Master “Biotechnology and Entrepreneurship”

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Contents

1. An introduction to Biotechnology

Biotechnology has been defined as the science using living systems, organisms, their parts or by-products to develop or make products intended to improve the quality of humanity life. Humans have been practiced biotechnology for thousands of years to make food such bread and cheese, to produce wine, or to obtain medicals products derived from plants, but in the last decades, biotechnology has experienced an impressive growth throughout the world.

The economy of the 21st century has to face important challenges regarding growing global population, climate changes and threats related to environmental protection. According to United Nations Report (2017), the current world population of 7.6 billion is estimated to increase every year with about 83 million people. The needs of this growing population can no longer be supported only by traditional primary and secondary business sectors based on finite or temporally limited resources of food and fuel. Population growth and reduction of available resources, problems arising from the use of traditional physical and chemical technologies, more complicated health problems and accelerated environmental degradation has caused the economic media and the policymakers to focus their attention on biotechnology as hopeful and promising solution for sustainable development.

Biotechnology is offering modern solutions for almost every aspect of human life: economic, social, health and environment. It promotes sustainable economic growth, increasing productivity and diversity, lowering by-products and wastes generation. Modern diagnostic approaches, therapeutic solutions, vaccines and other pharmaceutical products are generated by biotechnology. These achievements are intended to increase the survival rate and to lower the resources and pain associated with a non-suitable treatment. Biotechnology is offering also solutions for producing food enriched with specific nutrients, with significant contribution to a proper human health condition and even to malnutrition. Microbial processes are successfully used for improving the environmental quality by biodegradation and bioremediation. Economic prosperity is expected in rural areas or in developing countries based on agriculture, as well as in developed economies where biotechnology engender “high-tech” solutions.

It is generally accepted the classification of biotechnology sectors and their associated outputs, as it is presented in Table 1.

Table 1. Classification of biotechnology sectors (after DaSilva, 2004)

Sectors of Biotechnology	**Outputs**
Green Biotechnology (Agriculture/Plant Biotechnology)	Improvement of plants characteristics, as resistance to disease or hard environmental conditions, tolerance for herbicides, higher production yields, biofuels, biofertilizers
Yellow Biotechnology (Food & Nutrition Biotechnology)	Fermented food, enzymes and other active substances used in food industry
White Biotechnology (Industrial biotechnology)	Design and production of new plastics/textiles and the development of new sustainable energy sources such as bio-fuels
Grey Biotechnology (Environmental biotechnology)	Focused on the maintenance of biodiversity and the removal of pollutants/contaminants using microorganisms and plants to isolate and dispose of different substances such as heavy metals and hydrocarbons; biomass production for biofuel
Red Biotechnology (Pharmaceuticals, Diagnostics, Health)	Vaccines and antibiotics, developing new drugs, molecular diagnostics techniques, regenerative therapies and the development of genetic engineering to cure diseases through genetic manipulation
Blue Biotechnology (Aquaculture, Marine Biotech)	Engineered organisms from marine environment (algae, protozoa)

2. General microbiology

Microbiology means the study of microorganisms, those being unicellular (single cell), multicellular (cell colony), or acellular (lacking cells). The microorganisms are grouped in two main domains: **Prokaryote** (includes the group of Archaeabacteria - "ancient bacteria" and Eubacteria) and **Eukaryota** (includes all multicellular organisms and many unicellular protists and protozoans). Some protists are related to animals and some to green plants. Many of the multicellular organisms are microscopic, namely micro-animals, some fungi and some algae.

While some fear microbes due to the association of some microbes with various **human diseases**, many microbes are also responsible for numerous beneficial processes such as **industrial fermentation** (e.g. the production of alcohol, vinegar and dairy products), antibiotic production and act as molecular vehicles to transfer DNA to complex organisms such as plants and animals. Scientists have also exploited their knowledge of microbes to produce biotechnologically important **enzymes** such as Taq polymerase, reporter genes for use in other genetic systems and novel molecular biology techniques such as the yeast two-hybrid system. Also, bacteria can be used for the industrial production of **amino acids**. *Corynebacterium glutamicum* is one of the most important bacterial species with an annual production of more than two million tons of amino acids, mainly L-glutamate and L-lysine. Since some bacteria have the ability to synthesize

antibiotics, they are used for medicinal purposes, such as *Streptomyces* to make aminoglycoside antibiotics.

A variety of **biopolymers**, such as polysaccharides, polyesters, and polyamides, are produced by microorganisms; such biopolymers have tailored properties suitable for high-value medical application such as tissue engineering and drug delivery. Microorganisms are for example used for the biosynthesis of xanthan, alginate, cellulose, cyanophycin, poly(gamma-glutamic acid), hyaluronic acid, organic acids, oligosaccharides polysaccharide and polyhydroxyalkanoates.

Meanwhile, the microorganisms are beneficial for microbial **biodegradation or bioremediation** of domestic, agricultural and industrial wastes and subsurface pollution in soils, sediments and marine environments. The ability of each microorganism to degrade toxic waste depends on the nature of each contaminant. Since sites typically have multiple pollutant types, the most effective approach to microbial biodegradation is to use a mixture of bacterial and fungal species and strains, each specific to the biodegradation of one or more types of contaminants.

Symbiotic microbial communities confer benefits to their human and animal hosts health including aiding digestion, producing beneficial vitamins and amino acids, and suppressing pathogenic microbes. Some benefit may be conferred by eating fermented foods, probiotics (bacteria potentially beneficial to the digestive system) or prebiotics (substances consumed to promote the growth of probiotic microorganisms). The ways the microbiome influences human and animal health, as well as methods to influence the microbiome are active areas of research.

Prokaryote

Archaea

Archaea are prokaryotic unicellular organisms. A prokaryote is defined as having no cell nucleus or other membrane bound-organelle. Archaea share this defining feature with the bacteria with which they were once grouped.

Archaea differ from bacteria in both their genetics and biochemistry. Archaea possess genes and several metabolic pathways that are more closely related to those of eukaryotes, notably for the enzymes involved in transcription and translation. Archaea reproduce

asexually by binary fission, fragmentation, or budding; unlike bacteria and eukaryotes, no known species forms spores.

Archaea were originally described as extremophiles living in extreme environments, such as hot springs, but have since been found in all types of habitats. Only recently, scientists beginning to realize how common archaea are in the environment, with *Crenarchaeota* being the most common form of life in the ocean, dominating ecosystems below 150 m in depth. These organisms are also common in soil and play a vital role in ammonia oxidation.

The combined domains of archaea and bacteria make up the most diverse and abundant group of organisms on Earth and inhabit practically all environments where the temperature is below +120 °C. They are found in water, soil, air, as the microbiome of an organism, hot springs and even deep beneath the Earth's crust in rocks; they may play roles in the carbon cycle and the nitrogen cycle. No clear examples of archaeal pathogens or parasites are known. Instead they are often mutualists or commensals, such as the methanogens (methane-producing strains) that inhabit the gastrointestinal tract in humans and ruminants, where their vast numbers aid digestion. Methanogens are also used in biogas production and sewage treatment, and biotechnology exploits enzymes from extremophile archaea that can endure high temperatures and organic solvents.

Bacteria

Bacteria (know also of Eubacteria) are prokaryotic – unicellular, and having no cell nucleus or other membrane-bound organelle. Bacteria function and reproduce as individual cells, but they can often aggregate in multicellular colonies. Some species such as myxobacteria can aggregate into complex swarming structures, operating as multicellular groups as part of their life cycle, or form clusters in bacterial colonies such as *E.coli*.

Their genome is usually a circular bacterial chromosome – a single molecule of DNA, although they can also harbour plasmids. Some plasmids can be transferred between cells through bacterial conjugation. Bacteria have an enclosing cell wall, which provides strength and rigidity to their cells. They reproduce by binary fission or sometimes by budding, but do not undergo meiotic sexual reproduction. However, many bacterial species can transfer DNA between individual cells by a horizontal gene transfer, process referred to as natural transformation. Some species form extraordinarily resilient spores, but for bacteria this is a mechanism for survival, not

reproduction. Under optimal conditions bacteria can grow extremely rapidly and their numbers can double as quickly as every 20 minutes.

Importance for technology and industry. A large number of bacterial species are involved in natural degradative processes or are used for fermentations, for obtaining important products. For example, lactic acid bacteria, such as *Lactobacillus* and *Lactococcus*, in combination with yeasts and moulds, have been used for thousands of years in the preparation of fermented foods, such as cheese, pickles, soy sauce, sauerkraut, vinegar, wine, and yogurt.

The ability of bacteria to degrade a variety of organic compounds is remarkable and has been used in waste processing and bioremediation. Bacteria capable of metabolizing the hydrocarbons are often used to clean up oil spills. Fertiliser was added to some of the beaches in an attempt to promote the growth of these naturally occurring bacteria after some oil spill. Bacteria are also used for the bioremediation of industrial wastes. In the chemical industry, bacteria are most important in the production of enantiomerically pure chemicals for use as pharmaceuticals or agrichemicals.

Some bacteria can also be used as biopesticides in the biological pest control. This commonly involves *Bacillus thuringiensis* (also called BT), a Gram-positive, soil dwelling bacterium. Subspecies of this bacteria are used as a Lepidopteran-specific insecticides. Because of their specificity, these pesticides are regarded as environmentally friendly, with little or no effect on humans, wildlife, pollinators and most other beneficial insects.

Because of their ability to quickly grow and the relative ease with which they can be manipulated, bacteria are the workhorses for the fields of molecular biology, genetics and biochemistry. By making mutations in bacterial DNA and examining the resulting phenotypes, scientists can determine the function of genes, enzymes and metabolic pathways in bacteria, then apply this knowledge to more complex organisms. This aim of understanding the biochemistry of a cell reaches its most complex expression in the synthesis of huge amounts of enzyme kinetic and gene expression data into mathematical models of entire organisms. This is achievable in some well-studied bacteria, with models of *Escherichia coli* metabolism now being produced and tested. This understanding of bacterial metabolism and genetics allows the use of biotechnology to bioengineer bacteria for the production of therapeutic proteins, such as insulin, growth factors, or antibodies.

Eukaryotes

Most living things that are visible to the naked eye in their adult form are eukaryotes, including humans. However, a large number of eukaryotes are also microorganisms. Unlike bacteria and archaea, eukaryotes contain organelles such as the cell nucleus, the Golgi apparatus and mitochondria in their cells, as well as other organelles. The nucleus ~~is an organelle that~~ houses the DNA that makes up a cell's genome. DNA (Deoxyribonucleic acid) itself is arranged in complex chromosomes. Mitochondria are organelles vital in metabolism as they are the site of the citric acid cycle and oxidative phosphorylation; they contain specific DNA, named mitochondrial DNA, and ribosomes.

Unicellular eukaryotes consist of a single cell throughout their life cycle. This qualification is significant since most multicellular eukaryotes consist of a single cell called a zygote only at the beginning of their life cycles. Microbial eukaryotes can be either haploid (half the total number of chromosomes in a cell) or diploid (cell that contain two copies of each chromosome), and some organisms have multiple cell nuclei.

Unicellular eukaryotes usually reproduce asexually by mitosis under favourable conditions. However, under stressful conditions such as nutrient limitations and other conditions associated with DNA damage, they tend to reproduce sexually by meiosis and syngamy.

Fungi

A fungus is any member of the group of eukaryotic organisms that includes microorganisms such as **yeasts** and **molds**, as well as the more familiar mushrooms. These organisms are classified as a kingdom, Fungi, which is separate from the other eukaryotic life kingdoms of plants and animals.

A characteristic that places fungi in a different kingdom from plants, bacteria, and some protists is the presence of chitin in their cell walls. Similar to animals, fungi are heterotrophs; they acquire their food by absorbing dissolved molecules, typically by secreting digestive enzymes into their environment. Fungi are heterotrophic organisms, and are not able to realize photosynthesis. Growth is their means of mobility, except for spores (a few of which are flagellated), which may travel through the air or water. Fungi are the principal decomposers in ecological systems. These and other differences place fungi in a single group of related organisms, named the *Eumycota* (*true fungi* or *Eumycetes*), which share a common ancestor, an interpretation that is also strongly

supported by molecular phylogenetics. This fungal group is distinct from the structurally similar myxomycetes (slime molds) and oomycetes (water molds).

