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Thème

Screening of the biotechnological potential of some actinobacteria strains isolated in Bejaïa region

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Summary

LITERATURE REVIEW

Materials and Methods

Results and Discussion

List of tables

List of figures

List of Abbreviations

EMB: Eosin Methylene Blue

CMI: Minimum Inhibitory Concentration

TSB: Tryptic Soy Broth

TSBG: Tryptic Soy Broth with Glucose

CMC: Carboxymethyl Cellulose

UFC/mL: Colony Forming Units per Milliliter

SCA: Starch Casein Agar

ND: Not Determined / Not Detected

pH: Potential of Hydrogen

MH: Mueller-Hinton

ATCC: American Type Culture Collection

GC%: Guanine-Cytosine Content (in DNA)

GAE: Gallic Acid Equivalent

MIC: Minimal Inhibition Concentration

IC50: Inhibition Concentration of 50%

WHO: Word Health Organisation

Introduction

The widespread and intensive use of antibiotics in both humans and animals has led to the accelerated emergence of bacteria resistant to these drugs. Unfortunately, antibiotic resistance has rapidly become a growing global threat. For several decades, bacteria commonly associated with human infections have developed resistance mechanisms against every new antibiotic introduced **(Rolain and Berrazeg, 2014;Kokkini et al., 2022)**. The antimicrobial resistance represents a global health challenge considered as a hidden pandemic **(Adebisi et al., 2021)**.

In 2017, the World Health Organization (WHO) published a list of priority antibiotic-resistant pathogens to guide research and development of new antibiotics. This list includes critical, high, and medium priority pathogens known as ESKAPE bacteria, an acronym for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species **(WHO, 2017)**.

Thanks to their unique metabolism, actinobacteria are an attractive natural source of new bioactive metabolites with diverse activities, including antimicrobial, antioxidant, enzymatic, and antibiofilm properties **(Djinni et al., 2019a;De Simeis and Serra 2021).** These microorganisms, widely distributed in nature, are known to produce secondary metabolites that play a crucial role in combating pathogens, protecting crops, and serving various industrial and pharmaceutical applications **(Selim et al., 2021)**. For nearly 80 yearssince the discovery of streptomycinactinobacteria have remained the principal source of antibiotics, highlighting their important role in modern medicine **(De Souza-Rodriguez et al, 2024)**

Actinobacteria possess remarkable antibiofilm and antioxidant properties, enhancing their value in biomedical and industrial applications. Some strains can inhibit or disrupt biofilms formed by pathogenic microbes, addressing a major challenge in antimicrobial resistance. Additionally, they produce antioxidant compounds able of neutralizing free radicals, thereby reducing oxidative stress linked to various diseases. These combined activities make actinobacteria promising sources of therapeutic and protective agents **(Agatha and Waturangi, 2022;Rusyda et al., 2023;Fathoni et al., 2024)**.

The present study focuses on evaluating the bioactivity of actinobacteria strains derived from soil and water samples of Bejaia region, as well as their ethyl acetate crude extracts, specifically testing their antibacterial, antifungal, antibiofilm, antioxidant, and

enzymatic properties**.** This screening aims to identify the most promising strains fora more in-depth study of the active metabolites responsible for the various activities.

This manuscript is structured into three main chapters. The first one provides a general overview of actinobacteria, including their characteristics, taxonomy, and biological activities. The second chapter is devoted to the methodology, which covers the general principles of the materials and methods used in order to:

- Evaluate the antimicrobial activity of the studied strains as well as their ethyl acetate crude extracts ;
- Evaluate the minimum inhibitory concentrations (MICs);
- Evaluatetheirenzymaticpotential ;
- Evaluating the antibiofilmpotential ;
- Studying the antioxidantactivity.

Finally, the third part presents the obtained results along with their discussion.

LITERATURE REVIEW

1. General overview of actinobacteria

1.1 Definition and classification

The Actinobacteria is a phylum of Gram-positive bacteria and one of the largest taxonomic units within the domain Bacteria characterized by a high guanine-cytosine (G+C) content in their DNA, exceeding 55% **(Goodfellow, 1983 ;Barka et al., 2015)**. The majority of these bacteria are free-living organisms, being well known for their ubiquitous presence in soil and aquatic habitats, where they participate in numerous essential biological processes **(Lechevalier and Lechevalier, 1980;Goodfellow and Williams, 1983).** Although unicellular, particular species develop a fine, non-septate mycelium, resulting in a filamentous form that resembles fungi (**Ranjani et al., 2016).**

From a taxonomic perspective, actinobacteria belong to the phylum *Actinomycetota*, which includes six classes, 46 orders, 79 families, and 425 genera according to classification (2018) **(Salam et al., 2020).**

From a taxonomic perspective, actinobacteria belong to the phylum Actinomycetota, which includes six classes, 46 orders, 79 families, and 425 genera according to classification (2018), with the genus *Streptomyces* being particularly notable for its prolific production of antibiotics and other secondary metabolites (**Barka et al., 2015 ;Salam et al., 2020**).

1.2. Morphological and physiological characteristics

1.2.1. Morphological characteristics

Actinobacteria exhibit diverse morphological traits, including the "formation of complex structures such as spores, spore chains, sporangia, and sporangiospores, which can be observed microscopically" (**Li et al., 2016**). Their sporulation mechanisms are varied, with strategies such as "mycelial fragmentation, holothallic septation (as seen in *Streptomyces*), or the formation of *sporangia* (*Actinoplanes*)". The spores produced by these bacteria show diversity in both shape and organization, "occurring as single units, short or long chains, or thermoresistant endospores" (**Ranjani et al., 2016**). The microscopic observation of these features helps in identifying and classifying different species. Indeed, the ultrastructure of spores and hyphal morphogenesis are key features used in the taxonomic classification of Actinobacteria (**Waksman, 1940)**. Specialized structures, such as *synnemata* and *sclerotia*, are also produced by particular species, further contributing to their morphological diversity (**Ordóñez-Valencia et al., 2015).** Additionally, the presence of thermoresistant endospores underscores their ability to withstand harsh conditions (**Nichols et al., 2002**). Overall, the

complex sporulation and structural variety of actinobacteria underline their ecological versatility and significance.

The cellular and colonial morphology of actinobacteria is complemented by physiological and biochemical features, such as growth under various environmental conditions and the presence of specific enzymes (**Li et al., 2016**). Additionally, mycelial pigments, including melanoid and carotenoid types, serve as important taxonomic markers **(Dornelas et al., 2017)**. The morphological identification of actinobacteria is crucial for their classification, but it is often supplemented by chemotaxonomic methods due to the morphological similarities among different genera (**Wang and Jiang, 2016**).

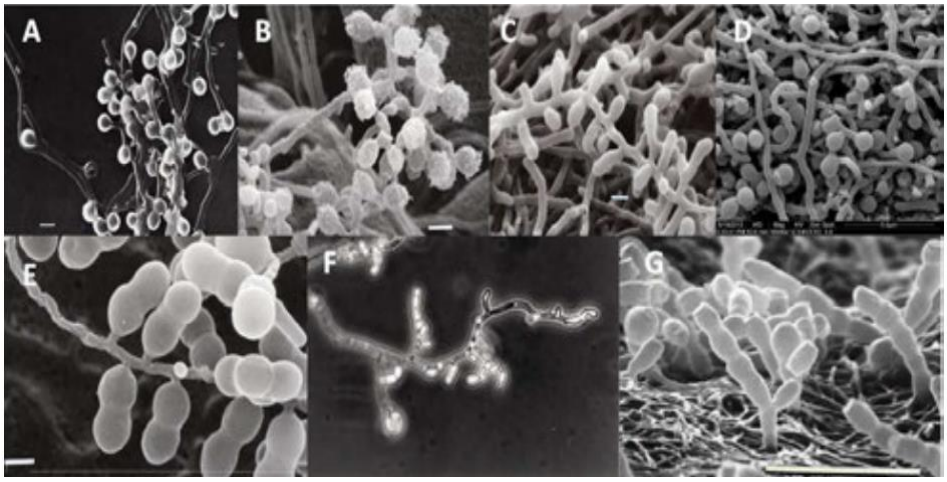

Figure 1: Micrograph of the production of single spores and spores in short chains**(Li et al., 2016).**

(A) *Micromonospora* sp. SF2259^{T}, (B) *Thermobifida alba* JCM 3077^{T}, (C) *Saccharomonosporaviridis* IFO 12207^{T}, (D) *Thermoactinomycesdaqus* H-18, (E) *Microbisporarosea* JCM 3006^{T}, (F) *Nocardia brevicatena* A444, (G) *Catellatospora* sp. MB-VE 1321.

