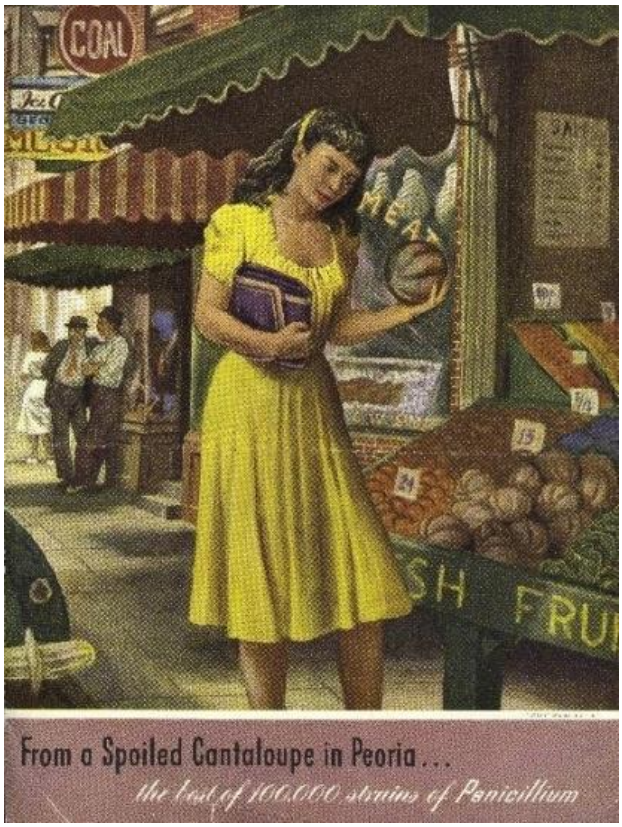
# Microbial production of antibiotics

## *Dad: the teacher told me that the greenish-yellow fluff that sometimes appears in melons save lives*





**José Luis García**

Centro de Investigaciones Biológicas Margarita Salas (CIB). Spanish National Research Council (CSIC). Madrid. Spain

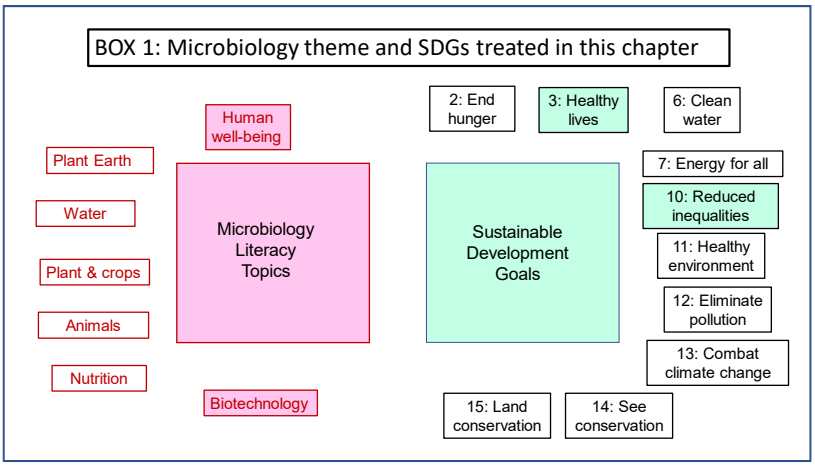
# Antibiotics

## Storyline

In June 1943, when researchers were trying to produce penicillin to treat the wounded of the second world war, Mary Hunt, a laboratory assistant at the National Center for Agricultural Utilization Research (NCAUR) (at that time called the Northern Lab. Northern Regional Research Laboratory (NRRL)) of Peoria (Illinois, USA), found a cantaloupe melon in a local market covered by a yellowish mould and brought it to the laboratory. This mould turned out to be a highly productive strain of the fungus *Penicillium chrysogeum* (NRRL 1951) and this discovery marked a turning point in mass production of penicillin. This strain produced 200 times more penicillin than that of the strain of *Penicillium notatum* that Alexander Fleming had discovered several years earlier, and 1,000 times more after being improved by mutations induced by ionizing radiation. Originally, penicillin was produced in surface cultures, but currently used submerged cultures soon became the method of choice. The history of the large-scale production of penicillin is in fact not only the starting point of the golden era of antibiotic therapy, but also a milestone of modern industrial biotechnology. It was also a case study of how to manage cooperative efforts among academia and industrial laboratories. After that, numerous studies were launched to find antibiotic substances and in a few years many antibiotics, produced mainly by a group of filamentous bacteria called the actinomycetes, were discovered and marketed. Today, the commercial development of a new antibiotic is a long and costly procedure, which unfortunately means that the development of new antibiotics is not among the main goals of large pharmaceutical companies. Modern experimental technologies and informatic tools are opening a new framework to facilitate the screening, discovery and production of new antibiotics faster and at lower costs. Learning how antibiotics are currently produced on an industrial scale is the topic of this chapter.

## The Microbiology and Societal Context

*Microbiology*: discovery of compounds with antibiotic activity; secondary metabolites; isolation of antibiotic-producing microbes; diversity of antibiotic-producing fungi and bacteria; beta-lactam antibiotics; large-scale production of antibiotics through fermentation or chemical synthesis; improved production of antibiotics through genetic modification of microbes; downstream processes for antibiotic purification; antibiotic resistance; improved activity of first-line antibiotic compounds through semi-synthesis to combat multiresistant pathogens; biocatalysis. And, *peripherally for completeness of the storyline*: the antibiotic business; low private investment in discovering new antibiotics; social inequalities in access to antibiotics. *Sustainability issues*: health; reduction of inequalities; economy and employment.




## Production of Antibiotics: The Microbiology

1. ***The importance of antibiotic use and misuse.*** Nowadays, it is not possible to imagine a world without antibiotics where any simple wound or a slight infection can endanger our lives. People in rich countries have not grown up with the idea that a simple infection could turn into something serious. If you have **pharyngitis** or streptococcal **pneumonia**, you can take oral antibiotics to solve the problem. If you have a skin infection, you may receive an antibiotic cream to rub in. Before the era of antibiotics, even simple surgery often led to fatal sepsis. Curiously, today the largest production of antibiotics is not made to fight infections in humans, but for consumption by farms that use them in the food of livestock to promote growth and reduce animal infections. The misuse and high consumption of antibiotics are promoting the appearance of multi-resistant bacteria, which are increasingly difficult to treat. For this reason, apart from favouring a more sustainable and rational use of existing antibiotics, we desperately need to discover and produce new antibiotics, if we do not want to return to a situation resembling the pre-antibiotic era.

