MEMORY

For the purpose of obtaining

PROFESSIONAL MASTER

in

Human Molecular Diagnostics

Presented and Supported by:

Alaa BZAL

October 10, 2022

Title

Production and Quantification of Ampicillin

Supervisor

Dr. Charbel Al-Bayssari

Readers

Dr. Bassel Ismail

Dr. Rim Al Safadi

CONTENTS

THANKS

I would like to begin by warmly thanking my memory director **Dr. Charbel Al-BAYSSARI,** professor-researcher and lecturer at the Lebanese University, for his welcome, patience, availability and above all his wise advice, which contributed to my reflection. Thank you for your time and encouragement. I express my deep gratitude to you and my great esteem.

I would also like to thank **Dr. Samar EL-HAMOUI**, who is in charge of this Master 2, for having ensured the smooth running of the theoretical part of my training and for having given all the attention to carry out this project.

I would like to express my sincere thanks to the members of the jury who agreed to evaluate my work, and who were kind enough to honor me with their presence.

In particular, I thank **Dr. Samir MOURAD**, Director of the MEGBI Institute Laboratory, for giving me the extraordinary opportunity to do my work. I would also like to thank the assistants of this laboratory for their help and guidance.

I would like to express my gratitude and respect to the teachers of the master's program "Molecular Diagnostics Applied to Man", I thank you for your quality teaching.

I warmly thank my dear parents for their trust and invaluable support. You are responsible for my success, I show you all my love. I also thank my sisters for their encouragement.

I would like to express my gratitude to my friends who have given me their moral support throughout my journey.

À all of these speakers, i offer my thanks, my respect and my gratitude.

ABSTRACT

Semi-synthetic LACTAMINES are one of the most important families of antibiotics on the world market. Among them, ampicillin is one of the most widely used in therapy, which has a wide spectrum of action in the prevention and treatment of various bacterial diseases. Currently, it is manufactured in bio-industry by a chemical route for the high yield obtained. The enzymatic synthesis of ampicillin is more environmentally friendly but also results in a much lower yield. It is mainly catalyzed by penicillin G acylase (PGA) and the 1,5-amino ester hydrolase (AEH) and can be carried out under thermodynamic control or kinetic control, knowing that the latter is more advantageous. Cascading conversions are increasingly being studied to make syntheses more environmentally friendly and economically advantageous. In this work, we carried out a cascade conversion with two biocatalytic reactions, following the synthesis method in one pot, two steps, in a fully aqueous medium to synthesize ampicillin. We have been able to produce ampicillin and quantify the ampicillin produced, but the purification and harvesting of this antibiotic have not been successful. Thus, our results obtained will be taken into account to improve the effectiveness of future trials of synthesis of this antibiotic.

Key words: [semi-synthetic elasticity-lactam antibiotics; ampicillin; bacterial diseases; enzyme synthesis; PGA; AEH; kinetic control; cascade conversion].

ABSTRACT

Semi-synthetic Lactam antibiotics are one of the most important antibiotic families in the world market. Among them, ampicillin is one of the most widely used in therapy, which exhibits a broad spectrum of action in preventing and treatment of various bacterial diseases. Currently, it is manufactured in bioindustry through a chemical route for high yield obtained. Enzymatic synthesis of ampicillin is much more environmentally benign but also results in a much lower yield. It is mainly catalyzed by penicillin G acylase (PGA) and 1-amino ester hydrolase (AEH), and can be carried out under thermodynamic control or kinetic control knowing that the last is more advantageous. Cascade conversions are increasingly studied to render syntheses more

environmentally benign and economically advantageous. In this work, a cascade conversion with two biocatalytic reactions according to the one pot, two steps method has been carried out in fully aqueous medium to synthesize ampicillin. We were able to produce ampicillin and quantify the ampicillin produced, however, purification and harvesting of this antibiotic were not successful. Thus, our results will be taken into account to improve the effectiveness of future synthesis trials of this antibiotic.

Key words: [Semi-synthetic elastomatic lactam antibiotics; ampicillin; bacterial diseases; enzymatic synthesis; PGA; AEH; kinetic control; cascade conversion].

CHAPTER 0: INTRODUCTION

Antibiotics are a group of drugs used in the treatment of various infections caused by bacteria and certain parasites. In nature, antibiotics are synthesized by living organisms, killing or stopping the growth of other microorganisms. The first modern antibiotic, penicillin G, was discovered by Alexander Fleming and introduced clinically in 1943 [1]. Today, antibiotics have affinity for various molecular targets and can therefore influence various properties of target cells [2]. However, the current state of antibiotic therapy is still not satisfactory. The main problem is the increasing number of multidrug-resistant bacteria, leading to the loss of the therapeutic properties of antibiotics. This is an important problem because bacteria have evolved in a way that allows them to slow down and eventually stop antibiotic activity. As a result, new antimicrobials are constantly being sought to reduce antibiotic resistance in bacteria. Antibiotics are anti-infective medicines widely used in the world. Semi-synthetic Lactamine-based antibiotics are the most important family of Lactamine-based antibiotics [3], they are classified as such because their Lactam-based fraction is obtained from a natural fermentation product and their acyl side chain is obtained from chemical or chemo-enzymatic synthesis. Thus, they were produced by coupling the acyl side chain with 6-Aminopenicillanic acid (6-APA) containing the enzyme-lactam fraction [4]. Among them, ampicillin is one of the most important semi-synthetic Lactamine antibiotics used today and the first broad spectrum penicillin used in the prevention and treatment of various bacterial diseases such as urinary [5] and respiratory [6] infections, endocarditis [7], salmonellosis [8], sepsis or meningitis [9]. The spectrum of activity of ampicillin is enhanced by the co-administration of sulbacam, or clavulanic acid, which inhibits the activity of the Lactamases produced by microorganisms as a mechanism of resistance to the antibiotic, [10], [11].

The chemical syntheses of ampicillin have developed since the early 1960s. Nowadays, they have dominated industrial production for the high yield obtained. But

Factors such as difficult reaction conditions, complicated process steps and a large volume of organic solvent required make them environmentally unsustainable [12]. Enzyme synthesis is an environmentally friendly alternative catalyzed primarily by penicillin G acylase (PGA), and the less studied 5-amino ester hydrolase (AEH) [13]. The enzymatic synthesis of ampicillin can be carried out under thermodynamic control, with an unactivated acyl side chain, or kinetic control using an activated side chain (mainly an ester or an amide). In general, kinetically controlled synthesis offers a higher yield, but still relatively low compared to the traditional chemical method [12]. Cascading conversions, which combine several reactions without going through intermediate recovery steps, are increasingly being studied to make syntheses more environmentally friendly and economically advantageous [14].

In this work, the aim has been to carry out the cascade conversion with two biocatalytic reactions, the first is the hydrolysis of penicillin G, and the second is the kinetically controlled enzymatic synthesis, catalyzed by immobilized penicillin G acylase, from the (R)-phenylglycine methyl ester side chain (more precisely by using D-phenylglycine methyl ester) and 6-aminopenicillanic acid containing the LETTER-LACTAM nucleus, in order to synthesize ampicillin in a fully aqueous medium according to the synthesis method in one pot, two steps. The second objective was to quantify the ampicillin produced. The production of ampicillin was completed and the ampicillin produced was then quantified. However, the purification and harvesting of this antibiotic have not been successful.

The results obtained will be taken into account in order to improve the effectiveness of the next synthetic trials of this antibiotic adopting our protocol.

Chapter l: STATE OF THE ART

1. ANTIBIOTICS

1.1. Definition of antibiotics

The term antibiotic (from the Greek anti: “against” and bios: “life”) derives from the word “antibiotic” which literally means “against life”. In the past, antibiotics were considered organic compounds produced by one microorganism that are toxic to other microorganisms [15]. Because of this concept, an antibiotic was originally broadly defined as a chemical substance, produced by a microorganism [16] or of biological origin [17], which, at very low concentrations, has the power to inhibit the growth of other microorganisms or even to destroy them, without intoxicating the host (eukaryotic cells)

[15]. However, this definition was changed in the modern era to include antimicrobials that are also produced in part or in whole by synthetic means. While some antibiotics are able to kill other bacteria completely, some can only inhibit their growth. Those that kill bacteria are called bactericides while those that inhibit bacterial growth are called bacteriostats [18]. Although the term antibiotic generally refers to antibacterial, antibiotic compounds are differentiated into antibacterial, antifungal and antiviral compounds to reflect the group of microorganisms they antagonize [15],[19].

1.2. Classification of antibiotics

Antibiotics can be classified according to several criteria but the most common classification methods are based on: origin, mode of action, spectrum of activity and chemical nature [20].

- Origin: produced by an organism (natural) or produced by synthesis (synthetic or semi-synthetic).
- Mode of action: action on wall, cytoplasmic membrane, protein synthesis, nucleic acid synthesis or key metabolic pathways [21].
- Activity spectrum: list of bacterial species with the majority of strains found to be susceptible. Where the spectrum of activity is limited to a certain number of species

It is said to be "narrow", while an antibiotic active on many bacteria is said to be "broad spectrum". Narrow-spectrum antibiotics act only on Gram + or Gram - (e.g. Penicillin G, Macrolides), while broad-spectrum antibiotics act on Gram + and Gram - (e.g. Tetracycline, Phenicolates) [21],[22].

- Chemical nature: very variable, often based on a basic chemical structure

  (e.g., cycle-lactam). Classification according to the chemical nature allows us to classify antibiotics into families (beta-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulfonamides, glycopeptides, oxazolidinones...) [21], [23], [24], [25].

The main classes of antibiotics are summarized in the following table:

Table 1: Classification of main classes of antibiotics [26], [27]

Classe	DCI
Bêtalactamines	
Pénicillines	G : benzylpénicilline (et forme retard) V : phénoxyméthylpénicilline M : cloxacilline, oxacilline A : amoxicilline, ampicilline Carboxypénicillines : ticarcilline Uréidopénicillines : pipéracilline Apparenté : pivmécillinam Témocilline
Inhibiteurs de bêtalactamases	Acide clavulanique, tazobactam – amoxicilline + acide clavulanique – ticarcilline + acide clavulanique – pipéracilline + tazobactam – ceftolozane + tazobactam
Céphalosporines	1re génération : céfaclor, céfadroxil, céfatrizine, céfalexine, céfazoline 2e génération : céfamandole, céfuroxime 3e génération (injectable) : céfotaxime, ceftazidime, ceftriaxone 3e génération (orale) : céfixime, cefpodoxime, céfotiam, ceftazidime-avibactam 4e génération : céfépime Autres céphalosporines : ceftobiprole, ceftaroline, ceftolozane + tazobactam
Céphamycine	Céfoxitine
Carbapénèmes	Imipénem, ertapénem, méropénem
Monobactame	Aztréonam
Glycopeptides	
Vancomycine, teicoplanine, télavancine (AMM européenne)	
Polypeptides	
Polymyxines	Polymyxine B, colistine (polymyxine E)
Lipopeptides	Daptomycine
Quinolones	
Quinolones 1re génération	Acide pipémidique, fluméquine
Fluoroquinolones	Norfloxacine, ofloxacine, péfloxacine, ciprofloxacine, lévofloxacine, moxifloxacine, loméfloxacine
Rifamycines	
Rifampicine, rifabutine	
Sulfamide	
Sulfaméthoxazole – triméthoprime	
Nitro-imidazolés	
Métronidazole, ornidazole	
Aminosides	
Tobramycine, gentamicine, amikacine	
Tétracyclines	
Cyclines	Minocycline, doxycycline
Glycylcyclines	Tigécycline
MLSK : macrolides, lincosamides, synergistines, kétolides	
Macrolides	Érythromycine, azithromycine, clarithromycine, josamycine, roxithromycine, spiramycine
Lincosamides	Clindamycine, lincomycine
Synergistine – Streptogramine	Pristinamycine
Kétolide	Télithromycine

1.3. Modes of action of antibiotics

Antibiotics generally act very specifically on certain structures of the bacterial cell. This high specificity of action explains why antibiotics are active at very low concentrations of the order of 100 $g.ml^{-1}$ on bacteria[22]. They may:

- Inhibit bacterial wall synthesis
- Disrupting the bacterial cell membrane
- Inhibit nucleic acid synthesis
- Inhibit protein synthesis
- Inhibit key metabolic pathways [28], [29], [30].

The following figure shows the mode of action of antibiotics in bacterial cells Gram positive and Gram negative:

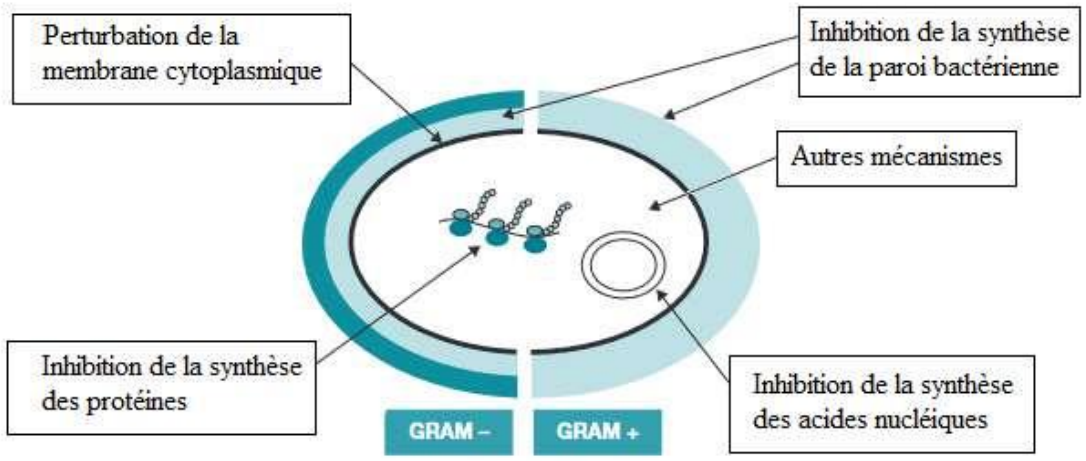

Figure 1: Mode of action of antibiotics in Gram-positive and Gram-negative bacterial cells [26]

❖ <u>Basic anatomy of the bacterial cell:</u>

Gram-positive bacteria consist of a cytoplasmic membrane surrounded by a hard, rigid mesh called a cell wall, essentially consisting of peptidoglycan. In contrast, Gram-negative bacteria consist of a thin cell wall surrounded by a second lipid membrane called the outer membrane (OM). The space between the outer membrane and the cytoplasmic membrane is called periplasm. OM is an additional protective layer in Gram-negative bacteria, consisting of phospholipids,

lipopolysaccharide (LPS) and proteins including porins. This membrane prevents many substances from entering the bacterium, however it contains channels called porins, which allow the entry of various molecules such as drugs and antibiotics [31]. The cell wall is a hard layer that gives the bacterium a characteristic shape, and plays both a nutritive role in allowing nutrients and waste to enter and leave, and a protective role in preventing the bacterium from osmotic and mechanical stresses. The cytoplasmic membrane prevents ions from entering or leaving the cell and maintains cytoplasmic and bacterial components within a defined space [32].

The following figure illustrates the cell wall structure of Gram-positive and Gram-negative bacteria:

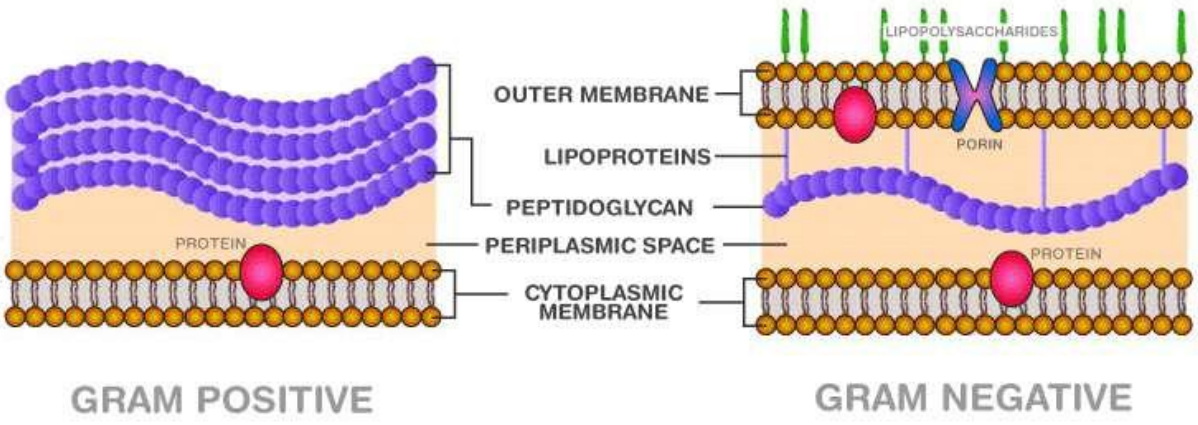

Figure 2: Cell wall structure of Gram-positive and Gram-negative bacteria [33]

In fact, the targets of action of antibiotics are of interest to the various elements of the bacterium and are represented in the figure opposite:

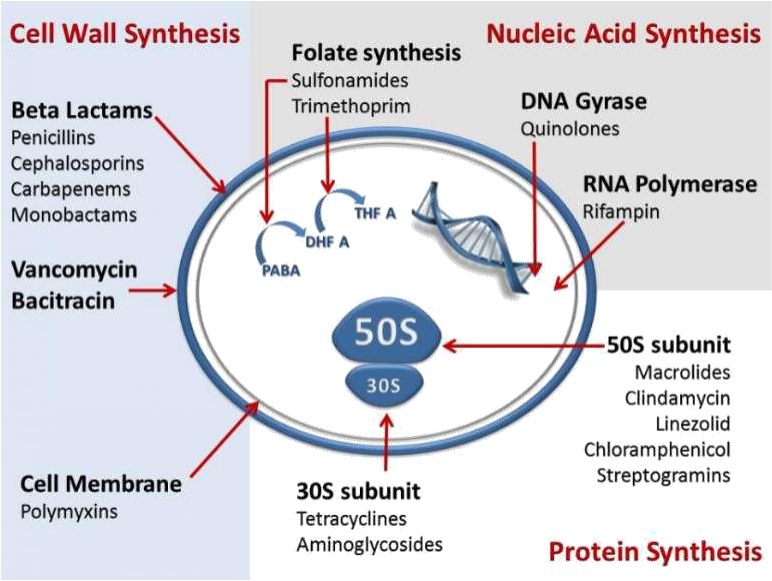

Figure 3: The different target sites of antibiotics in a bacterial cell [32]

A. Mode of action of wall synthesis inhibitors:

The basic structure of the bacterial wall consists of peptidoglycan (PG), a polymer with a complex structure. This polymer is composed of crosslinked chains of peptidoglycan monomers, which are formed of two alternating sugars, N-acetylglucosamine (NAG), and N-acetylmuramic acid (NAM), to which pentameric chains of amino acids are attached. The types and order of amino acids in pentapeptide, although almost identical in Gram-positive and Gram-negative bacteria, show slight variation between bacteria. The transpeptidase enzymes (otherwise known as penicillin binding proteins, PBP) in the cell wall catalyze cross-linking between adjacent glycan chains, which involves the removal of a terminal D-alanine residue from one of the peptidoglycan precursors. Transglycosylase enzymes (glycosyltransferases), which exist as separate subunits or closely associated with transpeptidases, create covalent bonds between adjacent sugar molecules NAM and NAG. The net result of covalent bonds between peptide chains and carbohydrate chains creates a rigid cell wall that protects the bacterial cell from osmotic forces that would otherwise lead to cell rupture. In a peptidoglycan monomer of *S. aureus* (FIG. 4), the pentapeptide exiting from NAM is composed of the amino acids L-alanine, D-glutamine, L-lysine and two D-alanines. Peptide crosslinking is formed by forming a short peptide bridge

consisting of 5 glycines, otherwise known as a pentaglycine chain. According to this process, the terminal D-alanine is cleaved from the pentapeptide to form a tetrapeptide in the peptidogycan, which leads to the formation of a network. In an *E. coli* peptidoglycan monomer (Figure 5), the NAM pentapeptide is composed of the amino acids L-alanine, D-glutamic acid, meso-diaminopimelic acid, and two D-alanines. Peptide cross-linking is formed between the diaminopimelic acid of one peptide chain and the D-alanine of another, and following this process, the terminal D-alanine is cleaved from the pentapeptide to form a tetrapeptide in the peptidogycan also leading to the formation of the network [34].

