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Abstract:

Pseudomonas aeruginosa is a dangerous opportunistic pathogen and its high resistance to traditional antibiotics prompts researchers to explore alternative strategies targeting virulence factors and bacterial growth. Among these alternatives there are marine-derived fungi, and they represent a promising source of bioactive secondary metabolites with antibacterial potential and anti-virulence properties.

This study aims to evaluate the antibacterial and anti-virulence activities of marine fungal extracts against clinical and environmental strains of *P. aeruginosa*, and comparing them to a standard reference strain (ATCC 27853). From the isolated marine fungi three species were selected for this study: *Penicillium sp.* (P1), *Aspergillus flavus* (A2), and *Aspergillus terreus* (A4), and their secondary metabolites were extracted using ethyl acetate. The antibacterial activity was evaluated using disk and well diffusion techniques, determining minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), as well as the anti-virulent effect on biofilm formation and pyocyanin production, an important virulent factor regulated by quorum sensing.

The results showed moderate antibacterial activity, with MIC values of 2.5 and 5 mg/mL, while MBC values were 10 and 20 mg/mL. In contrast, diffusion tests showed weak activity, indicating limited diffusion of the compounds in the solid medium. As the most active extract, *Aspergillus flavus* extract (A2) was selected for studying its effect on virulence factors, where it demonstrated a strong ability in inhibiting pyocyanin production by 85 to 99% in all strains, and reducing biofilm formation in the clinical strain by 24.4% and the ATCC 27853 strain by 28.5%, while a slight increase was observed in the environmental strain by 3.7%.

Finally, these results indicate that marine fungal extracts have moderate antibacterial activity and high efficacy in inhibiting virulence factors associated with the quorum-sensing system, highlighting their potential as alternative therapeutic agents that target bacterial virulence factors rather than bacterial growth.

Key words: *Pseudomonas aeruginosa*, marine fungi, Fungal Secondary metabolites, Antibacterial activity, Anti-virulence activity.

Résumé:

Pseudomonas aeruginosa est un pathogène opportuniste dangereux, et sa forte résistance aux antibiotiques traditionnels incite les chercheurs à explorer des stratégies alternatives ciblant les facteurs de virulence et la croissance bactérienne. Parmi ces alternatives il y a les champignons d'origine marine qui représentent une source prometteuse de métabolites secondaires bioactifs qui donnent un potentiel antibactérien et des propriétés anti-virulence.

Cette étude vise à évaluer les activités antibactériennes et anti-virulence d'extraits de champignons marins contre une souche clinique et environnementale de *P. aeruginosa*, avec une comparaison à une souche de référence standard (ATCC 27853). À partir des champignons marins isolés, trois espèces de champignons marins ont été sélectionnées pour cette étude : *Penicillium sp.* (P1), *Aspergillus flavus* (A2) et *Aspergillus terreus* (A4), et leurs métabolites secondaires ont été extraits à l'aide de l'acétate d'éthyle.

L'activité antibactérienne a été évaluée à l'aide des techniques de diffusion sur disque et en puits, et en déterminant les concentrations inhibitrices minimales (CMI) et les concentrations bactéricides minimales (CMB), ainsi que l'effet anti-virulence sur la formation de biofilm et la production de pyocyanine qui sont des facteurs de virulence importants régulés par le quorum sensing.

Les résultats ont montré une activité antibactérienne modérée, avec des valeurs de CMI de 2,5 et 5 mg/mL, et des valeurs de CMB de 10 et 20 mg/mL. Les tests de diffusion ont révélé une faible activité, indiquant une diffusion limitée des composés dans le milieu solide. En tant qu'extrait le plus actif, l'extrait d'*Aspergillus flavus* (A2) a été sélectionné pour étudier son effet sur les facteurs de virulence. Il a démontré une forte capacité à inhiber la production de pyocyanine de 85 à 99 % dans toutes les souches et à réduire la formation de biofilm chez la souche clinique par 24,4 % et chez la souche ATCC 27853 par 28,5 %, tandis qu'une légère augmentation a été observée chez la souche environnementale par 3,7%.

Enfin, ces résultats indiquent que les extraits de champignons marins possèdent une activité antibactérienne modérée et une efficacité élevée sur l'inhibition des facteurs de virulence associés au système de quorum sensing, mettant en évidence leur potentiel en tant qu'agents thérapeutiques alternatifs ciblant les facteurs de virulence bactériens plutôt que la croissance bactérienne.

Mots-clés : *Pseudomonas aeruginosa*, Champignons marins, Métabolites secondaires, Activité antibactérienne, Activité anti-virulence.

المخلص

البكتيريا *Pseudomonas aeruginosa* تعتبر ممرضاً خطيراً، وتدفع مقاومتها العالية للمضادات الحيوية التقليدية الباحثين إلى استكشاف استراتيجيات بديلة تستهدف عوامل الضراوة والنمو البكتيري. ومن بين هذه البدائل تظهر الفطريات المشتقة من البيئة البحرية، حيث تمثل مصدراً واعداً للمستقلبات الثانوية النشطة بيولوجياً والتي تمتلك قدرات مضادة للبكتيريا وخصائص مثبتة لعوامل الضراوة.

تهدف هذه الدراسة إلى تقييم الأنشطة المضادة للبكتيريا والمثبتة لعوامل الضراوة الخاصة بالمستخلصات الفطرية البحرية ضد سلالات سريرية وبيئية من البكتيريا *P. aeruginosa*، مع مقارنتها أيضاً بسلالة مرجعية قياسية (ATCC 27853). من بين الفطريات البحرية المعزولة، تم اختيار ثلاثة أنواع للدراسة: *Aspergillus flavus* (A2)، *Aspergillus terreus* (A4)، و *Penicillium sp.* (P1). واستُخلصت نواتجها الأيضية الثانوية باستخدام خلاص الإيثيل. تم تقييم النشاط المضاد للبكتيريا باستخدام طريقتي الانتشار بالأقراص والانتشار بالآبار وتحديد التركيزات المثبطة الدنيا للبكتيريا (MIC) والتركيزات القاتلة الدنيا للبكتيريا (MBC)، بالإضافة إلى تقييم تأثيرها المثبط لعوامل الضراوة على تشكل الغشاء الحيوي (Biofilm) وإنتاج البيوسيانين (Pyocyanin)؛ وهي أحد أهم عوامل الضراوة التي ينظمها نظام استشعار النصاب (Quorum sensing).

أظهرت النتائج نشاطاً مضاداً للبكتيريا بدرجة متوسطة، حيث تراوحت قيم التركيز المثبط الأدنى للبكتيريا (MIC) بين 2.5 و 5 ملغ/مل، بينما تراوحت قيم التركيز القاتل الأدنى للبكتيريا (MBC) بين 10 و 20 ملغ/مل. بينما، أظهرت اختبارات الانتشار نشاطاً ضعيفاً يشير إلى محدودية انتشار المركبات في الوسط الغذائي الصلب. بصفته المستخلص الأكثر فاعلية، تم اختيار مستخلص *Aspergillus flavus* (A2) لدراسة تأثيره على عوامل الضراوة، وقد أظهر قدرة قوية على تثبيط إنتاج البيوسيانين بنسبة تتراوح بين 85% و 99% في جميع السلالات، وإيضاً تقليل تشكل الغشاء الحيوي في السلالة السريرية بنسبة 24.4% وفي السلالة المرجعية ATCC 27853 بنسبة 28.5%، بينما لوحظت زيادة طفيفة في السلالة البيئية بنسبة 3.7%.

في الختام، تُشير هذه النتائج إلى أن مستخلصات الفطريات البحرية تمتلك نشاطاً مضاداً للبكتيريا بدرجة متوسطة، ولكن فعاليتها في تثبيط عوامل الضراوة المرتبطة بنظام استشعار النصاب عالية، وهذا يسلط الضوء على إمكانية استخدامها كعوامل علاجية بديلة تستهدف عوامل الضراوة البكتيرية بدلاً من التركيز فقط على تثبيط النمو البكتيري.

الكلمات المفتاحية: البكتيريا *Pseudomonas aeruginosa*، الفطريات البحرية، المستقلبات الثانوية الفطرية، النشاط المضاد للبكتيريا، النشاط المضاد للضراوة.

List of Abbreviations:

°C: Degrees Celsius

A: *Aspergillus*

A2: *Aspergillus flavus* extract

A4: *Aspergillus terreus* extract

A-498: Human Kidney Carcinoma Cell Line 498

AAC: Aminoglycoside Acetyltransferase

AHL: N-Acyl Homoserine Lactone

AmpC: Ampicillin C Beta-Lactamase

ampC: Ampicillin C Beta-Lactamase gene

ampD: Ampicillin D Regulatory Gene

ANT: Aminoglycoside Nucleotidyl transferase

APH: Aminoglycoside Phosphotransferase

AQ: 2-Alkyl-4-Quinolone

ATCC: American Type Culture Collection

B: *Bacillus*

Caco-2: Human Colon Adenocarcinoma Cell Line 2

Calu-3: Human Lung Adenocarcinoma Cell Line 3

CDC: centers for disease control

CF: Cystic Fibrosis

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

E: *Escherichia*

EPS: Extracellular Polymeric Substance

exlA: Exolysin A gene

exoS: Exoenzyme S gene

exoU: Exoenzyme U gene

FIM: Florence Imipenemase Metallo-Beta-Lactamase

MexCD-OprJ: Multidrug Efflux System CD - Outer Membrane Protein J

MexEF-OprN: Multidrug Efflux System EF - Outer Membrane Protein N

MexXY-OprM: Multidrug Efflux System XY - Outer Membrane Protein M

MICs: Minimum Inhibitory Concentrations

mm: Millimeter

MPa: Megapascals

MRSA: Methicillin-Resistant *Staphylococcus aureus*

NDM: New Delhi Metallo-Beta-Lactamase

NLRP3: NOD-like Receptor Protein 3

OprD: Outer Protein D

OprF: Outer Protein F

OprH: Outer Protein H

OXA: Oxacillinase

P: *Pseudomonas*

P1: *Penicillium sp.* extract

PA14: *P. aeruginosa* Strain 14

PA7: *P. aeruginosa* Strain 7

PAO1: *P. aeruginosa* Reference Strain O1

parC: Topoisomerase IV Subunit C gene

PAβN: Phenylalanine-Arginine Beta-Naphthylamide

pel: Pellicle

pH: Potential of Hydrogen

phz1: Phenazine Biosynthesis Operon 1

phz2: Phenazine Biosynthesis Operon 2

Pqs: *Pseudomonas* Quinolone Signal

pqsABCDHP: *Pseudomonas* Quinolone Signal Genes A, B, C, D, H

PqsR: Pqs Regulatory Protein

GIM: German Imipenemase Metallo-Beta-Lactamase

gyrA: DNA Gyrase Subunit A gene

H: *Helicobacter*

HGT: Horizontal Gene Transfer

Hhq: 2-Heptyl-4-Hydroxyquinoline

IL-22: Interleukin-22

IMP: Imipenem-Hydrolyzing Metallo-Beta-Lactamase

L: Liter

Las: LasR-LasI(Autoinducer System)

lasB: LasB Elastase gene

LasI: Las Synthase enzyme

LasR: Las Regulatory Protein

L-Pro: L-Proline

L-Tyr: L-Tyrosine

M: *Micrococcus*

MBCs: Minimum Bactericidal Concentrations

MexAB-OprM: Multidrug Efflux System AB - Outer Membrane Protein M

psl: polysaccharide synthesis locus

QS: Quorum sensing

QSIs: Quorum Sensing Inhibitors

Rhl: RhlR-RhlI Autoinducer System

rhlA: RhlA Rhamnolipid Synthesis gene

RhlI: Rhl Synthase Enzyme

RhlR: Rhl Regulatory Protein

RND: Resistance-Nodulation-Division

sp: Species (singular)

SPM: São Paulo Metallo-Beta-Lactamase

spp: Species (plural)

T3SS: Type III Secretion System

V: *Vibrio*

VIM: Verona Integron-Encoded Metallo-Beta-Lactamase

WHO: World Health Organization

µg/mL: Micrograms per Milliliter

µM: Micromolar

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APPENDICES



Introduction

Introduction

The World Health Organization identifies antimicrobial resistance (AMR) as “a natural process that happens over time through genetic changes in pathogens” (WHO, 2023). which is considered one of the severe threats to global public health, due to the development of infections that are difficult to treat, which can also result in increased spread of infection and increased mortality (Wong China *et al.*, 2021). Although the “golden era” of antibiotics discovery in the 1960s led to the development of multiple major antibiotic classes like cephalosporins, macrolides, and tetracyclines, however the clinical effectiveness of these antibiotics is threatened by the emergence of multiple resistant bacteria (Balouiri *et al.*, 2016). This problem has been getting worse and becoming harder to treat because of the overuse of antibiotics in medicine and agriculture. Especially since the discovery of new antimicrobial agents has not kept pace with the increasing rates of new resistant bacteria (Si, 2024; Hossain *et al.*, 2024). This calls for the discovery of effective antibacterial agents from new antimicrobial alternatives that have never been more urgent.

Among the many pathogens of concern, one that has received considerable focus is *Pseudomonas aeruginosa*, a Gram-negative, rod-shaped, and motile bacterium and known for its broad thermotolerance and ubiquitous ecological distribution, including soil, water, and animal tissues (Wu *et al.*, 2015; Diggle & Whiteley, 2020). *P. aeruginosa* is an opportunistic pathogen, causing many hospital-acquired infections, and represents a bigger threat to immunocompromised and critically ill patients, as treatment of these patients is often complicated (Alva *et al.*, 2018; Abdulhaq *et al.*, 2020). Pathogenicity of *P. aeruginosa* depends on several virulence factors including pyocyanin, which is known to increase both the severity and duration of infections. Its production is also regulated by a quorum-sensing (QS) system (Grosso-Becerra *et al.*, 2014; Mudaliar & Prasad, 2024). *P. aeruginosa* is known to exhibit both intrinsic and acquired resistance to a multitude of antimicrobial agents, including most classes of antibacterials, and further contributes to increased resistance of the bacterium to antimicrobial agents and to the host immune system. (Abdulhaq *et al.*, 2020; Diggle & Whiteley, 2020). These features of *P. aeruginosa* make it a particularly more opportunistic and dangerous pathogen in clinical settings.

The antibiotic resistance of *P. aeruginosa* can generally be categorized into two classifications: intrinsic resistance, which is present in all strains of the bacteria as a result of their genetic coding, and it relies on several mechanisms, including Mex-type efflux pump and AmpC β -lactamase activity, and outer membrane permeability. Acquired resistance, which is developed via genetic mutations that alter antibiotic target sites, and the transfer of antibiotic resistance genes that gain resistance plasmids, along with modifying enzymes (Behzadi *et al.*, 2021; Elfadadny *et al.*, 2024). The *P. aeruginosa* resistance is also promoted by biofilm formation. The bacteria can also form a protective matrix of polysaccharides that shields the bacteria from the action of antibiotics and the immune response of the host (El-Sapagh *et al.*, 2023; Qin *et al.*, 2022).

Historically, natural products provide the foundation for developing antibacterial products, and it is still important to this day despite the progress in synthetic chemistry (Yurchenko *et al.*, 2024). These compounds exhibit structural complexity and chemical diversity that can help target systems such as bacterial quorum sensing and are less likely to cause resistance among bacteria. Compared to conventional antibiotics (Almutairi & Edrada-Ebel, 2025). It is known that microbial secondary metabolites facilitate new structural diversity for antimicrobial compounds, and as a result, can provide numerous potential agents for developing new drugs that can combat infections (Si *et al.*, 2024). Natural product-based anti-biofilm agents have demonstrated a better efficacy profile and an improved safety profile, as compared to their chemically synthesized counterparts, by acting through various mechanisms such as disruption of quorum sensing (Mishra *et al.*, 2020). Marine organisms in particular have shown a promising future in antimicrobial drug discovery, with over 1,000 new compounds reported from marine habitats every year. (Song *et al.*, 2021).

Marine fungi are either obligate or facultative growing in marine environments (Rateb & Ebel, 2011). They are able to grow in barren and minimal environments and create metabolites, unlike terrestrial fungi which are unable to produce compounds in similar environments to those found in the sea. These compounds are complex and are produced at low concentrations (Debbab *et al.*, 2010; Hasan *et al.*, 2015). Among the marine fungi, *Aspergillus* species are known for being responsible for approximately 900 dazzling new compounds with a greater variety of pharmacological activities. (Alhadrami *et al.*, 2025). Even though marine fungi have significant potential and are considered a great source of secondary metabolites that have the

potential to battle multi-drug-resistant pathogens, they are still largely unexplored and less used (Sendekie *et al.*, 2024).

Despite their amazing and promising potential, research on marine fungi is restricted by several significant challenges that make the discovery and development of new antibacterial agents difficult. Most research focuses on isolating and identifying the structure of compounds with very few pharmacology studies, while the antibiofilm activity of seaweed-associated endophytic fungi remains largely unexplored. (Pan *et al.*, 2025; Almutairi & Edrada-Ebel, 2025). At the same time, there are also challenges in purification, inconsistency in bioactive compound production across fungal strains, and many fungal pathways are silent when grown in standard laboratory conditions, making it difficult to access useful fungal metabolites and leading to low rates of bioactive compounds yield. (AbdulQader *et al.*, 2023; Almutairi & Edrada-Ebel, 2025; Jin *et al.*, 2016; Yurchenko *et al.*, 2025). But at the same time, a major focus of marine mycology is expanding the biotechnology for large-scale marine fungi cultivation and developing innovative techniques to facilitate early detection of bioactive compounds targeting pathogens such as *P. aeruginosa*. (Yurchenko *et al.*, 2025; Tran Thi *et al.*, 2020).

This study aims to evaluate the antibacterial activity of secondary metabolites extracted from marine algal endophytic fungi and their potential against clinical and environmental isolates of *Pseudomonas aeruginosa* (including the reference strain *P. aeruginosa* ATCC 27853) alongside their anti-virulence properties against biofilm formation and pyocyanin production, which serve as indicators of quorum-sensing-related virulence.

To accomplish the objectives of this study, the following were done:

- Extraction of secondary metabolites from selected marine fungi.
- Evaluation of antibacterial activity against clinical and environmental *P. aeruginosa*, as well as ATCC 27853 bacteria.
- Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).
- Assessment of biofilm formation and inhibition capacity.
- Quantification of pyocyanin production.
- Analysis of the antimicrobial and anti-virulence effects of the fungal extracts.

The manuscript is divided into two parts: The first part is a bibliographic review of antimicrobial resistance in *P. aeruginosa*, marine fungi as a source of bioactive compounds, and the impact of these compounds on bacterial growth and virulence. The second part is the description of the experimental part, materials, methods, results, and discussion, including the general conclusion and outlook.

Litterateur
Review

Chapter 1

Pseudomonas aeruginosa:
Clinical, Environmental
Characteristics and
Antimicrobial Resistance

Chapter 1: *Pseudomonas aeruginosa*: clinical, environmental characteristics and antimicrobial resistance

1. General Overview of *Pseudomonas aeruginosa*

1.1. Historical background

Pseudomonas aeruginosa was first isolated in 1882 by Carle Gessard after he observed a blue-green coloration on a soldier's bandage. In 1894, Walter Migula introduced the genus *Pseudomonas* to classify Gram-negative, rod-shaped aerobic bacteria with polar flagella, although this classification included several unrelated organisms. Among this genus, *Pseudomonas aeruginosa* became one of the most important and widely studied species. (Moore & Flaws, 2011).

1.2. General definition

Pseudomonas aeruginosa is a Gram-negative bacterium with a rod-shaped and it could be found widely in the environment. It is considered as an opportunistic pathogen which typically causes disease in immunocompromised people (Moradali *et al.*, 2017). Including patients with cystic fibrosis, burn wounds, cancer, chronic obstructive pulmonary disorder (COPD), and also those receiving mechanical ventilation for conditions like COVID-19 (Qin *et al.*, 2022). And according to Wood *et al.* (2023), the mortality rates of these diseases can reach up to 40% in severe cases, highlighting a high level of clinical risk.

P. aeruginosa measures around 0.5 to 0.8 μm in width and 1.5 to 3.0 μm in length, and most of it strains carry a single polar flagellum that allows it movements and motility. Under normal conditions, it uses oxygen as its electron acceptor, but it can switch to nitrate or arginine when oxygen is limited, allowing it to survive across a wide range of environments (Moore & Flaws, 2011; Wood *et al.*, 2023). It is known for being saccharolytic, glucose and lactose non-fermenting, and non-spore forming. And it produces well-known pigments: pyocyanin, which gives colonies a blue-green color, and pyoverdine, a fluorescent yellow-green pigment. few strains also produce pyorubrin (red) or pyomelanin (brown). (Moore & Flaws, 2011).