However, since the 1990s, the pharmaceutical industry has not been very enthusiastic about the discovery of new antibiotics. Unlike medications for chronic diseases, people take antibiotics for a few days rather than for their entire lives, and that means lower revenues and return on the very considerable research and development investments. Modern medicine would become impossible if we lost our ability to treat infections. In poorer countries where the best antibiotics are not available, doctors are often forced to use less effective anti-infection medications, or to delay treatments, leading to an increase in antibiotic-resistant bacteria. Therefore, it is necessary that the price of modern more effective antibiotics ceases to be a problem for these less wealthy countries.

On the other hand, it is worth to mention that after the Second World War and during the second half of the 20th century, the production of antibiotics was considered of high strategic value by governments. States were very concerned with maintaining their own ability to produce these drugs. However, nowadays the production of antibiotics by fermentation is essentially displaced to Asia, because their production systems are cheaper. There are hardly any facilities in Europe and America capable of producing antibiotics on a large scale. This concentration of antibiotic raw material production in a few countries could in principle jeopardize the supply of these substances on a global scale should supply chain disruptions occur due, for example, to trade wars or unforeseen events such as pandemics.

2. ***Antibiotic production.*** Antibiotics can be produced at industrial scales mainly in three different ways, namely, naturally, by semi-synthesis or by chemical synthesis.

Natural antibiotics that are mainly biosynthesized by bacteria or fungi are usually produced by fermentation. A fermentation is simply growth of a microbial culture in a

liquid medium containing nutrients, starting with a small population and ultimately reaching a much higher one, during which a desired product is made (this can be the cells themselves, a large molecule like a protein, a small molecule like an antibiotic, or even a gas like methane, that can then be harvested. In the case of antibiotic fermentations, the microorganism is usually grown in submerged cultures using large **bioreactors**, some of them larger than 100,000 litters in capacity, containing a liquid growth medium (see below). During fermentation many operational parameters are continuously monitored and automatically adjusted, such as oxygen concentration, temperature, pH, nutrient levels, etc., to keep them optimal for microbial growth and production. Considering that most antibiotics are **secondary metabolites**, i.e., many of them are produced in the late or stationary phases of microbial growth, and thus, the cellular population size must be controlled very carefully to ensure that maximum yield is obtained before the cells die when nutrients run out. At the end of the fermentation process, the antibiotic must be extracted from the culture and purified. This so-called **downstream** processing may require the use of organic solvents, chemical precipitations and different chromatographic procedures to achieve a pure antibiotic product.

Many antibiotics are currently produced by semi-synthesis (see below). The production by semi-synthesis consists of combining a fermentation with a subsequent chemical modification of the molecule to maximize its properties. The chemical modification of the native molecule obtained by fermentation can be done to improve its potency or to change its spectrum of action, but also to increase its stability or solubility, to improve its pharmacokinetic and pharmacodynamics parameters or its way of being administered. Sometimes the semi-synthesis includes a **biocatalytic** step, i.e., the synthesis requires the use of enzymes. Semi-synthesized antibiotics also requires further downstream purification and control processes.

Some antibiotics can be also obtained by a complete chemical synthesis. However, considering that many natural antibiotics are very complex, chemical synthesis is only used for less complex molecules. It is important to note that some antibiotics are discovered by screening chemical libraries of compounds, this is, by screening thousands of previously chemically synthesized molecules that were originally prepared to be used by the pharmaceutical companies for different therapeutic uses. Once a successful molecule is found as a **lead compound** for a new antibiotic, it can be chemically modified to improve its pharmacological properties.

3. ***Antibiotic producer organisms.*** Antibiotics are products widely distributed in all type of habitats on the planet, where they play important roles in regulating the microbial diversity and populations in the environment. Thus, the majority of the lead compounds of new antibiotics have been discovered by screening natural producer organisms isolated from many different habitats. Only a few antibiotics have been selected from chemical libraries. Although many different microbes have the ability to produce antibiotics and, contrary to the widespread belief that most antibiotics are produced primarily by fungi, it is surprising that more than 50% of the antibiotics discovered in the golden age of antibiotic discovery (1940-1980) were produced by bacteria belonging to the genus *Streptomyces*. More than 5,000 antibiotic compounds were discovered. Unfortunately, only a few of these have been sufficiently non-toxic and efficient to be of use in clinical practice. Some examples of well-known antibiotics and their producer strains are: penicillin (*Penicillium chrysogenum*); cephalosporin (*Acremonium chrysogenum*); erythromycin (*Streptomyces erythreus*, *Saccharopolyspora erythraea*); streptomycin (*Streptomyces griseus*); chlortetracycline (*Streptomyces aureofaciens*); vancomycin (*Streptomyces orientalis*, *Amycolatopsis orientalis*); gentamicin (*Micromonospora purpurea*); polymyxin (*Paenibacillus polymyxa*); kanamycin (*Streptomyces kanamyceticus*); chloramphenicol (*Streptomyces venezuelae*);

neomycin (*Streptomyces fradiae*); bacitracin (*Bacillus licheniformis*); and mupirocin (*Pseudomonas fluorescens*).

It is not infrequent that the same antibiotic is found in different species. For instance, this is the case of clavulanic acid and cephamycin that are produced by *S. clavuligerus*, *S. jumonjinensis or S. katsurahamanus.* Moreover, a single strain can produce more than one useful antibiotic. This is one of the reasons that support the importance of a good **dereplication** system during the screening for new antibiotics (see below). The mixture of several antibiotics in the same microbial extract might introduce some confusion during the isolation process.

