

Synthetic Biology Textbook

The passion for the purpose of CBSin is something that we discovered right away in the first contact with the academic entity. Everyone who enters the Club has the sole desire to learn Synthetic Biology, but when inside, we are surrounded by welcoming people that make us love what we do. Every task becomes a passion and it is performed by interest in the constant academic entity development. We faithfully believe in the potential of the Club, in the events and projects that we elaborate, the feeling of personal development and professionalism is very striking for all members.

Relative to teaching, we believe that Synthetic Biology is a powerful transformation tool, both of science and of our society. Furthermore, we know that CBSin is notoriously an open door for Synthetic Biology to reach curious young people full of potential, in addition to being a reference for exploring this science responsibly and consciently. CBSin is transformation, union and commitment, we hope a lot for the success of our academic entity!

This textbook was prepared by students from the Engineering School of Lorena - USP as a project performed through the academic entity of the Synthetic Biology Club. The material created here is intended to provide a basis for all enthusiasts and beginners in the field of Synthetic Biology, as well as undergraduates, or even professors. who want to use the handout to organize and assemble your lesson plans. The work as a whole constitutes a wide range of topics covered, which had an extensive and careful literature review for each topic introduced. Thus, the textbook has an updated repertoire and an extension of deepening to then provide the best learning experience for the reader.

Easy Peasy Lemon Squeeze

Organelles _____ 2

Cell division _____ 3

What the f#@% is synthetic biology

Definition of Synthetic Biology _____ 13

History and Evolution of Synthetic Biology _____ 14

The IGEM _____ 17

Synthetic Biology Financial Market _____ 21

How to create a superhero

Molecular biology _____ 25

Genetic engineering _____ 33

Chassis and biobricks _____ 41

Matrix

Logical gates _____ 48

Biological circuits and gene regulation _____ 55

Mathematical modeling in mathematical biology _____ 59

Jurassic Park

Biosafety _____ 63

Microbiology _____ 68

Enzymology _____ 80

Cell Types

1. Summary

This topic will cover the contents of the two types of cells: eukaryotic and prokaryotic. We will talk about its main features and the difference between them.

2. Prokaryotes

Prokaryotes are the unicellular living beings that make up the Monera Kingdom, that is, the Bacteria and Archaea domains. Most everyday prokaryotes are from the Bacteria domain, while beings from the Archaea domain are best known for occupying environments considered hostile to other cells, such as concentrated salt water, acidic and hot springs of volcanic origin, in deep marine sediments, with little air, between others.

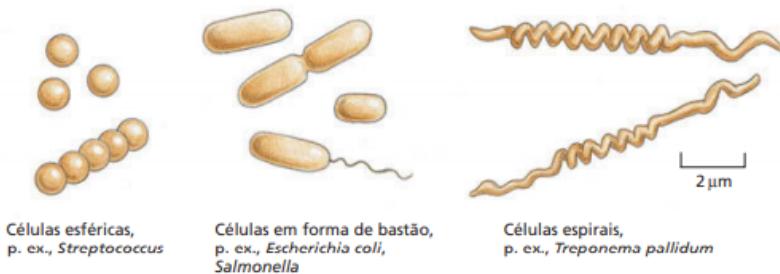
Thermal spring, place where one of the only beings that survive there is the Archaea group



<https://www.biologianet.com/biodiversidade/arqueas-arqueobacterias.htm>

But what exactly are prokaryotic cells like? To answer this question, let's organize its characteristics into topics:

- They represent the simplest way of life.
- They have a smaller structure, with a maximum diameter of 5 μm .
- Its shape can vary a lot, but the most common are: spherical, rod and spiral.

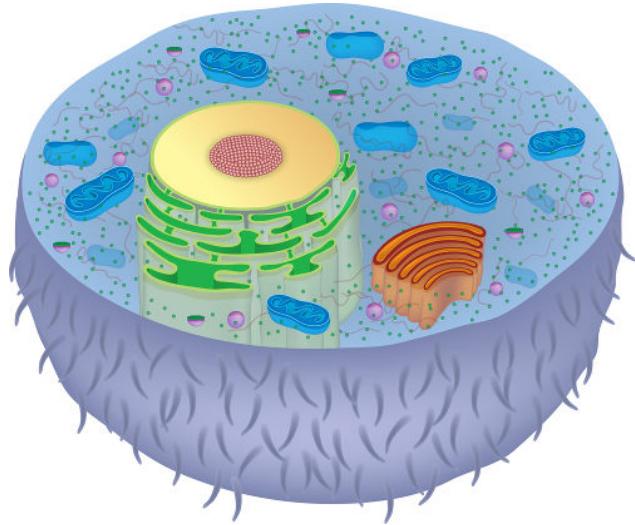


Fonte: Fundamentos da Biologia Celular. Alberts, Bray, Hopkin, Johnson, Lewis, Raff, Roberts & Walter

- They do not have membranous organelles.
- Nor do they have a nucleus that surrounds their DNA, so their genetic material is spread throughout the cytoplasm. In fact, this property is decisive for the classification of living organisms. This characteristic is reflected in its name, prokaryote, from the Greek words pro, meaning "before" and karyon, a "central part" or "core".
- They often have a protective layer surrounding the plasma membrane, called the cell wall. Many still have a polysaccharide layer.
- Most have a single circular chromosome. They can also contain smaller fragments of DNA circulating through the cytoplasm, called plasmids.
- They reproduce by asexual binary fission, so that, under favorable conditions, they can reproduce quickly.
- They are also able to exchange genetic material through structures called sexual pilis. This, together with the binary fission process, allows for rapid development and adaptation.

3. Eukaryotes

The main evolution of eukaryotic cells is the presence of a nuclear envelope, that is, they have a true nucleus, which protects the DNA, the karyotheca. The cytoplasm of this type of cell is subdivided into compartments, causing an increase in its metabolic efficiency. Fungi, algae, protozoa, plants and animals are eukaryotic organisms. That is, in general, these cells form complex organisms. However, some have independent life forms, such as amoebas and yeast (single-celled).



The presence of a plasma membrane allows the individualization of the cell, as it separates it from the environment.

Cytoplasm is composed of water and nutrients, structural biomolecules, organelles and supramolecular structures.

There is, for sure, no exactness as to the appearance of this type of cell. It is believed that, at first, the eukaryotic cell was a form of life that fed on other cells. In an evolutionary way, the enclosing structures were acquired by the cell.

4. Comparison of cell types

Prokaryotes	Eukaryotes
Extracellular envelope: capsule and bacterial wall (proteins and glycosaminoglycans)	Extracellular envelope: glycocalyx (glycoproteins, glycoproteins and proteoglycans) or cell wall (cellulose and pectin)
Abundance of lipopolysaccharide molecules in the plasma membrane, which provide protection such as resistance to hydrolytic enzymes and bile salts of enteric bacteria	Plasma membrane consisting of phospholipids, cholesterol, glycolipids, glycoproteins and proteoglycans
Absence of membranous organelles	Presence of membranous organelles
Respiratory chain molecules present in the inner membrane of the plasma membrane	Respiratory chain molecules located in the inner membrane of mitochondria
Nucleoid: absence of nuclear envelope, circular DNA, not associated with histone proteins and that does not condense into chromosomes	Nucleus: presence of nuclear envelope, linear DNA molecules, associated with histones and which condense into chromosomes at the time of division
Presence of extrachromosomal circular DNA strands (plasmids)	There are no plasmids
Free ribosomes; 70S ribosome sedimentation coefficient (ribosomal subunits: 50S +30S)	Free ribosomes or associated with the endoplasmic reticulum; 80S ribosome sedimentation coefficient (ribosomal subunits: 60S + 40S)
There is no separation between the processes of DNA duplication (replication), RNA synthesis from DNA (transcription) and protein synthesis from RNA (translation)	There is a separation between the processes of replication and transcription, which take place in the nucleus, and the translation that takes place in the cytoplasm
Absence of cytoskeleton	Presence of cytoskeleton
Do not perform endocytosis and exocytosis	Perform endocytosis and exocytosis
Filamentous extensions often depart from the surface: flagella and fimbriae. Flagella are rigid structures with a hook at the tip, which are used to move bacteria towards nutrients or away from	There are no fibers, and in those cells with flagella, their constitution involves the polymerization of the tubulin protein

toxic substances. Fimbriae are shorter and thinner than flagella and promote adherence of bacteria to host cells or transfer of DNA between two bacteria during conjugation	
Binary fission	Mitosis or meiosis

Organelles:

This topic will discuss how the cell works and what are the organelles, structures that do many essential functions to the cell. Understanding how a cell works has a high importance. to work with synthetic biology and use the cell machinery in our favor.

Introduction:

Just like superior organisms have organs that are fundamental to the functionality of the metabolism, cells contain many diverse and highly enzymatic structures in the cytoplasm. These structures are able to synthesize, or destroy compounds that are useful for the cell cycle.

Lysosome

Is an organelle responsible for intracellular digestion. Lysosomes contain about 40 different enzymes that are capable of acting in many different substrates.

Lysosomes are spherical and delimited by a membrane with carbohydrate, that protects the organelle against its enzymes. The Golgi apparatus synthesizes the lysosomes.

The digestion process consists in incorporating small particles through the invagination of cell membrane resulting in intracellular vacuoles. After that, lysosomes fund with these vacuoles and then release the enzymes. Useful molecules will be transferred to the cytoplasm.

Other functions are autolysis and autofagia. In the first case, damaged cells are digested spontaneously by the lysosomes. In the second case, organelles and other cell entities in excess are digested to generate energy and allow the organelle recycling.

Golgi apparatus

These are eukaryotic organelles with the function of processing and addressment of molecules to different parts of the cell.

Golgi apparatus has a structure with a cis and a trans surface that also have different functions and mechanisms.

Cis: Has the function of receiving vesicles from the endoplasmatic reticulum and releasing their content.

Trans: Synthetize the vesicles that will travel to different parts of the cell.

The Golgi apparatus is also related to the processament of lipids and protein, synthetizing mono and polysaccharides.

Peroxisome

It is an organelle related to the degradation of toxic compounds, and also possessing enzymes.

Catalase may be the most important enzyme once it is related with the protection against free radical formation like OH. Peroxisomes also degrade some lipids and proteins.

Vacuoles

This organelle is responsible for the hydric control, simple digestions, excretions, maintenance of the pH and also the storage of substances. In plant cells, the identification is easy, due to its characteristic size, although the organelle is still present in other cells. They are responsible for the flaccid and turgid state of plant cells that change according to the concentration of ionic compounds.

Rough endoplasmic reticulum

It is a structure attached to the external nuclear membrane, with a high amount of ribosomes. The main function is communications and socialization to the cell nucleus. It is important to declare that this organelle does not synthesize proteins.

Smooth endoplasmic reticulum

This organelle does not have a ribosome attached to it. The function of this organelle is the synthesis of lipids and sexual hormones. The smooth reticulum has also the ability to store some important substances on the tubular structure.

Plasma membrane

It is a lipid bilayer that separates the interior of all cells from the outside environment. An important characteristic is the selective permeability that ensures that important compounds are transferred into the cell and also to the outside. Other functions are protection and cell regulation. The current model that better explains how the membrane works, is the fluid mosaic, due to the mobility of the phospholipids, proteins and the carbohydrates. Also, the bilayer has a polar and an apolar side, which ensures the selectivity of the cell. The carbohydrates are only attached to the external side of the membrane, and have a function of recognizing the environment.

Ribosome

Ribosomes occur in all cells, and they are responsible for the synthesis of proteins. Their structure is made of two subunits, a bigger one and a smaller one, and the messenger RNA allocates between them for the translation.

Cytoskeleton

The Cytoskeleton is basically a web of fibers, that is in the cytoplasm. In Eukaryotic cells, there are microtubules made of actin fibers, therefore maintaining the shape and mechanical resistance of the cell. These microtubules also can contract, thereby deforming the cell in order to advance in different tissues. Prokaryotic cells also have a cytoskeleton with different fibers.

Cell Nucleus

It is presented in eukaryotic cells, and stores the genetic material, protecting it and not allowing it to be dispersed in the cytoplasm.

Cell Wall

The cell wall is an extracellular structure present in plant cells, bacteria and some fungi. And has the objective of protecting the cell, against all the outside adversities. With this structure, the cell becomes more rigid to avoid deformation of the cell. Its composition vary according to the organism

Chloroplast

Are exclusive in plants and some algae, This is a membranous organelle, responsible for different steps of photosynthesis. It is the organelle where the pigments are stored, and captures the photons, converting them into ATP and NADPH, releasing oxygen.

Mitochondria

The mitochondria is responsible for the generation of ATP, using NADH and FADH₂, to generate the gradient of protons so there can be the oxidative phosphorylation producing ATP.

CELL DIVISION

1. Menu

In this topic, will be discussed existing forms of cell division, how different organisms make it and how their mechanism works as a whole. Cell division is the process by which a mother cell can give rise to one or more daughter cells that have the same genetic code, that is, they maintain the same codon ratio as the mother cells.

The division can occur by binary fission, multiple fission, meiosis, mitosis, etc. Their study is important to understand why an organism performs a certain type of division. In

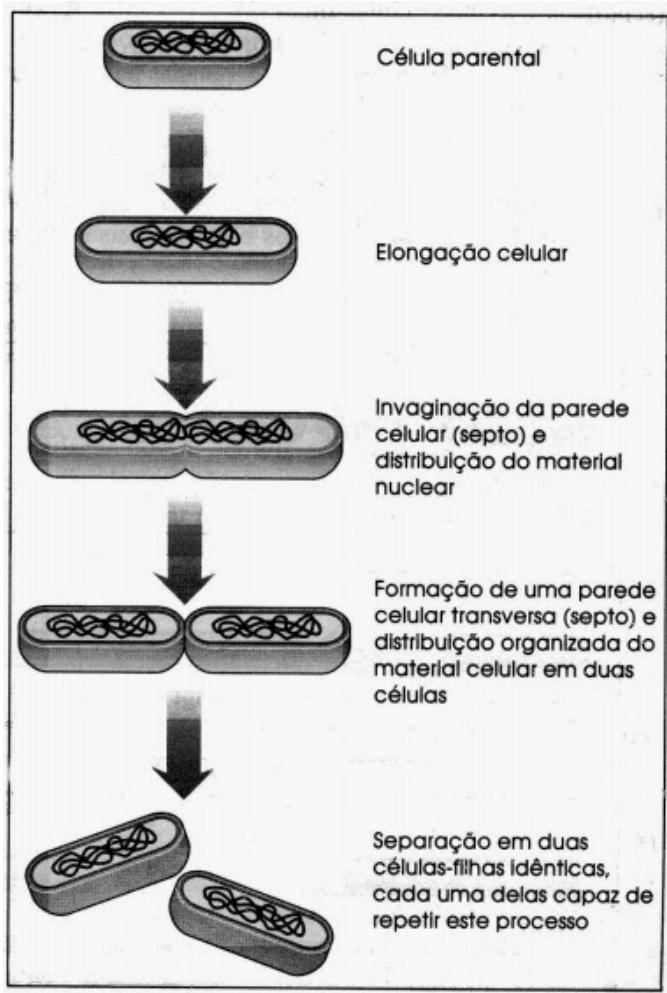
addition, it is possible to comprehend how division occurs and how its realization guides the cell's life cycle.

2. Binary fission

Fission is the biological process by which an individual entity originates two or more identical parts and by which the constituent parts are regenerated to resemble the original. In this part, binary fission, also called cissiparity, will be addressed, which is a simple, fast and conventional process, especially for bacteria.

It is common in archaea and bacteria, such as prokaryotes, they have fission as a central mechanism in their asexual reproduction. However, its presence is seen also in some eukaryotic organelles, such as mitochondria. The process begins with DNA replication: a single circular ribbon is unwound and replicated, with each one of the copies fixed at opposite points of the cell. The cell as a whole increases in length and its components, such as ribosomes, are duplicated. At the beginning of the division, the chromosomes are separated and the cell invaginates in the equatorial plane and, with that, there is the division of the cytoplasm, called cytokinesis. This mechanism continues until the new membrane forms between both new cells. These have a similar volume and a similar amount of ribosomes and plasmids to each other.

The binary fission is relatively simple and quick: a colony of E.Coli (common bacteria) at 37°C can be duplicated every 20 minutes. Others, like Mycobacterium tuberculosis can take up to 48 hours. This time is called the **Doubling time**.



Fissão Binária. Fonte: Pelczar *et al.*, 1996.

3. Interphase

The interphase is the entire period that the cell is not performing the dividing process itself, however it is the moment that the cell is preparing itself and increasing its volume and number of organelles. The interface can be divided into four distinct periods: G0, G1, S, G2.

G0-Rest

G0, from the english "Gap", is complementary to the G1. It's the moment when the cell is not willing to start dividing. The cell focuses on performing its vital functions and waits for a stimulus to proceed to the G1.

G1-Protein Synthesis

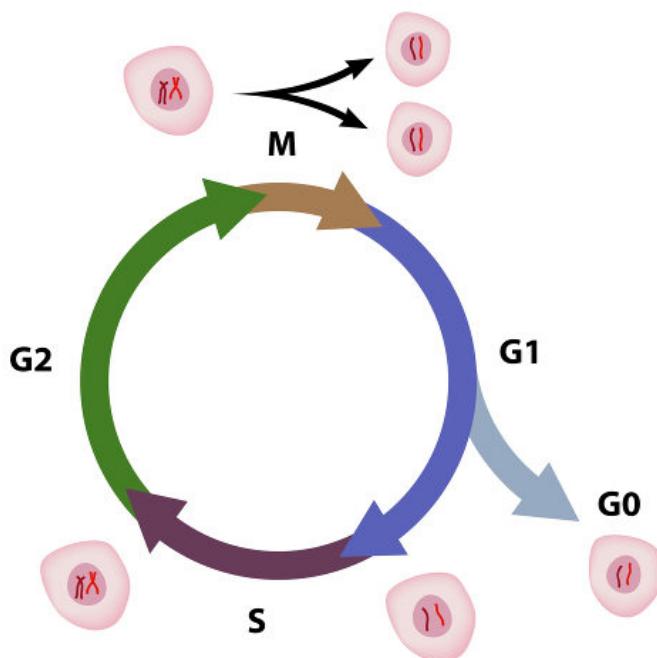
Cell growth period. There is an intense synthesis of RNA and proteins with the progressive increase in cell volume and the duplication of cell components.

S-Synthesis

In this part, the doubling of the chromosome's chromatids occurs, making the cell have twice the original DNA, that is, a cell that had n chromosomes now has $2n$. This step allows the cells generated in the future to have the same number of chromosomes.

G2- Duplication of centrioles

In this part, there is duplication of centrioles in organisms that have them. With its separation, there is the formation of the karyokinetic spindle. In this phase, the cell continues to expand its volume and increase the total number of organelles, making the final preparations so that it can begin its division.



Schematic representation of the interface. Fonte:

<https://www.biologianet.com/biologia-celular/interfase.htm>

4. The division itself

Mitosis is a division considered to be equational, where a mother cell generates two genetically identical daughter cells. We can divide mitosis into a few phases:

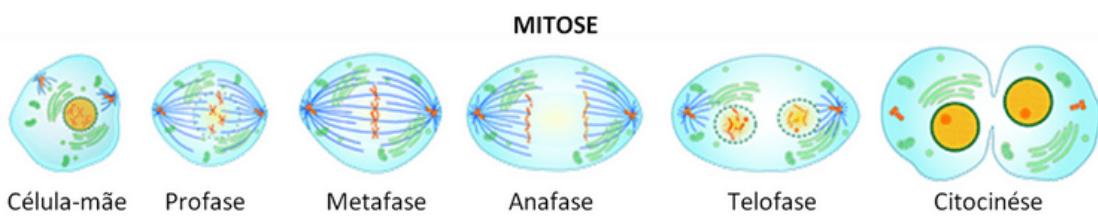
Prophase: At this stage, chromosomes start to become visible due to spiraling; The nucleoli begin to disappear and, from the centrioles, spindle fibers begin to come out. The core absorbs water and swells.

Metaphase: Chromosomes are clearly visible because they reach the maximum spiraling; chromosomes attach to spindle fibers in the equatorial region of the cell, one next to the other;

Anaphase: the spindle fibers start to shorten and, as a consequence, each filament is pulled towards the poles of the cell.

Telophase: Chromosomes begin to unwind; nucleoli and karyotheca begin to appear; and the centrioles are already located in pairs in each daughter cell.

Cytokinesis: is the breakdown of the cell in two. In animals, it occurs from the outside to the inside (centripetal) and in vegetables, from the inside to the outside (centrifuge).



In unicellular beings, mitosis is responsible for reproduction, and in multicellular ones, it is used to repair damaged tissues, replace dead cells and growth.

5. Meiosis

It is a type of cell division, exclusive to organisms that reproduce sexually, occurring only in germinative cells, for the formation of reproductive cells. Duplicate homologues do the pairing. In this phase, more specifically, in the pachytene stage, crossing-over occurs.

Prophase I: the chromosomes spiral, nucleoli disappear, spindle fibers appear and homologues are paired.

Metaphase I: homologous chromosomes in their maximum degree of spiraling arrange themselves in the middle region of the cell (but one on top of the other).

Anaphase I: the fibers are shortened and homologous chromosomes separate (the sister chromatids remain joined together).

Telophase I: the chromosomes unspiral and give rise to two haploid daughter cells (without homologous chromosomes).

Interkinesis: Interval between the two main stages of meiosis.

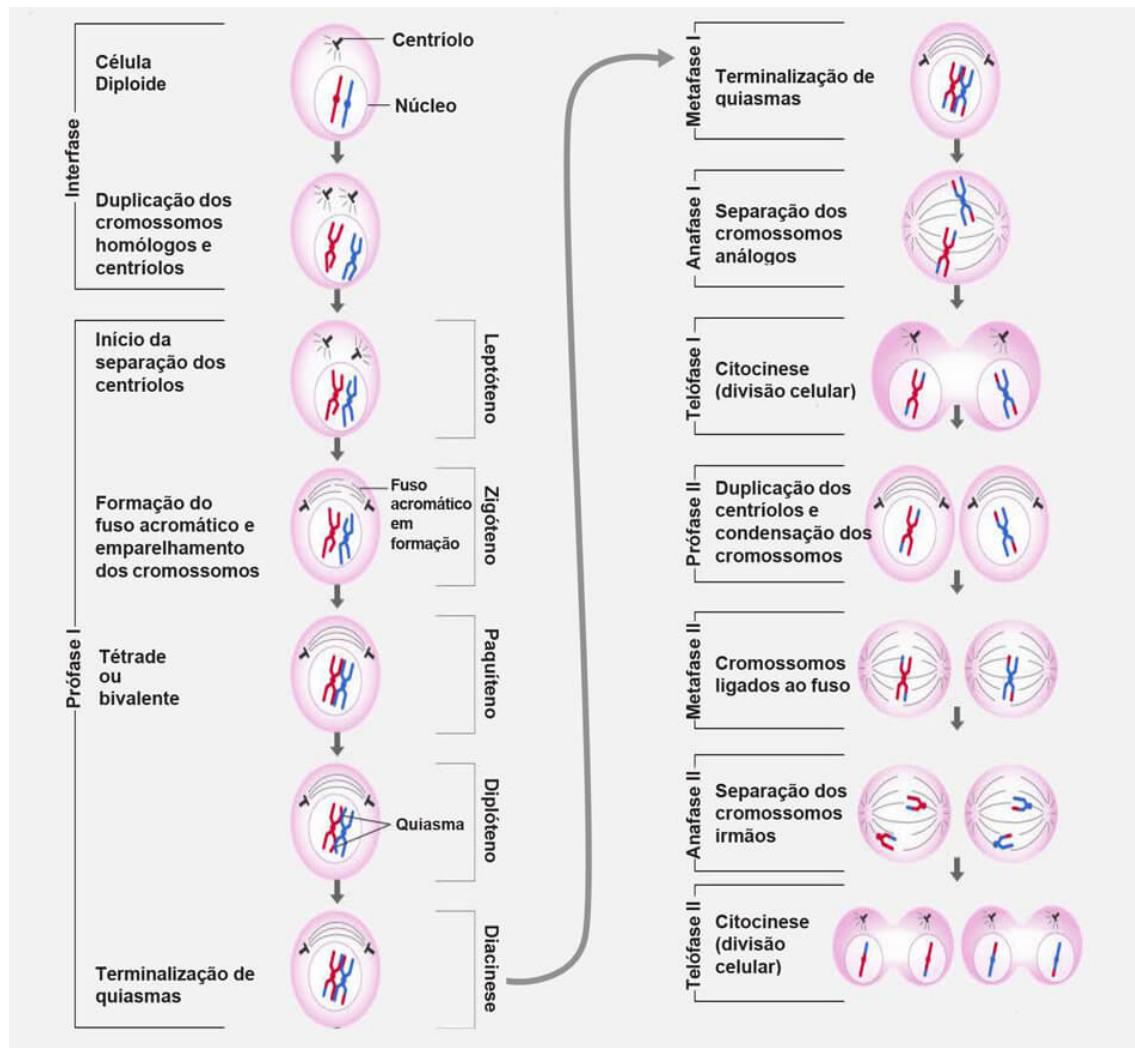
Prophase II: there is a new spiraling of the chromosomes, with spindle fibers.

Metaphase II: Chromosomes attach to spindle fibers by the centromere.

Anaphase II: duplication of the centromeres occurs and, with the shortening of the spindle fibers, only now the separation of the sister chromatids occurs.

Telophase II: there is a reorganization of the four haploid daughter cells and with half the initial chromosome number.

One of the main factors of meiosis is the emergence of diversity among the produced individuals, due to the random arrangement of chromosomes in meiosis I and Crossing-Over.



Definition of Synthetic Biology

1. Summary:

During the course, topics such as the definition and importance of synthetic biology, applications and examples in everyday life will be presented. It will be discussed why synthetic biology is such an interdisciplinary sector and how the advent of computing revolutionized the understanding of it.