Treating *P. aeruginosa* infections has become increasingly difficult, and even the World Health Organization (WHO) and the Centers for Disease Control (CDC) have placed it among the ESKAPE also called the priority pathogens, which refers to a group of bacteria including (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) for which new antimicrobial development is considered urgent (Wood *et al.*, 2023). *Pseudomonas aeruginosa* structure is showcasing in (Figure 1)

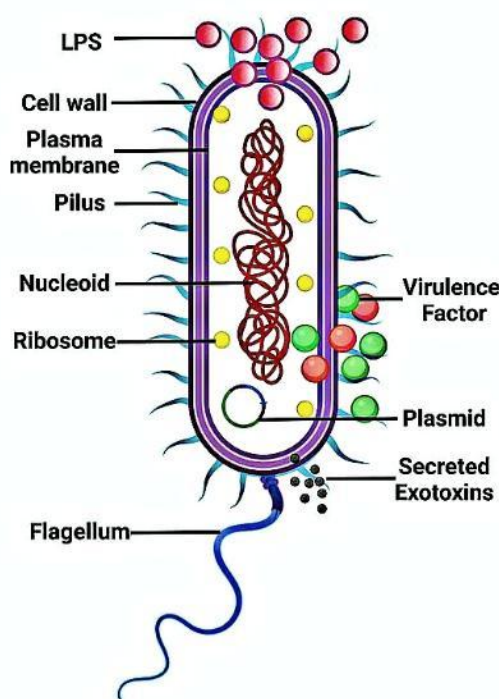


Figure 1: Schematic diagram of the structure of *Pseudomonas aeruginosa*, illustrating key cellular components. (Adapted from Anjum *et al.*, 2023).

2. Taxonomy and Classification

Pseudomonas aeruginosa belongs to the domain Bacteria, phylum Pseudomonadota, class Gammaproteobacteria, order Pseudomonadales, family Pseudomonadaceae, and genus *Pseudomonas* (NCBI, 2026). It is a Gram-negative, rod-shaped organism, consistent with the defining features of the family Pseudomonadaceae (Wood *et al.*, 2023).

The genus *Pseudomonas* was first described in 1894 and is now one of the largest known prokaryotic genera, with approximately 310 validly published species, and this number continues to grow. Its members have been found in soil, water, plant tissue, and animal hosts,

and some of them are opportunistic pathogens of humans, animals, or plants, as well as other species of broader ecological and economic relevance (**Rudra & Gupta, 2024**).

This genus is recognized as one of the largest genera among Gram-negative bacteria, with considerable genetic diversity across its members (**Girard *et al.*, 2021**). However, Genome-based analyses have revealed that not all species currently assigned to *Pseudomonas* share a similar common evolutionary origin, which has led researchers to propose reclassification of some misassigned species (**Lalucat *et al.*, 2020**).

Within this genus, *P. aeruginosa* is a unique and serves as the nomenclature type species. It anchors what researchers call the "*Aeruginosa* clade" or "*Pseudomonas sensu stricto*" (**Rudra & Gupta, 2024**). As the most studied member of the genus, *P. aeruginosa* is both a scientific reference organism and the defining example of what *Pseudomonas* means in clinical and evolutionary terms.

3. Environmental Distribution and Ecology

Pseudomonas aeruginosa is found in soil, water, on plants, and on animals. It has also been identified in hospital sinks, drains, and other clinical environments, which is why it is frequently described as ubiquitous (**Moore & Flaws, 2011; Crone *et al.*, 2019**). It can survive in oil-contaminated sites and other chemically harsh conditions, reflecting its broad metabolic range (**Crone *et al.*, 2019**).

The persistence of *P. aeruginosa* stems partly from its capacity to adapt. When nutrients become limited, it slows its metabolism by reducing the expression of genes involved in protein production, ribosome synthesis, cell wall construction, and nucleic acid activity. This metabolic slowdown allows the bacteria to enter a dormant state and survive conditions that would kill other organisms (**Moradali *et al.*, 2017**). According to **Cecil *et al.* (2025)**, he noticed that strains that grown in low-nutrient settings grow more slowly, reduce their cell size, produce more pyocyanin, and reduce their motility compared to nutrient-rich conditions.

The bacterium also faces oxidative stress in natural environments, where it is regularly exposed to oxidizing agents, and this happens by activating adaptive mechanisms that protect it against oxidative damage (**Cruz Nizer *et al.*, 2021**). These responses overlap with its reaction to

antibiotic exposure and nutritional starvation, and they all leads to the same outcome: metabolic dormancy and persister cell formation (**Moradali *et al.*, 2017**).

4. Virulence Factors

Pseudomonas aeruginosa produces a wide range of virulence factors that help it colonize host tissue, resist immune defenses, and cause damage. These factors include both cell-associated structures and secreted compounds. They support bacterial survival and growth, while still injuring the host tissue and interfere with the immune responses (**Moradali *et al.*, 2017**). The bacterium has multiple secretion systems for delivering these compounds into the host environment, which partly accounts for the diversity of infections it can cause (**Kerr & Snelling, 2009**). Among all of the virulence factors of *P. aeruginosa*, the three most important are: Biofilm formation and Quorum sensing and Pigment production.

4.1. Biofilm Formation

A biofilm is a structured community of bacteria attached to a surface and enclosed in a self-produced matrix called extracellular polymeric substances (EPS). When *P. aeruginosa* transitions from free-swimming to a surface-attached state, it stops producing flagella-driven motility and shifts its gene expression toward attachment, EPS production, and biofilm maturation. This transition involves coordinated regulation at multiple levels: transcriptional, post-transcriptional, and post-translational, affecting hundreds of genes at once. (**Moradali *et al.*, 2017**).

Biofilms offer substantial protection, as bacteria within biofilms tolerate phagocytosis, oxidative stress, nutrient limitation, competition from other species, and antibiotic treatment more effectively than planktonic cells, (**Moradali *et al.*, 2017**). In clinical settings, biofilms inside the host are linked to chronic lung infections in cystic fibrosis and bronchiectasis patients, as well as infections on medical devices and implants (**Kerr & Snelling, 2009**). Mucoid biofilm formation is considered a sign of chronic infection and disease progression *P. aeruginosa* can also form biofilms at the liquid-air interface, called pellicles, which are rich in glucose-based polysaccharides (**Wu *et al.*, 2015**).

The biofilm matrix composition varies by strain. Some strains produce alginate, while others rely on polysaccharides encoded by the *psl* and *pel* genetic loci. The *psl* locus encodes a mannose-rich polysaccharide involved in both cell-surface and cell-cell interactions, while *pel* encodes glucose-rich components. Quorum sensing, particularly the Las system, is also involved in the formation of mature biofilms (Wu *et al.*, 2015).

4.2. Quorum Sensing Systems

Quorum sensing (QS) is a system of bacteria used to detect population density. Bacteria releases small chemical molecules called autoinducers into the surrounding environment. Once the population reaches a certain density, the concentration of these molecules crosses a threshold, activating receptor proteins and triggering changes in gene expression across the population (Qin *et al.*, 2022).

P. aeruginosa has four QS systems that are connected and regulate each other. Two of them, Las and Rhl, are based on N-acyl homoserine lactone (AHL) signals. The other two, Pqs and its precursor Hhq, are based on 2-alkyl-4-quinolone (AQ) signals (Qin *et al.*, 2022; Wu *et al.*, 2015). Hierarchically, Rhl and Pqs are activated by the Las system. Rhl suppresses Pqs, while Pqs strengthens Rhl. Under certain nutritional or environmental conditions, Rhl and Pqs can also shift their relationship independently of Las (Qin *et al.*, 2022).

Each system follows the same basic structure: a sensor protein detects autoinducers and, once activated, it triggers the production of more autoinducers through a synthase enzyme. In the Las system, for example, LasR activates LasI; in the Rhl system, RhlR activates RhlI; and in the Pqs system, PqsR activates the pqsABCDH gene cluster (Moradali *et al.*, 2017).

QS controls a broad set of bacterial behaviors relevant to infection, including the production of extracellular enzymes, iron-chelating compounds, efflux pumps, biofilm development, swarming motility, and immune evasion mechanisms (Qin *et al.*, 2022). In chronic CF infections, QS has been shown to upregulate destructive virulence factors such as pyocyanin and other phenazines (Moradali *et al.*, 2017). Because QS operates through a non-lethal signaling pathway, researchers have explored it as a target for antivirulence drugs that would disrupt bacterial coordination without the selective pressure that conventional antibiotics create (Qin *et al.*, 2022).

4.3. Pigments (Pyocyanin and Pyoverdine)

P. aeruginosa produces several pigments. The main ones are pyocyanin, a blue-green compound, and pyoverdine, a fluorescent yellow-green pigment. Some strains also produce pyorubrin (red-brown) or pyomelanin (brown) (Moore & Flaws, 2011).

Pyocyanin is a redox-active secondary metabolite that can accept and donate electrons, generating reactive oxygen species inside host cells (Rada & Leto, 2013). It interferes with electron transport, energy metabolism, ciliary movement in the airways, calcium balance, and immune signaling (Wu *et al.*, 2015; Rada & Leto, 2013). In cystic fibrosis patients, pyocyanin can be present in airway secretions at concentrations up to 100 $\mu\text{mol/L}$ during periods of infection (Caldwell *et al.*, 2009). It also suppresses activation of the NLRP3 inflammasome, an intracellular protein complex that recognize danger signals by weakening a key part of the innate immune response (Qin *et al.*, 2022). pyocyanin production depends on the *phz1* and *phz2* operons, which are activated by QS regulatory proteins, placing pyocyanin under the control of the overall QS network (Mudaliar & Prasad, 2024).

Pyoverdine is a siderophore that binds iron in the surrounding environment and transports it back into the bacterial cell. In iron-depleted conditions, such as host tissue, this allows *P. aeruginosa* to obtain a nutrient that is otherwise unavailable, supporting bacterial survival and strengthening virulence. A second siderophore, pyochelin, performs a similar function but with lower iron affinity (Qin *et al.*, 2022).

5. Antimicrobial Resistance

Treating *P. aeruginosa* infections has become increasingly difficult over time. This bacterium carries a built-in resistance for many antibiotic classes, and multidrug-resistant strains have been rising globally for over two decades (Wood *et al.*, 2023; Moradali *et al.*, 2017). Resistance in this organism operates at three levels: intrinsic, acquired, and adaptive.

5.1. Intrinsic Resistance

According to Pang *et al.* (2018), intrinsic resistance refers to mechanisms built into the genome of *Pseudomonas aeruginosa*, present regardless of antibiotic exposure, and it relies on three main built-in mechanisms:

- a. Reduced outer membrane permeability: The outer membrane of *P. aeruginosa* is 12 to 100 times less permeable than that of *E. coli*. The major porin OprF, which would normally allow molecules to enter, remains mostly closed. The specific porin OprD normally allows carbapenems to pass through, but when OprD is lost or reduced, resistance to carbapenems increases. Another porin, OprH, is produced under magnesium starvation and stabilizes the outer membrane, raising resistance to polymyxin B and gentamicin.
- b. Efflux pumps: *P. aeruginosa* uses RND-family pumps to actively expel antibiotics from the cell. There are four main pumps that cover multiple drug classes:
 - MexAB-OprM removes beta-lactams and quinolones.
 - MexCD-OprJ removes beta-lactams.
 - MexEF-OprN targets quinolones.
 - MexXY-OprM handles aminoglycosides.

The overexpression of these pumps results in multidrug resistance. However, some efflux pump inhibitors such as PA β N can block these pumps and partially restore antibiotic activity.

- c. The third mechanism is the production of antibiotic-inactivating enzymes: AmpC, a class C beta-lactamase encoded in the chromosome, breaks down beta-lactam antibiotics, including cephalosporins. Some strains also produce extended-spectrum beta-lactamases of class A or D (OXA-type). For aminoglycosides, three types of modifying enzymes add chemical groups that prevent the drug from binding to its target, and they are: APH (phosphorylation), AAC (acetylation), and ANT (adenylation), each targeting different aminoglycosides.

5.2. Acquired Resistance

Acquired resistance arises during or after antibiotic treatment, either through mutations in bacterial DNA or through the uptake of resistance genes from other bacteria (Pang *et al.*, 2018). Unlike intrinsic mechanisms, this type of resistance happens because of antibiotic selection pressure, which is when the presence of an antibiotic creates an environment where resistant variants survive and spread (Moradali *et al.*, 2017).

According to **Pang *et al.* (2018)**, *P. aeruginosa* acquires antibiotic resistance through two main ways:

- a. Mutations can produce resistance in several ways, such as:
 - Loss or modification of OprD leads to carbapenem resistance.
 - Changes in the genes encoding DNA gyrase (*gyrA*), or topoisomerase IV (*parC*) reduce susceptibility to quinolones.
 - Ribosomal alterations that affect aminoglycosides and modifications of the penicillin-binding proteins affect susceptibility to beta-lactams.
 - Mutations in efflux pump regulator genes can cause overexpression of MexAB-OprM, MexXY-OprM, or MexCD-OprJ.
 - Mutations in *ampC* or *ampD* can also lead to overproduction of the AmpC beta-lactamase.
- b. Horizontal gene transfer (HGT) allows *P. aeruginosa* to acquire resistance genes from other bacteria through conjugation, transduction, or transformation (**Wood *et al.*, 2023**). For this process, integrons which are a genetic platform that collect and express resistance genes, are particularly important. Genes encoding metallo-beta-lactamases (IMP, VIM, SPM, GIM, NDM, FIM) and aminoglycoside-modifying enzymes are frequently found on integrons or plasmids, allowing several resistance mechanisms to be transferred together (**Pang *et al.*, 2018**).

5.3. Adaptive Resistance:

Adaptive resistance is a temporary and a reversible increase in tolerance to antibiotics that occurs in response to environmental conditions. It does not involve permanent genetic changes, and susceptibility returns when the activating condition is removed (**Moradali *et al.*, 2017; Pang *et al.*, 2018**).

According to **Pang *et al.* (2018)**, the main two adaptive mechanisms are:

The first mechanism is Biofilm formation, and it is the most documented mechanism of adaptive resistance. In this mechanism, bacteria inside a biofilm are surrounded by an EPS matrix made of exopolysaccharides, proteins, and extracellular DNA. This physical barrier reduces antibiotic penetration. Biofilm cells also show altered metabolic activity compared to free-swimming cells. Once the biofilm is removed, antibiotic sensitivity largely returns, confirming the adaptive rather than mutational nature of this resistance.

Second mechanism involves persister cells, which are a small fraction of the bacterial population, typically around 1% of biofilm cells, that shift into a dormant, metabolically inactive state. In this state, antibiotics are ineffective against them, because most drugs target active cellular processes. When antibiotic treatment ends, persister cells reactivate and repopulate the biofilm. This explains why chronic infections in CF patients often relapse after treatment.

Multidrug-resistant *P. aeruginosa* infections have risen steadily over the past 20 years, driven in part by increased antibiotic use and by the persistence of tolerant phenotypes under metabolic slowdown and oxygen-limited conditions (Wood *et al.*, 2023).

6. Clinical vs. Environmental Strains

Clinical and environmental strains of *P. aeruginosa* are genetically very similar, and whole genome analyses have shown that their genomes share a high degree of sequence conservation, and both types carry genes associated with virulence. This contrasts with many other bacterial pathogens, where clinical strains tend to have clearly distinct genomic features, but in *P. aeruginosa*, phenotypic differences between the two groups are not well reflected at the genomic level (Grosso-Becerra *et al.*, 2014).

However, some consistent differences do exist. Clinical strains showed greater adhesion to respiratory and intestinal cell lines (Calu-3 and Caco-2), while environmental strains adhere more strongly to kidney cell lines (Streeter *et al.*, 2016). Clinical strains carry specific virulence genes more frequently, and in most clinical isolates, virulence gene prevalence exceeds 50%, while in most environmental isolates it stays below 25%, with the exception of *exoS* (Alva *et al.*, 2018). Environmental strains showed higher adhesion to A-498 cells and a higher combined prevalence of both *exoS* and *exoU* toxin genes (Streeter *et al.*, 2016).

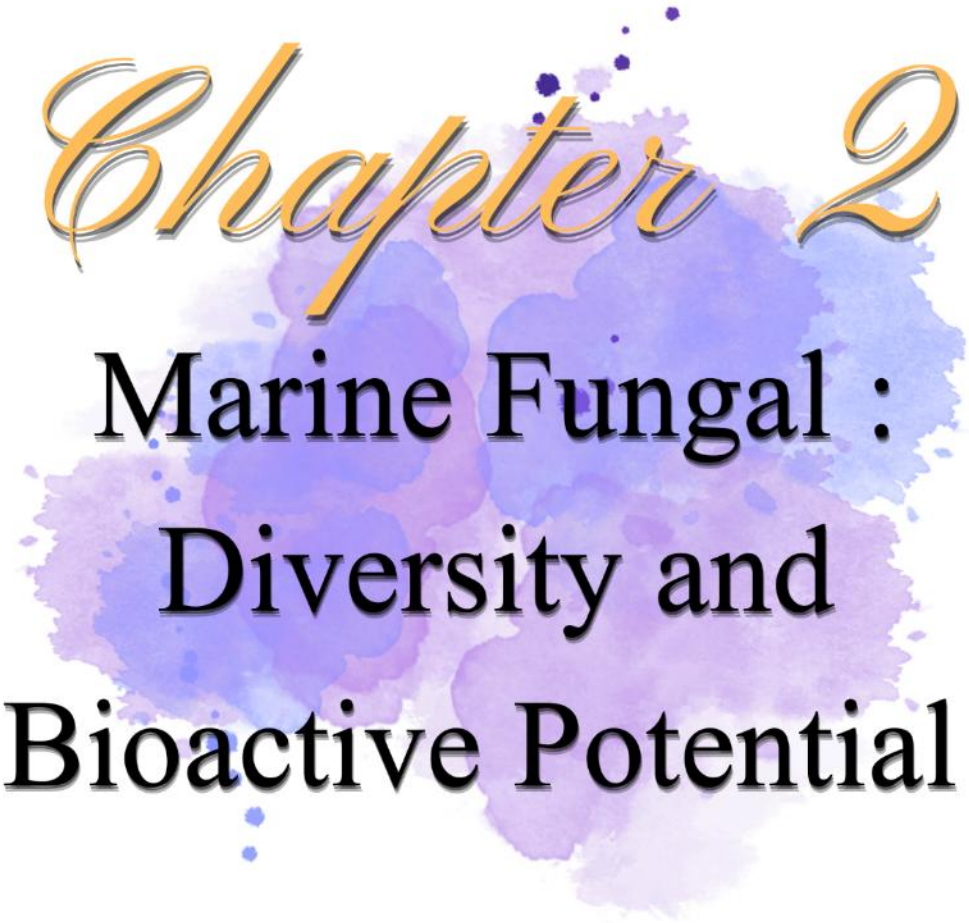
Regarding resistance, clinical strains showed reduced susceptibility to certain antibiotics but were more susceptible to ciliate predation than environmental strains. Clinical strains maintained higher cell density under starvation but were outcompeted when nutrients were available (Vasco *et al.*, 2025). Environmental strains also tended to produce higher levels of pyocyanin, in a study comparing soil and urinary tract isolates measured average pyocyanin concentrations of 9.3 µg/ml and 5.9 µg/ml respectively (Shouman *et al.*, 2023).

Environmental isolates tend to belong to different sequence types than clinical ones, suggesting separate evolutionary patterns (Aroca Molina *et al.*, 2024). Genome-based typing has also identified three broad resistotypes across both populations: PAO1, PA14, and PA7. PAO1 and PA14 both carry the Type III secretion system (T3SS), but differ in their toxin profiles, PAO1 carries *exoS* but not *exoU*, whereas PA14 carries *exoU* but not *exoS*. While PA7 lacks T3SS entirely and instead uses the two-partner secretion pathway to deliver the *exlA* exolysin (Behzadi *et al.*, 2021).

Phenotypic adaptation in clinical strains over time tends to include slower growth, reduced motility, biofilm formation, alginate overproduction, increased antibiotic resistance, and downregulation of QS systems (Moradali *et al.*, 2017).

All this information suggests that clinical and environmental strains are not different because of the presence of specific genes, but rather because of how they regulate and use these genes to survive under natural conditions and against different antibiotics. This highlights the adaptability and pathogenic potential of *P. aeruginosa*, which is a major clinical problem, especially with its high mortality rates in severe infections.

Overall, the need to explore alternative strategies other than traditional antibiotics is very urgent, especially strategies that target both the bacteria and its virulence factors such as biofilm formation and pigment production. In response to this urgent need, research has turned to natural sources of bioactive compounds. Among these, the marine environment represents one of the richest and largely unexplored sources, and within this environment, there is a wide range of microorganisms that exhibit significant antibacterial activity, such as marine fungi.

A decorative background element consisting of a purple watercolor splash with various shades of purple and blue, and several small purple dots scattered around it.