4. ***Brief description of a conventional antibiotic production microbial fermentation process.*** Many antibiotics are produced by submerged fermentation of microbes. Before fermentation can start, the desired antibiotic-producing microbe must be isolated and kept safe by cold storage in a -80ºC freezer, either freeze-dried **(lyophilized)** or suspended in a liquid containing glycerol, which protects it from freezing damage. Then, we need to create in the lab a starter culture using the cold stored sample of the isolated microbe. To begin this culture, firstly a sample of the microorganism is usually transferred to an agar plate containing the appropriate solid growth medium where the strain is cultured for some days. In this way we can observe the morphology of the colonies and confirm that the culture is not contaminated. Thereafter, the strain is transferred from the solid medium to a liquid medium in a shake flask, creating a growing suspension of the strain, that is further transferred to seed bioreactor tanks to scale up the culture. The seed bioreactors are glass (< 5 L volume) or steel (> 5 L volume) tanks designed to provide an ideal environment for growing microorganisms at medium scale. These tanks are filled with all the nutrients the specific microorganism needs to survive and thrive, including sources of carbon, nitrogen, phosphate, sulphur, or metals, together with growth factors like vitamins, amino acids, and other minor nutrients that may be required by the cells. The seed tanks are equipped with mixers, which keep the growth medium moving, and a pump to deliver sterilized, filtered air. Different sensors/**probes** and pumps control the maintenance of important parameters such as pH, oxygen consumption, etc. The general process is monitored and controlled by computers with sophisticated software. After about 24-48 hours, the microbial cell content – the biomass - of the seed tank is transferred to a large fermentation tank.

The large fermentation tank is essentially a larger version of the steel seed tank, which is able to hold thousands of litters of growth medium, even larger than 100,000 L volume. It is filled with the fermentation broth that contains the primary raw materials required for the production process. In submerged fermentations, this broth is an aqueous solution made up of all of the ingredients necessary for the proliferation of the microorganisms as well as for the antibiotic production. Typically, a production broth for large scale processes contains cheap sources of nitrogen as ammonia salts, phosphorous, and cheap sources of carbon like **molasses**, **corn steep liquor**, or **soybean meal**, sometimes together with other additional sugars (e.g., lactose, glucose). Trace elements needed for the proper growth of the antibiotic-producing microbe are included as salts such as phosphorus, sulphur, magnesium, zinc, iron, copper, and others. During this process, the strain should excrete large quantities of the desired antibiotic, mainly during the late or the stationary phases of growth. As in the seed bioreactors, the fermentation tanks are cooled or heated to keep the correct temperature, are constantly agitated, and a continuous stream of sterilized air is pumped into it to provide the required oxygen. Most of these fermentations are performed under aerobic conditions. To prevent foaming during fermentation, anti-foaming agents such as **lard oil**, octadecanol, and silicones can be periodically added. Since pH control is vital for optimal growth, acids or bases are also periodically added to the tank as necessary.

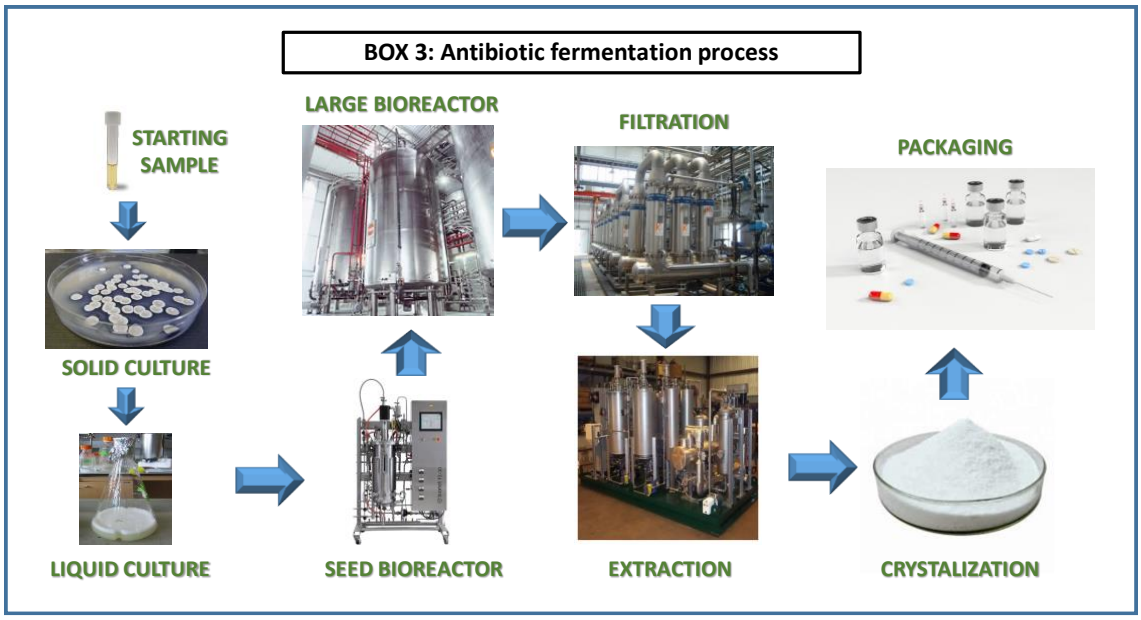
We should at this point make an aside to learn that in spite of the fact that anaerobic bacteria are the oldest terrestrial creatures and occur ubiquitously in soil and in the intestine of higher organisms, playing a major role in human health, ecology, and industry, and although some research had shown that anaerobic bacteria produce antibiotics, it was not until very recently that the first antibiotic from the anaerobic world, a new class antibiotic named closthioamide, effective against gonorrhoea, has been isolated in 2010 from the cellulose degrading anaerobic bacterium *Clostridium cellulolyticum*. Nevertheless, it is not still in the market.

After several days of fermentation, when the maximum amount of antibiotic has been produced, the fermentation is stopped and the isolation (downstream) process of the produced antibiotic starts. Generally, the antibiotic present in the culture broth is first separated from the insoluble materials in the broth by centrifugation or filtration, and then the cleaned culture broth is processed depending on the compound's properties using well-known chemical procedures such as solvent extraction, chromatography, and crystallization. At the end of the process, a purified powder form of the antibiotic is usually obtained, which can then be formulated into different types of products. The entire production process of fermentation, recovery, and processing can take at least one week.