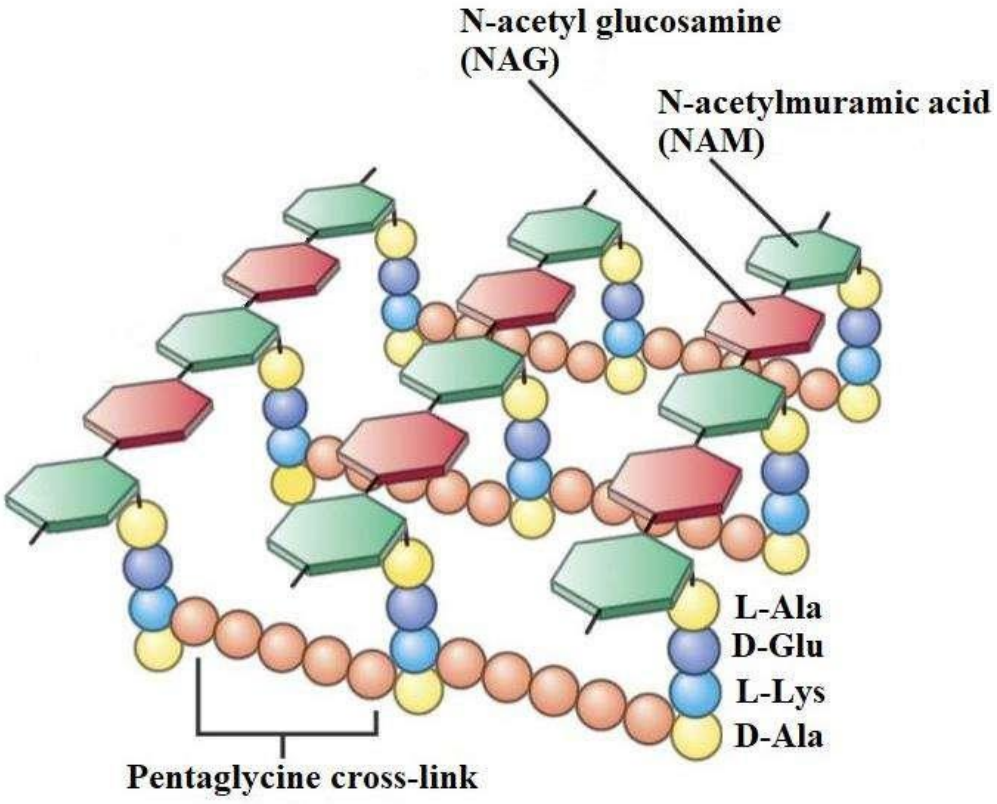

Figure 4: Structure of S. aureus peptidoglycan. L-Ala: L-Alanine; D-Glu: D-glutamine; L-Lys: L-Lysine; D-Ala: D-Alanine [35]

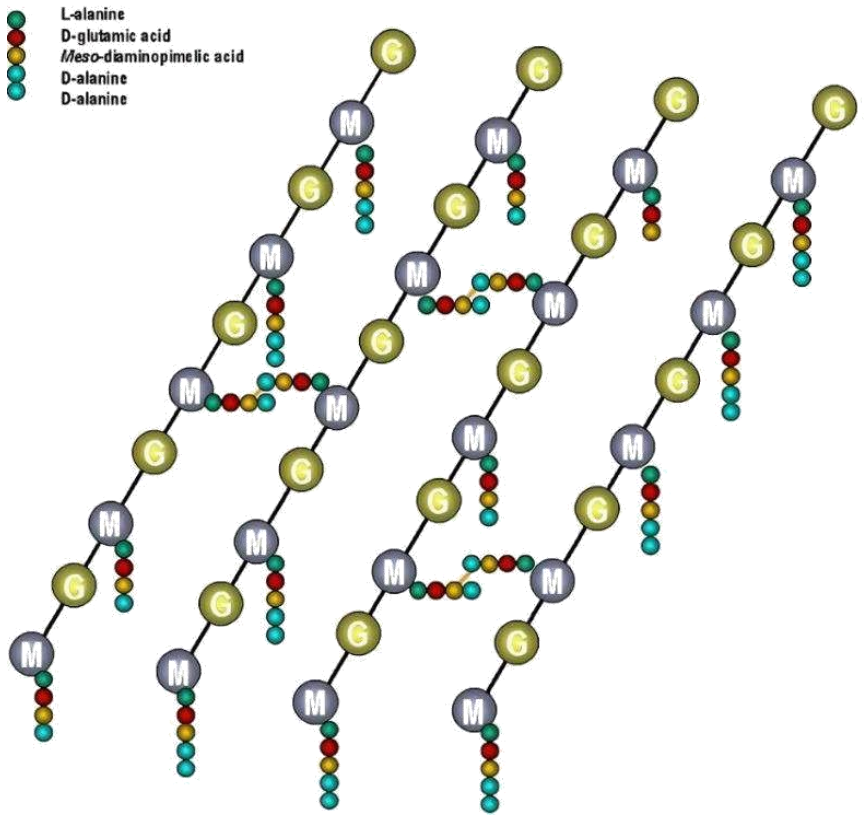

Figure 5: Structure of E. coli peptidoglycan. G: N-acetylglucosamine; M: N-acetylmuramic acid [36]

- **Beta-lactams:**

The action of these antibiotics is carried by their beta-lactam nucleus. In fact, this nucleus has a very high affinity for the catalytic site of the so-called penicillin binding proteins (PBPs). PBPs are transpeptidases and transglycosylases, enzymes responsible for the elongation and crosslinking of peptidoglycan in bacterial cell walls. The PG is the main component of the wall of all bacterial species, it supports the structure of the cells and protects them from the surrounding environment. During growth and division, the PG is continuously synthesized and remodeled. Failure to form PG thus leads to inhibition of growth, cell lysis and eventually cell death. The antibiotics of the enzyme-Lactamines mimic the D-alanyl D-alanine (DAla-DAla) portion of the peptide chain that is normally bound by PBPs, thus acting as irreversible inhibitors of transpeptidase enzymes. They then interfere with the structural crosslinking of the PG and subsequently prevent the final step of its synthesis in the bacterial cell wall. As a result, they weaken the cell wall of the bacterium and ultimately lead to cytolysis or death

à osmotic pressure. The antibiotics, which can act on both Gram-positive and Gram-negative bacteria, must penetrate the cell membrane to enter the cell periplasm in order to access penicillin-binding proteins. Although active against a wide range of bacterial pathogens, they have shown very little toxicity to mammalian cells, which has greatly contributed to their success in clinical practice [37], [38], [14] (Figure 6).

- **Glycopeptides:**

The molecular target of these antibiotics is the D-alanyl-D-alanine (DAla-DAla) end of the cell wall peptidoglycan precursor. Once the glycopeptides are linked to their target, they will prevent the action of PBPs by steric hindrance, thus blocking the elongation of the peptidoglycan. By way of example, the drug molecule vancomycin prevents the binding of this D-alanyl subunit with PBP, and therefore inhibits the synthesis of the cell wall (FIG. 6) [39], [40].

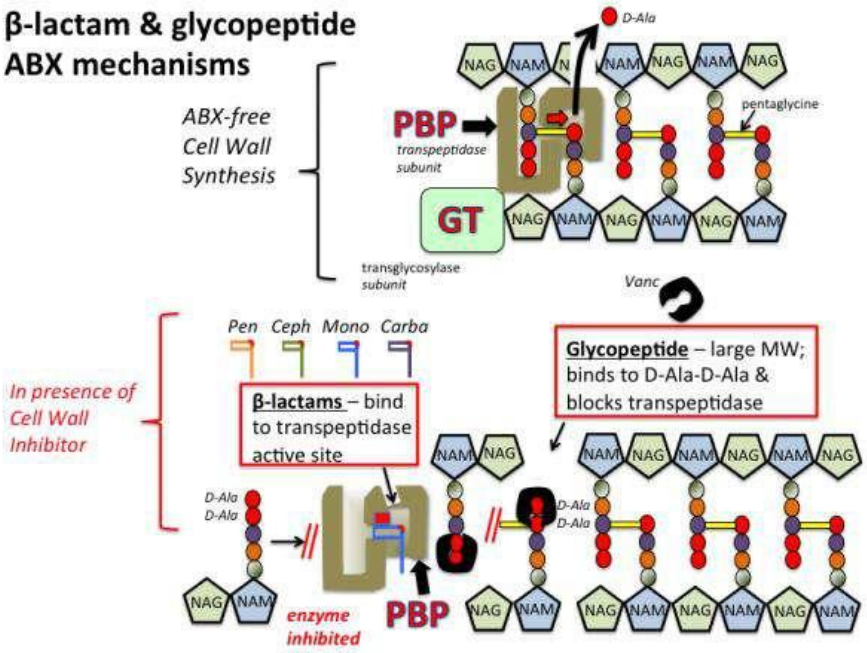

FIG. 6: Mode of action of the antibiotics of the LETTERA and of the glycopeptides in the bacterial cell. PBP: Protein from penicillin binding; GT: Transglycosylase; NAM: N-acetylmuramic acid; NAG: N-acetylglucosamine; D-Ala: D-Alanine; D-Ala-D-Ala: D-alanyl-D-alanine; Pen: Penicillins; Ceph: Cephalosporins; Mono: Monobactams; Carba: Carbapenems; ABX: Antibiotics; Vanc: Vancomycin; MW: Molecular weight [41]

B. Mode of action of antibiotics acting at cell membrane level:

- **Polymyxins:**

They act as cationic detergents: because of their amphipathic nature, they penetrate into the bacterium and are inserted among the phospholipids, thus disturbing the membrane permeability [26].

C. Mode of action of inhibitors of protein synthesis:

The information contained in the bacterial DNA is used to synthesize an RNA molecule called mRNA, a process known as transcription. Then, the macromolecular structure called ribosome synthesizes the proteins present in mRNA, a process called translation. Protein biosynthesis is catalyzed by ribosomes and cytoplasmic factors. The bacterial ribosome 70S is composed of two ribonucleoprotein subunits, the 30S and 50S subunits [42]. Antimicrobials inhibit protein biosynthesis by targeting the 30S or 50S subunit of the bacterial ribosome[43],[44] as shown in the following figure:

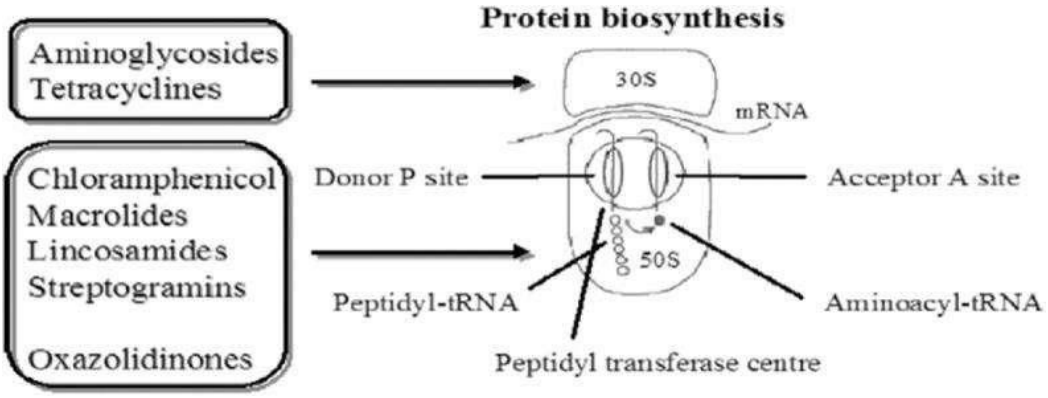

Figure 7: Sites of action of inhibitors of protein synthesis. mRNA: Messenger ribonucleic acid; 50S and 30S: Subunits ribonucleoproteins of bacterial ribosome 70S; t-RNA: transfer ribonucleic acid [32]

- **Aminoglycosides:**

They act by binding to 16S RNA, at the 30S subunit of the bacterial ribosome, near site A, resulting in a morphological change of the entire ribosome and an alteration of all the stages of protein synthesis. Due to the numerous reading errors, abnormal proteins are synthesized and then incorporated into the cytoplasmic membrane, which loses its integrity [32].

- **Tetracyclines:**

The tetracyclines bind to the 16S RNA of the 30S subunit of the bacterial ribosome. By steric hindrance, they thus prevent the binding of the aminoacyl-tRNA to the A site of the ribosome [42], [45].

- **Chloramphenicols:**

They attach to the 50S subunit of bacterial ribosomes. They inhibit protein synthesis by preventing the binding of the aminoacyl-tRNA complex to the ribosome site A, and consequently the transpeptidation reaction. [42], [43].

- **Macrolides:**

The macrolides bind to the 23S RNA of the 50S subunit of the bacterial ribosomes. They thus prevent the transfer of the peptidyl-tRNA complex from the P site to the A site, which results in an inhibition of the elongation of the peptide chain. Macrolides, lincosamides and streptogamins B have a similar mechanism of action. [42],[45].

- **Oxazolidinones:**

They bind directly to the 23S RNA of the 50S subunit of the bacterial ribosome, causing a distortion of the binding site of N-formyl-methionine-tRNA synthetase and preventing the formation of the 70S functional initiation complex which is essential for protein synthesis. [46], [47].

D. Mode of action of nucleic acid synthesis inhibitors:

- **Fluoroquinolones**

Quinolones enter the cytoplasm via the porine route and by passive diffusion. Their intracytoplasmic targets are type II topoisomerases: DNA gyrase and topoisomerase IV. They inhibit DNA transcription and replication by forming an indissociable DNA-gyrase-quinolone complex, resulting in rapid death of the bacterium [26].

- **Rifampycins:**

Rifampicin blocks the initiation of DNA transcription by covalently binding to the B subunit of bacterial DNA-dependent RNA polymerase, the enzyme responsible for the

transcript. The bactericidal effect could be explained by the stability of this bond which results in the formation of free radicals toxic to bacterial DNA [26].

E. Mode of action of folic acid metabolism inhibitors:

- **Sulfamides:**

Sulfamides are considered to be competitive antagonists of para-amino-benzoic acid (PABA), which is a necessary molecule for the synthesis of dihydrofolic acid (DHF) and tetrahydrofolic acid (THF), two precursors of folic acid. The latter is a cofactor in the synthesis of purine and pyrimidine bases to be incorporated into nucleic acids necessary for bacterial survival and replication. Sulfamides inhibit the synthesis of folates, puric acids and nucleic acids by binding to dihydropteroate synthase (DHPS) [42].

- **Trimethopremiums:**

Trimethopremia act at a later stage in folic acid synthesis and inhibit folate, puric acid and nucleic acid synthesis by binding to dihydrofolate reductase [42].

2. THE BETA-LACTAMINE FAMILY

2.1 Definition and Classification

The antibiotics, the LACTAMINE, have been used clinically for more than 60 years and are currently the most widely used group of antibiotics to treat bacterial infections. The common fraction of all the antibiotics, the 2-azetidinone ring, more commonly known as the 2-azetidinone ring (Figure 8), is responsible for their bactericidal capacities [48]. It is a ring of one to three carbon atoms and a highly reactive nitrogen atom [49].