Chapter 2

Marine Fungal : Diversity and Bioactive Potential

Chapter 2: Marine Fungi Diversity and Bioactive Potential

1. General Definition of Marine Fungi

The most widely used and accepted definition describes them as any fungus repeatedly recovered from marine habitats because it can grow and sporulate in marine environments, forms symbiotic relationships with other marine organisms, or shows genetic adaptation and metabolic activity suited to those conditions (**Pang *et al.*, 2016**).

Within this broad category, two types are recognized: Obligate marine fungi, those that grow and reproduce exclusively in marine or estuarine environments. Facultative marine fungi, the ones that originate from freshwater or terrestrial ecosystems but can still grow and sometimes sporulate when placed in marine conditions (**Bonugli-Santos *et al.*, 2015**; **Cunliffe, 2023**). Some fungi that were found in marine samples are the same or closely similar to non-marine taxa, which makes distinguishing between obligate and facultative species a persistent challenge in research (**Cunliffe, 2023**). For this reason, the neutral term "marine-derived fungi" is usually used to refer to any strain obtained from a marine environment through cultivation on marine media, without specifying whether it is truly marine or a terrestrial contaminant (**Jin *et al.*, 2016**).

Marine fungi are known for playing an important role in ecology such as breaking down lignocellulose, decomposing dead animal matter, and in some cases causing disease in marine plants and animals (**Hyde *et al.*, 2000**). As decomposers, they help convert polymeric compounds into forms that other organisms in the food web can use (**Hyde *et al.*, 1998**).

2. Taxonomy and Classification of Marine Fungi

A diverse marine fungal community is distributed across several phyla: Ascomycota, Basidiomycota, Chytridiomycota, Mortierellomycota, Mucoromycota, and Rozellomycota. Based on data compiled by Jones *et al.* (2019), there are 1,257 recognized obligate marine species distributed across 539 genera, 74 orders, 168 families, 20 classes, and five phyla. (**Wijayawardene *et al.*, 2022**). This highlights the high diversity in the marine environment.

Among all these, three phyla are frequently reported as the most dominant and representative groups, and they are:

2.1. Ascomycota

Ascomycota is the biggest phylum in the fungal kingdom, accounting for roughly 64% of all described fungal species and which corresponds to around 33,000 described species organized into three subphyla: Taphrinomycotina, Saccharomycotina, and Pezizomycotina (**Stajich *et al.*, 2009; Carris *et al.*, 2012**). Reproduction occurs sexually through the production of ascospores inside a sac-like structure named an “ascus”, and there are other species that reproduce asexually through spores called conidia, which vary widely in size, shape, color, and septation (**Carris *et al.*, 2012**).

In marine environments, ascomycetes are the dominant group. They appear to have evolved from terrestrial ancestors but have adapted well to aquatic conditions through small fruiting bodies, appendaged spores suited to transport and attachment, and tolerance to varying salinity (**Wijayawardene *et al.*, 2022**). Some members can even grow at higher salinity compared to the salinities of the seawater, are also adapted to low water activity or are genuinely halophilic (**Pang *et al.*, 2016**). Three broad groups exist among marine ascomycetes: those with terrestrial counterparts in the unitunicate lineage, marine Loculoascomycetes with terrestrial and freshwater relatives, and the exclusively aquatic family Halosphaeriaceae (**Wijayawardene *et al.*, 2022**).

On land, Ascomycota species live saprobes, mutualists in lichens and mycorrhizae, and parasites. And the pathogenic species within this phylum are known for causing serious threats to agriculture and account for nearly all severe systemic human fungal infections, as well as common conditions like athlete's foot (**Stajich *et al.*, 2009**).

Within the diverse genera classified in this phylum, two major genera of interest in this study are *Aspergillus* and *Penicillium*.

2.1.1. Genus *Aspergillus* in Marine Environments

Aspergillus is one of the most studied genera in marine fungal research. It is found primarily in mangrove substrates, coral reef systems, and deep-sea sediments. In these environments, it faces high osmotic pressure, high hydrostatic pressure, and low dissolved oxygen. In response to these pressures, marine *Aspergillus* strains have developed secondary metabolic pathways that differ from those of terrestrial relatives, partly through horizontal gene transfer and metabolic

network reorganization. This results in the production of natural products not found in terrestrial strains. *Aspergillus* accounts for 31% of new natural products reported from marine fungi, the highest proportion of any genus in this field (Wang *et al.*, 2025). (Figure 2).

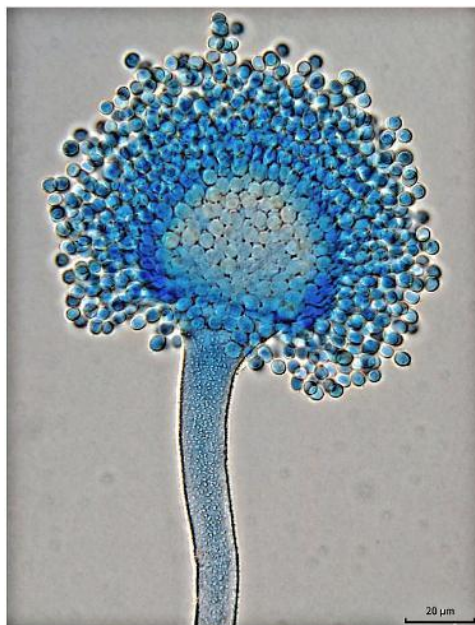


Figure 2 : Light microscopy of *Aspergillus flavus* stained with lactophenol cotton blue, showing the characteristic conidiophore structure. (Reproduced from Pickova *et al.*, 2021).

- **Photo credit:** Vladimir Ostry. Reproduced from **Pickova *et al.* (2021)** under CC BY 4.0 license.

2.1.2. Genus *Penicillium* in Marine Environments

Penicillium belongs to the family Aspergillaceae, order Eurotiales, class Eurotiomycetes, phylum Ascomycota. This genus is recognized by its colony texture (velutinous, floccose, or powdery), its range of colors, and its characteristic brush-like conidiophores visible under the microscope. At least 100 species have been reported from marine sources, including mangroves, seawater, sediments, sponges, and macroalgae. This confirms their presence across diverse marine substrates, suggesting active roles in organic matter breakdown and ecosystem function (Lee *et al.*, 2025).

The identification at the species level remains a challenge. Its colony morphology can suggest the genus, but differentiating between species who are closely-related requires more than visual inspection, given the high similarity among many taxa (Lee *et al.*, 2025). (Figure 3)



Figure 3 : Microscopic view of *Penicillium chrysogenum*, showing conidiophore structure with conidia (spores), (Reproduced from Harris, 2008).

* **Photo credit:** Image courtesy of CBS Fungal Diversity Centre. Reproduced from Harris, (2008).

2.2. Basidiomycota

Basidiomycota is the second largest fungal phylum, with close to 30,000 described species (Carris *et al.*, 2012; He *et al.*, 2019). Members produce basidiospores on club-shaped structures known as “basidia”. The mycelium of many species from this phylum shows two distinctive features: clamp connections (Hook-like hyphal outgrowths) and dolipore septa (barrel-shaped cross-walls). (Carris *et al.*, 2012). Basidiomycetes are almost entirely filamentous, and reproduce sexually and go through complex life cycles (De Mattos-Shipley *et al.*, 2016).

2.3. Chytridiomycota

Also called “Chytrids”, it forms a small group of about 900 identified species, and can be found in both aquatic and terrestrial habitats worldwide. A key feature that is shared by all species in this phylum is the production of zoospores, with each bearing a single posteriorly directed flagellum (Carris *et al.*, 2012).

3. Diversity and Ecological Distribution of Marine Fungi

Marine fungi inhabit a variety of substrates such as: submerged wood, leaves, sand grains, sediments, seagrasses, sponges, marine invertebrates, vertebrates, plankton, algae, mangrove plants, and even plastic materials found in marine environments. (Wijayawardene *et al.*, 2022).

The deep sea presents its own conditions: no sunlight, temperatures typically below 4°C, and hydrostatic pressures reaching up to 110 MPa. Fungi living there must cope with all three at once in order to survive in such conditions. Oceanic currents shape fungal diversity by moving nutrients, affecting productivity, and dispersing microbial populations across long distances (Wijayawardene *et al.*, 2022).

Ecologically, marine fungi serve several functions. They act as parasites of phytoplankton, recycle organic matter as saprotrophs by breaking down phytoplankton-produced polysaccharides, and form symbiotic associations with seaweeds and other marine life (Cunliffe, 2023). In mutualistic associations, marine fungi appear in three forms: lichens, mycophycobioses (relationships with macroalgae), and mycorrhizas (Hyde *et al.*, 1998).

Among productive isolates, sediments lead at 34%, followed by sponges at 19%, cnidarians at 16%, other invertebrates at 12%, and algae or seagrass at 10% (Carroll *et al.*, 2019). This highlights the variation in the distribution of marine fungi in marine environments.

For this study, marine fungi were isolated from different macroalgae, highlighting the importance of algae as a natural source of fungal diversity.

4. Marine Macroalgae as a Substrate for Fungal Isolation

Algae are photosynthetic organisms that contain chlorophyll and live in water or humid environments. They are divided into two broad size categories: microalgae, which are microscopic, and macroalgae, which are large enough to be visible and can reach over 50 meters in length. The marine environment contains an estimated 1,800 species of brown macroalgae, 6,200 species of red macroalgae, and 1,800 species of green macroalgae. Red algae are the most species-rich, but brown algae are the largest in physical size. The shape of the thallus and its texture are the main features used to distinguish between species (Pereira, 2021).

4.1. Green Algae (Chlorophyta)

Green macroalgae belong to the phylum Chlorophyta. Their pigmentation matches that of vascular plants, relying on chlorophylls a and b along with carotenoids (**Pereira, 2021**). The group includes marine, freshwater, and terrestrial species with diverse vegetative forms. When motile cells are present, they typically carry a cruciate root system. (**Leliaert, 2019**). (Figure 4)



Figure 4 : Representative examples of green seaweeds (Chlorophyta) exhibiting morphological diversity: (A) *Dasycladus vermicularis*; (B) *Ulva clathrata*; (C) *Ulva compressa*; (D) *Ulva intestinalis*; (E) *Ulva lactuca*; (F) *Ulva linza*. (Adapted from Cotas *et al.*, 2020).

- **Photo credit:** Portuguese Seaweeds Website (MACOI; <http://www.flordeutopia.pt/macoi/>). Reproduced from Cotas *et al.* (2020) under CC BY 4.0 license.

4.2. Red Algae (Rhodophyta)

Red macroalgae belong to the phylum Rhodophyta and use chlorophyll a, phycobilins, and some carotenoids for photosynthesis (**Pereira, 2021**). Red algae have relatively small, intron-poor genomes and no flagella or centrioles. But they have a highly efficient photosynthetic capacity. Multicellular red algae are among the earliest eukaryotic lineages to develop complex

multicellularity, yet despite their ecological, evolutionary, and commercial relevance, they remain understudied compared to other groups (**Borg *et al.*, 2023**). (Figure 5)



Figure 5 : Examples of red seaweeds (Rhodophyta) cited as sources of phenolic compounds: (A) *Asparagopsis armata*; (B) *Chondrus crispus*; (C) *Mastocarpus stellatus*; (D) *Palmaria palmata*; (E) *Solieria chordalis*; (F) *Pterocladia capillacea*; (G) *Porphyra umbilicalis*; (H) *Hypnea musciformis*. (Adapted from Cotas *et al.*, 2020).

4.3. Brown Algae (Ochrophyta)

Brown macroalgae belong to the phylum Ochrophyta, grouped entirely within the class of Phaeophyceae. Their pigments are chlorophylls a and c along with carotenoids, with fucoxanthin as the dominant one responsible for their characteristic brownish color (**Pereira, 2021**). They are widely distributed in temperate and subtropical marine environments, where they play important ecological roles, and also have substantial commercial value being used in food, pharmaceuticals, and biotechnology. (**Kim *et al.*, 2025**). (Figure 6).



Figure 6 : Representative species of brown seaweeds (Ochrophyta – Phaeophyceae) relevant to marine macroalgal waste valorisation: (a) *Alaria esculenta*; (b) *Ascophyllum nodosum*; (c) *Dictyota* sp.; (d) *Ecklonia maxima*; (e) *Fucus serratus*; (f) *Fucus spiralis*; (g) *Fucus vesiculosus*; (h) *Gongolaria abies-marina*; (i) *Halopteris scoparia*; (j) *Himanthalia elongata*; (k) *Laminaria digitata*; (l) *Laminaria hyperborea*; (m) *Laminaria ochroleuca*; (n) *Lobophora variegata*; (o) *Macrocystis pyrifera*; (p) *Pelvetia canaliculata*; (q) *Saccharina latissima*; (r) *Saccorhiza polyschides*; (s) *Sargassum muticum*; (t) *Undaria pinnatifida*. (Adapted from Pardilhó et al. (2022).).

4.4. Algae–Fungal Association

Algae are a major source of marine fungal diversity, where around one-third of all known marine fungal species are associated with algae. These fungi occupy a range of ecological roles such as mutualists, endosymbionts, parasites, pathogens, and saprobes. This makes algae-associated fungi relevant both ecologically and economically (Gnavi et al., 2019).

Fungi associated with seaweeds are predominantly ascomycetes, and the most reported genera include: *Aspergillus*, *Penicillium*, *Fusarium*, *Chaetomium*, *Alternaria*, and *Cladosporium*. many of which are also found as endophytes in terrestrial plants (Kim et al., 2025).

A number of studies have shown that some bioactive compounds previously attributed to the algal host were in fact produced by the associated fungi. Fungal abundance and species richness on certain algal species are comparable to levels recorded on brown and red seaweeds, which are generally considered the richest substrates for fungal diversity (Gnavi *et al.*, 2019).

5. Bioactive Potential of Marine Fungi

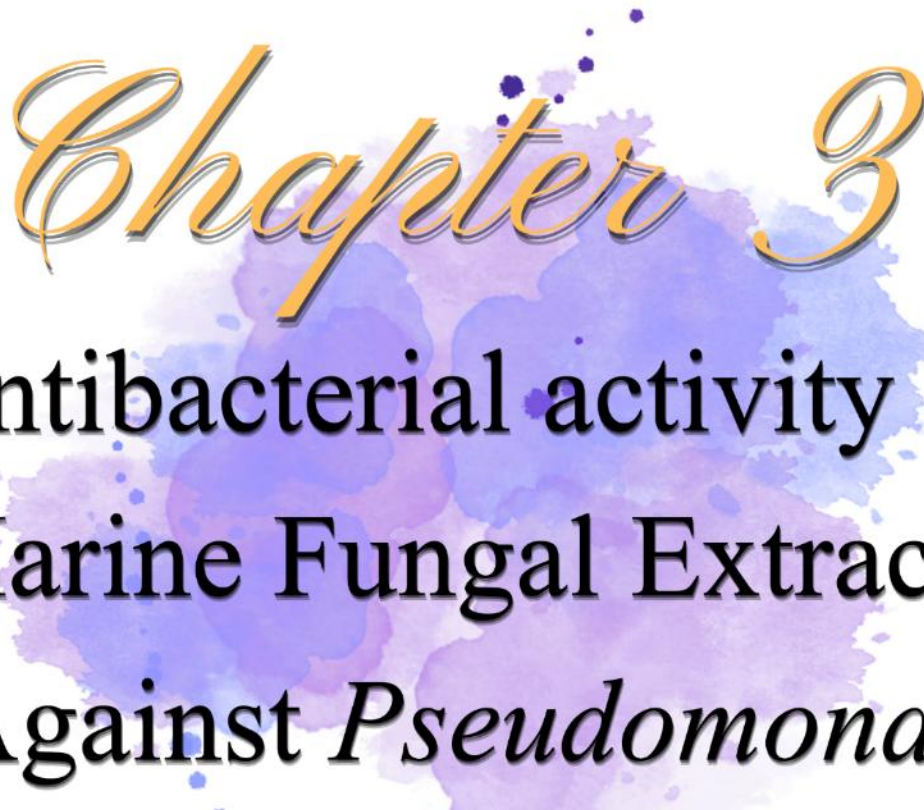
Marine-derived fungi have become an important source of new and effective bioactive compounds. Thousands of structurally distinct and biologically active molecules have been reported from marine fungi, many with properties favorable for pharmaceutical use, including suitable molecular weight and oral bioavailability (Jin *et al.*, 2016). These compounds have shown potential against cardiovascular disease and other chronic conditions (Papikinou *et al.*, 2024).

The production of secondary metabolites in fungi is generally linked to their ecological roles. Endophytic fungi isolated from healthy algae and plants have been reported to produce natural products at higher rates compared to related species isolated from soil (Tarman, 2020, citing Schulz *et al.*, 2002). These metabolites often serve to protect the host or the producing fungus against competitors and pathogens (Tarman, 2020).

More than 270 compounds with antimicrobial activity have been isolated from marine-derived fungi. Antifungal metabolites are also common among sediment fungi, likely playing a role in managing competition within fungal communities. These compounds are also relevant from a practical standpoint, as they may inhibit aquatic and plant fungal pathogens. The search for cytotoxic, antitumor compounds among marine fungal metabolites remains an active area of research (Yurchenko *et al.*, 2021).

Reported findings for some marine fungi include the following: *Aspergillus* leads in output: around 361 secondary metabolites were identified from marine *Aspergillus* species between early 2015 and December 2020 alone (Orfali *et al.*, 2021). *Penicillium* species produce a broad range of compound classes including polyketides, alkaloids, terpenoids, and peptides. Marine strains of *Penicillium* are responsible for 81 of the 180 cytotoxic compounds described from this genus (Grovel *et al.*, 2008).

These previous information about the bioactive potential of marine fungi highlights their role as important producers of a wide variety of secondary metabolites with diverse biological activities, particularly their antibacterial effects that may influence bacterial growth or survival. In this study, this effect was investigated against one of the famous multidrug-resistance bacteria, *Pseudomonas aeruginosa*.



Chapter 3

Antibacterial activity of
Marine Fungal Extracts
Against *Pseudomonas*
aeruginosa

Chapter 3: Antibacterial Activity of Marine Fungal Extracts Against *Pseudomonas Aeruginosa*

1. Overview of Bacteria–Fungi Interactions

Bacteria and fungi share the most natural environments. Their coexistence is not passive, rather it drives the ecosystem functions and affects the health of most of animals and plants worldwide (Deveau *et al.*, 2018). These interactions can take many forms, varying from cooperation to conflict, and the outcome often determines which organism survives and which does not (Arvanitis & Mylonakis, 2015).

The effects of these interactions go beyond simple growth dynamics, because bacteria and fungi can strengthen or weaken each other's virulence, and they also offer protection for each other against toxic compounds, and even reshape how a host organism responds to infection (Arvanitis & Mylonakis, 2015). At the same time the relationship is not always cooperative, instead bacteria and fungi frequently come into conflict (Zhang *et al.*, 2025).

1.1. Forms of Bacteria–Fungi Interaction

1.1.1. Physical Interactions

Some interactions between bacteria and fungi happen through direct physical contact. In wine fermentation, for instance, *Saccharomyces cerevisiae* consumes available nutrients in a way that limits the growth of the lactic acid bacterium *Oenococcus oeni*, which controls when the malolactic fermentation begins. In contrast, *P. aeruginosa* has been shown to physically attach to the hyphae of *Candida albicans*, which create a dense biofilm that eventually kill the fungus (Arvanitis & Mylonakis, 2015).

1.1.2. Interactions via Secreted Molecules

Both bacteria and fungi release molecules that affect their surroundings. Some act directly on neighboring microbes by promoting or limiting their growth, while others work indirectly by altering environmental conditions. This effect is bidirectional, which mean it effect both bacteria and fungi. Some bacteria produce molecules that kill fungi, at the same time,

Endophytic fungi have also been shown to reduce the growth of *Staphylococcus* species by their mycelial extract (Arvanitis & Mylonakis, 2015).

1.1.3. Alteration of Host Immune Response

A third type of interaction concerns the host. When one or more microbial species are present, they can change how the host's immune system responds by changing the intensity or type of defense mounted, which can shift the outcome of an infection. In one instance, a colonization of *Candida* spp. in a mice pneumonia model, raised levels of the cytokine IL-22, which led to the recruitment of natural killer cells thereby protecting the mice from *Pseudomonas pneumonia* (Arvanitis & Mylonakis, 2015).

1.2. Types of Bacteria–Fungi Interaction

1.2.1. Mutualism

Mutualism describes a relationship where both organisms benefit. In this case bacteria and fungi share resources and complement each other's functions, and the common examples include mycorrhizal symbiosis, lichen symbiosis, and the skin microbiota of animals (Zhang *et al.*, 2025).

1.2.2. Antagonism

Antagonism involves mutual inhibition, where one organism harms the other through infection, antibiotic production, or biological control mechanisms. These antagonistic relationships have significant applications in veterinary medicine and agriculture (Zhang *et al.*, 2025).

1.2.3. Competition

Competition happens when bacteria and fungi compete against each other for the same resources or space. But rather than directly harming each other, they limit each other's access to what they need, and this helps maintain the overall stability of microbial communities. As an example, fungi can regulate bacterial fitness by consuming shared resources and through chemical antagonism with releasing antibiotics (Zhang *et al.*, 2025).