2. What is synthetic biology?

Synthetic Biology is an area of biology in which engineering methods are used to design and alter biological functions and systems. It is often associated with genetic engineering techniques and has been increasingly improved with the introduction of computing, helping with projections, sequencing and contributing to the solution of various problems facing humanity. Synthetic biology can be understood as the use of a cell as a biological circuit analogous to a computer, which can be modeled and altered as desired.

An example:

A river is having problems due to the poor disposal of chemical substances, among them: endocrine disruptors, substances that mimic hormones, causing female fish to become male. One procedure that a professional familiar with synthetic biology could perform would be to modify a bacterial cell, such as E coli. This could modify the bacteria's genes to enable it to decompose this disruptor, eliminating this problem efficiently and without causing environmental impacts.

3. But what does synthetic biology do? How does it operate?

Synthetic Biology encompasses several areas of activity, involving different ways of life and the most varied tools; often relying on advanced computing and technologies. This science has as its main objective to design and modify living beings so that they can act in industry and research, enabling the creation of chromosomes and even artificial cells, being used as tools to solve several human problems.

There is a preference for using model organisms as the foundation of a project, for example *Escherichia coli*, a bacterium whose genome has already been entirely sequenced.

Biobricks are tools used to assemble genes that will be injected into model cells, their construction is done with logic analogous to computer programming. Thus, concluding the principle of assembling a biological circuit, the main tool in synthetic biology.

Synthetic Biology uses several tools, such as endonucleases (enzymes capable of cutting genetic material), primers, nucleotides, and enzymes such as polymerase in the PCR technique, the use of logic gates with concepts similar to programming, the issue of bioinformatics for make models that can predict the behavior of cells, among many others that will be discussed later. Therefore, it can be said that Synthetic Biology is an area of great interdisciplinarity, considered still very recent, being in constant evolution, and this discipline covers computing and its respective concepts as logical gates, as well as using the areas of knowledge more classical ones, such as biochemistry and molecular biology.

History and Evolution of Synthetic Biology

1. Summary

This topic will address a little about the great discoveries that drove the field of synthetic biology, in order to understand the contributions to the area and make the student understand their importance for the creation, from this, of new technologies.

2. Summary

"I need to tell you that I can make urea without requiring the kidney of an animal, neither man nor human." Today, this is a pretty obvious phrase for science. However, when it was first said, in 1828, it was a discovery that would put an end to the so-called "Theory of Vital Force". In short, the chemistry of the time understood that there were two types of molecules: the organic ones, found in living beings, and the inorganic ones, which are of inanimate origin. The theory consisted in the fact that it

was possible to convert organic molecules into inorganic ones through processes such as heating, but the reverse process, as there were, until then, no successful experiments, was considered impossible. The above quote is a speech by the physician and chemist Friederich Wöhler to his tutor Jons Jakob Berzelius when he said that he had managed to synthesize ammonium cyanate (inorganic) into urea (organic).

Interestingly, Wöhler's initial intention was to obtain ammonium cyanate itself as a final product. For this, he heated silver cyanide in the presence of oxygen from the atmosphere, obtaining silver cyanate $[AgCN(s) + \frac{1}{2} O_2(g) \rightarrow AgOCN(s)]$. This product was then reacted with ammonium chloride, resulting in a precipitate of silver chloride and an ammonium cyanate solution $[2 AgOCN(s) + NH_4Cl(aq) \rightarrow AgCl(ppt) + NH_4OCN(aq)]$. Wöhler, however, continued his experiment, subjecting the solution to a process of filtration and evaporation, making it solid. After that, still in solid state, it was subjected to heating. As a result, Wöhler observed the formation of white crystals, soon realizing that it was urea $[NH_4OCN(s) \rightarrow CO(NH_2)_2(s)]$.

Thanks to the demystification of the theory of vital force, scientists, in the following decades, were able to synthesize thousands of other molecules, thus beginning the dawn of the field of synthetic biology.

As seen, synthetic biology has its beginnings in the 19th century, but what is considered by many as the “starting point” or root of the branch is the discovery of the Operon system, which occurred in 1961 by scientists Francis Jacob and Jaches Monod. Basically, the model was proposed in order to explain some control over gene activity in prokaryotes. This subject, however, will be discussed in detail later, but it is important that there is initial knowledge about its existence and importance.

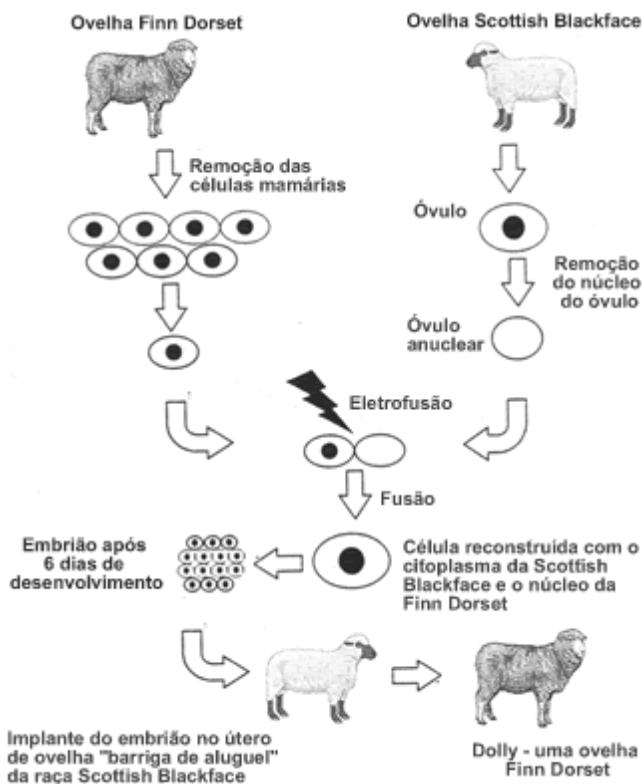
In 1985, in California, Kary B Mullis invented a technique of DNA amplification through cloning using enzymes found inside cells, the main one: polymerase. This enzyme is able to bind nitrogenous bases to a strand cleaved (by the helicase enzyme) starting from a primer. Bearing in mind these phenomena, the PCR technique was invented, from the English “Polymerase chain reaction” or simply polymerase chain reaction. With this advance, scientists were able to work with very small samples of DNA, it was possible to study fossils, analyze crime scenes more efficiently and even help diagnose diseases like AIDS in small samples.

The first laboratory dedicated to licensed synthetic biology research was opened in 2003 in the United States, the “Lawrence Berkeley National Laboratory”; however, the term “synthetic biology” was first used in a French scientific paper called “Théorie physico-chimique de la vie et générations spontanées” in 1910. 1978 Nobel Prize winners Werner Arber et al discovered an important tool for genetic engineering: endonucleases, with this advance it was possible to deepen the knowledge of genetics and, in the near future, to model and modify biological entities.

Essential for the advancement of synthetic biology and genetic engineering, the Human Genome Project (HGP), officially started in 1990 and completed in 2003, had as its main objectives the identification of all human genes and the determination of more than 3 billion pairs of component bases of the human genome. It is necessary, however, to emphasize that the function of approximately half of the identified genes is not known. It is due to the achievement and success of this project that laboratory tests can now be performed in order to diagnose diseases and/or mutations linked to genetic abnormalities in individuals, many of which are rare. Finally, it is worth remembering that the dysfunctions follow the pattern of genetic inheritance proposed by Mendel, considered the father of genetics.

With the advancement of computing and the creation of tools for automatic DNA sequencing, it was possible, in 1995, by TIGR (The Institute for Genomic Research) to publish the first complete sequencing of microbial genomes. Just 5 years later, scientists had already managed to reproduce the procedure and sequence the complete genome of another 29 microbes. The advantage of this feat is/was to obtain information about hundreds of thousands of genes (this number is even greater today). The challenge of synthetic biology is the search for knowledge about new genes and their functions and, thus, being able to develop and improve treatments, especially for diseases for which efficient prevention and cure have not yet been discovered.

In 1996, a Scotsman named Ian Wilmut and his team cloned the first mammal from a developed somatic cell, a sheep affectionately nicknamed Dolly. The process proved to be extremely complex because it was necessary to turn a mitotic cell into a stem cell, capable of differentiating itself.



It was only in 2009 that the definition of synthetic biology was solidified precisely: an area of biology that has many other areas of knowledge based on genetic engineering, bioinformatics and microbiology.

Over the years, researchers and computer developers (with considerable knowledge of biology) began to interpret a cell as hardware and software, where the software would be synthetic DNA. Based on these concepts, several computer simulations were carried out with the aim of sequencing the DNA, which resulted in a successful experiment in 2010: the first synthetic bacterial subspecies was created from a pre-existing one.

IGEM Competition

1. Summary

In this topic we will talk a bit about iGEM Foundation, the leading synthetic biology outreach and support organization that urges students around the world to explore and develop knowledge about synthetic biology. The format of the student-developed project and the contest format aims to be a motivating and effective teaching method.

Several synthetic biology companies have emerged in iGEM, highlighting its importance in the landscape of this new science.

2. What is iGEM?

The International Genetically Engineered Machine, or simply iGEM, is an international competition, organized by the non-profit iGEM Foundation, dedicated to sharing synthetic biology projects. This competition provides an opportunity for students at universities around the world to solve everyday problems by using and reinventing biological systems engineering.

2.1 Tracks

Tracks are equivalent to categories, and exist to help the team direct the project They are developing. Thus, competing projects can be grouped together. Another important piece of information is that teams can be awarded according to their category as the best project in that specific track.

The team itself chooses the track it wants to participate in, and can choose up to three different tracks in order of preference, but the team has an obligation to choose only one, selecting the other two is optional.

Tracks vary from year to year, but here is the 2020 list:

- Diagnostics - Disease diagnostics
- Energy
- Environment
- Food & Nutrition
- Foundational Advance
- Hardware
- High School
- Information Processing
- Manufacturing
- New Application - Innovation or New Applications
- Open - Open (Basically a track for projects that don't fit into other tracks)
- Software
- Therapeutics - Therapies for diseases

2.2 iGEM Registry

The Registry of Standard Biological Parts is a growing collection of standardized genetic "parts" that can be combined to build synthetic biology systems. Today, the Registry has more than 20,000 standard parts, and you can browse the site by searching by part type, chassis, and function, for example.

In addition, teams and researchers can also submit biological parts to the Registry, following the RFC10 submission standard, to help grow the collection.

2.3 iGEM Committees

The iGEM committees consist of more than 100 volunteers who seek to support and advise iGEM on various issues such as safety, ethics, and diversity. The members of the committees are experts from different fields of synthetic biology and from different countries.

There are six committees in total: Measurement; Safety and Security; Human Practices; Diversity and Inclusion; Responsibility Conduct; Jurors

3. What are iGEM's goals?

The competition aims, besides seeking solutions to local and global problems in order to sustain a better world, to promote a friendly environment for scientific dissemination, education, and sharing of knowledge and experiences within a "Giant Jamboree", where presentations and evaluations take place.

3.1 Importance of Human Practices

Human Practices is a medal criterion, however, its importance is not only in the awarding.

Human practices, sometimes affectionately called hp, aims to take each team's project beyond the laboratory, including society, situating the work in the environment, always looking for solutions to real problems.

The project is not limited to what is worked on in the laboratory and in theoretical research about synthetic biology, and is therefore interdisciplinary, in which real problems are explored and relationships can be established with many different fields, especially those related to the human sciences, such as ethics, law, and economics, for example.

So, the more interdisciplinary, inclusive and beneficial to society and the environment, the better your project is!

4. How does the awarding work?

It is important to clarify that the competition does not take place exactly among the registered teams. Therefore, the ranking is done based on evaluation parameters, not comparison. The teams can be awarded gold, silver, bronze, or none. If the team is aiming for a bronze medal, they must meet all the bronze requirements; if they are aiming for a silver medal, they must meet all the bronze and silver requirements; and for a gold medal, they must meet all the above requirements, plus at least two gold requirements.

5. A little about some projects developed by the CBSin team

- Lacquase Project (2018)

The project was based on studies that indicate the presence of a high concentration of estrogen in the water consumed in several Brazilian cities. At first, the project was based on two problems:

1. That large amounts of the hormone in question can cause health problems in humans and other animals;
2. The possibility of harming the environment by the mass reduction of fish, since such contaminants can cause only females to be born.

So, to circumvent these problems, it was proposed to develop a method for removing these hormones from water. For this, laccases from filamentous fungi were modified, cloned and expressed. Thus, these enzymes were explored as possible biocatalysts applied to biodegradation of pollutants in water and in effluent treatment plants, increasing water quality.

- Project E. Coil (2016)

Fossil fuel sources are running out. Analyses indicate that in about 30 to 40 years the reserves will dry up and it is unlikely that the planet will be able to replace these energy compounds. It is therefore necessary to look for alternative energy sources, such as biodiesel.

The current biodiesel production cannot supply the world's demand, one way to do this would be to allocate more land to increase production. Currently, about 95% of the world's biodiesel is produced from edible oils, such as canola (84%) and sunflower oil (13%). And these crops already use up all the suitable farmland the world has. Moreover, there is no high quality agricultural land to locate plant species for biodiesel production. Fuel crops will compete with food crops.

The project aims to develop a drop-in biodiesel that fits into regular combustion engines without the corrosion or malfunction that transesterified fuel normally causes. The transgenic E. coli will function as a catalyst factory, where vegetable oils will be converted into fuels with high mechanical quality and environmentally friendly properties.

When registering for iGEM, the team receives the iGEM Kit, which consists of six plates with several freeze-dried biobricks. Because it is a small quantity, it is necessary to make a transformation with the desired part, aiming to create a stock in glycerol



[Help:2019 DNA Distribution](#)

[About - igem.org](#)

Companies

1. Summary

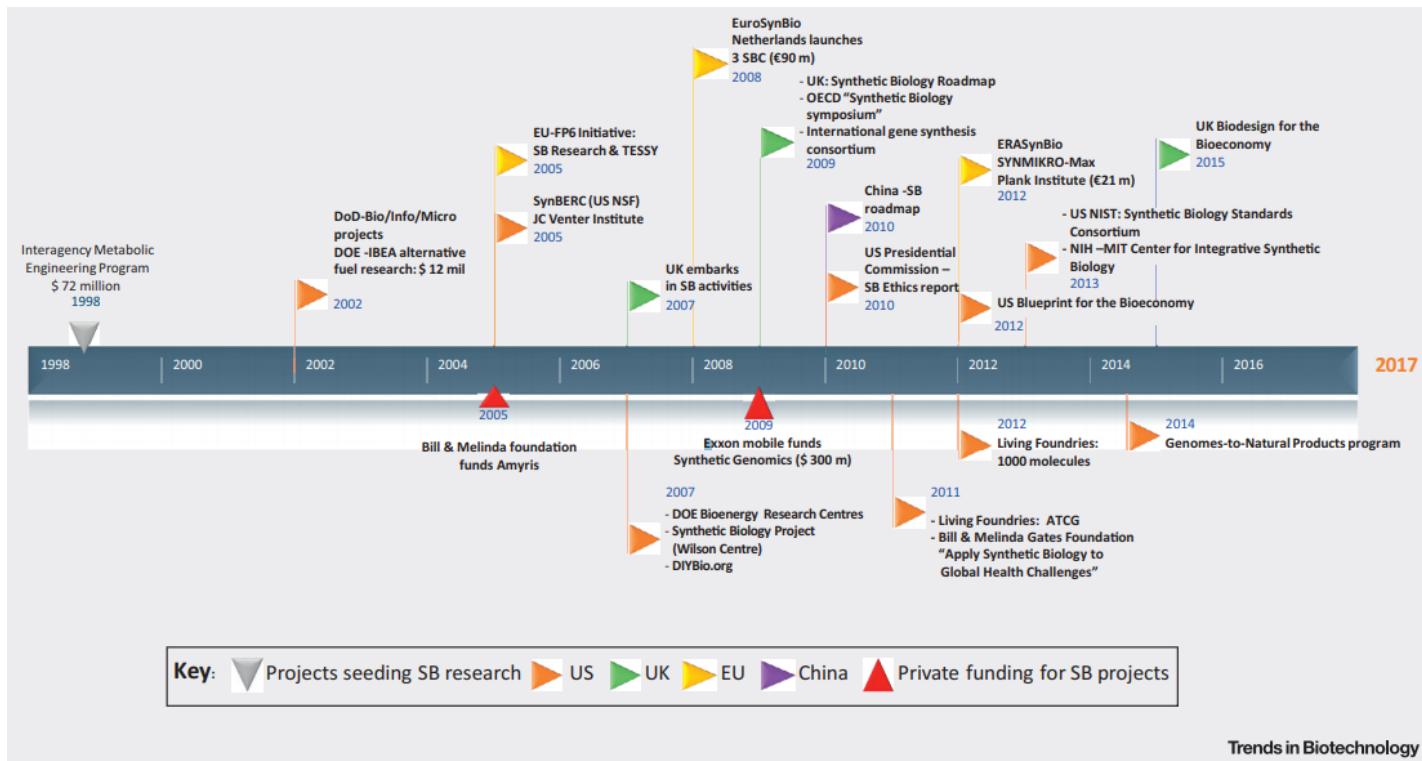
Although recent, the synthetic biology scenario is increasingly gaining its place in the market. Thus, we intend to explore the current situation of synthetic biology companies and some examples that are already well consolidated in the market. We also note the important understanding of the paths that lead a synthetic biology project to become a strong and persistent idea for the creation of a company.

2. Content

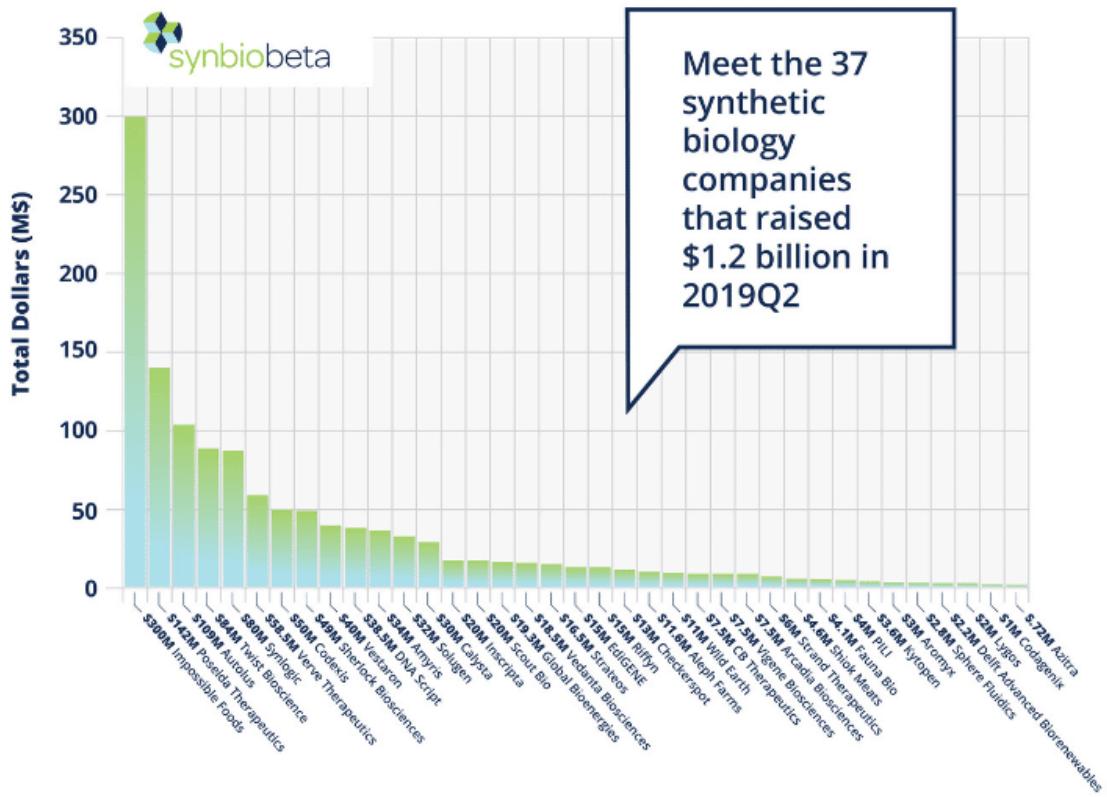
Synthetic biology has become a global enterprise, present in more than 40 countries, with some 700 organizations. Numerous initiatives and research have already been undertaken, with the financial support of 530 agencies. The promise, provided by synthetic biology, of advancement and breaking down barriers are evidence of the very importance of BS and government and agency support, as these supports sustain information of the advances of this new technology and what it can provide. These bodies and agencies, along with the assistance of inspection programs, are able to provide support not only financially, but also with a market direction and the creation of a heterogeneous community in synthetic biology entrepreneurship. Promising synthetic biology-related projects started around the 2000s, which by then had already encouraged some federal initiatives, in the case of the USA and Europe. In 2006 the National Science Foundation (NSF), funded with 40 million dollars for the Synthetic Biology Engineering Research Center (SynBERC), a multi institutional research center. In the USA, also in the same year, there was presidential support, through a mandate, for the development of biofuel technologies.

This was supported by federal funding of \$1 billion. Of this amount, \$400 million was earmarked for research in synthetic biology.

Likewise, Europe is not far behind, with a total of 450 million Euros over the period 2004 to 2013. In the UK, this funding reached 300 million pounds. Unlike in the USA, investment in synthetic biology is employed



through unified strategies and programs, focusing on developing a link between research development and its application in industry.



Industry Report

Investimentos na indústria de Biologia Sintética no segundo trimestre de 2019.

In 2018, over \$3.8 billion was invested in the synthetic biology industry, this mark is expected to be surpassed in 2019, which shows the growing interest from both public and private sector investors. Through the first half of 2019, 65 synthetic biology companies have obtained \$1.9 billion in funding, which shows how fast the field is growing.

Attractive Opportunities in the Synthetic Biology Market



The growth in the number of pharmaceutical and biopharmaceutical companies, medical facilities and research institutions in synthetic biology, high investment and government support in this area is expected to drive the synthetic biology market in the coming years. Forecasts point to the \$19.8 billion mark.

These are the companies that dominate the synthetic biology market: Thermo Fisher Scientific Inc. (US), Merck KGaA (Germany), Agilent Technologies, Inc. (US), Novozymes A/S (Denmark), Ginkgo Bioworks (US), Amyris, Inc. (US), Intrexon Corporation (US), GenScript Biotech Corporation (China), Twist Bioscience (US), Synthetic Genomics, Inc. (US), Codexis, Inc. (US), Synthego Corporation (US), Creative Enzymes (US), Eurofins Scientific (Luxembourg), Cyrus Biotechnology Inc. (US), ATUM (US), TeselaGen (US), Arzeda (US), Integrated DNA Technologies, Inc. (US), and New England Biolabs (US).

<https://www.marketsandmarkets.com/Market-Reports/synthetic-biology-market-889.html>

3. Spiber

Spiber company demonstrates efforts to pursue innovations in next generation sustainable materials: Proteins

Spiber has been around for 12 years, conducting studies to develop understanding and mastery of proteins. They have also developed technologies that allow them to increase protein design and production efficiency, aiming to reduce the production

cost of protein fermentation. In 2021, they should begin producing synthetic spider webs that will be used in a variety of products, from clothing to adding it to polymers to enhance them.

<https://www.spiber.jp>

4. Wild Earth

Wild Earth aims to produce animal feed in a healthier and more sustainable way. Using synthetic biology technologies, they can produce a wide range of proteins and carbohydrates, with different textures and flavors. The company is committed to producing healthier and safer food for pets. By using recombinant proteins, Wild Earth will avoid risks associated with traditional animal-based proteins.

<https://wildearth.tv>

5. New Harvest

New Harvest seeks to build and establish the field of cellular agriculture, seeking to revolutionize the animal products industry to provide affordable and sustainable food to an ever-growing population, using engineering technologies and synthetic biology. Using cellular agriculture, they can produce eggs, milk, meat, and more, without requiring intensive farming and animal husbandry.

<https://www.new-harvest.org/about>

6. Mammoth Biosciences

The company harnesses the diversity of nature to foster the next generation of CRISPR products. By using new CRISPR systems, the company is exploiting technology to read and write the code of life. They are working in several areas, such as health, agriculture, biodefense, and so on. It is worth mentioning that Jennifer Doudna, co-inventor of CRISPR-Cas genome editing, is a co-founder of the company.

<https://mammoth.bio>

7. Impossible Foods

Can you imagine eating a veggie burger whose flavor came from the genetic modification of a yeast? Well, the company Impossible Foods has examined several plants in search of molecules that mimic proteins present in meat responsible for the flavor.

It settled on a protein called leg-hemoglobin from soybeans, a hemoprotein

oxygen- or nitrogen-fixing hemoprotein. Impossible Foods has modified a yeast so that it produces this protein during fermentation, and they can use leg-hemoglobin to flavor their veggie burgers.

<https://www.youtube.com/watch?v=FjW2vNVZlhE>

<https://impossiblefoods.com/food/>

Molecular Biology

1. Summary

In this topic, we will be approaching the content referred to, mainly, the nucleic acids, its composition and the process of transferring genetic information that the DNA contains in itself forward.

2. What is it?

Molecular biology is a part of biology focused on the study of organisms in a molecular level, covering the functionality of nucleic acids (DNA and RNA).

The comprehension of the replication, transcription and translation processes is the main goal of understanding in this branch.

succinctly:

I. Replication: Through the separation of hydrogen bounds between two strands of DNA, new copies of genetic material are made. Each one of those molecules are através do rompimento das pontes de hidrogênio entre as duas fitas do DNA, há a formação de duas novas moléculas de DNA. This process is semiconservative because only one of the remaining DNA molecules is original from the mother cell.

II. Transcription: The information on the DNA is converted to a molecule called messenger RNA.

III. Translation: The message carried by the messenger RNA will be translated into a specific protein.

3. Why molecular biology?

Although it's a fairly new study field, molecular biology presents very positive results, being able to help in medical diagnosis and pharmaceutical development.

