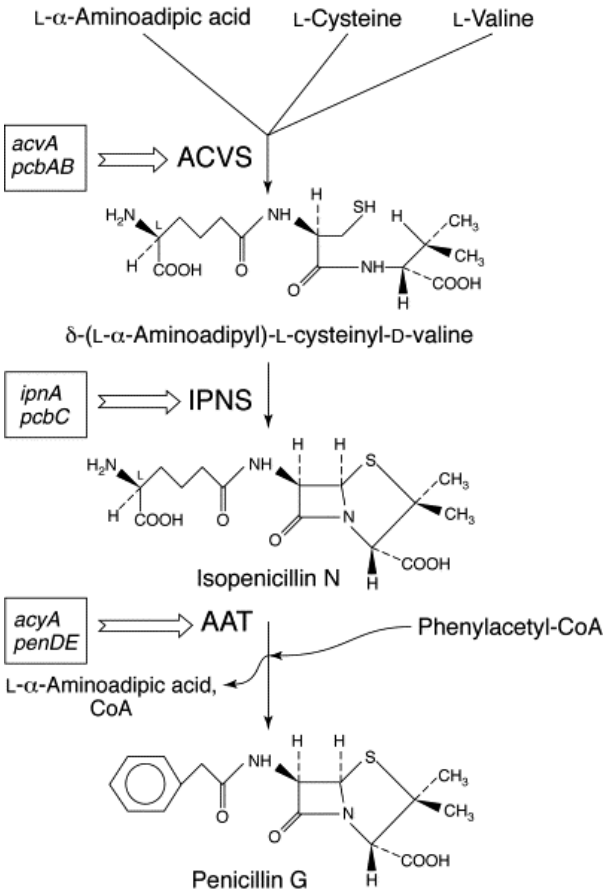

Figure V.2. Biosynthetic pathway of penicillin G (Online source 14).

Penicillins act by inhibiting bacterial cell wall biosynthesis through the inhibition of transpeptidase, a key enzyme involved in peptidoglycan synthesis, thereby leading to bacterial cell death. Most penicillins today are semi-synthetic, resulting from chemical modification of the naturally occurring penicillin produced by *Penicillium chrysogenum*. This modification is achieved by substituting the natural acyl group attached to 6-aminopenicillanic acid (6-APA) with other functional groups (Figure V.3), which imparts the molecule with new pharmacological properties.

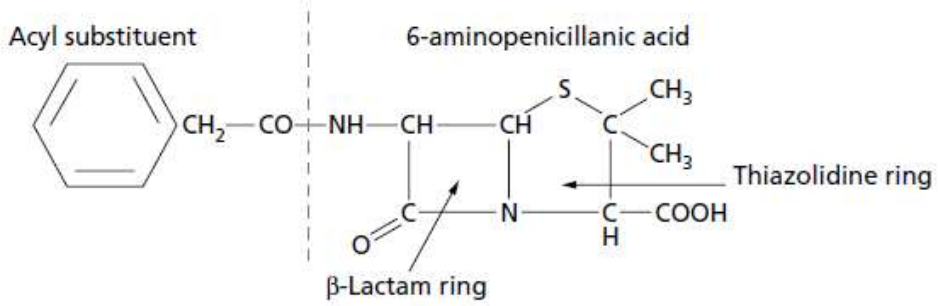

Figure V.3. Substitution of the acyl group of 6-aminopenicillanic acid (6-APA) by a new radical (Online source 14).

Semi-synthetic penicillins, such as methicillin, carbenicillin, and ampicillin, have various improvements over the original molecule, including resistance to gastric acidity allowing for oral administration, resistance to penicillinase, and activity against certain Gram-negative bacteria.

V.1.1.2. Fermentation of penicillin G

Industrial production employs submerged aerobic fermentation in large-scale bioreactors. The process begins with inoculum preparation from a pure spore culture, scaled up through seed flasks and seed fermenters to provide metabolically active biomass. The production medium contains carbon sources (glucose, lactose), nitrogen sources (corn steep liquor, ammonium salts), essential minerals, and phenylacetic acid to direct penicillin biosynthesis. Nutrient concentrations are carefully controlled to avoid repression of secondary metabolism[111].

Fermentation is maintained under controlled temperature (25–27 °C), pH (6.0–7.0), and high aeration and agitation to ensure sufficient oxygen transfer (DO >30%). The process is usually conducted in fed-batch mode (Figure V.4), allowing gradual nutrient addition to prolong the idiophase, the stage of maximal secondary metabolite production. The trophophase precedes the idiophase and is characterized by active fungal growth and biomass accumulation. The transition between these phases is tightly regulated through monitoring of nutrient levels, dissolved oxygen, and pH.

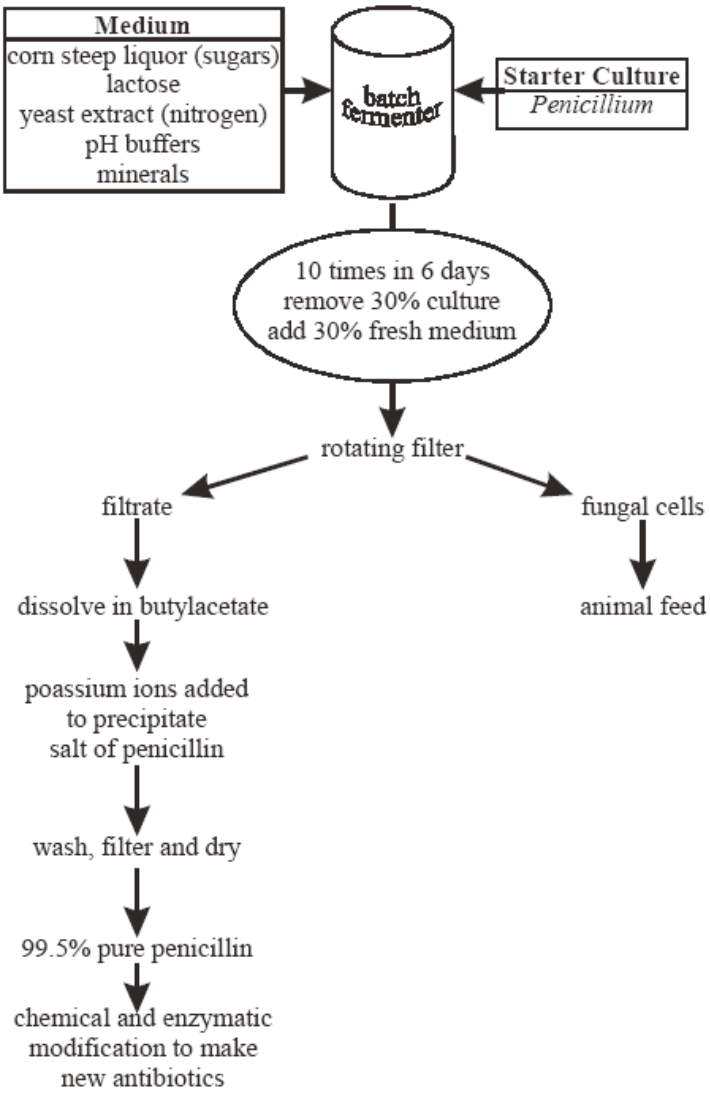

Figure V.4. Industrial production of Penicillin (Online source 15).

Modern production integrates strain improvement, medium optimization, fed-batch fermentation, and advanced bioreactor control, including automated monitoring of pH, dissolved oxygen, foam, and nutrient feeding. Scale-up strategies maintain oxygen transfer, mixing, and shear conditions to replicate laboratory results at industrial scale. These measures allow penicillin G to be produced at high yield, consistent quality, and in concentrations suitable for pharmaceutical use. This combination of biosynthetic pathway understanding, upstream fermentation technology, and downstream processing ensures efficient production of penicillin G as a clinically valuable antibiotic[111].