2. Antibacterial Compounds Produced by Marine Fungi

2.1. Alkaloids

Alkaloids are the most frequently reported class of antibacterial marine fungal metabolites. They account for nearly one-third of all antibacterial secondary metabolites listed in the literature on marine fungi (Wang *et al.*, 2024).

Wang *et al.* (2024), presented several examples that illustrate this range such as

- Cyclopiazonic acid and brevianamide F, from the marine-derived *Penicillium. vinaceum*, acted differently: brevianamide F was active against *Staphylococcus. aureus*, while cyclopiazonic acid only acted against *Escherichia. coli*.
- Pyrrospirones C, F, and I, from *Penicillium* sp. ZZ380, showed activity against both MRSA and *E. coli*, with MIC values of 2.0–5.0 mg/mL.
- Emethacin C, from *Aspergillus terreus* RA2905, inhibited *Pseudomonas aeruginosa* specifically.
- Cyclopiazonic acid (compound 35), from *A. flavus* SCSIO F025 isolated from South China Sea deep-sea sediments, inhibited *B. thuringiensis*, *M. lutea*, *S. aureus*, *B. subtilis*, and MRSA.
- Pyrrospirones K, O, C, D, and F from *Penicillium* sp. SCSIO 41512 showed significant activity against six pathogens including *B. subtilis*, *E. coli*, *S. aureus*, MRSA, and *Streptococcus agalactiae*, with MICs of 5.0–20 µg/mL.

2.2. Quinones

Wang *et al.* (2024), mentioned fifteen quinone compounds that were reported to have strong inhibitory effect against Gram-positive bacteria since 2012 all. Among the most recent findings: aspergiloxathene A, isolated from *Aspergillus* sp. IMCAS-MF180035, was active against *Staphylococcus aureus*, MRSA, *E. coli*, *Enterococcus faecium*, *P. aeruginosa*, and *H. pylori*.

2.3. Polyketides

Polyketides are built from the polymerization of acetyl and propionyl groups. As an example, two citrinin derivatives (penicitrinols J and K) from *Penicillium* sp. ML226 showed weak activity against *Staphylococcus aureus*. While Pleosporalones G and H, from *Pleosporales* sp. CF09-1, displayed moderate activity against *Vibrio anguillarum* and *V. parahaemolyticus* with MICs of 6.3–25 µg/mL. (Wang *et al.*, 2024).

2.4. Terpenoids

According to Wang *et al.* (2024), most antibacterial terpenoids from marine fungi are sesquiterpenes or tetracyclic triterpenes. A few key examples are:

- Aspergillusene E, from *A. versicolor* XS-20090066, inhibited *S. epidermidis* and *S. aureus*.
- Oxaliterpenoid, a sesterterpenoid from *Penicillium oxalicum* M893, showed strong activity against *E. faecalis*, *S. aureus*, and *B. cereus*, each with an MIC of 32 µg/mL.
- Two compounds from *A. fumigatus* MF071 showed strong activity against both *S. aureus* and *E. coli* with MICs of 3.13 and 6.25 µg/mL.
- Two drimane sesquiterpenoids, purpurides E and F from *Penicillium minioluteum* ZZ1657, inhibited both MRSA and *E. coli* with MICs of 3–12 µg/mL.

2.5. Coumarins

Among 16 coumarin analogs described in the literature, Asperpyranones A, from *A. terreus* RA2905, was active against *P. aeruginosa*. Citreisocoumarin, from *Penicillium vinaceum*, acted against *S. aureus*. Additionally, Nipyrone A–C and germicidin C, extracted from *A. niger*, which showed activity against *S. aureus*, *B. subtilis*, and *E. coli* with MICs in the range of 8–64 µg/mL. (Wang *et al.*, 2024).

2.6. Xanthoness

For the xanthoness, Wang *et al.* (2024), also mentioned some examples like:

- Purpureone, from *C. lunatus* SCSIO41401, inhibited two swine disease pathogens as well as *S. aureus*, *Erysipelothrix rhusiopathiae*, and *Pasteurella multocida*, with MICs of 13–50 µg/mL.
- Five bitetrahydroxanthone analogs from *A. brunneoviolaceus* MF180246 all showed activity against *S. aureus*, with MICs ranging from 6.25 to 50 µg/mL.
- Secalonic acid D, from *A. aculeatinus* WHUF0198, was active against multiple *H. pylori* strains as well as *S. aureus* USA300 and *B. subtilis* 168, with MICs as low as 1.0 µg/mL

2.7. Steroids

Wang *et al.* (2024), stated some steroids such as:

- Ergosta-5,7,22-triene-3 β -ol and volemolide, from *Aspergillus* sp. SCS-KFD66, inhibited *B. subtilis* ATCC 6633 with MICs of 128 μ g/mL.
- Aspergill steroid A, from *Aspergillus* sp. LS116, showed strong activity against the aquatic pathogen *V. harveyi*.
- The ergostane analog 4 α -hydroxy-17-methylincisterol, from *Trametes* sp. ZYX-Z-16, inhibited *S. aureus* ATCC 6538 (MIC 32 μ g/mL) and *B. subtilis* ATCC 6633 (MIC 16 μ g/mL).

2.8. Other Compounds

There are also other compounds documented by previous studies and they were mentioned in the review of Wang *et al.* (2024) including Benzoic acid derivatives, penicillin analogs, diphenyl ethers, glycosides, peptides, fatty acids, and related structures make up a smaller portion of the antibacterial marine fungal metabolite catalog.

This highlights the diversity of these compounds and also the ability of affecting several bacteria species.

3. Anti-Virulence Activity Against *P. aeruginosa*

3.1. Quorum Sensing Inhibition

Several marine fungal metabolites have been shown to interfere with *P. aeruginosa*'s quorum sensing (QS) systems. At subinhibitory concentrations (250 and 500 μ g/mL), *penicillium sydowiana* PPR extract reduced QS-regulated virulence phenotypes. Also, a computational docking analysis identified two active compounds in its metabolites (cyclo(Leu-Pro) and 4-hydroxyphenylacetamide), both of which bind to the QS receptor proteins LasR and RhlR in a manner similar to the natural signaling molecules (Parasuraman *et al.*, 2020).

Citrinin, from *Penicillium* sp. JH1, suppressed the transcription of genes in the Las, Rhl, and Pqs systems. Its proposed mechanism is competitive binding: citrinin appears to occupy the same binding sites as the natural autoinducer molecules, blocking receptor activation (Ji *et al.*, 2023).

Cladodionen, which was isolated from *Cladosporium* sp. Z148, bound to QS receptor proteins through hydrogen bonding and hydrophobic interactions. Docking analysis showed that cladodionen formed more stable complexes with LasR and PqsR than the natural ligands did. Its inhibitory effect was strongest on the Las and Rhl systems. It also downregulated the expression of receptor genes (*lasR*, *rhlR*, *pqsR*), autoinducer synthase genes (*lasI*, *rhlI*, *pqsA*), and virulence factor genes (*lasB*, *rhlA*) (Wang *et al.*, 2020).

Targeting QS rather than bacterial growth is considered a useful strategy because it reduces virulence without creating the same selection pressure that conventional antibiotics do, and this means bacteria are less likely to develop resistance to QS inhibitors (QSIs) compared to bactericidal or bacteriostatic compounds (Almutairi & Edrada-Ebel, 2025).

3.2. Inhibition of Biofilm Formation

Fungal extracts have shown measurable effects on *P. aeruginosa* biofilm formation, where a crude extract of *Penicillium sydowiana* PPR reduced biofilm development in *P. aeruginosa* PAO1 by 54.30% at 250 µg/mL and by 72.42 % at 500 µg/mL. At the same concentrations, EPS production was reduced by 60.80% and 74.99% respectively. (Parasuraman *et al.*, 2020). Other studies have reported that fungal extracts reduce EPS matrix formation and limit initial bacterial attachment during biofilm development (Behera *et al.*, 2025).

According to Ji *et al.* (2023), citrinin purified from *Penicillium* sp. JH1, inhibited *P. aeruginosa* FRD1 biofilm formation by 56.6%, which is considered to be stronger than the reference compound C-30 at the same concentration. And in another study by Wang *et al.* (2024), Cladodionen, isolated from the marine fungus *Cladosporium* sp. Z148, inhibited biofilm formation and motility of *P. aeruginosa* PAO1 at subinhibitory concentrations.

Among compound classes, polyketides appear most frequently in the literature in connection with biofilm inhibition (Almutairi & Edrada-Ebel, 2025).

3.3. Reduction of Pyocyanin Production

Pyocyanin production in *P. aeruginosa* was reduced by 54.22% and 64.38% when treated with *penicillium sydowiana* PPR extract at 250 and 500 µg/mL respectively (Parasuraman *et al.*, 2020).

Cladodionen reduced pyocyanin production by 23.6% at 400 µM (Wang *et al.*, 2020). Citrinin inhibited pyocyanin along with elastase and rhamnolipid in *P. aeruginosa* PAO1 at 30 µM, with

inhibition rates of 12.5%, 16.8%, and 25.6% respectively (Ji *et al.*, 2023). In another research, cyclic dipeptide cyclo(L-Tyr-L-Pro), isolated from *Penicillium chrysogenum* DXY-1, reduced pyocyanin production by 41% at a concentration below one-tenth of its MIC (Yu *et al.*, 2021). Also, an aqueous extract of *Penicillium tersa* inhibited pyocyanin and pyoverdine by 92.5% and 71.5% respectively (Behera *et al.*, 2025).

4. Evidence from Scientific Studies

Several studies have reported that marine fungal extracts inhibit both the growth and virulence activities of *Pseudomonas aeruginosa*, and some of these findings are:

In a study, of screening marine fungal extracts, three isolates (ZF 34, ZF 52, and ZF 91) showed significant activity against *e aeruginosa* with inhibition zones ranging from 0 to 40 mm, while most other extracts showed no effect. And the compound CJ-14445 was identified as a potential antimicrobial candidate specifically against *P. aeruginosa* (Bodede *et al.*, 2022).

A marine *Aspergillus* sp. isolated from sand in Sharm El-Sheikh produced a petroleum ether extract with antibacterial activity against *P. aeruginosa*, *E. coli*, *Klebsiella*, *Enterobacter cloacae*, *Salmonella*, and *Proteus vulgaris*, with an inhibition zones of 12, 27, 15, 26, 11, and 9 mm respectively. (Abdulbaqi *et al.*, 2024).

Dendryphiella salina, derived from the seaweed *Laminaria hyperborea*, achieved complete inhibition of *P. aeruginosa* ATCC 27853 biofilm at 100 µg/mL. And this showed that marine fungal metabolites that can disrupt both biofilm structure and QS pathways represent a useful combination of properties against virulent bacteria (Almutairi & Edrada-Ebel, 2025).

Cyclo(L-Tyr-L-Pro), from *Penicillium chrysogenum* DXY-1, reduced *P. aeruginosa* PAO1 QS-mediated activities such as pyocyanin production, and it disrupted biofilm formation and reduced QS gene expression. (Yu *et al.*, 2021).

5. Factors Influencing Antibacterial Activity

The production of bioactive compounds by marine fungi is not fixed. It responds to environmental conditions. Nutrient availability, pH, temperature, salinity, and the presence of

competing microorganisms can all trigger or suppress metabolite secretion. (**Martín-Rodríguez *et al.*, 2014**). And between these factors there are:

Growth medium composition directly affects both the quantity and quality of fungal extracts. In one study, *Aspergillus sydowii* produced different amounts of metabolites depending on the medium used, which in turn altered its ability to inhibit *E. coli* and *S. aureus* (**Widyaningsih *et al.*, 2018**). Fermentation scale and conditions also matter where certain compounds from strain SD-44 were only detected in a 500 L fermenter under shaking conditions and were absent in static rice medium cultures (**Pinedo-Rivilla *et al.*, 2022**).

The choice of solvent significantly shapes what is extracted. Ethyl acetate extracts tend to show higher antibiofilm activity because they capture moderately polar secondary metabolites including polyketides, terpenoids, and phenolic derivatives, that are often responsible for antibiofilm effects (**Almutairi & Edrada-Ebel, 2025**). In one direct comparison, ethyl acetate extract showed lower antibacterial activity than petroleum ether, chloroform, and methanol extracts (**Abdulbaqi *et al.*, 2024**). This suggests that no single solvent captures all relevant possible metabolites.

The growth environment also shapes metabolite output. Testing the ethyl acetate extract of *Mycelium sterillum* KT19 against *P. aeruginosa* produced an inhibition zone of 8 mm when the fungus was cultured in freshwater medium. The same extract produced a zone of 13.9 mm when the fungus was grown in seawater medium, this is a meaningful difference driven by a change in fermentation environment alone (**Tarman *et al.*, 2011**).

6. Therapeutic and Biotechnological Applications

Marine fungal metabolites offer a mode of action that differs from conventional antibiotics. Rather than killing bacteria directly, QS inhibitors reduce virulence and disrupt biofilm formation without affecting bacterial growth itself. This means they do not impose the same selection pressure that drives the development of resistance, which is a meaningful advantage as antimicrobial resistance continues to rise (**Almutairi & Edrada-Ebel, 2025; Parasuraman *et al.*, 2020**).

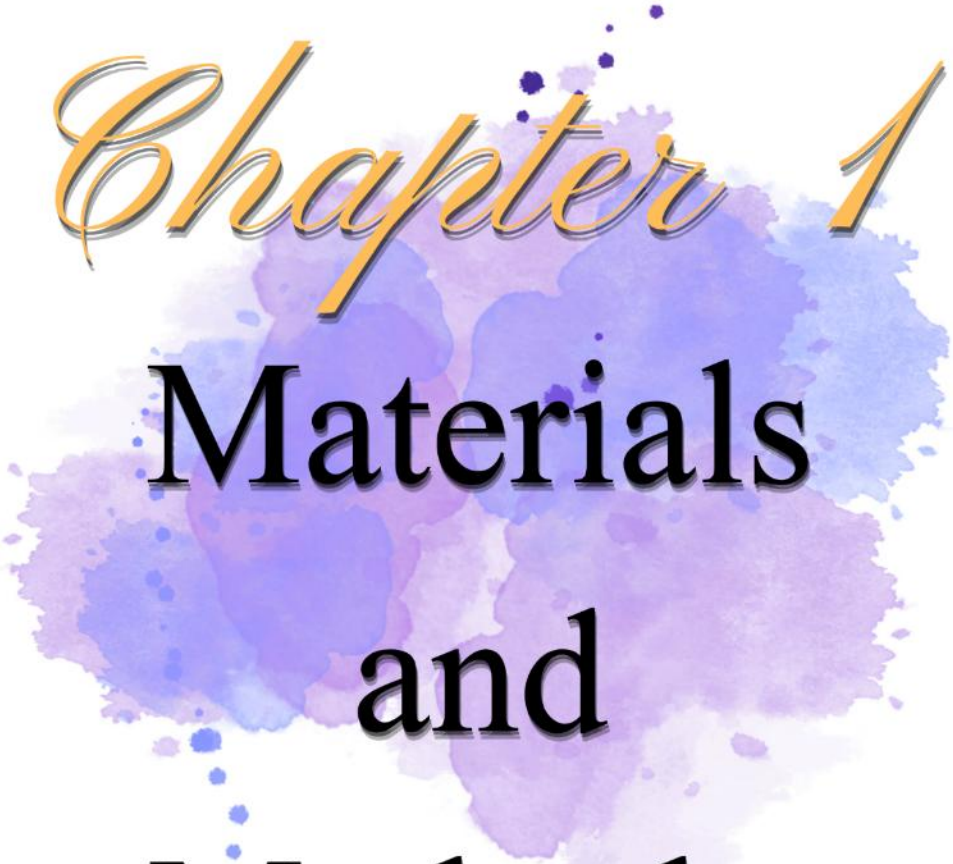
Marine fungi from extreme environments (deep-sea sediments, hydrothermal areas, high-salinity zones) have metabolic adaptations that make them useful for drug discovery. And their

secondary metabolite profiles differ significantly from those of terrestrial species (**Almutairi & Edrada-Ebel, 2025**). And they are also known for producing compounds of interest for antibacterial, anticancer, antiviral, and anti-neuroinflammatory applications, many with favorable properties such as low molecular weight (**Abdulbaqi *et al.*, 2024**).

Targeting QS systems directly is one of the most promising directions in managing infections caused by biofilm-forming pathogens. It disrupts cell-to-cell communication that prevents bacteria from coordinating virulence behaviors, which can limit infection progression without the drawbacks of bactericidal treatment (**Magalhães *et al.*, 2025; Almutairi & Edrada-Ebel, 2025**). The effect of QS inhibitors can also be enhanced through combination with other compounds, such as nitric oxide donors, increasing their antibiofilm effectiveness (**Almutairi & Edrada-Ebel, 2025**).

Collectively, the findings reported in these studies highlight the significant potential of marine fungal extracts as promising resources for addressing the growing challenge of multidrug-resistant microorganisms. Several compounds isolated from these fungi have demonstrated moderate to excellent antibacterial activity against a range of pathogens, including *Pseudomonas aeruginosa*. These results provide encouraging evidence for the development of novel antimicrobial agents that may contribute to improved global health outcomes through the reduction of infection-related morbidity and mortality.

Experimental
Part

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Chapter 1

**Materials
and
Methods**

Chapter 1: Materials and Methods

1. Objective

The main objective of the present study is to evaluate the antibacterial activity of marine-derived fungal extracts against environmental and clinical strains of *Pseudomonas aeruginosa* and to determine their potential as a source of alternative antimicrobial compounds

2. Non-biological materials

Culture media: The culture media used in this experiment included King A agar, King A broth, King B agar, nutrient agar, Luria-Bertani (LB) broth, Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), Mueller-Hinton agar with 2% NaCl, Sabouraud with chloramphenicol and seawater, Potato Dextrose agar (PDA) and Congo Red Agar (CRA). (**Appendix 1**).

3. Biological material

3.1. Bacterial Strains

The origin of *Pseudomonas aeruginosa* strains: 3 strains were used in this study:

- A clinical strain obtained from the Laboratory of Medical Analysis ETTALHI.
- An environmental strain obtained from the Microbiological Laboratory at the University of Abd El-Hamid Ibn Badis (Mostaganem).
- Reference strain (ATCC 27853) obtained from the Research Laboratory of Microbiology and Planten Biology at the University of Mostaganem was added to the study for comparative purposes.

3.1.1. Revival and purification (Streak Plating)

The bacterial strains were revived by streaking them onto selective culture media, King A agar and King B agar, to obtain fresh growth and well-isolated colonies. The plates were incubated at 37°C for 24 hours.

3.1.2. Macroscopic examination

The colonies were examined visually, and macroscopic characteristics were recorded by observing size, shape, margin, surface texture, elevation, and pigmentation.

3.1.3. Microscopic examination (Gram Staining)

A Gram staining was performed on selected colonies following standard coloration procedures. The prepared smears were examined under a light microscope to determine cellular morphology and Gram reaction. (Appendix 2)

3.1.4. Oxidase Activity Assay

The oxidase test was performed to detect the presence of the cytochrome C oxidase enzyme in the different bacterial strains studied. A fresh bacterial colony was transferred using a sterile loop onto oxidase reagent-impregnated strips. The color development was observed within 3–5 seconds for each strain. (Appendix 2)

3.1.5. Thermotolerance assessment (Growth at 42°C)

For more confirmation of identification, each strain studied was streaked onto nutrition agar and incubated at 42°C to assess its ability to tolerate this temperature, which allows differentiating *Pseudomonas aeruginosa* from other fluorescent *Pseudomonas* species. (Iglewski, 1996).

3.1.6. Conservation of *Pseudomonas aeruginosa* strains

The identified bacterial strains were preserved by inoculating pure colonies onto King B agar slants and incubating at 37°C for 24 hours, then storing at +4°C for further experiments.

3.1.7. Study of antibiotic sensitivity

The disc diffusion method on Mueller-Hinton agar was used to evaluate the antibiotic susceptibility of the two isolates (clinical and environmental), with *P. aeruginosa* ATCC 27853 as a quality control to ensure the validity of the assay, following the recommendations of the Antibiogram Committee of the French Society for Microbiology (CASFM, 2025).

Suspensions were prepared using fresh bacterial colonies in sterile physiological water, and their turbidity was adjusted to a 0.5 McFarland standard equivalent to $0.08 \leq OD \leq 0.1$ at 625 nm, which was confirmed by spectrophotometric measurements.

With a sterile swab, the bacterial suspension was applied evenly all over the surface of Mueller-Hinton agar plates. The five antibiotic discs chosen for this test were placed on the inoculated plates, then incubated at 37°C for 24 hours.

The inhibition zones were measured according to the standard disk diffusion procedure.

3.1.8. Biofilm formation

3.1.8.1. Crystal violet staining method

Biofilm formation was quantified using the crystal violet staining assay as described by **Henry *et al.* (2018)** with some modifications.

