MEMORY

For the purpose of obtaining

PROFESSIONAL MASTER

in

Human Molecular Diagnostics

Presented and Supported by:

Jana HAMZEH

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Title

Production and Quantification of Penicillin

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CONTENTS

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For your patience, encouragement, and wise counsel,

I want to tell you

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ABSTRACT

Alexander Fleming's discovery of penicillin G was one of the most important discoveries of the 20th century. *Penicillium chrysogenum* is widely used in the production of penicillin G. This study is done for the identification of penicillin-producing fungi, our study is limited to the genus *Penicillium*, specifically *Penicillium chrysogenum. Penicillium chrysogenum* is cultured in 2 nutrient broths to study which allows the strain to achieve its best antibiotic secretion after 10 days of incubation. Quantitative analysis by an antibiogram revealed the completion of penicillin G production from the 2 broths, with a higher concentration in broth 2 (1.64 IU) and very high antibacterial activity against *Staphylococcus aureus.* Thus, our results accentuate the idea of increasing the penicillin G production scale to an industrial scale using more improved *P. chrysogenum* strains to produce a large amount of penicillin G.

Key words: *Penicillium chrysogenum*, Penicillin G, beta-lactams, antibiotics.

ABSTRACT

The discovery of penicillin G by Fleming Alexander is one of the most important discoveries of the 20th century. *Penicillium chrysogenum* is widely used in the production of penicillin G. This study is for the identification of penicillin mushroom producers, our study is limited to the genus *Penicillium*, more precisely *Penicillium chrysogenum*. *Penicillium chrysogenum* is cultured in 2 nutrient broths in order to study which allows the strain to achieve its best antibiotic secretion after 10 days of incubation. The quantitative analysis carried out by an antibiogram revealed the completion of the production of penicillin G from the 2 broths, with a higher concentration in broth 2 (1.64 IU) and its antibacterial activity is very considerable against *Staphylococcus aureus*. Thus, our results emphasize the idea of scaling up the production of penicillin G towards an industrial scale using more improved *P. chrysogenum* strains to produce a large amount of penicillin G.

Keywords: *Penicillium chrysogenum*, Penicillin G, beta-lactams, antibiotics.

CHAPTER 0: INTRODUCTION

The term mold refers to filamentous microscopic fungi, some of which are of great economic and environmental interest [1].

Some molds can have magnificent effects: processing of food raw materials, production of antibiotics, enzymes or flavoring agents etc.

Others, however, are harmful: food alteration, causing mycoses and allergies and biosynthesis of mycotoxins [2].

Bacterial infections have been the main cause of death since antiquity (cholera, plague, tuberculosis...), but since 1928 the world has changed, finally we have a weapon against these bacteria, an antibiotic secreted by *Penicillium notatum* and *Penicillium chrysogenum,* called penicillin 3..

Since 1940, antibiotic manufacturing has taken a predominant part in the pharmaceutical industry accounting for nearly 25% of its turnover. Penicillin and streptomycin and its derivatives are 60% of antibiotics [2].

My work focuses on the production and quantification of penicillin G by *Penicillium chrysogenum* and involves the following steps:

- Culturing of *Penicillium chrysogenum* in liquid medium
- Preparation and filtration of nutrient broth
- Antibacterial activity of penicillin G on *Staphylococcus aureus* bacterium

Chapter I: STATE OF THE ART

1. MUSHROOMS

1.1. Definition

Fungi are living organisms consisting largely of filaments of cells of simple structure and a few more specialized cells that will give rise to spores. Fungi have genetic material confined to a nucleus in the same way as plants and animals. However, they have a number of characteristics that make them a separate group: walls containing cellulose and chitin, absence of chlorophyll and mobility [4].

1.2. Morphology and cellular organization

The basic organization of the mushrooms is the thallus which constitutes the vegetative apparatus. It is characterized by a wide variety of structures, ranging from a single-cell form (yeast) with a single nucleus per cell, to filaments (or hyphae) 2-10 ¯m in diameter.

These hyphae include the classical organelles of a cell: nucleus, mitochondria, cytoplasm, vesicles. They may or may not be partitioned, and their association forms the mycelium [5].

1.3. Life cycle of fungi

For most fungal species, unlike most animal species, reproduction, most can reproduce sexually or asexually, and a significant minority are classified as exclusively asexual [6].

The life cycle of fungi comprises two types of reproduction: i) asexual reproduction, during which a spore or fragment of mycelium grows and develops on a substrate (Figure 1). The mycelium emits conidiophores at the end of which conidia are emitted and then disseminated; ii) sexual reproduction involves the encounter of two mycelium of opposite sexual signs. One n-chromosome mycelium will encounter another mycelium with complementary polarity to give rise to cytoplasm fusion, which

generates a new 2n-chromosome mycelium. Life cycles differ from one fungus to another depending on the type of spores [7].

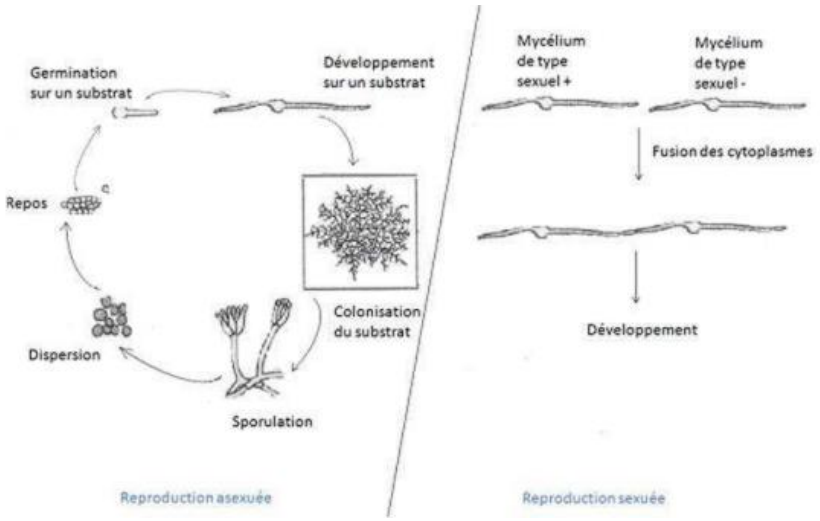


Figure 1 : Life cycle of mushrooms [8]

1.4. Classification of fungi

Fungi have been subject to complex classifications [9],10.,[11],[12].