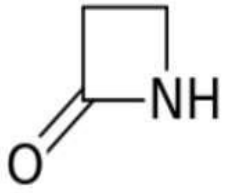

Figure 8: Chemical Structure of the Beta-Lactam Core [50]

There are four subfamilies of the family of the antibiotics, namely, the "Lactamine", which are currently used in clinical practice [51]:

- Penicillins
- Cephalosporins
- Carbapenems
- Monobactams

This classification depends on the chemical nature of the cycle fused to the cycle of the "Lactam", thus generating unique "Lactam" nuclei. In penicillin-based antibiotics, the lactam-derived ring is fused to a five-membered sulfur-containing ring called thiazolidine, which is the origin of the penam nucleus (FIG. 9). In cephalosporins, a six-membered sulfur-containing ring, dihydrothiazine, is fused to the Lactam-derived ring to create the cephem nucleus. In the carbapenem, the unit of the enzyme-lactam is fused to a cyclopentene. In monobactam antibiotics, no ring is fused to the system of the Lactam [52]. The nuclei are bound to different loop side chains to form different semi-synthetic antibiotics (e.g. ampicillin). Changes in the acyl side chain affect microbial activity and chemical stability, leading to expanded spectra of bactericidal activity [50].

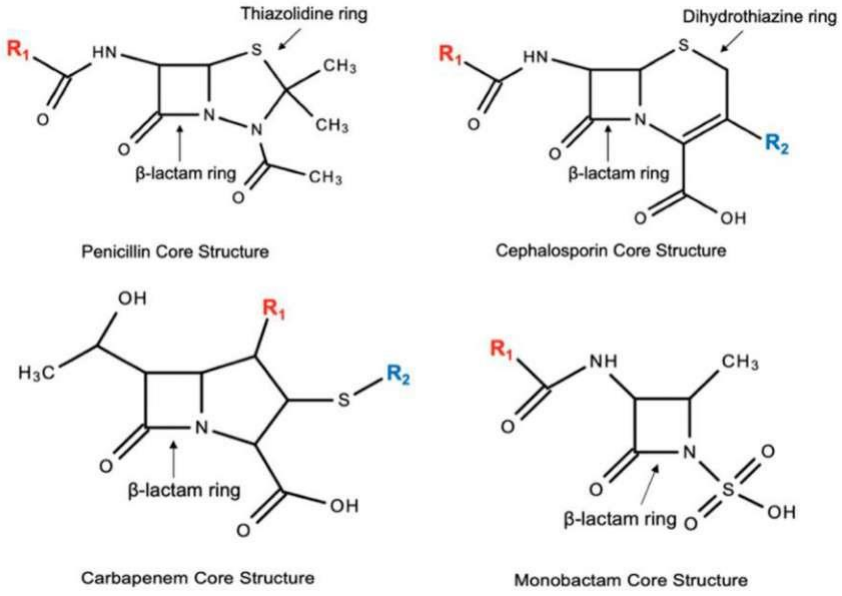

Figure 9: Chemical structure of the different subfamilies of the antibiotics: penicillins, cephalosporins, carbapenems and monobactams; R1 and R2: side chains [53]

3. PENICILLINS

3.1 Discovery

The first antibiotic discovered was penicillin, which is an antibiotic that is an Lactam-based antibiotic. In 1928, the Scottish scientist Alexander Fleming observed that some inoculated petri dishes of *Staphylococcus aureus* forgotten on the lab counter in early summer were accidentally infected with a fungus called *Penicillium notatum*. He also noted a transparent halo around the contaminating mold that indicated lysis and reduced growth rate of *Staphylococcus aureus* colonies. This seemed to indicate that the fungus could produce a bactericidal compound. In 1932, Fleming published the complete results of his work on the identification of a new antimicrobial agent from the metabolites of *Penicillium notatum*. He named the new antibacterial "Penicillin" in reference to the genus *penicillium*. At first, Fleming's discoveries were not of interest and there was no intention to use them for therapeutic purposes until the Second World War [54], [55], [56], [57], [58], [59].

Ernst Boris Chain and Lord Howard Florey at Oxford took over Fleming's research and successfully isolated penicillin and produced it on an industrial scale in 1940. One year later, Edward Abraham performed chromatographic purification of penicillin by conducting the first experiments focused on the evaluation of its antimicrobial properties in animals. In 1943, Robert Robinson elucidated the chemical structure of penicillin, which provided the means for a future synthesis. These findings earned Fleming, Chain and Florey the first Nobel Prize in Physiology or Medicine in 1945 [60], [61], [62], [63].

3.2 Chemical Structure

Penicillins are involved in a class of various groups of compounds, most of which end with the suffix "cillin". These are compounds containing a 6-animopenicillanic acid (6-APA) nucleus, consisting of the core of 6-lactam fused with a thiazolidine nucleus (a heterocyclic ring with five atoms), and other side chains (FIG. 10) [64].

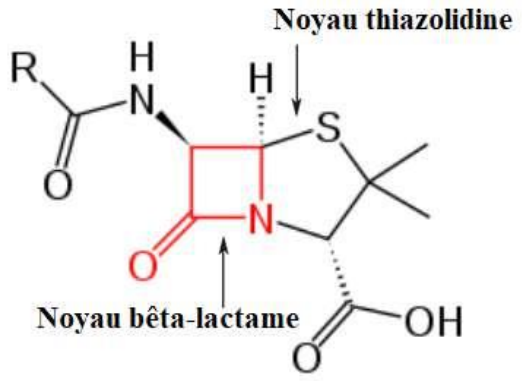

Figure 10: General structure of penicillins [65]

3.3 Method of production

Penicillins are a group of antibiotics derived from *Penicillium* mold (mainly *P. chrysogenum*, *P. notatum* and *P. rubens*). The different types of penicillins synthesized by various species of *Penicillium* mold can be divided into two classes:

- Natural penicillins: Produced from the mold fermentation process (exp: Penicillins G and V).
- Semi-synthetic penicillins: Prepared from 6-aminopenicillanic acid, which is present in all penicillins (exp: ampicillin, amoxicillin...) [52].

3.4 Classification and mode of action

The different penicillins differ in their structure of the side chain. Indeed, the side chain varies with each penicillin compound and generally determines the spectrum of activity and pharmacokinetic properties of the compound [21]. Penicillins are divided into several subgroups shown in the following table:

Table 2: Classification, spectra of activity and modes of action of penicillins [66], [67], [68]

Sous groupes	Antibiotiques (DCI)	Spectre d'activité	Mode d'action
Pénicilline G et ses dérivés	**Parentérales :** -Benzyl Pénicilline (pèni G) -Benzyl Pénicilline- procaine - Bénéthamine- benzylpénicilline -Benzathine- benzyl pénicilline	**Cocci Gram + :** Streptocoques (groupe A, C, G et B), Pneumocoques sensibles. **Cocci Gram- :** Neisseria (surtout le méningocoque). **Bacilles Gram+:** Corynebacterium diphteriae, Bacillus anthracis Listeria monocytogenes , Anaérobies.....	Paroi bactérienne, par toxicité sélective : Ils agissent sur la synthèse du peptidoglycane en inhibant les protéines liant la pénicilline (PLP). Les PLP ont une activité transpeptidasique, carboxypeptidasique et transglycolasique. L'inhibition des PLP aboutit à l'inhibition de la formation des ponts pentacycliques responsables de la structure réticulée de la paroi. On obtient ainsi des formes bizaroides (rondes ou filamenteuses) qui aboutissent à la lyse bactérienne.
	Orales : – Phénoxy méthyle pénicilline (pénicilline V) – Clométocilline		
Pénicillines M (antistaphylococciques)	– Méthicilline – Oxacilline – Isoxazolyl-pénicillines) : Cloxacilline, Dicloxacilline, Flucloxacilline.....	Staphylocoque producteur de pénicillinase. Staphylocoque MRSA- (sensibles à l'Oxacilline)	
Aminopénicillines (pénicillines à large spectre)	– Ampicilline – Dérivés de l'ampicilline : Bacampicilline,Métampicilli Pivampicilline, Pivampicilline – Amoxicilline, Epicilline	-Entérobactéries sauf : Klebsiella,Enterobacter, Serratia net Protéus indole+ . -Neisseria méningitidis,Haemophilus influenzae b sensible (pénicillinase-) -Inactifs sur Pseudomonas et Acinetobacter Streptocoquesa A, C, G	
Carboxy-pénicillines	– Carbénicilline, Ticarcilline	-Pseudomonas aeruginosa). -Bacilles à Gram- résistants à l'ampicilline. -Entérobactéries productrices de céphalosporinases : Citrobacter, Enterobacter, Serratia, Proteus indole+.	
Acyl-amino-pénicillines (Uréido-pénicillines)	– Azlocilline – Mezlocilline – Pipéracilline	Entérobactéries productrices de céphalosporinases. Pseudomonas aeruginosa, Acinetobacter	
Amidino-pénicillines	– Mécillinam -Pivmécillinam	Actifs uniquement sur les bacilles à Gram-, Pas d'action sur les Cocci à Gram+.	
Pénicillines sulfones : inhibiteurs de β lactamases utilisées en association avec une β lactamine	Ampicilline+**Sulbactam** Pipéracilline+**Tazobactam**	Bactéries à Gram- fermentaires Bactéries à Gram- oxydatifs	

4. AMPICILLIN

4.1 Discovery

In 1957, the isolation of 6-aminopenicillanic acid from penicillin fermentations led to the production of many semi-synthetic penicillins, of which ampicillin is the

more widely used [69]. In 1961, 1-amino-benzylpenicillin, known as ampicillin, was marketed [70].

4.2 Chemical Definition and Structure

Ampicillin (AMP) is a semi-synthetic, aminopenicillin-family, elasticated lactam antibiotic with a broad spectrum of action in the prevention and treatment of various bacterial diseases [71]. It is one of the most important anti-kinetic and broad-spectrum penicillins used today [71] that act on Gram-positive bacteria and some Gram-negative bacteria, but is inactivated by penicillinases, a class of enzymes that inactivate penicillin by hydrolysis of the kinetic-lactam ring
[69]. The chemical structure of ampicillin is illustrated in the following figure:

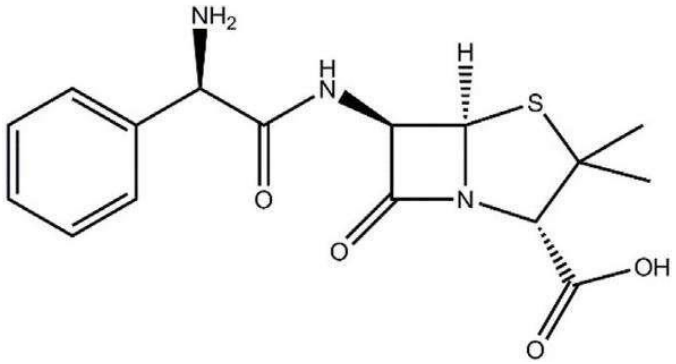

Figure 11: Chemical Structure of Ampicillin [71]

4.3 From penicillin to ampicillin

Although penicillins differ in their lateral chain structure [21], they all have the same antibacterial activity as benzylpenicillin or penicillin G, the original molecule extracted from *P. notatum*. Benzylpenicillin is narrow-spectrum, only Gram-positive bacteria (streptococci) and some Gram-negative bacteria such as *Treponema pallidum* are susceptible to it as a causal agent for syphilis and meningococci [72]. The inability to act against Gram-negative bacteria is observed not only among benzylpenicillins, but also among many different antibiotics. This is due to two factors: firstly, unlike Gram-positive bacteria, Gram-negative species contain the outer membrane, which acts as a selective barrier, blocking the penetration of penicillin; secondly, some Gram-negative bacteria have acquired specific genes that code for penicillinases. Given this, some antibiotics

such as ampicillin, carbenicillin and amoxicillin have been developed semi-synthetically with different side chains. These side chains give antibiotics the ability to escape the degradation capacity of certain enzymes produced by certain bacterial strains and to facilitate the movement of antibiotics through the outer membrane of these bacterial cell walls. This dual-component capability increases their spectrum of activity against Gram-negative bacteria [49].

Ampicillin is distinguished from penicillin G or benzylpenicillin by the presence of an amine group that helps to increase the hydrophilicity of ampicillin, allowing it to diffuse through the porins in most external membranes of Gram-negative bacteria (Figure 12). As a result, ampicillin has a broad spectrum of action, being active against non-lactamase-producing Gram-positive and Gram-negative bacteria, enzymes produced by microorganisms as a mechanism of resistance to and capable of hydrolyzing the antibiotics, namely *N. gonorrhoeae, N. meningitidis, H. influenzae, T. pallidum, Leptospira spp., E. coli, Salmonella spp., Shigella spp.* and *Proteus mirabilis*) [73].

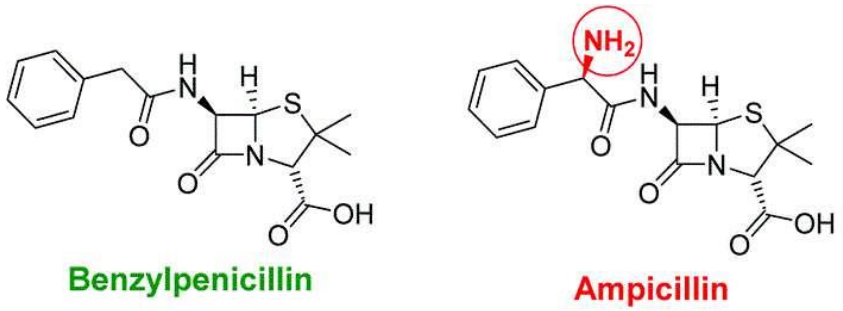

Figure 12: Structural relationship between penicillin G and its semi-synthetic antibiotic ampicillin [74]

4.4 Therapeutic Use

- Respiratory tract infections: Caused by: *Streptococcus pneumoniae*, penicillinase-producing and non-penicillinase-producing *Staphylococcus aureus*, beta-hemolytic group A streptococci, *Hemophilus influenzae*.

- Bacterial meningitis: caused by Gram-negative bacteria: *Neisseria meningitis*, *Escherichia coli*; and Gram-positive bacteria: *Listeria monocytogenes*, group B streptococci. The addition of aminoglycosides increases its effectiveness against gram-negative bacteria [75].

- Sepsis and endocarditis: Caused by Gram-positive bacteria, including *penicillin-sensitive Staphylococcus* species*, Streptococcus species, and Gram-negative Enterococcus species, including Escherichia coli*, *Salmonella* species, and *Proteus mirabilis*. Enterococcal endocarditis generally responds to intravenous ampicillin. The addition of aminoglycosides to ampicillin may increase its efficacy in the treatment of streptococcal endocarditis [76].

- Genitourinary tract infections: Caused by susceptible strains of *Escherichia coli* and *Proteus mirabilis* [77].

- Gastrointestinal infections: caused by *Salmonella typhi*, *Shigella* species and other *Salmonella* species , and *improve* generally with oral or intravenous treatment [78].

In addition, the spectrum of activity of ampicillin can be extended and improved by co-administration with inhibitors of spective lactamases, such as clavulanic acid, thereby allowing

à ampicillin is effective against penicillinase-producing bacteria (such as *H. influenzae, M. catarrhalis* and *Bacteroides fragilis)*; or sulbacam, which is also an inhibitor of beta-lactamases [79].

4.5 Mode of Action

As with any other antibiotic, the mode of action of ampicillin on susceptible organisms can be considered as a two-step process. In the first step, the drug binds to penicillin binding proteins (PBP) in the cytoplasmic membrane (Figure 13). These proteins play a vital role in the cell-cycle morphogenetic formation of cell wall peptidoglycan. Therefore, the inactivation of PBPs by an antibiotic is linked to the immediate cessation of their function.

The second step includes the physiological effects caused by this binding. Since penicillin binding proteins are involved in the late stages of PG synthesis, ampicillin then causes inhibition of the final stage of PG synthesis in bacterial cell walls. Peptidoglycan maintains the integrity of the cell wall, which resides in a hypotonic environment, and its disturbance causes lysis and cell death [80].

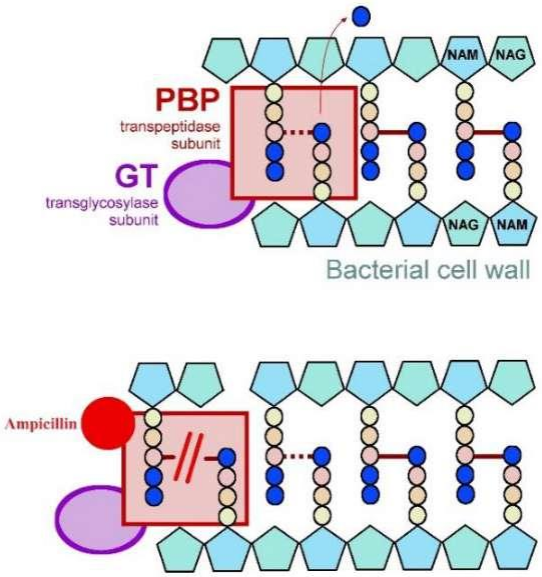

Figure 13: Mode of action of ampicillin in the bacterial cell. NAM: N-acetylmuramic acid; NAG: N-acetylglucosamine; PBP: Penicillin binding proteins; GT: Transglycosylases [81]

4.6 Synthesis pathways

Only two antibiotics, penicillin G and penicillin V, used in clinical practice are products of a fermentation process and are therefore considered natural molecules. The majority of the antibiotic-Lactams used in clinical practice are classified as semi-synthetic because the fraction of Lactam is obtained from the enzymatic hydrolysis of a natural fermentation product; penicillin G or penicillin V, while the acyl side chain is obtained by chemical or chemo-enzymatic synthesis [14].

A. <u>Chemical Synthesis:</u>

Chemical coupling of the alkyl-lactam moieties with an acyl side chain has dominated the industrial production of semi-synthetic alkyl-lactam antibiotics since their discovery

early 1960s. Ampicillin can be produced by chemical synthesis which can reach yields as high as 90% [13], but this technique generally involves expensive steps, such as temperatures as low as -30° C., the use of large volumes of toxic organic solvents (exp: methylene, dichloromethane . . . .) and the use of highly reactive compounds (exp: pivaloyl chloride). Despite recycling solvents and auxiliary reagents where possible, this technique still generates a large amount of non-biodegradable waste [4], [82].