Abundant worldwide, most fungi are inconspicuous because of the small size of their structures, and their cryptic lifestyles in soil or on dead matter. Fungi include symbionts of plants, animals, or other fungi and also parasites. They may become noticeable when fruiting, either as mushrooms or as molds. Fungi perform an essential role in the decomposition of organic matter and have fundamental roles in nutrient cycling and exchange in the environment. They have long been used as a direct source of human food, in the form of mushrooms and truffles; as a leavening agent for bread; and in the fermentation of various food products, such as wine, beer, and soy sauce. Since the 1940s, fungi have been used for the production of antibiotics, and, more recently, various enzymes produced by fungi are used industrially and in detergents. Fungi are also used as biological pesticides to control weeds, plant diseases and insect pests. Many species produce bioactive compounds called mycotoxins, such as alkaloids and polyketides, that are toxic to animals including humans. Fungi can break down manufactured materials and buildings, and become significant pathogens of humans and other animals. Losses of crops due to fungal diseases (e.g., rice blast disease) or food spoilage can have a large impact on human food supplies and local economies.

Many species produce metabolites that are major sources of **pharmacologically active drugs**. Particularly important are the antibiotics, including the penicillins, a structurally related group of β-lactam antibiotics that are synthesized from small peptides. Although naturally occurring penicillins such as penicillin G (produced by *Penicillium chrysogenum*) have a relatively narrow spectrum of biological activity, a wide range of other penicillins can be produced by chemical modification of the natural penicillins. Modern penicillins are semisynthetic compounds, obtained initially from fermentation cultures, but then structurally altered for specific desirable properties. Other antibiotics produced by fungi include: ciclosporin, commonly used as an immunosuppressant during transplant surgery; and fusidic acid, used to help control infection from methicillin-resistant *Staphylococcus aureus* bacteria. Widespread use of antibiotics for the treatment of bacterial diseases, such as tuberculosis, syphilis, leprosy, and others began in the early 20th century and continues to date. In nature, antibiotics of fungal or bacterial origin appear to play a dual role: at high concentrations they act as chemical defense against competition with other microorganisms in species-rich environments, such as the rhizosphere, and

at low concentrations as quorum-sensing molecules for intra- or interspecies signaling. Other drugs produced by fungi include griseofulvin isolated from *Penicillium griseofulvum*, used to treat fungal infections, and statins (HMG-CoA reductase inhibitors), used to inhibit cholesterol synthesis. Examples of statins found in fungi include mevastatin from *Penicillium citrinum* and lovastatin from *Aspergillus terreus* and the oyster mushroom. Fungi produce compounds that inhibit viruses and cancer cells. Specific metabolites, such as polysaccharide-K, ergotamine, and β-lactam antibiotics, are routinely used in clinical medicine. The shiitake mushroom is a source of lentinan, a clinical drug approved for use in cancer treatments in several countries, including Japan. In Europe and Japan, polysaccharide-K (brand name Krestin), a chemical derived from *Trametes versicolor*, is an approved adjuvant for cancer therapy.

The use of fungi in **food.** Baker's yeast or *Saccharomyces cerevisiae*, a unicellular fungus, is used to make bread and other wheat-based products, such as pizza dough and dumplings. Yeast species of the genus *Saccharomyces* are also used to produce alcoholic beverages through fermentation. *Aspergillus oryzae* is an essential ingredient in brewing Shoyu (soy sauce) and sake, and the preparation of miso, while *Rhizopus* species are used for making tempeh, a traditional Asian food made of fermented soy. Several of these fungi are domesticated species that were bred or selected according to their capacity to ferment food without producing harmful mycotoxins, which are produced by very closely related *Aspergilli*.

In **agriculture**, fungi may be useful if they actively compete for nutrients and space with pathogenic microorganisms such as bacteria or other fungi via the competitive exclusion principle, or if they are parasites of these pathogens. For example, certain species may be used to eliminate or suppress the growth of harmful plant pathogens, such as insects, mites, weeds, nematodes, and other fungi that cause diseases of important crop plants. This has generated strong interest in practical applications that use these fungi in the biological control of these agricultural pests. Some fungi, named entomopathogenic fungi, can be used as biopesticides, as they actively kill insects: *Beauveria bassiana*, *Metarhizium* spp, *Hirsutella* spp, *Paecilomyces* (*Isaria*) spp, and *Lecanicillium lecanii*.

Algae

The Algae is a collective name traditionally given to several phyla of primitive, and mostly aquatic plants, making up a highly diverse group of over 30 000 species. They display a wide variety of structure, habitat and life-cycle, ranging from single-celled forms to massive seaweeds tens of metres in length. Most algae share a number of common features which caused them to be grouped together. Among these are: possession of the pigment chlorophyll; deriving energy from the sun by means of oxygenic photosynthesis; fixing carbon from CO_2 or dissolved bicarbonate.
All algal types are eucaryotic, and therefore contain the internal organelles that is, nuclei, mitochondria, endoplasmic reticulum, ribosomes, Golgi body, and in most instances, chloroplasts. With the exception of one group (the Euglenophyta), all have a cellulose cell wall, which is frequently modified with other polysaccharides, including pectin and alginic acids.

The characteristics used to place algal protists into different taxa include the type of chlorophyll present, the form in which carbohydrate is stored, and the structure of the cell wall. A group not considered here are the cyanophytes, previously known as the blue-green algae; although they carry out oxygenic photosynthesis, they are procaryotes, and as such are more closely related to certain bacteria.

Important applications of algae group. Some tropical species of algae (named dinoflagellate) emit light, the only algae to do so. Due to an enzyme–substrate (luciferin–luciferase) interaction, this can cause a spectacular glow in the water at night, especially when the water is disturbed, for example by a ship. *Bioluminescence* of this kind has proved to be a useful 'tagging' system for cells in biological research.

The group of diatoms when die, their shells fall to the bottom of the sea, and can accumulate in thick layers where they represent a valuable mineral resource. This fine, light material (diatomaceous earth) has a number of applications, for example in filtration systems, and also as a light abrasive in products such as silver polish or toothpaste.

Red algae are the source of several complex polysaccharides of commercial value. Agar and agarose are used in the laboratory in microbial growth media and electrophoresis gels respectively, whilst carrageenan is an important thickening agent in the food industry. In addition, *Porphyra* species are cultivated in Japan for use in sushi dishes.

Algae can be converted into various types of fuels, depending on the technique and the part of the cells used. The lipid, or oily part of the algae biomass can be extracted and converted into

biodiesel through a process similar to that used for any other vegetable oil, or converted in a refinery into "drop-in" replacements for petroleum-based fuels. Alternatively or following lipid extraction, the carbohydrate content of algae can be fermented into bioethanol or butanol fuel.

3. Food biotechnology

One of the most demanding challenges of the food industry in the 21st century is the increasing demand for food, and specifically for healthy and nutritionally rich food products. Biotechnology is one of the solutions, its intention being to improve food, food ingredients, and functional food at the processing stage, beyond the agricultural production. In this respect, food biotechnology is dealing with current developments and applications of modern genetics, enzymatic, metabolic and systems-based biochemical processes in food and food-related biological systems. The economic importance of food biotechnology becomes particularly evident when we look at few food products produced in very large quantities worldwide using biotechnological methods: wine, beer and cheese.

The targeted use of biotechnological methods can, among other things, help reduce the quantity and number of unhealthy ingredients in foods as well as degrade allergenic substances. Genomic research and targeted breeding also greatly facilitate progress in agriculture. **Functional food**, a concept originated in the late 1980s in Japan, refers to a food product "designed" to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions and may be similar in appearance to conventional food and consumed as part of a regular diet. In order to produce functional and nutraceutical food, several techniques are available which include microbial and fermentation based metabolic processing. Fermentation has many applications related not only to improve food for health, but also in food waste remediation. Another examples of food biotechnology uses are the enzymatic degradation of lactose in dairy products or acrylamide precursors in bread and potato crisps, which results in a significant reduction of potentially carcinogenic acrylamide in the final product.

Biotechnology also offers new sustainable ways to preserve the existing resources. On the one hand, the addition of specific enzymes that stop biological degradation processes can prolong the shelf-life of food. This leads to lowering sales losses, as well as the quantity of food waste. On

the other hand, biotechnological methods that can break up nutrient-rich compounds such as woody plant constituents to make such compounds part of human and animal diets.

Fermentations

Fermented foods are usually defined as foods or beverages made through controlled microbial growth and enzymatic conversions of major and minor food components. Fermented foods, such as cheese and wine which are obviously food products produced by fermentation and some others that are not as obvious, are an important part of our diet. The transformation carried out by the microorganisms in fermented foods such as tea and bread can be delicate thus less obvious to lay people. In addition to fermented foods themselves, a big number of ingredients are produced using microorganisms' fermentation.

Fermentation is a technology that utilizes the growth and metabolic activities of microorganisms for the preservation and transformation of food materials. During food fermentation, the growth of spoilage and pathogenic organisms is inhibited by the metabolites generated by the fermenting organisms, thereby extending the shelf life of perishable produce. For instance, during lactic acid fermentation, lactic acid bacteria synthesize metabolites such as lactic acid, acetic acid, carbon dioxide, ethanol, hydrogen peroxide, bacteriocins, and antimicrobial peptides, which through synergy suppress the survival and growth of pathogenic and spoilage microorganisms.

The fermentation processes are wide, and the responsible microorganisms include bacteria, yeasts and fungi. Bacteriophages also play a role in modulating the microbial flora. Fermentation processes are sometimes simple involving one substrate component (e.g. milk) and one microorganism (e.g. *Lactococcus lactis*), but sometimes can involve a complex mixture of substrates and several microorganisms.

A great number of foods are transformed by the result of microbial action. Many of transformations represent spontaneous fermentations which occur when endogenous microorganisms can grow and metabolize the substrate under the influence of the extrinsic conditions. Food products such as sauerkraut and tea are transformed as a result of harvesting an agricultural commodity such as cabbage and tea respectively, and then man-made alteration of the environment to promote the action of these endogenous microorganisms. The cascade of seemingly innocuous events is now being dissected using high-resolution metagenomic analyses which reveal the succession of microbial populations including those previously unable to be cultured. The

complex micro-floras that are present in the raw ingredients and the dynamic nature of the population over the time of the fermentation contribute to the product. The deliberate introduction of microorganisms into food substrates was first reduced to practice by the work of scientists such as Christian Hansen. Starter cultures represent individual and collections of microorganisms that are deliberately added to induce a change in the food substrate. Some of these microorganisms, for example, lactic acid bacteria, are extraordinary in their capacity to convert substrates into products reaching efficiencies of over 98%. Fermentation end products include simple alcohols and acids, but the changes induced by these starter cultures range from taste to texture and nutritional content to safety. Besides preservation action, fermentation can improve and sometimes give characteristic aroma, flavor, texture, and nutritional profile into food.

Thus, although ancient civilizations developed fermentation primarily as a way of preserving perishable agricultural produce, nowadays the technology has become more and more a real tool for developing desirable organoleptic profiles in foods and improving their palatability. Bread is a good example, where the primary function of dough fermentation is to create the characteristic structure, texture, and organoleptic profile of bread after the baking process. Fermentation also may help to remove antinutritional factors and toxins in food materials and improve their nutritional profile. For instance, fermentation of soybean into products such as tempeh (fermented dehulled soybean with meat like flavor and texture), natto (a fermented soybean dish from Japan with strong smell and flavor and a slimy texture), and soy sauce (a dark brown condiment made from fermentation of soybean, wheat, and salt) leads to reduction of antinutritional factors such as phytic acid and trypsin inhibitors and results in the hydrolysis of complex soy proteins into more digestible and bioavailable peptides and amino acids.

Traditional food fermentation processes can be broadly classified into lactic acid fermentation, fungal fermentation, and alkaline fermentation. Examples of lactic acid fermented products, i.e., products primarily fermented by lactic acid bacteria, include yoghurt, sausages, cheese, sauerkraut (fermented cabbage from eastern and central Europe), and kimchi (fermented and spiced Napa cabbage from Korea). Yeast spp. are also involved in the fermentation of many of the lactic acid–fermented products, including kefir (a slightly alcoholic dairy beverage from the Caucasus), and kombucha (a fermented sweetened tea from China). Most of the well-known soy-based fermented foods from Asia such as tempeh and soy sauce are produced by fungal fermentation, except natto, which is produced by alkaline fermentation.

Industrial fermentation processes use either submerged or solid-state bioreactors that are operated in batch, semi batch, or continuous mode. Most food fermentation processes from sauerkraut and kimchi to miso and tempeh use solid-state fermentation processes operated in batch mode, where microorganisms are cultivated on the surface of a water-insoluble substrate. Submerged fermentation processes are used in the production of yoghurt and other dairy-based beverages, alcoholic beverages, and food condiments such as vinegar.