This content allows the reader to comprehend how a cell truly works, how proteins are synthesized. The vast applications of enzymes is of great importance to control,

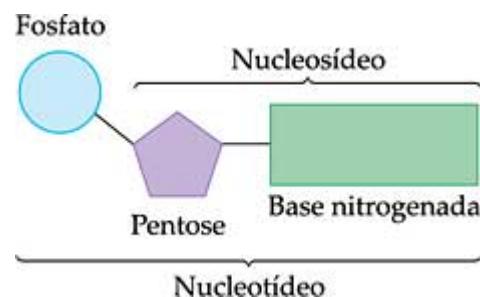
catalysis and inhibition of biochemical reactions, being that enzymes are increasingly being used in the industrial branch.

4. Molecular biology central dogma

On developing of molecular biology during XX century, the influence of genes on characteristics of a species became clearer. It was discovered that there is an intrinsic relation between proteins and biochemical processes as a whole. However, only with the molecular biology development it was possible to use the DNA technology to create useful proteins applicable in the industry. Molecular biology central dogma is to understand the steps which information is transmitted through a cell. DNA transcript into a messenger RNA, then that molecule is used to translate a protein through ribosomes. This statement was proposed in 1958 by Francis Crick, but some later information were essential for this formulation. Firstly, in 1942, Avery et al discovered that DNA was an important component of the genetic material. Then, in 1953, Watson and Crick published the tridimensional model of the DNA, the final piece to the discovery of molecular biology central dogma.

5. Nucleic acids and its functionalities

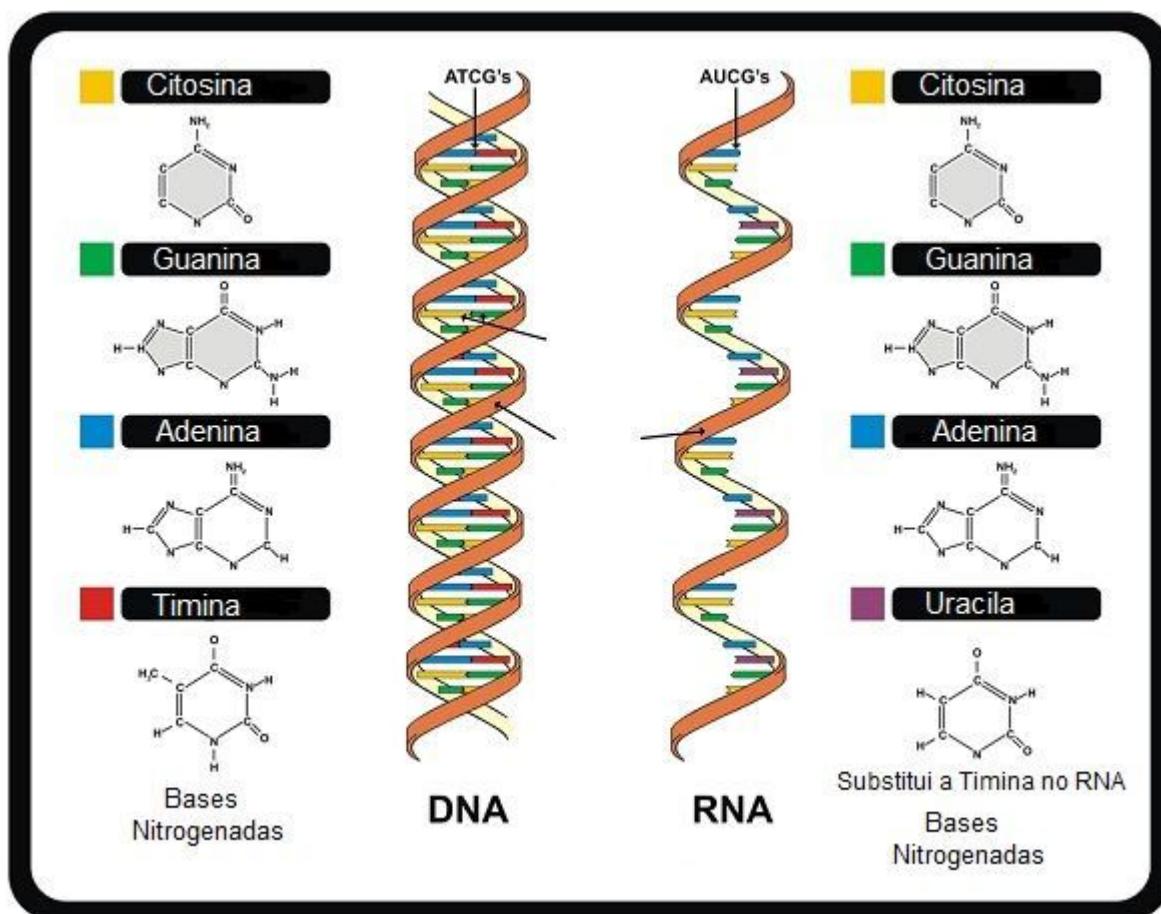
Nucleic acids are formed by nucleotides, which are the union of: phosphate group, pentose and nitrogenous base.



For the study of genetic sequencing, it is important to have knowledge about nitrogenous bases. Deoxyribonucleic acid (DNA) is composed of two pyrimidine bases, cytosine (C) and thymine (T), and two puric bases, adenine (A) and guanine (G). Ribonucleic acid (RNA) is composed of two pyrimidine bases, cytosine (C) and uracil (U), and two puric bases, adenine (A) and guanine (G).

Adenine is always paired with Thymine in the DNA double helix by two hydrogen bonds, while Cytosine is always paired with Guanine by 3 hydrogen bonds

(Chargaff's Rule). That is, a puric base always binds to a pyrimidine base. Thus, in RNA, adenine binds to uracil.

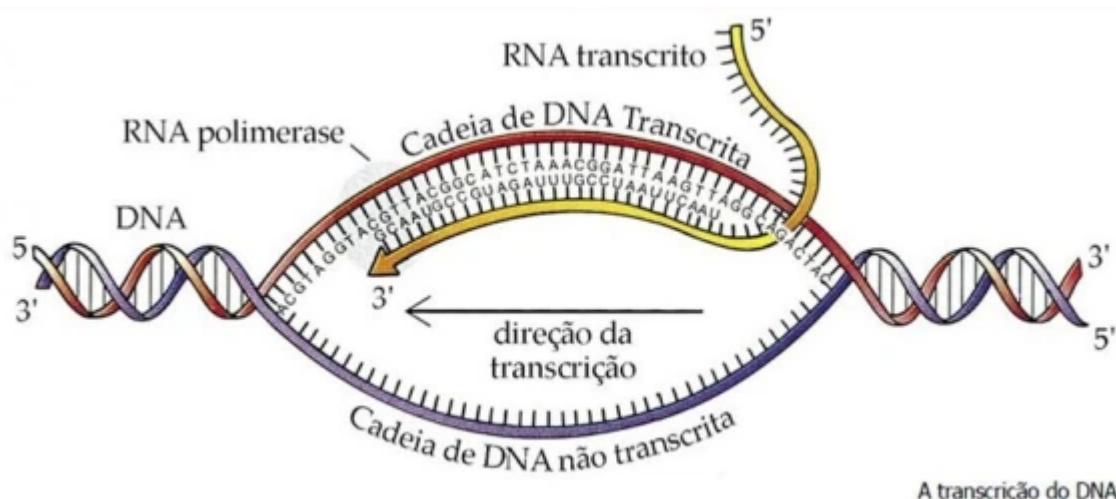


For a wide enough variation in the generation of amino acids for the creation of peptides, a combination of nucleotides, the basic form of information in a genetic code, is needed. A triplet of nucleotides is called a codon, which is how an organism transmits information.

		Segunda Base					
		U	C	A	G		
Primeira Base 5'	U	UUU UUC UUA UUG	UCU UCC UCA UCG	UAU UAC	Tirosina Stop codon Stop codon	UGU UGC	Cisteine
	C	CUU CUC CUA CUG	CCU CCC CCA CCG	CAU CAC CAA CAG	Histidina Prolina Glutamina	CGU CGC CGA CGG	Arginina
	A	AUU AUC AUA AUG	ACU ACC ACA ACG	AAU AAC AAA AAG	Asparagina Treonina Lisina	AGU AGC AGA AGG	Serina Arginina
	G	GUU GUC GUA GUG	GCU GCC GCA GCG	GAU GAC GAA GAG	Ácido Aspártico Acido Glutâmico	GGU GGC GGA GGG	Glicina
3' Terceira Base		UCAG	UCAG	UCAG	UCAG	UCAG	

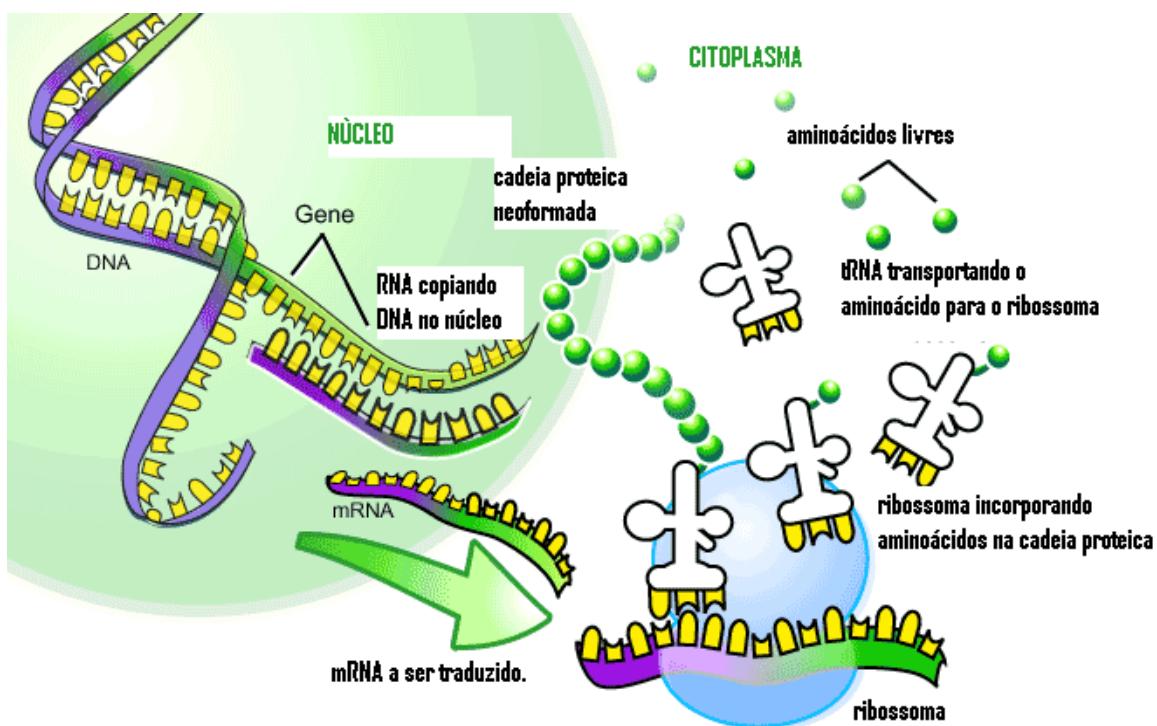
5.1. Transcription

Simply put, the DNA transcription process occurs with RNA polymerase, an enzyme that binds to the double helix whose structure is non spiralized. Through a promoter, where there is the Initiation Codon, the polymerase generates its complementary strand from an original strand, that is, for a TGATTA sequence, a strand with an ACUAAU sequence is generated (remembering that, in RNA , T is replaced by U). The enzyme goes through the DNA, generating the new strand, and the mother DNA coils as the process takes place, this continues until a stop region is reached: a stop codon. With this, the DNA returns to its original state and the RNA polymerase and the new strand are released.



5.2. Translation

The translation occurs in the ribosome with the strand of mRNA, generated in the transcription, being paired by the anti-codon (complementary) of a tRNA, which brings the amino acid relative to the codon. The Ribosome has 3 sites: E, P and A. The P site is the central one, where translation begins and where the formation of the polypeptide will occur; site A is where the new codons will be processed and, finally, the E site is where tRNAs will be released during the process. The mRNA strand and respective tRNAs follow the order A>P>E (exception: the first one). As Translation proceeds, the polypeptide is formed by the so-called peptide bond, a dehydration reaction, between amino acids. With the flow of tRNAs, new amino acids are brought in and the ribosome travels along the mRNA strand until it meets the stop codon, in which the process ends and the protein is released.

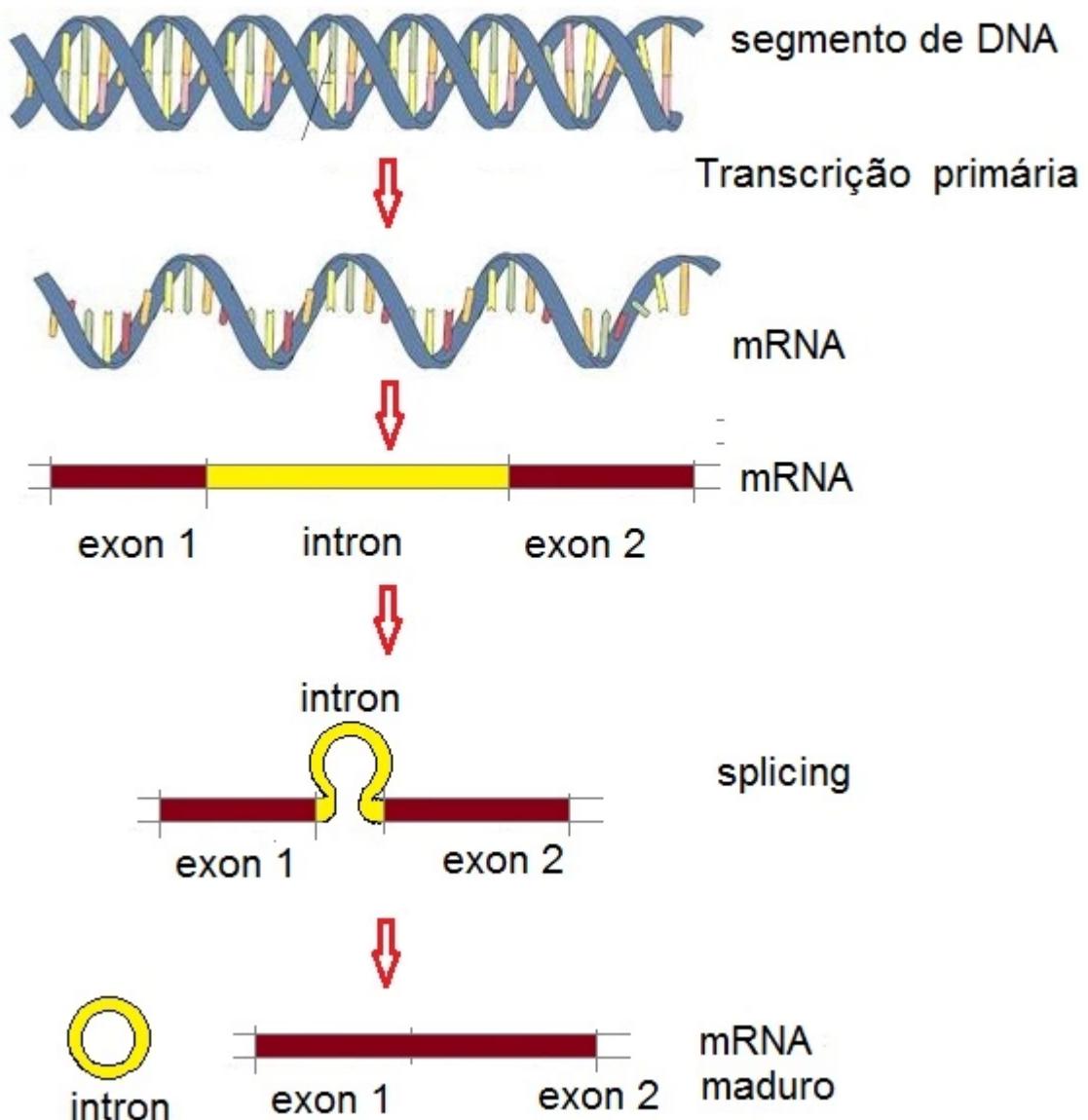


6. Exons and introns

It is also important to clarify that, in the genetic code, there are two types of regions. The coding regions (which carry genetic information), named “exons”, and the interspersed non-coding regions (which carry no genetic information), named “introns”.

These introns are transcribed into RNA in the cell nucleus, but are no longer present in the messenger RNA, meaning that the introns are not present in the synthesized protein. Introns are not present in mRNA due to the “cut”, called splicing, made by an

enzyme. The importance of this process is due to the fact that, if there is a failure and the intron is transcribed, the final product may be subject to mutations.



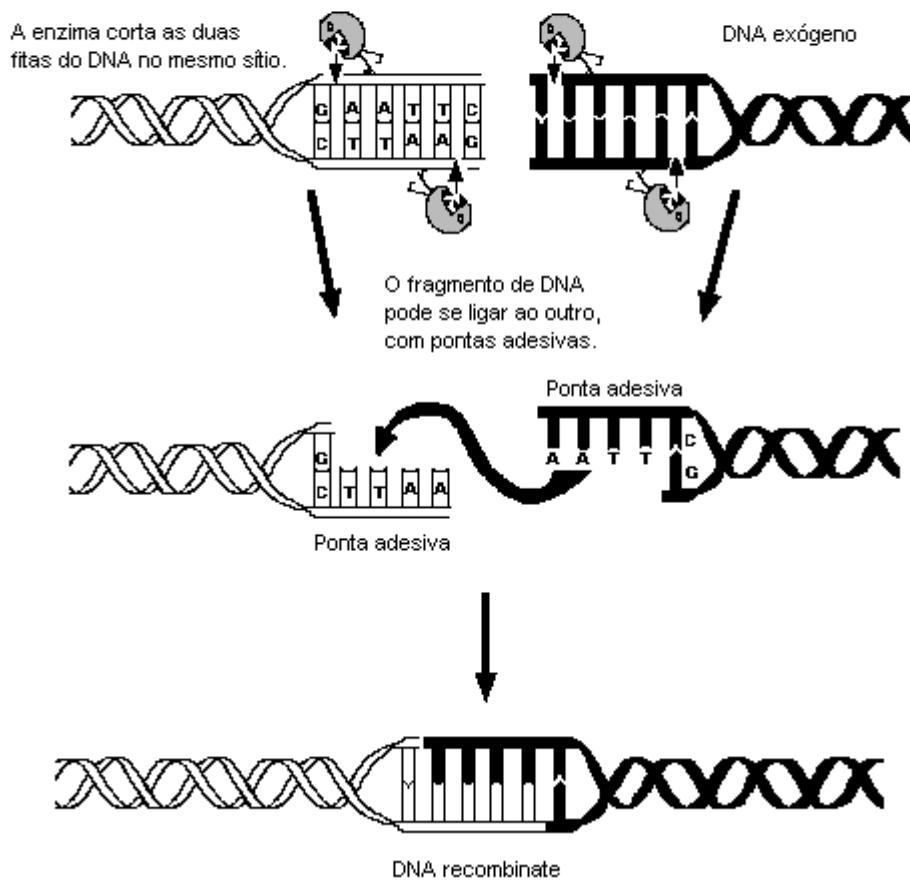
7. Endonucleases

Also known as restriction enzymes, endonucleases are, in general, produced by bacteria, which makes them able to defend themselves against foreign viruses to the organism.

These enzymes cut the strand of DNA at both ends of a sequence of bases, as the enzyme recognizes them. These cut ends are called "sticky ends" and can be attached to other DNA fragments cut by endonucleases as well. And the place where the cut was made is called "target site".

An example of an endonuclease is the EcoRI enzyme, produced by the E. Coli bacteria. It is able to recognize and cut only the GAATTC nitrogenous base sequence.

Enzima de Restrição Ação da EcoR1

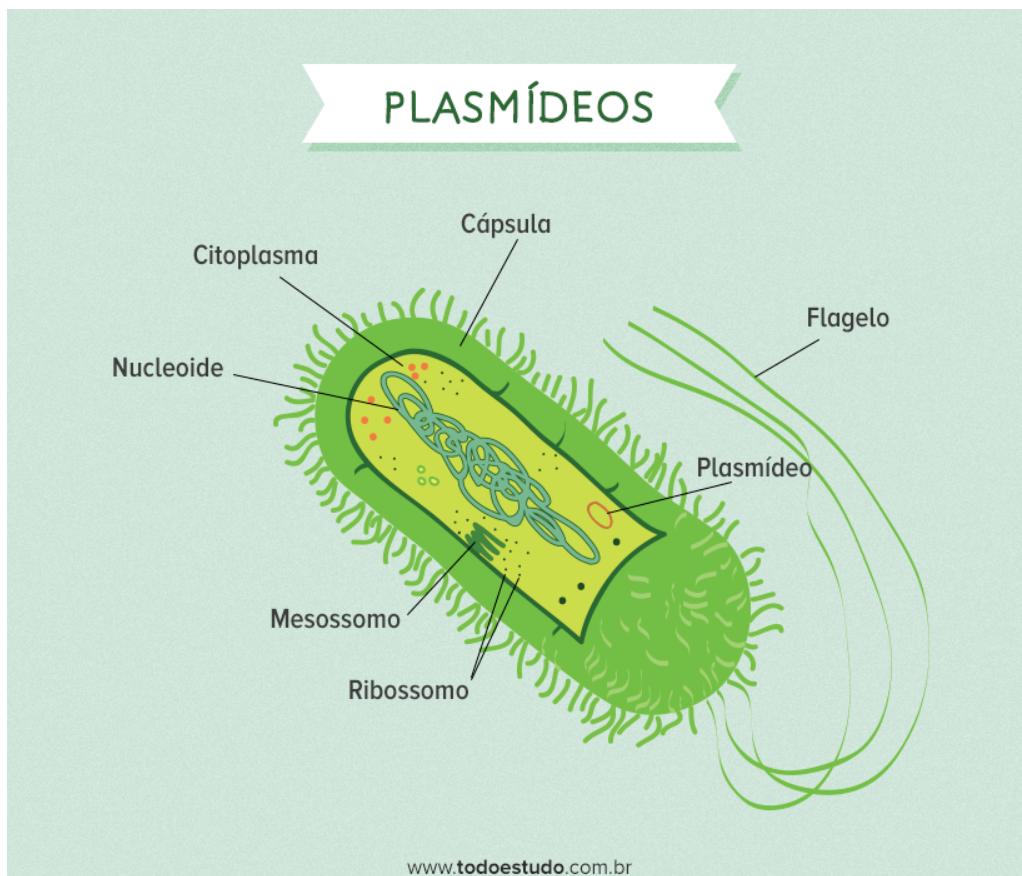


Still on EcoRI, the segment 5'-G A A T T C-3' is, in its complementary strand, equal to 3'-C T T A A G-5'. In other words, AATT/TTAA is a palindromic sequence, since each filament has, with reverse polarity, the same nucleotide sequence. The EcoRI enzyme cuts only between the G and A nucleotides of each palindrome. Which means that different endonucleases cut different palindromic sequences.

8. Plasmids

Plasmids are extrachromosomal DNA sequences, or simply circular pieces of DNA found in the cytoplasm of bacteria and carry their genes.

In the case of genetic engineering, the gene cloning process takes place inside bacteria. For this to occur, a vector is needed, which is the plasmid. The use of plasmid is due to the ease with which it can penetrate other cells.



Chassis and Biobricks

1. Summary

This topic will cover a subject more directly linked to synthetic biology: Chassis and BioBricks. In Chassis, an introduction will be given about the concepts of the theme and an exemplification of the types of chassis, showing which are the most common. It will also expose the factors that influence their choice, besides commenting on a more innovative technique: Cell-free.

In BioBrick, we will give its definition and discuss its importance in synthetic biology, also talking about more technical issues, such as its genetic structure (with the standardized restriction sites) and concepts of Prefix and Suffix, BioBricks in plasmids and their types. This topic is important to have an understanding of the main tools of synthetic biology and how they work.

2. Chassis

2.1 Introduction and concept

The languages of physics and engineering are usually quite precise, understood and recognized, even if partially, by lay people and professionals in other fields. Synthetic biology tries to do the same with its terms, avoiding words that evoke notions that are difficult to understand.

In an attempt to use a clearer and less specific jargon, the concept of chassis was incorporated. Originally, a chassis is the base of a car, where the components responsible for the car's characterization will be added; synthetic biology has appropriated this concept using the same term, which, in turn, has gained different meanings within the context of biological engineering.

However, the main concept of chassis, and the one that, for now, is of interest to the study proposed here, is that of a host that has part of its genome deleted, in order to leave only what is essential, such as the genes responsible for the basic metabolism, and then receives the synthetic genome, working as a machine for various purposes. This kind of base for genetic constructions can be of different types, among them bacteria, fungi, archaea, and even animal and plant cells. In short, the chosen organism functions as a shelter (chassis) for its simplified genome, for the purpose of maintaining basic activities, and its genetic apparatus of interest.

2.2 What are the most common types of chassis? Introduction to the most commonly used organisms

Despite the different chassis possibilities, the most common are bacteria, fungi, and plants. Organisms have different physiology and metabolism, so each can be used for a specific purpose according to its characteristics. Considering this, it is important to briefly understand, at least for now, these aspects of the most common chassis.

Bacteria are prokaryotic, having a circular chromosome contained in the cytoplasm (a gelatinous liquid rich in salts, glucose, and other organic molecules) where RNA and ribosomes are also contained; in addition, some bacteria have plasmids, which are small DNA molecules that do not encode essential information but do provide a selective advantage. This makes these microorganisms a potential chassis, due to the possibility of easy manipulation, relatively rapid reproduction, and low complexity.

In opposition to bacteria, fungi are eukaryotic beings that have a more complex cellular organization. There are both multi- and single-celled fungi, but the latter is the one of interest as a chassis, because it is a simple eukaryotic being.

Plants, on the other hand, although, like fungi, are eukaryotic, their level of complexity is higher, which can make prediction and manipulation difficult. However, plant cells can also be widely used as a chassis, with a wide range of species, from microalgae that function as model organisms for plants, to tomatoes biofortified with phytonutrients.

2.3 How to choose a chassis? What are the main differences? Examples

The choice of a chassis depends on several factors in addition to biophysical characteristics, such as metabolic resources and possibilities to be exploited. Among these factors is the need for the platform to be predictable, well-defined, and specific. The genetic variants of some species are used as a chassis.