V.1.1.3. Recovery of penicillin G

The first step in the downstream process is the separation of the biomass from the fermentation broth (Figure V.4). This is typically accomplished by filtration or centrifugation, which removes the *Penicillium chrysogenum* mycelium, leaving behind a clear supernatant rich in penicillin. The separated fungal biomass is often washed multiple times to eliminate residual traces of the antibiotic and can be subsequently used as animal feed due to its high protein content[111].

The clarified broth containing penicillin G is then subjected to acidification (usually to pH 2–2.5) using a mineral acid such as sulfuric or phosphoric acid. This converts the penicillin into its acidic form (penicillin acid), which is more soluble in organic solvents than in water. The acidified broth is then extracted using an organic solvent such as butyl acetate, amyl acetate, or methyl isobutyl ketone, which selectively dissolves the penicillin. This liquid–liquid extraction step effectively transfers the antibiotic from the aqueous phase into the organic phase.

Once extracted, penicillin is back-extracted (or re-extracted) from the organic solvent into an alkaline aqueous solution, typically containing sodium or potassium hydroxide. This step converts penicillin acid into its salt form (sodium or potassium penicillin G), which is stable in aqueous solution. The resulting solution is then concentrated under reduced pressure to remove residual solvent and water, crystallized under controlled conditions, dried, and formulated into pharmaceutical preparations[111].

Modern recovery processes have been optimized to minimize product loss and degradation, using continuous extraction systems, solvent recycling, and automated pH and temperature control. These improvements enhance both yield and product consistency, ensuring that penicillin G meets pharmacopoeial standards for clinical use.

V.1.2. Streptomycin

Streptomycin, the first aminoglycoside antibiotic discovered, is produced by *Streptomyces griseus* through a complex secondary metabolic process. Industrial production employs submerged aerobic fermentation in large bioreactors, where a pure spore culture is scaled up through several seed stages to generate an actively growing inoculum. The fermentation medium provides balanced nutrients to promote both growth and secondary metabolite formation. Common carbon sources include glucose, starch, and molasses, while nitrogen sources such as soybean meal, yeast extract, or ammonium sulfate supply amino nitrogen essential for microbial metabolism. Trace minerals like magnesium, phosphate, and iron are also required for enzymatic activity and antibiotic synthesis[112].

Fermentation typically proceeds under controlled conditions: temperature between 28–30 °C, pH maintained around 6.5–7.5, and continuous aeration and agitation to ensure adequate oxygen transfer.

The process is generally conducted in fed-batch mode, where controlled nutrient feeding prevents catabolic repression by excessive carbon or nitrogen sources. Streptomycin biosynthesis occurs predominantly during the idiophase, which follows the trophophase of active biomass accumulation. During this phase, key enzymes involved in the streptidine and N-methyl-L-glucosamine pathways are activated, leading to the formation of the aminocyclitol core structure characteristic of streptomycin.

At the end of fermentation, the first recovery step is the separation of mycelial biomass through filtration or centrifugation. The clarified supernatant contains the soluble antibiotic, while the biomass is washed to recover entrapped product and may be reused as a protein-rich supplement after detoxification. The purification process begins by acidifying the supernatant to facilitate adsorption of streptomycin onto ion-exchange resins, such as carboxymethylcellulose. The bound antibiotic is subsequently eluted using a buffered or mildly alkaline solution and then precipitated as streptomycin sulfate, the stable pharmaceutical form, by adding sulfuric acid or suitable organic solvents. The resulting precipitate is filtered, washed, and recrystallized from aqueous alcohol to increase purity and stability. Final drying under vacuum yields crystalline streptomycin sulfate, which is then formulated for medical applications. Modern downstream processes utilize membrane filtration, chromatographic purification, and process analytical technologies (PAT) to improve recovery efficiency, reproducibility, and compliance with Good Manufacturing Practice (GMP) standards[112].

Advances in fermentation engineering, including oxygen transfer optimization, real-time monitoring, and genetic improvement of *S. griseus* strains, have significantly enhanced streptomycin productivity. The integration of optimized fermentation control and efficient purification techniques ensures high yield, purity, and consistent quality of industrial streptomycin suitable for pharmaceutical applications[112].

V.1.3. Tetracyclines

Tetracyclines are broad-spectrum antibiotics produced mainly by *Streptomyces aureofaciens* and *Streptomyces rimosus* through a complex secondary metabolic process. Industrial production is carried out by submerged aerobic fermentation, where the microorganism is cultivated under carefully controlled conditions to maximize antibiotic yield. The inoculum, obtained from a pure spore culture, is sequentially scaled up through seed fermenters to ensure vigorous and homogeneous growth before introduction into production bioreactors[113].

The fermentation medium generally contains carbon sources such as glucose or starch, nitrogen sources including soybean meal or ammonium sulfate, and mineral salts such as calcium, magnesium, and phosphate. The process is conducted at 28–30 °C, with the pH maintained between

6.5 and 7.5, and sufficient aeration and agitation to support aerobic metabolism. Fermentation usually lasts 5–8 days and is often operated in fed-batch mode to sustain production and prevent repression of secondary metabolism. During the idiophase, enzymes such as polyketide synthases catalyze the condensation of malonyl-CoA units, forming the tetracyclic naphthacene structure characteristic of tetracyclines.

After fermentation, the broth contains the antibiotic, microbial biomass, and residual nutrients. The biomass is removed by filtration or centrifugation to obtain a clear supernatant containing dissolved tetracycline. The broth is then acidified to pH 2–2.5, converting tetracycline into its protonated form, which facilitates solvent extraction using butanol or ethyl acetate. The antibiotic is subsequently back-extracted into an alkaline aqueous phase (pH 7–8), yielding the stable tetracycline salt[113].

The solution is concentrated under reduced pressure and subjected to crystallization under controlled pH and temperature. The resulting tetracycline crystals are filtered, washed, and vacuum-dried, yielding a high-purity product ready for formulation. Modern recovery methods employ membrane filtration, ion-exchange chromatography, and solvent recycling to enhance yield, purity, and environmental sustainability. These integrated upstream and downstream processes, combining optimized fermentation, solvent extraction, and crystallization, enable large-scale production of high-quality tetracyclines for clinical and veterinary applications[113].

V.2. Enzymes

Enzymes are not classified as primary or secondary metabolites because they are biological catalysts that facilitate metabolic reactions rather than metabolites themselves. While they play essential roles in both primary and secondary metabolic pathways, enzymes are macromolecules synthesized from primary metabolites (such as amino acids) and therefore are not considered metabolites.

Enzymes are biological catalysts that accelerate biochemical reactions essential for life. They play a central role in metabolism, enabling organisms to efficiently carry out processes such as energy generation, biosynthesis, and degradation of complex molecules. Because enzymes operate under mild physiological conditions and exhibit high specificity, they are vital not only in biological systems but also in industrial, pharmaceutical, and environmental applications.Among enzyme sources, microorganisms, including bacteria, fungi, and yeasts, are the most significant producers due to their rapid growth, ease of genetic manipulation, and ability to secrete enzymes extracellularly. Microbial enzymes are preferred over plant or animal enzymes because they are more stable, easier to purify, and can be produced in large quantities through fermentation. For instance [114-115]:

- *Bacillus subtilis* and *Bacillus licheniformis* are well-known producers of amylases, proteases, and lipases used in the food, detergent, and textile industries.

- *Aspergillus niger*, a filamentous fungus, is widely used to produce glucoamylase, pectinase, and citric acid-related enzymes for food and beverage processing.

- *Trichoderma reesei* is an efficient producer of cellulases and hemicellulases, essential for biofuel production and paper industries.