In the last decade fermented food products make up a significant part of the diet in developing nations and the Far East. In the West, with the exceptions of bread, cheese, and sausages, fermented foods have largely faded to the sidelines with the advent of modern technologies such as refrigeration. Nevertheless, there is a renewed interest in traditional fermented foods in recent times, mainly driven by the purported health benefits of fermented foods both as vehicles of probiotic organisms and health-promoting metabolites.

Fermented foods are currently being promoted to prevent or cure a range of diseases from obesity to cancer. For instance, kimchi is claimed to have anticancer, antiobesity, antiaging, and anticonstipation effects, whereas kefir is claimed to reduce lactose intolerance symptoms, stimulate the immune system, and lower cholesterol and to have antimutagenic and anticarcinogenic properties. Although most of the health claims around fermented foods are based on folk beliefs with no scientific substantiation, findings from recent in vitro and animal models, as well as human intervention studies, support some of these claims.

Fermented foods are one of the top 10 food trends in 2016, continuing the trend over the last few years. Food companies are responding to this growing trend either by commercializing traditional fermented foods (e.g., kefir and kombucha) or developing novel fermented foods based on the traditional ones (e.g., Bionade, flavored malt-based beverages fermented using the starter culture of kombucha, and Rythem, coconut milk–based and fruit juice–based beverages fermented using kefir grains). Several soy- and cereal-based probiotic products are also in the market in response to the growing prevalence of allergies to dairy proteins, lactose and gluten intolerances, and life style choices such as veganism.

Beyond foods, **food ingredients** are also produced as a result of fermentation. Examples include amino acids, vitamins, and flavoring agents. Amino acids, notably glutamic acid, are produced by fermentation the current processes a result of intense research and development since the discovery in the 1950s that *Corynebacterium* could produce it. Increases in its production were

driven using this amino acid as a flavor enhancer initially in Japan, but eventually worldwide. Through a combination of classical mutagenesis and selection, molecular biology and fermentation the yields were currently optimized. New efforts are underway to elucidate fermentation processes using metagenomics and to develop novel fermented foods as part of the evolving culinary arts. Several world-class culinary institutions and their innovative chefs are bringing fermentation to a new level of fine dining.

Microbial food cultures

Fermented food is known since centuries considered as a feasible solution in preserving different perishable products. The need for an inoculum it was obvious and this need was covered by keeping a sample from the previous production. The deep scientific knowledge related to the microorganisms used for food fermentation came later on, around 1858 when Louis Pasteur demonstrated that the yeast are responsible for the alcoholic fermentation in wine and beer making. Since then, thousands of scientists and practitioners have been focused on the isolation and selection of microbial food cultures, known as "starter cultures" to be used in food manufacture.

Table 1 The use of starter cultures in fermented food products

Food product	**Microorganisms used as starter culture**
Bread	Yeast /Lactic acid bacteria
Wine	Yeast / Malolactic bacteria
Beer	Yeast
Dairy products	Lactic bacteria / Propionic bacteria Bifidobacteria / Yeast/ Moulds
Pickled vegetables	Lactic bacteria
Fermented sausages /salami	Lactic bacteria / Yeast / Moulds
Soy sauce	Lactic bacteria / Moulds (*Aspergillus*)

Starting with the 19th century the use of starter cultures became a norm for beer production, wine, vinegar and bakery yeast. Later on, after almost another century, the dairy and the meat industry started to use well characterized and defined starter cultures. **Yeasts, moulds and bacteria** are microorganisms used to make food products such as beer, bread, wine, vinegar, yoghurt, cheese, other dairy products, fermented meat and vegetables. The main food products obtained with the use of microbial starter cultures are presented in Table 1; conducting well the fermentation will favour useful flora, to the detriment of undesirable flora and consequently preventing spoilage and promote taste and texture. Still, should be mentioned that in the last years

has been noticed a trend in the production of “traditional” fermented food which keeps the autochthonous flavours and, in this case, the inoculation is not necessary as naturally occurring microorganisms in the raw materials could be a reliable source of the microbial flora, under proper conditions.

Basically, Microbial Food Cultures (MFC) are concentrates of one or more microbial species and/or strains including unavoidable media components carried over from the fermentation and components, which are necessary for their survival, storage, standardization and to facilitate their application in the food processing. These products may be classified as following: single-strain cultures, which contain one strain of a species; multi-strain cultures, which contain more than one strain of a single species; multi-strain mixed cultures, containing different strains from different species.

Relatively recently, practitioners have started to explore the so-called functional starter cultures. Compared to classical starter cultures, functional starter cultures offer an additional functionality and represent a way of improving and optimising the food fermentation process and achieving tastier, safer, and healthier products. These starters include microorganisms that generate health-promoting molecules, antimicrobials, including bacteriocins, aroma compounds or contribute to cured meat colour for example; very important, they possess probiotic qualities, or lack negative properties such as the production of biogenic amines and toxic compounds.

The production of starter cultures follows a simple and basic technology conducted in fermenters under strict hygienic conditions. Usually, the original microbial strains are stored in a microbiology laboratory as fresh or conditioned cultures; from the stock small quantity of microorganism, representing the inoculation material, is prepared to start the production process. This inoculation material is transferred in different growth media placed in fermenters for liquid fermentation. Media are tailored to the specific requirements of the microbial species and typically contain carbohydrates, proteins, vitamins and minerals. The culture is allowed to multiply and grow under carefully defined and monitored conditions. After the microbial growth, the cultured cells are harvested, usually by centrifugation, and the biomass is conditioned in liquid, frozen or powder form. This form may undergo a final formulation, which involve blending of multiple cultures, prior to shipment to the food manufacturer. As a general rule, the microbial load of the starter culture should be higher than 10^8 CFU/ml or mg for each microbial specie. The

manufacturer should know that is compulsory to verify the viable cells in the final matrix and to insert the information on the product label.

An important issue for the manufacturer is to maintain the genetic stability of the employed strains. In this sense here are some good practice to maintain this stability and to avid genetic drifting: the presence of an "in house" microbial bank to provide the original strain to each batch and to each production site; storage the "mother" culture below -80°C; setting a clear internal system for comprehensive documentation and traceability for each strain; a long-term storage plan that minimizes the number of generation times should be implemented; DNA-fingerprinting of new batches and comparison to previous batches should be routine; basic phenotypic characteristics of new batches should be verified.

Probiotics, prebiotics, synbiotics

Looking on the food market over the past years, it can be easily noticed the surge of health-conscious food choices making their way into the consumers' eyes. There is an increased demand for functional foods having positive effect beyond their basic nutritional value. Probiotic-rich drinks are nowadays popular beverages on the supermarket shelves. In the close past years, the beverage companies have been innovating around the probiotic products as fermented teas and drinks which have raise in popularity, like kefir; kombucha, one of the most popular beverages in the probiotic space, was reported for 25% annual market growth. Probiotic niche products include ice cream, cheese, candy, and chewing gum, although they do not play a major role in the European marketplace.

The FAO/WHO issued in 2001 a definition of **probiotics**, namely that they are live microorganisms which when consumed in adequate amounts confer a health effect on the host. It is important to notice that this statement is not covering the use of probiotics in feed, as pharmaceuticals, cosmetics or as food additives. Naturally, we can find probiotics in fermented foods like yogurt, kefir, cider, vinegar, pickles, some cheeses or fermented teas.

Compared with fermented foods, probiotics are relatively new products used for health maintenance which may not have a therapeutic claim. Probiotics may increase the resistance of the gut to invasion by pathogens, prevent the growth of pathogenic bacteria, enhance epithelial barrier function, or ameliorate disease processes by inducing the secretion of soluble factors. However, in people with a critical immune status, probiotics should only be used after careful consideration.

Probiotic foods are **manufactured** by adding the probiotic strains directly in the non-fermented food or simultaneously with the standard cultures in the case of fermented food, after carefully selection and studies proving beneficial effect to consumers. Some of the popularly used probiotic microorganisms are lactic acid bacteria, like *Lactobacillus rhamnosus, Lactobacillus reuteri,* and certain strains of *Lactobacillus casei, Lactobacillus acidophilus*-group*,* bifidobacteria, *Bacillus coagulans*, *Escherichia coli* strain Nissle 1917, certain enterococci, especially *Enterococcus faecium* SF68, and the yeast *Saccharomyces boulardii*. In an alternative process, fermentation takes place separately, and probiotic cultures (e.g., *Lactobacillus acidophilus* and *Bifidobacterium* species) are combined to form the final product.

According to WHO/ FAO guidelines, probiotic manufacturers should register their strains with an international depository. In SKLM's report from 2010 is emphasized the fact that only lactobacilli and bifidobacteria were present in probiotic foods in the European market at that time, and species like *Bifidobacterium*, *Enterococcus, Lactobacillus*, *Lactococcus, Streptococcus,* were listed especially in yoghurt 'like products. Still, the probiotic dietary supplements contain a wide variety of organism groups, e.g. *Bacillaceae* (e.g. *B. coagulans, B. subtilis),* enterococci *(E. faecium), Propionibacterium freudenreichii* and yeast *(S. boulardii).* On the market there are also available as probiotic foods other products, like breakfast cereals, muesli, ice cream, cheese, various beverages and uncooked sausages.

Several companies have developed innovative products to enhance their product portfolio. Product launch is considered the key strategy adopted by the manufacturers, followed by collaborations and agreement, expansion and acquisition. Based on end use, the market addresses human probiotics and animal probiotics; the first is the major revenue contributor in the overall probiotic market. According to some official reports the consumers option leans towards probiotics dairy products such as yogurt, ice cream, and cheese; the probiotic yogurt is the most common probiotic product preferred by consumers. Any business strategy should take into account several factors: the market niche; access to distribution channels; pricing, profit margins, and proprietary insulation in the marketplace. Hence, many companies underestimate the full impact of regulatory requirements on both business objectives and plans for product development.

In terms of probiotics, **regulation** varies between regions. Generally, probiotics are regulated as food supplements and regulation is focused on the legitimacy of any claims, rather than efficacy, safety and quality. Ingredients, manufacturing processes and conditions are

important determinants of product characteristics and changes of these factors may give rise to a product not identical to the "original" in efficacy and safety if proper measures are not taken. The lack of stringent regulation of probiotic manufacturing mean that the manufacturer (trademark owner) can commercialize any formulation under the same brand, even if significantly different from the original. In this respect, an important issue is to keep in the final product the ratio live/dead microorganisms; the whole technological process (harvesting; centrifugation; lyophilization, etc.) leads to an increase in dead microorganisms. The current regulations for labelling of probiotic products require that the consumer is informed about the number of live bacteria expressed as CFU per dose.

Generally, the **prebiotic** indicates dietary substances with the ability to modify host microbiota inducing benefit to the host; the most recent definition, proposed by the International Scientific Association for Probiotics and Prebiotics, a prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit". Generally, prebiotics derived from different fibre rich product that humans can't easily digest (Jerusalem artichoke, garlic and leeks). These prebiotic fibres pass through the human/animal digestive system with no immediate benefits, while the "good" bacteria in the gut actually consume this fibre which can lead to better digestive health. The most characterized prebiotics are galactans, fructans (fructo-oligo-saccharides and inulin), oligo-fructose, galacto-oligosaccharides, lactulose and breast milk oligosaccharides. These substances are used as food ingredients mostly consisting of non-starch polysaccharides and oligosaccharides and are able to stimulate lactobacilli, bifidobacteria and other beneficial microorganisms.

Despite the huge success of the probiotic, prebiotics haven't seen as much publicity until recently. The lack of consumers' demand might be the fact that haven't really been aware of prebiotics. The addition of chicory inulin on the prebiotic fibres list is estimated to impact positively the market. In the last years the market increased the offer in food supplements containing both probiotics and prebiotics, especially in non-traditional beverages. The research and innovation teams are looking now the find novel methods of inclusion to preserve the integrity of probiotics in such products.

The concept of **symbiotic** represent a combination of probiotic and prebiotics that affects the host beneficially by improving the survival and implantation of selected live microbial strains in gastrointestinal tract; their combination can cause the release of antibacterial substances such as

bacteriocin, which can retard the growth of pathogenic bacteria. Actually, symbiotics were developed to overcome possible survival difficulties for probiotics. Among the commonly used probiotic strains for symbiotic product formulations are *Bifidobacteria spp, Lactobacilli, Saccharomyces boulardii* and *Bacillus coagulans*, whereas the major prebiotics used include oligosaccharides such as fructo-oligosaccharide (FOS), galacto-oligosaccharides (GOS), xyloseo-ligosaccharides (XOS) or prebiotic from natural sources like inulin (Fazilah et al. 2018).