When the subject is chassis, the assimilation with *Escherichia Coli*, gram-negative, is almost automatic (more specifically the most common variant, the K12), because it is a bacterium that performs very well such function, since its genomic sequence is very well known, in addition to its rapid reproduction, ease of manipulation, and industrial and medical importance. However, there are other bacteria that also show promise in the area, such as the gram-positive *Bacillus subtilis*, well known for its ability to secrete protein, besides having the quorum-sensing system, which consists in communicating with other bacteria and the environment, restricting the expression of specific genes, and can be applied in several areas, such as chemical compounds, biopolymers and proteins. Since bacteria are most commonly used as chassis, here follows a comparative table between the different species (and variants) cited with parameters that can influence the choice.

Parameter / Consideration	<i>E. coli</i> (K12)	<i>B. subtilis</i> (168)
Natural environment	Water and the gastrointestinal tract	Water, soil, plant rhizosphere and

		gastrointestinal tract
Genome size	4.6 Mbp, 4288 CDS	4.2 Mbp, 4100 CDS
Genomic Complexity	Many repeated sequences, genes are scattered all over the genome	25% duplicated genes
Metabolic model	Available	Available
Databases	EcoliWiki, EcoCyc	SubtiWiki, BsubCyc
Time for generation	40 minutes	95 minutes
Average temperature for efficient growth	30 ~ 38°C/86 ~ 100.4°F	25 ~ 35°C/77 ~ 95°F
Substrate Type	Limited; simple sugars	Broad; simple and complex carbohydrates, peptides
Natural products	Ethanol, hydrogen	Antimicrobial compounds, butane-2,3-diol
Typical by-products (substrate usage)	CO ₂ / acetate	CO ₂ / lactate and acetate
Pathogenicity in humans	Only wild variants	None, potentially probiotic
Approval as Safe	Approved	Approved
Sensitivity to antibiotics	Sensitive to common antibiotics	Sensitive to common antibiotics
Availability of genetic tools	Widely available	Available

Tolerance to toxic compounds	Low	High
Research basis	Extensive	Extensive
Existing Applications	Numerous (recombinant protein production and biosensors)	Numerous (recombinant protein production)
Commercial availability considering patents	Heavily patented, a major problem	Widely patented

Source:Developing Synthetic Biology Tools and Model Chassis: Production of Bioenergy and HighValue Molecules. (Tampere University of Technology. Publication; Vol. 1288). Tampere University of Technology. Available at: <https://tutcris.tut.fi/portal/files/2460006/santala_1288.pdf>.

Eukaryotes also perform well as chassis, with fungi being a prominent one, as about 38% of microbial bioactive compounds come from them. As a consequence, it is natural that it is necessary to implement yeasts as chassis for the production of secondary metabolites, which can come to function as antibiotics and other drugs, pesticides and toxins. We have as an important chassis the fungal species called *Schizosaccharomyces pombe*, which has the ability to reproduce by fission and shares a considerably high amount of molecular, genetic, and biochemical information with more complex eukaryotes such as plants and animals; *S. pombe* has become attractive as a chassis by expressing a large number of membrane and secretion proteins.

As for plant cells that function as chassis, a discussion is established: which plant has the potential to be the best chassis? And as for other organisms, this answer is very complex, and it may even not exist, but there is the "original plant used as a chassis", *Arabidopsis thaliana*, which is usually the first choice for biological engineering, because it is one of the easiest plants to grow and genetically transform (under controlled conditions), besides having already had its genetic code widely studied. However, this plant lacks biomass, so another option for application as a chassis could be *Camelina sativa*, which despite obtaining much more biomass than the first, still remains somewhat mysterious.

- **Competent Cells**

The term "competent cell" refers to a cell that is able and well-functioning to incorporate an exogenous DNA in a simpler way, without harming the cell itself. Cells, such as *E. coli* bacteria, are made competent through a process with calcium chloride and heat shock. Additionally, cells that are in a faster growth phase (log phase) become competent more quickly.

- **Cell-free**

Chassis have served for years as hosts for biological circuits for a variety of purposes due to their ability to self-replicate. However, these living cells present some limitations regarding the objectives to be achieved, such as the complexity of the organisms, genetic mutations, and the impossibility of producing proteins that are toxic to the chassis in question. Thus, a method was developed that could use the machinery of the chassis and, at the same time, not suffer the impacts of the complexity and specificities of the cell.

This is an *in vitro* technique called cell-free, within this concept, different media are used, which can be summarized in three:

- Extract-based system

The system is composed of a "crude extract" of basic transcription and translation functions, DNA templates, amino acids, nucleotides, substrates for energy regeneration, non-protein enzymes (cofactors), and salts. Among the most common organisms that function as providers of these extracts are *Escherichia coli* and *Saccharomyces cerevisiae*.

- Purified System (PURE System)

It basically consists of a "toolbox" that contains essential components of *Escherichia Coli* for its replication. The process of developing the technique consisted of finding 25 proteins essential for *E. coli* replication. To reconstruct the entire procedure *in vitro*, only one circular DNA molecule with a sequence that indicates the start of replication of the genetic material was needed, so that they could apply PCR. This system proved to be very fast and efficient.

- Synthetic Enzymatic Pathway System

This system consists of a multitude of enzymes that have the ability to set complex bioreactions in motion.

Thus, the cell-free technique can contribute as an alternative to the in vivo model because it is easier to manipulate, since its control is done in an open environment, without cell membrane boundaries, besides the easier implementation of synthetic amino acids, ability to produce only the desired product, high toxic tolerance and integration with materials. Nevertheless, the in vitro technique also has its limitations, such as higher implementation cost and difficult self-replication. Here is a comparative table with the parameters cited and some others.

Characteristic	In vitro cell-free system	Cellular system in vivo
Transcription and translation handling	Easy to control in the open environment	Difficult to manipulate because of the cell membrane
Post-translation modification	Difficult	Easy
Self-replication	Difficult	Easy
DNA template	Plasmids or PCR products	Plasmids or genomes
Synthesis of membrane, proteins and complex proteins	Simple synthesis, through addition of surfactants or adjustment of the external system	Difícil síntese devido ao meio intracelular limitado
Incorporation of synthetic amino acids into proteins	Easy	Difficult
Ability to produce only	Easy, focusing on	Difficult, due to complex

the desired products	objective metabolic pathways	cellular metabolism
Tolerance to toxicity	High	Low
Integration with materials	Easy	Difficult
Design-build-test-learn cycle	About two days	About two weeks
Biomanufacture	High production rate; High product yield; Simple product purification process, without cell lysis	Medium production rate; Medium product yield; Lysis of the cell before purification of the product
Cost	Medium to high	Low to medium

Source: Cell-free synthetic biology: Engineering in an open world. Volume 2, Issue 1, March 2017, Pages 23-27. Available at: <<https://www.sciencedirect.com/science/article/pii/S2405805X1730008X?via%3Dihub#bib8>>

3. BioBrick

One of the goals of Synthetic Biology is to make the process of engineering biological systems easier, simpler, and more accessible to the entire community. In order to make this possible, BioBricks were introduced. The simplest definition of a BioBrick would be "standardized piece of nucleic acid or a biological part (a natural sequence of nucleic acids that encodes a certain biological function)".

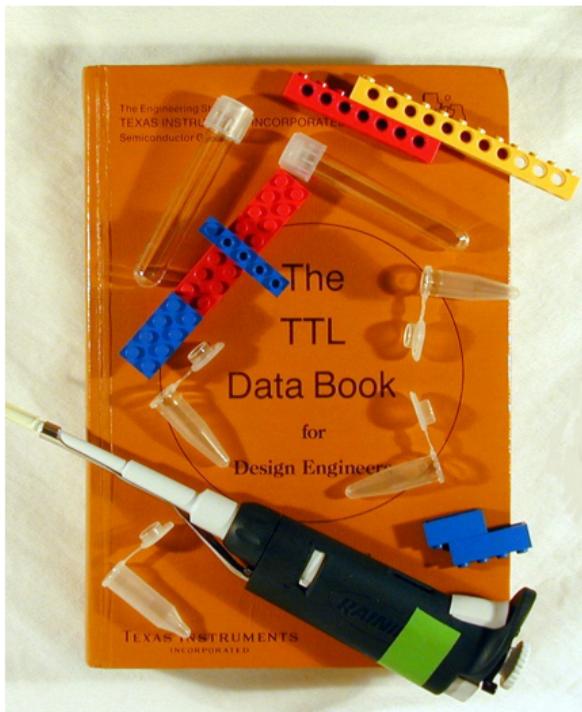
These parts are standardized, that is, refined and altered in order to allow their manipulation and use to be compatible with a standard of techniques used throughout synthetic biology. The innovation of the BioBricks standard was to allow the assembly between different parts, thus forming another BioBrick, which, in turn, can also be joined to another one.

In 1996, *Rebatchouk et al.* developed and implemented a general strategy for cloning and assembling nucleic acid fragments. Subsequently, there was an intent to create a physical composite of BioBricks, which at the time was not accepted by the scientific community. Only in 2003 did Tom Knight propose a physical composite standard, which was widely accepted.

3.1 The BioBricks Pattern, proposed by Tom Knight

Idempotent Vector Design for Standard Assembly of Biobricks

Tom Knight
MIT Artificial Intelligence Laboratory



Knight and his team set out to make a simple yet functional choice for standardizing BioBricks. Each component is present in a circular double-stranded DNA vector. On the 5' direction relative to the BioBrick of interest are EcoRI and XbaI restriction sites; and on the 3' side, SphI and PstI. It is important that the vector does not contain any other sites of these restriction enzymes.

Thus, a vector has the following format:

```

5' --gca GAATTC GCGGCCGC T TCTAGA G --insert-- T ACTAGT A GCGGCCG CTGCAG gct--
--cgt CTTAAG CGCCGGCG A ACATCT C ----- A TGATCA T CGCCGGC GACGTC cga--
EcoRI NotI XbaI SpeI NotI PstI

```

A few points are important to note. The unit base pairs, for example, are chosen carefully, with the goal of eliminating accidental generations of methylation sites in strains with EcoBI and EcoKI, such as E. coli Dh5 α .

```

5' --gca GAATTC GCGGCCGC T TCTAGA G --insert-- T ACTAGT A GCGGCCG CTGCAG gct--
--cgt CTTAAG CGCCGGCG A ACATCT C ----- A TGATCA T CGCCGGC GACGTC cga--
EcoRI NotI XbaI SpeI NotI PstI

```

Cuts with EcoRI and Spel generate "front inserts" (FI), as in the picture above:

On the other hand, cutting with XbaI and PstI generates "back inserts" (BI), which forms the parts to insert.

```

5' --gca GAATTC GCGGCCGC T TCTAGA G --insert-- T ACTAGT A GCGGCCG CTGCAG gct--
--cgt CTTAAG CGCCGGCG A ACATCT C ----- A TGATCA T CGCCGGC GACGTC cga--
EcoRI NotI XbaI SpeI NotI PstI

```

Below are the cuts for the "opening" of the vector

Cleavage with EcoRI and XbaI generates "front vector" (FV)

```

5' --gca GAATTC GCGGCCGC T TCTAGA G --insert-- T ACTAGT A GCGGCCG CTGCAG gct--
--cgt CTTAAG CGCCGGCG A ACATCT C ----- A TGATCA T CGCCGGC GACGTC cga--
EcoRI NotI XbaI SpeI NotI PstI

```

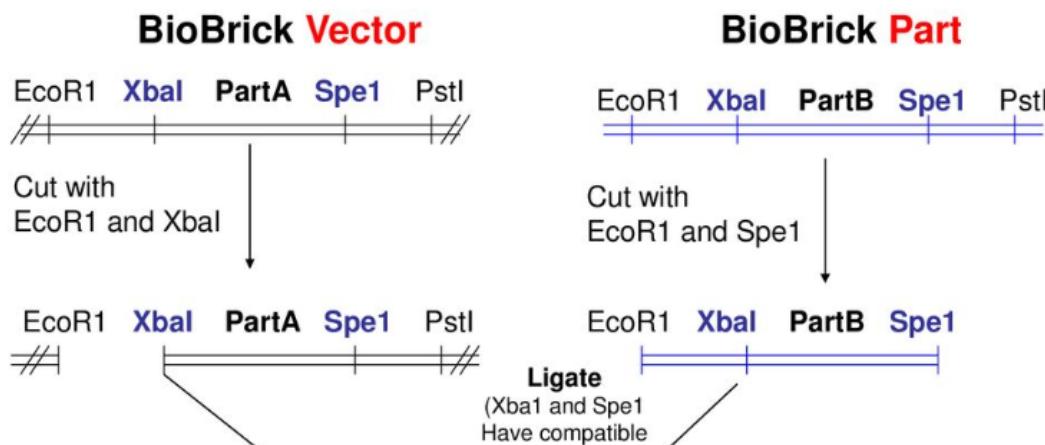
Cleavage with Spel and PstI generates back vectors (BV)

```

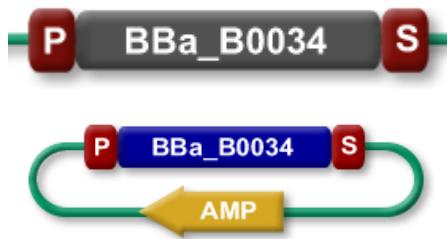
5' --gca GAATTC GCGGCCGC T TCTAGA G --insert-- T ACTAGT A GCGGCCG CTGCAG gct--
--cgt CTTAAG CGCCGGCG A ACATCT C ----- A TGATCA T CGCCGGC GACGTC cga--
EcoRI NotI XbaI SpeI NotI PstI

```

Thus, BIs can be inserted into BVs and IFs into VFs, which demonstrates that this standardization can be efficient, allowing the insertion of BioBricks into the vectors of interest. In the binding process, a mixed Spel/XbaI site is also formed which, because it is compatible, binds as shown in the image below." (BV)



A widely used term is the concept of Prefix and Suffix. In synthetic biology, the prefix refers to the pattern of bases that comes "before" the desired BioBrick, i.e., it links to the 5' direction; and the Suffix is the opposite, referring to the patterned sequence that follows the 3' direction of the Biobrick.



On the side, we have the organization of the Prefix(P) and Suffix(S) in a vector with the BioBrick BBa_B0034, which refers to a "Ribosome Binding Site" (RBS) which will be better explored later.

Below is the image of the sequence that refers to the prefix. We can see that, in simple terms, it is the sequence from the EcoRI to XbaI binding site.

BioBrick Prefix

The standard BioBrick prefix depends on the part that follows it.

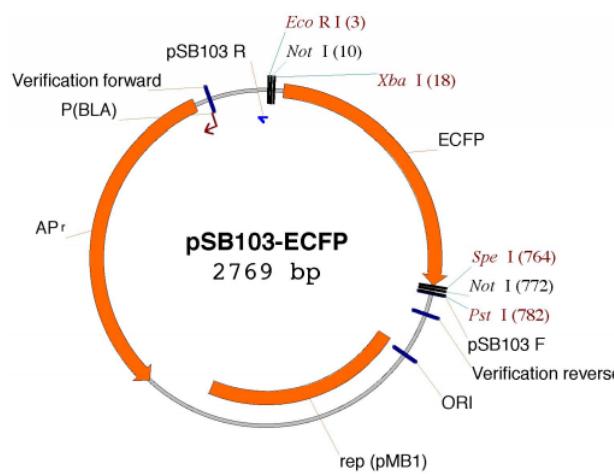
BioBrick Suffix

The standard BioBrick suffix is always:

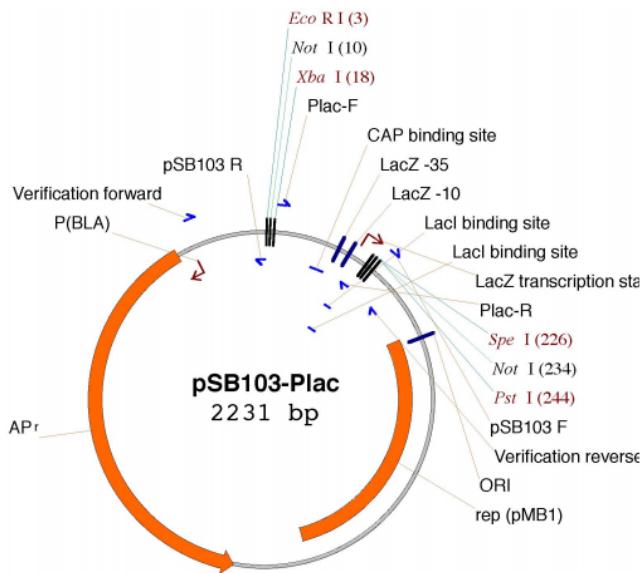
tactagtagcgccgctgcag

The suffix sequence also follows the same parameter, with sequence starting at the 3' end of BioBrick.

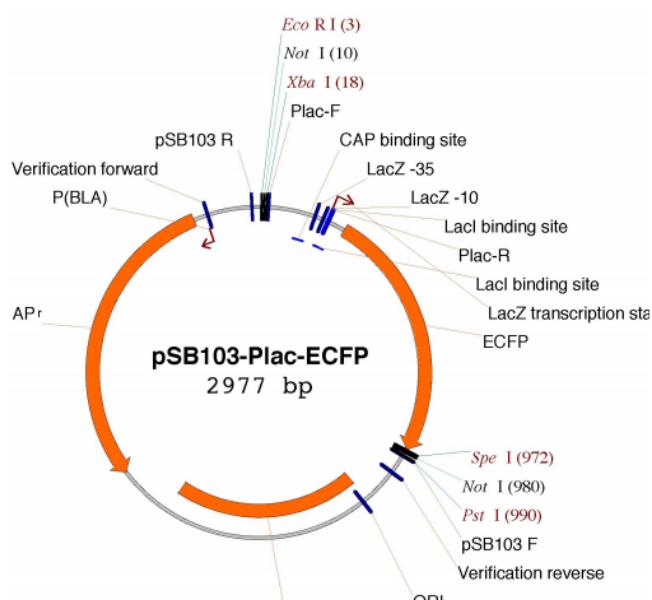
3.2 BioBricks in Plasmids



The vector (pSB103) on the side houses the BioBrick represented by the ECFP gene, which encodes the ribosome binding site, known as RBS, and also a sequence that encodes a cyan fluorescent protein (CFP). In the vector, we can identify the BioBrick between the prefix and suffix, which present the standard restriction sites.



This vector is of the same model as the previous one, pSB103. It has the ampicillin resistance gene and BioBrick Plac which encodes a promoter.



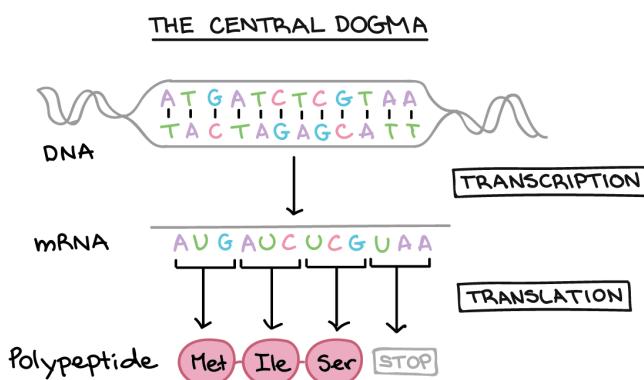
One can build a circuit that produces the CFP protein with a Plac promoter. For this purpose, from vectors containing BioBricks, it is possible to design the desired circuit.

For this, a few steps:

- Cutting of pSB103-ECFP with the enzymes EcoRI and XbaI, which generate a "front vector" (FV), that is, the opening of the vector containing the RBS and the CFP gene occurs;
- Then, pSB103-Plac is cut with the enzymes EcoRI and SpeI, which generates a "front insert" (FI), thus obtaining the BioBrick separated from the vector. Thus, it becomes possible, with the action of ligase, to insert the IF into the FV, obtaining pSB103-Plac-ECFP.

3.3 The types of BioBricks

First, we must remember the central dogma of molecular biology:



Source:

<https://pt.khanacademy.org/science/biology/gene-expression-central-dogma/central-dogma-transcription-a/intro-to-gene-expression-central-dogma>

Now we can talk about the parts (or BioBricks), which consist of stretches of DNA that form a functional unit. There are several units, however, we will focus on the following BioBricks:

Parts



- Promoters

The RNA polymerase enzyme builds a new RNA molecule from the DNA mold. Knowing this, it becomes easier to visualize that the RNA pol is joining a stretch of DNA, responsible for indicating the beginning of a gene to be transcribed, called promoter. In a more succinct manner, the promoter site is responsible for the beginning of the transcription, which, in its turn, is being carried out by the enzyme RNA polymerase.

- Types of promoters according to their functions

- Constitutive Promoters

These are those that operate constantly, so that it is dependent, in the case of prokaryotes, only on the presence of RNA polymerase holoenzyme, but not on transcription factors.

- Cell Signaling

These promoters are those that have their regulation as a function of cell signaling.

The mechanics, in a nutshell, is that a small molecule, or peptide, diffuses between cells and across cell membranes, then this signal molecule is recognized by a receptor, which in turn regulates the activity of the promoter.

- Metal-sensitive promoters

They are sensitive to various types of metal compounds, and their activity is regulated by the presence of these.

The regulation mechanism is given by a receptor that binds to a metal ion or complex.

- Bacteriophage promoters

They are generally used for the mass expression of a protein. They can operate in various chassis, but generally depend on a particular RNA polymerase present.

- Ribosome Binding Sites (RBS)

It is necessary to remember about the translation step, where basically the messenger RNA has its information decoded, so that finally the proteins that were there in code format are produced.

Basically, the RBS is a part of the RNA to which the ribosome binds, so that the site directs the ribosome to the start codon. For the process to be efficient, the ribosome and the RBS should be as complementary as possible, in order to increase the affinity and the efficiency of translation, in addition to the rather specific distance from the RBS to the start codon so that both sequences make contact with the right parts of the ribosome complex. RBS of prokaryotes, like promoters, can be constitutive (independent of regulators) or induced (regulated).

- Coding sequence

This is the sequence that contains the information needed to build functional protein chains. These BioBricks must have start and stop codons, so their pattern is: **ATG** (start codon) -[protein coding region]-**TAATAA** (stop codon). Remember that in mRNA ATG becomes AUG, which also codes for the amino acid methionine.

These BioBricks have the function of providing different functionalities for the DNA itself.

Below are some of the most used types of protein-coding sequences:

- Reporters

They are used to measure the expression of a gene or any other intracellular event. To indicate the measurement, they usually emit some signal, such as fluorescence.

- Transcriptional regulators

Proteins related to the activation or repression of transcription of a given gene.

- Selection markers

These are sequences useful for selection of cells with some characteristic of interest. They can confer some characteristic of selection advantage or disadvantage.

- Lysis proteins

These are the proteins involved in the lysis (death) of the cell by rupturing the cell membrane.

- Terminators

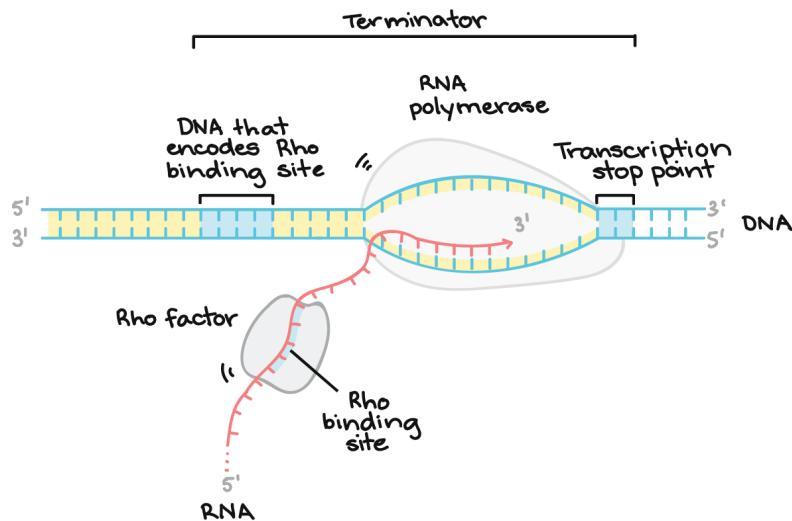
RNA pol continues transcription until it encounters a terminator sequence that, once transcribed, causes the enzyme to stop transcribing. There are two types of terminators in bacteria, Rho-dependent and Rho-independent.

The first consists of an RNA that has a site for binding to a protein called Rho factor, which binds to the sequence and starts moving the transcript toward the RNA polymerase. When the transcription bubble is reached, the factor pulls the transcript strand, separating it from the DNA template, which leads to the termination of the transcription process.

The second type of termination, on the other hand, depends on the DNA sequence, in which, close to the end of the transcription, there should be a region rich in C and G nucleotides, then the RNA transcribed from this region folds in on itself, causing the nucleotides to pair up, forming a structure similar to a clamp, which ends up trapping the RNA pol. Finally, a termination of the transcript is followed by a stretch rich in U nucleotides, which end up pairing up with the A nucleotides of the DNA template (poly-A tail), causing enough instability for the enzyme to detach itself and release the transcript.

It is important to stress that all *E. coli* terminators in the Registry are Rho-independent, because Rho-dependent are not sequence-dependent, making them infeasible to use.

