# 1

# Bio-industry in the Age of the Transition to Digital Technology: Significance and Recent Advances



## 1.1. Introduction

Bio-industry involves living organisms, mainly microorganisms, bacteria*[1] and fungi*, and develops products for the agrifood industry (yeasts*, lactic ferments*, enzymes*, etc.), the pharmaceutical industry (vaccines, antibiotics, etc.), agriculture (biopesticides*, biostimulants*, etc.), the environment (microorganisms for water, air and soil depollution, etc.), chemical industries (synthons*, biodegradable polymers, biosurfactants*, etc.). The markets concerned by these products are steadily increasing by several percent each year. For example, fine chemicals originating from fermentation were estimated at $24 billion in 2017, with a yearly increase of 3.4% over the 2017–2022 period (source: BCC).

The beginnings of bio-industry are probably in brewing beer in Mesopotamia, about 6,000 years ago, and wine production in Egypt as early as the Middle Predynastic period, more than 5,000 years ago (Figure 1.1). Over thousands of years, microorganisms have been used to produce (alcoholic beverages) or preserve (fermented products) numerous foods and have been used in processes such as flax retting (an operation that involves exploiting the production capabilities of hydrolytic enzymes in soil microorganisms to isolate the fibers of flax stems) without the people utilizing them even noticing their existence.

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Chapter written by Philippe JACQUES.

1 The asterisk symbols refer to the glossary at the end of the chapter.

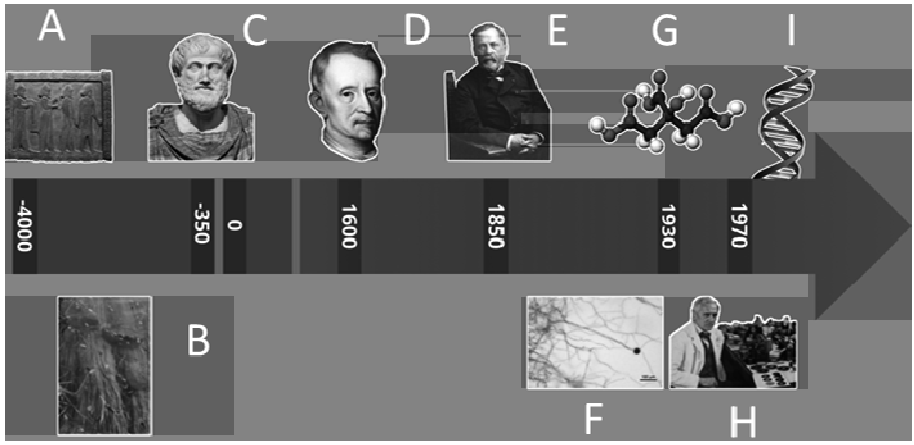


**Figure 1.1.** *Evolution of industrial microbiology. For a color version of this figure, see www.iste.co.uk/dalpont/process2.zip*

COMMENT ON FIGURE 1.1.– *The beginnings of industrial microbiology probably date back to the brewing of the first beers in Mesopotamia, some 6,000 years ago (A). During millennia, microorganisms have been exploited without knowledge of their existence, for example in flax retting (B). The spontaneous generation theory devised from Aristotle (C) to Van Helmont (D) has even been a major impediment to the demonstration of this form life invisible to the naked eye. This was definitively demonstrated due to the works of Antoni Van Leeuwenhoek and Louis Pasteur (E) during the 18th and 19th Centuries. During the first part of the 20th Century, various industrial applications involving microorganisms were created, such as for the production of amylase, citric acid (G) or penicillin, discovered by Fleming (H), using fungi (F). The discovery of DNA (I) in 1953 opened the field of genetic manipulation of microorganisms*.

This period precedes the works of Antoni Van Leeuwenhoek (1632–1723) and Pasteur (1822–1895). The first, through the invention of a tool, the microscope, capable of visualizing microscopic living beings, was able to highlight the presence of these microorganisms in many environments. The second has definitively demonstrated their role in the processes of processing organic matter and thus put an end to the theory of spontaneous generation (appearance of living beings from non-living matter) defended for centuries by renowned scientists.

Subsequently, researchers, now aware of the existence of life at the microscopic scale, were able to consider diversifying its exploitation for the production of organic acids, enzymes or antibiotics such as penicillin. The latter was discovered by chance in 1928 by a Scottish physician and bacteriologist, Sir Alexander Fleming. It

was, in fact, following contamination of his samples by a fungus called *Penicillium notatum* that he was able to identify a substance capable of inhibiting the growth of pathogenic bacteria under laboratory conditions. Fleming's work was pursued by an Australian and a German, Florey and Chain in 1939, who demonstrated the potential of penicillin to fight bacterial infections in animals. Penicillin was then produced in industrial quantities during World War II and thus saved thousands of lives on the battlefield. Fleming, Florey and Chain received the Nobel Prize for this discovery in 1945.

The discovery of deoxyribonucleic acid* (DNA) by Watson and Crick in 1953 and the development of molecular biology techniques have allowed the manipulation of the genetic heritage of microorganisms to increase the synthesis potential of enzymes and vaccines, among others. These technologies are probably reaching their peak today with the development of **synthetic biology**. The latter aims to design new microorganisms by modifying more or less profoundly the metabolic pathways of a natural microorganism. These microorganisms, which are true cell factories, should eventually be optimized for the production of many molecules of interest under perfectly defined conditions.

With the development of digital technology, a new era is opening up for industrial microbiology: that of **bioinformatics** applied to microorganisms and **systems biology**. The latter approach aims to develop a global view of how known microorganisms function by modeling, among other things, the sequence of multiple intracellular biochemical reactions, as well as their mode of regulation, in order to optimize their functioning in a given direction. Its aim is to manage and integrate the large amounts of data accumulated today, in particular as a result of the development of high-throughput approaches in so-called "omics": **genomics***, **proteomics***, **metabolomics***, **transcriptomics***, etc. To these approaches, one should also add the possibility offered by the potential of **microfluidics*** to analyze and control the behavior of each cell within a population, but also to revolutionize strategies for increasing the production volume by shifting from the notion of scaling-up to a concept of scale reduction.

Equipped with these new tools, **modern industrial microbiology** nowadays faces enormous challenges. High-throughput screening approaches to the potential for synthesis of microorganisms, of which only a tiny fraction is understood today, must strive to meet an ever-increasing demand for more biodegradable, less toxic products and whose production is based on eco-processes. These bioproducts should eventually replace a significant part of the synthetic molecules that, as a result of the development of chemistry, have invaded our environment. In addition to identifying suitable substitutes, engineers will therefore need to develop microbial strains* as well as bioprocesses* whose productivity will contribute to the achievement of cost

prices close to the prices of the synthetic molecules to compete with. At the moment of writing, this last challenge remains the major obstacle to the breakthrough of a number of products of microbial origin.