Quality control is of utmost importance in the production of antibiotics since we have to guarantee that contaminations are not introduced at any step during the production process. The culture media and all of the equipment have to be thoroughly sterilized. During manufacturing, the quality of all compounds is checked on a regular basis. Of particular importance are frequent checks of the microorganisms during fermentation. The quality controls are accomplished by using conventional microbiological and chemical analytic techniques. The main chemical properties of the finished antibiotic and the absence of undesired contaminants and microorganisms have to be checked. Only after the quality of each batch of produced antibiotic is certified, the antibiotic can be sold for consumption. Antibiotic production is normally regulated by Health Administrations which supervise that the rules are enforced.

From this step, the antibiotic product is transported to the final packaging or transformation companies, sometimes different from the fermentation company. Here, the products can be used as the starting material for the semi-synthesis of a new antibiotic (see below) or it can be directly formulated, this is, the antibiotics are mixed with the corresponding pharmaceutical **excipients** and put in boxes or in vials that are stored at room temperature or in different cold conditions. All these processes require additional quality controls.

Finally, the boxes or vials containing the antibiotics are further loaded up on trucks and transported to national or international distributors, hospitals, and pharmacies.




5. ***Semisynthesis of antibiotics.*** For decades, chemists have replenished the arsenal of antibiotics by semi-synthesis. Semi-synthesis, this is, a partial chemical synthesis of a molecule, is a chemical process that uses as starting material a compound previously isolated from a natural source, such as an antibiotic obtained from a microbial cell culture, to produce a new antibiotic with different properties. The new products generally acquire a molecular structure more complex or with some additional functional chemical groups or bonds than could not be easily acquired naturally. Semi-synthesis is used instead of complete synthesis when the final molecules are very complex and their synthesis will require many steps. Thus, semi-synthesis is the selected way of preparing complex compounds more cheaply than by total synthesis since fewer chemical steps are necessary to achieve the final product.

In some cases, the compound can be obtained both from a natural source or semi-synthesis. This is the case of tetracycline that can be obtained directly from *Steptomyces viridifaciens*, but that it was initially obtained by semi-synthesis in 1953 at Lederle Laboratories by a catalytic dehalogenation of chlortetracycline produced by *S. aureofaciens*, as well as being independently derived from oxytetracycline produced by *Streptomyces rimosus* at Pfizer Laboratories. Some natural tetracyclines used as antibiotics are chlortetracycline, oxytetracycline, tetracycline and demeclocycline, whereas the most used semisynthetic derivatives are methacycline, doxycycline, meclocycline, minocycline, lymecycline, rolitetracycline or tigecycline, among others. Doxycycline is a member of the second-generation of semisynthetic tetracyclines and is currently the most commonly used tetracycline, being considered as an essential drug by WHO. Tigecycline is an example of a third-generation of tetracyclines which has a broader spectrum of activity and is used for the treatment of complicated infections by multiresistant organisms. It is important to notice that there are other natural tetracyclines that are not used as antibiotics, like doxorubicin employed as antitumor drug.

A lead compound in drug discovery is a chemical compound that has pharmacological activity and could be therapeutically useful, but that still has suboptimal functional or clinical properties. The chemical structure of a lead compound serves as a starting point for chemical modifications to improve its potency, selectivity, or pharmacokinetic parameters. When we talk about generations of antibiotics, we call the natural product that acts as a lead compound the first generation of an antibiotic family. Thereafter, the following antibiotic generations are represented by structural chemical modifications that add or improve some properties of the natural molecules, for instance

that increase the solubility, or extend or reduce the bacterial spectrum of activity. Most of the new generations of antibiotics are produced by semi-synthesis, but others are produced by complete chemical synthesis depending of their complexity.

A special case of semi-synthesis of antibiotics at the industrial scale is the use of biocatalysis for the production of semisynthetic beta-lactam antibiotics (see below). Different hydrolytic enzymes such as amidases/esterases/acylases (e.g., penicillin G or V acylases, glutarylacylases), combined in some cases with D-amino acid oxidases have been used to produce the beta-lactam nucleus that are further used for the semisynthesis of the different generations of beta-lactams. The use at industrial scale of immobilized penicillin acylases obtained by recombinant technologies in the 80s to produce semisynthetic antibiotics represents one of the milestones of modern biotechnology. Moreover, some semisynthetic beta-lactam antibiotics can be also produced by enzymatic acylation using hydrolytic acylases in a reverse way by biocatalysis engineering.

**BOX 4: The case of beta-lactam antibiotics**

About half of all commercially available antibiotics in use are beta-lactam compounds. Beta-lactam antibiotics constitute a large family of molecules that contain a beta-lactam ring in their structure. This family includes penicillins (penams), cephalosporins (cephems), monobactams, carbapenems and carbacephems. The first beta-lactam antibiotic, penicillin, was discovered by Alexander Fleming in 1928. Penicillin is currently produced at industrial scale as penicillin G or penicillin V from the fermentation of fungus *Penicillium chrysogenum*. Cephalosporin C was discovered later by Giuseppe Brotzu in 1945. It is produced from the fermentation of the fungus *Acremonium crysogenum* (formerly *Cephalosporium*). However, most of the currently known beta-lactam compounds are produced by bacteria of the genus *Streptomyces*. These include clavulanic acid and cephamycin (*S. clavuligerus*, *S. jumonjinensis*, *S. katsurahamanus*, *S. griseus*, and *S. lactamdurans*), and thienamycin, one of the most potent natural antibiotics known to date, discovered in S. cattleya in 1976. But besides Streptomyces, there are other producing bacteria, such as *Nocardia uniformis*, which produces nocardicins, and *Pseudomonas acidophil*, which produces sulfazecine, among others.