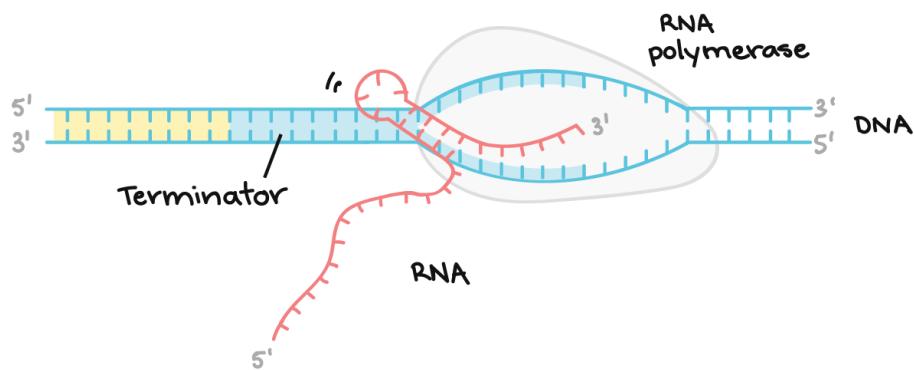
Termination with Rho factor



Source:

<https://pt.khanacademy.org/science/biology/gene-expression-central-dogma/transcription-of-dna-into-rna/a/stages-of-transcription>

Termination without Rho factor



Source:

<https://pt.khanacademy.org/science/biology/gene-expression-central-dogma/transcription-of-dna-into-rna/a/stages-of-transcription>

- E. coli terminators
 - Terminators with "forward" direction

This is the most common type of terminator in use, it terminates the tape transcription, but it is hardly 100% efficient.
 - Bidirectional terminators

They terminate the transcript both in the 5' → 3' direction and in the 3' → 5' direction. However, it can happen that the efficiency of termination differs between both strands.

- Reverse direction terminators

Because terminators are prone to errors, and their efficiency is often compromised, there are double terminators, which consist of two terminators joined together in order to make up for the deficiencies in the operation of a single terminator.

Genetic Engineering Applied to Synthetic Biology

1. Summary

In this topic, some molecular biology techniques will be discussed, such as cloning, DNA extraction and sample confirmation. These techniques are essential in a molecular biology project and there are different protocols to perform them. Then, the concept of some techniques and the purpose of using them will be discussed.

There are many amazing projects involving synthetic biology, such as clothes made from cobwebs, vegan burgers with meat flavor and aroma, trees with the ability to glow in the dark, and much more. For these ideas to come out of the paper, in addition to motivated and committed people, refined genetic engineering and molecular biology techniques are needed.

Molecular biology techniques have been developed since 1970 and perfected by researchers from different areas so that it is possible to obtain quality samples and enable the creation of more complex circuits. Below are various techniques pertinent to a molecular biology laboratory.

2.4. PCR

PCR, or polymerase chain reaction, is a genetic engineering technique with the main goal to amplify a sample of genetic material. It was discovered by Mullis in 1983, its importance is indisputable and has revolutionized several knowledge fields since medicine until archeology and advanced laboratory techniques.

Polymerase chain reaction is based on the following principles: primers (molecules that start the chain reaction), dntp (deoxyribonucleotides), among others biological entities.

The first step of PCR is the denaturation of the double strand of DNA through an increase in temperature (up to approximately 94 C), forming two single strands, as

the hydrogen bonds that used to exist between the nucleotides are broken by receiving external energy. The test tube is cooled (up to 50 C) and, shortly thereafter, the primers (structures composed of single strands of nucleic acids) are coupled to the DNA strands formed in the previous step. Primers are present in all living beings and are strongly associated with the natural process of DNA replication inside cells, they are the initiators of this phenomenon and can be of different types. The nucleotides of the primers are complementary to the DNA portion of interest, this fragment is called the target strand.

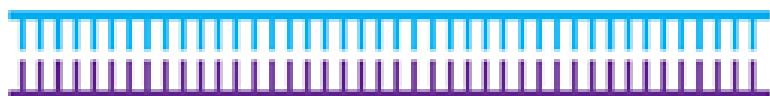
After coupling the primer, an enzyme called DNA polymerase goes into action, polymerizing a new strand of DNA from the primer and using dntp's from the environment to do so. The growth in the number of strands grows exponentially each time this technique is repeated, as the original number of strands always doubles after PCR. As there are several types of polymerase, it is advisable to use an enzyme capable of resisting high temperatures, as this will not denature and lose its function. Because of this, Tac polymerase is widely used, an enzyme obtained from a bacteria (*Thermus aquaticus*) that is even found in geysers in Yellowstone Park, in the United States. The ideal operating temperature for this enzyme is around 70 degrees Celsius, and the direction of new DNA synthesis is always in the direction of carbon 5' to 3'. It is noteworthy that because enzymes have a high specificity factor, the enzyme activation temperature must be adequate to ensure a good yield, this temperature is usually a little below the melting point of the primers.

There is also a variation of the PCR method known as colony PCR which aims to detect the presence of insert DNA when constructing plasmids for genetic transformation. This process occurs when the cell breaks down through hot water treatment and then specific primers will anneal to the DNA of interest. Once this is done, the PCR takes place normally. After performing the colony PCR, the samples obtained undergo a run in electrophoresis, determining the size of the strand in question.

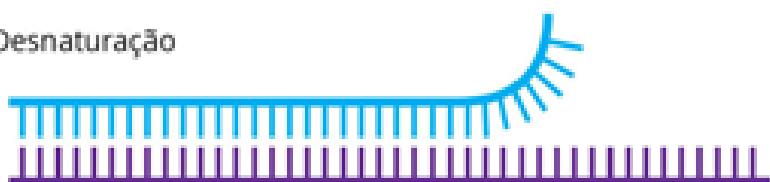
PCR is the most used technique in molecular biology, and has already proven its importance in the use of analysis on very small DNA samples, making it possible that forensic science could solve crimes through very scarce samples of genetic material, study evolutionary patterns and principles starting from fossils, performing clinical examinations without performing too invasive biopsies, and is being of great help for the sequencing of genomes of living beings.

There are other variations of the technique with similar objectives, nested PCR, for example, goes into sequencing or an electrophoresis at its end, while RT-PCR (Reverse Transcriptase-polymerase chain reaction) is used to identify and quantify mRNA. This process consists of obtaining complementary DNA through a single strand (usually of viral origin), this occurs with the use of an enzyme called reverse transcriptase. After using reverse transcriptase, the cDNA fragment is used in a PCR process, allowing studies related to diseases such as COVID 19 to be carried out, studying its functioning and ways of fighting it.

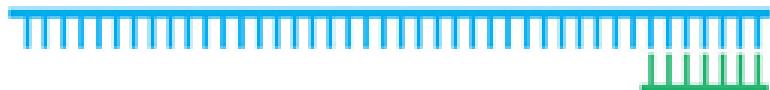
Dupla fita de DNA



Desnaturação



Anelamento



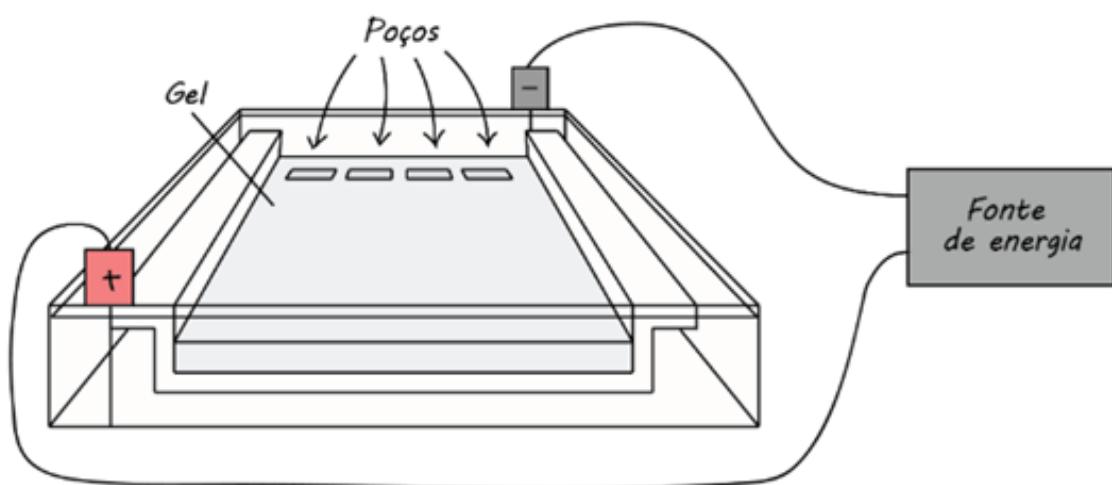
Extensão



3.0.Electrophoresis

Electrophoresis is a technique widely used in genetic engineering and aims to separate DNA strands through the difference in size of the fragments of genetic material. The most common type of electrophoresis is gel electrophoresis, which uses a device with two electrical poles, a few wells where the samples are deposited (wells), and a medium containing a gel (usually a carbohydrate called agarose). This procedure assumes that DNA has a negative electrical charge when in an aqueous medium, due to the ionization of phosphate groups present in the molecules. The electrophoresis device is prepared with a buffer solution (to avoid a change in pH), the addition of the samples and the gel, and then this is subjected to an electric current. The genetic material moves towards the positively charged circuit and is slowed down by the gel (hinder the passage of DNA). After the run is done, pigmentation occurs in the gel and exposure to ultraviolet radiation, making the DNA bands luminescent. The bands that are farther away are the smallest, while the ones closest to the wells are the largest, with this it is possible to separate and identify the sizes of the strips present in the sample.

The electrophoresis technique is not only highly related to other genetic engineering techniques, it is also complementary; among the related procedures, one can mention cloning, Southern blot, northern blot, the construction of genomic libraries and even the PCR itself.



4. Genetic transformation

By definition, genetic transformation is the process of inserting genes of interest into an organism in which you want to modify to obtain new useful characteristics. The inserted gene is called transgene and the modified organism is called transgenic or, even more broadly: genetically modified organism (GMO). There are still many discussions about the limits of genetic modification, even though several applications, such as transgenic foods, the use of genetic engineering to improve biotechnological processes in industry and even animal modifications, are present in everyday life. Even so, it is indisputable that the introduction of genetic transformation into society was revolutionary and for the better. Without the advent of this resource, society would probably be having even more problems related to fighting diseases and even with world hunger.

There are several techniques for inserting genes into cells, including:

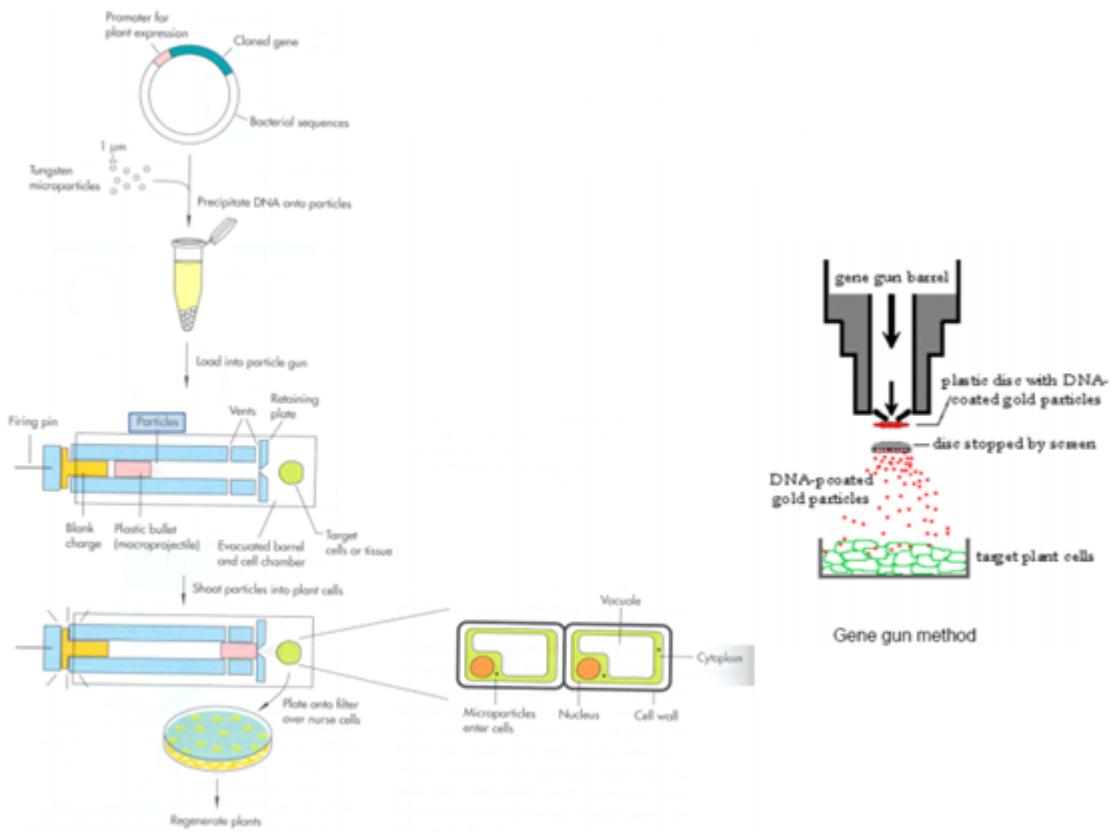
5. Microinjection

Microinjection is a method in which a device called a micromanipulator is used to inject DNA directly into the nucleus of a target cell. This method has a relatively low success rate among the cells tested, as it requires training for the operator of the device and, despite its simplicity, is lacking due to its specificity in animal cells and in tissues with a low content of restriction enzymes (endonucleases).

6. Biobalistics

It consists of firing small tungsten spheres (about $1\mu\text{m}$ in diameter), containing DNA on their surface, by the action of a device called a bomber. The functioning of this device is due to the expansion of compressed helium gas, causing several particles containing DNA to reach the target cells.

This technique is only recommended for cells with a thick cell wall, otherwise the cell breaks down and the attempt at genetic transformation becomes useless.



7. Agrobacterium

This method uses bacteria of the *Agrobacterium* genus. The modification is made by the action of an “extra”, self-replicating, circular genetic material present inside bacteria called plasmid, which must contain the desired gene. The operation of this method is quite simple, cultures of this bacterium are cultivated together with plant cells of the plant in which you want to modify, then the bacterium injects its DNA into the plant cell. After the multiplication of this cell, it is possible to create a plant with the desired characteristic, it is noteworthy that this method can only be performed on plants with damaged structure so the bacteria can recognize some phenolic components and attack.

8. Thermal Shock

This type of transformation is quite simple, it is also known as calcium chloride transformation. In this process, a calcium chloride solution is used to ease the repulsive effects between the plasmids and the bacterial membrane. After a sudden change in temperature, pores are opened in the bacterial structure, allowing the

plasmid to enter the cell and then transform it. The pores are reversible and the technique is highly recommended for its simplicity and low cost.

9. Electroporation

Protoplasts are plant cells without a cell wall due to an enzymatic treatment. During electroporation, these cells are subjected to an electric field with controlled properties that lead to the formation of (reversible) pores in the cell, allowing the passage of external DNA. This technique uses equipment called an electroporator, a device responsible for generating a successful frequency greater than 10 times that of biolistics. However, the regeneration process is slow and can lead to cell mutation by external factors.

10. DNA Extraction

DNA extraction is the process of separating deoxyribonucleic acid from proteins, membranes and other elements contained in the cell of interest. As there are different types of protocols, the time required for the process may vary.

There are DNA extraction kits available on the market for researchers. Careful handling of the material is necessary to avoid contamination and crossover of samples. Thus, it is necessary to follow the protocol with exactness and organization.

Despite variations in extraction protocols, kits generally follow some basic steps:

11. Cell Break

On the start of the process, the cell must be broken to release all the cellular components.

12. Separation of DNA from proteins and other elements of the cell:

To obtain a pure DNA sample, it is necessary to remove as much cellular debris as possible. Commonly, proteases are used to degrade DNA-associated proteins and others present. Some other debris can be removed by filtration.

13. DNA Isolation

This step consists of precipitating the molecule, purifying the sample and resuspension of an alkaline buffer solution.

It is necessary to confirm the DNA sample using electrophoresis. This sample can be used for various analyses, such as cloning and transformation for future work.

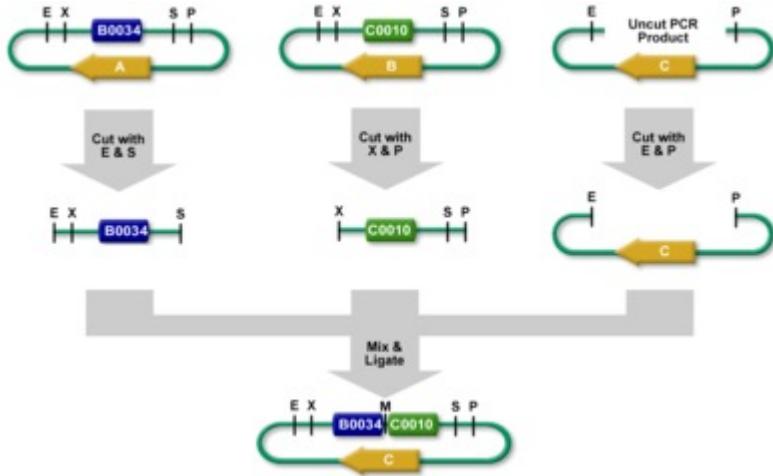
14. RNA Extraction

RNA extraction is similar to DNA extraction, but DNA extraction techniques cannot be directly applied to RNA as they differ in their chemical structure. It is usually more complex to work with RNA, as RNases, an enzyme that degrades RNA, are present in abundance in the environment, such as hands and laboratory surfaces. It is difficult to completely remove and degrade these enzymes. For RNA extraction, requiring aseptic care when handling the samples, using RNase-free materials and solutions (RNase free).

With the sample isolated, RT-PCR can be used to obtain a complementary DNA sequence. DNA is synthesized from a messenger RNA molecule, therefore introns free

15. 3A Assembly

3A Assembly is a method of joining two-part samples and selecting the correct assemblies through antibiotics. The technique uses restriction sites in the prefix and suffix to join the portions together. This new part will keep the same prefix and suffix as the previous parts and will contain a “scar” where the indented restriction sites were linked. The method uses antibiotics to eliminate unwanted colonies and eliminates the need for gel purification and colony PCR, having a higher success rate when compared to standard assembly.



16. CRISPR

A recent genome editing technique is called CRISPR- Clustered Regularly Interspaced Repeats Short Palindromic Repeats, this technique is adapted from a naturally occurring system in the bacterial genome. The bacterium is capable of integrating small stretches of viral DNA and uses them to integrate into its genome between the repeated stretches mentioned above. This allows for a kind of immunological memory. If the virus attacks it again, the bacterium is able to produce RNA segments to incapacitate the invader, using the Cas9 enzyme (or a similar one) to cut the corresponding stretch of viral DNA.

This technique works similarly in labs. A short stretch of RNA is synthesized, which will be used as a guide, attaching itself to a specific part of DNA in the genome to be edited and also to the Cas9 enzyme. Cas9 will cut the DNA in the desired stretch, thanks to the guide RNA. Once the DNA is cut, the cell's machinery can be used to add or delete genetic sequences.

It's worth noting that other enzymes can be used instead of Cas9.

LOGIC GATES

1. Summary

In this topic, content regarding logic gates, a subject contained in digital logic, will be covered. It is through these gates that the analysis of the electrical impulses transmitted by the genetic circuits can be done.

It is important to study this subject in order to gain insight into the parts that make up biological organisms. Metaphorically, gene gates are thought of as bricks used in the construction of circuits.

Thus, first the content used by electronic engineering will be covered, and then a connection will be made with synthetic biology, giving examples.

2. What are they?

It is worth noting that this is primarily a topic in the discipline of electronic engineering, but conceptually it is necessary for the study of synthetic biology. Before connecting the two areas, therefore, it will be explained about logic gates from an electronic point of view.

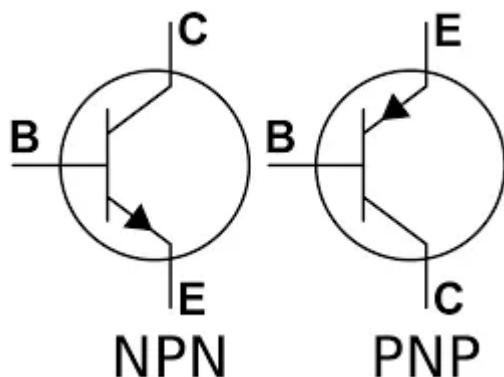
Logic gates are based on the so-called "Boolean logic", widely used in computing, and are electronic devices consisting basically of one or more inputs and usually a single output. In the inputs, the numbers 1 and 0 are used to indicate the digital logic levels, which are basically a mechanism that expresses whether the emanated voltage is high or low. 0 is used for zero (or near zero) voltages and 1 is used for voltages considered high.

These devices are used in order to create circuits that perform different functions, aiming at the functionality of the final product, such as microprocessors and computer memory.

Commercially, logic gates are basically divided into two types, based on which transistor has been used. Before illustrating which they are, it is necessary that the student knows that the transistor is a semiconductor device, composed of 3 plates, used for amplification of electronic signals and in switching.

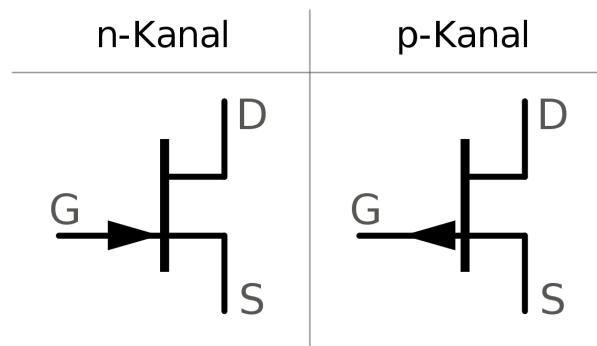
- **TTL (Transistor-transistor logic):** essentially uses a bipolar junction transistor (BJT). The three layers of the BJT are the base (B), controller of the electronic conduction, the collector (C) and the emitter (E), controllers of the conduction current input and output.

Within BJT'S there is a division into two subtypes: PNP, which has a current composed mostly of positive charges, and NPN, which has a current composed of negative charges (electrons).



- **CMOS (Complementary Metal-Oxide Semiconductor):** essentially uses the field-effect transistor (FET). The three layers of the FET are gate (G), source (S) and drain (D), structures analogous to the base, emitter and collector, respectively.

There are two subtypes of CMOS: N-kanal, electron conductors, and P-kanal, positive charge conductors.



For TTL input, voltages recorded between 0V and 0.8V are rated 0; voltages recorded between 0.8V and 2V are rated undetermined; and voltages recorded between 2V and 5V are rated 1.

In the case of the CMOS input, voltages recorded between 0V and 0.4V are rated as 0; voltages recorded between 0.4V and 2.7V are rated as undetermined; and voltages recorded between 2.7V and 18V are rated as 1.

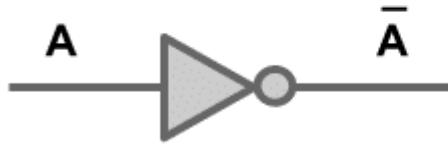
Thus, it can be assumed that the construction of systems with voltages considered indeterminate is not indicated, due to the difficulty in reading that this will cause.

3. Types of Logic Gates

It is worth noting that, in the examples used in this topic, the tables next to the figures are called "truth tables", used in order to indicate the output and input voltage readings in the circuit.

- NOT

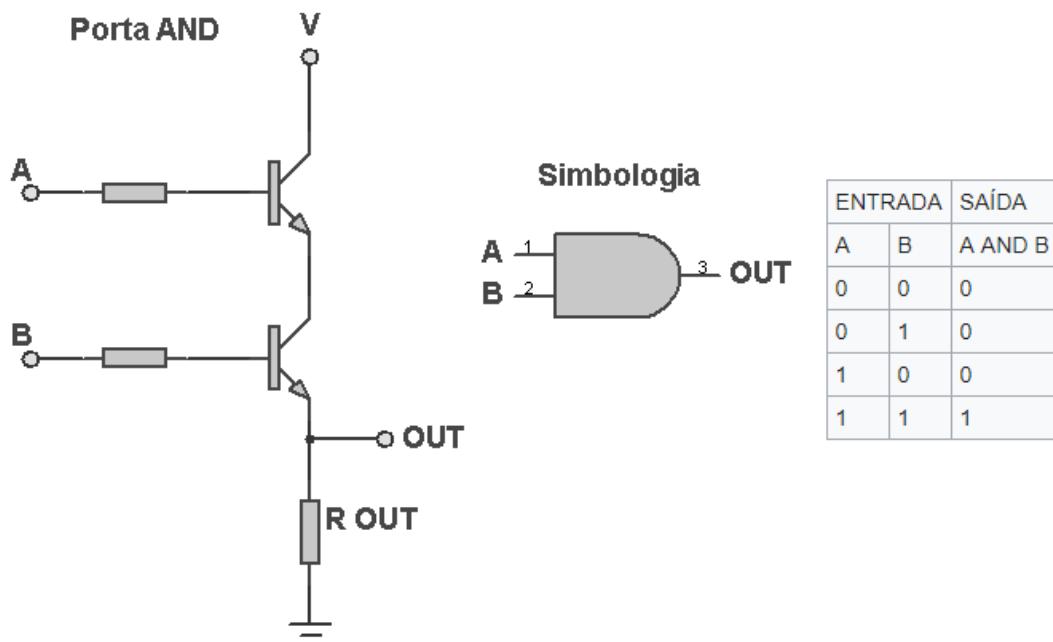
In this gate, the output reading is the inverse of the input reading.



A	A-bar
0	1
1	0

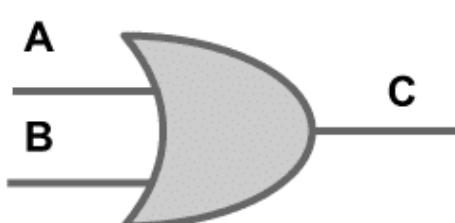
- AND

The output reading from this port will only be 1 if the reading at A and B is 1 simultaneously. Otherwise, the output reading will be 0.