1.2.2. Physiological characteristics

Actinobacteria exhibit remarkable physiological diversity, enabling them to thrive in a wide range of environments (**Li et al., 2016**). Their metabolism is primarily aerobic, although some species can tolerate anaerobic or microaerophilic conditions. These microorganisms are chemoorganotrophs, utilizing organic compounds as sources of carbon and energy. They are capable of degrading a wide range of substrates, including sugars like xylose, fructose, and mannose, although their ability to ferment lactose is limited, organic acids, alcohols, amino acids, and aromatic compounds, highlighting their significant role in nutrient cycling (**Varghese et al., 2012**).

Actinobacteria are also well-known for their ability to produce a vast array of secondary metabolites, including antibiotics, enzymes, pigments, and immunosuppressants

(Ranjani et al., 2016). This metabolic versatility is further enhanced by their ability to survive under adverse conditions and their potential to develop novel metabolic pathways (**Solyanikova and Golovleva, 2015**).Optimal growth conditions vary among species, but most prefer mesophilic temperatures (between 25°C and 35°C) and neutral pH **(Rammali et al., 2022)**.

Actinobacteria also exhibit diverse enzymatic activities, such as the decomposition of proteins and amino acids, and the production of extracellular enzymes that degrade complex polymers like cellulose, chitin, starch, lignin, and proteins, thereby contributing to nutrient recycling and humus formation, thus contributing to nutrient cycling and soil health and to their biodegradative capabilities (**Varghese et al., 2012 ;Solyanikova and Golovleva, 2015**). These enzymes have diverse industrial applications, notably in the food, pharmaceutical, and textile sectors **(Subash et al., 2024)**.

Moreover, some actinobacteria possess mechanisms that enable them to resist environmental stresses, such as desiccation and heavy metals and ionizing radiation, thereby facilitating their survival under extreme conditions **(Djinni and Djoudi, 2022;Reti et al., 2024).**

This physiological diversity, combined with their ability to produce bioactive compounds, makes actinobacteria microorganisms of great interest for biotechnological research and the discovery of new therapeutic products **(Djinni et al., 2019a).**Ongoing studies continue to reveal fascinating aspects of their biology, highlighting their ecological importance and potential for industrial and medical applications.

2.Biological activities of Actinobacteria

Actinobacteria are widely known for their metabolic versatility**.** They produce secondary metabolites with diverse biological activities, including antimicrobial, anticancer, antivirals, antiparasitics and antioxidant properties **(Almuhayawi et al., 2021)**.

2.1 Antibiotic activity

Actinobacteria possess exceptional potential in drug discovery due to their production of diverse secondary metabolites, particularly antibiotics and anticancer agents, with most clinically used antibiotics originating from *Streptomyces* and *Micromonospora* (**Ranjani et al., 2016**). During the "golden age of antibiotics," most major antibiotic families were discovered, and since then, over 12,000 antibiotics have been identified, about 70% of which are derived from actinobacteria (**Ranjani et al., 2016**). *Streptomyces* species alone produce around 7,600 compounds with significant antibiotic properties, which are effective against a

broad spectrum of pathogens, including Gram-positive bacteria such as *S. aureus* (including MRSA), certain Gram-negative bacteria, and fungi (**Berezin et al., 2019; Elshafie and Camele, 2022**). This group, especially *Streptomyces*, remains crucial in combating antimicrobial resistance due to their biosynthetic diversity and environmental adaptability (**Roohi and Bano, 2024;Cerqueira et al., 2025**).

These antimicrobials encompass several major structural classes, such as β-lactams, glycopeptides, lipopeptides, thiopeptides, actinomycins, aminoglycosides (e.g., streptomycin, kanamycin), and ansamycins (e.g., rifampicin), many of which utilize unique mechanisms to overcome microbial resistance (**Wang et al., 2017; DeSimeis and Serra, 2021; Liras et al., 2022**).In addition to antibacterial agents, actinobacteria have yielded potent antifungal compounds such as enduspeptide B, neomaclafungins A–I, and kribelloside, further underscoring their pharmaceutical relevance (**Jakubiec-Krzesniak et al., 2018**). These bioactive metabolites are typically extracted as crude or purified compounds following detailed chemical characterization, enabling their application in drug development (**Ranjani et al., 2016**).

2.2.Antioxidant activity

Actinobacteria, particularly the genera *Streptomyces* and *Rhodococcus*, are well known for their ability to produce secondary metabolites with antioxidant properties (**Rusyda et al., 2023;Fathoni et al., 2024)**. These compounds, including phenols, flavonoids, and carotenoids, that can neutralize free radicals and protect cells from oxidative stress, a key factor in the progression of many chronic diseases**(Rammali et al., 2024**). The antioxidant potential of rare actinomycetes and endophytic actinobacteria from *Harpagophytumprocumbens* further underscores the diverse and potent antioxidant capabilities of this bacterial group, with several strains demonstrating the ability to protect DNA from oxidative damage (**Mohammadipanah and Momenilandi, 2018; Magdalena et al., 2022).** Collectively, the findings results of diverse studies highlight the promising role of actinobacteria as a source of natural antioxidants, with potential applications in pharmaceutical and other industries.

2.3. Enzymatic activity

Actinobacteria represent a significant reservoir of biocatalysts with diverse industrial applications. These microorganisms possess the ability to produce a broad spectrum of extracellular enzymes involved in various biotechnological processes **(Azzouz et al., 2022;Subash et al., 2024),** including industrial applications and environmental

bioremediation **(Belabbas et al., 2025).** Among these enzymes, α-amylase is widely used in industries such as brewing, detergent formulation, pulp and paper production, and pharmaceutical. Additionally, β-glucosidase plays an important role in the food industry by enhancing the sensory qualities of fruit juices, tea, and wine **(Saez et al., 2025).**

Numerous studies have demonstrated the capacity of actinobacteria to synthesize hydrolases and oxidoreductases, such as cellulases, proteases, lipases, chitinases, and keratinases, which are crucial for the biodegradation of complex polymers **(Benhoula et al., 2024;Hamma et al., 2024).** Within the phylum Actinobacteria, several genera, including *Kytococcus*, *Actinomadura*, *Microbacterium*, *Kocuria*, *Streptomyces*, *Thermoactinomyces*, and *Nocardiopsis* have been identified as prolific producers of these enzymes **(Subash et al., 2024).**

2.4.Antibiofilm activity

Bacterial biofilms are protective structures that enhance resistance to antibiotics and immune defenses, thereby complicating the treatment of infections **(Gopikrishnan et al., 2019)**. Actinobacteria offer a promising solution due to their antibiofilm and anti-quorum sensing (QS) properties **(Agatha and Waturangi, 2022;Djinni et al., 2024)**. They primarily act by inhibiting quorum sensing**,** a bacterial communication mechanism essential for biofilm formation (**Figure 2**). By disrupting this signaling and degrading the extracellular matrix, the active metabolites produced by actinobacteriareduce bacterial adhesion and resistance, thereby enhancing the effectiveness of antimicrobial treatments **(Gopikrishnan et al., 2016)**.