## 1.2. Diversity of products and applications

The microorganisms used today in the industrial world belong to the domain of organisms known as prokaryotic or eukaryotic. The former is characterized by an absence of nucleus but also an intracellular organization poorly structured in organelles. These are bacteria and archaebacteria*. The latter include microorganisms whose cells contain a nucleus and a large number of various organelles. The main industrial eukaryotic microorganisms are yeasts, molds* and microalgae* (Figure 1.2).

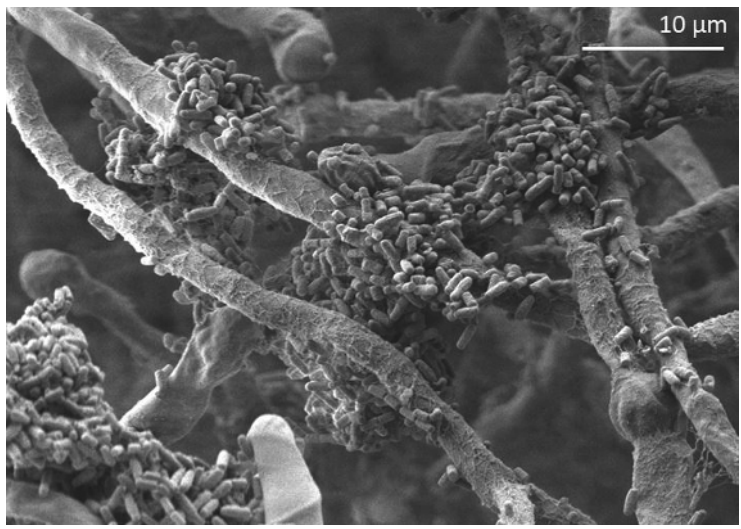


**Figure 1.2.** *Electron microscopy analysis of a bacterium and mold coculture. The bacterium is represented by the small sticks and the mold by the long filaments*

The applications of industrial microbiology cover a very large number of sectors of activities. At the European level, a color classification of biotechnology application sectors, including industrial microbiology, was proposed a few years ago (Figure 1.3). **Red biotechnology** concerns the healthcare sector; **green biotechnology** is linked to agriculture and agri-food sectors; **yellow biotechnology** includes applications related to the protection of the environment, to pollution treatment or elimination; **white biotechnology** develops bioprocesses and substitutes

for the chemical industry; finally, **blue biotechnology** studies products related to marine biodiversity. The evolution of the biotechnology market greatly varies depending on the sector concerned. More than 50% of new pharmaceutical products stem from red biotechnology. The development of **biopesticides** as a substitute for pesticides is an example of the development of green biotechnology. While these biopesticides currently only account for between 5% and 10% of synthetic pesticides, their market is growing by about 14% per year, while the global market for synthetic pesticides has reached a plateau in recent years. In the field of white biotechnology, enzyme* production is a booming market for textile, leather, biofuel, detergence, etc. industries. The other flagship of white biotechnology is the production of bioethanol. The latter must now evolve to make use of the processing of crop residues rather than compete with food-oriented crops. Yellow biotechnology, as well as green biotechnology for that matter, are facing cost prices too high compared to concurrent chemicals and restrictions on the European market related to the use of genetically manipulated microorganisms. Finally, the exploitation of the marine microbial potential still remains in its infancy.

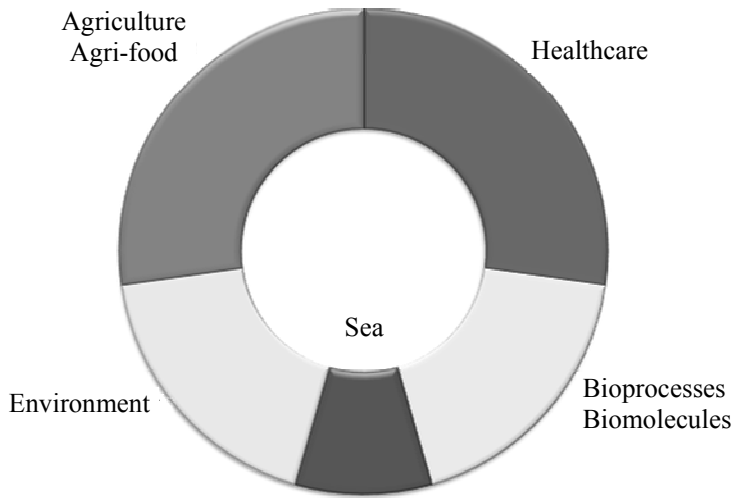


**Figure 1.3.** *Classification of biotechnologies according to a color code. For a color version of this figure, see www.iste.co.uk/dalpont/process2.zip*

### 1.2.1. *Fermentations in agri-food*

Industrial microbiology finds its origins in exploiting microorganisms for the processing and storage of products of plant or animal origins. Indeed, many microorganisms convert carbohydrates into organic acids (lactic fermentation for example) or alcohol (alcoholic fermentation). Other bacteria are also capable of

developing what is known as alkaline fermentation, which aims to produce ammonia from the degradation of protein sources. The various bacteria and yeasts involved in these processes have for many centuries been exploited through the use of part of the fermented product in which they are contained to inoculate a fresh product and repeat the transformation phenomenon. The needs of process standardization have led companies to use microorganisms themselves produced in dedicated companies to obtain reproducible fermentations that meet the quality criteria of the food sector. These products are now known as **starter cultures**, and they are living microorganisms sold in liquid or dry form and capable of starting a fermentation process. The quintessential example of this development is baking yeast (*Saccharomyces cerevisiae*) which is today the product based on the world's most produced living biomass of microorganisms. Other starter cultures are used in agri-food, such as lactic bacteria which ensure the fermentation of lactose of milk in lactic acid and constitute the basis of the production of numerous dairy products such as yogurts or cheeses, but also meat fermentation in sausage or that of cabbage for sauerkraut. Table 1.1 includes some examples of fermented products developed around the world.

| Product name | Microorganisms involved | Agro-resource | Type of fermentation |
|---|---|---|---|
| Sauerkraut | Lactic bacteria | Cabbage | Lactic |
| Sausage | Lactic bacteria | Meat | Lactic |
| Yogurt | Lactic bacteria | Milk | Lactic |
| Cheese | Lactic bacteria | Milk | Lactic |
| Beer | Yeast | Cereals | Alcoholic |
| Wine | Yeast | Grapes | Alcoholic |
| Bread | Yeast | Cereals | Alcoholic |
| Sourdough bread | Yeasts and lactic bacteria | Cereals | Alcoholic and lactic |
| Kefir | Yeasts and lactic bacteria | Milk | Alcoholic and lactic |
| Miso | Mushroom and lactic bacteria | Cereals | Lactic |
| Netetu | *Bacillus* | Parkia biglobosa seeds | Alkaline |
| Natto | *Bacillus* | Soy | Alkaline |

**Table 1.1.** *Examples of fermented products*

### 1.2.2. *Biomass-based products*

The starter culture concept has gradually expanded to other applications, such as enzyme-producing microorganisms whose hydrolytic properties are exploitable in environments such as septic tanks, grease traps used in water purification processes or for bioremediation* of polluted soils.