B. Biocatalytic synthesis:

The coupling of the crystals of the LETLAM with the acyl side chain can be carried out enzymatically using penicillin G acylase (PGA). A less studied enzyme, the acetate-amino ester hydrolase (AEH), may also be used for this reaction when the acyl side chain has an amine group in position 1. Furthermore, the coupling can be carried out under thermodynamic control, which uses an unactivated acyl side chain; or under kinetic control, which requires an activated side chain (typically an ester or an amide) [14]. Noting that thermodynamically controlled synthesis, also called direct synthesis or equilibrium controlled synthesis, is only capable of achieving minimum yields (1% or less) in aqueous medium. Under typical reaction conditions, the substrates are in their ionized forms, which PGA does not accept. Thermodynamically controlled synthesis is then unnecessary for the synthesis of semi-synthetic Lactamine-based antibiotics [83], [84].

C. Comparison of synthesis techniques:

The obvious disadvantage of the enzymatic coupling process compared to the chemical coupling process is the lower yield of the product. However, enzymatic coupling has undeniable advantages over chemical coupling in terms of raw material cost, environmental impact, product quality and ease of use; because it is carried out at ambient temperature, pressure and pH; and does not require toxic or dangerous reagents or solvents [13].

D. One-pot synthesis of ampicillin:

Cascade conversions, which combine several reactions without intermediate recovery steps, are increasingly being studied to make syntheses more respectful of

the environment is economically advantageous. The replacement of a multi-stage synthesis requiring intermittent isolation by a cascading process eliminates the need for isolation and purification of intermediates and thus results in smaller reactor volumes, shorter cycle times, higher volumetric and spatio-temporal yields and a reduction in the amount of waste generated [85],[12]. Cascade conversions may combine several biocatalytic steps, several chemocatalytic steps, or may combine both biocatalytic and chemocatalytic steps. In general, it is easier to combine several biocatalytic steps because most enzymes have similar operating conditions [85].

In this work, a cascade conversion was carried out with two biocatalytic reactions in an entirely aqueous medium to synthesize ampicillin (FIG. 13). In the first reaction, 6-APA containing the core of the LETTER-LACTAM was produced from the hydrolysis of penicillin G using immobilized PGA (iPGA). The by-product of this reaction, phenylacetic acid (PAA), is a known inhibitor of iPGA [86]. In the second reaction, ampicillin was produced by a **kinetically controlled** coupling of 6-APA and the (R)-phenylglycine methyl ester side chain, catalyzed by immobilized PGA (iPGA), (AEH could also be used in this 2^{nd} reaction) [87].

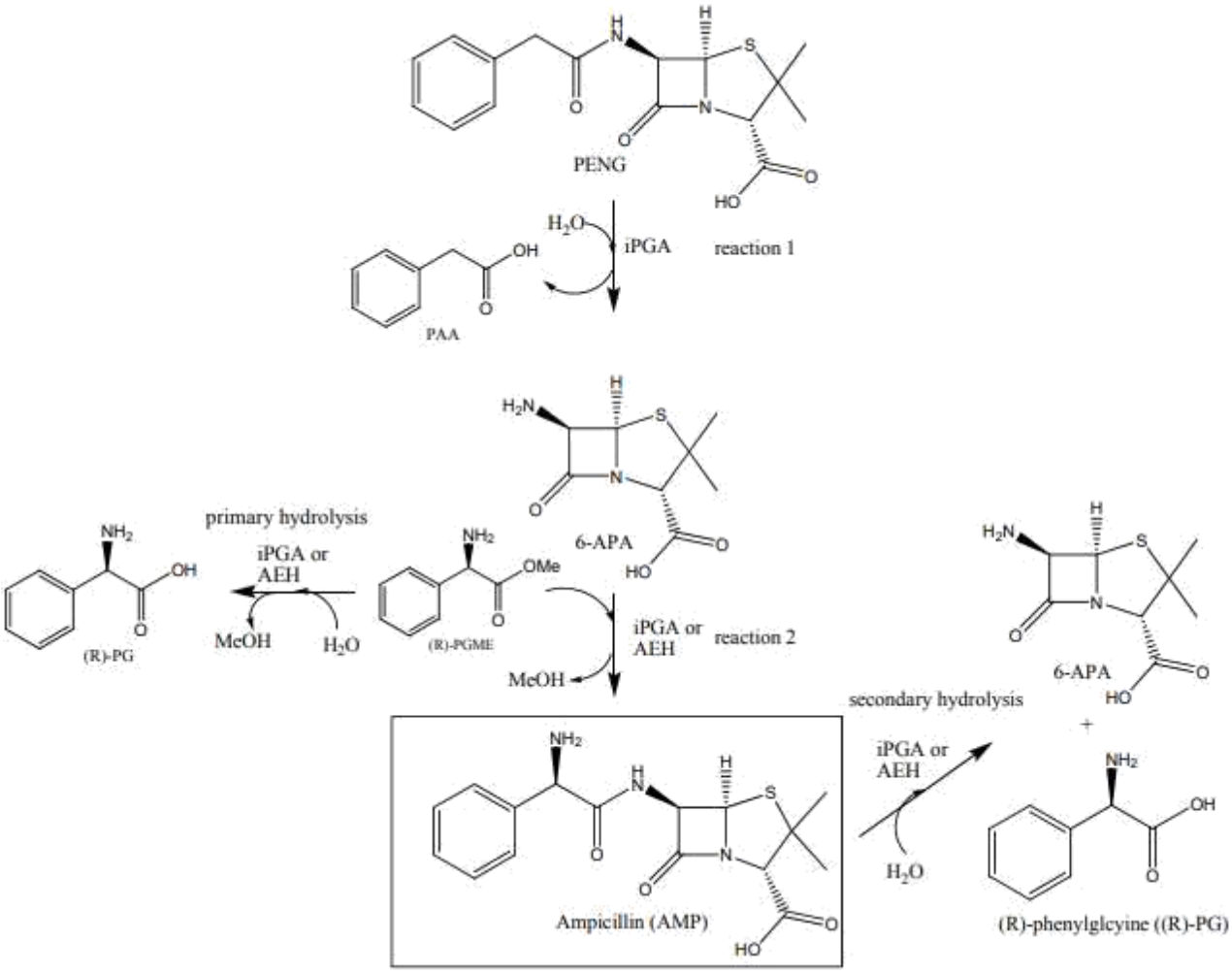

Figure 14: Direct two-enzyme, one-pot conversion of penicillin G (PENG) to ampicillin (AMP) using iPGA and AEH. PenG: Penicillin G; iPGA: Penicillin G immobilized acylase; PAA: phenylacetic acid; 6-APA: 6-aminopenicillanic acid; (R)-PGME: (R)-Phenylglycine methyl ester hydrochloride; (R)-PG: (R)-Phenylglycine methyl ester; AEH: 1-amino ester hydrolase; MeOH: Methanol; AMP: Ampicillin [14]

Penicillin G acylase (PGA) can act as a hydrolase as well as a transferase, which means that this same enzyme catalyzes the synthesis of ampicillin as well as the hydrolysis of the activated acyl donor R-PGME and the hydrolysis of the newly formed antibiotic. The low yield can thus be attributed mainly to the primary hydrolysis reactions of R-PGME and secondary hydrolysis of the ampicillin produced, which are catalyzed by iPGA [14], [88]. Since AEH enzymes are unique in their specificity to the acyl moiety with respect to the 1-amine groups, they cannot then catalyze the hydrolysis of penicillin G

to obtain 6-APA and they are not inhibited by the PAA, hence their advantage in this cascade [89].

In addition, there are two methods for the synthesis of ampicillin in one pot which can be proposed:

- **One-pot synthesis, 1 step (1P1S):**

This is a batch process. Penicillin G, (R)-PGME, and all enzymes (either iPGA or both iPGA and AEH) are added to the reaction and the reaction may continue.

- **One-pot synthesis, two steps (1P2S):**

Following which penicillin G and iPGA are added to the reaction and the hydrolysis reaction can proceed almost completely to produce 6-APA. When the hydrolysis reaction is nearly complete, (R)-PGME and either AEH or additional iPGA are added to the reaction mixture.

However, the one-pot two-step (1P2S) synthesis produces a higher overall yield [14].

Based on these data, the ampicillin synthesis technique chosen in my present study is **one-pot, two-stage** (1P2S) **synthesis**.

CHAPTER II: CONTRIBUTION

1. Framework

This work was initiated and carried out in the laboratory of the MEGBI institute of the association

« Aecenar", located in Tripoli, Ras maska, from 15 June 2022 until 5 August 2022.

2. Production of ampicillin by the one-pot, two-step method (1P2S)

This work has been done twice. The same experimental protocol was followed in Experiment 1 and Experiment 2, with the exception of a few modifications which will be clearly illustrated in the remainder of this part.

2.1 Bacterial strain used

A well-identified bacterial strain of *Escherichia coli* (ATCC 25922) obtained from the Doctoral School of Science and Technology in Tripoli, Northern Lebanon, was used in this work.

2.2 Products Used

Penicillin G, *Escherichia coli* penicillin G acylase immobilized on the Eupergit ® C commercial carrier and D-PGME were all purchased from Sigma Aldrich (St. Louis, MO, USA). Also, the ethyl acetate solvent, obtained from the Chamber of Commerce, Industry, and Agriculture of Tripoli and North Lebanon, was used. Finally, the commercial ampicillin which is the ampicillin 1 g Panpharma powder for injection, obtained from the pharmacy Maya, Tripoli, North Lebanon, was also used in this work.

2.3 Solutions Used

Potassium phosphate buffer solution, as well as pH, NaOH and H2SO4 adjustment solutions were used in this work. The protocols for the preparation of these solutions are detailed in the "Annexes" section of this report.

2.4 Ampicillin Synthesis

First, **a penicillin G solution (C=0.04 mol/L)** was prepared: A mass of 0.286 g (approximately 0.29 g) of Penicillin G (powder; M=356.4 g/mol^{-1}) was weighed using a digital balance, transferred into a beaker and then dissolved in 20 ml of the phosphate solution of

potassium previously prepared (pH=7 and C=0.1 mol/L). From this solution, a volume of 15 mL was taken and transferred to a new beaker of 100 mL.

To this same beaker, the enzyme iPGA (powder) has been added: In fact, 124 units of penicillin G require 1 g of enzyme (where 1 Unit of Penicillin G acylase is defined as 1.0 mol of hydrolyzed penicillin G per minute). 198.4 units of penicillin G are required, so a mass of 1.6 g of iPGA was measured using the digital balance and added. The whole was well mixed, the magnetized bar was added to the beaker which was then placed on the magnetic stirrer to stir for 60 minutes at room temperature (from 22 to 25° C.). One hour later, 6-APA and PAA were obtained in the solution following the completion of the ^{first} reaction, iPGA-catalyzed hydrolysis of penicillin G (Figure 15).

Figure 15: Solution containing 6-APA and PAA obtained by iPGA-catalyzed hydrolysis of Penicillin G

Next, **an ester solution (C=0.12 mol/L), D-PGME (powder, M=201.65 g.mol^{-1})**, was prepared: A mass of 0.48 g of ester was measured using a digital balance and then transferred to a beaker, to which a volume of 20 mL of the previously prepared potassium phosphate solution (pH=7 and C=0.1 mol/L) was added to ensure the total dissolution of the ester. From the above solution, a volume of 15 ml was taken using a graduated test piece and transferred to the beaker containing the reaction mixture. Finally, the iPGA was added to the mixture again: In fact, 30 units of penicillin G require 1 g of iPGA, 14.4 units of penicillin G are needed, so a mass of 0.48 g of iPGA was measured using the digital balance and added to the beaker of the reaction.

The contents of the beaker were well mixed and the pH of the solution was measured using a digital pH meter. The latter showed a value of 6.50, the pH of the solution was then adjusted to the

pH value=7 by adding a few drops of the strong base solution, NaOH, already prepared (FIG. 16).

FIG. 16: Adjustment of the pH of the reaction mixture to the value 7

The beaker containing the reaction mixture was again placed on the magnetic stirrer to ensure stirring for 1270 min (approximately 22.5 h) until the completion of the 2nd reaction, which is the coupling of the 6-APA and the acyl (R)-PGME side chain catalyzed by the iPGA. The total time required to produce ampicillin is then 1350 min.

2.5 Filtration of the reaction mixture

After completion of the reaction, the beaker containing the reaction mixture was moved under the hood, and a few drops of the previously prepared H2SO4 strong acid solution were added to this beaker in order to block the reaction completely and prevent the degradation of ampicillin. Then, using a glass container and a funnel with a sterile filter paper, the filtration was carried out in order to remove the enzyme from the filtrate (Figure 17).

Figure 17: Filtration of the reaction mixture

After that, a few eppendorf tubes were filled with 1 mL of filtrate each using a pipette. This first filtrate obtained will be called "**filtrate 1**" in the remainder of this work. The eppendorf tubes were subsequently used with the qualification and quantification tests in order to test the efficacy of the ampicillin produced in this **filtrate 1**, against *E. coli*, which is a strain sensitive to ampicillin. These tubes were kept cold until they were used.

2.6 Purification and harvesting of ampicillin produced

To the remainder of the filtrate, the ethyl acetate solvent was added with a different volume between the 1st and the 2nd experiment:

- **In experiment 1:** The remaining filtrate volume was 22.4 mL. Ethyl acetate has

  was added according to the ratio 14:2, thus: 14 mL of filtrate require 2 mL of solvent; therefore, for 22.4 mL of filtrate, a volume of 3.2 mL of this solvent was added to the container containing the filtrate.

- **In experiment 2:** The remaining filtrate volume was 6 mL. Ethyl acetate was

  added according to the ratio v:v, therefore 6 mL of filtrate require 6 mL of solvent. A volume of 6 ml of solvent was then added to the filtrate.

This step was carried out in order to make the ampicillin produced soluble in the ethyl acetate solvent in order to recover it subsequently in the organic phase only. Following the addition of this solvent, the mixture was divided into two phases: the aqueous phase and the organic phase. The mixture was then transferred to a glass tube in order to better visualize the separation (FIG. 18).

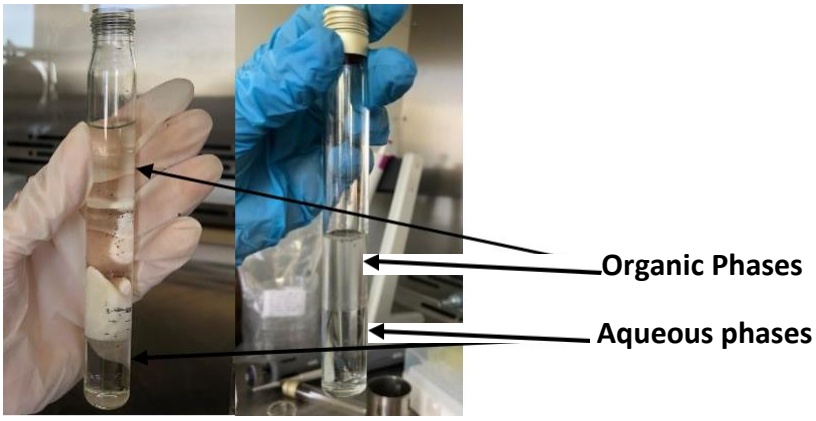

Figure 18: Separation of the reaction mixture into organic (top) and aqueous (bottom) phases from experiment 1 (left) and experiment 2 (right)

Then, using a pipette fitted with a pro-pipette, the organic phase was removed and transferred to a new tube. The aqueous phase, which should be eliminated, was also preserved and tested later for curiosity. Thus, from the aqueous and organic phases, eppendorf tubes were filled, each approximately 1 mL, using a graduated pipette equipped with a pro-pipette. These tubes were kept cold for later use in qualification and quantification tests to test the presence and efficacy of ampicillin in these phases.

The next step is to recover **pure ampicillin** from the remaining organic phase, while adding sodium bicarbonate to this phase. Following its addition, the crystallization process will take place finally producing pure ampicillin powder form (solid phase). **This step was carried out only in experiment 1.** Indeed, sodium bicarbonate was added with a ratio of 10:1.44; thus, 10 mL of the organic phase require 1.44 g of sodium bicarbonate (powder), therefore for 1.4 mL of the organic phase, a mass of 0.2016 g of sodium bicarbonate was added, and then the tube was preserved

à cold for 24 h until the crystallization process is complete. Following its completion, a white deposit was noticed at the bottom of the tube, thus the last step to be carried out

experiment 1 consists in filtering and drying the residue of the glass tube containing the organic phase (fig. 19), in order to obtain ampicillin powder (fig. 20).

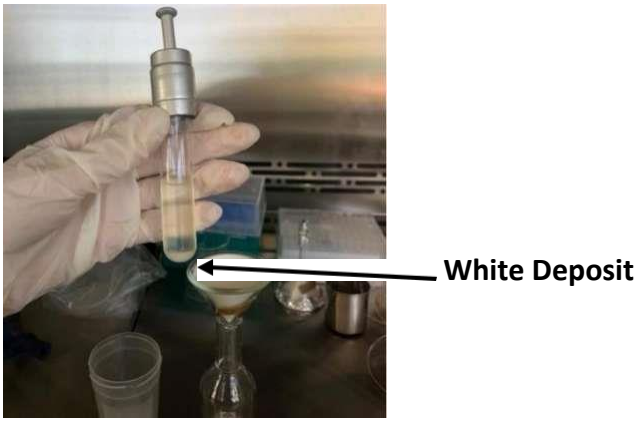

Figure 19: Filtration of the contents of the tube containing the organic phase following the crystallization process

Figure 20: Harvesting the Ampicillin Powder Produced

In order to preserve the efficacy of the antibiotic, the ampicillin powder was dissolved in 3 mL of the previously prepared potassium phosphate buffer solution (pH=7; C=0.1M). The last step of this **experiment 1** consists in carrying out the filtration, the filtrate obtained has been named **filtrate 2**. This filtrate was then transferred into 2 eppendorf tubes of volume 1 mL

each, which has been kept cold, and subsequently used to quantify ampicillin in this filtrate (figure 21).