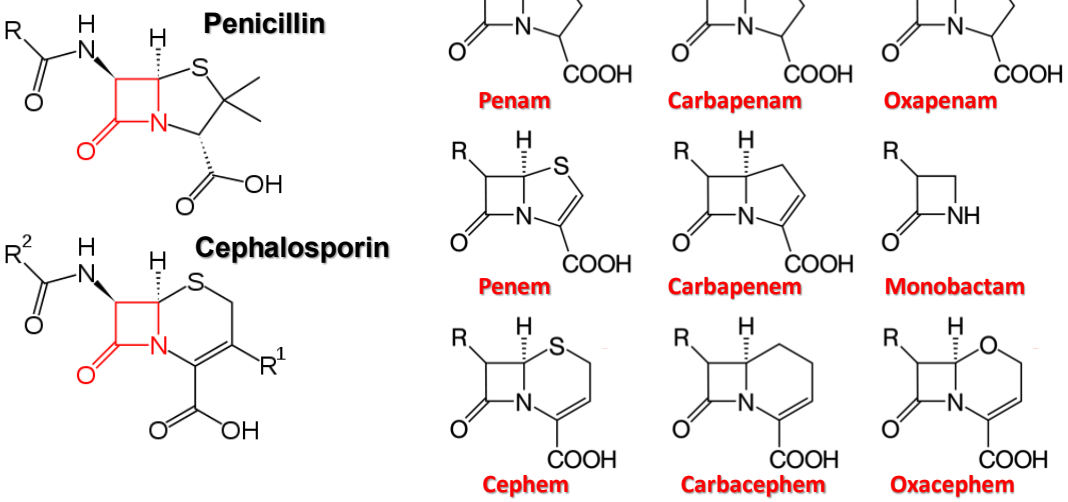
The biosynthesis of beta-lactam compounds is carried out by several specific enzymes. Perhaps the best known is isopenicillin N synthase, responsible for the formation of the beta-lactam ring in all penicillin/cephalosporin compounds, but different enzymes are used for the synthesis of the other beta-lactam compounds.

Bacteria and fungi that produce beta-lactam antibiotics have undergone various genetic improvement processes to increase fermentation yields. These processes include mutagenesis combined with high-throughput selection processes, as well as customized genetic engineering modifications. In some cases, production has been improved by increasing the copy number of biosynthetic genes in the genomes. To give an idea, fermentations of these antibiotics currently produce at least 50 g/L of penicillin and 25 g/L of cephalosporin C, but it is possible to produce even twice as much, as the real production yield is a secret firmly kept by the producing companies.

Although some of these natural beta-lactam molecules are used in clinic without further chemical modifications (e.g., clavulanic acid), the semisynthetic beta-lactam antibiotics are the most prescribed class of antibiotics in the world. Chemical coupling of a beta-lactam moiety with an acyl side chain has dominated the industrial production of semisynthetic beta-lactam antibiotics since their discovery. Chemical and enzymatic hydrolysis processes for releasing the beta-lactam nucleus of 6-aminopenicillanic (6-APA), 7-aminodesacetoxycephalosporanic (7-ADCA) or 7-aminocephalosporanic (7-ACA) acids are highly efficient with biocatalytic technologies leading the field. Penicillin G acylase that hydrolyses penicillin G has been used for decades to release the 6-APA nucleus required for the production of semisynthetic penicilllins (e.g., ampicillin, amoxicillin).

Today we recognize at least five different generations of semisynthetic cephalosporins with more than 70 different antibiotics containing the cephalosporin structure, although only about 50 have been commercialized. Some of these molecules were synthesized to increase the activity and extend the activity spectrum against different bacteria. Others have been created to avoid the activity of beta-lactamases which are enzymes that hydrolyse the beta-lactam rings and confer antibiotic resistance to many bacteria. The last generation of cephalosporins is able to fight methicillin-resistant *Staphylococcus aureus* (MRSA).
In some cases, the modifications have been done to facilitate the oral administration of the antibiotic. This is the typical case of amoxicillin, a derivative of penicillin, that alone or in combination with clavulanic acid (a beta-lactamase inhibitor) is one of the most frequently used oral antibiotics in the world.

**BOX 5: Molecular structures of beta-lactam antibiotics**




6. ***Main steps for developing a new lead antibiotic.*** The development of a new antibiotic involves several steps. The process begins with an *in vitro* screening research aimed to identify compounds that can kill or inhibit the growth of one or more bacteria used as targets. In some cases, these compounds have a chemical origin (chemical libraries), but in most cases they are produced by microorganisms which synthetize antibiotics as metabolites. In this last case, during this phase, thousands of microbial species have to be screened to find an antibacterial action on the microbes selected as target. The putative antibiotic producers must be firstly isolated and thereafter cultured in different media and conditions in order to force them to produce the antibiotics. This is not a simple task, since, as mentioned above, antibiotics are usually produced as secondary metabolites that are only secreted to the culture medium under very strict growth conditions. Moreover, this classical approach of accessing microbial diversity and screening for antibiotic substances tends to recover compounds that have already been found, so the number of new strains needed to be screened per new compound found increases rapidly over time: the 'Law of diminishing returns'. In order to increase the efficiency of antibiotic discovery, new approaches are needed.

The biosynthetic pathways of antibiotics are often 'silent' – the antibiotic is not made – under the lab culturing conditions used, so it can be helpful to stress the microorganisms to force them to produce these secondary metabolites that are usually synthesized as weapons against competitors in the natural habitat. Activating the expression of the silent gene clusters might allow the discovery of novel molecules. To this aim, the

use of metabolic engineering methods assisted by modern techniques of massive gene and protein analysis together with the use of new cultivation techniques have been developed to stimulate the expression of these clusters. The principles behind the cultivation-based techniques have been conceptualized in the "one strain many compounds" (OSMAC) framework, which assumes that a single strain can produce different molecules when grown under different conditions (e.g., nutrient content, temperature, aeration, co-cultivation, chemical elicitors).

Moreover, the secondary metabolites can be initially produced at very low concentrations and, because of that, it is necessary to concentrate the culture extracts as well as to use highly sensitive target cells. In some cases, it is possible to use very sensitive enzymatic assays when the molecular target is a known enzyme.