Nowadays, probiotic, prebiotic and synbiotic products are marketed towards use in gastroenterology, immunology, gynaecology, cardiology, urology, anti-ageing, skin care, dietetics and oral care. The broad applications of this limited group of organisms suggest that there is a need for more extensive clinical and epidemiological evaluation of these products and their efficacies. Generally, for probiotics to be marketed as pharmaceutical products, the burden of proof for efficacy will be much greater than for similar formulations marketed as functional food products or supplements. Most probably, in coming years, the research and regulations may shift the market share of probiotics towards pharmaceutical companies, which have infrastructure and revenue models to accommodate clinical trials.

4. Plant biotechnology

Plants have always been essential for humankind, and not only because they generate most of the oxygen we breathe and are our primary sources of energy – fossil fuels have been mostly formed by the decaying of plants – and food. Plants also provide us with raw materials for different industries, to produce, for example, paper, textiles, adhesives, dyes, or lubricants, and contain secondary metabolites that we use in cosmetics, perfumery, as medicines or in the chemical industry. Plant biotechnology directly impacts all the fields and industries listed above. Research and development activities in plant biotechnology have enjoyed a certain degree of freedom, if compared to the restrictions on human and, to a lesser extent, animal biotechnology; this has led to the development of a wide range of techniques and tools that can be applied to generate innovations, which may ultimately become the basis for a commercial company.

The plant biotechnology market is enormous. The industry is attracting much attention, and the number and amount of investments in plant biotechnology companies are rapidly growing. There are different approaches and methodologies in the sector, which include molecular markers,

mutagenesis, *in vitro* culture techniques, transgenesis, 'omics' technologies, and also organic agriculture and phytochemistry.

Applications of molecular markers in plant biotechnology

What are molecular markers?

As its name implies, a molecular marker is a characteristic or difference (polymorphism) in a biomolecule that can be used to reveal one or more features of a cell or individual carrying this marker. Molecular markers are a useful tool with many applications in plant biotechnology because they can be associated to a particular phenotype (marker-assisted selection) and/or used to establish relationships and evaluate differences among individuals, populations and species (genetic fingerprinting). Although the term molecular markers can refer to different types of biomolecules (e.g., proteins, nucleic acids), in recent times, it is mostly referred to DNA markers, for which there have been many technological developments in the last decades. Consequently, the cost of development and utilisation of molecular markers has decreased dramatically in the last years, thanks to the advances in genomics and bioinformatics.

Advantages of molecular markers in plant biotechnology

One significant advantage of the application of molecular markers in plants is that they allow increasing the efficiency of many processes in plant science and breeding without relevant ethical issues. Molecular markers do not increase genetic variability, as other techniques such as mutation, genetic modification, or gene editing can do; instead, they allow selecting and using the variation already available. This issue is important, as the application of molecular markers to provide services and products in plant biotechnology is not constrained by special regulations that apply to the techniques for increasing the variation mentioned above. This facilitates that entrepreneurs establish companies that do not require special and cumbersome regulations, other than those applicable to a company working with chemical reagents and live plant materials (and, where appropriate, plant pests and diseases).

Another clear benefit of DNA molecular markers is that, once we have a molecular marker linked to a trait, the selection for the trait can be performed without the need of observing the corresponding phenotype. This has many advantages because the selection of plants can be done at nurseries, using seedlings or small plantlets, for traits that are expressed in adult plants, and also

allows selection for tolerance or resistance to diseases without the need to manage pathogenic agents. For example, selection of tomato plants resistant to quarantine diseases such as *Tomato spotted wilt virus* (TSWV) can be performed by using molecular markers linked to resistance gene *Sw5*, without the need of inoculating plants with the virus. This possibility considerably reduces the costs involved in growing, phenotyping and selecting the plants, and eliminates those related to management of pathogenic agents. By increasing the efficiency of selection, molecular markers allow reducing the costs and speeding breeding programmes.

The increasingly reduced cost of screening with DNA markers is also a clear advantage that has facilitated and promoted their use. Nowadays, most laboratories can perform molecular marker genotyping in-house, even when only basic equipment is available. However, high throughput genotyping of plant samples with molecular markers is frequently done by specialised companies at very low costs. For example, some recent technologies, like Single Primer Enrichment Technology (SPET) genotyping, allow obtaining over 10,000 single nucleotide polymorphism (SNP) markers for less than 20 €/sample.

Types of molecular markers

Many different DNA molecular markers exist, based on the type of polymorphism which is targeted. For example, in restriction length polymorphisms (RFLPs), random amplified polymorphisms (RAPDs), and amplified fragment length polymorphisms (AFLPs) the polymorphism targeted is on the length of fragments generated after shearing the DNA with restriction enzymes, whereas in simple sequence repeats (SSRs) the target is the different number of repeats of short sequences of one or a few nucleotides, and in SNPs the target is a difference in a single nucleotide.

The technology used for detecting the molecular marker is also relevant, as it greatly influences the cost. For example, RFLPs were amongst the first developed DNA molecular markers, and their detection was based on hybridisation with labelled (quite often radioactively) probes, which is much more expensive than PCR-based detection methods. The reproducibility of molecular markers within and between laboratories is also an important characteristic that affects their applications in plant biotechnology. The more reproducible a molecular marker is, the better, in particular for applications based on obtaining highly reliable specific genetic fingerprints. Many other subsequent markers are based on the use of the polymerase chain reaction (PCR) technique,

which produces multiple (often millions) copies of specific target fragments of DNA. The abundance in the genome is also an essential factor to take into account when choosing a molecular marker. In this respect, thanks to transcriptome and genome sequencing and re-sequencing projects, it is frequent to have millions of SNP markers available. However, for other markers, such as SSRs, their abundance in the genome is generally much lower; nevertheless, even in this case, it is not uncommon to have thousands of SSR markers in species for which genome or transcriptome sequences are available. A summary of relevant characteristics of some of the most common DNA molecular markers is provided in Table 2.

Table 2 Main characteristics of some of the most common DNA-based molecular markers.

Characteristic	RFLPs	RAPDs	AFLPs	SSRs	SNPs
Target	Fragment length	Fragment length	Fragment length	Fragment length in tandem repeats	Nucleotide
Mode of action	Co-dominant	Dominant	Dominant	Co-dominant	Co-dominant
Reproducibility	High	Medium	Medium	Very high	High
Detection technology	Hybridisation	PCR	PCR	PCR	PCR
Genome abundance	High	High	High	Medium	Very high

Genetic fingerprinting

Many potential services and business opportunities are related to genetic fingerprinting, which consists of obtaining a genetic profile of individuals with molecular markers. Those molecular markers should be highly repeatable and stable, particularly when used for certification purposes. Among the potential applications of genetic fingerprinting, we can mention the detection of fraud and misuse of plant materials, the certification of authenticity of plant material, the certification of non-GMO material, the evaluation of diversity and relationships among and between samples or varieties and, when combined with the availability of phenotyping data, genome-wide association studies (GWAS).

The detection of fraud (i.e., unauthorised use of a plant variety, or illegal propagation) with morphological methods is problematic and can be ambiguous, as many morphological traits are influenced by the environment. Therefore, the use of specific genetic fingerprints that uniquely identify a variety can be used as proof to demonstrate an illegal use of plant material. Similarly,

certain varieties need authentication for particular uses (e.g., local varieties with specific characteristics), and here molecular markers can also provide a solution. An additional application of high interest of molecular markers is the detection of GMOs, whose cultivation and use as food are strictly regulated in many countries, including the European Union. In this case, it is common to use different PCR-based molecular markers for detecting particular genes and/or regulatory elements such as promoter or terminator sequences.

A widespread use of genetic fingerprinting is to evaluate genetic homogeneity or diversity in plant material of a sample, variety or population, which may be of interest to evaluate purity and uniformity. Also, genetic fingerprinting is useful for the establishment of relationships between different varieties or species, either for practical or basic research purposes. When phenotypic data of large collections of germplasm are available, genetic fingerprinting of the collection allows applying an analysis, which is a potent tool to detect genes of interest.

In vitro culture

Plant *in vitro* culture consists in cultivating under axenic conditions whole plants or, more commonly, parts of a plant (so-called 'explants'). Many plant cells are totipotent so that a differentiated cell can de-differentiate, reverting to a meristematic stage in which it can divide and differentiate again to form a new plant organ; for example, sections of a leaf blade can produce somatic embryos. Ultimately, it is possible to regenerate whole plants *in vitro* from those dedifferentiated cells. This process is called morphogenesis and is the base of a wide range of applications.

Two main morphogenetic routes are used for *in vitro* plant regeneration:

(1) the *embryogenic route*, in which the dividing cells organise into an embryo, which may give rise to the whole plant later on

(2) the *organogenic route*, in which the dividing cells organise to form a plant organ (mainly shoots and roots, but can also be flowers, tubers…).

In many cases, however, the cells fail to differentiate from the very beginning and grow in a disorganised manner, forming a callus. When the first divisions of a dedifferentiated cell form first a callus and then organs or embryos, it is said that the regeneration was indirect. When *in vitro* culture methods do not succeed in regenerating the desired organ and/or the whole plant in a particular species or genotype, it is said that the plant is recalcitrant.

Micropropagation: A quick method to reproduce plants

Micropropagation consists of cloning plants using the morphogenetic properties of the plants so that it is possible to produce thousands of plant clones in a short period and a relatively small space. Also, some micropropagation and/or micrografting techniques favour the elimination of virus from plants. Therefore, micropropagation allows the production of healthy plantlets at an industrial scale; this planned and controlled way of multiplying plant material is very beneficial for the agriculture sector. Actually, the establishment of micropropagation companies is one of the engines for increasing agricultural production in many regions of the world. Nowadays, micropropagation is especially used for the production of ornamental plants, fruit trees, such as citrus spp., and many other woody plants. This technique is by far the most widespread application of plant tissue culture, and one of the most profitable ones as long as a good market and socio-economic study is done prior to the establishment of the company.

Tissue culture techniques for improving plant-breeding processes

A breeder's work is based on selecting individuals with the best combination of traits. To perform this task, the breeder needs genetic diversity. Plant tissue culture offers a wide range of techniques to increase plant diversity. For example, by *protoplast fusion* plant protoplasts (that is, plant cells without their cell wall) are forced to fuse to produce either hybrids (allopolyploids) or higher ploidy products (autopolyploids); it should be remembered that many of our crops are polyploids (e.g., potato, wheat, strawberries). In some cases, the fusion of the two cells is not complete producing cybrids, which consist of cells with the nucleus of one protoplast and the cytoplasm of the other. Generation of cybrid plants has been a milestone for obtaining cytoplasmic male sterile (CMS) lines, which are very useful for hybrid seed production in self-pollinated species, as well as for the production of seedless *citrus* fruits. Another way to create genetic diversity is by generating somaclonal mutants, which can be forced to appear under *in vitro* conditions. Other useful techniques to generate diversity, such as *plant genetic transformation* and *induced mutations,* are explained elsewhere in this chapter and will not be mention here.

In some cases, breeders may find hybridisation barriers when using wild relatives to introduce interesting traits into elite cultivars; in such cases, *in vitro pollination* and *embryo rescue* can be very useful. In addition, embryo rescue can also speed up the breeding process by shortening the time from fruit set to planting the offspring . In any case, *double-haploid* production is one of

the most effective techniques to accelerate the breeding process, as it allows obtaining homozygotic plants for all their *loci* in a single generation and thus the quick production of pure lines. Generation of double-haploid plants is based on the regeneration of haploid plants from the plant gametes and its diploidisation, either spontaneous or induced by drugs such as colchicine. In most cases, haploids are formed from the immature male gametes, through androgenesis in *in vitro* anther or isolated microspore cultures, but can also be obtained from the female gametes by gynogenesis. *In vitro selection* is another technique which reduces breeding costs by allowing the analysis of thousands of plants in a small and very controlled space; it has been instrumental to select genotypes resistant to bacterial or fungal toxins or tolerant to salt stress.

Transgenic plants

A simple *technical* definition of a transgenic or 'genetically modified' (GM) plant would refer to a plant which contains one or a few foreign genes, of any origin, stably integrated into its genome, and thus transmitted to the plant progeny as any other endogenous gene. Since the aim of the genetic transformation is to confer a specific (and pre-designed) phenotype to the transgenic plant, the introduced genes are accompanied by the appropriate regulatory sequences (promoters and transcription termination signals recognised by the host plant) to allow expression of those genes.