Another example is that of microorganisms naturally present in so-called disease suppressing soils and therefore potentially capable of performing a controlling activity of predators in certain crops (biopesticides).

Table 1.2 includes some examples of such products.

| Name | Property | Microorganism |
|---|---|---|
| Lactic ferments | Sugar transformation into organic acids | *Lactobacillus, Lactococcus,* etc. |
| Probiotics | Easier food digestion, protection from pathogens | *Bifidobacterium* |
| Decontamination starters | Degradation of xenobiotic (polluting) substances | *Pseudomonas, Rhodococcus,* etc. |
| Degradation starters | Fat degradation | *Bacillus, Yarrowia lipolytica* |
| Biopesticide | Antagonistic activity against a plant crop predator | *Bacillus thuringiensis, Bacillus velezensis, Pseudomonas fluorescens, Beauveria* |
| Alcoholic ferments | Sugar transformation into alcohol and carbon dioxide | *Saccharomyces cerevisiae, Saccharomyces carlsbergiensis* |

**Table 1.2.** *Examples of starter cultures*

### 1.2.3. *Metabolite* based products*

The other large family of products of microbiological origin is that of biomolecules produced by microorganisms. Their very important biodiversity ranges

from simple organic acids created by the central metabolism to more or less complex polymers that act as energy storage for the cell, including the panoply of proteins or enzymes or even metabolites secondary to multiple structures and applications. An enzyme, amylase, capable of degrading starch, was one of the first metabolites produced on an industrial scale from the *Aspergillus niger* mold.

Genetic engineering techniques have also produced metabolites of plant or animal origin from genetically manipulated microorganisms. The first example is insulin, an animal hormone produced by a processed bacterium, *Escherichia coli.*

Nowadays, enzymes, metabolites and polymers of microbial origin are extremely varied. Table 1.3 takes a non-exhaustive view of their diversity by specifying their current market.

## 1.3. Traditional process for developing a product of industrial microbiology

The development of a product for industrial microbiology traditionally involves the following steps:

– **a selection stage** of a microorganism that meets a number of criteria. This organism often comes from a natural environment in which it exercises a property of interest;

– an **improvement** of the properties of the microorganism selected by a random mutagenesis or genetic manipulation approach;

– the development of a **small-scale production** and **purification** process;

– the large-scale **transfer** of this process;

– the product **formulation**.

For many decades, this strategy has been used with fairly similar tools regardless of the product or application under consideration. Today, technological developments are profoundly changing this strategy. In the rest of this chapter, these developments will be presented during the various stages of the process. Combined, they most likely constitute the typical blueprint **for the futuristic factory** in industrial microbiology.

| Ingredient type | Global market 2017 ($ millions) | Main products | Market by product ($ million) | Main application sector |
|---|---|---|---|---|
| Organic acids | 3119 | Citric acid | 1714 | Food and beverage, Pharmaceutical and cosmetics, Feed, Industrial and others |
| | | Gluconic acid | 232 | Food and beverage, Pharmaceutical and cosmetics, Industrial and others |
| | | Itaconic acid | 115 | Industrial and others |
| | | Lactic acid | 1010 | Food and beverage, Pharmaceutical and cosmetics, Industrial and others |
| | | Succinic acid | 48 | Food and beverage, Pharmaceutical and cosmetics, Industrial and others |
| Amino acids | 9271 | Glutamic acid | 3678 | Food and beverage |
| | | Lysine | 4410 | Feed, Industrial and others |
| | | Threonine | 1107 | Pharmaceutical and cosmetics, Feed |
| | | Tryptophane | 76 | Pharmaceutical and cosmetics, Feed |
| Vitamins and carotenoids | 1981 | Ascorbic acid | 954 | Food and beverage, Pharmaceutical and cosmetics, Feed, Industrial and others |
| | | Iso ascorbic acid | 125 | Food and beverage, Pharmaceutical and cosmetics, Industrial and others |
| | | Carotenoids | 270 | Food and beverage, Pharmaceutical and cosmetics |
| | | Riboflavin | 504 | Food and beverage, Pharmaceutical and cosmetics, Feed |
| Enzymes | 6287 | Carbohydrases | 1791.2 | Food and beverage, Pharmaceutical and cosmetics, Feed, Industrial and others |
| | | Proteases | 1344.4 | Food and beverage, Pharmaceutical and cosmetics, Feed, Industrial and others |
| | | Polymerases and nucleases | 932.4 | Pharmaceutical and cosmetics, Industrial and others |
| | | Lipases | 244.3 | Food and beverage, Pharmaceutical and cosmetics, Feed, Industrial and others |
| | | Phytases | 259.9 | Feed |
| | | Other enzymes | 681.9 | Food and beverage, Pharmaceutical and cosmetics, Feed, Industrial and others |
| Polysaccharides and polymers | 769 | Xanthan | 739 | Food and beverage, Pharmaceutical and cosmetics |
| Antibiotics | 2583 | Penicillin | 756 | Pharmaceutical and cosmetics, Feed |

**Table 1.3.** *Market for metabolites of microbial origin*

## 1.4. Strain selection and optimization

### 1.4.1. *Evolution of strain screening techniques*

It is very difficult to quantify the exploitable potential of microbiological life known today, but it is clear that it constitutes a tiny part of what remains to be discovered. The selection of microorganisms exhibiting certain properties thus remains one of the objectives of a large number of companies. Knowing that one gram or milliliter of organic medium can contain billions or tens of billions of microbial cells, selecting the best performing microorganism is often a laborious task which proves particularly labor intensive. However, this approach is now benefiting from advances in **robotization* and device miniaturization***.

In the past, the selection of microorganisms was primarily considered on solid medium. The objective was to develop a technique making it possible to identify a specific phenotype in a colony among tens or hundreds on a Petri* dish with an agar culture medium adapted to selection. The minimalist standard was the selection of the best strain from the analysis of 10,000 clones. The properties sought are very diverse: selection of lactic bacteria with a high acidification power by way of bringing forward a solubilization of a substrate or the evolution of pH by means of an indicator; identification of the hydrolytic properties (proteases on a casein-based medium, amylase on a starch-based medium, etc.) of antagonistic properties (inhibition of the growth of a bacterium, a yeast or a fungus (Figure 1.4)) or biosurfactant properties (colony size on an medium whose agar concentration is reduced).

The use of a robotic tool such as the **colony picker** (Figure 1.5) has allowed the automation of this approach and, speeding up the selection process or multiplying the number of clones analyzed. Using a camera, this tool is capable of analyzing changes in turbidity or the color of the environment of a colony, or even colony sizes, and is able to transfer the most efficient clones into a liquid medium for further testing or conservation. Depending on the equipment, this task can be performed at a speed of a few hundred to several thousand transplanted clones per hour.