It is divided into 4 sub-branches:

a) Zygomycetes

They include about 200 species, including saprophytic fungi (which feed on dead matter), as well as parasitic fungi of insects, nematodes and amoebas, and plants. They are characterized by gametocyst fusion sexual reproduction and sporocystospore asexual reproduction.

b) Ascomycetes

They include about 1,500 species. They are characterized by sexual reproduction by ascospore formation in the asci and asexual reproduction by conidia.

c) Basidiomycetes

There are about 20,000 species, which are the most advanced fungi. They are characterized by sexual reproduction by spore formation in basids and asexual reproduction by conidia.

d) Deuteromycetes

Still called Adéomycetes, they do not constitute a natural group, but an artificial group of about 1500 species. They never, or very unusually, exhibit any form of sexual reproduction. They reproduce only vegetatively using asexual spores (conidia).

The Deuteromycetes are divided into 3 classes:

- Blastomycetes: Yeasts
- Hyphomycetes: Filamentous fungi
- Coelomycetes [2]

1.5. Genus *Penicillium*

This genus combines filamentous fungi belonging to the phylum of the *Ascomycetes*. This genus consists of approximately 227 species [13]. The *Penicillium* are very common fungi in the environment, which can be responsible for many degradations. Their natural habitat is soil, food, organic matter, decomposing organic matter, compost, and cereals. They are common contaminants of temperate regions [2].

1.6. General cultural characteristics

The *Penicillium* grows quickly and easily on the culture media used routinely. They develop at moderate temperatures of the order of 20-27°C. After 2 days of incubation, small, flat colonies, usually white, are observed. After 3-4 days of incubation, the sporulation will give the colonies their hue, most often in the tones green, blue green, gray green, yellow green, gray, blue, but also, for some species, yellow, orange, pink, or red. This color allows a first orientation in the identification of species: gray green for P. citrinum, P. cyclopium, P. italicum; yellow green for *P. chrysogenum*; dark green for *P. roquefortii, P. fellutatum*; pale yellow for P. *nalgiovense*; bright yellow to red for *P. pupurogenum*.. [2].

1.7. Useful species

Many species of *Penicillium* are used industrially for the manufacture of cheeses or for the production of the various metabolites of interest:

- *Penicillium camembertii* is used in cheese making for soft cheeses;
- *Penicillium roquefortii* for the maturing of cheese with parsley paste;

- *Penicillium nalgiovense* for improving the organoleptic qualities of sausages;
- *Penicillium chrysogenum, Penicillium grisefulvum, Penicillium notatum,* are used to obtain different antibiotic substances 10..

1.8. *Penicillium chrysogenum*

a) Definition

The *Penicillium chrysogenum* are filamentous fungi, mold-like. The branched conidiophore has a brush-like shape. Conidia are arranged in long chains. The thallus is green/white [2].

b) Morphology

Like many other species of the genus *Penicillium, Penicillium chrysogenum* generally reproduces by forming conidiophoric brush-shaped spore chains (or conidia). Conidia are generally carried by air currents to new colonization sites. In *Penicillium chrysogenum,* the conidia are blue to blue-green and the mold sometimes gives off a yellow pigment (Figure 2). However, *Penicillium chrysogenum* cannot be identified on the basis of color, observations of morphology and microscopic characteristics are necessary to confirm its identity [14].

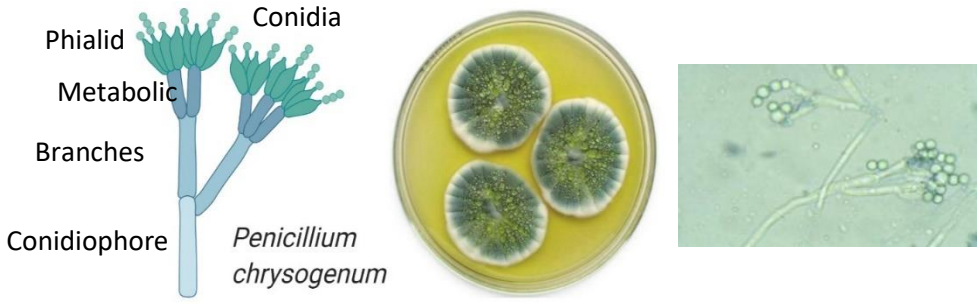


Figure 2 : Microscopic and macroscopic morphology of Penicillium chrysogenum [2],15.

c) Habitat and distribution

Penicillium chrysogenum is a very common species in soils, on organic matter, and food. This species can cause deterioration of textiles, papers, and food products.

Penicillium chrysogenum is a common fungus in subtropical temperate regions and can be found on food products 16., but it is mainly found in indoor environments, especially in damp buildings [17].

d) Industrial interest of *P. chrysogenum*

A study by Rodríguez-Sáiz et al. revealed that *P. notatum* does not produce as high amounts of penicillin as *P. chrysogenum* [18],[19]. Thus, *P. chrysogenum* was used industrially for the production of penicillin and xanthocillin X, for the treatment of infections and the production of enzymes: polyamine oxidase, phospho-gluconate dehydrogenase, and glucose oxidase 20.,[21].

Improved strains of *Penicillium chrysogenum* have been used industrially to increase penicillin production. This improvement was achieved by: (1) overexpression of penicillin genes, amino acids of the penicillin precursor and some proteins, (2) increased peroxisome abundance, (3) increased NADPH via pentose phosphate pathway and cysteine biosynthesis, (4) increased carbon catabolism and energy production, (5) improved response to oxidative stress, (6) decreased virulence mechanisms (Figure 3) [22].