From a *regulatory* point of view, however, the definition of a GM plant focuses on the *method of generation* rather than the *final product*, since genetic engineering techniques must be used in the process of genetic transformation for a plant to be legally transgenic. If classical breeding approaches – natural or forced sexual crosses and selection – are used to introduce foreign genes, or even complete genomes, in a plant variety, the product is not considered as transgenic. Moreover, if the genetic information of the plant is modified through spontaneous or induced mutations, the mutant plant is also not subjected to the regulations applied to GM plants, even though it is evident that it has been 'genetically modified'. There is, however, an exception (in Europe): in case mutations – indistinguishable from the latter – are produced by genome editing techniques (CRISPR/Cas9), then the plants obtained are considered transgenic, according to the recent EU legislation.

How are GM plants generated?

A three-step process is commonly used to obtain a transgenic plant. First, the foreign DNA, cloned in appropriate expression vectors, is introduced into plant cells, generally *in vitro* (in cell

cultures or plant explants), for which several procedures are available (see below). Second, through non-homologous recombination mechanisms, the foreign DNA gets integrated into the plant genome, at a single *locus* or several *loci* of the plant chromosomes, in one or more copies. Third, whole plants are regenerated *in vitro* from the transformed cells; depending on the starting plant material, different morphogenetic processes can be employed to obtain the transgenic plants: most often by organogenesis, but also through somatic embryogenesis or androgenesis. This last step usually represents the bottleneck of the entire procedure of plant genetic transformation, which has not yet been successful, or is still very difficult, in many 'recalcitrant' species or genotypes, because the lack of efficient *in vitro* regeneration protocols.

The construct used for plant transformation contains a selection marker gene conferring resistance to an antibiotic or herbicide; its expression during the process of *in vitro* regeneration, which is carried out under the appropriate selective conditions, allows elimination of the cells that have not been transformed. Therefore, the regenerated plant should be, in principle, the expected genetically modified product. Nevertheless, the transgenic nature of the original transformants must be confirmed by molecular analyses, as well as the expression of the transgene and its transmission to the plant progeny, after selfings and backcrosses. Finally, homozygous lines carrying a single copy of the transgene, which should behave as a dominant Mendelian character, are selected. Those plants will be subjected to agronomic analyses, in greenhouses and experimental fields, before they go through all further field tests and procedures required by regulatory agencies to be authorised for commercial production.

Transgenic plants as biofactories

Apart from generating crop varieties with enhanced agronomic characteristics or nutritional value, GM plants can be engineered to be used as *biofactories* for the production of recombinant proteins of interest for different industries. *Molecular farming* with the so-called 'third generation' of transgenic plants includes, among others: ***i)*** the production of pharmaceuticals, such as drugs, therapeutical proteins, vaccines or antibodies ('bio-pharma' or 'molecular *pharming*'); ***ii)*** production of industrial recombinant enzymes, for the detergents, leather tanning, paper, adhesives, paints or other chemical industries; ***iii)*** Proteins commercialised for experimental research, such as avidin, β-glucuronidase, aprotinin, trypsin, lysozyme or bovine serum albumin; ***iv)*** specialised food products: food additives, functional food, dietary supplements; ***v)***

biodegradable plastic, for example, polyesters of 3-hydroxy acids, such as polyhydroxy butyrate (PHB).

In addition, plant metabolism can be engineered to modify the composition of starch, cell walls or fatty acids in lipids, for the improvement of plants as raw material for industrial processes, in the production of adhesives, paper or lubricants. Furthermore, GM plants can be designed for enhanced activity in soil phytoremediation processes, for the decontamination of heavy metals or organic solvents.

'Omics' technologies

'Omics' technologies represent modern biotechnological tools, with numerous theoretical and practical applications. They are high-performance techniques that allow the analysis of a considerable volume of data. These technologies are essentially based on the (generally) non-targeted identification of all the genetic products (transcripts, proteins and metabolites) present in a biological sample. Numerous scientific advances are due to omics, such as the identification of signalling molecules associated with cell growth, cell metabolism or cell death. In addition to the theoretical advances achieved by these new technological approaches, they triggered a fundamental change in biomedical research, improving the diagnosis and treatment of diseases, contributing to the development of new drugs and starting the new epoch of personalised medicine. The 'panomic arsenal of omics' is extremely valuable not only in biomedicine but also in other fields such as food sciences and agriculture. The combination of these modern technologies is translated in better quality, taste and nutritional composition of food, an important role in crop protection, better understanding of insect resistance to pesticides, of plant resistance to herbicides, or plant breeding.

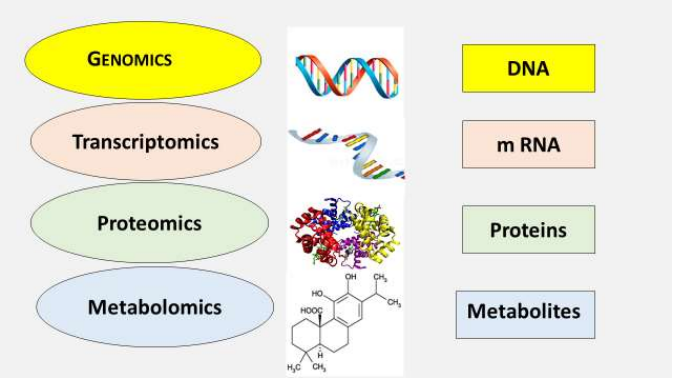

Fig. 1. Major 'omics' technologies

Omics represent a constellation of experimental disciplines (Fig.1), such as genomics, proteomics, transcriptomics, metabolomics, regulomics, spliceomics, lipidomics, phenomics, ionomics, microbiomics, metagenomics, phenomics, pharmacogenomics, toxicogenomics, introgressionomics among others.

Genomics

Genomics is the large-scale study of the genome, the complete set of an organism DNA, including all its genes and non-coding regions. From the first sequenced plant genome, that of *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000), followed by rice (International Rice Genome Sequencing Project, 2005), up to date the genomes of about 350 land plant species have been completed. The completion of the genome sequence of many crops has profound implications for agriculture, shedding light on the adaptation by natural and artificial selection to environmental constraints.

The high-performance whole genome sequencing machines permit fast genomic analysis at low costs. The knowledge of plant genome allows the understanding of the genetic and molecular basis of all biological processes in plants that are relevant to the species. By using genomics, thousands of genes can be quickly analysed in parallel, and complex crop traits, such as yield and yield stability may be unravelled. *Agrigenomics* or agriculture genomics is a new approach in plant biotechnology, with a high utility in molecular breeding and marker-assisted selection. The knowledge of genomics is a valuable tool for crop improvement. It contributes to obtaining higher quality germplasm and also to crop protection. The identification of genes that control economically important traits provides the basis for new progress in the genetic improvement of crop species, complementing traditional methods based on assisted crosses. Genomics is also useful for the biopharmaceutical industry and the conservation of biodiversity by identification the most relevant genome segments in relation to adaptation and evolution.

Transcriptomics

Transcriptomics is the study of the transcriptome, the sum of all of the RNA transcripts, including both mRNA and non-coding RNA (ncRNA) expression in a cell. The genetic information contained in the genome is expressed through transcription in the intermediary molecules of mRNA, whereas ncRNAs play additional regulatory roles. Transcriptomics give the possibility to

study gene expression in different tissues, conditions, or time points, explaining how genes are regulated.

The first transcriptomic analyses date back to the late nineties, using Northern blots and quantitative PCR, but due to the rapid evolution of technical methods such as next-generation sequencing (NGS) the functional elements of the genome are better understood. Transcriptomics represents an ideal tool for analysing the relations between the genotype and the phenotype and determines how the pattern of gene expression changes due to abiotic or biotic stresses, enabling the description of metabolites, transcription factors, and stress-inducible proteins in stress tolerant plants. The study of plants' responses to abiotic and biotic stresses is getting a special relevance, as agriculture is already affected by global warming in many parts of the world. Transcriptomics allows the identification of genes and pathways associated with responses to exogenous stresses in plants.

The major limitation of the 'classical' transcriptomics analysis using DNA arrays is that it requires to know the genomic sequence of the organism under study, therefore initially it could only be performed in model species. Although at present many plant genomes have been sequenced, the improvement and reduction in the price of NGS technology have led to the substitution of RNA hybridisation with the probes in the DNA arrays, by direct massive RNA sequencing. Thus, transcriptomics analyses can be now be carried out in all species, even if no genomic data are available.

Proteomics

Proteomics is the study of the proteome, the total set of proteins in a tissue or organism. Proteins are sequences of amino acids assembled according to templates of DNA and RNAs with structural or functional roles in cells. The proteome varies from cell to cell and in time, the majority of proteins suffering post-translational modifications, which produce different functional types of the same structure. For this reason, proteomics is more informative than genomics when dealing with environmental effects and it is continuously gaining a protagonist role in environmental monitoring and human health risk assessment.

The most popular proteomics methods are based on the combination of two-dimensional gel electrophoresis with mass spectrometry (MS). MS-based proteomics is becoming the standard approach for systematic characterisation of post-translational modifications, such as

phosphorylation, glycosylation, acetylation, or methylation among others. The identification of these modifications is relevant in the study of the phytochemicals produced by plants and of the effect of external factors on their composition. Through protein expression profiling, responses to stimuli such as pathogens or insect attack, or abiotic stresses can be analysed, and functioning of particular proteins can be elucidated. The highly complex interactions between plants and microorganism, such as symbiosis, can be better understood. Proteomics also contributes to the unravelling of mechanisms of resistance, mode of action, and biodegradation of pesticides, being a tool for the development of more effective and safe pesticides. The hundreds of thousands of different proteins in plants contribute to the texture, yield, flavour, and nutritional value of food products. Proteomics is also a useful tool for testing food authenticity, food security and safety.

Metabolomics

Metabolomics is one of the younger omics approaches, and refers to the study of the 'metabolome', a term coined in 1998 to designate the qualitative and quantitative analysis of all small molecules in an organism, with focus on intermediary metabolites, secondary metabolites and signalling molecules.

The metabolome is vast, including a huge number of molecules, which have disparate physical properties and are involved in many metabolic pathways and therefore its analysis is extremely complex; most metabolic profilings are usually performed by mass spectrometry (MS) or nuclear magnetic resonance (NMR). The metabolome is extremely dynamic, fluctuating according to environmental and internal conditions.

Metabolomics became an important diagnostic tool in medicine, but it is now used also in many other fields. Metabolite changes can be detected in response to variations in environmental conditions such as light, temperature, humidity, soil type and salinity, but also to pest attacks or the use of fertilisers or pesticides; specifically, monitoring of metabolic changes provides a good picture of how abiotic or biotic stresses affect crops. Metabolomics studies may lead to lesser pesticide usage, optimisation of trait development in agricultural products or increasing nutritional quality of food crops.

Food metabolomics evaluates food quality, in terms of composition and authentication, and allows the assessment of how processing and storage are affecting the bioactive compounds in food. The effect of environmental factors (climatic, pollutants) on plants can be monitored by

quantifying the changes in their metabolites. Of the around 200,000 known plant metabolites, new and improved medicinal and nutritional products will be discovered by the modern analytical techniques.

Plants as a source of compounds of commercial interest

Plants are a rich source of active compounds – **phytochemicals** – economically important because of their applications in different industries. They are secondary metabolites, with a wide array of biological functions, which the plants synthesise through diverse biochemical pathways. Many have protective roles in the plants, acting as antioxidants, free-radical scavengers or direct UV light screens in the mechanisms of response to abiotic stresses that generate oxidative stress in the plants, or as antiproliferative agents defending the plant against pathogenic microorganisms (bacteria, fungi, viruses and viroids) or nematodes. Other roles include feeding deterrence, for which many phytochemicals are bitter and/or toxic to potential herbivores, and this toxicity often extends to direct interactions with the herbivore's central and peripheral nervous systems. Secondary metabolites are also involved in the allelopathic interactions between plants or the establishment of symbiotic relationships, by attracting with colours and scents pollinators and animals responsible of fruit and seed dispersal; they can as well provide indirect defences for the plants by attracting natural enemies of their herbivorous attackers.

Phytochemical compounds are very diverse chemically, and the distribution of specific types of secondary metabolites is often restricted to taxonomically related species. They can be subdivided into a number of distinct groups by their chemical structure and synthetic pathways. The largest and most prevalent of phytochemical groups are the alkaloids, terpenes, and phenolic compounds.

The great diversity of plant secondary metabolites with different chemical structures is very interesting for different industries as they have shown to possess pharmacological activities and are useful as a source of pharmaceuticals, nutraceuticals, flavouring agents, food protectors, fragrances, cosmetics, insecticides, fungicides and other plant protection products, dyes and drugs.

Plant secondary metabolites are usually produced in low quantities; often they get accumulated in specific plant organs, at distinct developmental stages, in a particular agro-geo-climatic zone, or only in response to some external signal, such as exposure to a specific stress condition. Many commercially interesting metabolites like taxol, artemisinin or forskolin, among

many others, are very difficult to synthesise chemically, and the process is economically unviable.