- *Candida rugosa* and *Yarrowia lipolytica* (yeasts) produce lipases applied in pharmaceuticals and bioremediation.

These microbial enzymes are central to industrial biotechnology, offering cost-effective, renewable, and environmentally friendly alternatives to chemical catalysts. Their continued development through molecular biology and fermentation technologies supports the growth of a sustainable bioeconomy [115].

Enzymes are proteins that exhibit catalytic properties, facilitating biochemical reactions essential for cellular metabolism. Virtually all biomolecules capable of catalyzing chemical transformations within cells are enzymes. Microorganisms that secrete extracellular enzymes, typically hydrolases that are often substrate-induced (inducible enzymes), are generally preferred for industrial and biotechnological applications. For example, β-galactosidase production is induced in the presence of lactose. In contrast, intracellular enzymes, which remain confined within the cell, are less desirable due to the complex and costly extraction and purification processes required.

V.2.1. Proteases and other microbial enzymes

The production of bacterial proteases represents a major industrial process in terms of both volume and economic value. It is estimated that over 500 tons of these enzymes are produced annually worldwide. Bacterial proteases are widely applied in detergent formulations and cheese manufacturing due to their strong catalytic activity and stability under alkaline conditions. In detergent production, proteases derived from selected strains of *Bacillus amyloliquefaciens* are commonly used to enhance the solubilization and removal of protein-based stains from fabrics. However, a key challenge associated with their industrial use is the potential allergenic response caused by prolonged exposure to airborne bacterial proteins during handling and formulation[114-115].

Beyond proteases, several other microbial enzymes, including amylases, lipases, reductases, and glucoamylases, are commercially produced using bacteria and filamentous fungi. Species of the genus *Bacillus*, such as *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*, are efficient producers of alkaline proteases and amylases. Meanwhile, fungal species like *Aspergillus niger*, *A. oryzae*, and *Penicillium notatum* are exploited for the large-scale production of pectinases, glucosidases, and catalases.

Among the most economically significant enzymes are amylases and glucoamylases, which are used for the enzymatic hydrolysis of starch to produce glucose. The glucose obtained is subsequently converted into fructose through the catalytic action of glucose isomerase, resulting in high-fructose syrup (HFS), an important sweetener in the food and beverage industry. Derived mainly from corn, wheat, or potato starch, high-fructose syrup production exceeds ten million tons annually. These large-scale bioprocesses highlight the pivotal role of microbial enzymes in modern industrial biotechnology, providing sustainable, efficient, and environmentally friendly alternatives to traditional chemical processes [114-115].

V.2.2. Extremozymes: prokaryotic enzymes for extreme environments

Extremozymes are specialized enzymes produced by extremophilic prokaryotes, organisms that thrive under extreme environmental conditions such as high temperature, salinity, acidity, or alkalinity. Among these microorganisms, hyperthermophiles represent a remarkable group capable of growth at temperatures above 80°C. Their survival at such extreme temperatures is made possible by the production of thermostable macromolecules, particularly enzymes that maintain structural integrity and catalytic activity under heat stress. These heat-resistant enzymes are collectively referred to as extremozymes. A well-known example is pullulanase, an enzyme extracted from *Pyrococcus woesei*, a hyperthermophilic archaeon with an optimal growth temperature of around 100°C. This enzyme demonstrates remarkable stability in the presence of calcium ions, retaining its catalytic activity up to 110°C. Similarly, acid-tolerant enzyme mixtures derived from acidophilic microorganisms are used as feed additives in poultry, enhancing digestion by breaking down complex plant fibers under acidic gastrointestinal conditions[116].

Extremozymes are of significant industrial relevance because many chemical and biotechnological processes operate optimally under harsh conditions where conventional enzymes rapidly denature. For instance, thermostable DNA polymerases such as Taq polymerase from *Thermus aquaticus* and Pfu polymerase from *Pyrococcus furiosus* have revolutionized molecular biology through their application in the polymerase chain reaction (PCR). Additionally, heat-stable proteases, amylases, cellulases, pullulanases, and xylanases are extensively employed in the laundry detergent, textile, and biofuel industries, where high temperatures facilitate improved reaction kinetics and substrate solubilization.

Beyond thermophiles, other extremophiles, including psychrophiles (cold-loving), halophiles (salt-tolerant), and acidophiles or alkaliphiles (pH-adapted), produce enzymes that function efficiently under respective extreme conditions. Such cold-active, halotolerant, and pH-stable enzymes have gained attention for environmentally friendly industrial processes requiring mild reaction conditions or high osmotic stability. Consequently, extremozymes represent a powerful resource for

biotechnological innovation, providing robust biocatalysts adaptable to diverse and demanding industrial environments [116].

V.2.3. Enzyme production

Enzyme production is a central process in industrial biotechnology, involving the synthesis and recovery of biocatalysts from biological sources for use in various applications. Although enzymes can be derived from plants and animals, microorganisms, particularly bacteria, fungi, and yeasts, are the most preferred sources due to their rapid growth, ease of genetic manipulation, and capacity for large-scale production under controlled conditions. Microbial enzyme production offers a sustainable and cost-effective alternative to chemical catalysts, aligning with the goals of the green and circular bioeconomy[115].

V.2.3.1. Fermentation processes

Industrial enzyme production is primarily carried out through fermentation (Figure V.5), which provides optimal conditions for microbial growth and enzyme secretion. Two main types of fermentation are used [114-115]:

Submerged Fermentation (SmF): This process involves growing microorganisms in a liquid nutrient medium where enzymes are either secreted into the broth (extracellular) or retained within the cells (intracellular). SmF is widely applied for producing enzymes such as amylases, proteases, and lipases by species like *Bacillus subtilis*, *Bacillus licheniformis*, and *Aspergillus niger*. It is favored for its ease of monitoring and process control.

Solid-State Fermentation (SSF) : In this technique, microorganisms grow on moist solid substrates (such as wheat bran, rice husk, or sugarcane bagasse) with minimal free water. SSF mimics natural microbial habitats and is particularly suitable for filamentous fungi like *Trichoderma reesei* and *Aspergillus oryzae*. It often yields higher enzyme titers, greater stability, and simpler downstream processing compared to SmF.

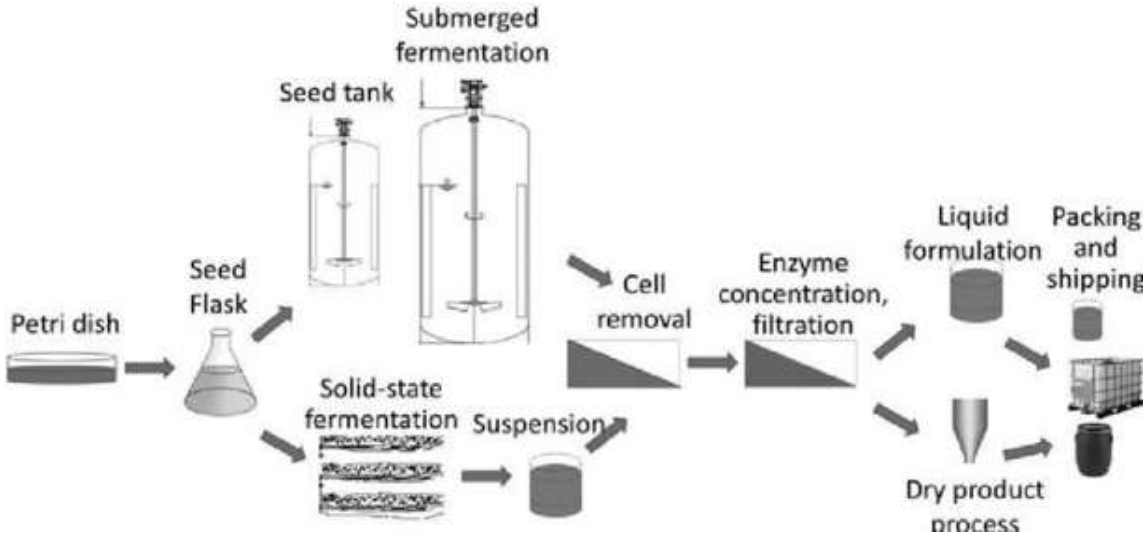

Figure V.5. General diagram for the manufacturing process of enzymes (Online source 16).