Nonetheless, the evolution of robotization and miniaturization technologies of reactors also makes it possible today to consider the selection of microorganisms of interest on the basis of their behavior in liquid environments. The possibility of cultivating strains in microtiter plates ranging from 24 to 396 wells (**microreactors**) from a few milliliters to a few dozen microliters allows one to consider testing a large number of clones in conditions that are often closer to those that will then be used for large-scale production.

These technologies also offer a larger selection test panel. Robotization now allows, among other things, to collect, add, mix, incubate, filter and automatically detect. The result is a multitude of possibilities for analyzing the potential of the strains to be selected (Figure 1.6).

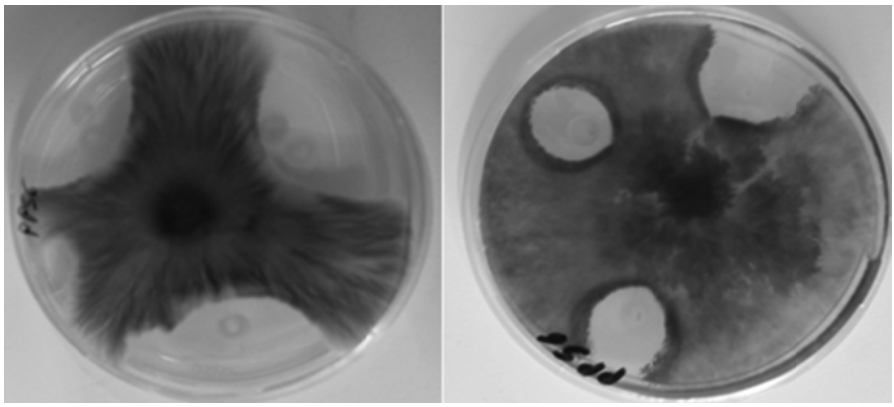

**Figure 1.4.** *Examples of selection techniques on solid medium. Research of antagonistic bacteria of the growth of a phytopathogenic fungus (*Fusarium oxysporum*) for the development of new biopesticides. For a color version of this figure, see www.iste.co.uk/dalpont/process2.zip*

**Figure 1.5.** *The colony picker is a piece of equipment that enables high-throughput screening of microbial strains possessing a particular property. It works by means of a camera that tracks colonies of microorganisms presenting a different phenotypic character (color, shape, pigment production, etc.)*


a)


b)

c)

**Figure 1.6.** *Examples of robots for microorganism culturing and the preparation of culture media*

COMMENT ON FIGURE 1.6.– *This type of equipment makes it possible to automatically prepare with a high-throughput culture media for reactors from a few hundreds of microliters to a few milliliters (c). These environments are then incubated and stirred in a device such as the "biolector" which allows for online monitoring of pH, dissolved oxygen, biomass growth and the concentration of a fluorescent probe ((a) and (b)).*

This device still naturally presents a number of limitations. For example, the potential of microbiological life in the seabed is nowadays practically unknown. Revealing it with robotic tools requires highly pressurized reactors. These devices are not yet available for high-throughput screening.

The explosion of genome sequencing of microorganisms and the improvement of bioinformatics prediction tools are now making it possible to consider new high-throughput screening* strategies. It is thus possible to search for a gene encoding a particular enzyme, allowing the expression of a particular phenotype* or a cluster of genes* encoding the enzymes responsible for the synthesis of a metabolite presenting a structure or particular structural features. This selection is thus achieved firstly *in silico**, that is to say without experimenting in a laboratory. It should enable saving valuable time in years to come.

### 1.4.2. *Evolution of genetic modification technologies, from random mutagenesis* to CRISPR-Cas9 technology*

The optimization of microbial strains has, for many years, been based on the use of random mutagenesis* obtained through cell treatment by irradiation (ultra-violet, X-rays, etc.) or by the use of chemical mutagenic agents (N-methyl-N'-nitro-nitrosoguanidine (NTG), ethyl methanesulfonate (EMS), etc.). Overproducing penicillin strains obtained after successive treatments of *Penicillium chrysogenum* by these different physical or chemical agents and the selection of hyperproducing mutants is a well-known example thereof. It is estimated that some twenty successive mutation stages have led to an increase from an antibiotic production of 60 mg/l to 7 g/l which corresponds to a productivity 100 times higher.

The advent of genetic engineering techniques and the in-depth knowledge of metabolic pathways of metabolite* biosynthesis of interest have gradually enabled a shift towards the targeted strain optimization by modifying the expression of certain genes (overexpression of an enzyme by using strong promoters*, for example) or by interrupting the latter. These approaches have led to metabolic engineering, which consists of directing the metabolism of a given strain in order to make it produce the maximum amount of metabolite under consideration. The latter makes use of the evaluation of metabolic flows that characterizes in moles/kg of dry matter and per hour the specific transformation rate of each reaction substrate in its product.

Three dimensions have recently revolutionized these approaches. On the one hand, advances in genetic techniques are now allowing genetic modifications to be multiplied while eliminating the selection markers used at the end of the operation (auxotrophy*, antibiotic resistance, etc.). Technologies such as **CRISPR-Cas9** (Clustered Regular Interspaced Short Palindromic Repeats) also make it possible to

limit genetic modifications to their strict minimum. These tools, which allow for minimal genome limitation without the addition of genes or foreign DNA to the strain, could ultimately constitute a response to requests for a reduced use of GMOs. A second dimension is the biology of systems, which consists of encompassing and modelling as many phenomena as possible occurring within a cell or a population of cells to optimize their functioning as part of a given objective. These models allow, with a number of strains well-controlled at the industrial scale such as *Escherichia coli*, *Bacillus subtilis* or bacteria or yeasts such as *Saccharomyces cerevisiae*, *Pichia pastoris* or *Yarrowia lipolytica*, to provide a global view of their metabolism or the regulation thereof and to define targeted modification strategies of their genetic heritage. A third approach is that of synthetic biology* which aims to induce the production by a given microorganism of a metabolite that it was unable to synthesize by transferring into this microorganism the genes encoding the enzymes responsible for the entire metabolic pathway necessary for this synthesis. Another approach to synthetic biology is one that consists in defining the minimum genome necessary to the growth of a microorganism in a given environment by eliminating all non-essential genes to cell life. This approach should eventually define "microbial chassis*" ready to synthesize metabolites of different origins. These microbial chassis could be adapted to a particular inexpensive substrate.