Plant tissue culture at an industrial scale presents itself as a commercially viable alternative for production of phytochemicals, considering the increasing demand for metabolites of interest, the long time required for certain slow-growing plants, the continuously reducing land availability for large-scale cultivation of plants, and the destruction of wild populations of medicinal plants through exploitation.

Only a few biosynthetic pathways, such as those involved in the production of cinnamic acid derivatives, anthraquinones, berberines, shikonins, or anthocynanins, are efficiently expressed in suspension cultures. Other compounds, such as morphinan alkaloids, tropane alkaloids (e.g. hyoscyamine and scopolamine), quinoline alkaloids, dimeric monoterpene indole alkaloids (e.g. vinblastine and vincristine), among others, are expressed only in traces in suspension cultures. Large-scale efforts to increase their expression through medium engineering and use of elicitors have not yielded results that can lead to commercial exploitation of tissue cultures for production of these compounds.

Organ culture has been explored for the production of those phytochemicals that cannot be obtained at profitable levels in suspension cultures. This is the case of morphinan alkaloids of *Papaver somniferum* (Papaveraceae), dimeric indole alkaloid (anhydrovinblastine, a direct precursor of vinblastine and vincristine) of *Catharanthus roseus* (Apocynaceae), sesquiterpene lactone (artemisinin) of *Artemisia annua* (Asteraceae), that are better produced in shoot cultures. Similarly, root cultures produce higher amounts of tropane alkaloids, such as hyoscyamine and scopolamine, as compared to suspension cultures.

Hairy root technology has found application in the scale-up production of many pharmaceutical plant secondary metabolites, such as ginsenoside and some alkaloids, and is increasingly getting considerable attention. Hairy root cultures show in many cases a rapid and plagiotropic growth, with branching on phytohormone-free medium. It has been extensively used to study root nodules formation and has found application in different species for the production of pharmaceutical plant secondary metabolites. This can be attributed to their ability to produce the compounds over successive generations without loss of biosynthetic capacity or genetic stability.

Among the metabolites with greatest commercial interest are the taxoids, which are secondary metabolites synthesised by *Taxus* spp. and found in the foliage and bark of these trees. The main pharmacological taxoid is Taxol, a polyoxygenated diterpene alkaloid approved by the FDA for use in the treatment of breast, ovarian and lung cancer, and of Kaposi's sarcoma – related to HIV. Due to the interest of this drug, several attempts have been made to find new sources to obtain Taxol, unsuccessfully. Only recently, the production of Taxol has been achieved by the submerged fermentation of one of the native strains of endophytic fungi of *Taxodium mucronatum*.

5. Environmental biotechnology

Environmental biotechnology applied in solid waste and wastewater treatment or remediation of contaminated soils have proven to be useful and efficient solutions for improving quality of living. Increased waste generation from anthropic activities requires adequate technologies based on microorganisms or enzymes for their biodegradation. Accumulation of toxic compounds in soil, due to industrial or agricultural activities, may be subjected to two main actions. The first one consists of bioremediation and phytoremediation, cleaning techniques based on microorganisms and respectively on plants. The second one is preventing of environmental degradation by replacing a conventional technology with a biotechnology. Biosensors are a biotechnological solution for monitoring the quality of air, water and soil. Environmental biotechnology can be considered emerging and growing-up challenge, stimulating enhanced biodegradation using new microbial strains, enzyme engineering, biosensor development, process engineering, waste assessment and recycling. *In situ* environmental biotechnology may bring prosperity because creates employment opportunities with increased incomes for high qualified specialist, gives products with added-value by waste valorization and creates a healthier environment.

Phytoremediation of polluted environments – a green alternative

The term "phytoremediation" comes from associating two other terms - the Greek prefix *phyto* which it means plant, and the Latin suffix *remedium,* meaning restoring balance or to correct or to remove something bad. Phytoremediation represents a group of technologies that use natural or genetically modified abilities of the (superior) plants to clean-up contaminated sites, by cleaning-up being understood the capacity to remove, degrade, detoxify or transform the

contaminant from polluted environments - soil, sediments, groundwater, surface water and/or atmosphere. Likewise, phytoremediation has been defined as the employment of science and engineering in order to study problems and provide solutions involving plants and contaminated environments. This technology has been used to remove heavy metals, such as Hg, Cr, Cd, Cu, Ni, Zn, Pb, As, Mo, Se, Pd), organic contaminants (alkylated polycyclic aromatic hydrocarbons, fungicides, pesticides, polychlorinated biphenyls, some radioactive isotopes – Cs, U. Phytoremediation strategies utilize trees, shrubs, crop plants, aquatic macrophytes and/or grasses from different species for treating contaminated air, soil or water. The option to clean the contaminated environment with plants became more attractive to the environmental scientists, as an alternative to the classic methods. These traditional technologies – excavation, chemical soil treatment, thermal treatment – proved to be expensive and destructive for the environment

A lot of researches was made in order **to improve the phytoremediation** process, especially the phytoextraction of heavy metals. These improvement efforts include genetic engineering of the plants, the addition of chelating agents or hormones and plant responses to those, the mycorrhizae formation, the exploitation of natural plant diversity, the interactions between plant roots and rhizosphere microorganisms, the use of endophytic bacteria that possess superior capacities for metal accumulation and/or degradation of organic contaminants.

Regarding phytoremediation process as a green alternative, it has to be mentioned that all the results obtained in the field might be different from those obtained at laboratory or greenhouse level. This is due to the fact that the field is a real world, a real environment, where different factors act simultaneously. Among these factors that interfere with phytoremediation in the field is also included variation of temperature, nutrients, precipitation and moisture, plant pathogens presence, uneven distribution of pollutants, soil type, soil pH, and soil structure. That's why the phytoremediation is an interdisciplinary domain and requires solid background knowledge in soil chemistry, plant biology, ecology, soil microbiology as well as environmental engineering

Microbial bioremediation

Microorganisms have biosynthetic and biodegradative abilities which proved very valuable in finding solutions for maintaining the quality of the environment or repairing the damaged ecosystems.

One of the main application of microorganisms in environmental protection and bioremediation is the creating of cleaning technologies in oil contaminated areas, but also in areas contaminated with polychlorinated biphenyl compounds (PCBs), hydrocarbons, dyes, pesticides, esters, heavy metals, or nitrogen containing chemicals (table 3). Compared with other methods, biological treatment using bacteria, fungi or microalgae is low in cost, highly efficient and prevent secondary pollution.

Table 3. Biological agents of bioremediation

Microorganism		Toxic compounds used	
		Organic pollutants	**Heavy metals**
Bacteria	*Bacillus spp.*	Cresol, phenols, aromatics, long chain alkanes, phenol, oil-based based paints, Textile Dye	Cu, Zn, Cd, Mn
	Pseudomonas spp	Benzene, anthracene, hydrocarbons, PCBs	U, Cu, Ni, Cr, Cd, Pb, Zn, As
	Pseudomonas putida	Monocyclic aromatic hydrocarbons, e.g. benzene and xylene	
	Xanthomonas sp	Hydrocarbons, polycyclic hydrocarbons	
	Streptomyces sp	Phenoxyacetate, halogenated hydrocarbon, diazinon	
	Mycobacterium sp	Aromatics, branched hydrocarbons benzene, cycloparaffins	
	Alcaligenes odorans, B.subtilis, Corynebacterium propinquum	Phenol	
	Acinetobactor sp., Pseudomonas sp., Enterobacter sp.,	Pesticides (chlorpyrifos, methyl parathion, malation, endosulfan)	
Fungi	*Coprinellus radians*	PAHs, methylnaphthalenes and dibenzofurans	
	A. niger, A. fumigatus, F. solani and P. funiculosum	Hydrocarbons	
	Aspergillus versicolor, Trichoderma sp., Microsporum sp., Cladosporium		Cd
	Saccharomyces cerevisiae		Pb, Hg, Ni
	Penicillium simplicissimum	Polyethelene	
	Rhizopus arrhizus		Ag, Hg
Algae	*Chlamydomonas sp*	Naphthalene	
	Dunaliella sp	Naphthalene, DDT	
	Euglena gracilis	DDT, Phenol	
	Spirogyra hyalina		Cd, Hg, Pb, As

Microbial biofertilizers for bioremediation

Biofertilizers are microbial enriched products, containing latent or living cells of selected beneficial microorganisms that are able to improve soil qualities and promote plant growth, mainly by increasing the nutrients' uptake. Biofertilizers accelerate certain bioconversion processes in the

growing substrate and increase nutrients' bioavailability for plants. They can be applied to the soil, seed or plant surface, enriching the microbial communities of the rhizosphere and colonizing the inner and external parts of the plants.

In the sustainable agriculture, biofertilizers are cost efficient supplements of plant nutrients that increase the efficacy of chemical fertilizers or reduce their application requirements.

Among the beneficial microorganisms used as biofertilizers are: mycorrhyza, several soil and plant inhabiting fungi, most of the plant growth promoting bacteria and some blue-green algae. Based on their function, they can be classified as nitrogen fixers, phosphorus solubilizers, phytohormone and enzymes producers and other.

The **nitrogen-fixing microorganisms** can convert the atmospheric nitrogen (unavailable for direct plant nutrition) into organic nitrogen compounds, which are available for plants. Such biofertilizers can substitute nitrogen fertilization in some cultivated plants. The microorganisms used as nitrogen-fixing biofertilizers include symbiotic bacteria and free-living or non-symbiotic microorganisms (bacteria, actinomycetes and blue-green algae). Among the symbiotic bacteria, *Rhizobium* and related genera (*Azorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Ensifer* etc) are able to fix nitrogen in leguminous plants, producing nodules on their roots. Various other nitrogen fixing microorganisms were found as both symbiotic and free-living bacteria and actinomycetes. In such case were found *Acetobacter*, *Azotobacter*, *Azospirillum*, *Paenibacillus* and *Frankia*.

Among other beneficial microorganisms used as biofertilizers are **phosphorus solubilizing microorganisms,** which increase phosphorus uptake from phytic acid and phytate organic phosphorus and improve the solubility of inorganic phosphates. Available commercial biofertilizers with P-solubilizing activity are based on bacterial strains, like BIOPHOS and GET-PHOS containing *Bacillus megaterium* var. *phosphaticum, and others such as* JumpStart® contain the soil fungus *Penicillium bilaii*.

Mycorrhizal fungi are also very good soil fertilizers. Mostly they are efficient in phosphorus uptake from insoluble sources but due to their colonization capacity they improve plant nutrition with several other nutrients from sources generally unavailable for host plants. Moreover, they positively influence soil aggregation and water dynamics.

Energetic plants feasible resources for biofuel production

In the past decades, biofuels have attracted a lot of attention due to the increasing demand on energy resources as well as increasing concerns about greenhouse gas emissions due to the fossil-fuels use. Based on the type of the used feedstock, biofuels are classified into four generations. First generation biofuels make use of edible biomass which raised controversy because it competes with global food needs. The second generation biofuels are based on non-edible biomass but some limitations are concerned, related to the cost-effectiveness when scaling-up the production to a commercial level. The third generation biofuels use as feedstock the microorganisms, while in the case of the fourth generation biofuels the focus is on genetically modifying microorganisms able to achieve a preferable yield in the ratio hydrogen/carbon to eliminate or minimize carbon emissions.

The **second generation biofuels**, which are based on renewable alternatives by utilizing non-edible lignocellulosic biomass such as annual or perennial plants which may lead to economic income of the farmers, because this biomass is considered to be an inexpensive and attractive biofuel resource. Bioethanol can be produced from lignocellulosic biomass through hydrolysis and subsequent fermentation; this is why in **bioethanol** production the use of fermentative microorganisms is a must. Such examples of microorganisms can be yeast (*Saccharomyces*), bacteria (*Zymomonas*) or even moulds.

In the past decades, most of the **biodiesel** was currently made from soybean, rapeseed, sunflower, and palm oils; while soybean oil was commonly used in the United States, about 80% of the European Union's total biofuel production was based of biodiesel produced from rapeseed and sunflower seeds. Because of socio-economic issues, nowadays biodiesel produced from edible vegetable oils is currently considered as non-feasible and solutions have been proposed. Apart of different agricultural waste, a wide variety of plants can be used as lignocellulosic biomass for biofuels production like, poplar trees, willow and eucalyptus, miscanthus, switchgrass, reed canary grass, camelina, Jatropha jojoba oil, etc.