For each strain, three replicates were prepared. Sterile test tubes filled with LB broth were inoculated with the bacterial isolates (clinical, environmental, and *P. aeruginosa* ATCC 27853 as a reference) and incubated at 37°C for 48 hours.

After incubation, the culture medium was carefully poured into a proper waste container, and the tubes were washed three times with distilled water to remove any nonattached planktonic cells. The tubes were then inverted on a paper towel and air-dried for 20 minutes. Then, a 0.1% crystal violet solution was added to each tube to stain the adhered cells for 20 minutes. Excess stain was removed, and the tubes were rinsed again three times with distilled water. The bound dye was solubilized using 70% ethanol, and the optical density of the resulting solution was measured at 595 nm using a spectrophotometer.

3.1.8.2. Congo Red Agar method

Following the procedure outlined by **Freeman *et al.* (1989)**, Congo red agar medium was prepared and poured into sterile petri dishes. Fresh bacterial cultures were streaked onto the prepared medium surface and incubated at 37°C. Colony morphology was examined after 24 h and 48 h of incubation to evaluate biofilm formation.

3.2. Marine fungal

3.2.1. Sampling

The algae samples used for this experiment were collected on 19 February 2026 from two marine regions of Mostaganem Province:

- Red algae from Sidi Lakhdar (Dianci Beach). (Figure 7)
located in: 36.21097421633252, 0.383607809750258
- Green and brown algae from Salamander (Salamander beach). (Figure 7)
located in: 35.91024426330873, 0.05462049624387763



Figure 7 : Sampling sites for algae (Diancy Beach and Salamandre Beach). Source: Google Maps.

These Algae were classified into three major groups according to **Kilinc *et al.* (2013)**:

- Green algae (phylum Chlorophyta) are rich in chlorophyll and commonly found in sandy and rocky beaches with good lighting.
- Brown algae (phylum Ochrophyta) are widely found in coastal waters and are used in several fields.
- Red algae (phylum Rhodophyta) are characterized by phycobilin pigments which allow them to survive low light and deep marine environment, and they are considered to be highly divers in comparison with Green and Brown algae.

All these algal samples were transported to the laboratory under sterile condition for fungal isolation

3.2.2. Isolation of Fungi

The fungal isolation was performed using two different methods. However, a cleaning and preparation procedure was first applied to the algae before the fungal isolation experiment.

Following the protocol described by **Kamat *et al.* (2020)**, the algae samples were washed with sterile seawater to eliminate any surface contaminants then rinsed for 60 seconds with 70% ethanol, followed by rinses with sterile distilled water and dried on sterile tissue paper. The extraction methods were the same for all algae (green, red, and brown).

3.2.2.1. Dilution method

Using a clean mortar and pestle, 1 g of algae was crushed, and then 10 mL of sterile seawater was added and mixed well to obtain a suspension. 1 mL of the mixture was used with sterile seawater to prepare serial dilutions up to 10^{-3} . Subsequently, 0.1 mL from each dilution was inoculated onto Sabouraud dextrose agar (SDA) medium. A sterile spreader made from a Pasteur pipette was used to evenly distribute the sample across the medium surface. Lastly, the plates were incubated for five to seven days at 28°C to promote the growth of fungal colonies. (**Kjer *et al.*, 2010**)

3.2.2.2. Tissue segment plating

Using a sterile blade, the algae were aseptically cut into segments of about 2 cm. Five segments were placed on SDA medium supplemented with chloramphenicol and seawater. Care was taken to ensure that the agar was in contact with the cut surface. The Plates were incubated at 28°C for 5-7 days. (**Kjer *et al.*, 2010**)

3.2.3. Purification of Fungi

All the filamentous appearance colonies observed after the incubation period were selected and purified on the same medium used for isolation by streaking. Then the plates were incubated at 28°C for 7 days.

The resulting isolates were studied macroscopically and microscopically as it is described by Salvamani and Mohammad Nawawi, (2014) and Pitt and Hocking, (2022).

3.3. Antimicrobial activity of marine Fungi against *Pseudomonas aeruginosa*

3.3.1. Antagonism Test (cross-streak method)

Following the method explained by **Hossain (2024)**, the antagonism test was performed using two media: Mueller-Hinton agar and Mueller-Hinton agar with 2% NaCl. The use of Mueller-Hinton agar supplemented with 2% NaCl was intended to simulate a marine environment conducive to marine fungi while maintaining conditions suitable for bacterial growth.

The fungal isolates were inoculated in a single line onto each medium at the edge of a Petri dish. After seven days of growth of the fungi at 28°C the target microorganisms were inoculated in lines perpendicular to the fungi isolate culture. Antimicrobial activity was assessed by measuring the length of the zone of inhibition between the edges of the fungi and those of the target microorganisms, after 24 hours. (Figure 8).

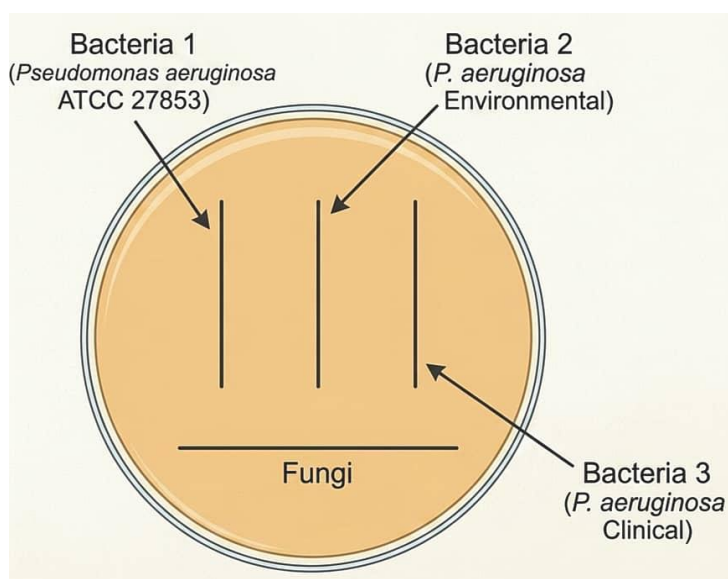


Figure 8: Schematic representation of the Cross-streak assay on Mueller-Hinton medium

3.3.2. Extraction of fungal secondary metabolites

After seeing the results of the antagonism test, each fungus giving bacterial inhibition was inoculated on 8 petri dishes filled with PDA medium and incubated at 28°C for 14 days. The fungi were then cut into small pieces and put in Erlenmeyer flasks with the same volume of ethyl acetate and agitated for 24 hours. The suspensions were filtered using Wattman filter

paper, and then evaporated using a rotary evaporator at a temperature of 40°C. (**Campos *et al.*, 2015**)

The resulting crude extracts were weighed to determine the yield obtained.

3.3.3. Evaluation of antibacterial activity of fungal extracts

A stock solution of the crude fungal extract was prepared by dissolving 20 mg of the crude extract in 1 mL of DMSO. The solution was stored at -20°C until use for future antibacterial assays. (**Sendekie *et al.*, 2026**). A DMSO control was tested on the bacterial strains.

3.3.3.1. Disk diffusion method

The antibacterial activity was evaluated using a modified disk diffusion method adapted from **Bauer *et al.* (1966)**. A fresh bacterial culture was used to prepare an inoculum equivalent to the 0.5 McFarland standard. This inoculum was then swabbed evenly across the entire surface of the Mueller-Hinton agar plate using a sterile swab.

On the same plate, an antibiotic disk served as a positive control, and a DMSO disk as a negative control. The extract was tested at the same concentration using two methods: a paper disk impregnated with the extract and an agar well filled with 10 µL of the extract, both at a concentration of 20 mg/mL. This setup was carried out for each bacterial strain.

The inhibition zone measurements were taken after 24 hours of incubation at 37 °C.

3.3.3.2. Minimum inhibitory concentration (MIC):

The minimum inhibitory concentration (MIC) of the marine fungal extract against *Pseudomonas aeruginosa* was determined using a broth dilution method in a 96-well microplate adapted from the protocol described by **Wiegand *et al.* (2008)**, with the addition of TTC for endpoint detection.

A series of twofold dilutions of the extract was prepared using sterile MHB in a 96-well microplate. Started by adding 200 µL of the extract to the first well, while 100 µL of the medium MHB was added to the remaining wells. Then 100 µL was transferred sequentially from one well to the next to obtain a serial dilution, with thorough mixing at each step.

Each well was inoculated with 10 μ L of a standardized bacterial suspension adjusted to a 0.5 McFarland, then a 90 μ L of MHB medium. The microplate was incubated at 37 °C for 24 hours. Bacterial growth was assessed visually by adding TTC (2,3,5-triphenyl tetrazolium chloride), which gives a red color to the wells in the presence of viable bacteria, to confirm whether growth has occurred.

The MIC is defined as the lowest concentration of the extract that shows no visible growth compared to the control.

3.3.3.3. Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) was determined following the MIC test as described by **Balouiri *et al.* (2016)**.

A sterile loop was used to streak from each of the wells with no visible bacterial growth onto nutrient agar plates. Each streak was labeled according to its concentration, and then incubated at 37 °C for 24 hours. The MBC was defined as the lowest concentration of the extract that resulted in no bacterial growth, indicating complete bacterial killing.

3.3.4. Anti-virulence and quorum-sensing inhibition assays

3.3.4.1. Biofilm formation and inhibition assay

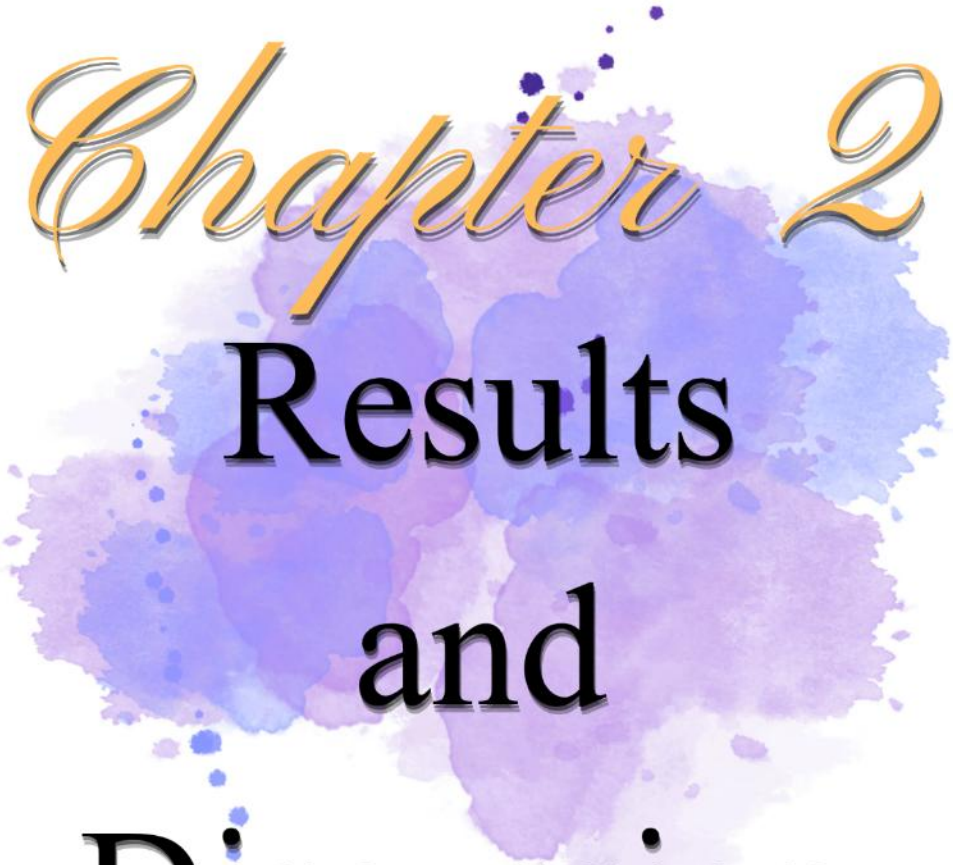
The test was done in triplicate, with and without the extract to compare its effect on the biofilm formation. Sterile hemolysis tubes were filled with LB medium supplemented with sub-MIC extract concentration according to the MIC test results for each bacterial strain, while control tubes contained no extract. The same amount of bacterial suspension of 0.5 McFarland standard was added to all tubes before incubating them at 37°C for 48 hours.

After incubation, the planktonic cells were discarded, and the tubes were gently washed with distilled water to remove non-adherent cells. The attached biofilm was stained with 0.1% crystal violet, followed by gently washing and air-drying. The bound dye was measured with a spectrophotometer at 595nm after solubilization with ethanol 70%. (**Henry *et al.*, 2018**)

3.3.4.2. Pyocyanin production assay

Pseudomonas aeruginosa was cultured in King A broth containing sub-inhibitory concentrations of the extract in sterile hemolysis glass tubes and incubated at 37 °C for 48 hours

with shaking. After incubation, cultures were centrifuged, and pyocyanin was extracted from the supernatant using chloroform, followed by re-extraction into acidic solution (0.2 N HCl). The resulting pink-colored solution was measured spectrophotometrically at 520 nm. A reduction in absorbance compared to the control indicated inhibition of pyocyanin production (Essar *et al.*, 1990).

A decorative background element consisting of a purple watercolor splash or ink blot, centered behind the chapter title. It has various shades of purple and blue, with some darker spots and a soft, irregular edge.

Chapter 2

Results and Discussion

Chapter 2: RESULTS AND DISCUSSION

1. Characterization of *Pseudomonas aeruginosa* isolates

1.1. Confirmation of isolates identification

1.1.1. Macroscopic examination

After 48 hours of incubation at 37 °C, all three isolates exhibited noticeable growth on both King A and King B agar media. Characteristic greenish-blue pigmentation was observed on King A agar, whereas greenish-yellow pigmentation developed on King B agar. In addition, colonies grown on King B agar exhibited marked fluorescence under UV light (Figure 9; 10).

Pseudomonas aeruginosa produces two distinct pigments, pyocyanin and pyoverdine. pyoverdine is a yellow green pigment that serves as a fluorescent siderophore involved in iron acquisition under iron-limited conditions, while pyocyanin is a blue green phenazine compound associated with virulence (Murray *et al.*, 2021; Cornelis, 2010). The production of pyocyanin and pyoverdine is a characteristic phenotypic trait of *Pseudomonas aeruginosa* that supports the identification of the studied isolates.



Figure 9: macroscopic aspect of *P. aeruginosa* Clinical strain



Figure 10 : macroscopic aspect of *P. aeruginosa* Environmental strain

- **Macroscopic Colony Morphology**

Colonies appeared to be small, mostly circular with irregular margins, and minor shape variations with a smooth surface. The colonies appeared to have the same color as the pigment produced in the culture media.

1.1.2. Microscopic Examination (Gram Staining)

Gram staining revealed all 3 isolates to have a thin rod-shaped bacillary morphology with a pink color that matches the known morphology of *Pseudomonas aeruginosa*. (Figure 11)

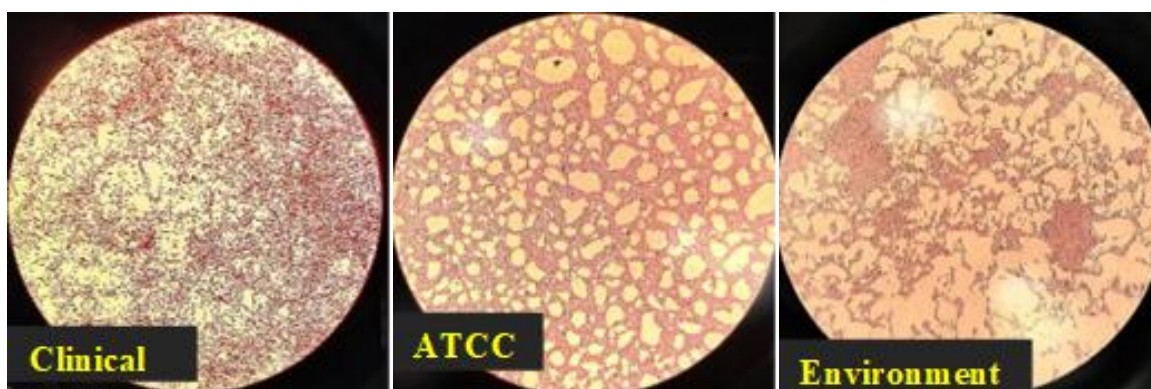


Figure 11: microscopic aspect of all tested strains of *P. aeruginosa* (Gram staining)

1.1.3. Oxidase Test

All three bacterial isolates showed a rapid positive oxidase reaction after fresh colonies were applied to the oxidase reagent strips, with a visible blue coloration appearing within 3–5 seconds (Figure. 12).

This quick positive reaction indicates the presence of the enzyme cytochrome C oxidase, a defining enzymatic characteristic of *Pseudomonas aeruginosa*, and other non-fermenting Gram-negative bacilli (Forbes *et al.*, 2016). The uniform oxidase positivity observed among the clinical, environmental, and reference strains further supports their phenotypic identification as *P. aeruginosa*.

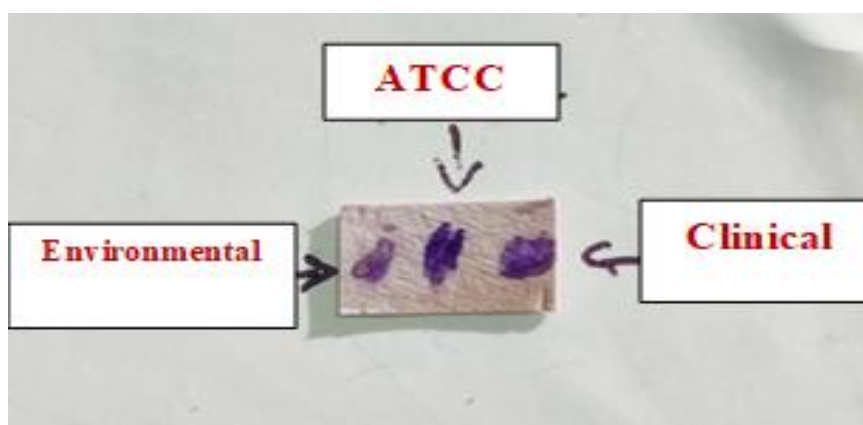


Figure 12 : Results of oxidase test of all of *P. aeruginosa* strains

1.1.4. Thermotolerance Assessment (Growth at 42°C)

All 3 strains of *Pseudomonas* showed visible growth at 42 °C (Figure 13). This result supports their identification as *Pseudomonas aeruginosa*, which is in line with previous reports describing the ability of this species to grow at 42°C, unlike another fluorescent *Pseudomonas* spp. (Iglewski, 1996).

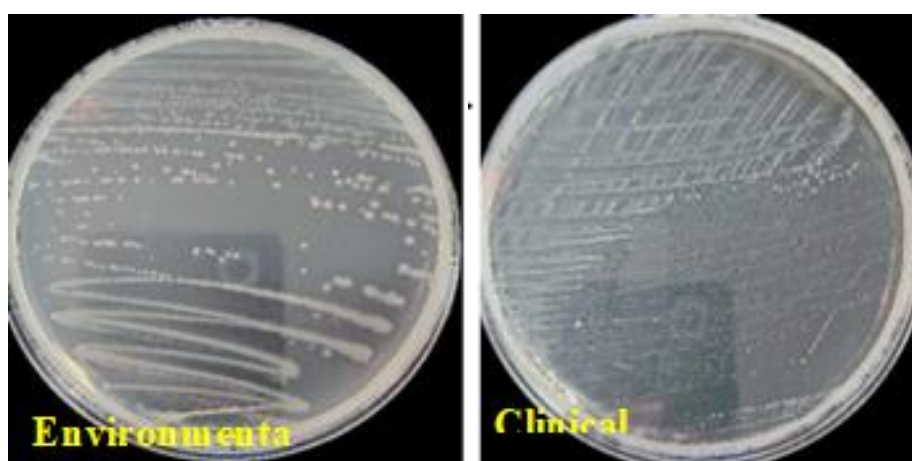


Figure 13 : *P. aeruginosa* clinical and environmental strains growth on Nutrient Agar at 42°C

All obtained results of the phenotypic and physiologic characteristics of the isolated strains were summarized for a comprehensive overview. (Table 1).

Table 1 : Identification results of *Pseudomonas aeruginosa* isolates

Strain	ATCC 27853	Clinical	Environmental
Cell form	Rod shaped	Rod shaped	Rod shaped
Gram staining	Gram negative	Gram negative	Gram negative
Oxidase	Positive	Positive	Positive
Pigment on King A	Blue-green	Blue-green	Blue-green
Pigment on King B	Yellow-green	Yellow-green	Yellow-green
Growth at 42°C	Positive growth	Positive growth	Positive growth

1.2. Study of antibiotic sensitivity

The antibacterial susceptibility profile of the clinical and environmental *P. aeruginosa* isolates were evaluated in a comparison with the reference strain ATCC 27853. (Figure 14). (Table 2)

The results were interpreted through a comparative quantitative analysis of inhibition zone diameters rather than standard clinical categories since these selected antibiotics have no establishment in CLSI M100 break point for *P. aeruginosa*.