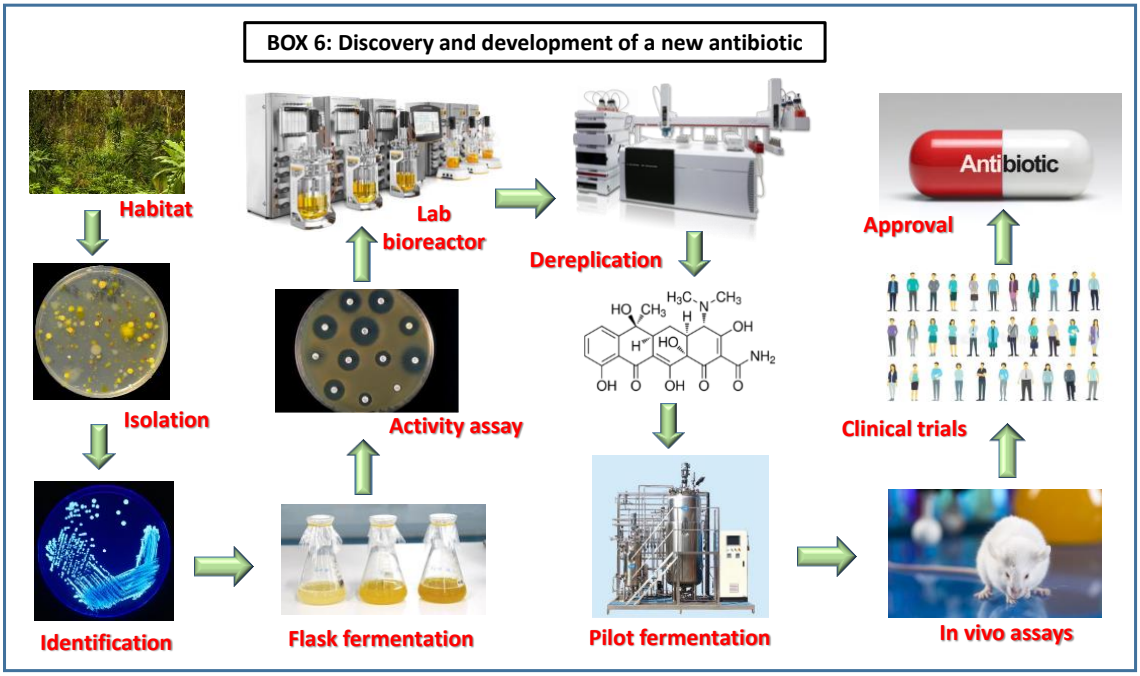
On the other hand, we know that very few microorganisms contained in the microbiome of a particular habitat can be cultured and thus, they cannot be isolated and assayed by conventional methods. Therefore, considerable effort is being invested in the development of new growth media and new strategies to culture difficult-to-culture microorganisms using innovative devices that try to mimic the same environmental conditions where microorganisms live.

When an active compound is detected, it is tested against a battery of known infectious bacteria. If the results are promising, the newly isolated microorganism is cultured at medium scale to facilitate the isolation and characterization of the chemical antibiotic structure. This process is called “dereplication”. Dereplication is an essential step in natural product discovery to prevent re-isolation and re-characterization of already well-known bioactive compounds. Dereplication is used for the rapid identification of the major active compounds, whatever their chemical class, in a single sample or for the acceleration of bioactivity-guided fractionation procedures. A conventional dereplication method consists in an analytical separation of the active compounds by HPLC followed by a structural characterization using mass spectrometry and NMR (Nuclear Magnetic Resonance). However, many other procedures can be used for this objective including platforms of bacteria resistant to known antibiotics. In general, this is a very time-consuming and laborious step because thousands of compounds have to be assayed and structurally characterized. Unfortunately, in many cases one discovers at the end of the process that the supposed new antibiotic is already known. If the new compound passes this phase, it is normally patented and submitted to further *in vivo* tests. These tests typically involve the conventional phases of clinical trials to prove that the antibiotic works in animals and humans, is not harmful and is very efficient to treat a disease. To carry out these clinical trials is necessary to scale up the production process even if the product could not be approved. To develop the clinical trials is very expensive and time consuming. Finally, if all the clinical phases are successfully accomplished, the Health Administrations must approve the antibiotic for commercial use. The entire process can take many years.

In principle, the new techniques for massive sequencing of metagenomes and bacterial genomes open the door to find new biosynthetic gene clusters encoding pathways of putative new antibiotics that are not expressed in the isolated microorganism under laboratory conditions or, even more, that cannot be expressed because it is impossible to culture the microorganism. Using bioinformatics tools, it is now possible to at least partially predict the structure of the compound that putatively could be synthesized by this pathway. In theory, these new gene clusters can be chemically synthesized and cloned to be expressed in an appropriate heterologous host where the new product can be synthesized and assayed. This approach although possible is expensive and time consuming, and at this moment the success rate for producing a new antibiotic through this way is very low.

A new way to discover new antibiotics is based in computational tools and artificial intelligence. A trained deep neural network can predict antibiotic activity in molecules that are structurally different from known antibiotics. On the other hand, by 3D modelling of

a bacterial protein that can be selected as a target, it is possible to test *in silico* the binding or interaction capacities of many different molecules that theoretically can block its activity. In this way, it could be possible to find molecules that can now act as antibiotics, but that are in use or that have been used for other clinical purposes. This process is called **drug repositioning** or **repurposing.**




### *7. Improvements of the antibiotic producer strains. Classical and modern tools.*

Microorganisms used in industrial scale fermentations are rarely identical to the original strain isolated during the screening steps. This is because species have been routinely genetically modified to yield the maximum amounts of antibiotics per litre of culture and in the shortest fermentation time. Random mutagenesis protocols using ultraviolet radiation, x-rays or certain chemicals are often employed to modify the strains. Potent high throughput selection systems based in the use of robots are used to isolate the best producer mutants. Further cultivation and evolution of the higher yielding strains over many generations can raise yields even more. These methods of random mutagenesis and selection of the best producers require time and labour, and although they may give some improvements, significant increases in yield do not always occur in the short-term. The major advantage of these methods is that you do not need to know much about the producing organism. The organism works as a kind of black box that responds better or worse to our inputs. Man power and serendipity are important to achieve good results.