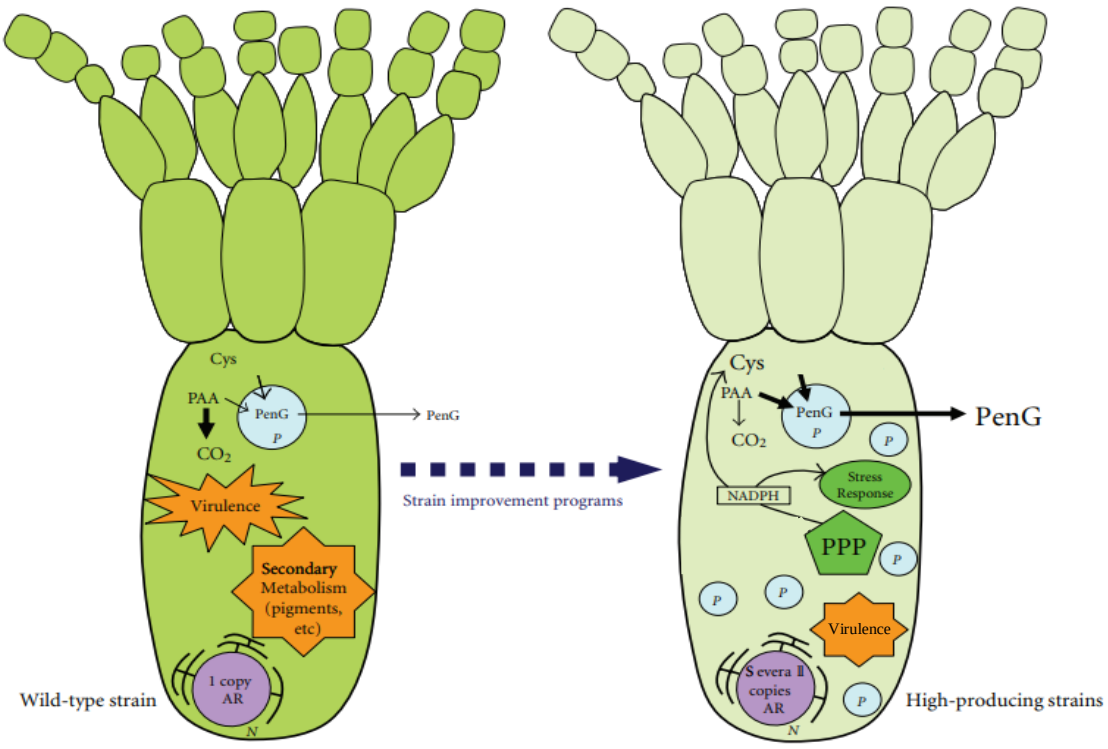


Figure 3 Summary of the main results on penicillin production showing changes in P. chrysogenum throughout its breeding program [23]

2. ANTIBIOTICS

2.1. Definition

The antibiotic (from the Greek anti: counter, biotikos: concerning life) first used in 1889, with reference to a substance synthesized by one organism to destroy another, would later become more precise, as a chemical substance produced by a microorganism having the capacity to selectively inhibit growth or even destroy other microorganisms. Compounds used for therapeutic purposes in bacterial diseases in humans and animals are frequently called antibiotics by health professionals. However, the term is often misused and is subject to a regular broadening of meaning. Indeed, the definition of the word antibiotic refers strictly to antimicrobial substances of natural origin, and according to that concept, that term should therefore not be used to describe synthetic substances such as sulfonamides and quinolones, or semi-synthetic substances such as amoxicillin 24.. For ease of reference in the remainder of this article, the term antibiotic will nevertheless refer to these three categories of compounds [25].

2.2. Mode of action of antibiotics

The antimicrobial potency of most classes of antibiotics is directed towards a unique characteristic of bacterial structure or their metabolic processes. The most common antibiotic targets are illustrated in (Figure 4). The mechanism of action of antibiotics is as follows:

a) Inhibition of cell wall synthesis
b) Failure of the structure or function of the cell membrane
c) Inhibition of nucleic acid structure and function
d) Inhibition of protein synthesis

e) Blocking of key metabolic pathways 26.,27.,28.

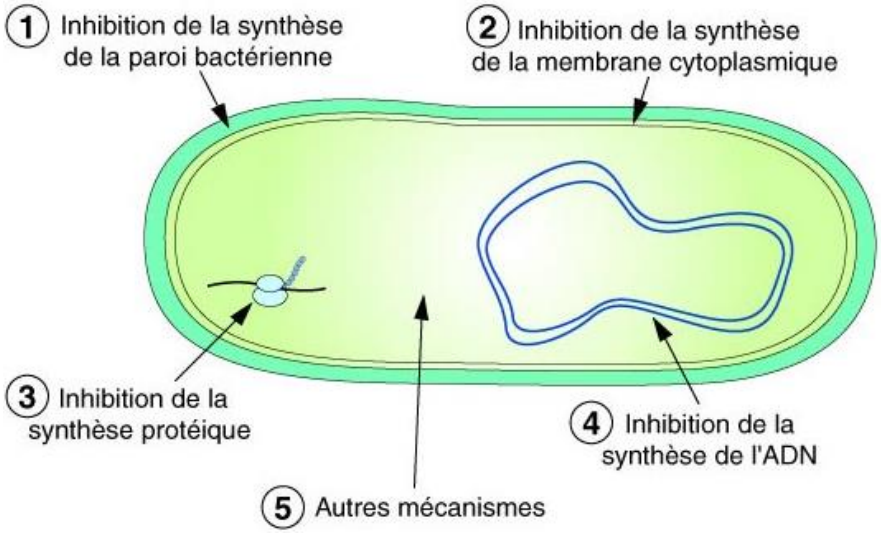
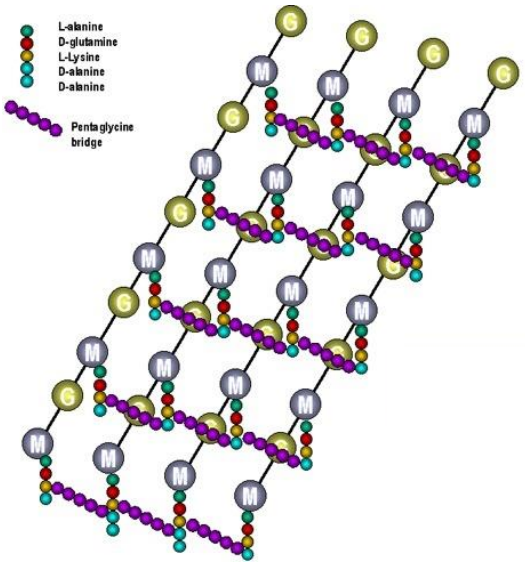


Figure 4 bacterial cell and antibiotic target sites 29.

a) Inhibition of cell wall synthesis

Most bacterial cells are covered with a rigid layer of peptidoglycan (PGN) 30.,31., which gives the bacterium its shape and its rigidity, allowing it to withstand the high intracytoplasmic osmotic pressure [2]. To stay alive, bacteria must synthesize



peptidoglycan; they do so by the activity of PBPs, which are transglycosylases and transpeptidases. These two enzymes play a very central role by adding disaccharide pentapeptides to extend the glycan strands of the peptidoglycan molecule (FIG. 5) 32.. Some antibiotics inhibit the final step of peptidoglycan biosynthesis during cell multiplication [2] by inhibiting PBP-catalyzed peptide bond formation 33., the new bacterium is no longer protected, leading to bacterial lysis [2]. The kinetic lactams (family to which penicillin belongs) act according to this mode of action 34..

Figure 5 : Schematic representation of the structure of the peptidoglycan network. N-acetylglucosamine (G) and acid N-acetylmuramic (M) 35.

b) Failure of the structure or function of the cell membrane

The antibiotic has surfactant properties* which allow it to be inserted among the phospholipids of the outer membrane. This disrupts the membrane permeability which increases abnormally. This allows the diffusion of water-soluble substances out of the bacterium, resulting in its destruction. The polymyxins (cyclic peptides) act according to this mode of action.