The observed data of most disks revealed a measurable *in vitro* activity unlike the expected resistance that is typically associated with *P. aeruginosa*. Both clinical and environmental showed inhibition zones bigger compared to those of ATCC 27853 for Colistin (CS) and Triple Sulfonamides (SSS). While Vancomycin (VA) showed the expected total resistance across all strains. In another way, the Aminoglycosides disks Kanamycin (K) and Neomycin (N) showed a different pattern compared to the ATCC 27853 strain where environmental strain was more susceptible, producing a bigger inhibition zone than the ATCC 27853, In contrast the clinical isolate was more resistant showing a smaller zone for N and total resistance for K. this suggests that the clinical strain has likely developed stronger resistance mechanism compared to the environmental strain who remains relatively sensitive.

This observed result highlights the variability of *P. aeruginosa* in non-clinical laboratory settings. Since these antibiotic disks are not clinically recommended for *Pseudomonas aeruginosa* therapy, except for Colistin, and Vancomycin who give a total resistance.

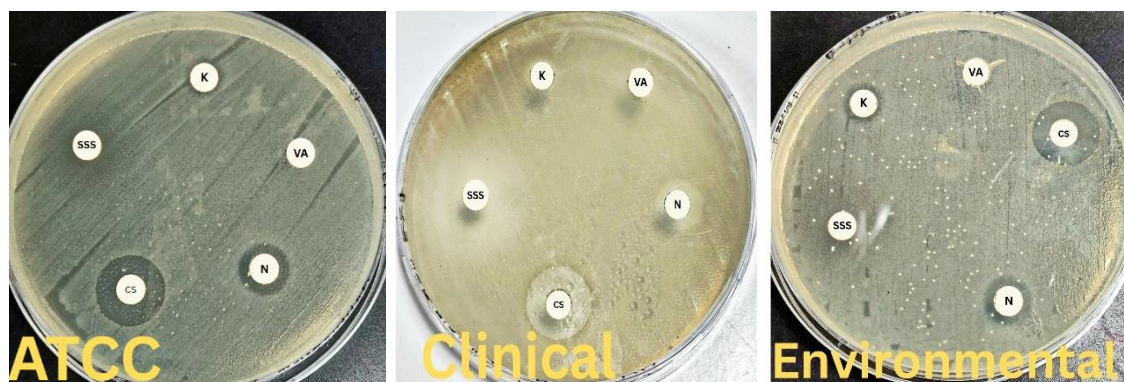


Figure 14 : Antibacterial susceptibility profiles of clinical, environmental, and ATCC strains of *Pseudomonas aeruginosa* by disk diffusion assay

Table 2 : Antibiotic inhibition zone diameters (mm) of clinical, environmental, and ATCC 27853 strains of *Pseudomonas aeruginosa*.

Strain	Family	Polymyxins		Sulfonamides	Aminoglycosides		Glycopeptides	
	Disk code	CS (mm)		SSS (mm)	K (mm)	N (mm)	VA (mm)	
Clinical		15	S	21	/	9.4	/	R
Environmental		15	S	18	7	11	/	R
ATCC 27853		14.8	S	15	6.4	10	/	R

1.3. Biofilm formation

1.3.1. Crystal violet staining method

Several previous studies have used the crystal violet staining assay to assess the ability of each strain in forming biofilms (Stepanović *et al.*, 2007; Turu & Canh, 2025). This method uses optical density (OD) values to categorize strains as non, weak, moderate, or strong biofilm producers. The assay was performed in triplicate for each strain.

All tested strains showed visible staining of crystal violet on the inner surfaces of the tubes, confirming their biofilm production ability. (Figure 15)

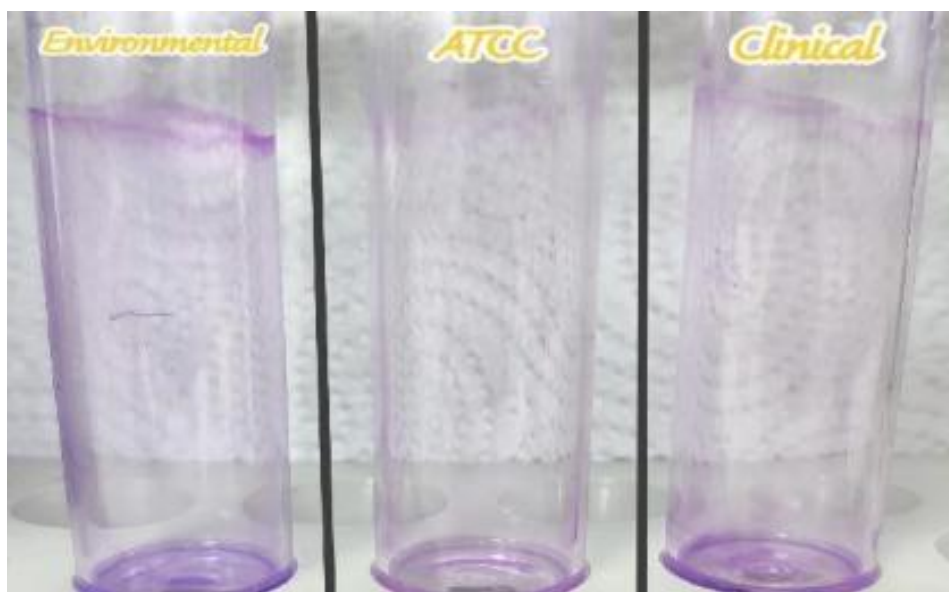


Figure 15 : Crystal violet staining of biofilm formation in test tubes for *P. aeruginosa* (clinical, environmental, and ATCC strains).

The cut-off value (OD_c) is calculated based on the negative control according to **Turu & Canli, (2025)**, using the following formula:

$$OD_{\text{cutoff}} = \text{Mean OD of negative control} + (3 \times \text{Standard deviation (SD) of negative control})$$

The mean OD of the negative control was 0.0025, with a standard deviation of 0.00056, resulting in a standard deviation of **0.0042**.

Based on the results of the ability to form biofilms, strains were categorized into four groups:

- $OD \leq OD_c$: Non-biofilm forming.
- $OD_c < OD \leq 2 \times OD_c$: Weak biofilm producer.
- $2 \times OD_c < OD \leq 4 \times OD_c$: Moderate biofilm producer.
- $4 \times OD_c < OD$: Strong biofilm producer.

The quantitative results of biofilm formation and their classification are in Table 3.

Table 3 : Quantitative results of biofilm production ability of *P. aeruginosa* strains

Strain	Mean OD	Biofilm production
ATCC	0.009	Moderate biofilm producer
Clinical	0.010	Moderate biofilm producer
Environmental	0.0147	Moderate biofilm producer

A graphical representation of mean OD values illustrates the similarity in biofilm formation values among the tested strains (Figure 16).

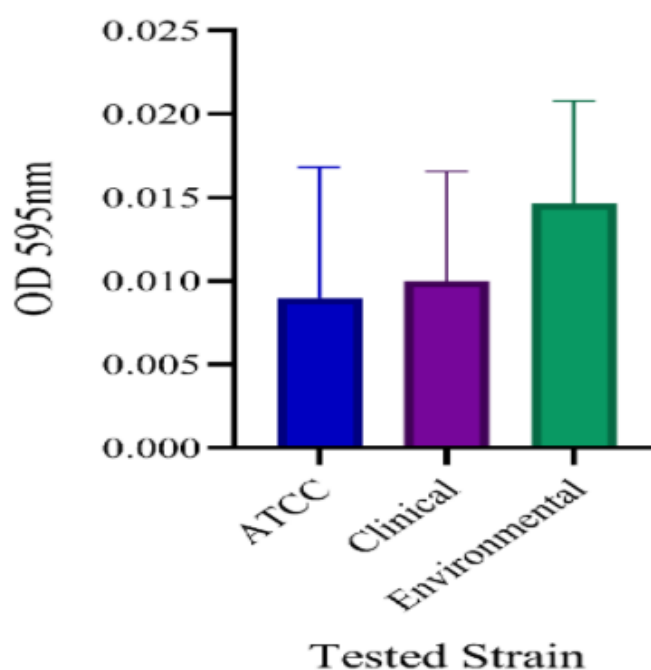


Figure 16 : Mean biofilm formation (OD595 ± SD) of *Pseudomonas aeruginosa* strains determined by crystal violet assay

Statistical analysis using the Kruskal–Wallis test showed no statistically significant difference in biofilm formation between clinical, environmental, and ATCC 27853 strains ($H = 1.098$, $p = 0.6321$).

These results indicate that all tested strains have a moderate ability in forming biofilm under the experimental conditions, with no significant variation observed, confirming limited but present biofilm biomass production.

1.3.2. Congo Red Agar method

The biofilm-forming ability of the clinical, environmental, and ATCC strains was assessed on Congo red agar after 24 and 48 hours of incubation. No noticeable differences were observed between the two-incubation periods while evaluating the colony's morphology.

Both clinical and the ATCC 27853 strains produced smooth, red colonies that matched the color of the culture medium, with no visible signs of black coloration or rough texture, suggesting an absence to weak biofilm formation. In contrast, the environmental strain showed small, red crystalline colonies, with a hint of black color in the plate, which may indicate weak to moderate biofilm formation ability. (Figure 17)

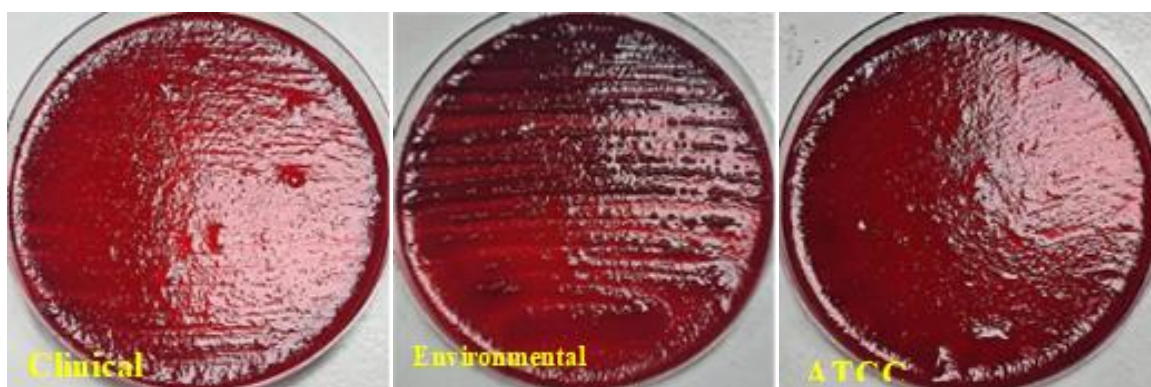


Figure 17 : Colony morphology of *Pseudomonas aeruginosa* strains on Congo red agar after 48 h incubation

Overall, the Clinical and ATCC strains were classified as absent to weak biofilm formation, while the environmental strain showed weak to moderate biofilm formation according to the criteria outlined by **Turu & Canli (2025)**. (Table 4).

The absence of strong visualized dark or rough colonies suggests that not all the strains of *Pseudomonas aeruginosa* produce a detectable biofilm using this method of Red Congo agar.

Table 4 : Congo Red agar test results for biofilm formation of *Pseudomonas aeruginosa* strains

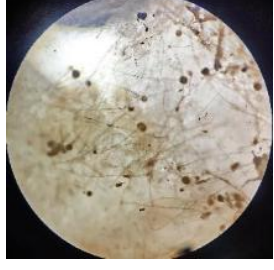
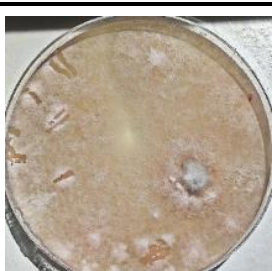
Strain	Colony color	Morphology	Interpretation
ATCC	Red	Smooth	Non to weak biofilm producer
Clinical	Red	Smooth	Non to weak biofilm producer
Environmental	Dark Red to black	Dotted (Crystalline)	Weak to moderate biofilm producer

2. Identification and characterization of fungal isolates

The isolation of fungi from marine algae (green, brown, and red seaweeds) using Sabouraud dextrose agar supplemented with chloramphenicol and seawater, resulted in filamentous fungal colonies.

The purified isolates were identified both macroscopically based on morphology, color, texture, and growth patterns, and microscopically based on structural features observed under the microscope, including hyphal organization, conidiophore structure, and the type and arrangement of spores. These characteristics are widely used in classical fungal taxonomy for preliminary identification of filamentous fungi, as described by Salvamani and Mohammad Nawawi, (2014) and Pitt and Hocking (2022). (Table 5).

Table 5 : Macroscopic and Microscopic Characteristics of Purified Fungal Isolates from Marine Algae

Presumptive Identification	Macroscopic Characteristics (SAB + chloramphenicol + seawater)	Microscopic Characteristics	Image		Algae source
			Macroscopically	Microscopically	
<i>Aspergillus niger</i>	- Fast-growing colony. - initially white then turning black. - granular texture. - circular growth with defined margins.	- Septate hyphae. - long conidiophores terminating in globose vesicles. - biseriate phialides. - black conidia in radiating chains.			Red and Brown algae
<i>Aspergillus flavus</i>	- Yellow-green to olive colonies. - powdery to granular texture. - moderate growth.	- Septate hyphae. - rough conidiophores. - globose vesicles. - uni- or biseriate phialides. - spherical conidia.			Red algae
<i>Aspergillus terreus</i>	- Cinnamon to brown colonies - velvety texture - moderate growth - compact morphology	- Septate hyphae. - smooth conidiophores. - small vesicles. - biseriate phialides. - smooth conidia.			Red algae
<i>Penicillium sp.</i>	- Green to bluish-green colonies - velvety to powdery texture - radial growth - white margins	- Septate hyphae. - branched conidiophores forming a brush-like (penicillus) structures. - chains of round conidia.			Green, Red and Brown algae
<i>Sterile mycelium</i> (unidentified filamentous fungus)	- Fast-growing - fluffy cotton-like texture - white to pale pink colonies - aerial mycelium	- Hyaline, filamentous hyphae - Absence of conidiophores and spores			Green and Red and Brown

Marine algae were used in this study because they are rich sources of endophytic marine fungal isolates, and these fungi are known to have the capability to synthesize metabolites to inhibit bacteria such as *Pseudomonas aeruginosa*. (Harikrishnan *et al.*, 2021). The additional of seawater in the isolated medium increases salinity that mimic the marine environment influencing the bioactive profile of these fungi which aligns with the approach used in this study. (Notarte *et al.*, 2018)

Based on the results, the red algae were the one with the highest fungal diversity, followed by brown algae then green algae which is similar to what Sahoo *et al.* (2021) found. The isolated fungi were assigned to two genera *Aspergillus* and *Penicillium*, while some isolates exhibited sterile mycelium. These are among most frequently isolated filamentous fungi from green, brown, and red seaweeds, and are known for their adaptability to diverse environmental conditions (Jones *et al.*, 2019).

The addition of seawater to the isolation medium increases salinity, mimicking the marine environment and significantly influencing the bioactive profile of these fungi, which aligns with the approach used in this study

3. Antimicrobial activity of marine Fungi against *Pseudomonas aeruginosa*

3.1. Antagonism Test

No clear inhibition zones were observed after 24 hours of incubation. However, a noticeable modification in bacterial growth pattern and appearance were detected, characterized by directional spreading away from the fungal colonies, with increased pigmentation. (Figure 18).

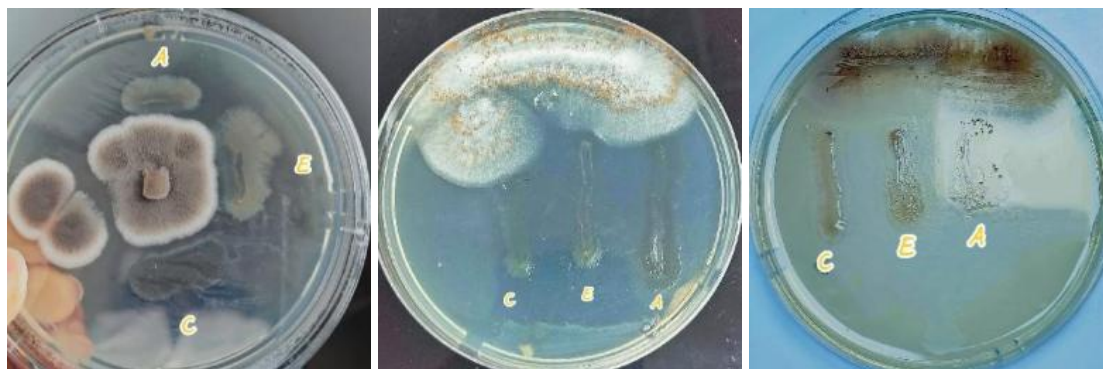


Figure 18 : Interaction between fungal isolates and *Pseudomonas aeruginosa* (C: clinical, E: environmental, A: ATCC)

This behavior suggests a weak antagonistic interaction between the fungal isolates and *Pseudomonas aeruginosa*.

This observation may be explained by the production of fungal secondary metabolites at low concentrations that could affect bacterial behavior instead of inhibiting growth. And that could be because some filamentous fungi such as *Aspergillus*, secondary metabolite production is often regulated during specific stages of growth and may remain limited under certain culture conditions (Calvo *et al.*, 2002; Keller, 2019). Also *P. aeruginosa* has been associated with the production of defensive metabolites such as pyocyanin (Kerr *et al.*, 1999).

The antagonism activity was observed only on standard Mueller-Hinton medium. While no positive results were detected on Mueller-Hinton supplemented with 2% NaCl. which may be resulted to an osmotic stress that affected both fungal metabolism by possibly reducing or their secondary metabolite production (Huang *et al.*, 2011), and bacterial stress responses by possessing complex regulatory systems for rapid responses to environmental stress (Moradali *et al.*, 2017).

The inhibitory interaction between *Pseudomonas aeruginosa* and marine fungi observed during direct contact in this cross-streak assay usually results in the inhibition of fungal growth by *P. aeruginosa*, mainly due to the production of pyocyanin, which is considered a major antifungal compound (Kerr *et al.*, 1999). However, other studies have also reported the opposite effect, where fungi inhibit *P. aeruginosa*. For example, Ahmed *et al.* (2019) found that some marine fungal isolates produced clear inhibition zones when grown on the same plate with *P. aeruginosa*. Both of these interactions were observed during this study suggesting that the inhibitory interaction between marine fungi and *P. aeruginosa* could be strain-dependent or related more to the experimental conditions.

3.2. Extraction of fungal secondary metabolites

Based on the results of the antagonism test, three fungal isolates were selected for metabolite extraction: *Penicillium* sp. (P1), *Aspergillus flavus* (A2), and *Aspergillus terreus* (A4).

These isolates showed an inhibitory potential and were considered a suitable candidate for secondary metabolite production.

Fungal cultures were chosen to be cultured on potato dextrose agar (PDA), which provides a rich source of carbohydrates and nutrients that support fungal growth and metabolite biosynthesis. PDA has been widely used for cultivating filamentous fungi, because of its ability to promote sporulation and metabolic activity (Frisvad *et al.*, 2012).

Following extraction, the yield of crude metabolites was calculated using the standard formula:

$$\text{Yield (\%)} = (\text{Weight of crude extract} / \text{Initial dry weight of fungal biomass}) \times 100$$

The extraction yield varied between the fungal isolates, with *Aspergillus flavus* (A2) producing the highest yield (0.0847%), followed by *Aspergillus terreus* (A4) with (0.055%), and finally *Penicillium* sp. (P1) showed the lowest yield of (0.0244%). The detailed extraction yields are presented in (Table 6).