However, there are other ways to improve the producer strains. Due to the advances in molecular biology and genetic engineering that occurred in the 1980s, antibiotic production companies have been introducing new improvement procedures based on these modern techniques. In this sense, a typical technique used to increase yields is gene amplification, where many copies of the genes coding for the enzymes involved in the antibiotic production pathway can be inserted back into the genome of the original strain by using different recombination procedures. Moreover, the synthetic genes can be modified by site directed mutagenesis to generate more efficient enzymes. The expression of these genes can be also modified not only in order to increase their transcription levels, but also to control the growth phase for optimal transcription to make the production of the antibiotic constitutive or regulated. The biggest problem that exists to apply these techniques is that the antibiotic producing strains that are natural isolates might not be

manipulated by genetic engineering easily, and we have to spend time to develop efficient genetic tools to modify them.

The genetic engineering manipulations not only allow to increase the antibiotic yield, but they can also be used to produce new compounds. **Combinatorial biosynthesis,** i.e., combining biosynthetic genes from different antibiotic clusters and pathways, and **mutasynthesis**, i.e., blocking the biosynthesis of a precursor and feeding analogous or synthetic precursors, are strategies that allow the production of novel analogues of antibiotics with modified functionalities that otherwise could be very difficult to obtain by chemical synthesis.

On the other hand, as mentioned above, it may also be possible, although not without difficulty, to transfer the antibiotic the genetic cluster responsible for the antibiotic biosynthesis from the producer organism to another heterologous host that can be more easily manipulated and cultivable. In fact, today, the gene cluster can be chemically synthesized de novo with the most appropriate codon usage of the host.

During the first decade of the 21st century, several new analytical procedures have been developed around the so-called omics technologies (genomics, transcriptomic, metabolomics, proteomics, fluxomics). The combination of these technologies with the possibility to create metabolic models at genomic scale using systems biology tools allows to develop rational approaches to modify the production process from a holistic point of view. Using these models is possible to determine the metabolic bottlenecks and thefafter, to eliminate them by using metabolic engineering techniques.

The combination of all these technological approaches can facilitate a faster development of the production processes once the new antibiotic has been found though the screening procedures. However, considering that the currently used antibiotics have been described many years ago, only few attempts are now devoted to improve the producer strains since most of the processes are now well consolidated.

## Relevance for Sustainable Development Goals and Grand Challenges

- **Goal 3. Ensure healthy lives and promote well-being for all at all ages.** Antibiotic misuse, combined with an overuse in the livestock sector, is accelerating the emergence of antibiotic multi-resistant bacteria. Thus, natural microbial selection, assisted by global misuse of existing antibiotics, and the slowing pace of discovery of new antibiotics conspire to place society at or near a crisis point. Our current arsenal of antibiotics is steadily losing its efficacy and there is little sign that it will be adequately replenished in the near future. Global efforts have to be made to invest more R&D funds to discover and produce new antibiotics.
- **Goal 10. Reduce inequality within and among countries.** It is clear that not all people on the planet who suffer from infectious bacterial diseases have the same access to antibiotics as have the patients from the richest countries. Especially the most modern and expensive antibiotics that protect against multi-resistant bacteria are the ones that do not reach these patients. This problem not only generates a higher mortality in these countries but, in addition, in many cases also increases bacterial resistance, because these patients have to use less effective antibiotics. It is necessary to implement political measures and economic aids on a global scale, so that the poorest countries can have access to these antibiotics and thus, reduce these inequalities that harm the health of the entire planet.

## Potential Implications for Decisions

### *1. Individual*

It is important to be aware that antibiotics are drugs of high value to maintain our health and therefore, we have to contribute to develop citizen platforms to make campaigns to

request the pharmaceutical companies and the administrations for the investment in R&D so that new antibiotics can be discovered. These platforms could develop crowdfunding programs so that academia could conduct research for the discovery of new antibiotics.

### *2. Community policies*

Concerning the need for new antibiotics, at the community level, workshops and campaigns would be held in schools, neighbourhoods, and associations with health professionals to explain what antibiotics are, how to use them properly, and the need to invest in science to discover new products to combat the resistance that is emerging in pathogens.

Concerning the reduction of inequality within and among countries, it is important to carry out social campaigns to raise awareness among pharmaceutical companies that they should include in their corporate social responsibility plans the possibility of making a portion of their antibiotic production available to poor communities, either free or at low prices, so that patients in poorer countries can access them. Likewise, rich communities and organizations that practice social patronage could purchase these antibiotics to provide them free of charge wherever needed. However, for these types of actions to be truly effective, these antibiotics should be supplied through international non-profit medical organizations as intermediaries, thus preventing not only their misuse but also their diversion and resale in illegal markets.

### *3. National policies related to production of antibiotics*

Pharmaceutical companies could be incentivised through tax breaks or direct aid to change their business approach to the profitability of antibiotic production. Administrations need to convince them that some fraction of their profits should be invested in R&D activities to discover new antibiotics resulting in social benefits that do not necessarily generate high incomes, like is the case drugs for chronic or cancer diseases, where the continuous prescription or high prices of drugs rapidly compensate investments in R&D.

On the other hand, given that most large pharmaceutical companies have little interest in the search for new antibiotics, another option is that the national administrations or international organizations would create specific R&D plans and programs to finance R&D activities via academic-industry partnerships.

Moreover, considering the fact that antibiotics have a great strategic value to maintain the health of the population, nations should formulate and implement policies with contingency plans that allow them to be able to produce at least some of them in case of extreme need or shortage, and not leave their production in the hands of other countries.

## Pupil Participation

1. **Class discussion of the issues associated with production of antibiotics**
    a. Consider the list of antibiotic-producing microorganisms and try to find a reason that can explain why *Streptomyces* are such good antibiotic producers.

2. **Pupil stakeholder awareness**
    a. Make a list of the biggest pharmaceutical companies that sell antibiotics.
    b. Make a ranking of the countries that produce antibiotics by fermentation in greater quantities.
    c. Discuss why so few new antibiotics have been discovered in the current century.