*A surfactant is an amphiphilic molecule, i.e., that has a polar head that has affinity for water and an apolar tail that has affinity for apolar substances such as oil 34..

c) Inhibition of nucleic acid synthesis

The antibiotic will bind to the DNA and prevent the progression of the DNA polymerase. This inhibits DNA replication, which is essential for the formation of new bacteria, as well as transcription. The fluoroquinolones act according to this mode of action.

d) Inhibition of protein synthesis

This leads to the cessation of protein biosynthesis or the formation of abnormal proteins [2]. Tetracyclines and macrolides act according to this mode of action 34..

e) Blocking of key metabolic pathways

By causing bacterial enzymes to attach to the antibiotic rather than to the normal substrate 26.. The antibiotic is a structural analog of a base precursor molecule used in nucleic acid composition. The bacterium will insert it into its metabolism, but the

slight differences in structure between the antibiotic and the precursor will cause the metabolic pathways to be blocked. Sulphonamides act according to this mode of action 34..

2.3. Classifications of antibiotics

There are several ways to classify antibiotics, but the most common classification schemes are based on their molecular structures, mode of action and spectrum of activity. Others include the route of administration (injectable, or oral...). Antibiotics belonging to the same structural class generally have a similar pattern of efficacy, toxicity and side effects with allergic potential. Some common classes of antibiotics based on chemical or molecular structures include: beta-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulfonamides, glycopeptides, oxazolidinones.

2.4. Beta-lactams

Members of this class of antibiotics contain a highly reactive 3-carbon and 1-nitrogen ring (Figure 6). They interfere with proteins essential for bacterial cell wall synthesis and thereby kill or inhibit their growth. More specifically, certain bacterial enzymes called penicillin binding protein (PBP) are responsible for the crosslinking of peptide units during the synthesis of peptidoglycan. Members of beta-lactam antibiotics are able to bind to these PBP enzymes and, in doing so, interfere with peptidoglycan synthesis leading to lysis and cell death. The most important representatives of the beta-lactam class include:

a) Penicillins
b) Cephalosporins
c) Monobactams
d) Carbenems 36. (Figure 7)

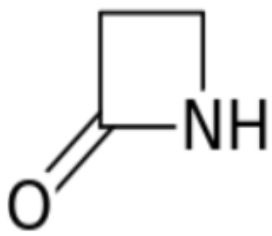


Figure 6 : Chemical structure of the beta-lactam ring 37.

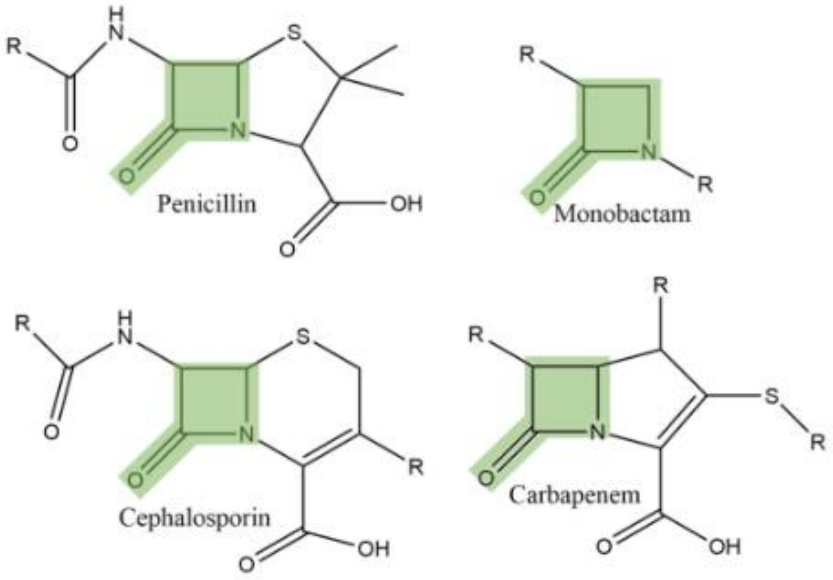


Figure 7 : Examples of structures of the different beta-lactam classes 38..

2.5. Penicillins

The chemical structure of penicillin is composed of a beta-lactam nucleus, a thiazolidine nucleus, and other side chains (Figure 8).

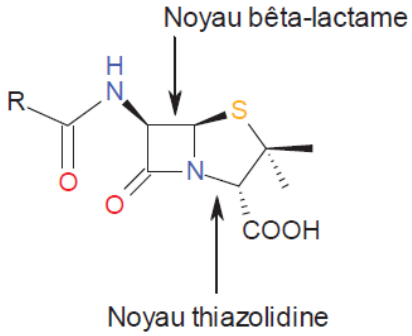


Figure 8 : Structure of penicillin 39.

Members of the penicillin class include: penicillin G, penicillin V, oxacillin (dicloxacillin), methicillin, nafcillin, ampicillin, amoxicillin, carbanecillin, piperacillin, mezlocillin, ticarcillin (Figure 9).