## 1.5. Production and purification processes

### 1.5.1. *Needs of microorganisms*

The growth of a microorganism and/or the production of a metabolite of interest depend heavily on the environment in which the microorganism is located. The first need therefore is to have access to an energy source. For a certain number of microorganisms exploited in industrial microbiology, this energy is drawn from the oxidation of an organic substrate such as glucose throughout a process called respiration*: they are called chemoorganotrophic* organisms. For so-called aerobic microorganisms, the final electron acceptor is oxygen. The latter is not very water-soluble (7.55 mg/l at 30$^{o}$C and 1 bar), it is the transfer rate from a gas phase to a liquid phase that often constitutes a limiting factor. For optional anaerobic microorganisms (namely those that are able to develop in the absence and presence of oxygen), the electron acceptor is an organic molecule. Finally, strict anaerobic microorganisms use a mineral molecule (nitrate, sulphate, etc.) as an electron acceptor. Chemolithotrophic* organisms draw their energy from the oxidation of a mineral substance. Finally, the third major source of energy used is light for photo-organotrophic* organisms.

The second essential element is to be able to extract from the culture medium all the elements (C, H, O, N, P and S) necessary to manufacture the set of monomers

(amino acids, monosaccaride, fatty acids, puric and pyrimidic bases, vitamins, etc.) and polymers (proteins, polysaccharides, lipids, nucleic acids, etc.) that compose a microorganism. In addition, there is a certain number of trace elements necessary to ensure ion and osmotic equilibrium in the cell but also the realization of a number of reactions that require their presence as a cofactor. They are often classified according to the needs of the cell, in major (Na, K, Cl, Mg, Mn, etc.) and minor (Fe, Cu, Co, etc.) trace elements. Some microorganisms may merely require a source of organic carbon and different mineral salts containing nitrogen, phosphorus, sulfur and trace elements to develop. Others require a much richer medium in organic molecules that they have lost the ability to manufacture on their own. Photosynthetic microorganisms such as microalgae are able to use $CO_2$ as a carbon source. Finally, every strain of microorganism develops within a pH and temperature range that is specific to it. This mix of different information conditions the environment in which microorganisms are able to develop optimally and thus the processes developed to produce them.

### 1.5.2. *Production processes*

Depending on the nature of the microorganism produced and its application, production processes will have to meet all or part of the following criteria:

– allow a pure microorganism culture or a controlled culture of a microbial consortium;

– ensure a controlled supply of oxygen or $CO_2$ in gas form;

– allow pH control;

– allow temperature control;

– allow continuous substrate feeding;

– allow the control of foam formation;

– ensure adequate contact with a light source (for microalgae cultures);

– ensure a homogeneous mixture of a system containing three phases: a solid phase (microorganisms), a liquid phase (the culture medium) and a gas phase (compressed air, oxygen or carbon dioxide).

Inherited from chemical engineering, the traditional bioreactor used today in the vast majority of bio-industries meets the majority of these criteria. In short, its mirror-polished stainless-steel structure allows it to avoid the adhesion of microorganisms on the reactor walls while ensuring resistance to the steam sterilization process of the equipment. Equipped with a heat exchanger (usually a double envelope) to maintain the temperature, it possesses a gas supply system (air, oxygen, $CO_2$, etc.) most often

equipped with a sterilizing filter and a mixing system that ensures a homogeneity of the three phases existing within the bioreactor. This system also plays an important role in optimizing the transfer of oxygen or $CO_2$ from gas bubbles to the culture medium. The reactor also includes a sample collection valve, a drain valve, as well as one or two valves for adding liquid. An air outlet often equipped with a sterilizing filter ensures the evacuation of gases. A number of sensors allow the online monitoring of certain parameters such as pH, temperature, dissolved oxygen concentration, volume, presence of foam, etc. Some of these parameters can be regulated. These sensors are therefore connected to an automat which, under the control of supervising software, sends messages to actuators, which are most often valves.

Since microalgae culturing requires a significant amount of light, it employs very different types of reactors. These latter must ensure a maximum transfer of photons and are therefore often based on maximal exposure (with a large surface-to-volume ratio) of culture to light. In addition, the absence of carbon source in the medium reduces the risk of contamination and therefore makes it possible for some productions of open-pit cultures to be considered.

Three traditional cultivation techniques (Figure 1.7) are being considered in industrial microbiology. In the so-called "batch" culture, a bioreactor containing all the elements necessary for the growth of a microorganism and/or the production of a metabolite is seeded by a preculture (inoculum). No additional culture medium is added or extracted during production. This simple process is often the one used in a first production approach. In continuous cultivation, fresh culture medium is continuously brought and the fermentation medium is also extracted with an identical flow to ensure a constant culture volume. The dilution rate used imposes the growth rate of the microorganism if it is lower (chemostat-type process) or equal (turbidostat-type process) to the maximum growth rate of the microorganism. This process is relatively unused at the industrial level. In addition to technical and logistical difficulties (maintaining the sterility of a complex process and the need for continuous monitoring), it poses a significant risk for certain strains of genetic drift following the appearance of spontaneous mutants potentially better adapted to the culture mode. Rather, it is used at the laboratory level to better understand the physiological behavior of a given microbial strain. The third process, "fed-batch", is therefore the one traditionally employed by manufacturers to ensure increased productivity compared to batch culture. It consists of ensuring a gradual feeding of the culture medium without removing culture broth. It can be used to avoid too high a concentration of substrate in the culture medium, which would directly or indirectly cause a potential inhibition of the microorganism growth (such as in the production of bakery yeast). The other application of this device is the possibility of ensuring an optimal growth in batch conditions for a given microorganism and in order to then bring it under different culturing conditions, by adding a particular medium, to optimally produce a metabolite of interest (such as for the production of penicillin).