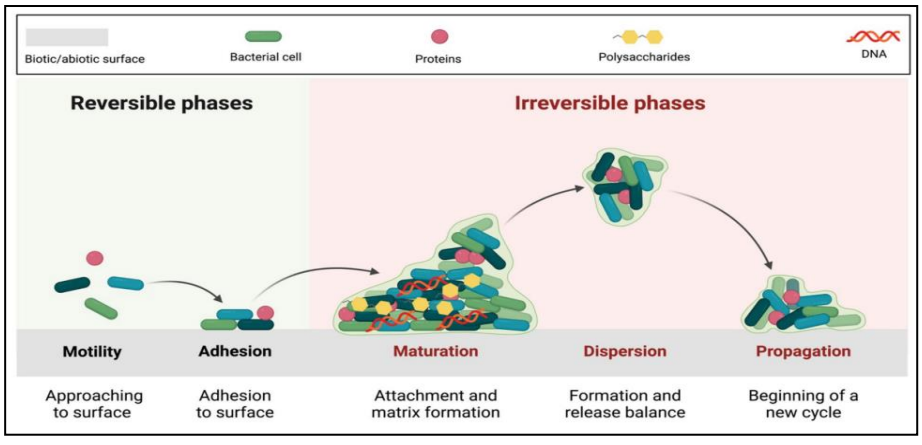

Figure 2: Structure and biofilm formation cycle **(Al-Madboly et al., 2024).**

The anti-QS activity of actinobacteria, which disrupts communication among bacteria, further enhances their antibiofilm efficacy, as demonstrated by their ability to inhibit quorum sensing in *Chromobacteriumviolaceum* and other pathogens (**Wijaya et al., 2023**). These findings underscore the potential of actinobacteria as a natural source of antibiofilm agents, offering promising applications in food safety, aquaculture, and the development of new disinfectants to manage biofilm-related challenges in various industries (**Azman et al., 2019**).

2.5. Plant growth promoting Rhizobacteria (PGPR) activity

Actinobacteria play a significant role in enhancing agricultural productivity and maintaining soil health (**Franco-Correa and Chavarro-Anzola, 2016**). These microorganisms are characterized by their ability to produce a wide array ofmetabolites with antifungal, antibacterial, and insecticidal properties while simultaneously promoting plant growth. Consequently, they represent an eco-friendly alternative to chemical agricultural inputs, sustainably supporting crop protection and contributing to environmentally responsible farming practices. Furthermore, actinobacteria play a key role in maintaining rhizosphere balance and nutrient cycling in soils **(Subramaniam et al., 2016).** These bacteria can promote plant growth andprotect direct actions, such as synthesizing phytohormones that stimulate crop development, and indirect processes, including nitrogen fixation, phosphate solubilization, and iron assimilation **(Sathya et al., 2017)**. More specifically, theyenhance plant growth by directly producing compounds such as indole-3-acetic acid (IAA) and hydrolytic enzymes **(Subramaniam et al., 2016).**

The integration of Actinobacteria into agricultural systems not only enhances crop yield and quality but also contributes to the long-term sustainability of farming practices by improving soil fertility and plant health (**Andrade et al., 2023;Laskar, 2024**).

2.6.Anticancer activity

Despite advances in medical science, cancer remains a major global health challenge, affecting millions of individuals worldwide. Chemotherapy remains a cornerstone of cancer treatment, while compounds derived from plants and microorganisms have significantly contributed to the development of anticancer drugs **(Parthasarathy and Krupakar, 2024)**.

Actinobacteria, particularly those from the genus *Streptomyces*, have emerged as a significant source of anticancer compounds, offering promising avenues for cancer treatment and prevention **(Noufal et al., 2022;Abdel-Fattah et al., 2024)**.

Among the well-known actinobacterial-derived anticancer agents, actinomycin D and doxorubicin have been widely used in clinical settings for several decades **(Djinni et al., 2019b).**

Despite the promising experimental results of diverse studies, further research is necessary to validate the efficacy and safety of these compounds *in vivo* and to develop efficient production methods for clinical applications (**PereiraCardoso et al., 2024**). Overall, the diverse and potent anticancer activities of actinobacteria-derived compounds underscore their potential as a valuable resource in the ongoing search for effective cancer therapies (**Silva et al., 2020;Bano et al., 2024).**

2.7. Antiviral activity

Actinobacteria are known to produce bioactive secondary metabolites, including AhmpatinineiBu, antimycin A1a, and pentapeptide 4862F, which are natural compounds exhibiting significant antiviral activity **(Jakubiec-Krzesniak et al., 2018).** Several studies have reported the production of novel antiviral compounds by these microorganisms, targeting pathogenic viruses such as Western equine encephalitis virus, HIV-1, Zika virus, acyclovir-resistant herpes simplex virus type 1, as well as influenza A and B viruses **(Berezin et al., 2019)**. The table I summarizes some antiviral molecules produced by actinobacteria.

Table I: Antiviral compounds produced by Actinobacteria, their producer species, target viruses, and corresponding references.

Molecule	**Producer (species)**	**Targeted virus**	**Reference**
Antimycin A1a	*Streptomyces sp.*	influenza A and B viruses	Katarzyna andJakubiec-Krzesniak, 2018
AhmpatinineiBu	*Streptomyces sp.*	HIV-1, Zika	Katarzyna andJakubiec-Krzesniak, 2018
Pentapeptide 4862F	*Streptomyces sp.*	Herpès simplex virus type 1 (HSV-1)	Katarzyna andJakubiec-Krzesniak, 2018
unspecifiedmolecule	*Kazakhstan'sActinomycetes*	Western equineencephalitis, Influenza	Berezin et al., 2019

2.8. Bioremediationactivity

Actinobacteria are key microorganisms in bioremediation processes due to their ability to produce a wide array of enzymes and secondary metabolites, which enables the degradation of various organic pollutants, including hydrocarbons, pesticides, and synthetic dyes **(Reti et al., 2024; Belabbas et al., 2025; Saez et al., 2025).** Their resilience to extreme environmental conditions, combined with their metabolic versatility, also enables them to remove a variety

of inorganic contaminants, thereby contributing effectively to ecosystem restoration **(Djinni andDjoudi, 2022;Behera and Das, 2023).** Moreover, actinobacteria are known to interact synergistically with other microorganisms, such as fungi, promoting the formation of microbial consortia that can enhance the efficiency of decontamination processes and strengthen sustainable remediation strategies **(Saez et al., 2025).**In addition, several genera of actinobacteria such as *Streptomyces*, *Rhodococcus*, *Nocardia*, and *Arthrobacter* have demonstrated a remarkable ability to biodegrade persistent organic compounds, including polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides, and industrial dyes.

This efficiency relies not only on their enzymatic arsenal but also on sophisticated cellular mechanisms such as biosurfactant production, pollutant adsorption by the extracellular polymeric substance (EPS) matrix, and the expression of genes involved in oxidative stress resistance **(Behera and Das, 2023).** Some actinobacterial strains, such as *Thermobifidacellulosilytica*, *T. halotolerans*, and *Streptomyces coelicolor*, have demonstrated effective lindane degradation in contaminated soils, particularly under optimized conditions. *T. cellulosilytica* achieved the highest removal rates. Additionally, tests on *Lactuca sativa* confirmed a reduction in soil toxicity following treatment. These results highlight the potential of these strains for pesticide bioremediation **(Usmani et al., 2021)**.