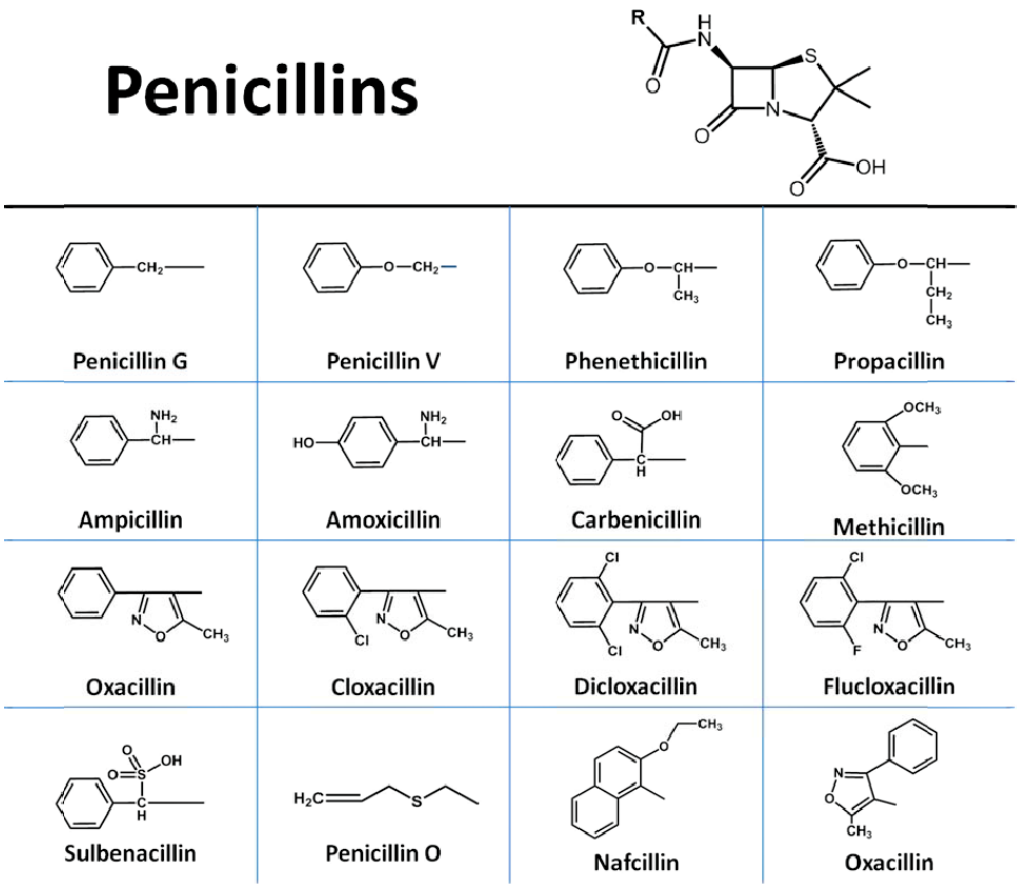


Figure 9 : Structure of penicillin class members [40]

a) Fleming's discovery and Oxford breakthrough from 1928 to 1941

The discovery of penicillin was more than a fluke, but it began with a fluke. In 1928, upon returning from vacation, Alexander Fleming, a bacteriologist practicing at St. Mary's Hospital in London, noticed that one of his petri dishes containing staphylococci he left on a bench was contaminated. He observed that a fungal contaminant affected the growth of neighboring bacteria. The fungus was found to be *Penicillium notatum* and the antibacterial molecule it produced was named penicillin. Penicilliun *chrysogenum* is a close relative of *P.notatum* and is the preferred source choice. Fleming recorded his findings in the 1929 British Journal of Experimental Pathology article, where he showed that penicillin is capable of inhibiting bacterial growth in vitro. Fleming believed that penicillin could be useful as a local antiseptic, but failed to purify penicillin or characterize its activity.

Fleming's paper on penicillin was the basis for scientists at Oxford to begin research on new antimicrobials in 1939. At that time, Howard Florey was working on lysozyme

and its ability to kill bacteria. Along with Ernst Chain, a laboratory chemist, Florey became interested in Fleming's observation of *Penicillium'*s antimicrobial capacity. Chain and Florey decided to design a method to grow the fungus and aimed to produce it in sufficient quantities to allow additional testing of its antimicrobial roles. Norman Heatley, a young chemist from Florey's laboratory, played a key role in the penicillin purification process. Heatley and Chain developed the first methods of extracting penicillin, which were necessary to obtain sufficient material to conduct the first trials with the drug. By the mid-1940s, sufficient penicillin was available at the Sir William Dunn School of Pathology in Oxford to conduct trials of its efficacy in mice. It was this experiment conducted by Heatley and colleagues that provided key data to demonstrate the effect of penicillin in vivo. Eight mice were injected with a lethal dose of group A streptococcus. After one hour, two mice received a single dose of penicillin (10 mg) and two received 5 mg of penicillin, plus three additional doses of 5 mg at 3, 5, 7 and 11 hours post-infection. Four mice were used as controls and were not given penicillin. Seventeen hours after the initial infection, all mice in the control group died, while all mice given a dose of penicillin survived. This remarkable observation provided key evidence that penicillin had the potential to save lives [41].

b) Microorganism growth and penicillin production by *P. chrysogenum*

- **Growth of micro-organisms**

Measurement of microorganism cell growth can be performed based on viable culturable cell count (UFC) or total cell count. It is possible to plot a growth curve

that indicates the synchronized development of the cells as a function of time (Figure 10).

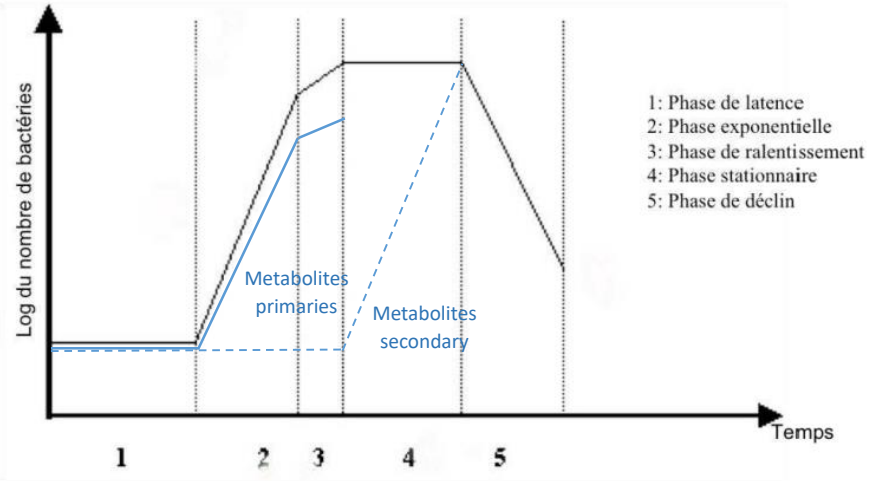


Figure 10 : Change in the number of cells in the microorganism over time in days [42]

The growth curve comprises 5 phases, more or less long, depending on the different microorganisms. (1) During the latency phase, the number of micro-organisms hardly increases because the cells do not divide. This is a period of adaptation of microorganisms to the environment. (2) The exponential phase is the time when microorganisms divide exponentially. It is in this phase that the rate of growth of the microorganisms is determined, and the primary metabolites are produced which are necessary for the survival of the microorganisms. (3) During the downturn, the growth rate is lower. (4) The stationary phase often reflects a balance between multiplication and cell death, the population stabilizes. For a large proportion of microorganisms, this time of growth is favorable to the production of secondary metabolites such as toxic compounds, antibiotics (e.g. penicillins G, V, and O produced by *P. chrysogenum*), enzyme inhibitors etc. (5) the phase of decline is due to nutrient depletion and waste accumulation. The number of micro-organisms that die exceeds the number of new micro-organisms from cell division [42].