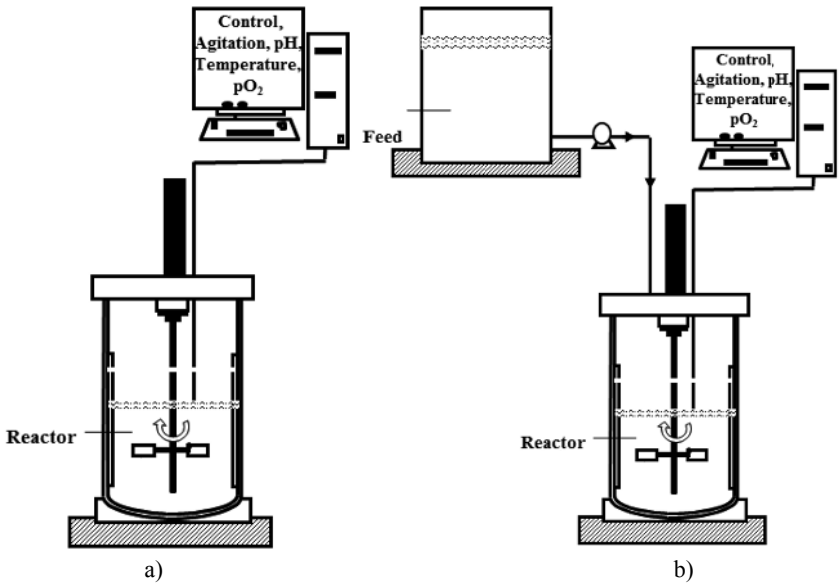


**Figure 1.7.a.** *Conventional bioreactor culture processes: a) batch culture; b) fed-batch culture*

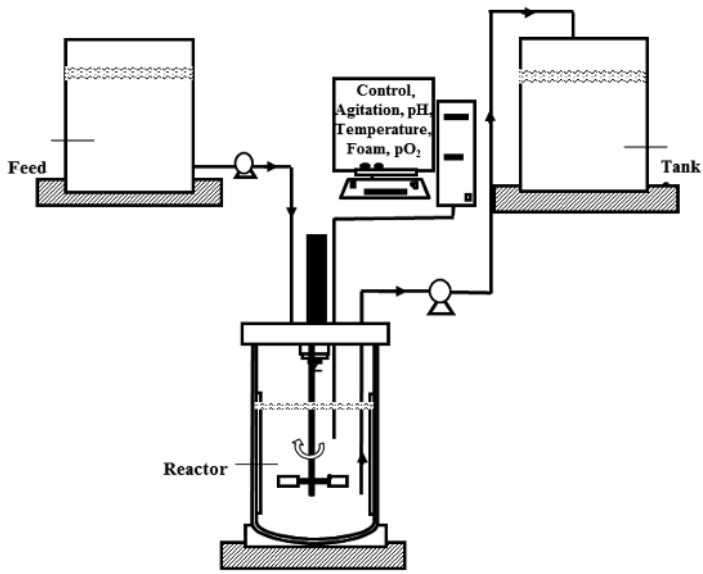


**Figure 1.7.b.** *Conventional bioreactor culture processes: continuous culture (continued)*

In order to ensure process scaling up (Figure 1.8), a number of equations making use of adimensional figures and the theory of similarities have been developed. The utilization of these equations requires that a standard reactor geometry and their agitation system be satisfied (see Chapter 5 of the first volume). The main adimensional numbers considered are Reynolds (action of viscosity forces), power (action of inertia forces), Froude's (gravity force action), and Weber's numbers (action of surface tension forces), etc. However, scaling up does not make it possible to satisfy the constraints of the process in an equivalent way according to the volume. This leads to a choice for engineers that will have consequences on the performance of their large-scale processes. A good example is the need to maintain a high oxygen transfer while limiting shear forces harmful to microorganisms. This dilemma is particularly acute for filamentous microorganisms that are most often more sensitive to shear forces. These difficulties associated with upscaling have, in a number of cases, resulted in abandoning very promising small-scale projects that are unfortunately difficult to transfer to larger scales.

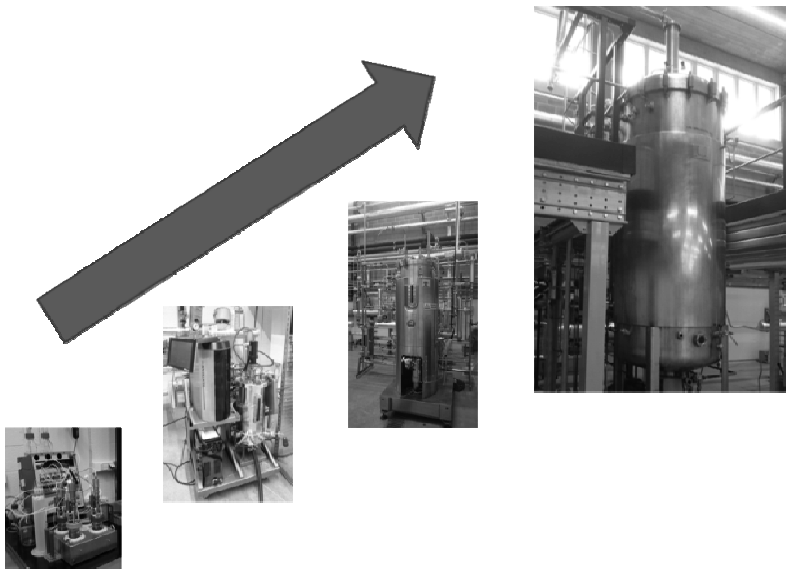

**Figure 1.8.** *Upscaling of the bioreactor culturing process (200 ml to 2 $m^3$)*

### 1.5.3. *Downstream fermentation processes (downstream processing, DSP)*

Downstream fermentation processes are dependent on the nature of the product to be recovered (cells, intracellular or extracellular metabolites) and its application.

A first solid-liquid separation step is always required. As a general rule, it makes use of sedimentation, accelerated (centrifugation) or not, or filtration. When the product to be recovered is intracellular, an operation for bursting cells is necessary: their destruction may be mechanical, by solid (namely grinding) or liquid (such as pressure difference), or non-mechanical constraints (physical, chemical or enzymatic lysis).

Subsequently, a number of unit operations also used in chemical engineering (see Chapter 5 of the first volume) can be chained up to yield a more or less pure product in dry or non-dry form (extraction, precipitation, ultrafiltration, chromatography, drying, etc.).

### 1.5.4. *Coupled procedures*

The first unit purification operations can, in a number of processes, be directly coupled to production and thereby improve productivity. This may include, for example, the continuous extraction of a compound that is toxic to the cell or likely to be rapidly degraded in the culture medium. A fine example of a coupled process is the membrane-based bioreactor (Figure 1.9). In such a device, the reaction medium is in permanent contact with a filter membrane (immersed into the bioreactor or connected through an external circulation loop) that allows the continuous extraction of the culture medium free of its cells (microfiltration) or of the latter as well as molecules larger than the cut-off threshold of the membrane (ultrafiltration).

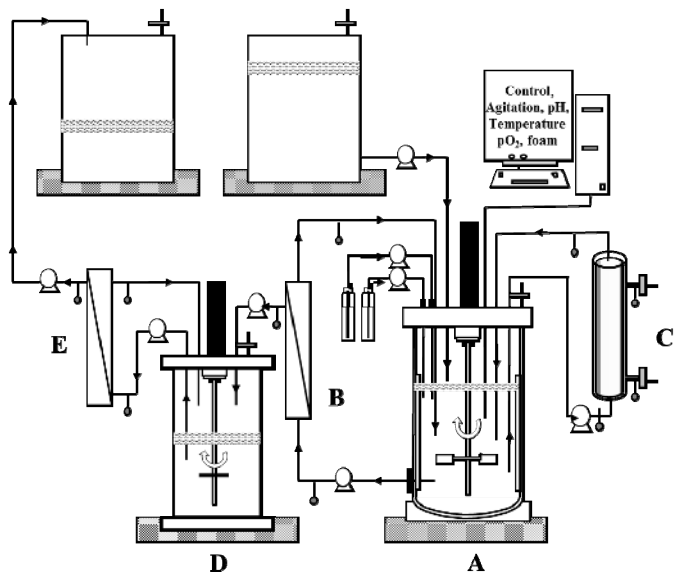


**Figure 1.9.** *Coupled membrane process for biosurfactant production*

COMMENT ON FIGURE 1.9.– *Microorganisms are cultured in a first reactor (A) connected to a microfiltration membrane (B). The latter allows the extraction of the biosurfactant produced continuously. The bioreactor aeration is achieved by way of an air-liquid membrane contactor (C) which severely restricts the presence of foam due to the biosurfactant (absence of bubbles in the bioreactor). Finally, the microfiltrate recovered from an ancillary tank (D) is purified by ultrafiltration on a membrane (E) with a cut-off threshold of 10,000 daltons that lets small molecules pass through, but not micelles formed by the biosurfactant*.