- OR

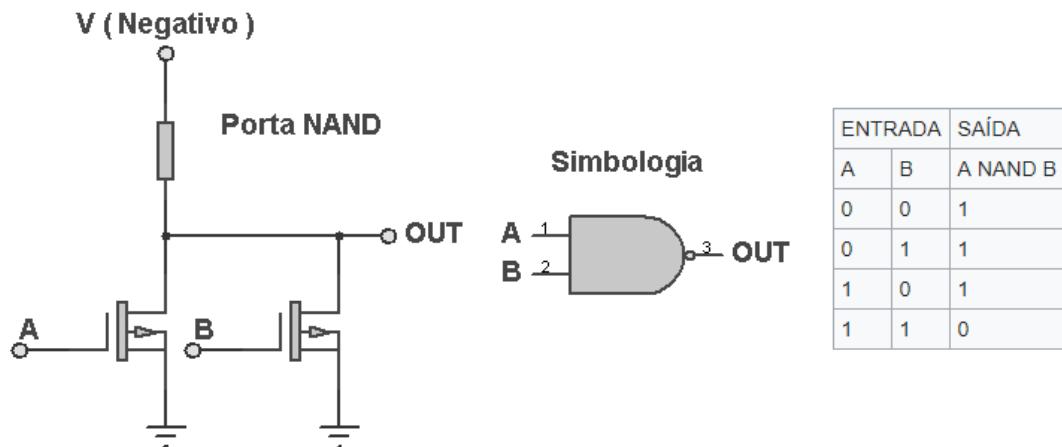
The output reading from this port will be 1 if the reading of A or B, or A and B, is 1. In case A and B are 0 simultaneously, the output reading will also be 0.



A	B	C
0	0	0
0	1	1
1	0	1
1	1	1

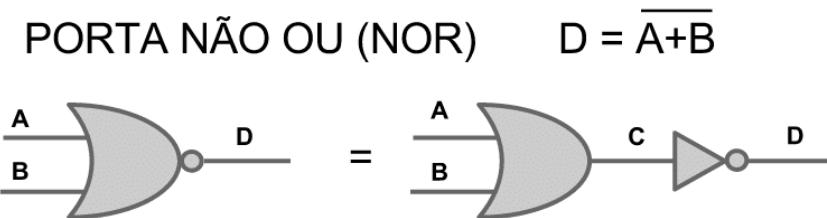
- NAND

In this port, the output reading will be 0 only when the readings of A and B are 1 simultaneously.



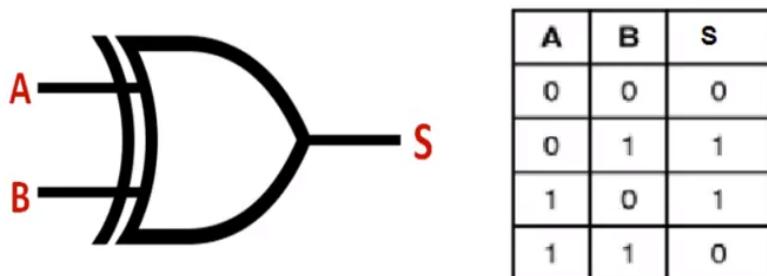
- NOR

The output reading, on this port, will be 1 only when A and B are 0 simultaneously.



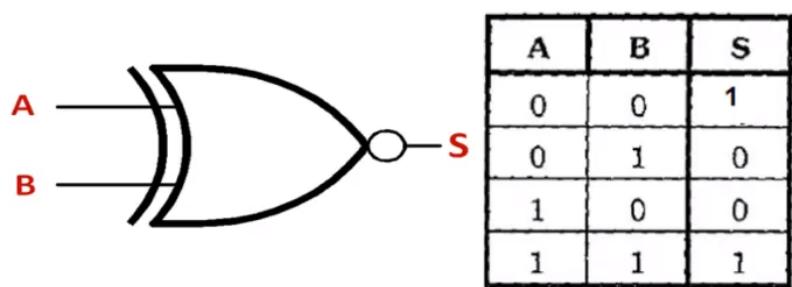
- XOR

In this port, the output reading will only be 0 when the readings at A and B are equal (1 and 1, simultaneously, or 0 and 0, simultaneously). If A and B have divergent readings, the output reading will be 1.



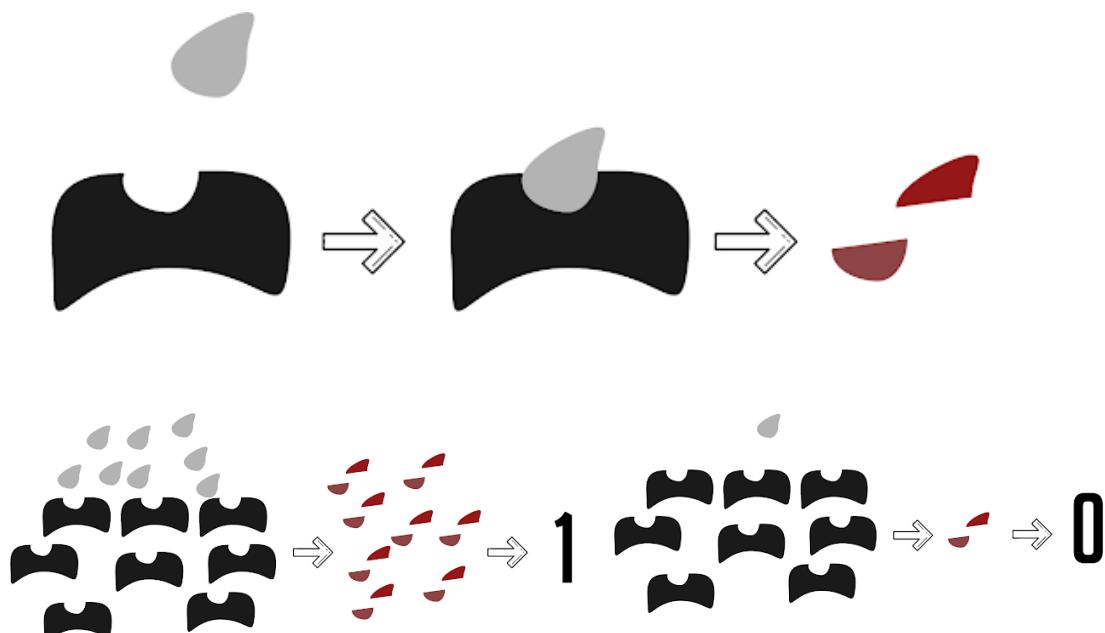
- XNOR

In the case of this port, the output reading will only be 1 when the reading at A and B are equal (0 and 0 simultaneously, or 1 and 1 simultaneously). The output reading will only be 0 when the readings at A and B are divergent.

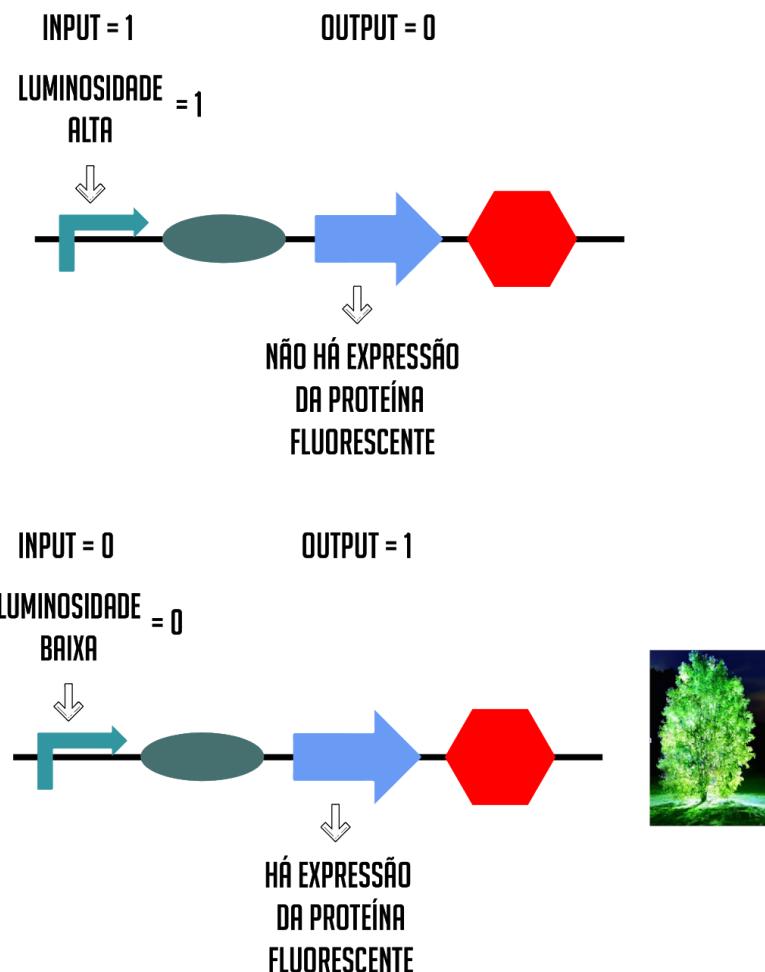


4. Biology and circuits

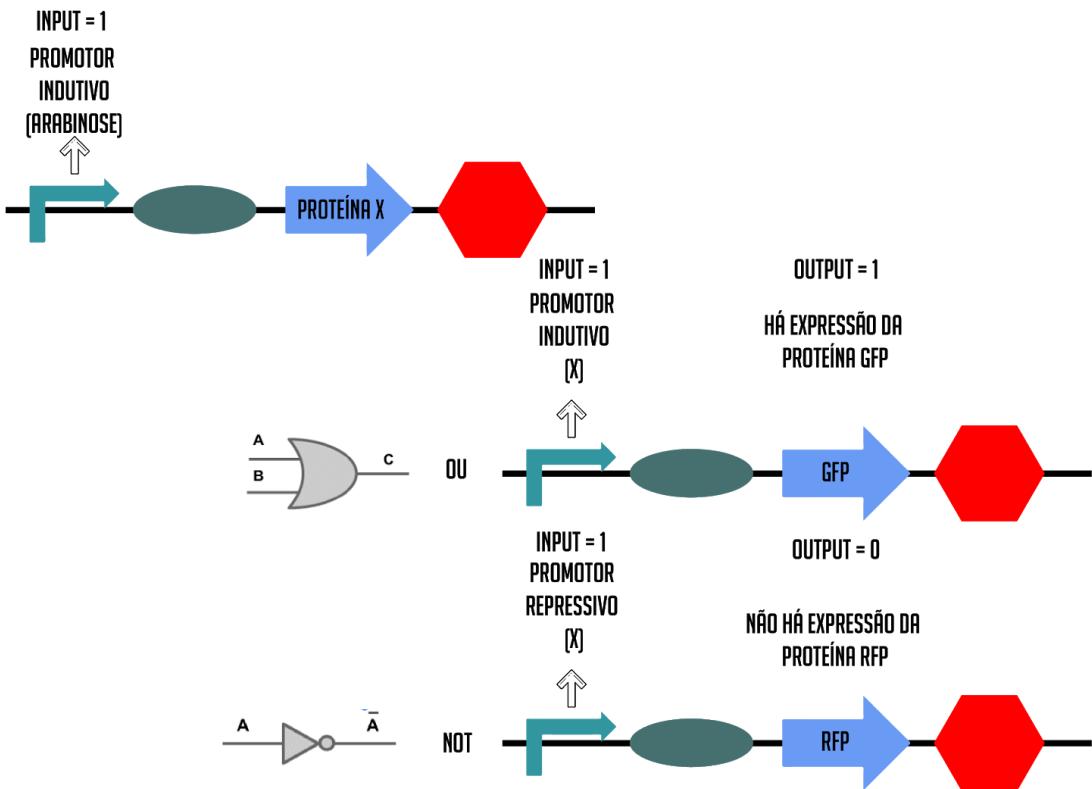
As said before, the binary system is represented by 0 or 1, depending on the signal received. For example, an off source has a value of 0, if it is on it will be 1. It is possible to use this logic with biological systems; if in a medium there is an enzyme that is sensitive to a specific substrate and generates a product. When the substrate concentration is low in relation to the enzyme, we will have little product, so the signal will be 0. If we have a high substrate concentration, then we have a high product concentration.



With this logic, it is possible to build more complex circuits with appropriate gene regulation.



In the image above, we have, in simplified form for didactic purposes, a circuit from an iGEM project that aims to replace lamp posts with glow-in-the-dark trees. The promoter is repressive and sensitive to light, and it is possible to relate it to the NOT gate; when there is high luminosity (input = 1), the expression of the fluorescent protein is inhibited (output = 0). When the brightness is low (input = 0), expression of the protein occurs (output = 1).



In this other example we have a circuit with an arabinose inductive promoter, stimulating the production of protein X. We have two other circuits; one with an "OR" gate, which is inductive to protein X; the other with a "NOT" gate, which is inhibited in the presence of protein X.

If input = 0 in the first circuit, the scenario is reversed in the other two. There will be expression of RFP and expression of GFP will be inhibited.

BIOLOGICAL CIRCUITS AND GENE REGULATORY CIRCUIT

1. Summary

In this topic, content relating to the functioning of natural and/or synthetic biological circuits, the mechanisms of gene regulation that govern the functioning of cells and their metabolism will be covered.

From the simplest single-cell organisms to the most complex multi-cell organisms, all need ways to maintain their metabolism and functioning in the face of the various situations to which they may be exposed. In this context, circuits create logical routines that dictate the way in which the organism will function and how the expressed genes will be regulated with the internal and external variables that the cell may witness.

The methods of natural regulation are extremely vast and complex, with interactions intrinsic to the cellular metabolism itself and responses to external stimuli such as pH, temperature, salinity, and concentration of compounds in the intra and extracellular environment. Studying the methods allows not only to understand, but to apply the existing circuits to synthetic organisms, as well as to create synthetic variants of the circuits, so that the organism can be "programmed" to operate according to the needs of what is desired.

2. Introduction to biological circuits

When thinking about circuits, the idea of an electric circuit comes to mind, and, in principle, it is not totally wrong to think that a biological circuit is functionally equivalent in logic to an electric one. An electric circuit is composed of a source that emits an electric current, information, which is interpreted by a sequence of resistors, capacitors, inductors and transistors that govern the logical operation of an item such as a light bulb. On the other hand, the biological equivalent would be the information of a chemical component in the medium that, according to its concentration (the intensity of an electric current), governs a sequence of proteins, genes and enzymes that lead the biological circuit to, for example, the production of a protein X. In this part of a circuit, the understanding of Boolean Algebra, the understanding of binary codes of 0 and 1 (true or false) that through logic gates will dictate the driving of a given process.

Just as the complexity required by electronics does not end in a simple circuit, biochemistry contemplates a larger arrangement of circuits that regulate existing processes. A small electrical circuit such as a chip represents a simple system that, when joined with other chips, forms a board that can interpret and output a range of information. Similarly, a sequence of biological circuits operates in the regulation of metabolism/catabolism within a specific cellular process. The complexity achieved by a union of several different boards constitutes a computer that can read and output a large amount of data. In this way, a living organism operates in the union of several simultaneous catabolic and metabolic processes that form life as we know it.

It is important to understand that the intrinsic complexity of life and its adaptation to a gigantic variety of resources and external agents. Therefore, it is a common reality to keep a natural operon functioning optimally in isolation. Studying the gene variance of organisms of the same species and the reaction of the desired processes in the

presence of various chemical markers is crucial when aiming to use a biological circuit, be it natural, modified or highly synthetic.

3. Operon Lac

Operon Lac is the gene regulator that governs the ordering of lactose synthesis in E. Coli, its understanding has been of paramount importance for the understanding of biological circuits. Consequently, several synthetic circuits have their functioning based and/or inspired on it.

Lactose is a glycogen capable of providing energy to the cell, but it is a non-preferential metabolic pathway, since life prefers to use resources strategically rather than efficiently and sparingly. The Lac operon genes are responsible for governing this metabolism and the mRNA strand governing this operation is made up of a promoter, an operator that naturally represses transcription of the strand, and the lac Z, Y, and A genes. The lacZ gene is responsible for the production of an enzyme whose function is to break lactose into simpler sugars, proper for the cell; lac Y is responsible for the absorption of lactose by the PM; lac A is not fully understood and still aroused great interest, but it is beyond the scope of this example to discuss it in more detail.

To illustrate the functioning of this Operon, a glucose-rich, lactose-free medium is imagined. The RNA-polymerase binds to the promoter to start transcription, however, there is the presence of the repressor Lac, which is naturally bound to the operator, preventing the continuity of transcription.

Suppose that the concentration of lactose increases and that of glucose also remains high. An isomer of lactose, allolactose, binds to the Lac Repressor, which leaves the Operator, allowing transcription to continue. But still a negligible catabolism of the enzymes destined for lactose is observed. The cell is parsimonious and prefers glucose digestion as a primary energy source. Attention must be paid to cyclic adenosine monophosphate, cAMP, a chemical signaling agent that is intrinsically linked to both the lac operon and the presence of glucose in the medium. In case there is little glucose in the medium, there is an increase in the presence of cAMP, previously low due to the higher concentration of glucose. In the CAP (Catabolic Activator Protein) site, anterior to the promoter, one notices the presence of an activator protein that attaches to this site when it is already linked to cAMP, promoting several transcriptions of the gene in question.

Therefore, the catabolism in question does not depend only on the presence of lactose in the medium, which inhibits the action of the repressor. But also the low presence of glucose, which increases cAMP concentrations and thus promotes the process.

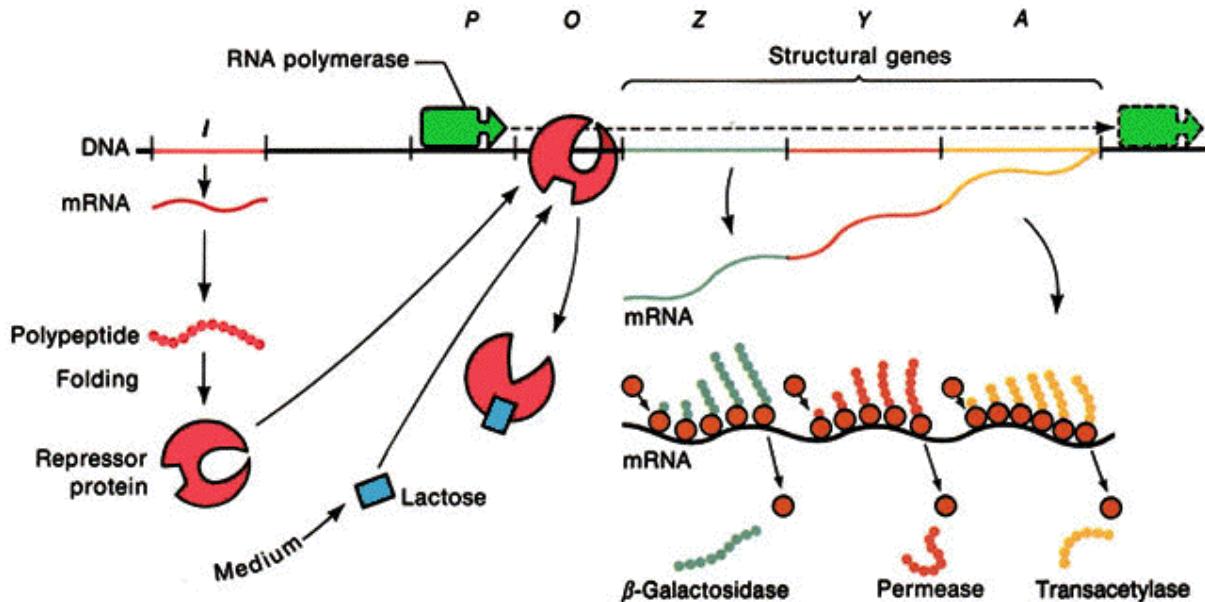


Illustration depicting the gene responsible for catabolism and the corresponding enzymes. The promoter, the operator with its repressor and the lac genes Z, Y and A are observed.

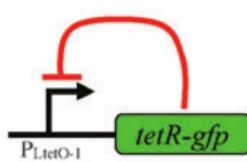
4. Regulators and Operators

Gene regulation is a very important factor in the act of engineering biological circuits. The fact that cellular noise exists makes this regulation even more important, but what is cellular noise?

Cellular noise is a random variation in cells, for example, cells of the same type may have different protein expression rates. These variations can be impacted by many factors, such as the interaction of the molecules themselves, and fluctuating environmental conditions, be these pH, temperature, or nutrient availability.

These noises can alter the viability and optimal functioning of a genetic circuit, so there are some strategies to minimize and even regulate expression to what is desired. One example of regulation is negative feedback regulation, where expression is repressed by the transcript itself.

In this circuit below, there is the *tetR-gfp* gene, which encodes TetR, a repressor with the GFP protein.



All noise in a biological system means, in simple terms, deregulation in the rates of protein production. Therefore, we can say that the correlation of expression fluctuation between 2 genes would reveal the regulatory architecture between them. This point can be demonstrated by the analysis of the following image, where you have a circuit with a gene encoding CI fused with YFP, repressor of RFP expression. Thus, the relationship of YFP and RFP, is different from RFP and CFP.

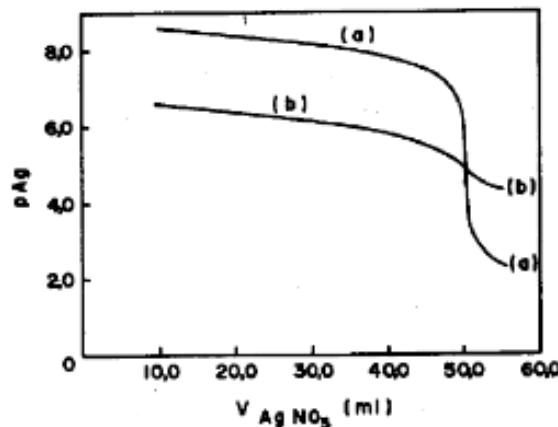
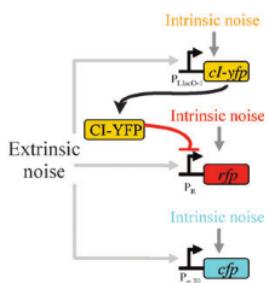
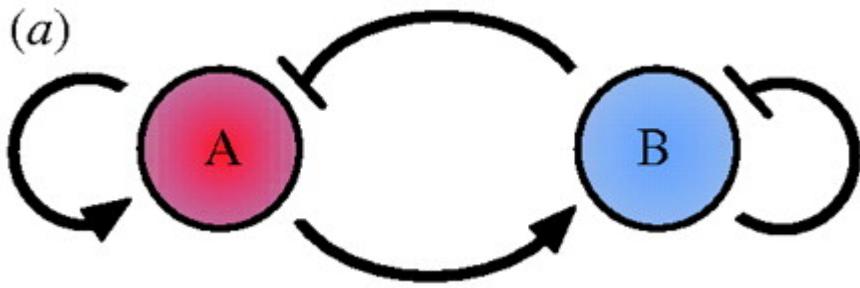


Figura 4.2 – Dependência do perfil da curva de titulação com a concentração dos reagentes
 a) Titulação de NaCl $0,100\text{ M}$ com AgNO_3 $0,100\text{ M}$
 b) Titulação de NaCl $1,00 \times 10^{-3}\text{ M}$ com AgNO_3 $1,00 \times 10^{-3}\text{ M}$

Oscillation can be defined as a periodic variation of a protein over time, where its concentration reaches maximum and minimum values at regular time intervals. This dynamic is regulated by agents, which, in this case, are the

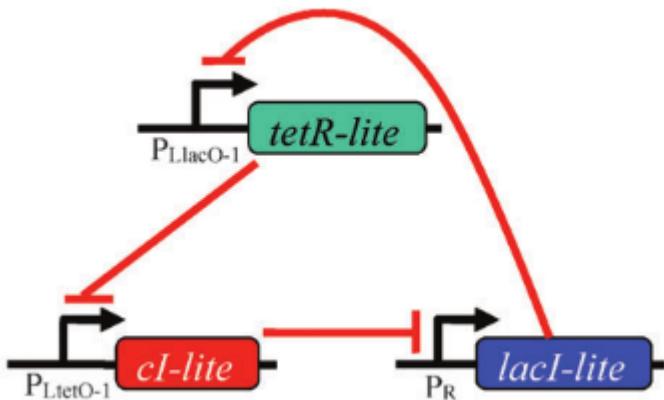
genes together with their transcription factors. With this as a basis, we deepen the study in one of the possible architectures of oscillators, the Smolen-type oscillator.



In this network we have two genes, an activator (A) and a repressor (B). The activator is responsible for performing positive feedback: when the concentration of its protein increases, the synthesis rates also increase. This generates the peak points in the oscillation. On the other hand, the repressor forms a negative feedback: when its protein increases in concentration, synthesis rates decrease. This part is associated with the minimum points (valleys) on the oscillation graph. The combination of these two functions generates oscillation.

A self-sustaining oscillator is, for example, a repressor, which can be defined as a gene regulation network with a feedback loop having at least 3 genes. In the case below, 3 transcriptional repressor genes lacI, tetR cl were put into *E. coli*, such that the expression of each gene represses the expression of the respective LacI, TetR and Cl genes.

This transcriptional repression cycle generates a delayed negative feedback loop, which is a minimum requirement for an oscillator.



MODELAGEM MATEMÁTICA EM BIOLOGIA SINTÉTICA

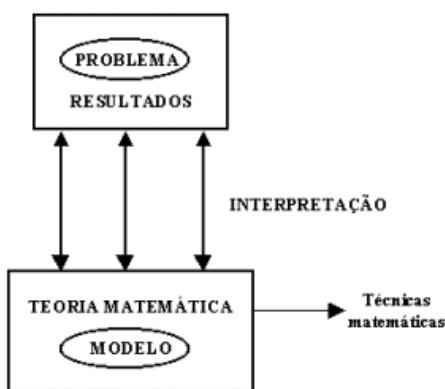
1. Ementa

In this topic, we will discuss the central concepts of mathematical modeling and how it is inserted in biology, especially in synthetic biology. In addition, it is expected to provide a basic understanding of how modeling works for biological systems.

2. O que é modelagem matemática?

Modeling is the proposition of a mathematical model that seeks to contemplate reality, or at least a fraction of it, within the limitations of the conditions adopted. Modeling aims to mathematically represent a phenomenon with the help of formulas and developed methods. Among these, differential equations are widely used. Nowadays it is common to use computation as a tool to help describe phenomenon that are difficult to determine.

The mathematical modeling has to match what is of interest. Briefly, the construction of a mathematical model aims to represent the relations between chemical, biological and physical variables given as important to the objective of a project. The way modeling works can be explained in the following scheme:



In general, the construction of a mathematical model should consider conducting experiments and formalizing the chemical and physical properties of the system under study. This is very important so that there can be a comparison between the results obtained experimentally and the predictions of the model, and there may or may not be validation of the proposed mathematical model.