- **Penicillin production by *P. chrysogenum***

When *P. chrysogenum* detects the presence of a carbon energy source preferred to lactose, it does not use lactose until the preferred substrate (e.g. glucose) is fully

consumed [43]. Once glucose is fully consumed, *P. chrysogenum* uses lactose which acts as a satisfactory carbon compound when used as a food source for the microorganism with a slow feeding rate, because lactose is a complex sugar. Thus, there is an intense synthesis of penicillin in this phase, due to lactose consumption [44] (Figure 11)

Figure 11 : Variation in lactose, cell count, and penicillin over time [45]

c) Mode of action of penicillin

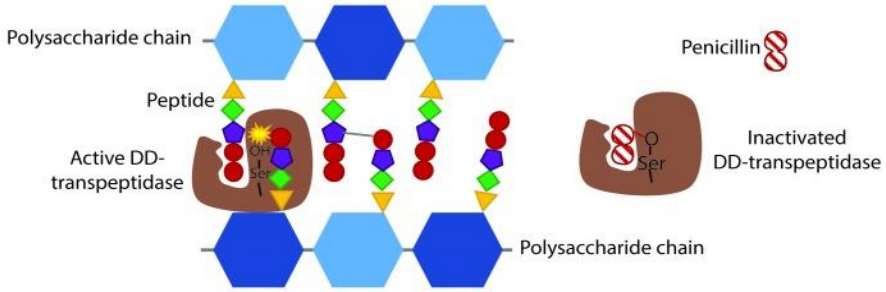
An essential structural element for most bacteria is the cell wall, a protective layer of peptidoglycan (PGN) whose main function is to preserve cell integrity and prevent macromolecules from entering the cell. The PGN is located just outside the cytoplasmic membrane and is composed of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) chains, which are covalently cross-linked via short peptides. During growth and division, PGN is continuously synthesized and remodeled. It is therefore essential that bacteria be able to synthesize the components of PGN and assemble them into a single macromolecule. The characteristic strength of PGN lies in its mesh-shaped conformation, which is mainly derived from peptide bonds. These bonds are formed by the activity of specific enzymes called transpeptidases or penicillin binding proteins (PBPs). Penicillin, like other components of beta-lactam antibiotics, contains a four-membered beta-lactam ring, which is responsible for inhibiting transpeptidase. By mimicking the last two D-alanine residues of the peptide, penicillin is able to bind irreversibly to the active site of transpeptidase, preventing the enzyme from cross-linking the peptidoglycan strands. Therefore, by blocking the formation of peptide bridges, penicillin prevents the formation of new PGNs and the cell is sensitive to lysis, since PGN is no longer able to offer resistance against osmotic stress. In addition, penicillin specifically targets

bacteria because eukaryotic cells lack both PGN and the enzymes responsible for PGN synthesis [41] (Figure 12)

Figure 12 : Schematic representation of penicillin mechanism of action [41]

d) Classification and spectrum of activity of natural penicillins

Currently, the name penicillin is used generically to refer to different molecules that have beta lactam structures and the same antibacterial activity as benzylpenicillin (penicillin G) - the original molecule extracted from *P. notatum*. The classification of penicillins is based on chemical substitutions on the residue attached to the beta lactam ring, which confer different activities (Figure 13).



- Penicillin V or phenoxymethylpenicillin, has a narrower spectrum than penicillin G, it is active on:
  - Gram (+) shells: *Streptococcus pneumoniae, Streptococcus pyogenes*
  - Gram bacteria (+): *Corynebacterium diphtheriae*
  - Gram (-) bacteria: *Fusobacterium nucleatum*

- Penicillin O or almecillin may be useful in patients who are hypersensitive to penicillin G. It has a spectrum of activity very similar to penicillin G.

- Penicillin G or benzylpenicillin, is more active against Gram (+) bacteria and less active against Gram (-) bacteria:
  - Gram (+) shells: staphylococci, pneumococci, and other streptococci
  - Gram bacteria (+): *Bacillus anthracis*, *Clostridium perfringens*, and *Corynebacterium diphtheriae*
  - Gram (-) hulls: *Neisseria gonorrhoeae* and *Neisseria meningitides*

- Gram bacteria (-): *Streptobacillus moniliformis* [46]

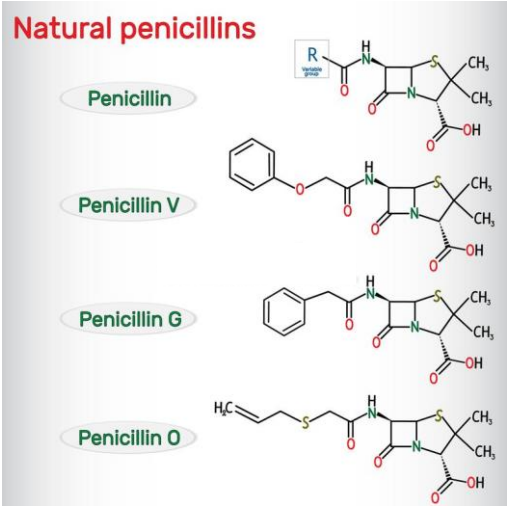
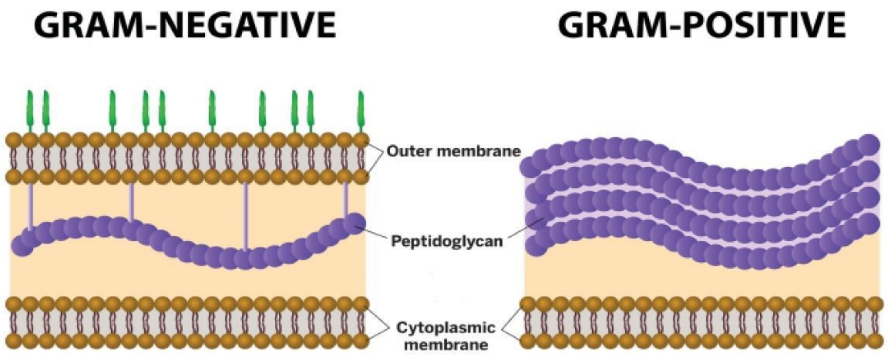


Figure 13 Benzylpenicillin, phenoxymethylpenicillin, almecillin [47]

The inability to act against Gram-negative bacteria is observed not only among benzylpenicillins, but also among many different antibiotics. This is largely due to two factors: firstly, unlike Gram-positive bacteria, Gram-negative species contain the outer membrane, which acts as a selective filter, blocking the penetration of penicillin (Figure 14); secondly, some Gram-negative bacteria have acquired specific genes that encode penicillinases (also called beta-lactamases), a class of enzymes that inactivate penicillin by hydrolysis of the beta-lactam ring.

Following extensive use of "natural penicillins", penicillinase-producing strains have also appeared among Gram-positive species. This pharmacological shift is research towards the development of semi-synthetic penicillins [41] with different side chains such as ampicillin, carbenicillin and amoxicillin. These side chains provide antibiotics with 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 capacity increases their spectrum of activity against Gram-negative bacteria 36..