V.2.3.2. Factors affecting enzyme production

Several parameters significantly influence enzyme yield and activity during fermentation [114-115]:

Nutrient composition : Carbon and nitrogen sources affect both cell growth and enzyme induction. For example, starch and casein can act as inducers for amylases and proteases, respectively.

pH and temperature : Each microorganism has an optimal pH and temperature range (*Bacillus subtilis* produces protease optimally at pH 8–9 and 37–40 °C).

Aeration and agitation : Oxygen transfer and mixing affect microbial metabolism and enzyme secretion, especially in SmF systems.

Inducers and inhibitors : The presence of specific substrates or metal ions can enhance or suppress enzyme synthesis.

Fermentation time : Enzyme production often peaks during the late exponential or early stationary growth phase, depending on the microorganism.

V.2.3.3. Advances in enzyme production

Significant progress in enzyme production technology has revolutionized the efficiency, stability, and industrial applicability of microbial enzymes. Through genetic and metabolic engineering, microorganisms have been optimized to overproduce enzymes with improved catalytic properties. For instance, *Bacillus subtilis* and *Bacillus licheniformis* have been genetically modified to yield high levels of alkaline proteases used in detergents and leather processing. Similarly, *Aspergillus niger* and *Trichoderma reesei* are engineered to enhance the secretion of cellulases and xylanases employed in biofuel and paper industries [114-115].

The application of recombinant DNA technology has allowed heterologous expression of industrial enzymes in robust hosts. For example, recombinant lipase from *Candida rugosa* expressed in *Pichia pastoris* exhibits superior stability and productivity compared to the native strain. Likewise, recombinant amylases and laccases have been produced in *Escherichia coli* and *Saccharomyces cerevisiae* to improve starch processing and textile bleaching efficiency.

Protein engineering and directed evolution have also led to enzymes with enhanced thermostability, altered substrate specificity, and tolerance to extreme pH or solvents, critical for industrial applications. For instance, engineered phytases from *Aspergillus fumigatus* show higher thermostability for use in animal feed, while glucose oxidase mutants with increased activity are applied in biosensors and food preservation.

In addition, advances in fermentation technology, such as fed-batch and continuous bioreactors, solid-state fermentation (SSF) for fungi, and high-cell-density cultivation for bacteria, have greatly improved enzyme productivity. The incorporation of omics-based tools (genomics, proteomics, metabolomics) and computational modeling allows precise control of metabolic fluxes, optimizing yield and reducing production costs. These innovations, combined with bioprocess automation and eco-friendly production strategies, have positioned microbial enzyme production as a key pillar of modern industrial biotechnology, supporting global needs in food, pharmaceuticals, detergents, biofuels, and environmental bioremediation[114-115].

V.2.4. Enzyme extraction and purification

Following microbial fermentation, the recovery of enzymes involves a series of extraction and purification steps designed to isolate the active protein in a stable and concentrated form. The extraction process begins with the separation of biomass from the culture medium, typically achieved through centrifugation or filtration, depending on whether the enzyme is intracellular or extracellular. Extracellular enzymes, which are secreted into the culture broth, are more easily recovered from the supernatant, whereas intracellular enzymes require cell disruption techniques such as sonication, high-pressure homogenization, or enzymatic lysis to release the enzyme from the cytoplasm[117-118].

Once released, the crude enzyme extract undergoes clarification to remove cell debris and other insoluble materials. The subsequent purification process employs a combination of physicochemical and chromatographic techniques aimed at achieving the desired purity, activity, and stability. Commonly used methods include ammonium sulfate precipitation, dialysis, and ultrafiltration, followed by ion-exchange chromatography, gel filtration (size-exclusion chromatography), and affinity chromatography. Each step is optimized to balance enzyme yield and purity while minimizing denaturation or loss of activity[117-118].

Purification strategies often depend on the enzyme's molecular weight, isoelectric point, and hydrophobicity. In industrial applications, a partial purification may be sufficient, whereas research or pharmaceutical uses often require highly purified enzyme preparations. The progress of purification is routinely monitored using specific activity assays, protein concentration measurements, and SDS-PAGE analysis to confirm enzyme integrity and homogeneity.

Finally, purified enzymes are typically stabilized through lyophilization, cryopreservation, or formulation with stabilizing agents such as glycerol or calcium ions, ensuring long-term storage and functional stability. The integration of downstream processing automation and membrane-based separations continues to enhance enzyme recovery efficiency and reduce production costs, thereby strengthening the economic viability of industrial enzyme manufacturing[117-118].

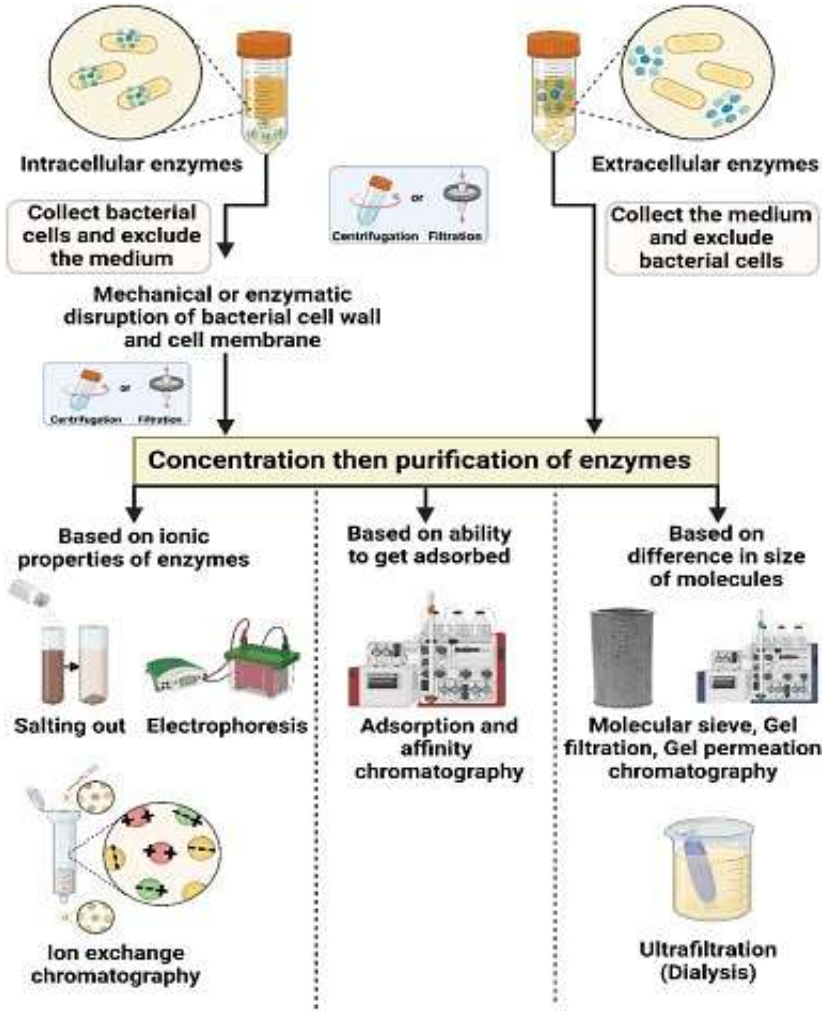

Figure V.6. Process of microbial enzyme recovery [118].