2.9. Other biological activities

Actinobacteria, especially *Streptomyces* species, are prolific producers of secondary metabolites with diverse biological activities. Indeed, their antiparasitic potential is exemplified by compounds such as ivermectin, derived from *Streptomyces avermitilis*, which is widely used in medicine, as well as by novel metabolites from marine actinobacteria showing efficacy against Leishmania (**Davies-Bolorunduro et al., 2021**). Moreover, insecticidal activities are well documented, with isolates like *Streptomyces griseoplanus, S. bacillaris,* and *S. albolongus* exhibiting broad-spectrum toxicity against major lepidopteran pests, highlighting their promise as biocontrol agents (**Jun Young Kim et al., 2022**). Furthermore, certain actinobacteria disrupt insect juvenile hormone activity, making them candidates for novel insect growth regulators (**Jong-Hoon Kim et al., 2024**). In addition, the immunomodulatory capacity of actinobacteria is attributed to metabolites from genera such as *Streptomyces* and *Rhodococcus,* which possess anti-inflammatory and immunosuppressive properties, offering prospects for managing autoimmune and inflammatory disorders (**Zhao et al., 2022;Duangupama et al., 2023**).Notably, probiotic actinobacteria, especially

Bifidobacterium longum, modulate inflammation and gut microbiota, supporting intestinal health and therapeutic interventions in gastrointestinal diseases (**Lin et al., 2023**). Their neuroprotective activity is also demonstrated by metabolites like agmatine for potential treatment of neurodegenerative diseases (**Zhao et al., 2022;Duangupama et al., 2023**). Finally, actinobacteria are significant producers of bioactive pigments, such as prodigiosin, which possess antimicrobial, antioxidant, and anticancer activities, and offer sustainable alternatives to synthetic dyes for industrial applications (**Celedón and Díaz, 2021;Díez et al., 2025)**

Materials and methods

This study was conducted at the Laboratory of Applied Microbiology (LMA), University A. Mira of Bejaia, between March 13 and June 19, 2025.

1. Material

1.1. Analytical material

The analytical material used in this study are listed in Annex 1.

1.2. Biological material

1.2.1. Actinobacteria strains

A total of ten actinobacteria strains (OCdS3, OZnS3, S22, S23, BZnS4, BZnS5, BPbS2, BCrS4, OPbS3 and BPbS6) were provided by Dr. SOUAGUI Samiha,belonging to the Laboratory of Applied Microbiology. These strains were originally isolated from soil and water samples collected in the Bejaia region.

1.2.2. Target microorganisms

twelve reference strains were used in this study, provided by the Laboratory of Applied Microbiology.

Table II: The tested target microorganisms.

Gram-positive bacteria	**Gram-negative bacteria**	**Moulds**
Bacillus subtilis ATCC6633	*Agrobacterium sp*	*Candidaalbicans ATCC10231*
Bacillus cereus	*E. coli ATCC 25922*	*Aspergillus niger*
Micrococcus luteus	*E. coli ST131*	*Penicillium sp.*
Staphylococcusaureus ATCC25923	*Salmonella entirica*	
	Vibriocholerae ATCC10231	

2. Methods

2.1. Subculturing of strains

The studied actinobacteria strains were subcultured on Starch Casein Agar (SCA) medium (see Annex II) and incubated at 30°C for 7 days.

2.2. Antimicrobial activities evaluation

2.2.1. Agar cylinders method

Bacterial and fungal cultures were prepared by incubating bacterial strains and *C. albicans* for 24 hours at 37°C and molds strains for 72 hours at 25°C on nutrient agar, EMB agar, or Chapman agar. From each culture, three to four colonies were aseptically collected using a sterile Pasteur pipette and transferred into tubes containing 5 mL of sterile physiological water. The cell suspensions were homogenized by vortexing and then adjusted to a final concentration of 10^7 colony-forming units (CFU)/mL. The standardized inocula were then uniformly spread onto the surface of Mueller-Hinton (MH) agar plates using a sterile swab to produce a consistent microbial lawn.

Actinobacteria strains were cultivated at 30°C during 7 days on SCA medium until well-developed mycelial growth was obtained. The evaluation of their antimicrobial activity was performed using the agar cylinder method. Agar cylinders of 6 mm in diameter were formed from the actinobacteria plates and placed at regular intervals on Mueller-Hinton plates previously inoculated with the target microorganisms. The plates were kept at 4°C for 2 hours to allow diffusion of the metabolites, then incubated at 37°C for 18 to 24 hours for bacteria and *C. albicans* and at 25°C/ 72h for molds. Antimicrobial activity was assessed by measuring the diameters of the inhibition zones formed around the cylinders, expressed in millimeters.

2.2.2. Agar well diffusion method

2.2.2.1. Culture of the strains and extraction of bioactive metabolites

Based on the most active strains following the results of the agar cylinder test, the active strains were cultured on 30 modified Mincer agar medium (Annex II) plates each. The plates were then incubated at 30 °C for 7 days. After cultivation, the extraction of active compounds was performed by maceration (solid-liquid extraction). For this purpose, the cultured strains and agar media were cut into small pieces and transferred into 2-liter Erlenmeyer flasks containing 900 mL of ethyl acetate. The mixture was macerated overnight at room temperature and protected from light. The crude extract was obtained by filtration using Whatman filter paper (number 1) to separate the solvent from the agar and biomass, then concentrated to dryness using a rotary evaporatorwith a water bath set at 40 °C and a medium rotation speed. The resulting dry extract was subsequently resuspended in methanol and the mass concentration was determined. The extract was stored at 4 °C until further use.

Standardized inocula and the target microorganisms were spread on Petri dishes, and wells of 6 mm diameter were formed using a sterile Pasteur pipette. Each well was then filled with 50 µL of the crude extracts. Inhibition zones were measured after 24 hours of incubation at 37 °C for bacteria and *C. albicans* and after 72h of incubation at 25°C for moulds. Methanol was used as a negative control.

2.2.3. Determination of the Minimum Inhibitory Concentration (MIC)

The analysis was performed using the broth microdilution method as defined in the Clinical and Laboratory Standards Institute (CLSI) guidelines (Figure 3).

The target pathogens (*S. aureus*, *V. cholerae*, and *C. albicans)* selected for their sensitivity to the tested crude extracts,were cultured in 10 mL of Tryptic Soy Broth (TSB) supplemented with 2.5% glucose (TSBG) and incubated at 37 °C for 24 hours.

A volume of 50 µL of TSBG was distributed into the 96 wells of a microplate. Subsequently, 50 µL of one of the tested crude extracts (SOZnS3, M22, or MBCrS4) was added to the 50 µL of TSBG in the first well of each row. Serial dilutions (1/2) were then performed by transferring 50 µL from the first well to the adjacent one, and this process was repeated along the entire row.Thisserial dilutions (1/2) procedure allowed the generation of decreasing concentrations of the tested extracts. To verify the sterility of the medium, two rows were used as negative controls: one containing only TSBG and the other containing methanol along with the bacterial cultures. The positive control consisted of wells containing the bacterial suspensions mixed with TSBG. Three replicates were performed for each test. The absorbance of each well was measured using a BIOTEK microplate reader at a wavelength of 630 nm.

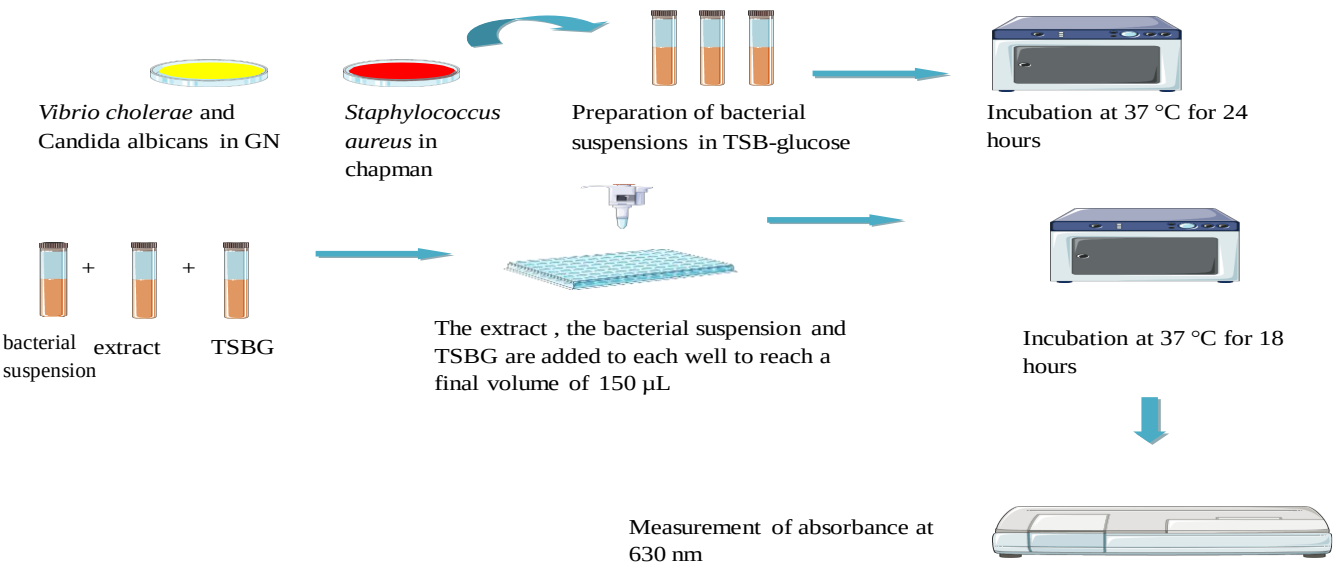

Figure 3: Schematic representation of the experimental protocol for the determination of the Minimum Inhibitory Concentration (MIC) using the microdilution method.