It may be necessary to develop new mathematical techniques and methods in order to simulate what is desired; or, rarely, there may be no mathematical theory capable of describing the problem. However, this is unusual to occur, since mathematical modeling aims to simplify a complex system in order to manipulate its results.

Thinking about this need for simplification, there is a principle for modeling very similar to the philosophical concept of Occam's Razor, or principle of economy, which aims to always keep that system simpler.

3. Why Study it?

In many situations, it can be complex to determine the instantaneous concentration of a substrate, product, or any other variable in a system that is not saturated or in equilibrium. Modeling allows, through certain already obtained data and considerations, to propose formulas and equations that contribute to estimate a certain value, such as the general behavior of a certain quantity. Although, occasionally, the model cannot fully correspond to the real data, it helps to estimate how such a phenomenon occurs numerically.

But how and why is modeling so useful? Why, in general, if well structured with important and well defined variables, is the model such an important and inherent tool for the study and development of projects within synthetic biology? As mentioned in Mancera's lecture notes, all these questions can be answered in a very compact way by the following topics:

- Simplification

Modeling helps in dividing complex systems into subunits, studying their interactions in order to maintain as much as possible the integrity of the system in question. This implies the possibility of studying and experimenting with specific properties.

- Manipulation

The visualization of the interactions within the model and, consequently, its understanding is easier, and it is possible to add and remove elements that cause disturbances in the system, something that is difficult to do in the real world.

- Organization

The mathematical model is built with what is known about the system and what is deemed essential to its functioning, aiming at one or more specific objective(s). However, vague and hidden variables must also be elucidated. Thus, it is necessary to outline an organized system that allows the visualization of the system both for the model and for those who build it. Paying attention to a few details brings a greater possibility of test validation of the project.

4. Modeling in Synthetic Biology

It is common for biology professionals to try to separate mathematics from the biological sciences. However, this science is done through observation, which in turn generates data, so the inherent relationship with mathematics is evident. This becomes even clearer when we think about the field of applied sciences, such as biological engineering.

Within the field of biology, mathematical application always begins with problematization, that is, before describing in numbers, it is necessary that the problem be thought and translated into words, always keeping in mind the objectives of the research and how they will be achieved.

Synthetic biology works alongside engineering, so it emerges from biology as a distinct discipline, based on quantification, often thinking about efficiency, speed, and production. Therefore, the use of mathematical modeling is essential to what synthetic biology is all about.

5. Modeling examples

To rationalize and demonstrate how a model is formulated, suppose a simple problem in which you want to estimate the concentration of a certain protein using differential equations. You want to estimate the amount of Cre-Recombinases in an *E. coli*. For this, it is assumed that the bacteria are in a controlled system with constant temperature and food/substrate availability in a homogeneous solution. It is necessary to estimate which factors are most relevant for the production of the protein. From the knowledge and everything that has been discussed in this workbook, it can be inferred that the amount of mRNA is relevant for the production of the protein. To analyze the problem, we observe the variation of the amount of mRNA by time, that is, mathematically represented by the derivative:

$$\frac{d}{dt}[mRNA]$$

To visualize the variation, one pays attention to the amount being generated as well as consumed/degraded. First, the amount of mRNA produced is analyzed, which is inherently linked to the transcription rate ($k=40$ base pairs/second) and the size of the targeted protein ($N=1032$ base pairs). Since in chemistry and biochemistry you work in concentration, you have to divide by volume, so there is:

$$\frac{k_{trans}}{V n_{bp}}$$

This would be the production-related term, as there are no other known and/or significant sources of mRNA generation, this becomes the only one. Now, it is necessary to analyze the mRNA decline that is linked to the degradation rate (K_d) and the instantaneous mRNA concentration. Therefore, can be stated that:

$$\frac{d}{dt}[mRNA] = \frac{k_{trans}}{V n_{bp}} - k_{dRNA} \cdot [mRNA]$$

The term is negative, because it refers to the consumption, the decrease of mRNA. Having established a ratio for the mRNA, one looks at how the protein is generated. The amount that the protein is bound to the translation process that is proportional to the concentration of mRNA and at a constant rate of ($K_{trad}= 15$ AA/second). Since each amino acid is generated by one codon (3 base pairs) and mRNA has 1032 pairs, there will be 344 amino acids per protein. In line with the previous equation, the protein will be degraded at a rate proportional to its concentration in the medium. Therefore, it follows that:

$$\frac{d}{dt}[Prot] = \frac{k_{trad}[mRNA]}{n_{aa}} - k_{dProt}[Prot]$$

Assuming that the initial concentration of both mRNA and protein were zero at the beginning, their concentration would continue to increase continuously. On the other hand, the degradation of both increases in proportion to the concentration. A point must be reached where the speed of degradation equals the speed of generation. At this instant, one can say that the system is saturated or in equilibrium. Therefore, there is no longer any variation in the concentration of either of the two items discussed:

$$\frac{d}{dt}[Prot] = 0 \quad \frac{d}{dt}[mRNA] = 0$$

By applying the above condition to both equations, isolating the [mRNA] term and combining them, the final protein value can be found.

$$[Prot] = \frac{k_{trad}k_{tran}}{k_{dProt}k_{dRNA} \frac{n_{bp}^2}{3}} \simeq 2000 \text{ nM}$$

You were able to solve the system to estimate a value. However, if a number n of samples were taken, would this be the average value? Not necessarily, but within the limits of the considerations adopted, the value serves as a "guide", a reference. It can be said that an order of magnitude estimate around 10~100 or 500k~1M would be far from the operational reality.

Example and images taken from:

<https://symbiobrasil.wordpress.com/2012/09/18/modelagemsynbio/>

<https://bionumbers.hms.harvard.edu/search.aspx>

6. Possibilities and limitations of a model

The above example demonstrates the logical line that a model follows in order to be practical and functional. It is reasonable to think that it is limited by the various considerations. When proposing a model, it is necessary to think about what the purpose of its use will be. In this case, a constant temperature was assumed, but what if it is varying with the environment? Or if it were necessary to operate at a higher temperature? One could consider that it is always necessary to act according to the order of the literature and pay attention to what is significant for the system.

Adding numerous variables that only create noise (minimal or negligible variations) only complicates the system without significantly affecting the result. It is necessary to consider and find the balance between simplicity and applicability to reality; to contemplate reality in its entirety is difficult in any area of knowledge, but a specific and practical representation is a powerful tool.

An estimate is not an exact value, but it can be accurate and meaningful to protocol procedures and tolerances. It is worth pointing out that for multivariate systems whose complexity can no longer be reduced, the use of computational methods is valuable to solve the systems.

Biossecurity

1.Summary

In every work it is essential to pay attention to the safety of the individuals involved, and synthetic biology is no different. It is extremely important to guarantee the safety of the technicians and of the environment, since an irresponsible project may generate irreversible consequences. Thus, this topic will discuss important points about biosafety and standards in the field of biotechnology.

2.Introduction:

The advent of genetic engineering has enabled an immense technological development. Cells have begun to be used as reactors, making it possible to synthesize compounds that previously would not have been possible or would have been too labor-intensive. Despite the countless advantages that the manipulation of organisms at the genetic level has brought with it, it is necessary to be wary. Biosafety aims to minimize or eliminate these risks, always seeking to ensure that laboratory technicians and the public are free from these threats.

3.Biosecurity

Biosecurity is understood as the set of attitudes that reduce biological risks related, mainly, to food and agriculture. Among the risks that can be listed in relation to the subject, we can mention the dispersion of pests, mad cow disease, erosion, loss of biodiversity or loss of genetic resources. It is important to note that biosafety differs from biosecurity, since the latter is specific to GMOs.

Many methods of preventing accidents involving biological entities in agriculture are developed and regulated by Embrapa (the Brazilian Agricultural Research Corporation). This company has several research units spread throughout Brazil, always seeking to expand genetic variability to increase productivity through exchanges of genetic resources.

Another resource used is the plant quarantine of all foreign species brought to Brazil for research purposes. This technique consists in the careful analysis of the plants in

order to eradicate any virus, bacterium, fungus, nematode, insect or mite that poses a risk to native vegetation. If this is not possible, the species is either returned or incinerated.

Biological safety can also address risks with microorganism infections in the laboratory. To this end, there are a large number of regulatory norms (NR) that must be followed to ensure the safety of all technicians and related personnel.

<https://www.who.int/csr/resources/publications/biosafety/BisLabManual3rdwebport.pdf>

4.Killswitch:

In the process of engineering a cell, an ever-present concern is the possibility that the modified organism will get out of control and harm the process it was supposed to help with. With this in mind, the concept of the killswitch was created. This term refers to any device designed to stop something from functioning if it is not following its guidelines correctly, and in GMOs this is no different.

The killswitch in GMOs may be genes, endonucleases, or some other biological entity that, in response to specific signals, self-destructs and prevents the cell from doing any kind of damage. These signals are quite varied, and can be the presence of light, specific proteins, metal ions, temperature, etc.

In the case of cell development and manipulation at the genetic level, there is great concern that a GMO is released into an environment where it cannot be controlled. Because of this concern, killswitches are often used in the form of genes that synthesize some toxin that kills the engineered cell as soon as it loses its isolation, preventing collateral damage.

5.Descartes and waste:

Chemical and biological industries and laboratories have several procedures to avoid the bad disposal of substances. Even small amounts of chemical agents or microorganism cultures can have a major environmental and even clinical impact. To avoid this, laboratory safety and biosafety have several regulations to be followed in

order to avoid such damage. Among these regulations, we can mention the norms of signaling, asepsis, adequate disposal, use of protection equipment, etc. Among the examples that can be mentioned, we can mention the creation of super bacteria due to the misuse of antibiotics, a phenomenon that will be better explained below.

In 1928, Alexander Fleming discovered the first antibiotic by studying bacteria of the *Staphylococcus aureus* type; an accidental contamination led him to realize that fungi of the genus *Penicillium* grew without suffering any kind of intervention from the bacteria present on the plate. This highlighted the discovery that it is possible to produce substances that can kill bacteria: antibiotics.

However, nowadays, there is great concern about the overuse and bad disposal of this compound, since bacteria are able to create resistance, forming the so-called super bacteria. These biological entities, because they can be pathogenic, cause several complications in their combat. An example that has brought great concern to health professionals is MRSA (Methicillin Resistant *Staphylococcus aureus*), which has a high resistance to methods to fight bacterial infections. To avoid this serious problem, it is necessary that both the population and the professionals in these areas are aware of the damage that the bad disposal of substances can cause, because with the exacerbated use of these compounds it is possible that medicine will soon be unable to fight these infections.

In the laboratory, in order to prevent infectious diseases, some of the material used is often incinerated - depending on the risk posed by the microorganism in question. Besides the bad disposal of antibiotics, biosecurity also aims to avoid other problems, such as the conversion of the fish population from male to female due to improper disposal of hormones, the spread of diseases by the action of bacteria improperly disposed of in laboratories, etc.

5.Bioethic:

Bioethics is, by definition, a study of the moral problems and consequences arising from research and development in the biological sciences, such as medicine, genetic engineering, and the legitimacy of conducting tests on living beings. Due to scientific advances in the field of genetics, it can be said that this topic has never been more hotly debated than it is now. Debates about the use of genetic engineering to suppress disease, modifications of physical and intellectual characteristics in human

beings, and in general the reconciliation of ability with the moral limits of science have been increasingly explored. Among the topics addressed by bioethics are abortion, in vitro fertilization, euthanasia, cloning, transgenics, and the use of stem cells.

In a post World War II context, with Nazism, genocide, countless deaths, and experiments on humans still very recent in people's memories, the United Nations Organization (UNO) presented, on December 10, 1948, the Universal Declaration of Human Rights, aiming to reaffirm the importance of human life and prevent such a disaster from happening again. Scientific research must be carried out with the aim of developing humanity without making a moral sacrifice to do so. In this way, the importance of bioethics in scientific development is reaffirmed.

Chinese researcher He Jiankui in 2018 created the first genetically modified human children from CRISPR-Cas9. The twins were born healthy and well. The researcher's intention was to make the children resistant to HIV infection. However, this decision opened several discussions covering the limits of science and the use of genetic manipulation in human beings. This news shook the entire scientific community and the researcher was heavily criticized for violating various academic ethics; the consequences of this act will probably still be echoed. The modification was made in germ cells, so the characteristics manifested by the children will be passed on to the next generations, and may cause various problems and side effects.

6.Níveis de segurança

The laboratory environment involves several risks for workers and the environment, so it is necessary that laboratories follow rules established by ANVISA in order to minimize possible risks.

In this topic, we will talk about the four levels determined by Anvisa in the Collegiate Directorate Resolution (RDC) No. 50.

Biosafety level 1 (NB 1)

Determined for handling risk class 1 organisms, since they have a low probability of causing injury to humans or animals. It is not necessary to use specific protection equipment, only open workbenches with sinks nearby.

The work must be done by trained individuals and supervised by a biosafety professional, besides being guided by Good Laboratory Practices, such as

- hand washing;
- not eating, drinking and smoking. Food should be kept out of the work area;
- avoid wearing open shoes;
- use mechanical devices for pipetting, and it is forbidden to do it by mouth;
- Do not wear accessories such as rings, watches and bracelets during laboratory activities;
- have a first-aid kit;
- perform disinfection of work surfaces at the end of use or when there is direct contact with viable material;
- have an insect and rodent control program.

Biosafety level 2 (NB 2)

With this level, you can handle microorganisms of risk class 2, so the use of PPE (personal protective equipment) is recommended, such as gloves and face protection. The use of safety booths is also required. The installation should follow the same requirements as NB1, and an autoclave is added to the environment.

- have limited access;
- have a Biohazard sign;
- have precautions for sharp objects;
- present a Biosafety Manual that defines any waste decontamination or medical surveillance rules.

Biosafety level 3 (NB 3)

With this level, you can handle microorganisms of risk class 3. It has the same requirements as NB 2, with the addition of the use of safety cabins in all manipulations with the microorganisms. There must be physical separation of

the access corridors, double doors with automatic closure, exhaust air, which must not recirculate, and the air flow must not leave the laboratory.

- have strictly controlled access;
- disinfect all waste and clothing used in the laboratory before being washed;
- collect and store serological samples from each employee exposed to risk as reference.

Biosafety level 4 (NB 4)

At this level, individuals will be exposed to highly dangerous microorganisms, those of risk class 4. The use of class III safety cabins, use of positive pressure overalls with air supply is required. The laboratory must be totally isolated from the rest of the building. The supply and exhaust systems must be vacuum and have efficient decontamination systems, in addition to other specifications described to be followed due to the risk involved.

The safety practices must also be carefully performed and involve

- change of clothes before entering;
- showering upon leaving;
- decontamination of all material on leaving the laboratory.

Classification of Microorganisms

The risk classification for a particular microorganism is based on criteria driven by the potential risk it poses to the individual, the community, and the environment.

- Risk class 1

The individual and community risk is absent or very low, i.e., these are microorganisms that have a low probability of causing infections in humans or animals. Examples: *Bacillus subtilis*.

- Risk class 2

The individual risk is moderate, and for the community it is low. These are microorganisms that can cause infections, but efficient therapeutic and prophylactic measures are available, and the risk of spread is limited. Examples: Yellow Fever Virus and Schistosoma mansoni.

- Risk Category 3

The risk to the individual is high and to the community is limited. The pathogen can cause serious infections in humans and animals and can spread from individual to individual, but therapeutic and prophylaxis measures exist. Examples: Venezuelan Equine Encephalitis Virus and Mycobacterium tuberculosis.

- Risk Class 4

The risk for the individual and the community is high. These are microorganisms that pose serious risk to humans and animals, being highly pathogenic, easily spread, and there are no prophylactic or therapeutic measures. Examples: Marburg virus and Ebola virus.

1. Summary

This part of the block aims to introduce some concepts of microbiology and its techniques, in addition to clarifying its importance for synthetic biology. To do so, we are going to go through some important points, such as the different groups of microorganisms and what their main characteristics are. Also, talking a little about the importance and giving some examples of application within synthetic biology.

Starting from this point, we will address some basic issues of laboratory practice, ranging from the importance of asepsis, passing through basic laboratory materials and going to classification of culture media. Having introduced these questions, we end this part of the block with some laboratory techniques of microbiology, explaining the concept and procedures.

2. Introduction

Microbiology is the branch of biology that studies microscopic life forms and how they relate to each other and to the environment. The functions of these beings are very important for the environment, within biogeochemical cycles, for example. With the rise of biotechnology, these microorganisms have been given leading roles within

the industrial sector. There are also those that are pathogenic to other organisms, such as animals, fungi and plants.

3. Groups of microorganisms: biodiversity, characterization and applications

When talking about biotechnology, it is important to think about bioinformatics and genomics, which aim at integration and objectivity in the use of data. In this way, they dialogue with microbial biodiversity, while it is not interesting to be restricted to a small group of microorganisms, since this restriction leads to a scenario of low prospectation for industrial biotechnology. Therefore, it is important to explore known organisms and also seek to catalog new ones, which, changing the paradigm of an exclusively taxonomic view, can bring new possibilities to industrial microbiology research and market.

To understand the applications of these microorganisms and explore the biotechnological potential of each one, it is necessary that we seek to know a little about the main groups of microorganisms, which are: viruses, bacteria, algae, protozoa and unicellular fungi.

3.1. Virus

Entities considered as a boundary between living and non-living beings. They are acellular and consist of a nucleic acid (DNA or RNA) surrounded by a protective cover called a capsid. They depend on living cells to use their machinery, as they do not have their own metabolism and, outside of hosts, they crystallize.

As a very diverse group, viruses do not have a pattern, so they are classified according to the type of nucleic acid, means of infection and hosts.

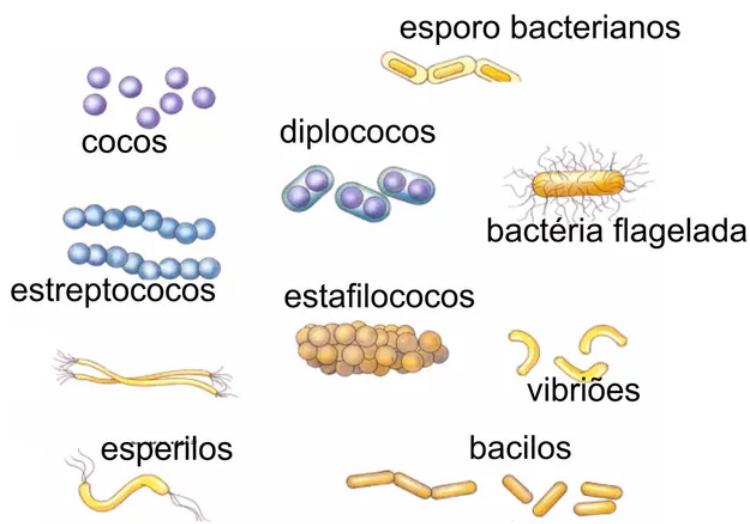
- Adenovirus: Its nucleic acid is DNA;
- Retroviruses: Its nucleic acid is RNA;
- Bacteriophage: Type of virus that infects bacteria;
- Mycophagus: Type of virus that infects fungi;
- Arbovirus: It is one that is transmitted through insects.

A good example of the application of viruses in biotechnology is the use of synthetic bacteriophages to combat biofilm-producing bacterial infections. This alternative

treatment is called phagotherapy and has been increasingly studied in an attempt to deal with the resistance that bacteria have created to antibiotics.

3.2.Bacteria

These unicellular beings are prokaryotes and their differentiation criteria are many, among the most important are morphology, chemical composition, nutritional needs, biochemical activities and energy sources.



Font: <http://educacao.globo.com/biologia/assunto/microbiologia/bacterias.htm>

The best known example of engineered bacteria is *Escherichia coli*. As its genome is well known, there is a great possibility of carrying out genetic modifications, such as gene deletion due to genomic reduction.

Metabolic engineering techniques are used to modify *E. coli* so that it produces, for example, artemisinic acid, through fermentation and photochemical transformation. Artemisinin acid is the precursor of artemisinin, an active substance used in the treatment of malaria.

3.3.Protozoa

They are unicellular, eukaryotic and generally heterotrophic. They are unable to form tissue, but they can form colonies.

An example of application is the genus *Tetrahymena*, ciliated and non-pathogenic protozoa, which has been studied because of its possibilities. They have been engineered to efficiently bioconvert cholesterol to provitamin D₃ for application in the chemical and food industries.

3.4. Microalgae

Single-celled algae are eukaryotic and photosynthetic autotrophic, they can be found in aquatic bodies or moist environments. The structure of these organisms is relatively simple, allowing rapid growth and easy genetic and metabolic manipulation, in addition to not having high nutritional requirements and easy biomass duplication.

Thus, microalgae are strong candidates for the production of biodiesel, since one of the components of their biomass are lipids, macromolecules that are raw material for the production of this alternative fuel. Studies indicate that the efficiency of these organisms, in this production process, is quite high, once again proving to be a strong alternative.

3.5. Fungi

Filamentous fungi are of great importance for biotechnology. However, here we will focus on yeast, unicellular microscopic beings, eukaryotes and heterotrophs.

The yeast *Saccharomyces cerevisiae* is the best known microscopic fungus with a genome, having several applications in industry. Two examples of application in industrial biotechnology of this yeast are in the production of the compound taxol, an important compound for the production of antitumor, and in the synthesis of hydrocortisone acetate, in the production of steroid anti-inflammatory drugs from glucose.

4. Nutrition and Cultivation of Microorganisms

It is necessary to know the nutritional needs of the organism of interest for cultivation, in order to mimic its growth medium, in addition to considering issues such as temperature and pH.

4.1. Culture Medium

The culture medium is the nutrient material that is prepared for microbial growth in the laboratory. Some microorganisms can grow well in any culture medium, while others require special conditions. Some microorganisms cannot grow on any culture media developed to date.

The culture medium, initially, must be sterile, that is, not present living organisms, in order to avoid contamination of the study material. Microorganisms introduced into the culture medium are called inocula, while those that develop in the medium are called culture.

4.2. Classification of culture media for consistency:

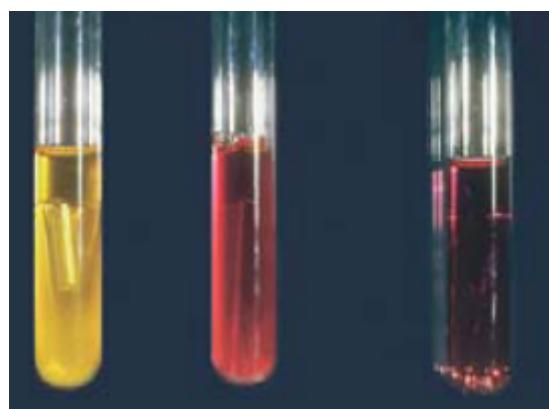
4.2.1. Solid medium:

Microorganisms consume agarose to grow. It is an extremely effective medium for isolating pure colonies. It does not promote population growth as large as the liquid medium, but allows colonies to be formed for further study.



4.2.2. Liquid medium:

Promotes mass and dispersal growth, but without colony formation. Nutrients are dispersed in the solution. This method is very useful for storing microorganisms for later sample collection.



4.2.3. Semi-solid Medium:

It guarantees the possibility of motility, this medium is obtained from the addition of small amounts of agar.

There are different classifications of culture medium:

- Defined medium

The defined medium is one in which the addition of substrate is of exact chemical composition.

- Complex medium

In the complex environment, its exact composition is not known. Most bacteria and fungi are generally grown in complex media.

- Enriched medium

Enriched means favor the growth of a population that is at a disadvantage in relation to others.

- Selective medium

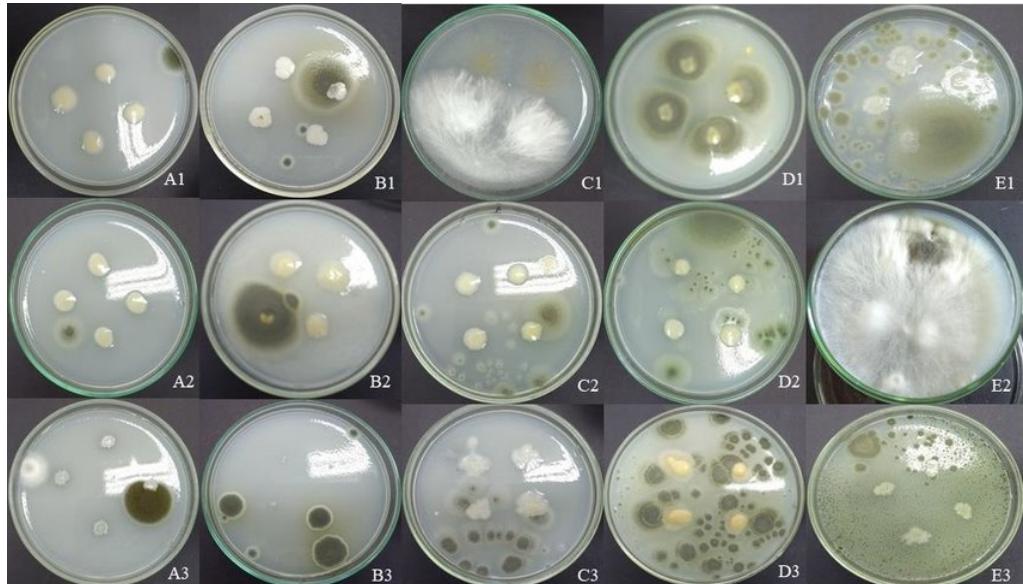
Selective media are thought to prevent the growth of unwanted microorganisms and favor those of interest.

- Differential medium

Differential media make it easy to distinguish multiple microbial colonies that are growing on the same plate.

Visual scale of the degree of fungal contamination in the culture medium.

Scale: The letters A, B, C, D and E numbered 1 to 3 represent examples of the degree of contamination in ascending order: degree 1 (+); 2 (++) ; 3 (+++); 4 (+++); 5 (++++).