Figure 14 : Cell wall structure of Gram-positive and Gram-negative bacteria [48]

e) Diseases treated with penicillin

Penicillin is the largest known family of antibiotics. It acts on bacteria in a targeted way, preventing their development or destroying them. It can fight bacteria that cause infections:

- airways: like nasopharyngitis, bronchitis, sinusitis, pneumonia.
- gastrointestinal tract: like listeriosis, acute diarrhea.
- skin and soft parts: such as impetigo, skin abscess, cellulite (skin and tissue infection just below), erysipelas.
- of the genital and urinary tract: such as cervicitis (cervical inflammation), urethritis, bacterial vaginosis [49].

CHAPTER II: CONTRIBUTION

1. Framework of work

This work was initiated and carried out in the Aecenar laboratory in Tripoli.

2. Production

2.1. Strain collection

A strain of *Penicillium chrysogenum* has been preserved at the Doctoral School at the Lebanese University in Tripoli (Figure 15).

Figure 15 Penicillium chrysogenum

2.2. Preparation of the medium and preparation of the inoculum

Preparation of the liquid medium (Broth): 0.5 g peptone, 0.1 g tryptone, 0.1 g glucose, 0.1 g yeast extract, 0.25 g NaCl, 50 ml distilled water were mixed and heated in a beaker. The pH was measured and adjusted to 7 by adding a few ml of H_2SO_4. The medium was then placed in 4 tubes, autoclaved for 1 hour and then cooled. An inoculum is taken from the strain *Penicillium chrysogenum* using a sterile loop and then transferred and mixed in the liquid medium. The tube is incubated at room temperature for 4 days.

2.3. Culturing

2 nutrient media were prepared in 100 ml of distilled water for each experiment (Figure 16).

Medium 1: 2g glucose, lactose (2.7g for experiment 1; 0.8g for experiment 2), 1g yeast extract, 0.5g corn oil, 0.8g peptone, 0.1g KH2PO4, 0.15g MgCl2, 0.2g CaCl2, 0.2g $Na2CO_3$, 0.8ml phenyl acetate (in experiment 2 only)

Medium 2: 10g glucose, lactose (10.7g for experiment 1; 3g for experiment 2), 4g peptone, 1g CaCl2, 1g $Na2CO_3$, 0.4g KH_2PO_4, 0.8ml phenyl acetate (in experiment 2 only)

The pH of the 2 media was measured and then adjusted to 7 by adding a few ml of H_2SO_4.

10 ml of each of the 2 mixtures were then added to a test tube each. The broth was then mixed and 2 ml were added to the 2 tubes containing medium 1 and 2 so that *P. chrysogenum* could adapt to the medium. Finally, the contents of the tubes were

returned to the 2 Erlenmeyer flasks containing media 1 and 2 and they were incubated at 26°C with stirring for 10 days.

Figure 16 : Middle 1 left and middle 2 right

2.4. Filtration

Filtration is a technique that involves passing a liquid through a filter whose pores have a diameter of 0.2 ⁻m. The microorganisms are too large and are therefore retained by the filter. After filtration, the pH of the filtrate was adjusted to 2-2.5 in order to stop any reaction that could degrade penicillin. The filtrate was then kept cold.

3. Sensitivity test

This step was carried out in experiment 2 only.

A *Staphylococcus aureus* bacterium has been preserved at the Doctoral School, Lebanese University, Tripoli.

A solid medium (agar gel) was prepared. A few ml of the filtrates were then poured into the petri dish and *S. aureus* was cultured. The box was incubated at 37°C for 24 hours.

4. Purification

3 ml of each filtrate were added with 3 ml of butyl acetate which serves as solvent for separating the organic phase and the aqueous phase using a separating funnel. The organic phase was recovered in a tube and a few ml of $NaSO_4$ were added in order to carry out the crystallization. The tube was kept cold for 24 hours for the crystallization step to be completed. Finally, the contents of the tube were filtered and dried in order to collect penicillin in powder form.

5. Quantification

Dilution was carried out in 9 test tubes containing NaCl and commercial penicillin (1,000,000 IU) as shown in Table 1:

Table 1 : Dilution of commercial penicillin

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9
Concentration of commercial penicillin G (IU)	1 00 000	10,000	1,000	100	30	25	20	16	10
Penicillin G volume (ml)	9	9	9	7	3	5	5	4	2.5.
Volume of NaCl (ml)	1	1	1	1	7	1	1	1	1.5.

The amount of penicillin produced from the 2 filtrates was dissolved in a phosphate buffer and filtered to get rid of the impurities. Then, a colony of S. aureus was dissolved in 5 ml NaCl, then NaCl was continued until its turbidity became similar to that of the standard (0.5 McFarland) (Figure 17).

The Mueller-Hinton medium was prepared in a petri dish. A swab was immersed in the tube containing S. aureus and inoculated into the petri dish. An antibiogram was carried out by applying 20 ⁻l of each antibiotic concentration (30, 25, 20, 16, 10) to the disks present in the petri dish and then incubated for 24 hours.

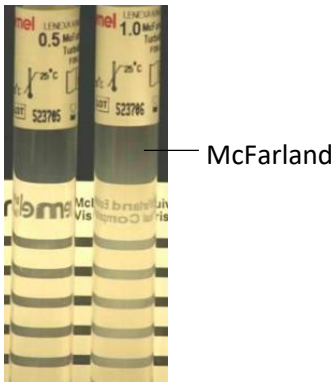


Figure 17: McFarland turbidity test

CHAPTER III: RESULTS AND DISCUSSION

1. Experience 1

The filtrate 1 and 2 antibiograms showed that different concentrations of commercial penicillin caused inhibition of bacterial growth by the formation of the inhibition zone. However, there is no zone of inhibition at the disk corresponding to the penicillin G produced, which indicates that our experiment has failed (FIGS. 18 and 19).