2.3. Enzymatic activities evaluation

In order to evaluate the enzymatic activities of the studied isolates, nine different enzymes were investigated using solid media, each containing the specific substrate for the enzyme being tested (Annex II).

2.3.1. Cellulase activity

Strains were cultured on cellulose agar medium following the method described by **Carder (1986)**(Annex II). The plates were incubated at 30°C for 7 days. After incubation, 1% aqueous Congo red solution was applied over the colony surfaces. After 20 minutes of contact, the plates were flooded with 1 M NaCl solution and kept at 5 °C overnight. The presence of cellulase activity was revealed by the formation of a clear halo surrounding the colonies.

2.3.2. Protease activity

Protease production was assessed on skim milk agar (Annex II). After sterilization the nutrient agar medium at 120 °C for 20 minutes, 10% of « Candia » skim milk was added aseptically. The inoculated plates were incubated at 30 °C for 7 days. The formation of a clear halo around the discs indicated proteolytic activity, as described by **Ningthoujam et al. Munch (2009)**.

2.3.3. Amylase activity

Amylolytic activity was assessed on Gausse agar medium (Annex II). After the incubation period at 30 °C for 7 days, a previously prepared Lugol's iodine solution (Annex

II) was applied to the surface of the plates and allowed to react for a few minutes. The excess solution was discarded, and the plates were washed with distilled water. The formation of a clear halo around the discs indicated the presence of amylase activity **(Vinoth Raj et al., 2009)**.

2.3.4. L-Tyrosinase activity

To evaluate L-tyrosinase activity, the strains were inoculated onto L-tyrosine agar medium (see Annex II) and incubated at 30°C for 7 days. The appearance of brown to black pigmentation around the colonies indicated positive tyrosinase activity (**Gourdonet al.,1974**).

2.3.5. Gelatinase activity

Gelatinase activity was evaluated on gelatin agar medium (see Annex II). The strains were inoculated and incubated at 30 °C for 7 days. After incubation, gelatin hydrolysis was indicated by the appearance of a clear or liquefied zone around the colonies, resulting from enzymatic degradation of the gelatin (**Williams et Cross, 1971)**.

2.3.6. Pectinase activity

Pectinase activity was assessed on pectin agar medium (see Annex II). The strains were inoculated and incubated at 30 °C for 7 days. The pectinolytic activity was evident from the formation of a clear halo surrounding the colonies, indicating enzymatic degradation of pectin in the medium **(Brühlmann et al., 1994).**

2.3.7. L-Asparaginase activity

L-asparaginase activity was evaluated using L-asparagine agar medium (see Annex II). The strains were inoculated and incubated at 30 °C for 7 days. The presence of L-asparaginase activity was indicated by the formation of a clear or altered zone around the colonies, resulting from the enzymatic breakdown of L-asparagine**(Imada et al., 1973).**

2.4. Antioxidant Activities

2.4.1. Scavenging activity against DPPH free radical

A volume of 100 µL of the extract was added to 3 mL of DPPH solution. The mixture was vortexed and incubated in the dark at 37 °C for 20 minutes. The absorbance was then measured at 517 nm. A decrease in absorbance indicates the scavenging of DPPH free radicals. (**Lopes-Lutz et al. 2008)**

$$\mathbf{PI\% = (1 - \frac{A sample}{A control}) \times 100}$$

2.4.2. Determination of total polyphenols

100 µL of extract was vortexed at room temperature with 2.5 mL of Folin-Ciocalteu regent. The mixture was vortexed, followed by the addition of 2 mL of sodium carbonate (7.5%), and vortexed again for 2 minutes. After 40 minutes of incubation at 45 °C, the absorbance was measured at 765 nm as described by **Junaid *et al.* (2013)**. The phenolic compounds content is expressed in mg gallic acid equivalent per 100 g dry matter (mg GAE/100 g DM)

2.5. Antibiofilm Activity

The evaluation of antibiofilm activity was conducted through a serie of steps following a standardized protocol using flat-bottom 96-well microplates. This assay is based on the ability of microorganisms to form biofilms at the solid-liquid interface (**Driche et al., 2017**). The microbial strains selected for this assay (*S. aureus*, *V. cholerae*, and *C. albicans*) were chosen based on their high sensitivity observed in previous antibacterial tests. Each target strain was cultured in 10 mL of Tryptic Soy Broth (TSB) enriched with 2.5% glucose (TSBG) and incubated at 37 °C for 24 hours to promote biofilm formation.

The effect of crude extracts (SOZnS3, M22, and MBCrS4) derived from the three tested strains on biofilm formation was evaluated**.**Bacterial suspensions were previously standardized to 10^8 CFU/mL in TSBG medium, with an optical density adjusted to 0.1 at 630 nm and incubated for 24 hours. A volume final of 200 µL of each suspension was dispensed into the wells of the microplate, except for the wells designated as the negative control.

Three experimental conditions were established:

- **Negative controls** : TSBG + methanol (without bacterial inoculum), TSBG + bacterial suspension +methanol
- **Positive control** : TSBG + bacterial suspension (without extract),
- **Test wells**: TSBG + bacterial suspension + crude extract (SOZnS3, M22, or MBCrS4).

The microplates were then incubated at 37°C for 24 hours to allow biofilm formation.

After 24 hours of incubation at 37°C, the contents of each well were carefully removed. The wells were then washed three times with 350 µL of sterile distilled water to eliminate non-adherent planktonic bacteria. Subsequently, the microplates were placed in an oven at 60°C for 45 minutes to fix the formed biofilm. Biofilm detection was performed by adding 200 µL of 0.2% crystal violet solution (prepared in sterile distilled water) to each well, followed by

incubation at room temperature for 15 minutes. To remove excess, non-bound dye, the plates were washed three times with sterile distilled water. Finally, the fixed biofilm-bound dye was solubilized using 150 µL of 95% ethanol. The absorbance of each well was measured using a BIOTEK microplate reader at a wavelength of 595 nm (Figure 4).