3. **Exercises**

a. Discuss the differences between producing an antibiotic by fermentation in a flask or in a bioreactor.

b. Propose unconventional niches/habitats where you can search and find microorganisms that can produce new antibiotics. Explain the reason for your proposals.

c. Look up the timeline on the discovery of antibiotics of clinical use and draw some conclusions.

d. Discuss why so few antibiotics have been discovered in anaerobic bacteria so far.

## The evidence base, further reading and teaching aids

### SCIENTIFIC LITERATURE


- Brüssow H. (2024) The antibiotic resistance crisis and the development of new antibiotics. Microb Biotechnol. 17(7):e14510. https://enviromicro-journals.onlinelibrary.wiley.com/doi/10.1111/1751-7915.14510
- Farha MA, Tu MM, Brown ED. (2025) Important challenges to finding new leads for new antibiotics. Curr Opin Microbiol. 83:102562. https://www.sciencedirect.com/science/article/pii/S1369527424001383?utm_source=chatgpt.com
- Hug JJ, Bader CD, Remškar M, Cirnski K, Müller R. (2018) Concepts and methods to access novel antibiotics from actinomycetes. Antibiotics (Basel) 7(2):44. https://www.researchgate.net/publication/325316257_Concepts_and_Methods_to_Access_Novel_Antibiotics_from_Actinomycetes
- Lewis K (2020) The Science of Antibiotic Discovery. Cell. 181:29-45. https://www.cell.com/cell/fulltext/S0092-8674(20)30233-6
- Pham JV, Yilma MA, Feliz A, Majid MT, Maffetone N, Walker JR, Kim E, Cho HJ, Reynolds JM, Song MC, Park SR, Yoon YJ. (2019) A Review of the Microbial Production of Bioactive Natural Products and Biologics. Front Microbiol. 10:1404. https://www.frontiersin.org/articles/10.3389/fmicb.2019.01404/full
- Ribeiro da Cunha B, Fonseca LP, Calado CRC. (2019) Antibiotic Discovery: Where Have We Come from, Where Do We Go? Antibiotics (Basel) 8(2):45. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6627412/


### DIVULAGTION LITERATURE


- WELCOME. Why is it so hard to develop new antibiotics? https://wellcome.org/news/why-is-it-so-hard-develop-new-antibiotics?utm_source=chatgpt.com
- Pbslearningmedia. Producing Penicillin. https://www.pbslearningmedia.org/resource/odys08.sci.life.gen.producing/producing-penicillin/
- Pbslearningmedia. The discovery of penicillin. https://www.pbslearningmedia.org/resource/odys08.sci.life.gen.discovery/the-discovery-of-penicillin/
- BOC Sciences. Antibiotic Production via Fermentation - How Do Antibiotics Work? https://www.youtube.com/watch?v=foaqaQDm04M
- Jonh Innes Centre. Why do microbes produce antibiotics? https://www.jic.ac.uk/blog/why-do-microbes-produce-antibiotics/
- Letek M. Alexander Fleming, The Discoverer of the Antibiotic Effects of Penicillin. https://kids.frontiersin.org/articles/10.3389/frym.2019.00159

- Karwa S. et al. How Do Scientists Develop Antibiotics? https://kids.frontiersin.org/articles/10.3389/frym.2020.532921
- Takano E. Synthetic Biology: Production of Novel Antibiotics. https://www.youtube.com/watch?v=7Sk0TCo-qoQ

## Glossary

**Antibiotic** – A substance that kills or inhibits the growth of bacteria/fungi, used to treat bacterial/fungal infections.

**Biocatalytic/Biocatalysis** – The use of natural catalysts, such as enzymes or whole cells, to speed up chemical reactions.

**Bioreactors** – Controlled vessels or systems where biological processes (e.g., fermentation) are carried out to produce products like medicines, enzymes, or biofuels.

**Chiral molecules** – Molecules that exist in two mirror-image forms (like left and right hands), important in drug design since different forms can have different biological effects.

**Combinatorial biosynthesis** – A technique that combines genes or enzymes from different organisms to create new natural product derivatives.

**Corn steep liquor** – A nutrient-rich byproduct of corn wet-milling, used as a cheap growth medium in fermentation.

**Dereplication** – The process of quickly identifying known compounds in a sample to avoid re-isolating them in drug discovery.

**Downstream** – Refers to the purification, processing, and formulation steps that occur after the main production process (e.g., after fermentation).

**Drug repositioning/repurposing** – Finding new medical uses for existing drugs.

**Excipient** – An inactive substance used in drug formulations to aid delivery, stability, or taste (e.g., fillers, binders).

**Infection** – The invasion and multiplication of microorganisms (such as bacteria, viruses, or fungi) in the body, often causing illness.

**Lard oil** – A traditional nutrient source derived from animal fat, sometimes used in microbiological media.

**Lead compound** – A chemical with promising biological activity that serves as the starting point for drug development.

**Lyophilized** – Freeze-dried; a process that removes water from a product to preserve it.

**Molasses** – A thick byproduct of sugar refining, rich in nutrients, often used as a fermentation substrate.

**MRSA (Methicillin-Resistant *Staphylococcus aureus*)** – A type of bacteria resistant to many antibiotics, making infections difficult to treat.

**Multiresistant** – Refers to microorganisms that are resistant to multiple antibiotics or drugs.

**Mutasynthesis** – A method where mutant organisms are fed modified precursors to produce novel natural products.

**Omics** – A collective term for large-scale biological studies such as genomics (genes), proteomics (proteins), and metabolomics (metabolites).

**Pharyngitis** – Inflammation of the throat (pharynx), often causing a sore throat.

**Pneumonia** – Infection of the lungs, leading to symptoms such as cough, fever, and difficulty breathing.

**Probes/Sensors** – Tools used to detect, measure, or monitor specific biological or chemical processes.

**Secondary metabolites** – Molecules produced by organisms that are not essential for growth but often have ecological functions (e.g., antibiotics).

**Semi-synthesis** – A method of producing drugs by chemically modifying natural compounds.

**Soybean meal** – A byproduct of soybean oil extraction, rich in protein, often used as a fermentation medium.

**Systems biology** – An approach that studies interactions within biological systems (genes, proteins, pathways) to understand how they work together as a whole.