When making the choice on which energetic plant should be cultivated, apart from the plants' adaptability to different European climatic areas, it is important to have already a well-defined cultivation and harvesting technology. Generally, it is recognised that grass-plants (non-woody) are preferable in terms of cultivation technology because in their case can be employed common agricultural techniques, which are not bringing complications to farmers. Still, some of

the farmers consider that perennial crops are more simple to be cultivated and harvesting, being more profitable; in this last case, high costs are involved only in the first year, when setting up the perennial plantation; costs are assumed to be 1.5 to 3 times higher than in the case of analogical costs for annual planting/seeding this is why incentives/subsidies are required.

6. Industrial biotechnology

Industrial biotechnology is defined as any application of biochemical, molecular biology and microbiology techniques aimed at facilitating industrial processes, producing bio-products and bioenergy and reclaiming environmentally compromised areas.

Industrial biotechnology uses microorganisms or enzymes to obtain a large variety of products ranging from primary metabolites like organic acids, alcohols, amino acids, nucleotides, vitamins, to complex products as biopolymers, detergents, biofuels with diverse applications in food sector, chemical and pharmaceutical industry, environmental, bioenergy. Developing such new technologies based on biological systems, with respect of low resource-consuming and environmental protection, stimulates scientific research and innovation.

The direct economic effect of the Industrial Biotechnology sector is defined by its in-house activities, i.e. the people it employs and the turnover and added value it creates as a sector. The largest employment is generated in the market of bio-based chemicals, followed by bioplastics and biofuels. Also a number of pharmaceutical applications, notably antibiotics, account for a substantial share of industrial biotechnology employment.

Vitamins production

Microbes produce seven vitamins or vitamin-like compounds commercially: beta-carotene, vitamin B12, vitamin B13, riboflavin, vitamin C, linolenic acid, vitamin F, and ergosterol. More than half of vitamins produced commercially are fed to domestic animals.

Riboflavin (vitamin B2) overproducers include two yeast-like molds, *Eremothecium ashbyii* and *Ashbya gossypii*, which synthesize riboflavin in concentrations greater than 20 g per L. A riboflavin-overproducer such as *A. gossypii* makes 40,000 times more vitamin than it needs for its own growth. The biochemical key to riboflavin overproduction appears to involve insensitivity to the repressive effects of iron. Riboflavin formation by *A. gossypii* is stimulated by precursors hypoxanthine and glycine. A newer process using a recombinant *B. subtilis* strain yields

20–30 g riboflavin per L. Resistance to purine analogs has improved production in *Candida flareri* and *B. subtilis*, as has resistance to roseoflavin, a riboflavin antimetabolite. Mutation of *A. gossypii* to resist- ance to itaconic acid and aminomethylphosphonic acid (glycine antimetabolite) has yielded improved riboflavin producers.

Vitamin B12 (cyanocobalamin) is produced industrially with *Propionibacterium shermanii* and *Pseudomonas denitrificans.* Such strains make about 100,000 times more vitamin B12 than they need for their own growth. The key to the fermentation is avoidance of feedback repression by vitamin B12. Of major importance in the *P. denitrificans* fermentation is the addition of betaine. Vitamin B12 overproduction is totally dependent upon betaine but the mechanism of control is unknown. *Propionibacterium freudenreicheii* can produce 206 mg per L but is not yet a major industrial producing organism.

Traditionally, biotin has been produced chemically but new biological processes are becoming economical. In the production of *biotin*, feedback repression is caused by the enzyme acetyl-CoA carboxylase biotin holoenzyme synthetase, with biotin 5-adenylate acting as corepressor. Strains of *Serratia marcescens* obtained by mutagenesis, selected for resistance to biotin antimetabolites and subjected to molecular cloning, produce 600 mg per L in the presence of high concentrations of sulfur and ferrous iron.

Vitamin C (L-ascorbic acid) is used for nutrition of humans and animals as well as a food antioxidant and has been produced almost completely by chemical synthesis (Reichstein process) for many years. This otherwise chemical process utilizes one bioconversion reaction, the oxidation of D-sorbitol to L-sorbose. It has been shown to proceed at the theoretical maximum, i.e., 200 g per L of D-sorbitol can be converted to 200 g per L of L-sorbose, when using a mutant of *Gluconobacter oxydans* selected for resistance to high sorbitol concentration. The Reichstein process will probably have to compete with commercial fermentation approaches in the next few years. A novel process involves the use of a genetically engineered *Erwinia herbicola* strain containing a gene from *Corynebacterium* sp. The engineered organism converts glucose to 2-ketogulonic acid, which can be easily converted by acid or base to ascorbic acid. Another process involves cloning of the gene encoding 2,5-diketo-D-gluconate reductase from *Corynebacterium* sp. into *Erwinia citreus*. Plasmid cloning of the genes encoding L-sorbose dehydrogenase and L-sorbosone dehydrogenase from *G. oxydans* back into the same organism yielded a strain capable of converting 150 g per L of D-sorbitol into 130 g per L of 2-keto-L-gulonate.

Organic acids production

Microbes have been widely used for the commercial production of organic acids. Citric, acetic, lactic, gluconic, and itaconic acids are the main organic acids with commercial application. Other valuable organic acids are malic, tartaric, pyruvic, and succinic acids.

Citric acid is easily assimilated, palatable, and has low toxicity. Consequently, it is widely used in the food and pharmaceutical industry. It is employed as an acidifying and flavor-enhancing agent, as an antioxidant for inhibiting rancidity in fats and oils, as a buffer in jams and jellies, and as a stabilizer in a variety of foods. The pharmaceutical industry uses approximately 15% of the available supply of citric acid.

The commercial process employs the fungus *Aspergillus niger* in media deficient in iron and manganese. Manganese deficiency has two beneficial effects in the citric acid fermentation: (i) it leads to high levels of intracellular NH4 which reverses citric acid inhibition of phosphofructokinase; and (ii) it brings on the formation of small mycelial pellets which are the best morphological form for citric acid production. The morphological effect is due to a change in cell wall composition caused by growth in low Mn+. A high level of citric acid production is also associated with an elevated intracellular concentration of fructose 2,6-biphosphate, an activator of glycolysis.

High concentrations of citric acid can also be produced by *Candida oleophila* from glucose. In chemostats, 200 g per L can be made and more than 230 g per L can be produced in continuous repeated fed-batch fermentations. This compares to 150–180 g per L by *A. niger* in industrial batch or fed-batch fermentations for 6–10 days. The key to the yeast fermentation is nitrogen limitation coupled with an excess of glucose. The citric acid is secreted by a specific energy-dependent transport system induced by intracellular nitrogen limitation. The transport system is selective for citrate over isocitrate.

Vinegar (acetic acid) has been produced since 4,000 BCE. A solution of ethanol is converted to acetic acid in which 90–98% of the ethanol is attacked, yielding a solution of vinegar containing 12–17% acetic acid. Vinegar formation is best carried out with species of *Gluconacetobacter* and *Acetobacter*. An interesting application of genetic engineering in the acetic acid fermentation was the cloning of the aldehyde dehydrogenase gene from *Acetobacter*

polyoxogenes on a plasmid vector into *Acetobacter aceti* subsp. *xylinum*. This manipulation increased the rate of acetic acid production by over 100% and the titer by 40%,

Fermentation has virtually eliminated chemical synthesis of *lactic acid*. Whereas lactobacilli produce mixed isomers, *Rhizopus* makes L-(+)-lactic acid solely. *Rhizopus oryzae* is favored for production since it makes only the stereochemically pure L-(+)-lactic acid. It is produced anaerobically with a 95% (w/w) yield based on charged carbohydrate, a titer of over 100 g per L, and a productivity of over 2 g per Lh. This is comparable to processes employing lactic acid bacteria. It is polymerized into polylactide which is a new environmentally favorable bioplastic. Also of importance is the non-chlorinated environmentally benign solvent, ethyl lactate.

Production of *gluconic acid* amounts to 150 g per L from 150 g per L glucose plus corn steep liquor in 55 hours by *A. niger*. Titers of over 230 g per L have been obtained using continuous fermentation of glucose by yeast-like strains of *Aureobasidium pullulans*.

Antibiotics

The best known of the secondary metabolites are the antibiotics. This remarkable group of compounds form a heterogeneous assemblage of biologically active molecules with different structures and modes of action. They attack virtually every type of microbial activity such as synthesis of DNA, RNA, and proteins, membrane function, electron transport, sporulation, germination, and many others. Since 1940, we have witnessed a virtual explosion of new and potent antibiotic molecules which have been of great use in medicine, agriculture, and basic research. However, the rate of discovery drastically dropped after the 1970s. The search for new antibiotics must continue in order to combat evolving pathogens, naturally resistant bacteria and fungi, and previously susceptible microbes that have developed resistance. In addition, new molecules are needed to improve pharmacological properties; combat tumors, viruses, and parasites; and develop safer and more potent compounds.

About 6,000 antibiotics have been described, 4,000 obtained from actinomycetes. Certain species and strains are remarkable in their ability to make a multiplicity of compounds. *Streptomyces griseus* strains produce over 40 different antibiotics and strains of *B. subtilis* make over 60 compounds. Strains of *Streptomyces hygroscopicus* make almost 200 antibiotics. One *Micromonospora* strain can produce 48 aminocyclitol antibiotics.

The antibiotic market includes about 160 antibiotics and derivatives such as the β-lactam peptide antibiotics, the macrolide polyketides and other polyketides, tetracyclines, aminoglycosides, and others. The anti-infective market is made up of antibacterials, sera, immunoglobulins and vaccines, anti-HIV antivirals, antifungals, and non-HIV antivirals.

In the pursuit of more-effective antibiotics, new products are made chemically by modification of natural antibiotics; this process is called semisynthesis. The most striking examples are the semisynthetic penicillins and cephalosporins, erythromycins, tetracycline, and the relatively recently introduced tigecycline.

For the discovery of new or modified products, recombinant DNA techniques are being used to introduce genes coding for antibiotic synthetases into producers of other antibiotics or into non-producing strains to obtain modified or hybrid antibiotics. There are over 50 such antibiotics on the market today. Titers of penicillin with *Penicillium chrysogenum* have reached 70 g/ L, whereas those of cephalosporin C by *Acremonium chrysogenum* are over 30 g per L. Published data on clavulanic acid production by *Streptomyces clavuligerus* indicate the titer to be above 3 g/L. A relatively recently approved antibacterial is daptomycin, a lipopeptide produced by *Streptomyces roseosporus*. It acts against Gram-positive bacteria including vancomycin-resistant enterococci, methicillinresistant *Staphylococcus aureus*, and penicillin-resistant *Streptococcus pneumonia*.

Enzymes and bioconversions

Enzymes are biochemical molecules showing high catalytic power. These active proteins can be obtained from different sources like microorganisms, plants and animals, but the most attractive are the microbial ones, mostly because they are less expensive and may be subjected to genetic improvement for higher production yields.

Modern genetic engineering techniques are also applied for obtaining enzymes with improved characteristics and able to act in extreme conditions in terms of pH, temperature and saline concentration, enlarging their possible applications. In the last 50 years the great potential of biocatalysts is used for producing food, animal feed, detergents and cleaners, biofuels, textiles and leather, cellulose and paper, cosmetics and pharmaceuticals. Experiencing immediate impact from the developments in recombinant DNA technology was the industrial enzyme industry, which had been supplying enzymes with a market of about $300 million in the 1980s. Enzyme companies,

realizing that their products were encoded by single genes, rapidly adopted recombinant DNA techniques to increase enzyme production and to make new enzymes.

Biocatalysis is now used in various fields and there are numerous examples that can be reported, such as the production of acrylamide by the nitrile hydratase of *Rhodococcus rhodochrous*, and the production of lactose-free milk through the use of β-galactosidase, which splits lactose into glucose and galactose; similarly, fructose is produced by different companies starting from glucose through the use of glucose isomerase.

In the textile industry, enzymes such as proteases and lipases are used instead of chemical additives and allow to wash at low temperatures with considerable energy savings and reduced environmental impact. Enzyme inhibition studies are currently used for developing new and specific therapies, more targeted and with less side effects, with important contribution to human health. The continuous expansion of enzymes applications may be considered as an opportunity for developing new business in this field. Enzymatic technologies are more environmental friendly, generating higher quality and safer products with minimum wastes.

The world markets for some major products of enzymatic reactions are as high as $1 billion. *Streptomyces* glucose isomerase is used to isomerize D-glucose to D-fructose, to make 15 million tons per year of high fructose corn syrup. The high intensity sweetener market is comprising the production of aspartame, saccharin, cyclamate, neohesperidine DC, acesulfame-K, and thaumatin. *Pseudomonas chlorapis* nitrile hydratase is produced at 100,000 tons per year and employed to produce 30,000 tons/year of acrylamide from acrylonitrile.