ÄKTA purification platform

The crude extract is subsequently clarified to remove cell debris and insoluble particles, followed by concentration using ultrafiltration or precipitation (ammonium sulfate). The next step involves chromatographic purification, where high selectivity and reproducibility are achieved through automated systems such as the ÄKTA chromatography platform (Cytiva). This platform allows for precise control of flow rates, gradient formation, pressure, and UV detection, enabling the application of multiple chromatographic methods, including ion-exchange chromatography, size-exclusion chromatography, and affinity chromatography, under optimized and reproducible conditions[119].

The ÄKTA system is equipped with UNICORN™ software, which facilitates real-time monitoring, method programming, and data analysis, significantly improving purification efficiency and consistency. These automated features minimize human error, reduce purification time, and ensure high product quality. Purification progress is evaluated by specific activity assays, protein quantification, and SDS-PAGE analysis to assess enzyme purity and molecular integrity. Finally, the purified enzyme is stabilized through lyophilization, cryopreservation, or formulation with stabilizing agents such as glycerol, calcium ions, or sugars to maintain long-term activity. The integration of automated chromatography platforms like ÄKTA into downstream processing has greatly advanced enzyme purification, providing a scalable, reproducible, and efficient approach aligned with modern biopharmaceutical and industrial enzyme production standards[119].

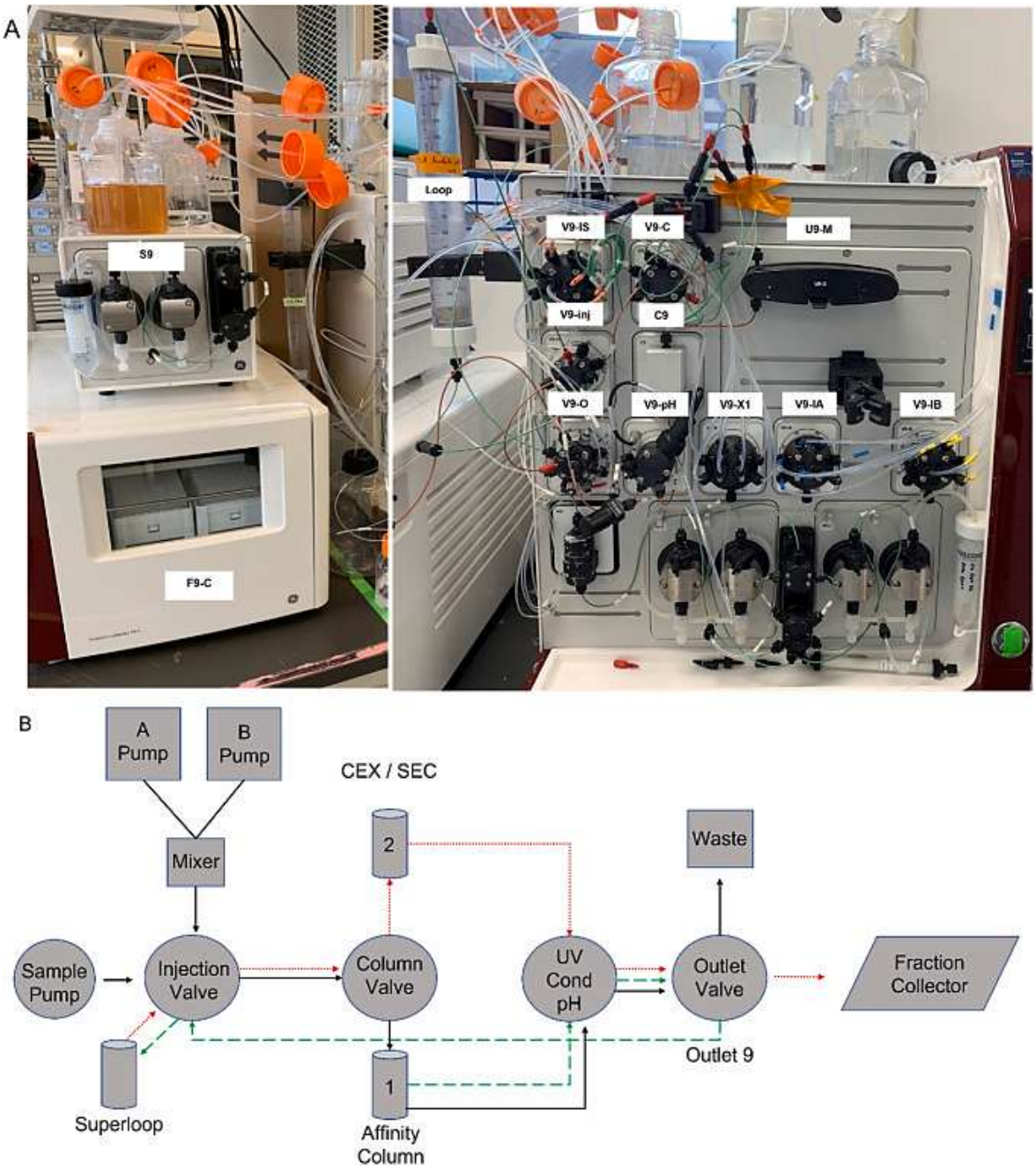

Figure V.7. ÄKTA setup and diagram of flow path [119].

A) The ÄKTA components included in this system described are labeled with identifying text above their respective locations. The ÄKTA pure 25 M shown is equipped with a sample pump (S9) with 7-port inlets on the sample inlet (V9-IS), X-valve (V9-X1), Inlet A (V9-IA), Inlet B (V9-IB), a variable wavelength detector (U9-M), pH monitor (V9-pH), conductivity monitor (C9), injection valve (V9-inj), 5-column valve (V9–C), and 10-outlet valve (V9–O). ***B)*** The flow of the sample and buffer lines are indicated by the solid black lines with culture media flowthrough passing through the outlet valve to waste. The dashed green flow path outlines the elution path from the primary capture columns (ProA/Fab Capture/IMAC) to the outlet 9 position and into the Superloop. The dotted red lines represent the flow path of the Superloop load being applied to the polishing column (CEX/SEC) and resulting in final fractionation[119].

V.2.5. Enzyme immobilization techniques

Enzyme immobilization refers to the confinement of enzyme molecules onto or within a solid support while maintaining their catalytic activity. This strategy enhances enzyme stability, reusability, and operational efficiency, making it a cornerstone of modern industrial biocatalysis. Immobilized enzymes are widely used in the food, pharmaceutical, biofuel, and environmental industries, where process stability and cost-effectiveness are essential. Several techniques have been developed for enzyme immobilization (Figure V.8), classified broadly into physical and chemical methods [119]:

Physical adsorption involves weak interactions, such as van der Waals forces or hydrogen bonding, between the enzyme and the support matrix (silica gel, activated carbon, or polymer beads). Although simple and cost-effective, this method may suffer from enzyme leaching under changing reaction conditions.

Ionic bonding is a simple, low-cost, and reversible enzyme immobilization method based on electrostatic interactions between oppositely charged enzymes and support materials. The binding can be easily reversed by adjusting pH, temperature, or ionic strength. Although this technique allows mild immobilization and easy regeneration, excessive surface charge may distort enzyme conformation or alter kinetic behavior, thereby reducing catalytic efficiency and product yield.

Entrapment, Entrapment involves enclosing enzymes within a semi-permeable gel or matrix such as calcium alginate, polyacrylamide, or sol–gel. The matrix allows substrates and products to diffuse while retaining the enzyme inside. This method ensures the stability and reusability of enzymes in repeated reactions. Entrapment also reduces enzyme loss and improves process efficiency. It is widely used for industrial biocatalysis and biosensor applications.