Figure 21: Eppendofs tubes containing filtrate 2 from the 1st experiment

All the above-mentioned steps following that of the addition of the ethyl acetate solvent, therefore starting from the sodium bicarbonate addition step, were not carried out in the 2nd experiment.

2.7 Qualification testing of ampicillin produced

A. <u>Qualitative antibiogram</u>

This test was carried out **only for experiment 1**. The aim is to test the efficacy of ampicillin produced against *E.coli*, which is a strain susceptible to ampicillin. Thus, a qualitative antibiogram was carried out, just to see the appearance or not of an inhibition zone

bacterial growth following the application of: **filtrate 1**, the organic phase and the aqueous phase, which have been stored cold in eppendorf tubes, on petri dishes containing the Mueller-Hinton medium (prepared in advance).

For this purpose, a well-identified *E.coli* bacterial strain (ATCC 25922) was used (Figure 22).

Figure 22: Well-identified bacterial strain of E. coli (ATCC 25922)

Using a sterile loop, a bacterial colony was taken from this strain and grown on a petri dish containing the standard agar medium prepared in advance. The culture was ready 24 h after incubation and thus bacterial regeneration was carried out. From this bacterial culture of E. coli, an isolated colony was collected using a sterile loop, the loop was then soaked in a sterile saline solution, NaCl 0.9% (hence 9 g of NaCl in 100 mL of distilled water), and the contents of the tube were well mixed. The turbidity of this tube was compared with that of the McFarland Standard tube (containing a standard 0.5 MF $BaCl_2$ solution).

The same turbidity should be obtained by adding more colonies if the suspension is too light or by diluting with a sterile saline solution, 0.9% NaCl (already prepared in a separate tube), if the suspension is too heavy, until this condition is met (Figure 23).

Figure 23: Comparison of turbidity between the bacterial suspension tube (right) and the standard McFarland tube (left).

A sterile swab was soaked in the bacterial suspension tube. Press firmly against the inner wall of the tube just above the liquid level and turn the swab to remove excess liquids. Then the spreading was done three times, turning the can at about 60°C after each application to obtain an equal distribution of the inoculum over the entire surface of the Mueller-Hinton agar. Finally, swabs were performed all around the edge of the agar surface. These steps were carried out for 3 petri dishes. The cans were inverted and each divided into 2 parts: a box for the comparison between the **filtrate 1** and the organic phase (1^{st} box); a second for the comparison between the **filtrate 1** and the aqueous phase (2^{th} box) and finally a control box. After that, the disks were deposited using a sterile clamp. In addition, a 20 L volume of the eppendorf tube containing the filtrate 1 was deposited on the disk at this filtrate of the 1^{st} and 2^{th} cans, respectively, by means of a pipette; a 20 L volume of the eppendorf tube containing the organic phase was taken and then deposited on the disk corresponding to this phase on the 1^{st} cans and finally, a 20 L volume of the eppendorf tube containing the aqueous phase was taken and then deposited on the disk corresponding to this phase on the 2^{nd} box (Figure 24). The 3 boxes were then placed in the incubator for 18 to 24 h at 37°C.

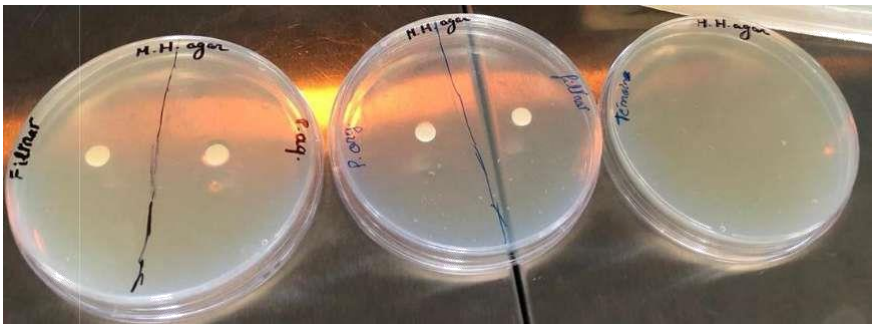

Figure 24: Qualitative antibiogram for filtrate 1, aqueous phase and organic phase of experiment 1 on petri dishes containing Mueller Hinton agar

B. <u>Sensitivity test</u>

This test was carried out **only for experiment 2**. The aim is also to test the presence and efficacy of ampicillin produced against *E.coli*, an ampicillin-sensitive strain.

The standard agar medium was prepared in advance and poured into 3 petri dishes. On the first box, the **filtrate 1** (which is the only filtrate obtained in this experiment) stored in the eppendorf tube was poured. You have to move the box to cover the whole middle. After 2 to 3 minutes, the excess filtrate was discarded from the surface, then, using a sterile loop, a colony of *E. coli* was taken from a bacterial culture already prepared, and was striated on the surface of the medium. Spreading was carried out from the top of the box downwards, covering the entire surface. In the same way, the two other cans, one corresponding to the organic phase and the other to the aqueous phase, were prepared. The cans were then placed in the incubator for 18 to 24 h at 37°C.

2.8 Quantification Tests for Ampicillin Produced

The quantification of the ampicillin produced was carried out using the disk diffusion method, the principle of which is as follows: Cellulose disks, impregnated with a given quantity of an antibiotic, are placed on an agar, previously inoculated by the strain

à test (in our case, an *E.coli* strain). The antibiotic diffuses from the disk into the agar, with a decreasing concentration gradient, from the disk to the periphery. After incubation for 18 hours at 24 hours at 37° C., a measurement of the zones of inhibition of bacterial growth is carried out for each of the disks. The larger the diameter, the more sensitive the bacterium is to the antibiotic tested. This method is illustrated in the following figure:

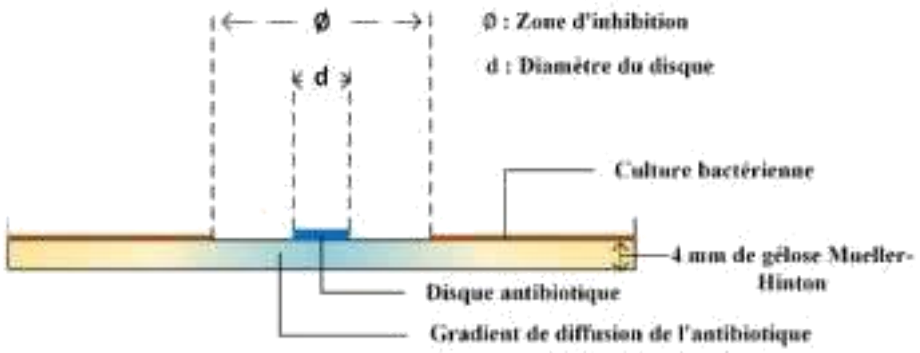

Figure 25: Disk Broadcast Principle [90]

A. Preparation of a standard ampicillin solution and carrying out a series of dilutions

To quantify our ampicillin produced, first of all, it is necessary to have a standard ampicillin solution that will be used as a standard to draw a standard curve. Thus, this solution was first prepared by dissolving 1 g of commercial ampicillin (powder) in 5 ml of the previously prepared potassium phosphate solution (pH=7; C=0.1M). The solution obtained is a standard ampicillin solution of 200 mg/ml. This ampicillin solution was then diluted 6 times to obtain the different concentrations: 20; 15; 12; 10; 8 and 5 mg/ml.

There are 6 sterile glass tubes, each containing a specific volume of distilled water. The series of dilutions is carried out from one tube to another (as explained below), starting from the eppendorf tube containing the standard ampicillin solution (C=200 mg/ml).

- According to the following equation, the calculation is carried out:
  =

  Where Cf: final concentration desired; Ci: initial concentration and f: dilution factor

- **Eppendorf tube** (containing standard ampicillin solution): = 200 /
- **1st tube**: =so 20 / ~~= 200~~ / then
  $= 200^{20} = 10^{1}$

  Thus, **1 mL of the eppendorf tube is taken and transferred into the first tube containing 9 mL of distilled water**; Final volume: 10 mL

- **2nd tube**: =so 15 / = 20 / then
  = 15 20 = 7 10 5

  Thus, **7.5 mL of the first tube are taken and transferred into the 2nd tube containing 2.5 mL of distilled water**; Final volume: 10 mL
- **3rd tube**: =so 12 / = 15 / then
  = 12 15 = 7 6 .5

  Therefore **6 mL of the 2nd tube are taken and transferred into the 3rd tube containing 1.5 mL** distilled water; Final volume: 7.5 mL
- **4th tube**: =therefore 10 /=12 / then
  = 10 12 = 5 6

  Thus, **5 mL of the 3rd tube are taken and transferred into the 4th tube containing 1 mL of distilled water**; Final volume: 6 mL
- **5th tube**: =so 8 / = 10 / then
  =10 8 =4 5

  Thus, **4 mL of the 4th tube are taken and transferred into the 5th tube containing 1 mL of distilled water**; Final volume: 5 mL
- **6th tube**: =so 5 / =8 / then
  = 5 8 = 2 4 .5

  Thus, **2.5 mL of the 5th tube are taken and transferred into the 6th tube containing 1.5 mL of distilled water**; Final volume: 4 mL

Thus, the concentrations 20; 15; 12; 10; 8; 5 mg/ml necessary for carrying out the antibiogram are ready.

B. Preparation of the inoculum

Using a sterile loop, 3-5 colonies were collected from colonies isolated from an already prepared *E.coli* bacterial culture. The loop was then introduced into a tube containing 2 mL of a sterile saline solution, 0.9% NaCl. The turbidity of this suspension was calibrated to 0.5 MF when compared to a McFarland Standard tube. The same turbidity must be obtained, in

adding more colonies if the suspension is too light or diluting with a sterile saline solution, NaCl 0.9% (placed in a separate tube), if the suspension is too heavy, until the

à this condition. The swab was then immersed in the suspension. Firmly press against the inner wall of the tube just above the liquid level and rotate

swab to remove excess liquids. Spreading was carried out three times on the entire surface of the Mueller Hinton agar (already prepared) of each dish, turning at about 60° C. after each application to obtain an equal distribution of the inoculum. Finally, swabs were performed all around the edge of the agar surface.

The boxes were overturned and divided into several regions, each of which was copied. **In this stage, there is again a difference between the 1st and the 2nd experience:**

- The test for quantification of the ampicillin produced was carried out first for the **filtrate 2** of **the experiment 1**; and then for the **filtrate 1** and the organic and aqueous phases. Whereas for **experiment 2**, the quantification of the ampicillin produced was carried out for the **filtrate 1** and the organic and aqueous phases. Thus:
  - **Quantification of filtrate 2 from experiment 1**: The boxes were divided into 4 regions:

    3 for known concentrations and 1 for unknown ampicillin concentration in **filtrate 2.** The 1st can and its copy were then divided for the concentrations: 20, 15, 12 mg/mL and the unknown concentration of ampicillin in **filtrate 2**. The second box and its copy for concentrations 10, 8, 5 mg/mL as well as the unknown concentration of ampicillin in **filtrate 2**.
  - **Quantification of filtrate 1 and the organic and aqueous phases of experiment 1 and experiment 2:**

    One can was divided into 4 regions for concentrations 20, 15, 12 mg/mL and the unknown concentration of ampicillin in **filtrate 1** (same for its copy can); and one can was divided into 5 regions for concentrations 10, 8, 5 mg/mL and the unknown concentration of ampicillin in the aqueous phase and in the organic phase (same for its copy can).

The boxes are allowed to stand at room temperature for at least 3-5 minutes to dry the agar plate surface before proceeding to the next step.

C. Preparing disks

Using a sterile clamp, the disks were distributed into the boxes. Using a pipette, a volume of 20 L was taken each time from the desired concentration (20; 15; 12; 10; 8; 5 mg/ml, filtrate 1, filtrate 2, organic phase, aqueous phase) and deposited on the corresponding disk in the box. Once all the disks were in place, the boxes were closed, inverted and placed in the incubator at 37°C for 18 to 24 hours.

D. Quantification of ampicillin produced

After incubation, the inhibiting zones that appeared were measured using a ruler. Next, a graph showing the ampicillin concentration as a function of the diameter of the inhibition zone (Log C as a function of the diameter) was drawn in order to be able to quantify the ampicillin produced.

CHAPTER III: RESULTS AND DISCUSSION

1. Results of the experiment 1

1.1 Qualification Test for Produced Ampicillin

The result of the qualitative antibiogram shows the appearance of a zone of inhibition of bacterial growth with the **filtrate 1**, the aqueous phase and the organic phase. This confirms the presence of ampicillin produced in these 3 phases, as well as its efficacy since it inhibited the bacterial growth of *E. coli* contrary to the result obtained in the control box where there was normal bacterial growth (FIG. 26).

Figure 26: Result of the qualitative antibiogram of filtrate 1, the aqueous phase and the organic phase of experiment 1

1.2 Quantification Tests for Ampicillin Produced

A. <u>Quantification of ampicillin produced in filtrate 2:</u>

Firstly, the quantification test for the ampicillin produced was carried out for filtrate 2. The antibiogram showed the appearance of bacterial growth inhibition zones with all concentrations (20; 15; 12; 10; 8 and 5 mg/ml) obtained from the dilution of the standard ampicillin solution (C=200 mg/ml), however no inhibition zone appeared with **filtrate 2** (Figure 27).

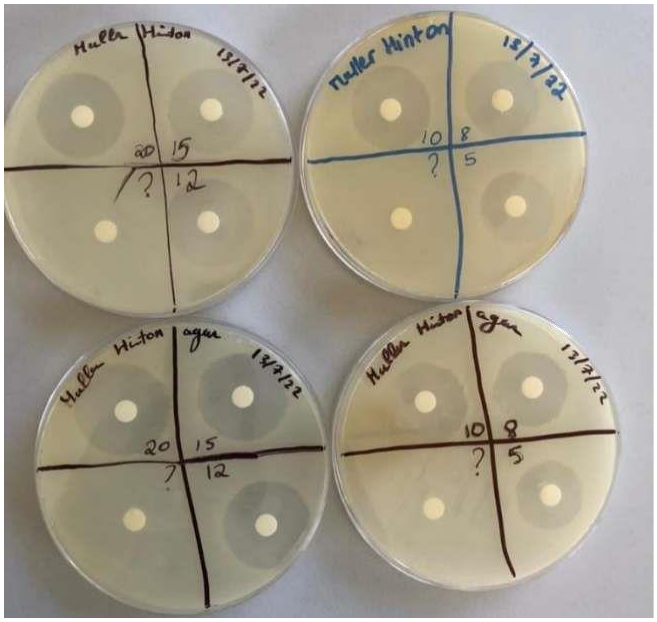

Figure 27: Result of the Quantitative Antibiogram of the filtrate 2 of Experiment 1

On the basis of the above-mentioned results, the 2th experiment was limited to carrying out the qualification and quantification tests just for the **1 st filtrate**, obtained following the production and filtration of the reaction mixture, and for the aqueous and organic phases obtained following the first step of the purification which consists in adding the ethyl acetate solvent.

Thus, for the 2nd experiment, qualification and quantification tests were carried out after each stage directly, without going through the process of obtaining **the ampicillin produced pure** from the organic phase which requires adding the sodium bicarbonate to this phase, carrying out the filtration and recovering the ampicillin powder, which in turn must be dissolved in the buffer solution in order to preserve its effectiveness. Also, the 2nd experiment was carried out by adjusting a parameter of the purification of the ampicillin produced, which is the increase in the volume of solvent added, with the aim of optimizing the purification and recovering the ampicillin produced only in the organic phase.

B. <u>Quantification of the ampicillin produced in filtrate 1 and the organic and aqueous phases:</u>

The antibiogram showed the appearance of bacterial growth inhibition zones at all concentrations (20; 15; 12; 10; 8 and 5 mg/ml) obtained from the dilution of standard ampicillin solution, as well as the appearance of growth inhibition zones

bacterial cells which are reduced, with **filtrate 1**, and the aqueous and organic phases. The same result was obtained for each copy box (FIG. 28).