Figure 19 : Antibiogram showing results of filtrate 2 quantification

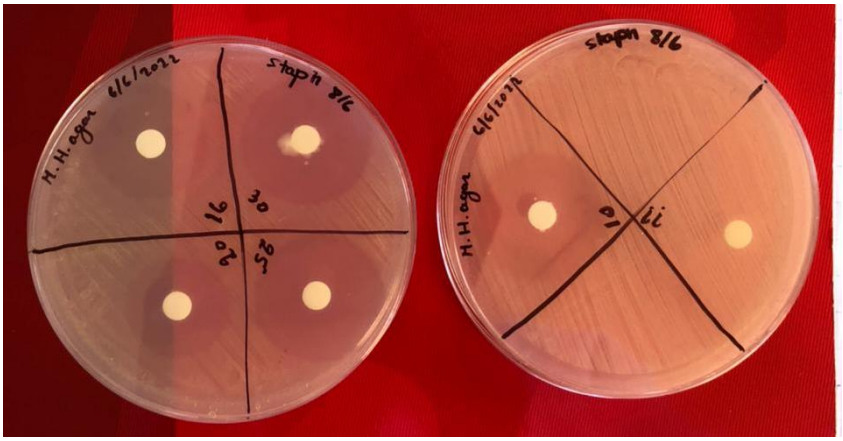
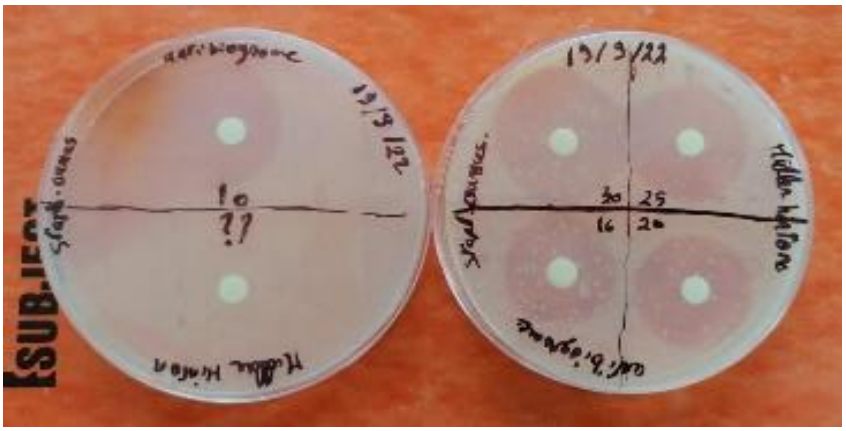


Figure 18 : Antibiogram showing the results of the quantification of filtrate 1



2. Experience 2

A sensitivity test was performed prior to the quantification step to investigate the presence or absence of penicillin G. Figure 20 shows the growth of the bacterium thus confirming the absence of penicillin G.

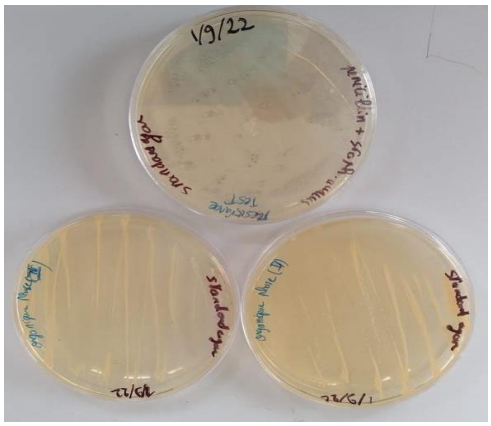


Figure 20 Result of sensitivity test of experiment 2. (a) control, (b) filtrate 1, (c) filtrate 2

3. Discussion

Our work focused on the cultivation of *Penicillium chrysogenum* on a liquid medium, producing and quantifying penicillin G by conventional methods. In the presence of glucose and lactose in the nutrient broth, glucose is preferentially consumed first by *P. chrysogenum* since it is considered to be the most important energy source. Lactose is consumed secondarily in a more difficult way, thus inducing the production of penicillin. In the first experiment, lactose was greater than glucose in both media, inhibiting penicillin formation. According To [44] The presence of a high amount of lactose inhibits the production of penicillin G. In addition, phenyl acetate, the precursor that leads *P. chrysogenum* to produce penicillin G, was absent in our first test. Yet, [44] stresses that it is a key product during fermentation for the production of Penicillin G.

In the light of the 1st experiment, the amount of lactose was significantly less than that of glucose in our 2nd test. Also, in the 2nd experiment, 0.8 ml of the phenyl acetate precursor was added once in order to direct *Penicillium chrysogenum* to produce penicillin G only. [44]However, the phenyl acetate precursor is the best precursor used to date, but it is preferable to add it several times in small amounts during fermentation in order to avoid toxic effects and thus improve the results of

the experiment, but this has not been achieved in our experiment. However, in all cases, the production of penicillin G failed, suggesting that the laboratory temperature was not suitable because of the sunlight that affected both mixtures of the incubator causing the destruction of *Penicillium chrysogenum*. In addition, for greater precision in the production of penicillin, HPLC or TLC techniques could be used prior to quantification by the antibiogram to determine the presence of penicillin G and other active products that may be present. According To [2]However, these 2 analytical methods are commonly used in laboratories for the separation and rapid identification of the constituents of a given extract.

CHAPTER IV: CONCLUSIONS AND PROSPECTS

The purpose of our study was the production and quantification of penicillin G by *Penicillium chrysogenum.* In the search for a better organism for penicillin production, none was found to be greater than *P. chrysogenum*.

The amount of lactose less than glucose promotes the production of penicillin by *P. chrysogenum*. The presence of the phenyl acetate precursor is essential for the specific production of penicillin G.

Finally, given that penicillin G production has failed and that its antibacterial activity is absent with respect to the *S. aureus* strain tested in the laboratory, this work opens up new perspectives such as changing the laboratory conditions under which one works in order to realize the production and quantification of penicillin G and then convert the work to a laboratory scale to a larger industrial scale using more improved *P. chrysogenum* strains to produce a large quantity of penicillin G, which is considered to be one of the most important discoveries of the twentieth century and certainly the one of the most important discoveries in modern medicine.

LIST OF ABBREVIATIONS

- DNA: Deoxyribonucleic acid
- RNA: Ribonucleic acid
- rRNA: Ribosomal ribonucleic acid
- tRNA: Transfer ribonucleic acid
- G or GlcNAc: N-acetylglucosamine
- *H. influenzae: Haemophilus influenzae*
- LSU: Large subunit
- M or MurNAc: N-acetylmuramic acid
- SSU: Small Subunit
- *S. aureus: Staphylococcus aureus*
- *Streptococcus pneumoniae*
- PBP: Penicillin binding protein
- *P. camembertii: Penicillium camembertii*
- *P. chrysogenum: Penicillium chrysogenum*
- *P. citrinum: Penicillium citrinum*
- *P. cyclopium: Penicillium cyclopium*
- *Penicillium notatum*
- *P. expansum: Penicillium expansum*
- *P. fellutatum*: Penicillium *fellutatum*
- *P. fuinculosum: Penicillium fuinculosum*
- *P. griseofulvum: Penicillium griseofulvum*
- pH: Hydrogen Potential
- *P. italicum: Penicillium italicum*
- *P. nalgiovense*: *Penicillium nalgiovense*
- *P. pupurogenum: Penicillium pupurogenum*
- *P. roquefortii: Penicillium roquefortii*
- PGN: Peptidoglycan

LIST OF SYMBOLS

- cm: Centimeter
- ˚C: Degree Celsius
- g: Gram
- (-): Negative
- ★ m: Micrometer
- ·L: Microliter
- mg: Milligram
- ml: Milliliter
- %: Percent
- (+): Positive
- S: Svedberg
- IU: International Unity

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