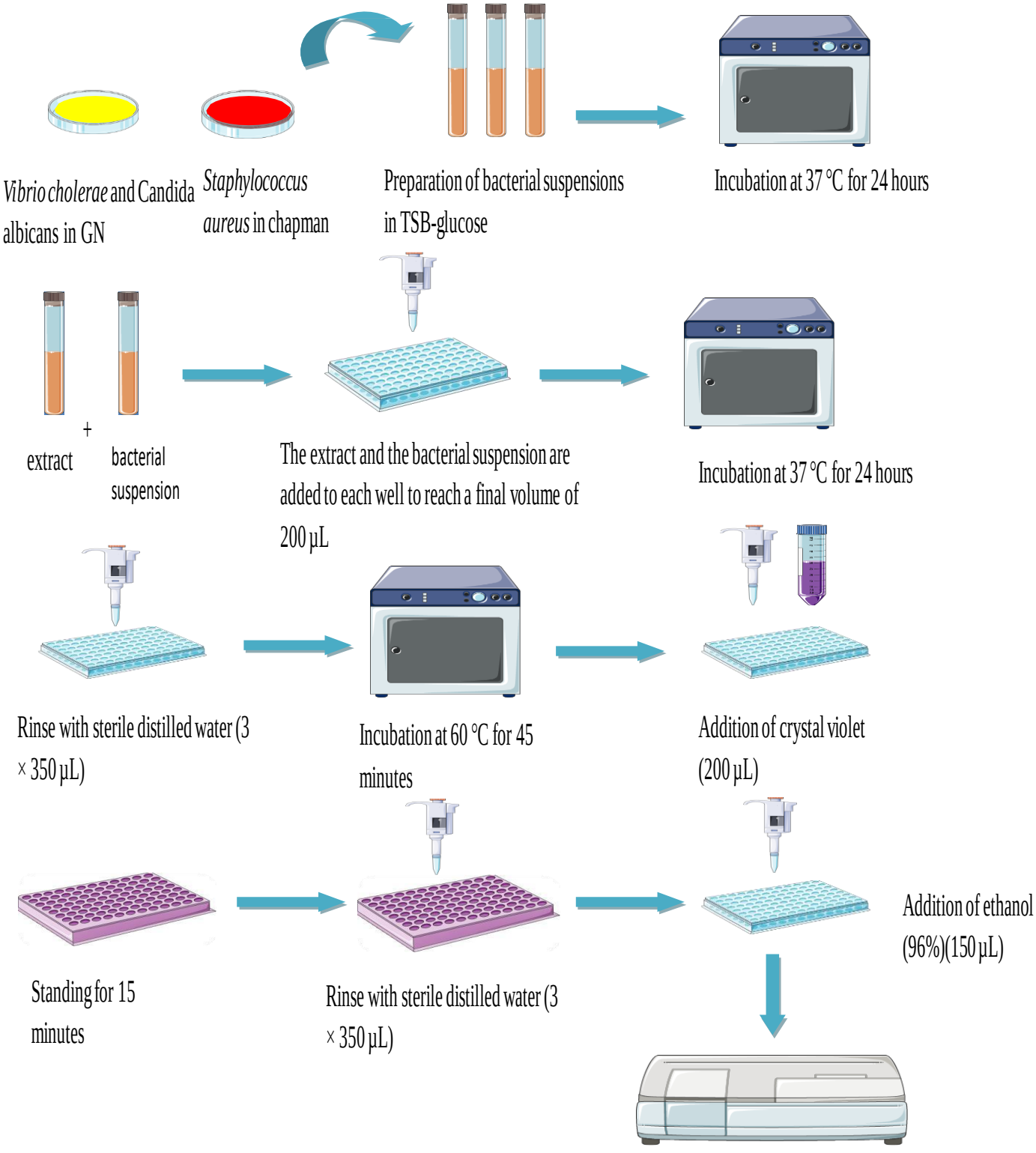

Figure 4: Schematic representation of the experimental protocol for the determination of the antibiofilm activity.

Results and discussion

This chapter presents the results and discussion of the different parts of this study, which include the morphological features characterization of the strains, followed by the evaluation of their biological activities including, antagonistic activity determination of both the strains and the ethyl acetate crude exracts, antibiofilm potential along with the evaluation of the minimum inhibitory concentrations as well as the antioxidant and enzymatic activities.

1.Analysis of the morphological characteristics of actinobacteria strains

1.1.Macromorphological features

The analysis of the morphological characteristics of the eight strains was conducted on their respective culture media. Strains OCdS3, OZnS3, BZnS5, BZnS4, BPbS2, and S23 were cultivated on SCA medium, while strains BCrS4 and S22 were grown on Mincer medium for7 days at 30°C. The obtained results are presented in the table III. The figure 5 illustrates the macromorphological features of two strains cultured on Mincer medium.

Table III: Macromorphological characteristics of eight strains.

CharactersStrains	**Medium**	**Growth**	**Color of aerialmycelium**	**Production of diffusible Pigments**
OCdS3	SCA	++	white	-
OZnS3	SCA	++	darkgrey	+
BZnS5	SCA	++	white	-
BZnS4	SCA	++	white	-
BPbS2	SCA	++	white	-
S23	SCA	++	white	-
BCrS4	Mincer	+++	white	-
S22	Mincer	+++	white	-

Key : ++average growth , +++ important growth , - no pigment production .

BCrS4 S22

Figure 5: Cultural characteristics of studied actinobacteria.

1.2.Micromorphological characters

After 7 days of incubation at 30°C, some colonies were directly examined on Petri dishes using a light microscope equipped with a 40x objective lens. The figure6 showed the micromorphological structures of three selected strains among the eight studied. The aerial mycelium observed is characterized by branched, straight filaments bearing chains of non-motile spores.

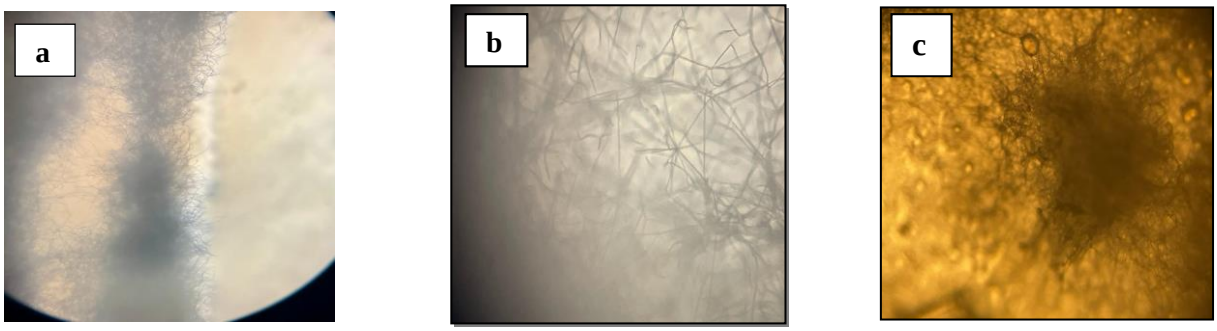

Figure 6: Micromorphological observation of the studied strains under optical microscope with Gx40. **a)**: Strain BZnS4; **b)** Strain S22; **c)** Strain BZnS5.

2. Antimicrobial activity evaluation of the studied strains

The antimicrobial activity of the tested strains (OCdS3, OZnS3, BZnS5, BZnS4, BPbS2, Bpbs6, OPbS3, S23, BCrS4, and S22) was evaluated against various pathogenic microorganisms. These included four Gram-positive bacteria (*B. subtilis, B. cereus, M. luteus, and S. aureus*), five Gram-negative bacteria (*Agrobacterium* sp., *E. coli ATCC25922, E. coli* ST131, *S. enterica,* and *V. cholerae*), and three fungal species (*C. albicans, A. niger, and Penicillium* sp.). The results were obtained after incubation at 37 °C for 24 hours for bacteria and *C. albicans* and at 25 °C for 48-72h hours for molds.

The obtained results **(Figure 7)** demonstrated a notable variability in the antimicrobial activity among the tested strains. The strains OZnS3, OCdS3, and S23 showed moderate antimicrobial activity against *E. coli*, with inhibition zone diameters measured at 14 mm for OZnS3 and S23, and 16 mm for OCdS3. The latter showed a strong inhibitory activity against *A. niger* (18 mm), *K. pneumoniae* (16 mm), and *S. enterica* (23 mm). Moreover, the isolates BPbS2, BZnS4, BZnS5, and BCrS4 showed marked antimicrobial activity. As for the BZnS4 isolate, which exhibited a particularly strong effect, with inhibition zones of 49 mm against *S. aureus* and 42 mm against *B. subtilis*, indicating a high antagonism against the target Gram-positive bacteria. BZnS5 showed considerable activity against *C. albicans* (35 mm), while

BPbS2 and BZnS4displayed notable inhibition against *E. coli* 39 mm and 34 mm, respectively. Regarding *A. niger*, inhibition zones of 26 mm and 24 mm were recorded for BZnS4 and BCrS4, respectively. Additionally, BPbS2 showed strong activity against *Agrobacterium sp.*, with an inhibition zone of 29 mm. These findings suggest that some strains, particularly BZnS4, BZnS5, BPbS2 and BCrS4 are able to produce secondary metabolites with high antimicrobial potential. They display a broad spectrum of activity against Gram-positive and Gram-negative bacteria, as well as pathogenic fungi. Conversely, other strains exhibited more limited or no antimicrobial effects.Our study revealed remarkable antimicrobial activity in certain strains, particularly BZnS4 and BZnS5, which exhibited inhibition zones of up to 49 mm against *S. aureus* and 35 mm against *C. albicans*, clearly outperforming the results reported by **Janatiningrum et al. (2024),**where only 50% of the isolates showed activity with lower indicesIn comparison, although the COL22 isolate from the study by **Meliani et al. (2022)** demonstrated significant antifungal activity against *A. flavus* and *F. oxysporum* (~23 mm), its spectrum of action remains more limited than that observed in our isolates.The isolates from these studies are of rhizospheric origin.