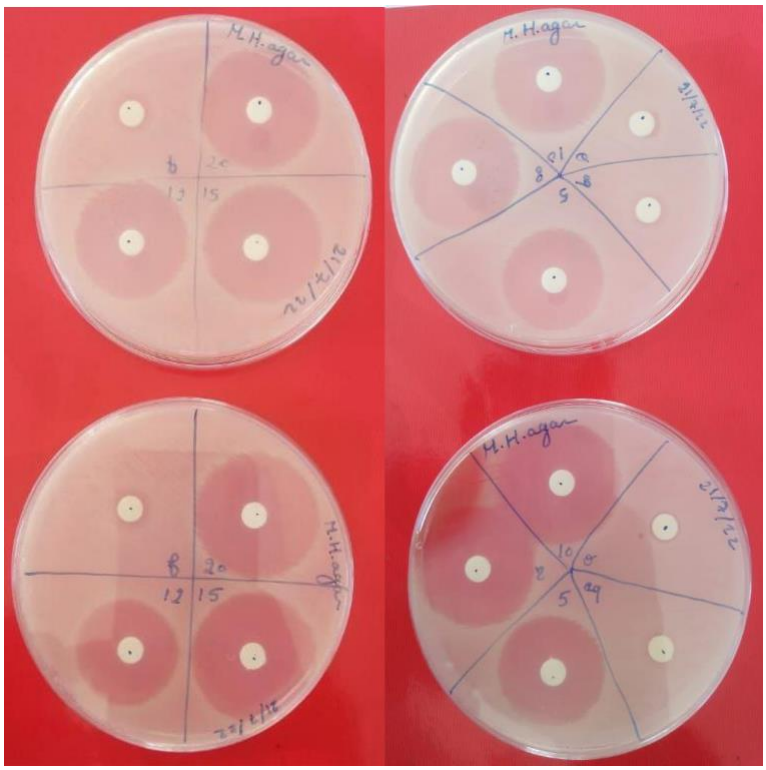

FIG. 28: Result of the quantitative antibiogram of filtrate 1 and of the aqueous and organic phases of experiment 1

Subsequently, the diameters of the inhibition zones relative to the different concentrations of standard dilute ampicillin, as well as those of filtrate 1 and of the organic and aqueous phases were measured by means of a ruler in each box and its copy, and then the mean was calculated (Table 3):

Table 3: Measurements of diameters of bacterial growth inhibition zones for different concentrations of standard dilute ampicillin and filtrate 1, aqueous phase and organic phase of experiment 1

Concentration (mg/ml)	**20**	**15**	**12**	**10**	**8**	**5**	**Filtrate 1**	**Phase organic**	**Phase watery**
Log C	1.3.	1.18	1.08	1	0.9	0.69	?	?	?
Diameter (cm)	$\frac{3.1+3}{2} = 3.05$	$\frac{2.9+3.1}{2} = 3$	$\frac{2.8+2.5}{2} = 2.65$	$\frac{2.9+2.8}{2} = 2.85$	$\frac{2.8+2.8}{2} = 2.8$	$\frac{2.5+2.6}{2} = 2.5$	$\frac{1+1}{2} = 1$	$\frac{0.8+0.8}{2} = 0.8$	$\frac{0.8+0.8}{2} = 0.8$

Next, the graph showing the concentration of ampicillin as a function of the diameter of the inhibition zone (Log C as a function of diameter) was drawn in order to be able to quantify the ampicillin produced in filtrate 1, the organic phase and the aqueous phase (FIG. 29):

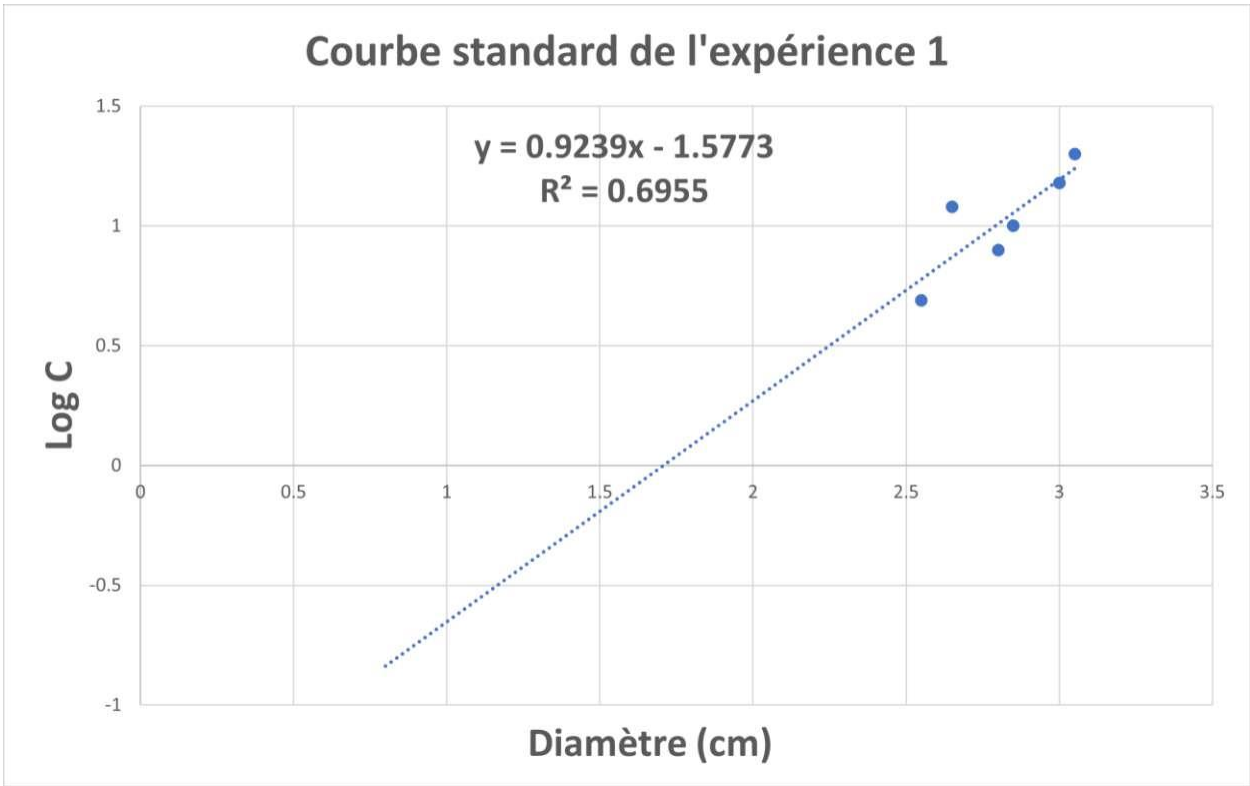

Figure 29: Graph showing the variation in ampicillin concentration (Log C) as a function of the diameter of the inhibition zone, corresponding to experiment 1

From the following equation the concentration of the ampicillin produced in **filtrate 1**, the aqueous phase and the organic phase is determined:

= 0.9239 - 1.5773

- For **filtrate 1**:= 0.9239 1 - 1.5773 = -0.6534; = 0.222 /
- For organic and aqueous phases having the same diameter of their inhibition zones:
  = 0.9239 0.8 - 1.5773 = -0.83818; = 0.145 /

2. Results of the experiment 2

2.1 Qualification Test for Produced Ampicillin

The result of the bacterial sensitivity test carried out for **filtrate 1** (the only filtrate obtained in this experiment) and the aqueous and organic phases shows an inhibition of the bacterial growth thus confirming again the presence of ampicillin in filtrate 1 (FIG. 30) and in the other two phases (FIG. 31), as well as its effectiveness.

Figure 30: Bacterial susceptibility test to ampicillin contained in the filtrate 1 of experiment 2

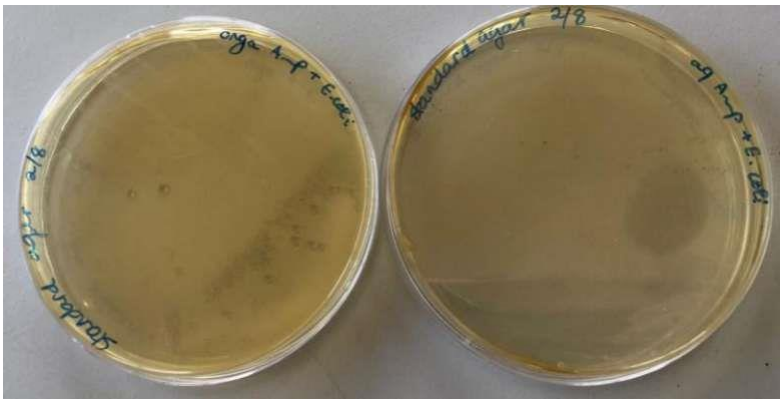

Figure 31: Bacterial susceptibility test to ampicillin contained in the organic and aqueous phase of experiment 2

The purification has not been improved since ampicillin is always obtained in the aqueous phase.

2.2 Quantification Test for Ampicillin Produced

Also in this experiment, the antibiogram showed the appearance of the bacterial growth inhibition zones with all the concentrations (20; 15; 12; 10; 8 and 5 mg/ml) obtained

à from the dilution of the standard ampicillin solution, as well as the appearance of reduced bacterial growth inhibition zones with filtrate 1 and the aqueous and organic phases. The same result was obtained for each copy box (FIG. 32).

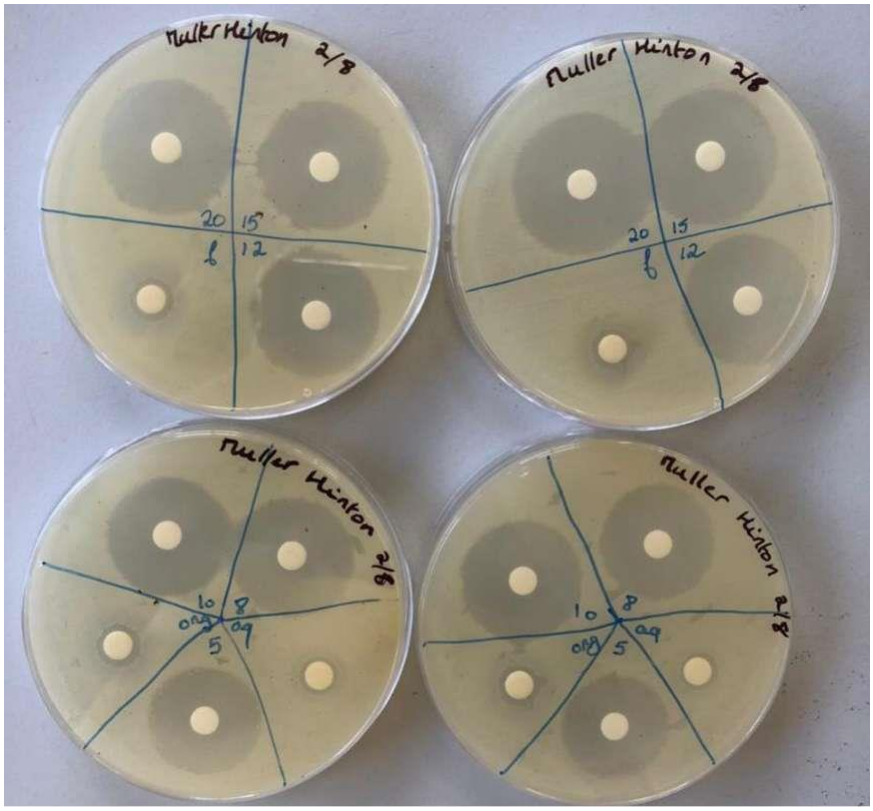

FIG. 32: Result of the quantitative antibiogram of filtrate 1 and of the organic and aqueous phases of experiment 2

Then, in order to quantify the ampicillin produced in each of these phases, the diameters of the inhibition zones relating to the different concentrations of standard diluted ampicillin as well as those of **filtrate 1** and of the organic and aqueous phases were measured by means of a ruler in each box and its copy, and then the mean was calculated (Table 4):

Table 4: Measurements of diameters of bacterial growth inhibition zones for different concentrations of standard dilute ampicillin and filtrate 1, aqueous phase and organic phases of experiment 2

Concentration (mg/ml)	20	15	12	10	8	5	Filtrate 1	Phase organic	Phase watery
Log C	1.3.	1.18	1.08	1	0.9	0.69	-	?	?
Diameter (cm)	$\frac{3+3}{2} = 3$	$\frac{2.9+2.85}{2} = 2,875$ (≈ 2.9)	$\frac{2.8+2.85}{2} = 2,825$ (≈ 2.8)	$\frac{2.7+2.65}{2} = 2,675$ (≈ 2.7)	$\frac{2.65+2.6}{2} = 2,625$ (≈ 2.6)	$\frac{2.5+2.4}{2} = 2.45$	$\frac{1.3+1.2}{2} = 1.25$	$\frac{1+1}{2} = 1$	$\frac{0.9+0.9}{2} = 0.9$

Next, the graph showing the ampicillin concentration as a function of the diameter of the inhibition zone (Log C as a function of the diameter) was drawn (FIG. 33):

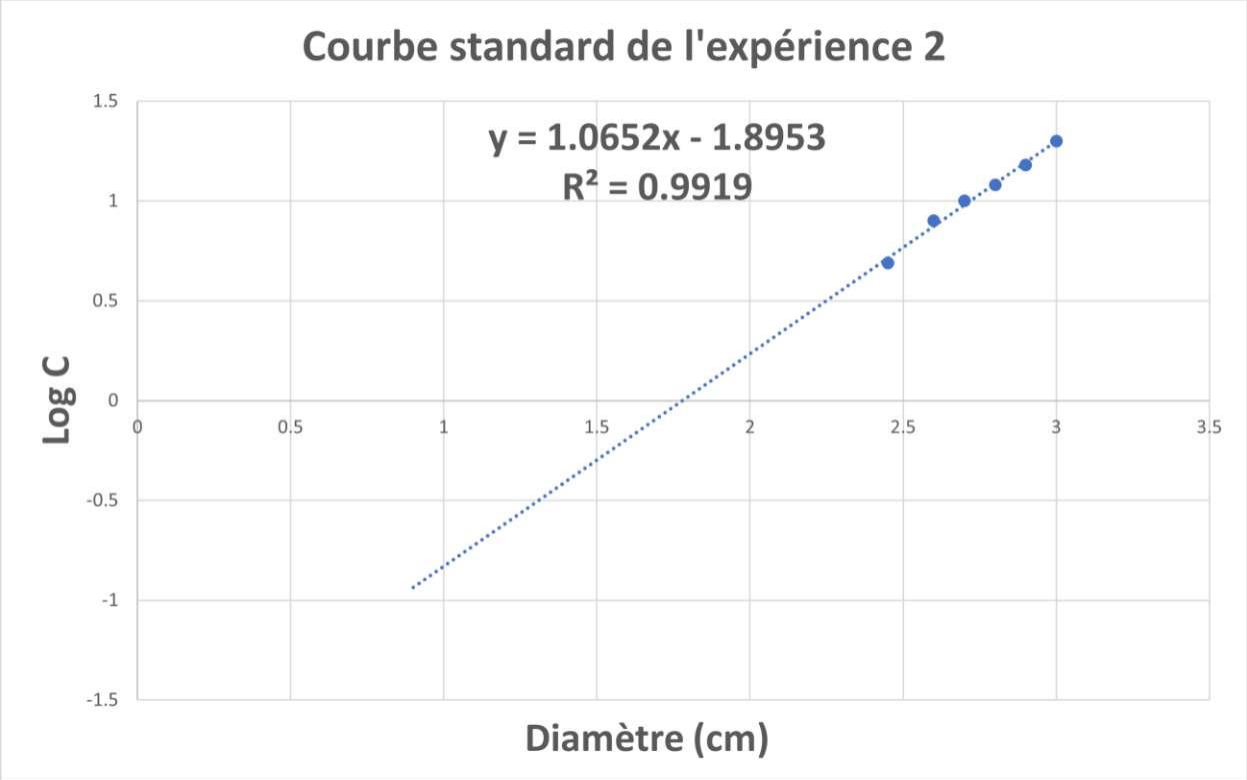

Figure 33: Graph showing the variation in ampicillin concentration (Log C) as a function of the diameter of the inhibition zone, corresponding to experiment 2

The concentration of ampicillin produced in filtrate 1, the aqueous phase and the organic phase is determined from the following equation:

$= 1.0652 - 1.8953$

- For **filtrate 1**:= 1.0652 1.25 - 1.8953 = - 0.5638; = 0.27 /
- For the organic phase: = 1.0652 1 - 1.8953 = - 0.8301 then = 0.15 /
- For the aqueous phase: = 1.0652 0.9 - 1.8953 = - 0.93662 then = 0.12 /

3. Discussion

Ampicillin is one of the most widely used antibiotics in therapy because it is suitable for a wide spectrum of bacterial infections and a good level of activity and tolerance. To date, numerous developments have been acquired in the enzymatic synthesis of ampicillin and the kinetically controlled strategy has proved effective [13],[91]. The aim of this work is to carry out cascade conversion with two biocatalytic reactions: the hydrolysis of penicillin G, and the kinetically controlled enzymatic synthesis catalyzed by penicillin G acylase immobilized from the (R)-phenylglycine methyl ester side chain and 6-aminopenicillanic acid containing the core of the lactam, in order to synthesize ampicillin in a fully aqueous medium according to the two-step, one-pot synthesis method. The aim was then to quantify the ampicillin produced.

With regard to our ^{first} production test for ampicillin **(experiment 1)**, the qualification test for the ampicillin produced confirmed the presence of this antibiotic in **filtrate 1**, the aqueous phase and the organic phase. However, the appearance of a zone of inhibition of bacterial growth with the aqueous phase was a surprising and unexpected result. Indeed, this result suggests the possibility of having a problem in the purification, since the addition of ethyl acetate was carried out in order to make the ampicillin produced soluble in this solvent, which should then extract the ampicillin from the aqueous phase and transfer it to the organic phase, thus making it possible to **recover this ampicillin only in the organic phase**. Thus, a loss of the amount of ampicillin produced in the aqueous phase is predicted. On the other hand, since the ampicillin produced was present in the **filtrate 1** and the aqueous and organic phases, its presence in the filtrate 2 which must contain the purified produced ampicillin was predicted, so it went directly to the stage of its quantification, without carrying out a qualification test for this filtrate 2. In this context, the antibiogram carried out showed the absence of the ampicillin produced in **filtrate 2.** In fact, this result was also unexpected since **filtrate 2** is supposed to contain **the pure produced ampicillin**, unlike **filtrate 1** which contains the unpurified produced ampicillin, and it is for this reason that attempts were made to quantify the produced ampicillin contained in **filtrate 2** first. However, this result could be explained as follows:

➢ In the light of the result of the qualitative antibiogram of this first experiment, a small diameter of the inhibition zone corresponding to the **filtrate 1 is** noted, which already reflects a low concentration of ampicillin produced in the reaction. On the other hand, since the volume of the reaction mixture was so small, this suggests that the amount of ampicillin produced may be small. Furthermore, the problem in the first stage of purification, that of adding the ethyl acetate solvent, seems to cause, from our point of view, the distribution of the ampicillin produced between the aqueous phase and the organic phase, thus further reducing the amount of ampicillin that should be obtained in the organic phase, which seems to lead to a reduction in the concentration of ampicillin produced in this phase, compared with a normal case where there is no purification problem. This seems to be logical, given the diameter of the inhibition zone corresponding to the organic phase which is reduced, or even smaller than that of the **filtrate 1**. In recalling the process of obtaining the **2nd filtrate**, which is a rather lengthy process: given that it was from the organic phase that the second filtrate was obtained, following the crystallization process with the addition of sodium bicarbonate to this phase, filtration, recovery of the pure ampicillin in powder form, dissolution of this powder in the potassium phosphate buffer solution and finally filtration again which gave this **filtrate 2,** it is noted that there has been a **significant dilution,** in our opinion, of the concentration of the pure ampicillin produced from the organic phase, and this dilution has taken place more precisely with the stage of dissolution of the pure powder obtained with the potassium phosphate buffer solution, which could be the origin of the obtaining a concentration of pure ampicillin in **filtrate 2 which is even** lower than the concentration of ampicillin in the organic phase. It seems to us that the concentration of pure ampicillin contained in filtrate 2 was then very low, so that it became lower than the minimum concentration of inhibition of bacterial growth, which could explain the absence of the appearance of zone of inhibition of bacterial growth with **filtrate 2**.