Encapsulation, confines enzymes inside membranes, fibers, or microcapsules, offering a protective environment. These structures act as physical barriers against temperature, pH, or chemical fluctuations. Encapsulation enhances enzyme activity preservation and long-term storage stability. Common materials include polymeric membranes and lipid-based systems. This technique provides effective protection under harsh environmental conditions.

Affinity bonding is a highly specific immobilization technique that relies on selective interactions between an enzyme and a complementary ligand (substrate analog, cofactor, or dye) attached to a support matrix. This method ensures strong and oriented binding, preserving enzyme activity and allowing controlled elution under mild conditions. Although precise and efficient, it can be costly due to complex ligand synthesis and support functionalization.

Covalent binding represents a more stable immobilization approach, involving the formation of covalent bonds between enzyme functional groups (amino, carboxyl, or thiol) and activated

supports, such as agarose or epoxy resins. This technique minimizes enzyme leaching and enhances thermal and pH stability.

Cross-linking, another chemical method, involves the use of bifunctional reagents like glutaraldehyde to form enzyme aggregates or cross-linked enzyme crystals (CLECs), which display excellent mechanical strength and stability.

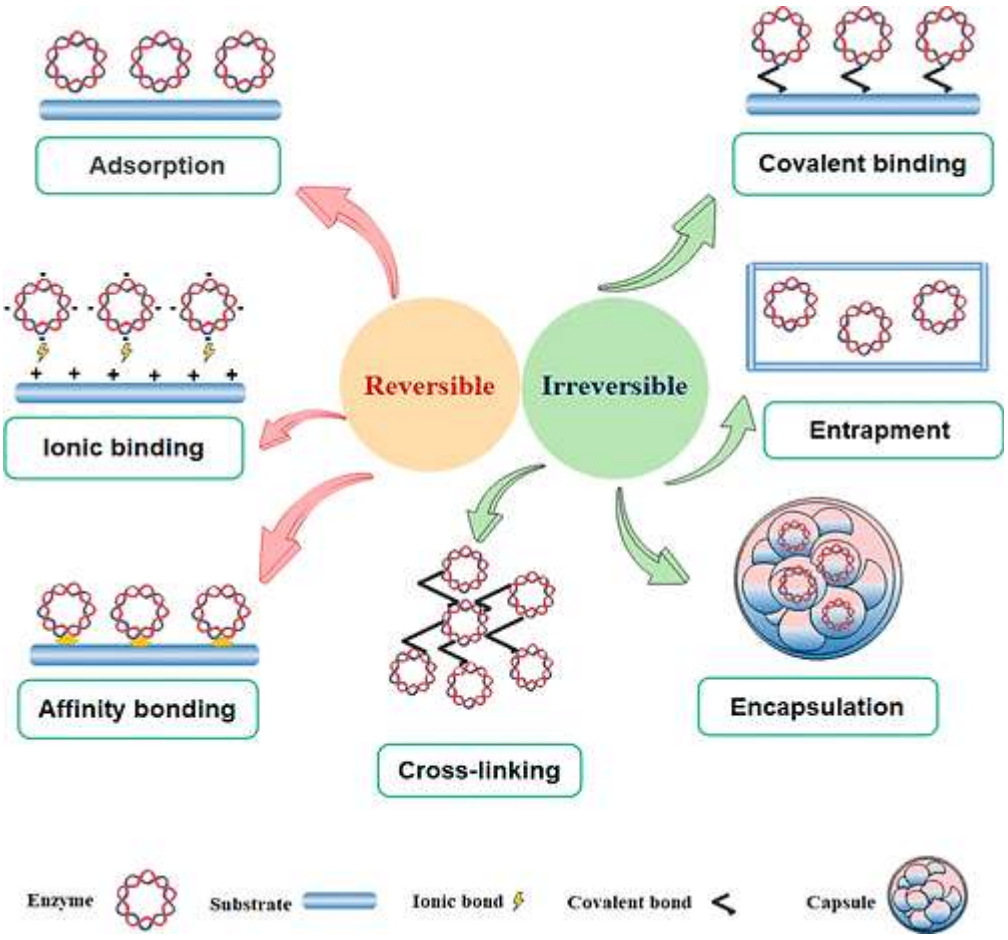

Figure V.8. Schematic representation of major enzyme immobilization techniques [119].

Reversible immobilization methods include adsorption, ionic bonding, and affinity bonding, which rely on noncovalent interactions allowing enzyme recovery. In contrast, irreversible methods such as covalent binding, entrapment, encapsulation, and cross-linking form stable associations between the enzyme and the support matrix, providing enhanced durability and operational stability.

The choice of immobilization technique depends on factors such as enzyme type, desired stability, process conditions, and cost considerations. Immobilized enzymes can be used in continuous flow reactors, facilitating product recovery and enzyme reuse across multiple cycles, thus reducing production costs. Advances in nanomaterial supports, magnetic carriers, and microreactor technologies have further enhanced immobilization efficiency, mass transfer, and catalytic

performance. Collectively, these developments have transformed enzyme immobilization into a vital tool for sustainable and scalable bioprocessing [119].

V.3. Toxins

Toxins are secondary metabolites, they are molecules synthesized by living organisms that can disrupt the normal functioning of certain cells. They are also variably immunogenic, meaning they are capable of inducing an immune response. Toxins can be classified according to their origin. Those produced by bacteria are called bacteriotoxins, while those secreted by microscopic fungi are known as mycotoxins, such as those produced by molds belonging to the *Aspergillus* and *Penicillium* genera. Among bacterial toxins, two major categories exist: endotoxins and exotoxins. Endotoxins are structural components of lipopolysaccharides, whereas exotoxins are proteinaceous in nature. The main exotoxins include the diphtheria toxin (*Corynebacterium diphtheriae*), staphylococcal enterotoxins (*Staphylococcus aureus*), tetanus toxin (*Clostridium tetani*), and botulinum toxins (*Clostridium botulinum*). These toxins are used as sources of antigens, and more importantly, for the production of toxoids used in vaccines [121].

The most notable endotoxins are the cholera enterotoxin (*Vibrio cholerae*) and the typhoid endotoxin (*Salmonella typhi*). Some of these toxic compounds can also play significant roles in biological control. Furthermore, various molds excrete toxic substances such as alkaloids. These compounds, produced by *Claviceps purpurea*, possess pharmacological properties and are of medical interest. Aflatoxins, produced by *Aspergillus flavus*, represent another important group of mycotoxins with notable implications for food safety and public health[121].

V.3.1. Industrial production of toxins

The industrial production of toxins is primarily conducted for medical, pharmaceutical, and research purposes, under controlled and biosafe conditions. In biotechnology, bacterial and fungal toxins are produced using fermentation processes that optimize microbial growth and toxin yield. For bacteriotoxins, industrial production often involves aerobic or anaerobic fermentation using specific pathogenic or genetically modified strains under biocontainment. Examples include *Clostridium botulinum* for botulinum toxin, used in medicine and cosmetics, and *Corynebacterium diphtheriae* for diphtheria toxin, which is further detoxified to produce the diphtheria toxoid used in vaccines[121].

Mycotoxins, produced by filamentous fungi such as *Aspergillus*, *Penicillium*, and *Fusarium*, are usually obtained through solid-state or submerged fermentation on agricultural substrates. Industrial production of these toxins is generally limited to analytical and research purposes, as they are hazardous. However, controlled synthesis of related compounds contributes to the development of pharmaceuticals (ergot alkaloids from *Claviceps purpurea*).

The industrial handling of toxins requires strict biosafety and regulatory compliance, including adherence to Good Manufacturing Practices (GMP), biosafety level containment (BSL-2 or BSL-3), and oversight by agencies such as the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC)[121].