Font:

https://www.researchgate.net/figure/Figura-1-Escala-visual-do-grau-de-contaminacao-fungica-em-meio-de-cultura-Escala-As_fig1_330655187

5. Techniques for cultivation of microorganisms

5.1. Introduction:

In laboratories where microorganisms work, the most diverse techniques for cell isolation and cultivation are always present. The importance of understanding how these methods work has been increasingly highlighted in industries, as biotechnology has gained a lot of presence by replacing chemical pathways (which are often aggressive, expensive and harmful to the environment). Thus, it is possible to use relatively simple laboratory analysis to investigate the possibility of application in industry.

5.2. Importance of asepsis and care to avoid contamination:

Single-celled bacteria and fungi are present in all types of environments on the planet. Therefore, for an experiment involving microorganisms to be successful, it is

necessary that all experimental steps are carried out with utmost precaution to avoid external contamination.

From an industrial point of view, a contamination in one of the biotechnological processes can cause a huge failure in production, or be harmful to the health of some of the employees and customers. Because of this, everyone involved in the experiments must carry out the activities in a cautious and systematic way.

5.3.Materials used:

Before discussing the cultivation techniques themselves, it is necessary to know the instruments used:

5.3.1. Petri dish:

It is a flat cylinder made of glass or plastic and contains a lid to prevent external contamination. In this plate, a solid culture medium (gelatinous) will be placed where microorganisms will be inoculated for the beginning of cultivation. This gel can be composed of several substances, the main one being a carbohydrate called agarose.



5.3.2.Drigalski Handle:

It can be made of metal, glass, or some other material that makes it difficult to contaminate unwanted microorganisms. It is used to spread suspensions of microorganisms in the petri dish.



5.3.3. Automatic pipette:

Apparatus with the objective of transferring precise volumes of liquids, and may contain a liquid culture medium, suspended microorganisms or even liquid reagents. It is extremely important in cell culture and is present in basically all techniques. At the end of this device, there is a plastic tip that is discarded after use, preventing contamination.



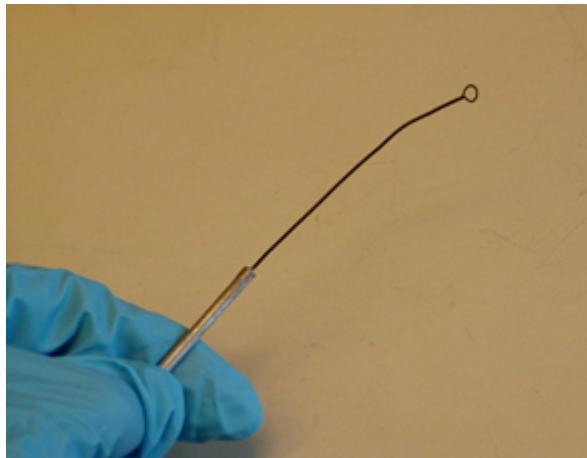
5.3.4. Bunsen burner:

Instrument that provides heat in the form of an intense flame, widely used in chemistry laboratories. In the microbiology laboratory, it will be used mainly for instrument sterilization and fixation for viewing under a microscope.



5.3.5. Inoculation loop (or loop):

Laboratory instrument that aims to transfer small amounts of microorganisms in liquid suspension. It is the main isolation tool for pure cultures on solid media. It has a metallic ring at its end, which must be treated in the Bunsen burner whenever there is a transfer.



5.4.6. Autoclave:

It basically works like a huge pressure cooker. Its function is to sterilize glassware and instruments for use on microorganisms. Its internal temperature and pressure are capable of killing (almost) all microorganisms that were present on the surface of the devices.



5.3.7. Laminar flow:

A structure with the function of preventing the handler (or its environment) from contaminating your sample. Laminar flow uses filters to sterilize the outside air and, once sterilized, the air is directed into the working chamber. The air enters with a laminar flow and is then expelled to the outside. The laminar regime prevents the air from suspending contaminating particles and, if this happens, it will be expelled before it contaminates the sample.



5.3.8. And also...not least:

In the laboratory it will be necessary to use instruments whose function is more intuitive, such as tweezers, volumetric beakers, test tubes, water distillers, magnifying glasses, microscopes, ovens, bain-marie and so on.

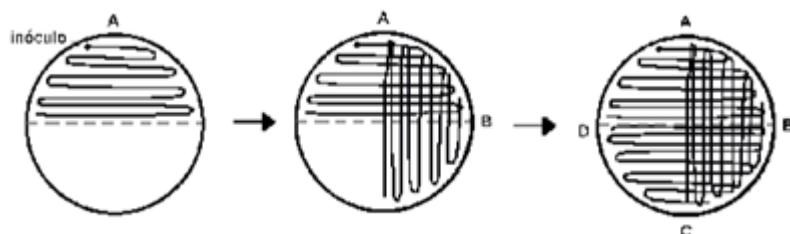
5.4. Plate Sowing:

5.4.1. Inoculum depletion technique: Isolation of colonies

When faced with a test tube containing several colonies of species of microorganisms, often you want to obtain a colony with a single cell type. From this lack comes the need for the inoculum depletion isolation technique.

This technique assumes that each time the inoculation loop passes through the gel in the petri dish, the amount of cells present in the loop decreases. Soon, at the end of the journey there will be pure colonies.

Once the proper sterilization is done, the loop must go through the dry plate (without damaging the gel) repeatedly, forming zigzag figures and covering the largest possible area. After this initial inoculum, the same must be done, but perpendicular to the lines already drawn. Turn the plate over and make new lines parallel to the initial inoculum, as shown in the diagram below:



After performing this process, it is expected that pure cultures will be visualized in the final portion of the plate (after the growth of microorganisms with the aid of an incubator).

This same technique can be applied in test tubes with the gel inclined in relation to its cross section.



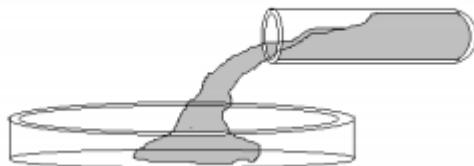
5.4.2. Spreading with handle:

This technique requires the use of a Drigalski loop, or a swab, a structure very similar to a cotton swab (transfers more volume than the inoculation loop), to form a surface containing bacteria. The transfer of the inoculum is done with an automatic pipette and then the pipetted volume is spread. This method can be used to isolate colonies by diluting the bacteria in test tubes.



5.4.3. Pour-plate or depth seeding:

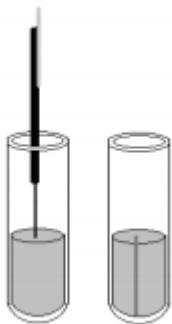
It is a technique used to quantify bacteria on a plate. It basically consists of adding a small volume of a bacterial suspension to a cooled molten culture medium (followed by the homogenization of the resulting mixture).



5.5. Seeding in Test Tube:

5.5.1. Pitching sowing technique:

As the name suggests, an instrument, called an inoculum needle, is inserted into a test tube containing solid medium to determine specific metabolic functions of bacteria. The method consists of perforating the gel by the action of the inoculation needle, removing it and finally observing the growth of bacteria after a certain time in the incubator.



5.5.2. Diffusion:

It is a technique that uses liquid culture media, involves the addition of microorganisms with a handle to a test tube. This technique allows some biochemical properties of bacteria to be analyzed, through tests that will be presented later. The method also allows for large microbial growth when in a medium that allows for enrichment.



5.6. Gram staining method:

As the name suggests, this method aims to identify morphological characteristics of bacteria through a pigmentation involving lugol (a solution containing KI) and other dyes. This technique was developed by a Danish doctor named Hans Cristian Joaquim Gram, in 1884, when he observed that when coloring the bacterial cells, some turned purple and others red.

This phenomenon is due to some characteristics that differentiate bacteria, such as the thickness of the cell wall and the presence of an outer membrane covering the microorganism. Gram positive bacteria (G+) are able to retain the violet color due to the presence of an outer membrane composed of peptidoglycans, while Gram negative (G-) receive their reddish color at the end of the process.

Procedure:

- 1- The slide containing the sample to be analyzed must be fixed with a Bunsen burner, breaking the cells so that the dyes can enter them.
- 2- A solution of methyl violet is applied on the slide, for approximately one minute, so that the cells interact with the dye.
- 3- Wash the sample with distilled water.
- 4- Lugol is added to the sample for one minute and then the slide is washed with ethyl alcohol.
- 5- Wash the lens with plenty of distilled water.
- 6- The slide must be covered with safranin for 30 seconds and then washed with running water.
- 7- The slide must be air-dry, and can finally be viewed under the microscope.

5.6.1. Completion of Gram analysis:

If the sample turns red, the test is considered negative and it can be said that the microorganism in question does not have an outer membrane composed of peptidoglycans. If it turns purple, the conclusion is that the bacteria has an outer membrane and the test is considered positive.

Gram's method is of fundamental importance for taxonomy (the science that classifies living beings) and for microbiological tests, whether clinical (such as in the examination of STDs) or in research.

ENZYMOLOGY

1. Summary

In this topic, the contents referring to the subject of enzymology will be addressed, explaining what enzymes are and what types they could be. Furthermore, it will be discussed enzyme purification processes and chemical kinetics, since enzymes are basically biological catalysts.

2.What are enzymes?

Using a general concept, enzymes are proteins that act as catalysts for chemical reactions in the cellular environment, mostly. In other words, while chemical catalysts act in more extreme conditions, enzymes act at a temperature of 37 C and pH 7.4 (in this example). It is noteworthy, however, that these conditions are for humans and microorganisms that live inside them, such as E. Coli. Before going into the properties of enzymes, it is necessary to resort to a concept already given in another topic of the book: Substrate, chemical compound under which an enzyme acts. That is, it can be said that enzymes have specificity for their substrate. Enzymes can accelerate by 10^{17} times the catalytic activity of a cell. This property is so important because it shows that most chemical reactions in a cell would not occur nearly quickly enough under ambient conditions.

Although every enzyme is a protein, some depend on a non-protein component called a "cofactor". Therefore, enzymes receive two classifications: conjugated ones, which require cofactors, and simple ones, which do not. The part of the enzyme that is not the cofactor (protein part) is called apoprotein or apoenzyme. And the set of the protein part with the cofactor firmly attached is called the prosthetic group.

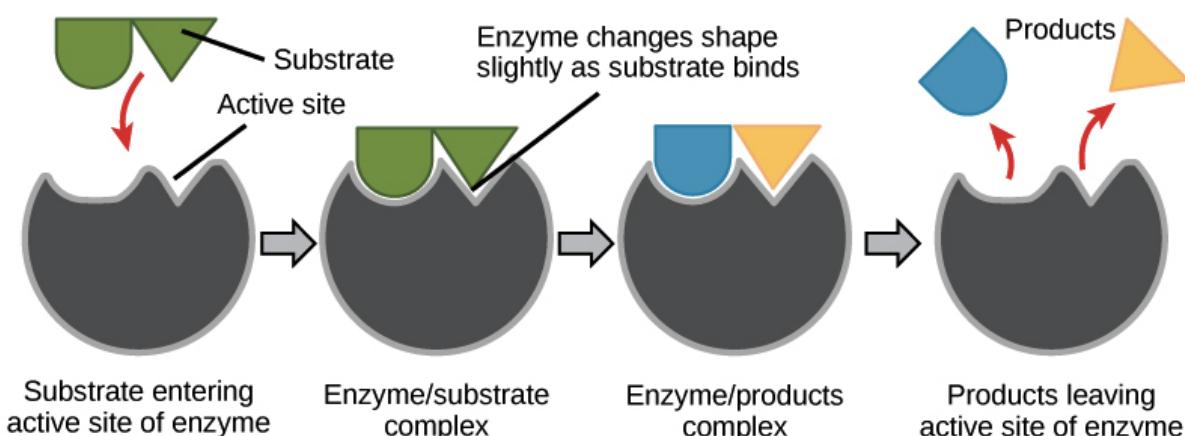
Cofactors bind to the active site of the enzyme temporarily, through non-covalent interactions, or permanently, through covalent chemical bonds.

3.Active site of enzymes

In the topics above, the active site of enzymes was mentioned, but a topic is needed to highlight some concepts.

First-hand, the activation site is nothing more than the site of the enzyme where the substrate attaches. Initially, scientists used a model called "key-lock" to explain how the connection between the two parts occurred. As the name implies, in this model the substrate and enzyme were molded like puzzle pieces so that there was a fit between the pieces. Currently, it is known that this join follows the model called "induced fit". Basically, the enzyme is able to quickly change its shape in order to bind to the substrate, forming an even tighter/still joint than predicted in the key-lock model.

It is important to clarify that, at the end of the reaction, in all cases, the enzyme detaches itself from the substrate, returning to its original state. Also, when a catalysis reaction ends, it releases the formed products. Once it is done, it becomes able to carry out a new reaction.



4. Enzyme types

- Hydrolases: associate with water molecules in order to break covalent bonds.
They are mainly divided into:
 - Hydroxylases: Add a hydrogen atom taken from H₂O to the substrate to produce a hydroxyl group.
 - Glycosidases: perform hydrolysis of covalent bonds that join monosaccharides (glycosidic bonds).
 - Peptidases: perform hydrolysis of peptide bonds.
 - Ligases: Form new molecules by linking two or more existing ones.
- Oxidoreductases: perform an electron transfer (oxireduction). They are mainly divided into:
 - Reductases: add hydrogen atoms to the substrate (reduction)
 - Oxygenases: accelerate the addition of molecular oxygen to the substrate.
 - Dehydrogenases: remove electrons from their substrates in the form of hydride ions (oxy-reduction).
- Transferases: Translocate functional groups from one molecule to another, such as carboxyls, amines and phosphates.
- Liases: break covalent bonds, removing water, carbon dioxide or ammonia.
- Isomerases: accelerate reactions that lead to the production of isomers.

5. Protein structure

As every enzyme is a protein, it is necessary to talk about the structure of proteins, which are macromolecules that make up more than half of the net weight of cells.

Proteins can be subdivided into fibrous and globular:

Fibrous proteins: they are insoluble in water, physically resistant and play a structural role. Keratin and collagen are examples of fibrous proteins.

Globular proteins: These are normally water soluble and can be crystallized. They play a structural role in cells. Every enzyme is a globular protein.

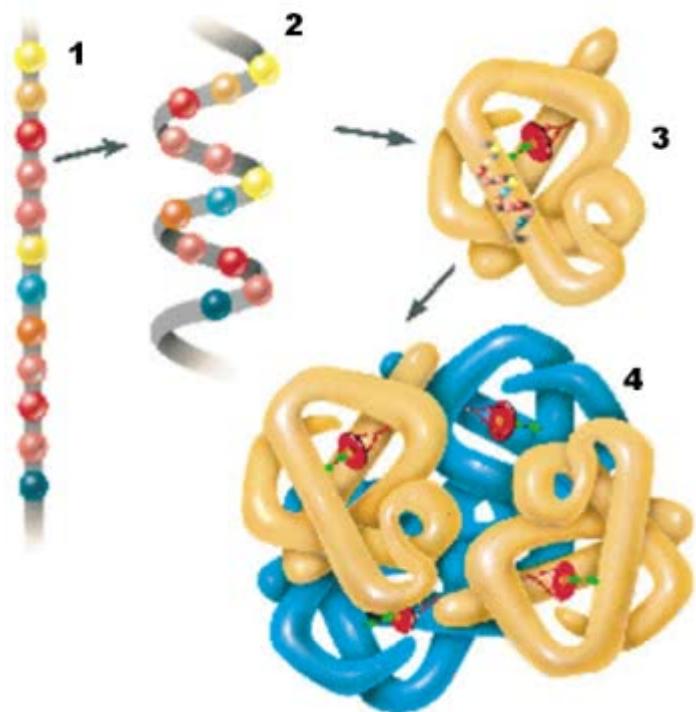
Amino acids are units that form proteins, each with specific properties and functions.

Another subdivision that the protein can receive is between simple and conjugated.

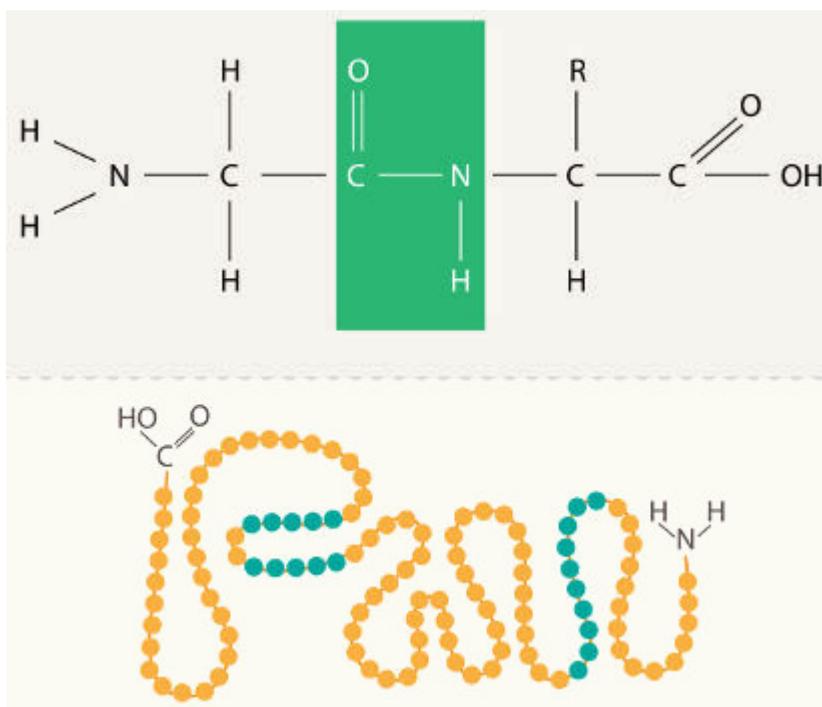
Simple proteins: are composed only by amino acids.

Conjugated proteins: in addition to amino acids, they have another component, such as lipids (lipoproteins) and oligosaccharides (glycoproteins).

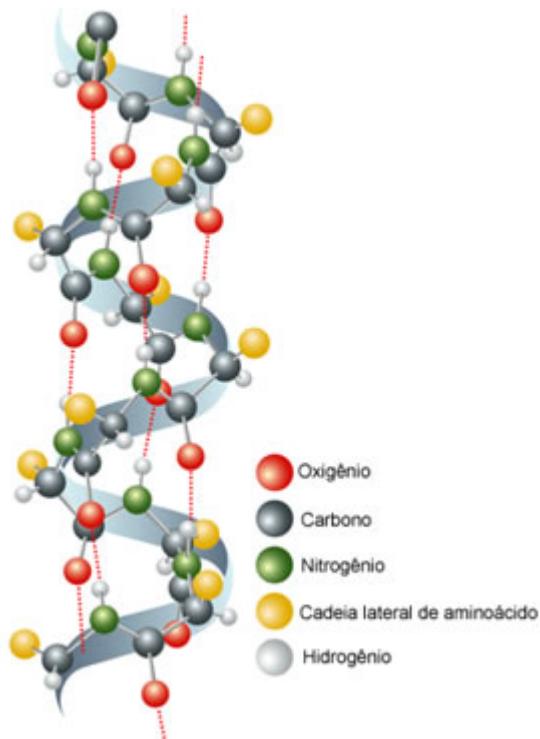
The structure of proteins can be subdivided into primary(1), secondary(2), tertiary(3) and quaternary(4).



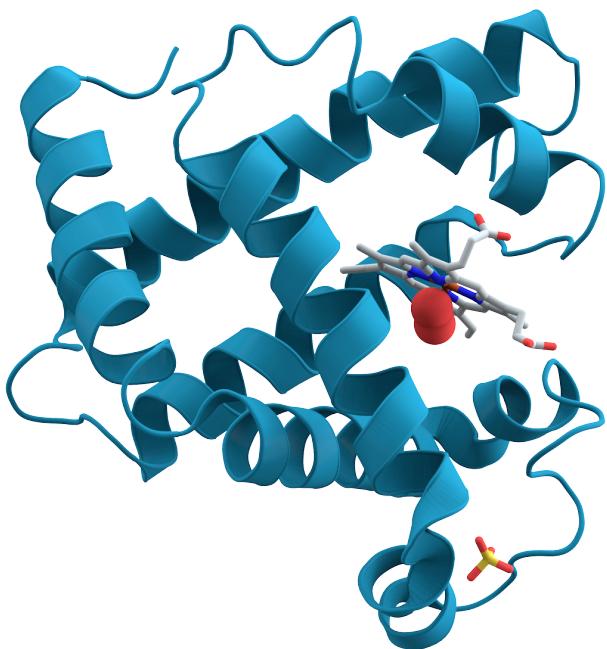
- Primary proteins: these are a sequence of amino acids in a peptide chain. This bond connects the carboxyl groups of each amino acid to each amino group of the next ones in the chain.



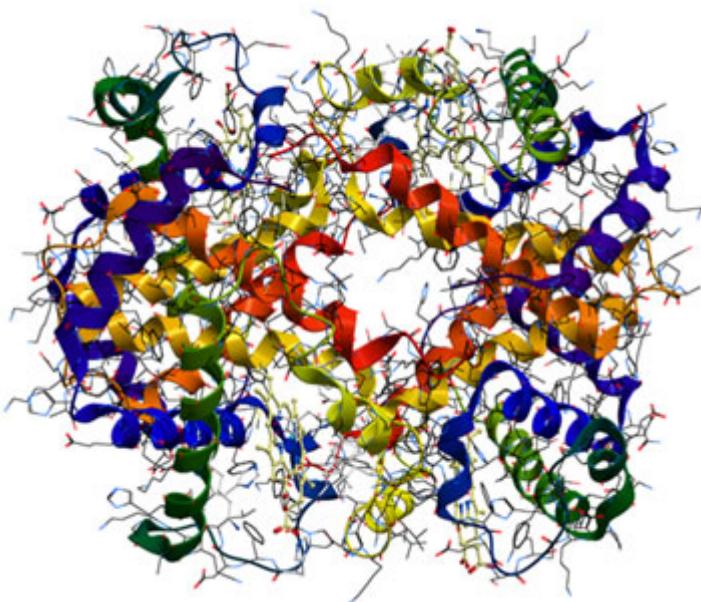
- Secondary proteins: when the peptide chain grows, the parts of the amino acids will interact with each other. The bond between amino acids occurs through sulfur bonds or hydrogen bonds. (hydrophobic interactions)



- Tertiary proteins: are formed by non-covalent bonds between the amino acid side chain.



- Quaternary proteins: are the result of the joining of several tertiary proteins.



6. Enzyme purification

The level of purification of an enzyme depends on its intended use.

Enzymes used in industrial applications, such as the food industry, are obtained in large quantities and purity is considered secondary when compared to costs.

For therapeutic applications, high purity is essential, obtaining small quantities.

6.1. Chromatography:

It is defined as a differential separation of the components of a sample between a mobile phase and a stationary phase.

The stationary phase is formed by spherical particles of an insoluble material that is placed ("packed") in a column.

The enzyme mixture to be separated is introduced through the mobile phase and forced to migrate through the column.

Enzymes that have greater interaction through the stationary phase will migrate differently (fix themselves or move more slowly) from those that have greater affinity for the mobile phase.

Stationary phase – matrix or resin that can be modified by linking chemical groups to give it certain physicochemical characteristics suitable for each process.

Most common matrices – cellulose, dextran, agarose, polyacrylamide and polystyrene and must have high chemical, mechanical and biological stability.

6.1.1. Ion exchange chromatography

This technique involves adsorption to the resin's charged groups, followed by its elution with fractionation.

Enzymes – carry ionized groups on their surface due to aa residues.

Positive charges – due to histidine, lysine and arginine residues.

Negative charges - are due to aspartic and glutamic acids and the C-terminal carboxyl group.

The charge balance depends on the relative amounts of these charged groups, varying with pH; when these groups are present in equal numbers, we have the ip (isoelectric point). Above ip the enzyme has a negative charge and below it a positive charge.

Therefore, there are matrices loaded with positive groups like DEAE (diethylaminoethyl), called anionics or anion exchangers, and with negative groups, like CM (carboxymethyl), called cations or cation exchangers.

In selecting the appropriate resin for enzyme purification, the stability pH of that enzyme must be considered to determine the working range.

If the enzyme is more stable above pI, an anionic resin should be chosen, and if the stability range is below pI, a cationic resin is chosen.

The mobile phase must always be buffered in order to minimize pH fluctuations, which could affect the interactions between the mobile and stationary phases.

6.1.2. Size-based purification

Basic principle – it is a partition of molecules between solvent and a stationary phase of defined porosity; is a form of partition chromatography for separating molecules of different sizes and has various denominations such as gel filtration and gel exclusion chromatography.

The separation process is carried out using a matrix with controlled porosity, packed in a column and surrounded by the mobile phase.

When a sample consisting of a mixture of molecules of different sizes is applied, the smaller molecules will penetrate the pores of the matrix and, as a result, will have a slower movement through the column, with the components being eluted at the end of the separation process.

Larger molecules pass through the column together with the mobile phase and are eluted first, while those of intermediate size can enter the gel and are eluted in direct order in relation to size, with those of smaller MW leaving in a smaller elution volume.

The volume in which the sample is eluted is called the elution volume (V_e); and the volume in which particles larger than the pore are eluted, that is, the mobile phase volume, is called the exclusion volume or void volume (V_0).

The column bed volume is called the total volume (V_t). For a given particle, the elution conditions within a given gel must be constant. In this way, you can calculate a coefficient called the partition coefficient (K_{av}), which is given by the following formula:

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}$$

most of the supports used are made of polymers such as dextran, agarose and polyacrylamide.

The choice of gel will depend on the purpose to be achieved (when you want to increase gel resolution, you choose smaller particles).

If the objective is to separate large molecules, such as enzymes, from small molecules, such as salts or other solutes weighing less than 3000 Da, gels with small pores are used. In this case, enzymes are excluded from the pores and are eluted at V₀. Suitable gels for this purpose are Sephadex G – 25 or G – 50 (Pharmacia) and bio gel P – 6 or P – 10 (Bio – Rad).

Equipment for performing a gel filtration includes: a suitable column, a UV detector, a fraction collector and a means of properly controlling the flow (peristaltic pump). Choosing long columns increases resolution but increases separation time and dilution.