Then, after obtaining a negative result with the test for quantification of the ampicillin produced in filtrate 2, and since the qualification test previously carried out for the filtrate

1 and the aqueous and organic phases confirmed the presence of the ampicillin produced in these phases, it was then sought to quantify the ampicillin produced in each of these phases. As a result, the concentration of the produced ampicillin obtained in the **filtrate 1** is low, and it is close to the sum of those obtained in the organic and aqueous phases. Our hypothesis, which has already been put forward as to the existence of a problem in the purification, which leads to the distribution of the produced ampicillin between the organic phase and the aqueous phase, thus seems to be logical, while recalling that the aqueous phase must not contain the ampicillin produced under normal conditions of the purification. Indeed, since the ethyl acetate solvent used was unable to extract the ampicillin from the aqueous phase to the organic phase, it was then necessary to carry out the experiment a second time by trying to optimize the 1^{st} stage of the purification, while increasing the volume of the solvent added to the
filtrate 1.

When our 2^{nd} experiment was carried out, the qualification test of filtrate 1 and of the aqueous and organic phases showed the presence of ampicillin in these phases. Thus, despite the adjustment of the volume of the solvent added, the problem in the purification of the ampicillin produced has not been solved since ampicillin is still obtained in the aqueous phase. Then, the quantitative test was carried out, the concentration of the ampicillin produced obtained in the **filtrate 1** is also low, and almost equal to the sum of those obtained in the organic and aqueous phases, a result similar to that obtained with the ^{1st} experiment. Next, it should be noted that the adjustment of a parameter in the purification of the ampicillin produced, which consists in increasing the volume of the ethyl acetate solvent added to the filtrate 1 of this 2^{th} experiment could not correct the problem of the purification since ampicillin is always obtained in the aqueous phase, the ampicillin produced seems to be always distributed between the organic and aqueous phase. However, the concentration of the ampicillin recovered in the organic phase was this time greater than that obtained in the aqueous phase, which is more logical. Also, the second experiment showed a better result with respect to the standard curve with R^2=0.9919 compared to that of the ^{first} experiment where R^2=0.6955. It can be concluded that the optimization which was carried out by adding a volume of solvent according to the ratio v:v in the ^{1st} stage of the purification improved the results, but these results do not deny

the assumption that the ethyl acetate solvent is unable to completely extract ampicillin from the aqueous phase. In this context, the production of ampicillin and the quantification of the ampicillin produced have been completed, but the purification and harvesting of this antibiotic have not been successful.

In 2003, Wei, Dong-Zhi and Liu Yang in their study on the effect of ethylene glycol on ampicillin synthesis using penicillin G acylase showed that the yield of ampicillin synthesis can be significantly improved in the presence of ethylene glycol. Ethylene glycol was found to increase ampicillin yield by 39-50%. Indeed, the yield of ampicillin synthesis depends on the three different kinetic processes catalyzed by penicillin G acylase: ampicillin synthesis and enzymatic hydrolysis reactions. Therefore, the prevention of hydrolysis of the acyl donor substrate (D-PGME) and the product (ampicillin) is desirable for increasing the yield of ampicillin synthesis. With this strategy, organic solvents, including ethylene glycol, are often added to the aqueous medium to suppress enzyme hydrolysis reactions [92].

In 2009, a one-pot, two-step enzymatic synthesis of ampicillin in aqueous phosphate buffer using ethylene glycol as solvent was demonstrated for the first time by Du, Li-Li, et al. The ethylene glycol solvent was added in the v:v ratio and the maximum yield was 57.3%. Thus, it has also been demonstrated that the use of ethylene glycol has improved the yield of the synthesis. We have not used this solvent, it is then desirable to carry out the synthesis of ampicillin subsequently following the cascade conversion, in one pot, two steps using this solvent [93].

In 2010, Blum, Janna K., et al. conducted a study on the synthesis of ampicillin using a two-enzyme cascade, using the enzyme 15-amino ester hydrolase (AEH) and penicillin G acylase. The one-pot, two-step synthesis system gave an optimal ampicillin yield of 46%. Maximum conversions were obtained in one to two hours, significantly reducing the reaction times previously observed in systems using iPGA and ethylene glycol. It has been shown that the two-enzyme system with the iPGA

and AEH outperformed systems that used only the iPGA, reporting the obvious benefit of using the AEH enzyme. In fact, AEHs are unique in their specificity to the phenyl-amine groups on the acyl moiety so that they cannot catalyze the hydrolysis of penicillin G to give 6-APA and are not inhibited by PAA, hence their advantage in this cascade [94].

In short, the cascade conversion was carried out with two biocatalytic reactions in a fully aqueous medium to synthesize ampicillin, following the two-step, one-pot synthesis method catalyzed by the iPGA. In light of these data, our work could be improved with the use of the ethylene glycol co-solvent or by performing the cascade

à two enzymes using iPGA and AEH at the same time, in order to be able to increase the synthesis yield.

CHAPTER IV: CONCLUSIONS AND PROSPECTS

Semi-synthetic Lactamine antibiotics are one of the most important families of antibiotics in the global market. Among these antibiotics, ampicillin has a wide range of actions in the prevention and treatment of various bacterial diseases. Nowadays, the chemical synthesis of this antibiotic has dominated industrial production due to the high yield obtained, but enzymatic synthesis is an environmentally friendly alternative. Since kinetically controlled enzyme synthesis provides a higher yield than thermodynamically controlled enzyme synthesis, numerous approaches have been reported to improve the efficiency of kinetically controlled enzyme synthesis of ampicillin.

In our present study, we performed cascade conversion with two biocatalytic reactions: the hydrolysis of penicillin G and the kinetically controlled enzymatic synthesis of ampicillin, in a fully aqueous medium to synthesize ampicillin, following the two-step one-pot synthesis method, knowing that the biocatalyst was immobilized penicillin G acylase only. In the step of purification of the reaction mixture, the solvent used was ethyl acetate. The results obtained confirm the completion of the production of ampicillin and the ampicillin produced was then quantified, but the purification and harvesting of ampicillin were not successful. However, our study is a promising alternative to current ampicillin synthesis methods such as: thermodynamic synthesis, one-pot synthesis, one step...

On the other hand, this work could be improved by adding organic solvents, such as ethylene glycol, to the aqueous medium to suppress enzymatic hydrolysis reactions, thus contributing to the increase in the yield of ampicillin synthesis. Moreover, since cascade conversion with two reactions, catalyzed by both iPGA and AEH, outperformed systems using only iPGA, the use of AEH has been shown to be very advantageous since it is not inhibited by the PAA, hence the need for its use. In this context, future tests for the production of ampicillin in an aqueous medium using the co-solvent ethylene glycol,

or performing the two-enzyme cascade using both iPGA and AEH, may lead to a much higher yield.

Finally, given the importance of ampicillin in the treatment of various bacterial diseases, and given that it is inactivated by penicillinases, this work opens the doors to other perspectives such as combining this antibiotic with natural antibiotics, such as oil, or honey, to prevent resistance to ampicillin and reduce the therapeutic dose of this antibiotic.

LIST OF ABBREVIATIONS

- DNA: Deoxyribonucleic acid
- Aecenar: Association for Economic and Technological Cooperation in the Euro-Asian and North African Region
- AEH: Cl-amino ester hydrolase
- AMP: Ampicillin
- RNA: Ribonucleic acid
- mRNA: Messenger ribonucleic acid
- tRNA: Transfer ribonucleic acid
- D-ala: D-alanine
- DAla-DAla: D-alanyl-D-alanine
- INN: International non-proprietary name
- D-Glu: D-glutamine
- DHF: Dihydrofolic acid
- DHPS: Dihydropteroate synthetase
- D-PGME: D-phenylglycine methyl ester hydrochloride
- *E. coli: Escherichia coli*
- Example
- *H. influenzae: Haemophilus influenzae*
- iPGA: Penicillin G immobilized acylase
- L-Ala: L-alanine
- L-lys: L-lysine
- Lipopolysaccharide
- MEGBI: Middle East Institute of Genetics and Biotechnology
- MeOH: Methanol
- NAG: N-acetylglucosamine
- NAM: N-Acetylmuramic acid

- *N. gonorrhoeae: Neisseria gonorrhoeae*
- *N. meningitidis: Neisseria meningitidis*
- OM: External membrane
- PAA: Phenylacetic acid
- PABA: Para-amino-benzoic acid
- PBP: Penicillin binding protein
- *P. chrysogenum*: Penicillium chrysogenum
- PENG: Penicillin G
- PG: Peptidoglycan
- PGA: Penicillin G acylase
- pH: Hydrogen potential
- *Penicillium notatum*
- *P. rubens: Penicillium rubens*
- (R)-PG: (R)-phenylglycine methyl ester
- (R)-PGME: (R)-phenylglycine methyl ester hydrochloride
- *S. aureus: Staphylococcus aureus*
- Spp: Cash
- S: Svedberg
- THF: Tetrahydrofolic acid
- *T. pallidum: Treponema pallidium*
- 1P1S: 1 pot, one step
- 1P2S: 1 pot, 2 steps (one-pot, two-steps)
- 6-APA: 6-aminopenicillanic acid

LIST OF SYMBOLS

- Beta
- +: Positive
- -: Negative
- {g}:Microgram
- Cu mol: Micromole
- IPCC: Alpha
- %: Percent
- °C: Degree Celsius
- IV: Number four in Roman numerals
- II: Number two in Roman numerals
- L: liter
- M: Molar
- Ml: Milliliter
- Min: Minute
- v:v: Volume to Volume Ratio
- MF: MacFarland
- Mg/ml: Milligram per Milliliter
- Cf: Final concentration
- Ci: Initial Concentration
- F: dilution factor
- cm: centimeter
- h: time
- g: Gram
- g/mol: Gram per mol
- ≈: About

LIST OF FIGURES

LIST OF TABLES

APPENDICES

1. Protocol for the preparation of potassium phosphate buffer solution

The preparation of a volume equal to 0.5 L of a potassium phosphate buffer solution, pH=7 and concentration 0.1 M, requires the use of the following components:

Table 5: Essential components for the preparation of potassium phosphate buffer solution

Components	Quantity	Concentration
Phosphate of potassium dibasic (M =174.18 g/mol)	4 672 g	0.05364 M
Phosphate of Potassium monobasic (M=136.09 g/mol)	3 154 g	0.04636 M

Using a sterile sampling spatula, a mass of 4,672 g of dibasic potassium phosphate was taken and then measured using a digital balance. Similarly, using a new sterile sampling spatula, a mass of 3.154 g of monobasic potassium phosphate was taken and measured using the digital balance. These measured masses were poured into a beaker, distilled water was added and the whole was mixed using a glass stirring rod. The mixture was then poured into a 250 mL graduated test piece. Distilled water was poured to this volume of 250 mL. The mixture was then transferred to a 500 mL volumetric flask, where distilled water was added until it reached the mark. The flask was plugged and spilled several times to ensure good homogenization. The mixture obtained was poured into a suitable container and then autoclaved. After autoclaving, the buffer solution was ready for further use (Figure 34).

Figure 34: Potassium phosphate buffer solution

2. Protocols for the preparation of solutions necessary for pH adjustment of the reaction mixture

A. Preparation of the strong base solution, sodium hydroxide (NaOH):

A mass of 20 g of sodium hydroxide 'NaOH' was weighed using a digital balance. In a 500 mL volumetric flask, a volume of 250 mL of distilled water was poured, and NaOH was added to the same container. This reaction is exothermic, so any material used must be made of glass since the reaction releases heat when it occurs. The flask was immersed in a beaker filled with distilled water to cool it (Figure 35).

Figure 35: Cooling of Distilled Water and Sodium Hydroxide Mixture

Once cooled, distilled water was added to the gage line. The flask was then plugged and spilled several times to mix well and ensure complete dissolution of the NaOH. The solution obtained, 1M NaOH, was then stored in a glass container for any subsequent use (FIG. 36).

Figure 36: 1M sodium hydroxide solution

B. Preparation of the strong acid solution, sulfuric acid (H2SO4):

Using a pipette fitted with a propipette, a volume of 28 mL of 98% sulfuric acid (M=98.08 g/mol) was taken (FIG. 37), and then introduced into a 500 mL volumetric flask half-filled with distilled water.

Figure 37: 98% sulfuric acid sampling

The volumetric flask was then filled to the mark with distilled water. This flask was then placed in a beaker filled with distilled water to cool it down as it is an exothermic reaction. The flask was plugged and spilled several times to homogenize well. The sulfuric acid solution obtained, H2SO4 1M, was then poured into a glass container for further use (Figure 38).

Figure 38: 1M sulfuric acid solution

BIBLIOGRAPH

[1] R. Gaynes, "The Discovery of Penicillin—New Insights After More Than 75 Years of Clinical Use," *Emerg. Evil. Dis.*, vol. 23, no. 5, pp. 849-853, May 2017, doi: 10.3201/eid2305.161556.

[2] G. Kapoor, S. Saigal, and A. Elongavan, "Action and resistance mechanisms of antibiotics: A guide for clinicians," *J. Anaesthesiol. Clin. Pharmacol.*, vol. 33, no. 3, p. 300, 2017, doi: 10.4103/joacp.JOACP_349_15

[3] A. K. Chandel, L. V. Rao, M. L. Narasu, and O. V. Singh, "The realm of penicillin G acylase in anti-lactam antibiotics," *Enzyme Microb. Technol.*, vol. 42, no. 3, pp. 199-207, Feb. 2008, doi: 10.1016/j.enzmictec.2007.11.013.

[4] A. Bruggink, Ed., *Synthesis of [beta]-lactam antibiotics: chemistry, biocatalysis & process integration*. Dordrecht: Boston: Kluwer Academic Publishers, 2001

[5] K. J. Shah, K. Cherabuddi, J. Shultz, S. Borgert, R. Ramphal, and K. P. Klinker, "Ampicillin for the treatment of complicated urinary tract infections caused by vancomycin-resistant Enterococcus spp (VRE): a single-center university hospital experience," *Int. J. Antimicrob Agents*, Vol. 51, No. 1, pp. 57-61, Jan. 2018, doi: 10.1016/j.ijantimicag.2017.06.008.

[6] H. Lode, "Role of sultamicillin and ampicillin/sulbacam in the treatment of upper and lower bacterial respiratory tract infections," *Int. J. Antimicrob Agents*, vol. 18, no. 3, pp. 199-209, Sep. 2001, doi: 10.1016/S0924-8579(01)00387-9.

[7] M. T. Castro-Lainez *et al.*, "A rationale for combination ampicillin and daptomycin in renal transplant patients with enterococcal infective endocarditis," *IDCases*, vol. 14, p. e00460, 2018, doi: 10.1016/j.idcr.2018.e00460

[8] S.-K. Eng, P. Pusparajah, NS Ab Mutalib, HL. Ser, K.-G. Chan, and L.-H. Lee, "*Salmonella*: A review on pathogenesis, epidemiology and antibiotic resistance," *Front. Life Sci.*, vol. 8, no. 3, pp. 284-293, Jul. 2015, doi: 10.1080/21553769.2015.1051243.

[9] G. M. Pacifici, "Clinical Pharmacology of Ampicillin in Neonates and Infants: Effects and Pharmacokinetics," *Int. J. Pediatr.*, no. Online First, Oct. 2017, doi: 10.22038/ijp.2017.26942.2320.

[10] M. Akova, "Sulbacam-containing lactamase inhibitor combinations," *Clin. Microbiol. Infect.*, vol. 14, pp. 185-188, Jan. 2008, doi: 10.1111/j.1469-0691.2007.01847.x.

[11] K. Bush, "Beta-lactamase inhibitors from laboratory to clinic," *Clin. Microbiol. Rev.*, vol. 1, no. 1, pp. 109-123, Jan. 1988, doi: 10.1128/WRC.1.1.109.

[12] R. A. Sheldon, "E factors, green chemistry and catalysis: an odyssey," *Chem. Common. Camb. Engl.*, 29, pp. 3352-3365, Aug. 2008, doi: 10.1039/b803584a.

[13] M. A. Wegman, M. H. A. Janssen, F. van Rantwijk, and R. A. Sheldon, "Towards Biocatalytic Synthesis of Eco-Lactam Antibiotics," *Adv. Synth Catal.*, vol. 343, no. 6-7, pp. 559-576, Aug. 2001, doi: 10.1002/1615-4169(200108)343:6/7<559::AID-ADSC559>3.0.CO;2-Z.

[14] A. L. Deaguero, "Improving the enzymatic synthesis of semi-synthetic beta-lactam antibiotics via reaction engineering and data-driven protein engineering," School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, 2011

[15] A. D. Russell, "Types of Antibiotics and Synthetic Antimicrobial Agents," in *Hugo and Russell's Pharmaceutical Microbiology*, S. P. Denyer, N. A. Hodges and S. P. Gorman, Eds. Oxford, UK: Blackwell Science Ltd, 2004, pp. 152-186. doi: 10.1002/9780470988329.ch10.

[16] S. P. Denyer, N. A. Hodges, and S. P. Gorman, "Pharmaceutical Microbiology," p. 494.

[17] H. G. Schlegel and C. Zaborosch, *General Microbiology*. Cambridge University Press, 1993

[18] C. Walsh, *Antibiotics: actions, origins, resistance*. Washington, D.C: ASM Press, 2003.

[19] S. Morse, G. Brooks, and J. Butel, *Jawetz, Melnick & Adelberg's Medical Microbiology, 23rd edition*. 2004.