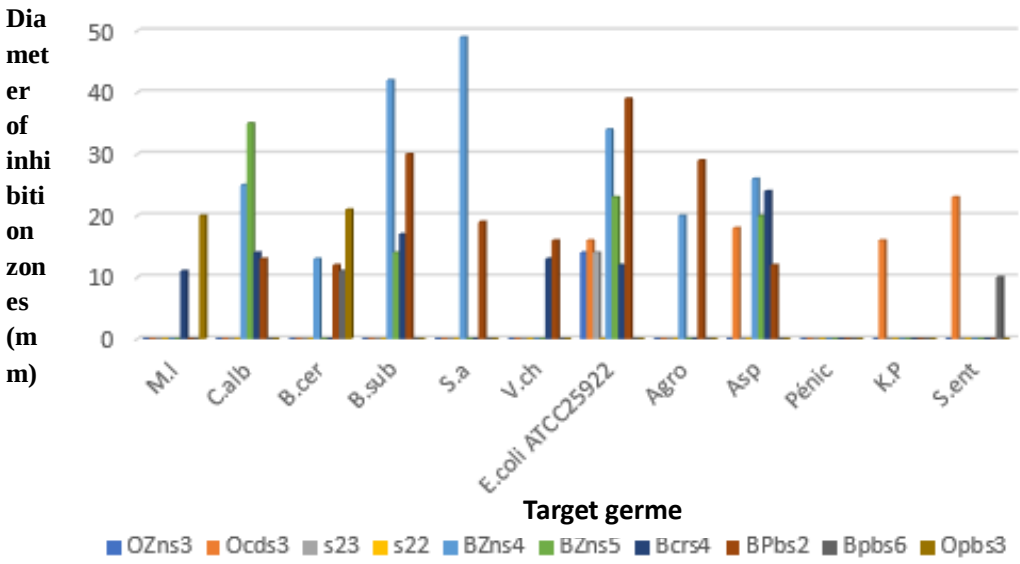

Figure 7: Histogram ofantimicrobial activity of actinobacterial studied strains.

We selected four strains for further investigation: BCrS4 and BZnS5, due to their strong ability to inhibit the tested pathogenic microorganisms, as well as OZnS3 and S22, which, although they did not show significant inhibitory activity (except against *E. coli ATCC 25922* for OZnS3). These isolates were retained to evaluate their effectiveness under different experimental conditions.

2.1.Production and extraction of active metabolites

The extraction of bioactive compounds produced by the strains S22, BZnS5, OZnS3 and BCrS4 resulted in the obtention of fiveethyl acetate crude extracts, including MBCrS4 and MS22 (obtained from cultures on Mincer medium) with concentrations of 53 mg/ml and 46 mg/ml, respectively, as well as three other extracts obtained from cultures of the strains on SCA medium. These are SBCrS4, SOZnS3, and SBZnS5, with concentrations of 13.5 mg/ml, 32 mg/ml, and 25 mg/ml, respectively.Each extract was recovered in an appropriate volume of methanol. Due to the limited amount of ethyl acetate and time, it was not possible to extract active compounds from the other strains (BPbS2, BPbS6, S23, OCdS3, OPbS3, and BZnS4). The obtained crude extracts are shown in Figure 8.

Figure 8: Ethyl acetate crude extracts of the three strains (MBCrS4, SBCrS4, SOZnS3, and MS22) from left to right.

2.2. Agar well diffusion assay

The antimicrobial assays of actinobacterial extracts showed significant variability in activity, influenced by both the strain, the extract concentration and the target pathogen. Among the tested extracts, MBCrS4, with a concentration of 53 mg/ml,exhibited the broadest and most potent antimicrobial spectrum, with large inhibition zones observed against a wide range of the target-tested strains. It exhibited strong antifungal effects against *A. niger* (30 mm), *Penicillium sp.* (28 mm) and *C. albicans* (24 mm), as well as antibacterial activity against both Gram-positive and Gram-negative bacteria, including the multidrug-resistant strain *E. coli* ST131 (15 mm) as illustrated in **Figure 9**. The SBCrS4 crude extract**,** with a concentration of 13.5mg/ml,also demonstrated significant antimicrobial activity, particularly against *C. albicans* (30 mm), *Agrobacterium* sp. (30 mm), *B. subtilis* (29 mm), and *M. luteus* (31 mm) as illustrated in **Figure 9**. However, it showed no activity against *E. coli*and *E. coli* ST131. These results demonstrate the potential of strain BCrS4 to produce broad-spectrum antibiotics when cultivated on two distinct culture media (modified Mincer or SCA). Other

extracts, such as MS22 and SBZnS5**,**with a concentration of 46 and 25 mg/ml respectively,displayed a moderate activity against several pathogens, while SOZnS3, with a concentration of 32 mg/ml exhibited less effects that are limited. Overall, the findings indicate that several extracts possess notable antimicrobial potential, with effectiveness against both Gram-positive and Gram-negative bacteria, as well as fungal pathogens. The activity observed against resistant strains such as *E. coli* ST131 underscores the promising therapeutic value of these actinobacteria metabolites. In our study, MBCrS4 extract at 53 mg/ml exhibited strong antimicrobial activity, with inhibition zones reaching up to 30 mm against *A. niger*, 28 mm against *Penicillium* sp., 24 mm against *C. albicans*, and 15 mm against *E. coli* ST131. These results are higher than those reported by **Dhaini et al. (2025)**, who observed inhibition zones of up to 25 mm against *C. albicans*, *E. coli*, and *S. aureus* from marine strains. These comparisons highlight the strong potential of BCrS4 strain, which demonstrate effective activity against a broad spectrum of pathogens, including multidrug-resistant strains such as *E. coli* ST131.

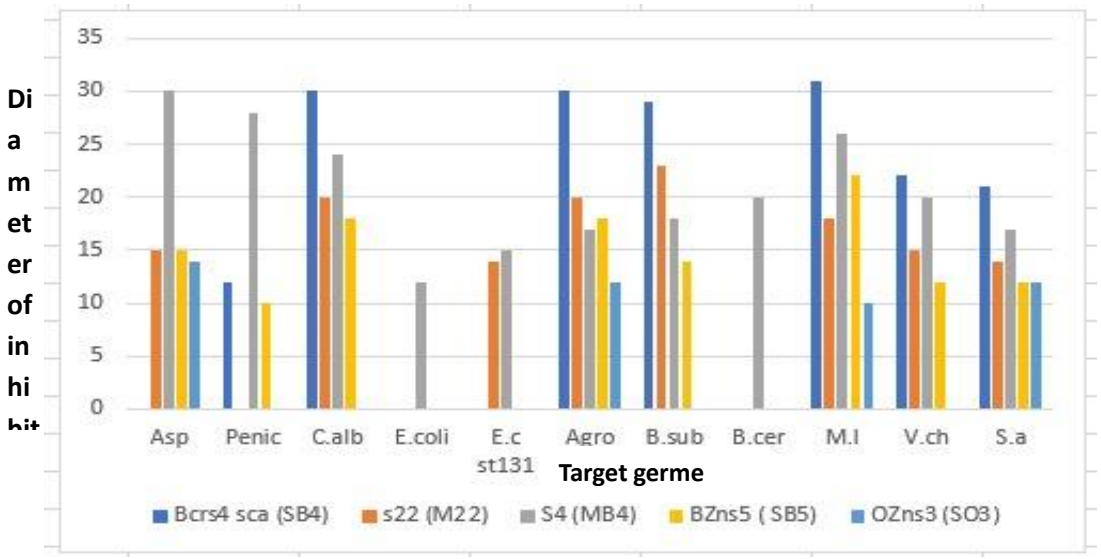

Figure 9: Histogram showing the antimicrobial activity of ethyl acetate crude extracts of five actinobacteria strains against various target microorganisms, evaluated by the well diffusion method.