### 1.5.5. *Microfluidic* intake (scale-up/scale-down)*

Microfluidics is gradually invading the industrial microbiology sector. Its use has several vocations: to miniaturize bioreactors to facilitate high-throughput studies of optimal culture conditions or the screening of mutants as mentioned above, to study the behavior of cells individually and to collect the most efficient cells.

The development of **optodes**, which are miniaturized sensors based on the emission of a light source according to the variation of a culture parameter such as pH or dissolved oxygen concentration, has enabled the development of miniature bioreactors of a few hundred microliters. In addition, advances in molecular biology are now generating genetically modified strains in which genes encoding fluorescent proteins are placed downstream of a promoter involved in the expression of a gene of interest. This “reporter gene” logic is a means to follow the expression of a given gene in different circumstances by a global measure of the fluorescence emitted by all cells in culture. The behavior of each cell can be analyzed using flow cytometry. This technique, also used to count cells in culture, enables the analysis of the morphology or of the phenotypic behavior of every cell by making them pass through a very fine capillary in the beam of a laser. Microfluidics, in connection with time-lapse microscopy, allows one to follow the development of a colony from a single cell and the evolution of a possible phenotypic heterogeneity based on this mother cell. These very promising technologies have made it possible, in recent years in particular, to add to the problem of physical and chemical heterogeneity of the cell environment in a bioreactor, a dimension of cellular heterogeneity.

### 1.5.6. *Process intensification*

The scaling-up constraints mentioned above, the progress of small-scale tools and the high productivity achieved through cell optimization are likely to completely revolutionize the bio-industry of the future. One of the solutions increasingly envisaged is the multiplication of small volume production tools, operated in parallel

and in which process optimization has been validated. This approach could increase the success rate of transferring bioprocesses from the pilot lab scale to the company's scale.

## 1.6. Innovative concepts

### 1.6.1. *Biofilm reactors*

The development of immobilized cell reactors is a fairly old concept that has been used for many years in industrial microbiology. In a number of cases, these reactors provide increased productivity for a metabolite or enzyme of interest. They also facilitate the development of continuous processes for the permanent extraction of a culture medium virtually devoid of cells. Conventional approaches used are fixation on a support by adsorption, encapsulation in gel or the exploitation of the natural or induced formation of cellular aggregates. In recent years, the discovery and characterization of biofilm growth of a number of microorganisms has led to the development of a new approach to processes that incorporate this differentiated form of growth. Although still complex to control, the formation of biofilm provides a natural form of cellular immobilization that responds in nature to a number of stresses. In the long run, biofilms may eventually be of interest, particularly in the production of certain secondary metabolites.

### 1.6.2. *Mixed cultures and cascades of microorganisms*

For many years, the logic of pure, perfectly controlled culture has predominated in most industrial microbiology processes. Advances achieved in microbial ecology and in characterizing microbial ecosystems* are paving the way for a new era that could eventually create more resilient processes, integrating a microbial community rather than a pure microorganism. Another evolution in microbial processes is the development of cascades of microorganisms with, in particular, different but complementary enzymatic capabilities for obtaining a particular product.

## 1.7. Towards a digital bio-industry

The biodiversity of microorganisms and the complexity of their operating mode are driving forces underlying the development of digital technology in bio-industry (see also Chapter 5 of this volume on the impact of digital transition on industry). Both involve the creation of databases that gather information collected due in particular to the explosion of genomics*, proteomics*, transcriptomics*, etc. These

data also constitute a source that feeds into numerous increasingly complex models based on biostatistical analyses or learning techniques. It should lead to the possibility of predicting the behavior of a microorganism in a given environment and thus controlling that behavior. This is notably the subject of **systemic biology*** which attempts to include as much information available as possible into these models (Figure 1.10).

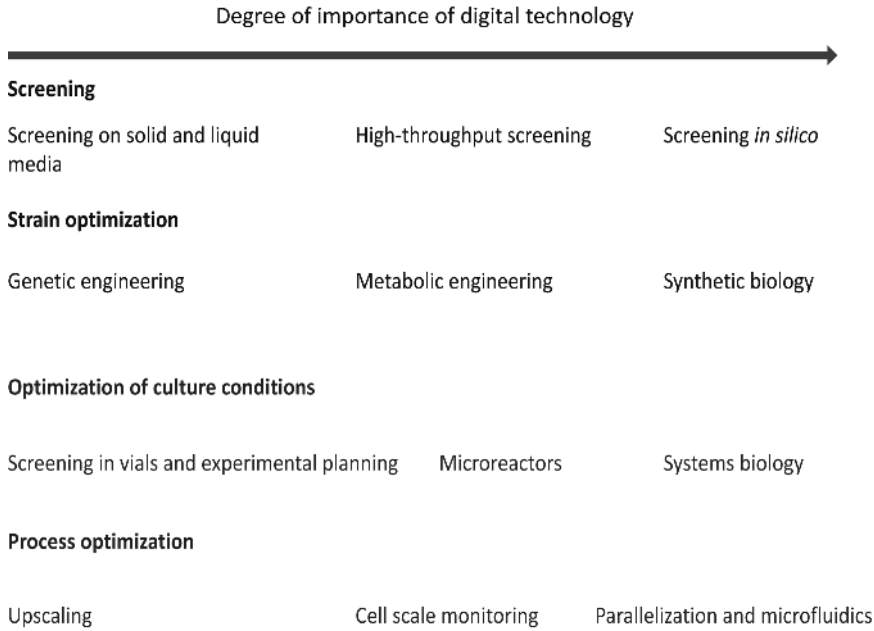


**Figure 1.10.** *Evolution of approaches to industrial microbiology and the significance of digital technology*

## 1.8. Acknowledgements

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## 1.9. Glossary

### *Archaebacteria*

Or *Archaea*, formerly known as archaeobacteria, are prokaryote single-celled microorganisms, or more specifically living beings composed of a single cell that does not include a nucleus or organelles, like bacteria.

### *Auxotrophy*

The inability of a living organism to synthesize an organic compound necessary for its development. that is characterized by this defect. Conversely, the term prototrophy is employed.

### *Bacteria*

Single-celled organisms, with a very simple structure, devoid of nucleus and organelles, with diffuse genetic material, usually without chlorophyll and reproduces by scissiparity.

### *Biopesticide*

A living organism or one of its derivatives capable of controlling the activity of a pathogen organism or that is harmful to plants. It works by reducing inoculum and/or interfering with one or more stages of the infectious cycle of the phytopathogen.