Significant markets exist for specialty enzymes such as recombinant chymosin for cheese making, restriction enzymes for molecular techniques, and Taq polymerase for PCR applications.

In addition to the multiple reaction sequences of fermentations, microorganisms are extremely useful in carrying out **biotransformation processes**, in which a compound is converted into a structurally related product by one or a small number of enzymes contained in cells. Bioconverting organisms are known for practically every type of chemical reaction. Transformed steroids have been very important products for the pharmaceutical industry. One of the earliest and most famous is the biotransformation of progesterone to 11-α-hydroxyprogesterone. The reactions are stereospecific, the ultimate in specificity being exemplified by the steroid bioconversions. This specificity is exploited in the resolution of racemic mixtures, when a specific isomer rather than a racemic mixture is desired.

Bioconversion has become essential to the fine chemical industry, in that customers are demanding single-isomer intermediates. These reactions are characterized by extremely high yields, i.e., 90–100%. Other attributes include mild reaction conditions and the coupling of reactions using a microorganism containing several enzymes working in series. There is a tremendous interest in **immobilized cells** to carry out such processes. These are usually much more stable than either free cells or enzymes and are more economical than immobilized enzymes.

Biocatalysis mediated by immobilized enzymes is now used in various industrial fields starting from the pharmaceutical one, for the production of drugs such as β-lactam or anti-thrombotic antibiotics; in the food industry where they can be used both as biosensors and as catalysts of reactions of production, processing, and degradation; and in the biofuels synthesis industry, where biodiesel is produced, as well as through the classic chemical way, also through reactions based on the use of immobilized enzymes. This guarantees greater selectivity and specificity, the occurrence of reactions under mild conditions of temperature, pH and pressure and, in addition, the absence of by-products which should otherwise be removed.

Recombinant DNA techniques have been useful in developing new bioconversions. For example, the cloning of the fumarase-encoding gene in *S. cerevisiae* improved the bioconversion of malate to fumarate from 2 g per L to 125 g per L in a single manipulation. The conversion yield using the constructed strain was near 90%.

Biofuels production

Biofuels production has expended rapidly, encouraged by the regulations concerning renewable energy. According to European 2020 strategy (2010) the target for energy obtained from renewable sources is 20% of total energy. If the first generation of biofuels was based on starchy (corn, wheat, sugar cane, sugar beet) and oily (soybean, sunflower, rapeseed) raw materials competing with food, the second generation is oriented to renewable lignocellulosic materials and waste oil feed-stocks with low economic value. The third generation biofuel is produced using microalgae fermented with microorganims, converting CO_2 and producing O_2. Applying biotechnology on agricultural and food processing wastes and by-products for biofuels has also an important environmental contribution towards mitigation the impact of climate changes. Economic impact of biofuels depends a lot on the availability and price of the used feed-stock. Regarding the social aspect, alternative obtaining solutions for fuels and energy require high qualified human

resources, encouraging academia and industry collaboration, and generate independence on limited fossil carbon resources.

In most cases, lipases from different sources such as *Thermomices lanuginosus*, *Candida antarctica* and *Candida rugosa*, *Pseudomonas fluorescens*, *Pseudomonas cepacia* and *Saccharomyces cerevisiae* are used as enzymes to be immobilized for the production of biodiesel. Lipases are the chosen enzymes because they are able to conserve their activity even in means with low water content, such as organic solvents, and because, in addition to catalyzing the hydrolysis of triglycerides, they also catalyze esterification and transesterification.

7. Healthcare biotechnology

Healthcare biotechnology refers to a medicinal or diagnostic product or a vaccine that consists of, or has been produced in, living organisms and may be manufactured via recombinant technology (recombinant DNA is a form of DNA that does not exist naturally. It is created by combining DNA sequences that would not normally occur together). This technology has a tremendous impact on meeting the needs of patients and their families as it not only encompasses medicines and diagnostics that are manufactured using a biotechnological process, but also gene and cell therapies and tissue engineered products. Biotechnology offers patients a variety of new solutions such as: Unique, targeted and personalized therapeutic and diagnostic solutions for particular diseases or illnesses, An unlimited amount of potentially safer products, Superior therapeutic and diagnostic approaches, Higher clinical effectiveness because of the biological basis of the disease being known, Development of vaccines for immunity, Treatment of diseases, Cultured Stem Cells and Bone Marrow Transplantation, Skin related ailments and use of cultured cell, Genetic Counseling, Forensic Medicine, Gene Probes, Genetic Fingerprinting, Karyotyping.

Gene therapy

Gene therapy is an experimental technique that uses genes to treat or prevent disease. In the future, this technique may allow doctors to treat a disorder by inserting a gene into a patient's

cells instead of using drugs or surgery. Researchers are testing several approaches to gene therapy, including:

- Replacing a mutated gene that causes disease with a healthy copy of the gene.
- Inactivating, or “knocking out,” a mutated gene that is functioning improperly.
- Introducing a new gene into the body to help fight a disease.

Although gene therapy is a promising treatment option for a number of diseases (including inherited disorders, some types of cancer, and certain viral infections), the technique remains risky and is still under study to make sure that it will be safe and effective. Gene therapy is currently being tested only for diseases that have no other cures.

How does gene therapy work?

Gene therapy is designed to introduce genetic material into cells to compensate for abnormal genes or to make a beneficial protein. If a mutated gene causes a necessary protein to be faulty or missing, gene therapy may be able to introduce a normal copy of the gene to restore the function of the protein.

A gene that is inserted directly into a cell usually does not function. Instead, a carrier called a vector is genetically engineered to deliver the gene. Certain viruses are often used as vectors because they can deliver the new gene by infecting the cell. The viruses are modified so they can't cause disease when used in people. Some types of virus, such as retroviruses, integrate their genetic material (including the new gene) into a chromosome in the human cell. Other viruses, such as adenoviruses, introduce their DNA into the nucleus of the cell, but the DNA is not integrated into a chromosome.

The vector can be injected or given intravenously (by IV) directly into a specific tissue in the body, where it is taken up by individual cells. Alternately, a sample of the patient's cells can be removed and exposed to the vector in a laboratory setting. The cells containing the vector are then returned to the patient. If the treatment is successful, the new gene delivered by the vector will make a functioning protein.

Researchers must overcome many technical challenges before gene therapy will be a practical approach to treating disease. For example, scientists must find better ways to deliver genes and target them to particular cells. They must also ensure that new genes are precisely controlled by the body.

Ethical issues surrounding gene therapy

Because gene therapy involves making changes to the body's set of basic instructions, it raises many unique ethical concerns. The ethical questions surrounding gene therapy include:

- How can "good" and "bad" uses of gene therapy be distinguished?
- Who decides which traits are normal and which constitute a disability or disorder?
- Will the high costs of gene therapy make it available only to the wealthy?
- Could the widespread use of gene therapy make society less accepting of people who are different?
- Should people be allowed to use gene therapy to enhance basic human traits such as height, intelligence, or athletic ability?

Current gene therapy research has focused on treating individuals by targeting the therapy to body cells such as bone marrow or blood cells. This type of gene therapy cannot be passed to a person's children. Gene therapy could be targeted to egg and sperm cells (germ cells), however, which would allow the inserted gene to be passed to future generations. This approach is known as germline gene therapy.

The idea of germline gene therapy is controversial. While it could spare future generations in a family from having a particular genetic disorder, it might affect the development of a fetus in unexpected ways or have long-term side effects that are not yet known. Because people who would be affected by germline gene therapy are not yet born, they can't choose whether to have the treatment. Because of these ethical concerns, the U.S. Government does not allow federal funds to be used for research on germline gene therapy in people.

Toxicity Screening and Drug Discovery

As a result of the many progresses that have been made in the field of Biotechnology, Pharmacology underwent significant remodeling. A shift occurred from a predominant trial-and-error approach (known as **forward pharmacology**) to more accurate methods, using the latest discoveries of molecular biology in order to discover new pharmaceutical entities (known as reverse pharmacology).

In forward pharmacology, also called **phenotypic drug discovery** (PDD), compounds are screened in cellular or animal disease models to identify molecules that have beneficial effect: only

after an active drug has been identified, an attempt is made to identify the biological target of the drug.

In **reverse pharmacology**, also known as **target based drug discovery** (TDD), a biological target is hypothesized to be disease modifying. A high throughput screening (HTS) of compound libraries against the purified protein target is completed, identifying hit compounds that are then optimized and, differently than the forward pharmacology, tested for *in vivo* efficacy in the final drug discovery stages.

This new approach satisfied the demands related to the increased complex knowledge of biological systems enlightened by the new "omics" sciences, which enhanced our capability to link diseases to their causes and therefore led to an exponential rise in drug targets. In light of the new discoveries and approaches in pharmacology and molecular biology, biotechnologies became the leading force of drug discovery, and one of the main research fields of new start-ups and companies all over the world.

Drug discovery is the process by which new pharmaceutical compounds are discovered and brought to the market. It is a process made up of several stages that takes an average of fifteen years to be completed (see Figure 2). The first step is to decide which pathology to study, and identifying and validating the target that might be disease modifying. Afterwards, the exploratory research starts: with the first large screening tests it is possible to identify HIT molecules (chemical entities that have a promising affinity with the target). After more accurate investigations, there is the selection of a molecule that binds specifically and selectively to the target and is able to modify its normal mechanism-of-action, the LEAD compound. The latter is rationally modified in order to improve biological activity and ADME (absorption, distribution, metabolism, and excretion): if a promising compound is found during the screening, the preclinical and clinical phase will be started. Upon finalization of the clinical trials an approval of the drug by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA) has to be given before the drug can be brought to the market: during the years of its distribution, the safety of the drug will be continuously monitored thanks to pharmacovigilance.

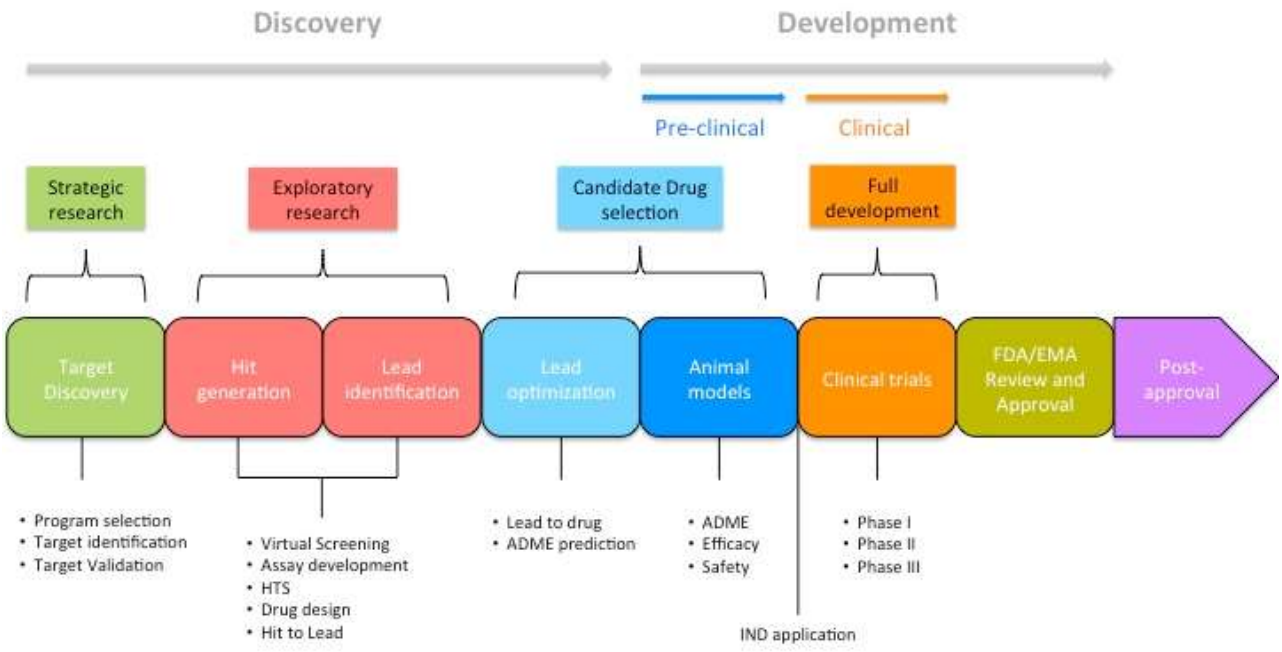

Fig. 2. Schematic representation of the drug discovery process. The two main phases, discovery and development, are articulated in sub-phases: for each sub-phase the major strategies and aims are listed. (IND, investigational new drugs).