[20] H. Ludman, P. J. Bradley, and H. Ludman, Eds., *ABC of ear, nose and throat*, 5th ed. Malden, Mass: Blackwell Pub.: BMJ Books, 2007.
[21] D. Mohsaturn, "Classification and mode of action of antibiotics."
[22] S. Torshe, “Antibiotics,” Mentouri Brothers University CONSTANTINE 1, 2020 2019, p. 31.
[23] A. H. A. M. van Hoek, D. Mevius, B. Guerra, P. Mullany, A. P. Roberts, and H. J. M. Aarts, “Acquired Antibiotic Resistance Genes: An Overview,” *Front. Microbiol.*, vol. 2, p. 203, Sep. 2011, doi: 10.3389/fmicb.2011.00203
[24] U. Frank and E. Taconelli, *The Daschner Guide to in-hospital antibiotic therapy*. Springer, 2012.
[25] F. Adzitey, "Antibiotic Classes and Antibiotic Susceptibility of Bacterial Isolates from Selected Poultry; A Mini Review," *World Vet. J.*, vol. 6, no. 1, p. 36, 2015, doi: 10.5455/wvj.20150853.
[26] National Association of Teachers of Clinical Pharmacy, Ed., *Clinical and Therapeutic Pharmacy*, 5th ed. Issy-les-Moulineaux: Elsevier Masson, 2018
[27] “2018-01-11 -Antibiotics table.pdf.” Accessed: Sep. 09, 2022. [Online]. Available: https://www.antibio-responsable.fr/antibiotiques/-/media/2018-01-11%20-Tableau%20des%20antibiotiques.pdf
[28] K. P. Talaro and B. Chess, *Foundations in Microbiology*. McGraw-Hill Education, 2011.
[29] M. T. Madigan and J. M. Martinko, *Brock Biology of Microorganisms*. Pearson Prentice Hall, 2006
[30] G. D. Wright, "Q&A: Antibiotic resistance: where does it come from and what can we do about it?," *BMC Biol.*, vol. 8, no. 1, p. 123, Dec. 2010, doi: 10.1186/1741-7007-8-123.
[31] A. R. Hauser, *Antibiotic basics for clinicians: the ABCs of choosing the right antibacterial agent*, 2nd ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, 2013.
[32] G. Kapoor, S. Saigal, and A. Elongavan, "Action and resistance mechanisms of antibiotics: A guide for clinicians," *J. Anaesthesiol. Clin. Pharmacol.*, vol. 33, no. 3, p. 300, 2017, doi: 10.4103/joacp.JOACP_349_15
[33] A. Ghimire, “Bacterial Structure Explained in Simple,” *The Nepali Doctor*, Jun. 25, 2021. https://thenepalidoctor.com/bacterial-structure/ (accessed Sep. 11, 2022).
[34] P. M. Tulkens, “GENERAL PHARMACOLOGY OF ANTIBIOTICS,” p. 212.
[35] C. Proteomics, “Peptidoglycan Structure Analysis,” *Creative Proteomics*. https://www.creative-proteomics.com/services/peptidoglycan-structure-analysis.htm (accessed Sep. 22, 2022).
[36] ‘BACTERIAL CELL ENVELOPE, SPORES AND MACROMOLECULAR BIOSYNTHESIS.’ https://www.microbiologybook.org/fox/cell_envelope.htm (accessed Sep. 22, 2022).
[37] L. P. Kotra and S. Mobashery, "Intellectual-Lactam antibiotics, intellectual-lactamases and bacterial resistance," *Bull. Inst. Pasteur*, vol. 96, no. 3, pp. 139-150, Jul. 1998, doi: 10.1016/S0020-2452(98)80009-2.
[38] J. E. Bennett, R. Dolin, and M. J. Blaser, Eds., *Mandell, Douglas, and Bennett's principles and practice of infectious diseases*, Ninth edition. Philadelphia, PA: Elsevier, 2020.
[39] “Glycopeptide antibiotics and development of inhibitors to overcome vancomycin resistance,” *Nat. Prod. Rep.*, vol. 19, no. 1, pp. 100-107, Jan. 2002, doi: 10.1039/b100912p.
[40] I. Klare, C. Konstabel, D. Badstübner, G. Werner, and W. Witte, "Occurrence and spread of antibiotic resistances in Enterococcus faecium," *Int. J. Food Microbiol.*, vol. 88, no. 2-3, pp. 269-290, Dec. 2003, doi: 10.1016/S0168-1605(03)00190-9.
[41] A. Forouzesh, P. A. Moise, and G. Sakoulas, "Vancomycin ototoxicity: a reevaluation in an era of increasing doses," *Antimicrob. Agents Chemother.*, vol. 53, no. 2, pp. 483-486, Feb. 2009, doi: 10.1128/AAC.01088-08.
[42] H. Yoneyama and R. Katsumata, “Antibiotic resistance in bacteria and its future for novel antibiotic development,” *Biosci. Biotechnol Biochem.*, vol. 70, no. 5, pp. 1060-1075, May 2006, doi: 10.1271/bbb.70.1060.

[43] P. Vannuffel and C. Cocito, “Mechanism of action of streptogramins and macrolides,” *Drugs*, vol. 51 Suppl 1, pp. 20-30, 1996, doi: 10.2165/00003495-199600511-00006.

[44] N. J. Johnston, T. A. Mukhtar, and G. D. Wright, “Streptogramin antibiotics: mode of action and resistance,” *Curr. Drug Targets*, vol. 3, no. 4, pp. 335-344, Aug. 2002, doi: 10.2174/1389450023347678.

[45] R. Wise, "A review of the mechanisms of action and resistance of antimicrobial agents," *Can. Breathe. J.*, vol. 6 Suppl A, pp. 20A-2A, Feb. 1999.

[46] P. A. Lambert, "Bacterial resistance to antibiotics: modified target sites," *Adv. Drug Deliv. Rev.*, Vol. 57, no. 10, pp. 1471-1485, Jul. 2005, doi: 10.1016/j.addr.2005.04.003.

[47] B. Bozdogan and P. C. Appelbaum, "Oxazolidinones: activity, mode of action, and mechanism of resistance," *Int. J. Antimicrob Agents*, vol. 23, no. 2, pp. 113-119, Feb. 2004, doi: 10.1016/j.ijantimicag.2003.11.003.

[48] S. C. Abeylath and E. Turos, "Drug delivery approaches to overcome bacterial resistance to beta-lactam antibiotics," *Expert Opin. Drug Deliv.*, vol. 5, no. 9, pp. 931-949, Sep. 2008, doi: 10 1517/174 25 247 5 931.

[49] E. Etebu and I. Arikekpar, “Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives,” p. 12.

[50] T. T. Tidwell, "Hugo (Ugo) Schiff, Schiff Bases, and a Century of Spirit-Lactam Synthesis," *Angew. Chem. Int. Ed.*, vol. 47, no. 6, pp. 1016-1020, Jan. 2008, doi: 10.1002/anie.200702965.

[51] A. L. Demain and R. P. Elander, “The beta-lactam antibiotics: past, present, and future,” *Antoine Van Leeuwenhoek*, vol. 75, no. 1-2, pp. 5-19, Feb. 1999, doi: 10.1023/a:1001738823146.

[52] L. M. Lima, B. N. M. da Silva, G. Barbosa, and E. J. Barreiro, "Intellectual-lactam antibiotics: An overview from a medicinal chemistry perspective," *Eur. J. Med. Chem.*, vol. 208, p. 112829, Dec. 2020, doi: 10.1016/j.ejmech.2020.112829

[53] S. B. Chaudhry, M. P. Veve, and J. L. Wagner, “Cephalosporins: A Focus on Side Chains and Lactam Cross-Reactivity,” *Pharmacy*, vol. 7, no. 3, p. 103, Jul. 2019, doi: 10.3390/pharmacy7030103.

[54] L. Pasteur and J. Joubert, *The first documented discovery of an antibiotic*. Gauthier-Villars, 1877.

[55] K-F. Kong, L. Schneper, and K. Mathee, "Beta-lactam antibiotics: from antibiosis to resistance and bacteriology: BETA-LACTAM RESISTANCE,” *APMIS*, vol. 118, no. 1, pp. 1-36, Jan. 2010, doi: 10.1111/j.1600-0463.2009.02563.x.

[56] S. Selwyn, “Pioneer work on the penicillin phenomenon, 1870-1876,” *J. Antimicrob. Chemother.*, vol. 5, no. 3, pp. 249-255, 1979, doi: 10.1093/jac/5.3.249.

[57] S. Ch, “Alexander Fleming and Penicillin. The chance of a lifetime?," *N. C. Med. J.*, vol. 53, no. 6, Jun. 1992, Accessed: Sep. 15, 2022. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/1614562/

[58] F. A, "On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. 1929,” *Bull. World Health Organ.*, Vol. 79, No. 8, 2001, Accessed: Sep. 15, 2022. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/11545337/

[59] R. Hare, "New light on the history of penicillin," *Med. Hist.*, vol. 26, no. 1, pp. 1-24, Jan. 1982, doi: 10.1017/S0025727300040758.

[60] E. Chain, “The early years of the penicillin discovery,” *Trends Pharmacol. Sci.*, vol. 1, no. 1, pp. 6-11, Jan. 1979, doi: 10.1016/0165-6147(79)90004-X.

[61] E. P. Abraham and E. Chain, “An Enzyme from Bacteria able to Destroy Penicillin,” *Nature*, vol. 146, no. 3713, pp. 837-837, Dec. 1940, doi: 10.1038/146837a0.

[62] A. Ep *et al.*, "Further observations on penicillin. 1941,” *Eur. J. Clin Pharmacol.*, vol. 42, no. 1, 1992, Accessed: Sep. 15, 2022. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/1541313/

[63] M. E. Florey, M. B. Adelaide, H. W. Florey, and M. B. Adelaide, “GENERAL AND LOCAL ADMINISTRATION OF PENICILLIN,” *The Lancet*, vol. 241, no. 6239, pp. 387-397, Mar. 1943, doi: 10.1016/S0140-6736(00)41962-8.

[64] H. Zähner and W. K. Maas, *Biology of Antibiotics*. New York, NY: Springer US.

[65] K. B. Holten and E. M. Onusko, “Appropriate prescribing of oral beta-lactam antibiotics,” *Am. Fam. Physician*, vol. 62, no. 3, pp. 611-620, Aug. 2000.

[66] P. Thévenot, *From Antibiogram to Prescription*, 2nd ed. Marcy-l'Étoile: Biomérieux, 2003.

[67] L. Le Minor and M. Véron, *Medical Bacteriology*, 2nd ed. Flammarion, 1989.

[68] D. Yala, D. Mohassé, and M. Ouar Korich, “Classification and mode of action of antibiotics,” *Médecine Maghreb*, vol. 91, pp. 5-12, 2001.

[69] D. M. Bear, M. Turck, and R. G. Petersdorf, "Ampicillin," *Med. Clin. North Am.*, vol. 54, no. 5, pp. 1145-1159, Sep. 1970, doi: 10.1016/S0025-7125(16)32583-4.

[70] D. J. Waxman and J. L. Strominger, “Sequence of active site peptides from the penicillin-sensitive D-alanine carboxypeptidase of Bacillus subtilis. Mechanism of penicillin action and sequence homology to beta-lactamases,” *J. Biol. Chem.*, vol. 255, no. 9, pp. 3964-3976, May 1980.

[71] Mr. Gawrońska, Mr. Kowalik, and Mr. Makowski, "Recent advances in medicinal chemistry of ampicillin: Derivatives, complex metal, and sensing approaches," *TrAC Trends Anal. Chem.*, vol. 155, p. 116691, Oct. 2022, doi: 10.1016/j.trac.2022.116691.

[72] K. P. Talaro and B. Chess, *Foundations in Microbiology*, 8th ed. McGraw-Hill, 2012.

[73] L. L. Brunton, B. C. Knollmann, and R. Hilal-Dandan, Eds., *Goodman & Gilman’s the pharmacological basis of therapeutics*, Thirteenth edition. McGraw Hill Medical, 2018.

[74] A. Müller and B. König, “Preparation of luminescent chemosensors by post-functionalization of vesicle surfaces,” *Org. Biomol. Chem.*, vol. 13, no. 6, pp. 1690-1699, 2015, doi: 10.1039/C4OB02327G.

[75] B. A. Biggs and A. Kucers, "Penicillins and related drugs," *Med. J. Aust.*, vol. 145, no. 11-12, pp. 607-611, Dec. 1986.

[76] W. R. Wilson *et al.*, "Antibiotic treatment of adults with infective endocarditis due to streptococci, enterococci, staphylococci, and HACEK microorganisms. American Heart Association,” *JAMA*, vol. 274, no. 21, pp. 1706-1713, Dec. 1995.

[77] B. H. Heintz, J. Halilovic, and C. L. Christensen, “Vancomycin-resistant enterococcal urinary tract infections,” *Pharmacotherapy*, vol. 30, no. 11, pp. 1136-1149, Nov. 2010, doi: 10.1592/phco.30.11.1136

[78] B. Rowe, L. R. Ward, and E. J. Threlfall, "Multidrug-resistant Salmonella typhi: a worldwide epidemic," *Clin. Evil. Say. Off. Published. Evil. Say. Soc. Am.*, vol. 24 Suppl 1, pp. S106-109, Jan. 1997, doi: 10.1093/clinids/24.supplement_1.s106.

[79] *American hospital formulary service: a collection of drug monographs and other information*. Washington, D.C.: The Society.

[80] R. B. Ghooi and S. M. Thatte, "Inhibition of cell wall synthesis—is this the mechanism of action of penicillins?," *Med. Hypotheses*, vol. 44, no. 2, pp. 127-131, Feb. 1995, doi: 10.1016/0306-9877(95)90085-3.

[81] “Amoxicillin - an antibiotic | ari.info,” *ari.info*. https://www.animalresearch.info/en/drug-development/drug-prescriptions/amoxicillin/ (accessed Sep. 19, 2022).

[82] M. Roman and D. Selişteanu, “Enzymatic Synthesis of Ampicillin: Nonlinear Modeling, Kinetics Estimation, and Adaptive Control,” *J. Biomed. Biotechnol.*, vol. 2012, pp. 1-14, 2012, doi: 10.1155/2012/512691

[83] R. Fernández-Lafuente, C. M. Rosell, B. Piatkowska, and J. M. Guisán, "Synthesis of antibiotics (cephaloglycin) catalyzed by penicillin G acylase

approaches," *Microb Enzyme. Technol.*, vol. 19, no. 1, pp. 9-14, Jul. 1996, doi: 10.1016/0141-0229(95)00179-4.

[84] C. G. P. H. Schroën *et al.*, "Thermodynamically controlled synthesis of anti-lactam antibiotics. Equilibrium concentrations and side-chain properties," *Enzyme Microb. Technol.*, vol. 24, no. 8-9, pp. 498-506, Jun. 1999, doi: 10.1016/S0141-0229(98)00140-9.

[85] A. Bruggink, R. Schoevaart, and T. Kieboom, "Concepts of Nature in Organic Synthesis: Cascade Catalysis and Multistep Conversions in Concert," *ChemInform*, vol. 34, no. 48, Dec. 2003, doi: 10.1002/chin.200348270.

[86] W. B. L. Alkema, R. Floris, and D. B. Janssen, "The Use of Chromogenic Reference Substrates for the Kinetic Analysis of Penicillin Acylases," *Anal. Biochem.*, vol. 275, no. 1, pp. 47-53, Nov. 1999, doi: 10 1006/abio 1999 4300.

[87] J. K. Blum and A. S. Bommarius, "Amino ester hydrolase from Xanthomonas campestris pv. campestris, ATCC 33913 for enzymatic synthesis of ampicillin," *J. Mol. Catal. B Enzym.*, vol. 67, no. 1-2, pp. 21-28, Nov. 2010, doi: 10.1016/j.molcatb.2010.06.014

[88] H. Marešová, M. Plačková, M. Grulich, and P. Kyslík, "Current state and prospects of penicillin G acylase-based biocatalyses," *Appl. Microbiol. Biotechnol.*, vol. 98, no. 7, pp. 2867-2879, Apr. 2014, doi: 10.1007/s00253-013-5492-7.

[89] J. J. Polderman-Tijmes *et al.*, "Identification of the Catalytic Residues of 1,2-Amino Acid Ester Hydrolase from Acetobacter turbidans by Labeling and Site-directed Mutagenesis," *J. Biol. Chem.*, vol. 277, no. 32, pp. 28474-28482, Aug. 2002, doi: 10.1074/jbc.M204143200.

[90] 'Antibiogram | Protocol | Interpretation." https://microbiologie-clinique.com/antibiogramme.html (accessed Sep. 28, 2022).

[91] M. I. Youshko, H. M. Moody, A. L. Bukhanov, W. H. J. Boosten, and V. K. Svedas, "Penicillin acylase-catalyzed synthesis of beta-lactam antibiotics in highly condensed aqueous systems: beneficial impact of kinetic substrate supersaturation," *Biotechnol. Bioeng.*, vol. 85, no. 3, pp. 323-329, Feb. 2004, doi: 10.1002/bit.10863.

[92] D.-Z. Wei and L. Yang, "Effects of ethylene glycol on the synthesis of ampicillin using immobilized penicillin G acylase," *J. Chem. Technol. Biotechnol.*, vol. 78, no. 4, pp. 431-436, Apr. 2003, doi: 10.1002/jctb.749.

[93] L.-L. Du, Q. Wu, C.-X. Chen, B.-K. Liu, and X.-F. Lin, "A two-step, one-pot enzymatic synthesis of ampicillin from penicillin G potassium salt," *J. Mol. Catal. B Enzym.*, vol. 58, no. 1-4, pp. 208-211, Jun. 2009, doi: 10.1016/j.molcatb.2008.12.020.

[94] J. K. Blum, A. L. Deaguero, C. V. Perez, and A. S. Bommarius, "Ampicillin Synthesis Using a Two-Enzyme Cascade with Both-Amino Ester Hydrolase and Penicillin G Acylase," *ChemCatChem*, vol. 2, no. 8, pp. 987-991, Aug. 2010, doi: 10.1002/ctc.2010 0135.