V.3.2. Examples of industrial toxin production

The industrial production of microbial toxins plays an essential role in medicine, research, and biotechnology. These toxins, originally harmful biological molecules, can be purified, modified, or detoxified to obtain valuable products such as vaccines, pharmaceuticals, and diagnostic tools. Industrial production is carried out under controlled fermentation conditions and follows strict biosafety and quality standards to ensure safety and efficacy. For instance [122]:

Botulinum toxin (*Clostridium botulinum*): Produced under strictly controlled anaerobic fermentation conditions using *Clostridium botulinum*. The toxin is then purified and processed to obtain Botox®, used in medicine (for muscle spasms, migraines) and cosmetics (for wrinkle reduction). Production is carried out in biosafety level 3 (BSL-3) facilities following Good Manufacturing Practices (GMP).

Diphtheria toxin (*Corynebacterium diphtheriae*): Industrially produced via aerobic submerged fermentation. The crude toxin is chemically detoxified (using formaldehyde) to produce diphtheria toxoid, which serves as an antigen in DTP vaccines (Diphtheria-Tetanus-Pertussis). This process combines microbial fermentation and biochemical purification steps.

Tetanus toxin (*Clostridium tetani*): Cultivated under anaerobic batch fermentation, followed by precipitation, filtration, and detoxification to obtain tetanus toxoid for vaccines. Industrial production is regulated under World Health Organization (WHO) and pharmaceutical standards to ensure safety and consistency.

Ergot alkaloids (*Claviceps purpurea*): Produced by solid-state fermentation of rye or synthetic media infected with *Claviceps purpurea*. These fungal toxins (mycotoxins) are purified and used in pharmaceuticals such as ergotamine, applied in migraine treatment and obstetrics[122].

VI. Bioconversion and enzyme engineering

Bioconversion and enzyme engineering are key aspects of industrial microbiology. Bioconversion uses microorganisms or their enzymes to transform raw materials into valuable products. Enzyme engineering optimizes enzyme activity, stability, and specificity, enhancing bioconversion efficiency. Together, they enable microbial processes to function under harsh industrial conditions and increase product yield. Their integration supports sustainable, cost-effective, and high-value bioprocesses. Overall, they form the foundation of modern industrial biotechnology, ensuring efficiency, versatility, and innovation[123].

VI.1. Bioconversion

Bioconversion refers to the use of microorganisms or enzymes to transform raw materials into valuable products (Table VI.1). It plays a key role in producing biofuels, pharmaceuticals, and food ingredients. This process often replaces harsh chemical methods with eco-friendly and energy-efficient alternatives. Microbial enzymes catalyze specific reactions with high selectivity and mild operating conditions. Therefore, bioconversion is a cornerstone of industrial biotechnology and sustainable production [123].

Early biocatalytic processes in synthetic chemistry primarily involved fermentation-type bioconversions using whole microorganisms. Over time, the isolation and purification of enzymes enabled their application under diverse reaction conditions and led to the incorporation of reactor-based processes and hybrid systems that alternate in vitro chemical steps with whole-cell catalysis. In such hybrid strategies, one or multiple steps of a biosynthetic pathway are executed within whole cells capable of bioconversion to yield either a native or a synthetic product, a practice commonly referred to as *enzymation*. The selection of whether to use whole cells or purified enzymes depends on factors including the type of reaction, the necessity for cofactor recycling, the intended production scale, and the stability of the enzyme outside its native cytoplasmic environment. When employing whole-cell systems, additional considerations such as substrate- or product-mediated interference with the host strain's metabolism must be addressed. These challenges mirror those encountered in fermentation strain development and often necessitate similar interventions in metabolic engineering. While many applications of these biocatalytic and bioconversion processes target fine chemicals and pharmaceuticals, enzymes and microbial conversion systems are increasingly being applied to bulk and commodity products as well[124].

Table VI.1. Microbial and enzymatic bioconversions of complex molecules

Complex Molecule	Substrate Source	Microbial /Enzyme Biocatalyst	Bioconversion Reaction	Product	Added Value	Fermentation / Process
Lignin	Lignocellulosic biomass	*Phanerochaete chrysosporium*, laccase, peroxidases	Oxidative depolymerization	Aromatic monomers (p-coumaric acid, ferulic acid)	Yes, precursors for flavonoids, biofuels	Solid-state or submerged fermentation
Cellulose	Plant biomass (cellulose)	Cellulases from *Trichoderma reesei*	Hydrolysis of β-1,4-glycosidic bonds	Glucose	Yes, feedstock for bioethanol	Submerged fermentation / enzymatic reactor
Starch	Corn, potato	α-Amylase, glucoamylase (*Aspergillus niger*)	Hydrolysis of starch	Maltose, glucose	Yes, used in food, fermentation, bioethanol	Enzymatic saccharification
Aromatic hydrocarbons	Polycyclic aromatic hydrocarbons (PAHs)	*Pseudomonas putida*, dioxygenases	Ring-cleavage oxidation	Catechol, protocatechuate	Yes, intermediates for bio-based chemicals	Bioreactor / bioremediation
Proteins / Peptides	Whey proteins, casein	Proteases (*Bacillus subtilis*, *Aspergillus oryzae*)	Hydrolysis of peptide bonds	Amino acids, peptides	Yes, food additives, flavor, nutraceuticals	Submerged fermentation / enzymatic reactor
Aromatic compounds	Flavonoids / polyphenols	Laccase, peroxidase (*Pleurotus spp.*, *Trametes spp.*)	Oxidative cleavage	Smaller phenolic acids	Yes, antimicrobial, antioxidant compounds	Solid-state or submerged fermentation
Complex lipids	Triacylglycerols, waste oils	Lipases (*Candida antarctica*, *Rhizopus oryzae*)	Hydrolysis / transesterification	Glycerol + fatty acids / biodiesel	Yes, biofuel or feedstock	Batch or continuous enzymatic reactor

Bioconversion process of lignin derivatives into homoeriodictyol

The bioconversion of lignin-derived aromatic monomers into value-added compounds such as homoeriodictyol is achieved using engineered microbial cell factories, in this case, *Saccharomyces cerevisiae*. Lignin-derived monomers are first taken up by the yeast cells and directed into a heterologous biosynthetic pathway designed for flavonoid production. Key enzymes within this pathway are engineered through enzyme optimization techniques, including directed evolution and protein engineering, to enhance catalytic efficiency and substrate specificity. Competing metabolic branches are suppressed to increase flux toward the target product, while cofactor availability, such as NADPH and CoA, is optimized to support enzymatic reactions. Through these integrated strategies, the engineered yeast efficiently converts lignin monomers into homoeriodictyol with high yield and specificity, demonstrating the synergy between bioconversion and enzyme engineering in industrial biotechnology. This approach illustrates how pathway design, enzyme optimization, and metabolic regulation collectively enable the transformation of complex biomass into high-value chemicals [123].

VI.2. Enzyme engineering

Enzyme engineering involves modifying enzyme structure to improve performance, stability, or specificity. It uses advanced tools like site-directed mutagenesis and directed evolution to design superior biocatalysts. Engineered enzymes can function under extreme temperatures, pH, or solvents, expanding their industrial use. This field bridges molecular biology and computational modeling for precise enzyme optimization. Ultimately, enzyme engineering enhances bioconversion efficiency and product yield in biotechnology [123].

Enzyme engineering enables the development of enzymes capable of withstanding extreme temperatures, pH variations, or organic solvents, and supports applications ranging from drug synthesis and biomass conversion to environmental remediation.