### *Bioprocess*

Bioprocesses refer to, according to the most widely accepted and shared definition in the field of biotechnology, all the implementations of living systems, or their constituents, for the production of knowledge, goods or services.

### *Bioremediation*

A technique consisting of increasing biodegradation or biotransformation, by inoculating specific microorganisms (bio-augmentation) or by stimulating the activity of indigenous microbial populations, by biostimulation, by nutrient intake and by adjusting the conditions of the medium.

### *Biostimulant*

In the areas of human or animal health and agriculture, a biostimulant or plant defense stimulant (PDS) is a product for the preventive stimulation of the activity of an organism (vegetable in the case of PDS) or for the stimulation of its immune system.

### *Biosurfactant*

An active biological compound of detergents that dissolves or suspends stains adhering to surfaces. It promotes humidification, solubilization and the emulsion of organic compounds.

### *Chemolithotroph*

A chemolithotrophic organism, typically a bacterium, uses accordingly an organic molecule or mineral compound as energy source (chemosynthesis) and oxidizing mineral compounds such as $NO_3$ nitrates, $O_2$ dioxygen, etc., as electron donors.

### *Chemoorganotroph*

An organism that derives its energy from the metabolism of an organic substrate (sugar, amino acid, fatty acid, etc.).

### *Deoxyribonucleic acid (DNA)*

A nucleic acid characteristic of chromosomes, consisting of two strands coiled in double helix and each formed by a succession of nucleotides. As a carrier of genetic information, DNA ensures the control of cell activity.

### *Ecosystem*

A system formed by an environment (biotope) and by the set of species (biocenosis) that live, feed and reproduce therein.

### *Enzyme*

A protein that accelerates chemical reactions.

### *Fungi*

A group that includes single-celled eukaryotic organisms (yeasts) or multicellular organisms (molds) of microscopic size as well as macroscopic fungi.

### *Gene*

A segment of DNA that conditions the synthesis of one or more proteins and thus the manifestation and transmission of a specific hereditary character.

### *Gene cluster*

A set of genes encoding enzymes involved in the synthesis of the same molecule.

### *Gene expression*

Refers to all of the biochemical processes by which the hereditary information stored in a gene is read to lead to the production of molecules that will have an active role in cellular functioning, such as proteins.

### *Genomics*

Genomics is the science of genomes. It studies DNA sequences in living beings. A genome is made up of the set of genetic information contained in the cell. For example, in a human cell, the genome is composed of information carried by the 23 pairs of chromosomes of the nucleus as well as by the DNA present in mitochondria. Genomics helps, for example, to better understand the diversity of life, build phylogenetic trees or identify genes associated with diseases.

### In silico

Standing for “in silicon”, *in silico* is a Latin-inspired neologism designating research or trials performed by means of complex computerized calculations or computer-based models. This expression is mainly used in the fields of genomics and bioinformatics.

### In vitro

Used to describe an experiment carried out in an artificial medium, in the laboratory.

### *Lactic acid ferments*

Lactic acid ferments are bacteria used for processing milk into dairy products, such as yogurt and cheese, but also for processing other foods.

### *Metabolite*

A metabolite is an intermediate organic compound or it originates from the metabolism. This term is generally reserved for small molecules and monomers, as opposed to macromolecules. Therefore, unlike glycogen, glucose is a metabolite which is a polysaccharide of very high molecular weight.

### *Metabolomics*

Metabolomics is a very recent science that studies all primary and secondary metabolites in the case of plants present in a cell, organ or organism. This is the equivalent of genomics for DNA.

### *Microalgae*

The term microalgae, sometimes called a microphyte, refers to microscopic algae. Microalgae have been consumed around the world for thousands of years. For example, traces of the consumption of various species of microalgae in Mexico during the Aztec period have been found.

### *Microbial chassis*

A model microorganism genetically manipulated to be able to produce different types of heterogeneous metabolites.

### *Microfluidics*

Microfluidics is the science and technology of fluid-handling systems, of which at least one of the characteristic dimensions is in the order of a micrometer.

### *Miniaturization*

The creation of mechanical, optical or electronic products and their devices at increasingly smaller scales.

### *Mold*

A small-sized fungus that causes a chemical modification in the medium on which it grows.

### *Mutagenesis*

Action of modifying the sequence of a gene.

### *Petri dish*

A box of glass or plastic material used in bacteriology for culturing on solid media.

### *Phenotype*

In genetics, the phenotype is the set of all observable characteristics of an organism. Very often, the use of this term is more restrictive: the phenotype is considered at the single character level, at the cellular or even molecular level.

### *Photo-organotrophic*

Photo-organotrophic organisms are photosynthetic bacteria that utilize organic molecules as a carbon source, an organic molecule as an electron source for reducing this carbon, and light as an energy source (some cyanobacteria, for example).

### *Promoter*

A genetic sequence located downstream of a gene and that manages its expression.

### *Proteomics*

The study of proteins existing in a cell or tissue, for the purpose of identifying those that are, for example, specific to a pathology. Its applications concern in particular medical diagnostics, biotechnological and pharmaceutical research.

### *Respiration*

A set of phenomena that enable oxygen absorption and the release of carbon dioxide from living beings.

### *Robotization*

The substitution of human operators by robots for performing industrial tasks. The action of replacing someone's reasoned behaviors with automatisms.

### *Screening*

Techniques in the field of pharmacology, biochemistry, genomics and proteomics techniques that aim to study and identify in chemical libraries and target libraries, molecules biologically active that present new properties.

### *Strains*

A set of cells derived from the same clone and thus presenting the typical characteristics of the original bacterium or virus.

### *Synthetic biology*

Synthesis biology, or synthetic biology, is an emerging scientific and biotechnology field that combines biology and engineering principles, with the aim of designing and building new living organisms.

### *Synthon*

A "fictitious molecule" whose representation symbolizes reactivity. This concept is used when planning for the retrosynthesis of complex organic compounds. They are derived from a simple fragmental decomposition of the molecule which is to be

assembled. In biotechnology, synthons are "building blocks" that could be used for the biosynthesis of biopolymers.

### *Systemic biology*

The biology of systems (or systemic biology, or integrative biology) is a recent field of biology that studies living organisms as systems since that is what they actually are, as opposed to historical approaches that tend to break down the study at every level, into biology, physiology, biochemistry, etc.

### *Transcriptomics*

A study of all messenger RNAs produced during the process of genome transcription. It relies on the systematic quantification of these mRNA, which makes it possible to obtain a relative indication of the transcription rate of different genes under given conditions.

### *Yeast*

Eukaryotic microorganism member of the fungus kingdom. The most famous industrial yeast, *Saccharomyces cerevisiae*, is mainly used for its ability to transform carbohydrates into carbon dioxide and alcohols. It is used for baking and the production of alcoholic beverages.

## 1.10. References

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