Table 6 : Extraction yield (%) and crude extract quantity of fungal isolates

Extract code	Fungal identification	Biomass (g)	Crude extract (g)	Yield (%)
P1	<i>Penicillium</i> sp.	200 g	0,0488 g	0.0244%
A2	<i>Aspergillus flavus</i>	142 g	0.1203 g	0.0847%
A4	<i>Aspergillus terreus</i>	137 g	0.0754 g	0.0550 %

3.3. Antibacterial activity of fungal extracts

3.3.1. Disk and well diffusion assay

The antifungal extracts of (P1, A2, and A4) were evaluated for their antibacterial activity against ATCC 27853, clinical and environmental strains of *Pseudomonas aeruginosa*, through agar disk diffusion and agar well diffusion assays. (Figure 19; 20; 21)

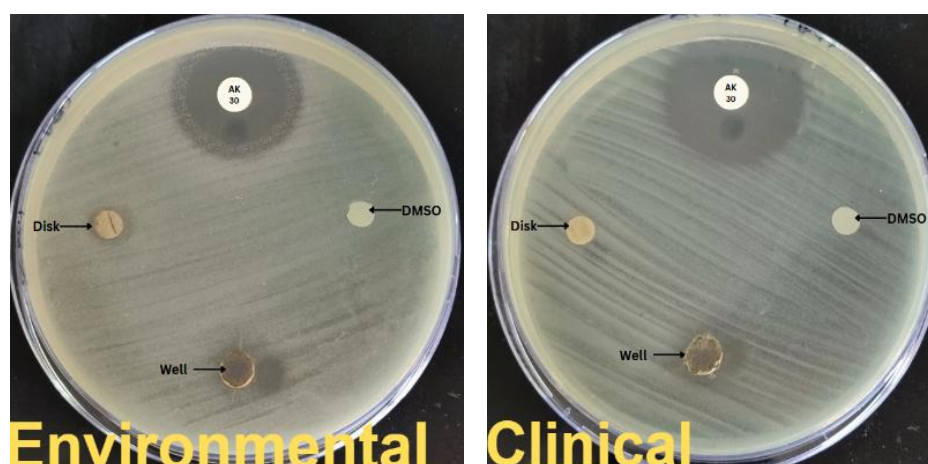


Figure 19 : Well and disc diffusion results of extract P1 against clinical and environmental *Pseudomonas aeruginosa* strains

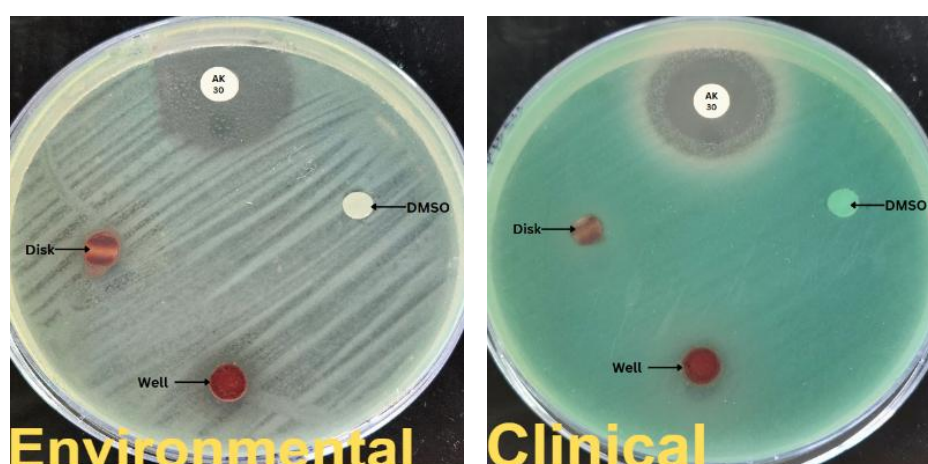


Figure 20 : Well and disc diffusion results of extract A4 against clinical and environmental *Pseudomonas aeruginosa* strains

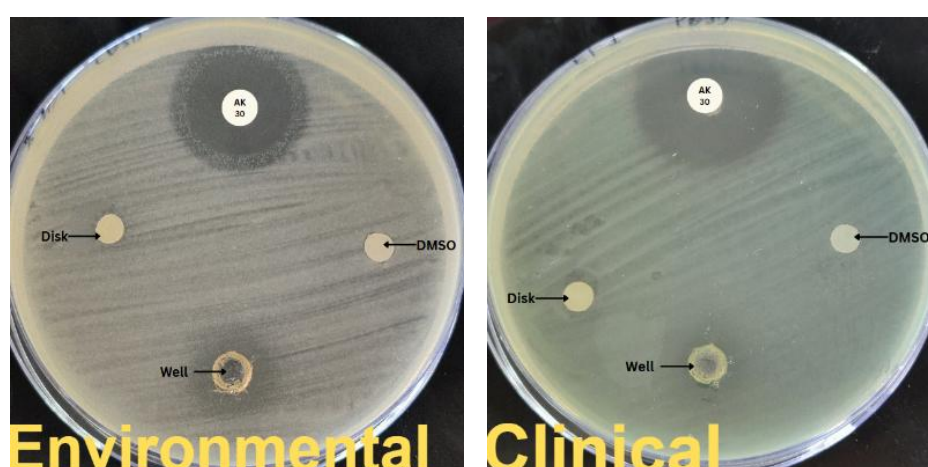


Figure 21 : Well and disc diffusion results of extract A2 against clinical and environmental *Pseudomonas aeruginosa* strains

Overall, the P1 extract showed a low activity, with an inhibition zones limited to values close to the disk diameter, indicating weak or marginal inhibition, while no measurable activity in the well diffusion assay. The A2 extract demonstrated moderate antibacterial activity exclusively in the well diffusion method, while no significant inhibition was observed in disk diffusion. The A4 extract showed variable activity depending on the strain, with strong inhibition observed against the reference ATCC 27853 strain, but weak activity against the clinical isolate and no detectable inhibition against the environmental strain. (Table 7).

These findings indicate that the antibacterial effect of the tested fungal extracts is both extract-dependent and strain-dependent. Also, agar well diffusion generally resulted in bigger inhibition zones than disk diffusion, which may be related to improved diffusion of the extract in the agar medium.

This variation aligns with the known resistance nature of *Pseudomonas aeruginosa*, especially among non-reference strains, as well as the heterogeneous nature of bioactive compound production in fungal extracts (Lambert, 2002; Moradali *et al.*, 2017; Balouiri *et al.*, 2016).

Table 7 : Inhibition zones (mm) of fungal extracts against *Pseudomonas aeruginosa* strains

Extract	Method	ATCC 27853 strain (mm)	Clinical strain (mm)	Environmental strain (mm)
P1	Disk diffusion	6.0	6.0	6.2
	Well diffusion	6.0	6.0	6.0
A2	Disk diffusion	6.0	6.0	6.0
	Well diffusion	12.0	12.1	12.4
A4	Disk diffusion	24.7	6.1	6.0
	Well diffusion	25.7	8.2	6.0
Mean of Antibiotic disk Amikacin 30µg		25.5	33.4	19.8

3.3.2. Minimum inhibitory concentration (MIC)

The values of MIC for all tested strains ranged between 2.5 and 5 mg/mL depending on which extract was tested against them, with an initial concentration of 20 mg/mL. The P1 extract showed lower MIC values against ATCC and environmental strains, while a higher value was observed against clinical isolate. The A2 extract showed a variable pattern, with lower MIC against the clinical strain and higher values for the other strains. In contrast, the A4 extract

showed a similar MIC value across all tested strains, indicating a stable but moderate inhibitory effect. (Figure 22; 23; 24). The observed results may be related to strain-dependent susceptibility of *Pseudomonas aeruginosa*. Overall, these results indicate that the tested extracts exert a mainly inhibitory (bacteriostatic) effect at the tested concentrations, with limited strain specificity.

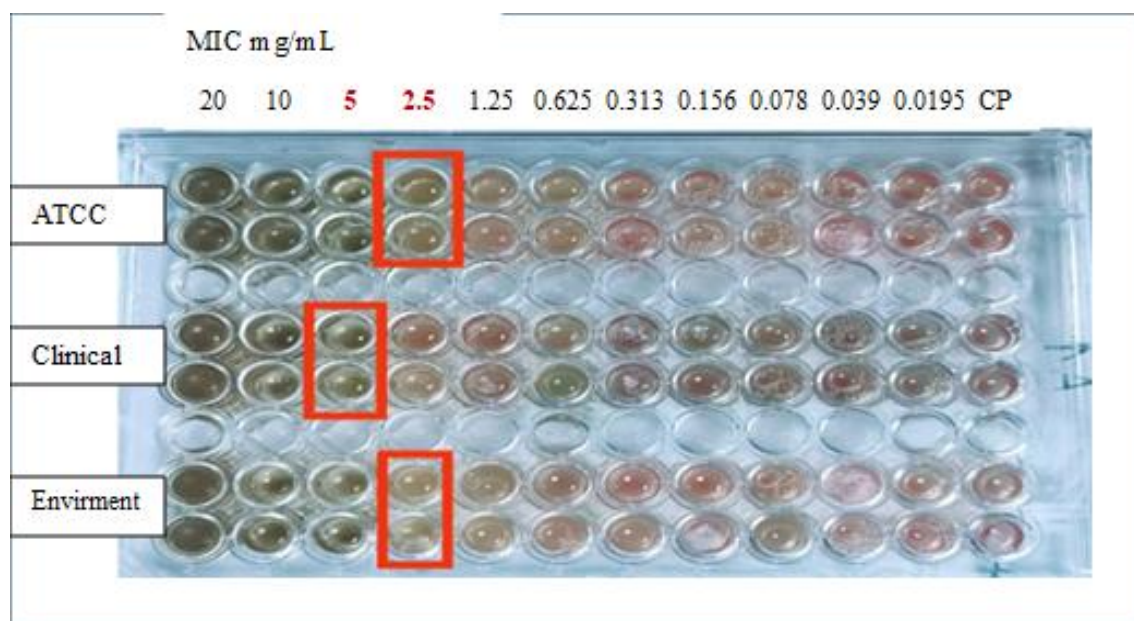


Figure 22: MIC results of extract P1 against ATCC 27853, clinical and environmental *Pseudomonas aeruginosa* strains

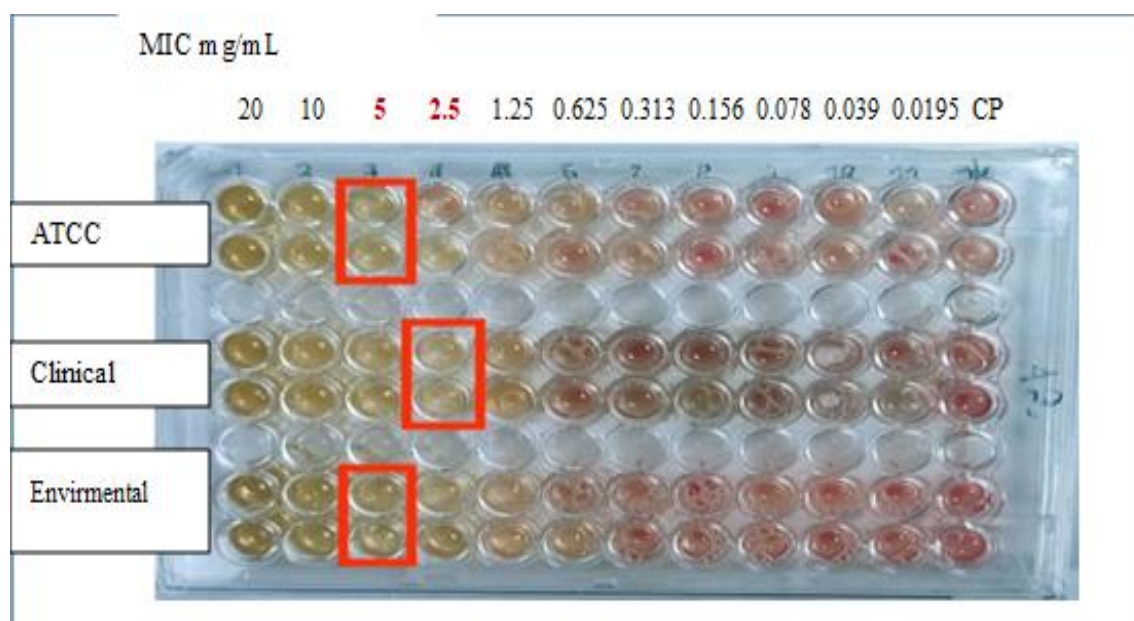


Figure 23 : MIC results of extract A2 against ATCC 27853, clinical and environmental *Pseudomonas aeruginosa* strains



Figure 24 : MIC results of extract P1 against ATCC 27853, clinical and environmental *Pseudomonas aeruginosa* strains

3.3.3. Minimum bactericidal concentration (MBC)

MBC values ranged between 10 and 20 mg/mL. Where P1 extract exhibited a uniform MBC of 20 mg/mL across all tested strains, indicating lower bactericidal efficiency. The A2 extract showed lower MBC values (10 mg/mL) against ATCC and clinical strains, while a higher concentration was required for the environmental isolate. The A4 extract showed mixed behavior, with lower MBC against the ATCC 27583 strain and higher values for the clinical and environmental strains. (Figure 25; 26; 27)

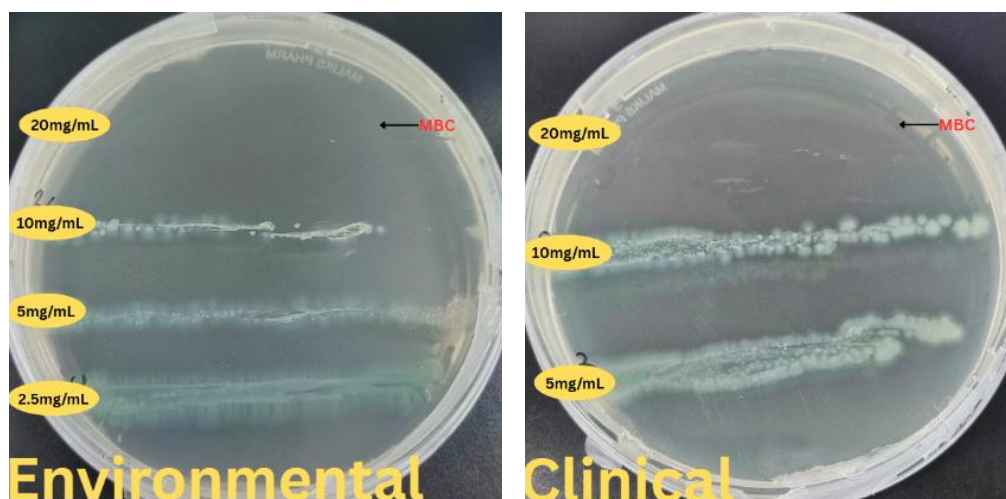


Figure 25 : MBC results of extract P1 against clinical and environmental *Pseudomonas aeruginosa* strains

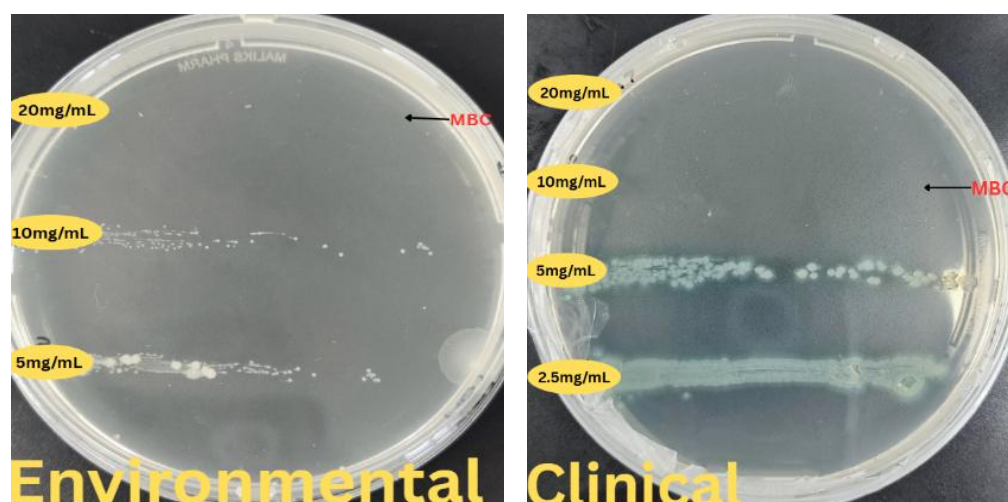


Figure 26 : MBC results of extract A2 against clinical and environmental *Pseudomonas aeruginosa* strains

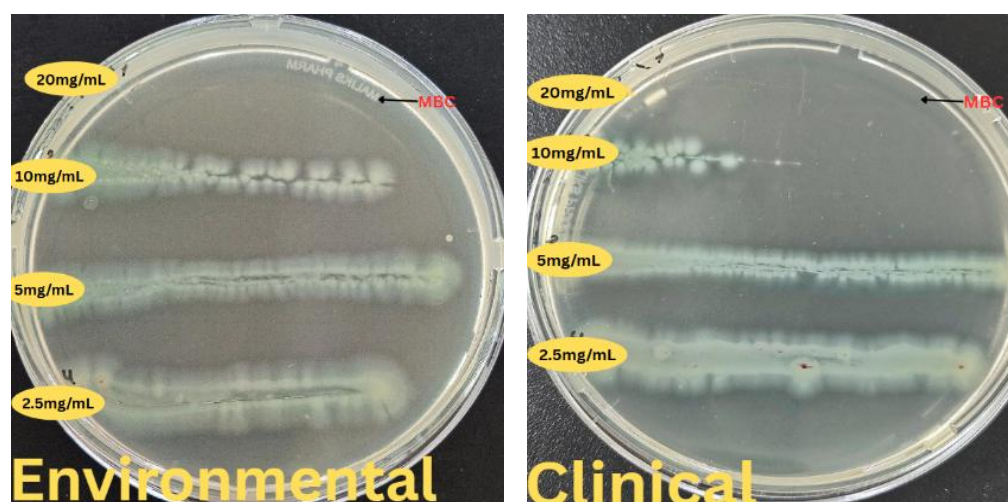


Figure 27 : MBC results of extract A4 against clinical and environmental *Pseudomonas aeruginosa* strains

Most of the MBC values were higher than the corresponding MIC values, suggesting that the tested extracts were primarily inhibitory at lower concentrations and required higher doses to achieve bactericidal activity. This pattern is commonly observed in crude natural extracts due to the presence of mixed bioactive compounds with varying antimicrobial potency.

For further evaluation of the antibacterial nature of the extracts, the MBC/MIC ratio was calculated for each extract–strain combination. (Table 8)

Table 8 : MIC and MBC values and antibacterial activity classification of fungal extracts

Extract	ATCC 27853				Clinical				Environmental			
	MIC	MBC	MBC/MIC	Activity	MIC	MBC	MBC/MIC	Activity	MIC	MBC	MBC/MIC	Activity
P1	2.5	20	8	bacteriostatic	5	20	4	bactericidal	2.5	20	8	bacteriostatic
A2	5	10	2	bactericidal	2.5	10	4	bactericidal	5	20	4	bactericidal
A4	5	10	2	bactericidal	5	20	4	bactericidal	5	20	4	bactericidal

- MIC: minimum inhibitory concentration
- MBC: minimum bactericidal concentration
- MBC/MIC ratios were calculated to evaluate the bacteriostatic or bactericidal nature of the extracts.
- Values ≤ 4 suggest bactericidal potential, while values >4 indicate a bacteriostatic tendency.

In a comparison with previous studies, the MIC values ranging between 2.5 and 5 mg/mL of crude extract that was observed in this study is considered weak antibacterial activity (**Tapfuma et al., 2022**). But there were studies such as **Baspinar et al. (2018)** saw that same extract had a strong activity against certain bacteria but was considered weak against *Pseudomonas aeruginosa*, which show that the effect depends not only on the extract but on the bacteria tasted against as well. At the same time there are studies that found similar results such as **Chin et al. (2024)** who found MIC of 0.313-2.5 mg/mL for marine fungal extracts against some Gram-negative bacteria and **Maadane et al. (2017)** who found MIC of 2.6-4.3 mg/mL of marine microalgae extracts against *P. aeruginosa*. While for MBC values that varied between 10-20 mg/mL, most of the research does not provide exact value for direct comparison, however the MBC/MIC ratios obtained in this study ranged above 4 for most tested extracts, which indicate a bacteriostatic effect according to standard antimicrobial classification criteria, where values greater than 4 are generally associated with inhibitory rather than bactericidal activity (**Pankey & Sabath, 2004**). which classify the extracts in this experiment as bacteriostatic that inhibit bacterial growth and reproduction more than bactericidal that cause bacterial death.

3.3.4. Anti-quorum sensing activity

3.3.4.1. Effect on biofilm formation

Extract A2 was chosen to test its effect at sub-MIC concentration on the biofilm formation ability of the tested strains of *Pseudomonas aeruginosa*.