VI.2.1. Methods of enzyme engineering

Various strategies have been developed to engineer enzymes, depending on the level of knowledge about the enzyme structure and the desired property. The main approaches include directed evolution, site-directed mutagenesis, rational design, and semi-rational/combinatorial engineering, each with specific advantages and applications (Figure VI.1). For instance [125]:

Directed evolution: it mimics natural evolution in the laboratory by introducing random mutations into the enzyme gene and selecting variants with improved properties. It is particularly useful when the structural information of the enzyme is limited. Example: engineering lipases with enhanced thermal stability for industrial biodiesel production.

Site-directed mutagenesis: This method introduces specific amino acid substitutions in known active or structural sites of the enzyme to improve its properties. It relies on structural or mechanistic knowledge of the enzyme. Example: modifying cytochrome P450 monooxygenases to increase regioselectivity in drug metabolite synthesis.

Rational design: Rational design uses computational modeling and structural analysis to predict beneficial mutations that improve enzyme function. Example: engineering glucose oxidase to retain activity at higher pH for biosensor applications.

Semi-rational / Combinatorial approaches: Semi-rational strategies combine directed evolution with rational design by focusing mutagenesis on selected regions of the enzyme while preserving structural integrity. Example: enhanced β-glucosidases for hydrolysis of lignocellulosic biomass.

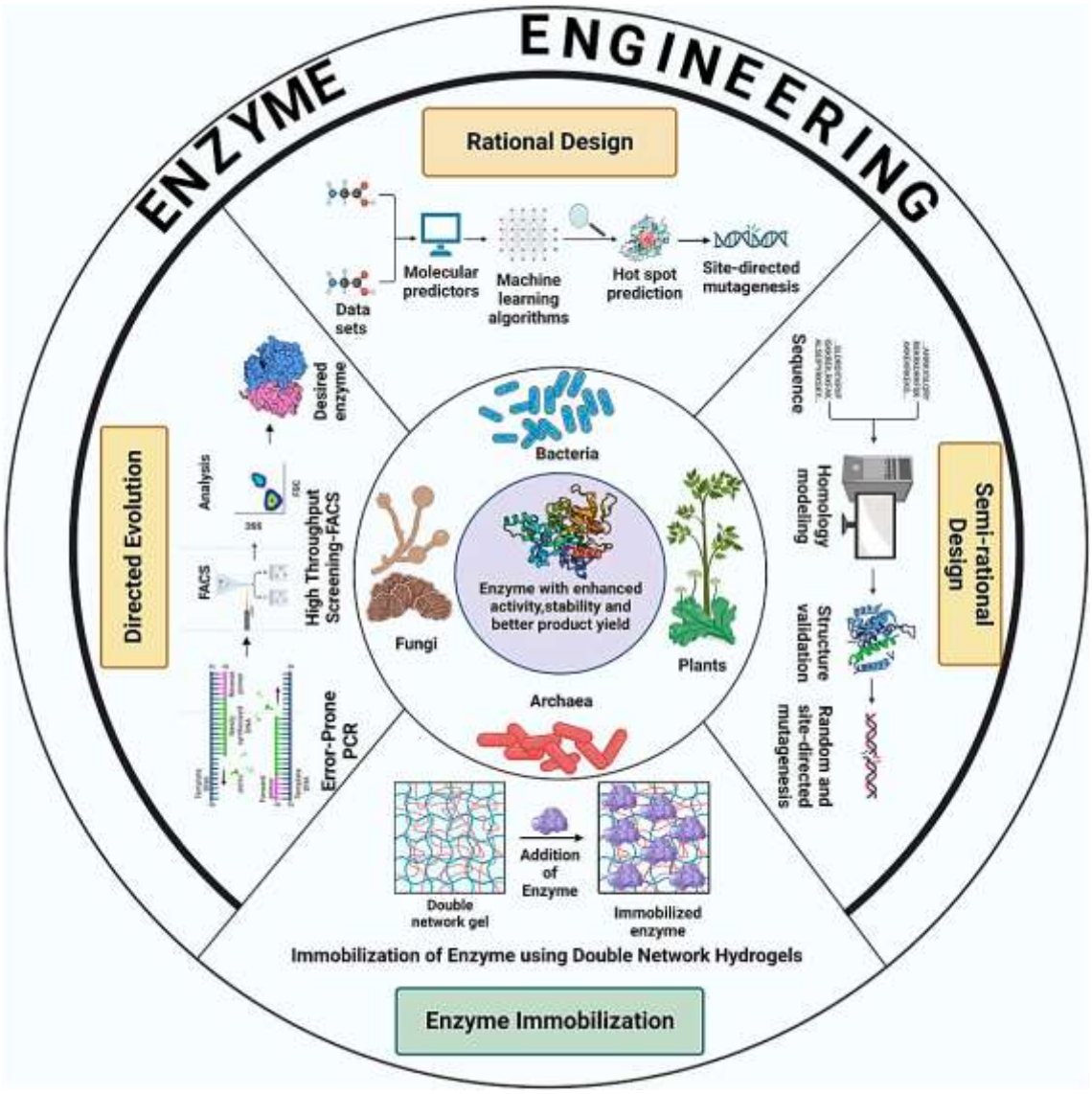

Figure VI.1. Strategies for enzyme engineering from various enzyme classes (Online source 17).

VI.2.2. Applications of enzyme engineering

Engineered enzymes have widespread applications in industry, healthcare, energy, and environmental biotechnology. By tailoring enzymes to specific tasks, they enable efficient, sustainable, and selective biocatalysis. For instance [125]:

Industrial Biocatalysis: Thermostable amylases and proteases in detergents and food processing.

Pharmaceuticals: Engineered penicillin acylases for semi-synthetic antibiotic production.

Biofuels and bioconversion: Lipases and cellulases optimized for biodiesel and biomass conversion.

Environmental remediation: Laccases and peroxidases engineered to degrade pollutants or remove phenolic compounds from wastewater.

This educational document has been specifically designed for second-year Master's students in microbial biotechnology, with the goal of providing them with a thorough understanding of bioengineering and industrial microbiology. The fundamental knowledge presented is intended to strengthen students' understanding of core principles and concepts that are central to these fields, thereby equipping them with the necessary tools to excel in both theoretical and practical aspects of microbial biotechnology.

The bioengineering section is a crucial foundation for mastering bioengineering techniques and managing bioreactors effectively. The content focuses on providing students with essential operational skills, particularly in understanding the variability of culture processes and controlling kinetics. This knowledge is indispensable for students who aim to work in industrial microbiology or related sectors. It covers the fundamental principles on fermentation, its various processes, and models for different fermentation systems including its various processes and modeling approaches, as well as the design and hydrodynamic behavior of fermenters, and how these principles are applied to optimize fermentation processes.

The industrial microbiology part of this handout offers an overview of industrial microbiology, and its diverse applications. The traits and selection of industrial microbes, the production of primary and secondary metabolites of industrial significance, and the basic concepts of bioconversion and enzyme engineering are all introduced. When taken as a whole, these subjects give students a strong grasp of microbial processes and how they are used in industry, emphasizing the crucial role that microorganisms play in the creation of biotechnological products and environmentally friendly industrial processes.

A distinctive feature of this document lies in its use of clear and precise language, carefully selected to enhance the accessibility of complex scientific concepts. To facilitate understanding, the text integrates a variety of visual aids, including simplified diagrams, figures, and summary tables. These graphical elements serve to clarify intricate ideas and promote a deeper conceptual grasp among readers.

The document is intended to function as a comprehensive educational resource, providing an in-depth understanding of fermentation processes and bioreactor management. It aims to equip students with both a solid theoretical foundation and practical insight in the fields of bioengineering and industrial microbiology. By combining rigorous theoretical content with illustrative materials, this resource seeks to foster intellectual development and technical proficiency, thereby preparing future professionals to apply their knowledge effectively within industrial and biotechnological contexts.

I would like to express my profound and heartfelt gratitude to everyone who contributed to the completion of this course material.

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