The results showed a strain-dependent response to the extract. Where a reduction in biofilm formation was observed for the ATCC 27853 and Clinical strains, while the environmental strain showed a slight increase in biofilm production in the presence of the extract. (Figure 28; 29) (Table 9)

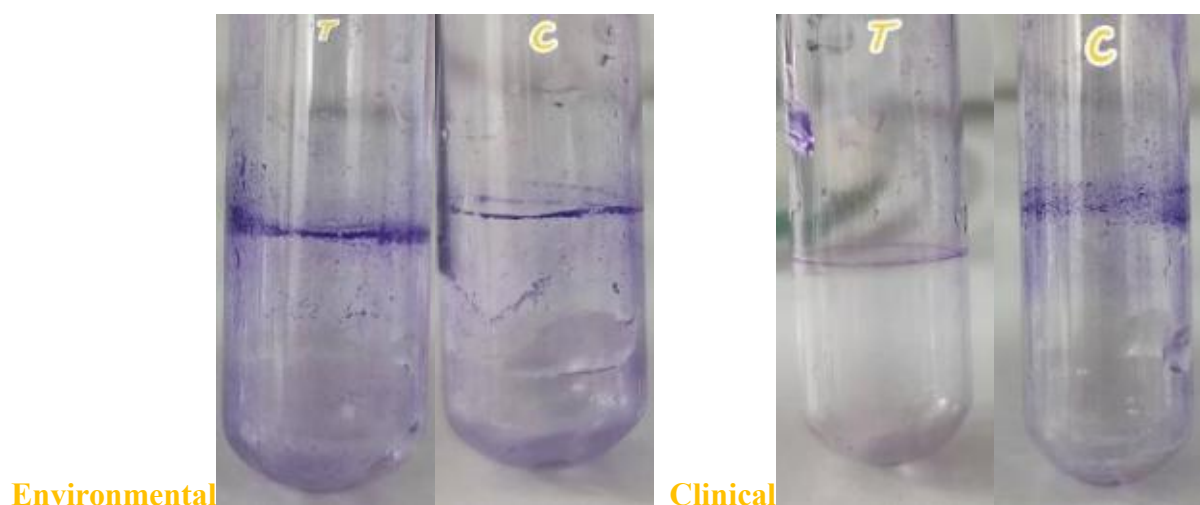


Figure 28 : Visual comparison of biofilm formation by Environmental and Clinical strains of *P. aeruginosa* (C: control tubes, T: treated tubes).

These results suggest that the quorum sensing inhibitory potential of the extract is dependent on the bacterial strain and its biofilm-forming capacity. Such variability has been previously reported, where sub-inhibitory concentrations of antimicrobial agents can reduce biofilm formation in some strains while having no effect or even promoting biofilm development in others due to adaptive stress responses and regulatory differences in quorum sensing pathways. (Lattab A. 2018).

Table 9 : Effect of extracts on biofilm formation and percentage change in *P. aeruginosa* strains

Strain	Control mean	+ Extract mean	% Change	Effect of the extract
ATCC	1.021	0.730	–28.5 %	Reduce
Clinical	1.125	0.850	–24.4 %	Reduce
Environmental	1.270	1.317	+3.7 %	Promote

- Values are the means of three independent measurements.
- % Change = ((Extract – Control) / Control) × 100

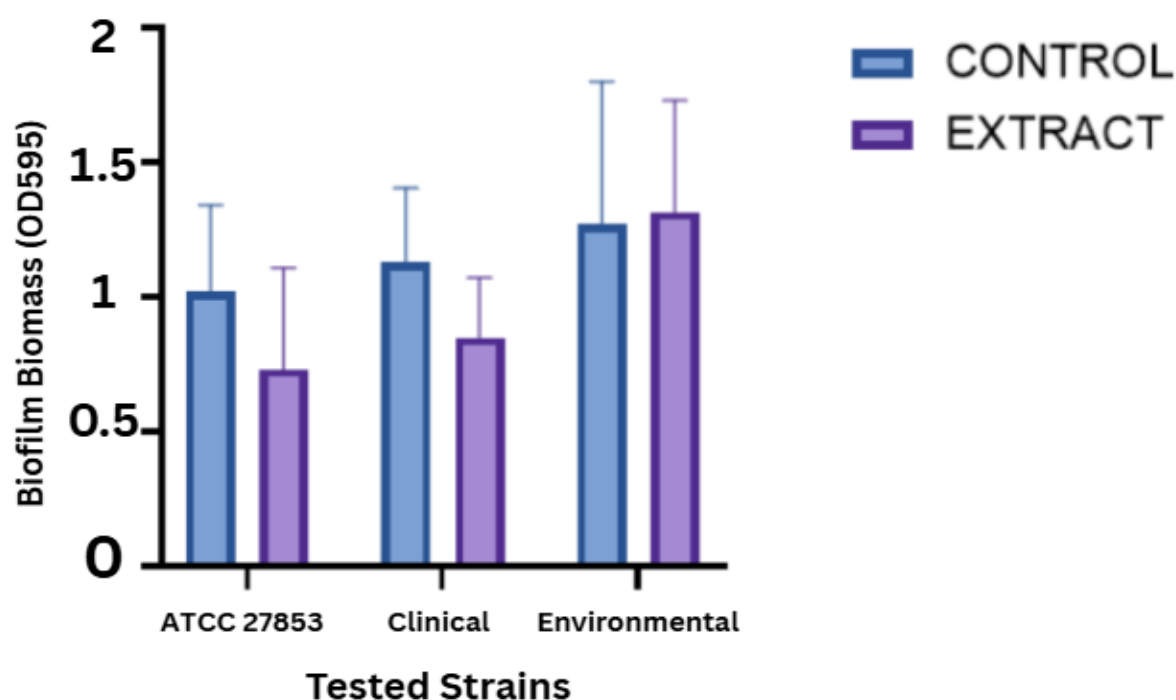


Figure 29 : Biofilm formation of *Pseudomonas aeruginosa* strains with and without A2 extract (sub-MIC)

3.3.4.2. Effect on pyocyanin production

A clear reduction in pyocyanin levels was observed in all tested strains with the presence of the extract compared to the control conditions (Table 10). These results indicate a consistent inhibitory effect of the extract on pyocyanin production across all strains tested. (Figure 30)

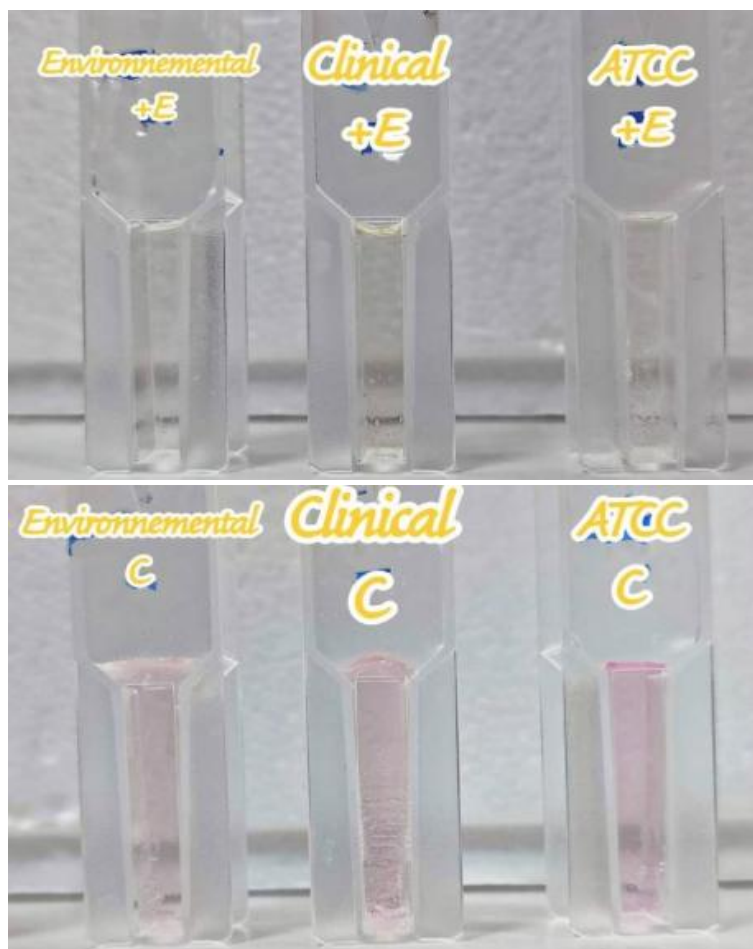


Figure 30 : Visual assessment of pigment production (pyocyanin) in control and treated bacterial cultures (C: control, +E: treated with extract A2)

Pyocyanin is a key virulence factor regulated by quorum sensing in *Pseudomonas aeruginosa*, and its reduction suggests a potential interference with bacterial virulence regulation. (Rabani *et al.*, 2025). Similar results by previous studies show that some marine-derived fungi extraction can effectively inhibit the biofilm by effecting the QS (Tran Thi *et al.*, 2020).

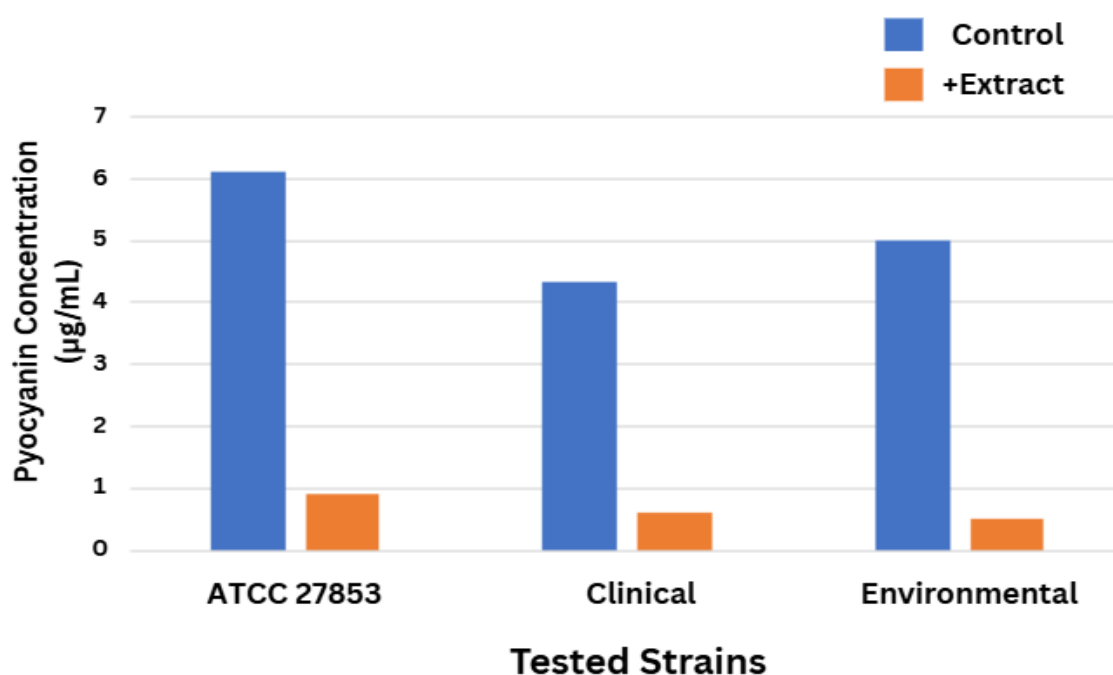
The quantity of pyocyanin produced by *P. aeruginosa* was calculated using the equation followed by (Essar *et al.*, 1990) and visually represented in (Figure 31):

$$Q = A \times f$$

- Q: quantity of pyocyanin
- A: Absorbance
- f: factor of conversion (17,072)

Table 10 : Effect of fungal extracts A2 on pyocyanin production in the bacterial strains

Strain	Condition	Absorbance (A)	Pyocyanin ($\mu\text{g/mL}$)	% Change	Effect of the extract
ATCC	Control	0.357	6.09	-84.9 %	Reduce
	+ Extract	0.054	0.92		
Clinical	Control	0.252	4.30	-85.6 %	Reduce
	+ Extract	0.036	0.62		
Environmental	Control	0.293	5.00	-90 %	Reduce
	+ Extract	0.029	0.50		

Figure 31 : Effect of A2 extract on pyocyanin production in *Pseudomonas aeruginosa* strains (ATCC 27853, Clinical, Environmental)

Marine fungal metabolites can be therapeutically valuable due to their anti-virulence mechanisms that primarily function through quorum sensing (Ji *et al.*, 2023; Wang *et al.*, 2020). Biofilm protect bacteria from immune system and antibiotics (Luo *et al.*, 2017; Thi *et al.*, 2020) while pyocyanin is an important virulence factor making *P. aeruginosa* harder to treat (Mudaliar & Prasad, 2024; Abdelaziz *et al.*, 2023), Therefore, compounds capable of

inhibiting these virulence factors are considered promising anti-virulence therapies, which is the case for extract A2 in this study

This anti-virulence effect is highly valuable because it may slow the evolution of resistance and could also be used as an alternative or complementary therapeutic strategy.



Conclusion

The purpose of this study is evaluating the antibacterial activity of marine fungal extracts against clinical and environmental strains of *Pseudomonas aeruginosa*, alongside a reference (ATCC 27853) strain. As well as their effect on the virulence factors regulated by quorum sensing (QS), such as biofilm formation and pyocyanin production.

Based on the results found during this study, the fungal extracts have moderate antibacterial activity, with a value of 2.5 and 5 mg/mL for the minimum inhibitory concentration (MIC) and a value of 10 and 20 mg/mL for the minimum bactericidal concentration (MBC), which indicate an inhibitory effect (bacteriostatic) at low concentrations and a killing effect (bactericidal) at high concentrations. Among all tested extracts, the extract (A2) of *Aspergillus flavus* showed strong activity in inhibiting the virulent factors regulated by the quorum-sensing system, where it reduced pyocyanin production by 85 to 90% in all tested strains, and reduced biofilm formation in the reference strain clinical and in the ATCC 27853 strain by 24.4% and 28.5%, while a slight increase with 3.7% was observed in the environmental strain.

These results highlight the potential of marine endophytic fungi as a promising source of bioactive compounds capable of affecting bacterial growth and virulence of *Pseudomonas aeruginosa*. But at the same time, there are some limitations to this study, including the reliance on a few strains (one strain per source), which limits the generalization of the results found on the bacterial populations. Also, the limited number of fungal species isolated does not completely represent the diversity of marine fungi, without forgetting the in vitro conditions used in this study cannot fully replace the complex marine environment that these marine fungi live in.

Future studies could expand the number of bacterial strains and the fungal species tested, and do further investigations on the bioactive compounds responsible for the antibacterial and anti-virulence effects observed in this study.



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A decorative watercolor splash in shades of purple and blue, with the word "Appendices" written in a gold, cursive font across it.

Appendices

Appendix{1}

Culture Medium Composition

Luria Bertani Broth, Miller (Miller Luria Bertani Broth)	
Ingredients	g / L
Tryptone	10.000
Yeast extract	5.000
Sodium chloride	10.000
Final pH (at 25°C)	7.5±0.2

Nutrient Agar	
Ingredients	g / L
Peptone	5.000
Sodium chloride	5.000
HM peptone B	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

Mueller Hinton Agar	
Ingredients	g / L
HM infusion solids B (from 300g)	2.000
Acicase	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

Mueller Hinton Broth	
Ingredients	g / L
HM infusion B from 300 g	2.000
Acicase®	17.500
Starch	1.500
Final pH (at 25°C)	7.3±0.1

Sabouraud Chloramphenicol Agar	
Ingredients	g / L
Tryptone	5.000
Peptone	5.000
Dextrose (Glucose)	40.000
Chloramphenicol	0.050
Agar	15.000
Final pH (at 25°C)	5.6±0.2

Kings Medium A Base	
Ingredients	g / L
Proteose peptone	20.000
Potassium sulphate	10.000
Magnesium chloride	1.640
Agar	15.000
Final pH (at 25°C)	7.3±0.1

King's Medium B Base	
Ingredients	g / L
Proteose peptone	20.000
Dipotassium hydrogen phosphate	1.500
Magnesium sulphate heptahydrate	1.500
Agar	20.000
Final pH (at 25°C)	7.2±0.2

Mueller-Hinton Agar Supplemented with 2% NaCl	
Ingredients	g / L
Beef extract	2.000
Acid hydrolysed casein	17.500
Starch	1.500
Sodium chloride	20.000
Agar	17.000
Final pH (at 25°C)	7.3±0.2

Potato Dextrose Agar	
Ingredients	g / L
Infusion from potatoes from 200g	4.000
Dextrose (Glucose)	20.000
Agar	15.000
Final pH (at 25°C)	5.6±0.2

Congo Red Agar (CRA)	
Ingredients	g / L
Brain heart infusion broth	37
Sucrose	50
Agar	10
Congo red	0.8
Final pH (at 25°C)	7.3±0.2

Note: Congo red dye is heat-sensitive and should not be autoclaved with the complete culture medium. A sterile Congo red solution is added aseptically to the basal medium after steam sterilization, either by membrane filtration (0.22 µm filter) or as a separately sterilized solution prepared in distilled water. The dye should be protected from light and added just before use .

Appendix{2}

Gram Staining and Oxidase Assay

1. Gram Staining according to the routine laboratory procedure

- A bacterial smear was prepared on a clean glass slide and heat-fixed prior to staining. Then the Gram staining procedure was performed as follows:

1. The smear was flooded with crystal violet solution and left for 1 minute, then rinsed gently with distilled water.
2. The slide was treated with Gram's iodine (Lugol's solution) for 1 minute, followed by rinsing with distilled water.
3. Decolorization was carried out using 95% ethanol for approximately 10 seconds, then immediately rinsed with distilled water to stop the reaction.
4. The smear was counterstained with safranin for 1 minute, then rinsed with distilled water.
5. The slide was air-dried.
6. The microscopic observation was performed using a light microscope, initially under low magnification and then under oil immersion (100× objective) for detailed examination of bacterial morphology and Gram reaction.

7. Record result:

- Gram Positive: purple/violet color
- Gram Negative: pink/red color

2. Oxidase Test according to the routine laboratory procedure

1. Select a well-isolated colony from a fresh agar plate.
2. Use a sterile platinum loop to collect a small amount of the colony.
3. Smear the colony onto an oxidase test disc/paper.
4. Gently spread the colony on the disc (avoid excessive pressure).
5. Observe the reaction immediately (within less than 10 seconds).

6. Record result:

- Positive: dark purple/blue color within 10 seconds
- Negative: no color change within 10 seconds
- Late color change (>10–30 s): invalid result (ignored)

Appendix{3}

Laboratory Materials and Equipment

1. Culture and Microbiology Consumables

English	Français	العربية
Distilled water	Eau distillée	ماء مقطر
Petri dishes	Boîtes de Pétri	علب بيتري
Platinum inoculating loop	Anse de platine	لووب بلاتين
Sterile cotton swab	Écouvillon stérile	ماسحة قطنية معقمة
Microscope slides	Lames de microscope	شرائح زجاجية
Coverslips	Lamelles	غطاء الشريحة الزجاجية
Test tubes (standard size)	Tubes à essai (taille standard)	أنابيب اختبار عادية
Micro test tubes (hemolysis tubes)	Microtubes (hémolyse)	أنابيب اختبار صغيرة (هيموليز)
Antibiotic discs	Disques antibiotiques	أقراص مضادات حيوية
Micropipette tips	Embouts de micropipettes	رؤوس الميكروبيبات
Pasteur pipettes	Pipettes Pasteur	بيبات باستور
Microplates with lids	Microplaques avec couvercles	ميكروبلاك مع غطاء
Whatman filter paper	Papier filtre Whatman	ورق ترشيح واتمان
Membrane filters (0.22 µm)	Filtres membranaires (0,22 µm)	فلتر غشائية 0.22 ميكرومتر
Syringe filters	Filtres seringues	فلتر المحاقن
Glass bottles (clear/amber)	Flacons en verre (clairs/ambres)	قارورات زجاجية (شفافة/غامقة)
Syringe (5mL and 10mL)	Seringues (5mL and 10mL)	حقن

2. Equipment and Laboratory Instruments

English	Français	العربية
Light microscope	Microscope optique	ميكروسكوب
Spectrophotometer	Spectrophotomètre	سبكتروفوتومتر
Spectrophotometer cuvettes	Cuvettes	كيوفيت (أنابيب القياس)
Micropipettes (20, 100, 1000 µL)	Micropipettes (20, 100, 1000 µL)	ميكروبيبات
Autoclave	Autoclave	أوتوكلاف
Vortex mixer	Agitateur vortex	فورتكس
Magnetic stirrer	Agitateur magnétique	مخلاط مغناطيسي
Orbital shaker incubator	Incubateur agitateur	حاضنة مهتزة
Incubator (static)	Étuve	حاضنة
Refrigerator (4°C)	Réfrigérateur (4°C)	ثلاجة 4 درجات مئوية
Freezer	Congélateur	مجمد
Water bath (50–95°C)	Bain-marie (50–95°C)	حمام مائي
Analytical balance	Balance analytique	ميزان دقيق
Precision balance	Balance de précision	ميزان إلكتروني
Graduated cylinder	Éprouvette graduée	مخبار مدرج
Beaker	Bécher	بيشر
Erlenmeyer flask	Erlenmeyer	دورق إرلنماير
Volumetric flask	Fiole jaugée	دورق حجمي
Magnifying glass	Loupe	مكبرة

3. Chemicals and Staining Reagents

English	Français	العربية
Ethanol	Éthanol	إيثانول
Chloroform	Chloroforme	كلوروفورم
Ethyl acetate	Acétate d'éthyle	أستات الإيثيل
Crystal violet	Cristal violet	كريستال فيوليت
Fuchsin	Fuchsine	فوشين
Lugol's iodine	Lugol	محلول لوغول
Immersion oil	Huile d'immersion	زيت الغمر