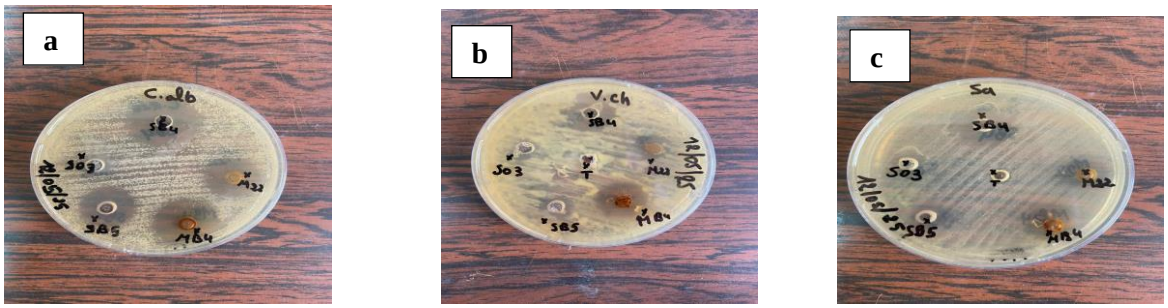

Figure 10: Antimicrobial activity of the five studied Crude Extracts against ***(a)*** :*C.albicans,* ***(b)*** *: V. cholerae and (c) : S. aureus.*

2.3. Determination of the Minimum Inhibitory Concentration (MIC)

The table IV presents the minimum inhibitory concentrations (MIC) of the three studiedethyl acetate crude extracts of the strains BCrS4 and S22, cultivated respectively on Mincer medium, as well as from the strain OZnS3 cultivated on SCA medium, against three pathogenic microorganisms (*S. aureus, V. cholerae and C. albicans).* The remaining extracts were not tested due to the limited amount available.

Table IV: Minimum inhibitory concentrations (MIC) of ethyl acetate crude extracts MBCrS4, MS22, and SOZnS3 against selected pathogenic microorganisms.

Extracts/germ	***MIC value (mg.ml^{-1})***		
	S.aureus	***V. cholerae***	***C.albicans***
MBCRS4	< 0,2		
MS22	< 0,18		
SOZnS3	< 0,125		ND

ND :not determined.

The crude extracts of BCrS4 (MBCrS4)**,** S22 **(**MS22), and of OZnS3 **(**SOZnS3) exhibited verystrong broad-spectrum antimicrobial activity against the tested bacteria: *S. aureus* and *V. cholerae*. The SOZNS3 extract demonstrated the highest efficacy, with a MIC value < 0.125 mg/ml, followed by S22**(**MIC < 0.18 mg/ml) andBCrS4 (MIC < 0.2 mg/ml), as illustrated in the Table III. Additionally, MS22 and MBCrS4 demonstratedinhibitory activity against *C. albicans*, while SOZnS3 was not evaluated for antifungal activity due to an insufficient amount of extract. These findings suggest that the BCrS4 and S22strains cultivated on modified Mincermedium**,** as well as OZnS3on SCA medium, are promising producers of bioactive metabolites with strong antimicrobial potential, with OZnS3 standing out for its slightly superior efficacy.In our study, the crude extracts of SOZnS3, MS22, and MBCrS4

exhibited very low Minimum Inhibitory Concentrations (MICs) against *S. aureus* and *V. cholerae*, with respective values of <0.125 mg/ml, <0.18 mg/ml, and <0.2 mg/ml, highlighting a strong antimicrobial potential. In comparison, the study by **Cheruiyot Koech et al. (2025)**reported MICs against methicillin-resistant *S. aureus* (MRSA) ranging from 625 µg/ml to less than 9.9 µg/ml, depending on the actinobacterial strain tested. Thus, our results demonstrate comparable or even superior efficacy, particularly for the SOZnS3 extract, confirming the promising potential of actinobacteria metabolites in combating resistant bacterial pathogens.Also, several studies have demonstrated that some actinobacteria strains exhibit significant minimum inhibitory concentrations (MICs) against various pathogenic microorganisms, underscoring their potential as promising sources of antimicrobial compounds **(Djinni et al., 2023 ;Dhainiet al., 2025).**

3. Enzymatic activities of the studied strains

Actinobacteria produce a variety of extracellular enzymes of industrial importance, including amylases, cellulases, lipases, and proteases (**Sahu et al, 2024).**These enzymes facilitate the degradation of complex substrates and are widely employed in various sectors such as food processing, textiles, paper manufacturing, biofuels, detergents, pharmaceuticals, and waste treatment. Investigating their enzymatic activity, particularly in strains isolated from specific environments such as arid soils, enables the identification of enzymes adapted to extreme conditions (Extremozymes) and supports their biotechnological exploitation for sustainable industrial applications**(Smati et al, 2025).** In this study, six enzymatic activities were investigated on solid media for eight actinobacteria strains (S22, S23, OCdS3, OZnS3, BZnS4, BZnS5, BCrS4 and BPbS2).

3.1. Screening for cellulolytic activity

Actinobacteria play a key role in the degradation of carboxymethylcellulose (CMC), a cellulose-derived compound commonly found in industrial waste. Their ability to produce cellulases enables the conversion of these organic-rich wastes into less polluting substances, thereby contributing to the reduction of industrial pollution. This enzymatic activity is particularly relevant in the agri-food sector, where it facilitates the treatment of effluents and the valorization of residues into useful products such as fertilizers **(Fitri et al, 2023)**.

To evaluate cellulases production, the studied strains were cultivated on carboxymethylcellulose (CMC) agar and incubated for 7 days at 30 °C. After incubation, the plates were flooded with a 0.1% Congo red solution. Clear zones **(Figure11)** appeared around

all colonies, indicating the ability of the eight actinobacteria isolates to hydrolyze cellulose. The enzymatic index (EI) of each strain is presented in **table V**. In our study, all eight tested strains exhibited positive cellulolytic activity on CMC medium, with enzymatic indices (EI) ranging from 2.4 to 6.2. The strain OZnS3 showed the highest EI value (6.2), followed by BPbS2 and S22 (EI = 4), while BCrS4 exhibited the lowest EI (2.4). These results are comparable to those reported by **Bhakyashree and Kannabiran (2020),** in which *Streptomyces* strains isolated from halophilic soils exhibited cellulase enzymatic indices ranging from 3.4 to 7.0, depending on growth conditions and strain characteristics. For example, *Streptomyces* sp. S1 had an EI of 5.1, close to that of our strain OZnS3, whereas *Streptomyces* sp. S4 showed an EI of 3.7, similar to the values observed for BZnS5, S23, or OCdS3. In addition, according to **Gorrab et al. (2024),** some extremophilic strains such as *Streptomyces* DSK59 or *S. drozdowiczii* M7a are known to produce thermostable cellulases with strong activity on lignocellulosic substrates.

Figure 11: Cellulolytic activity of the studied strains on CMC medium

3.2. Screening for amylolytic activity

Among the numerous enzymes produced by actinobacteria, amylase holds a significant position due to its wide range of industrial applications. This enzyme is extensively utilized in various sectors, including the food, textile, and paper industries. Through its ability to hydrolyze starch, amylase plays a crucial role in facilitating key processes in these fields, thereby contributing to improved manufacturing procedures and the valorization of raw materials **(Smati et al., 2025).**As part of the assessment of amylase production by actinobacteria, the nine strains were cultivated on Gause's starch medium and incubated for 7 days at 30 °C. After incubation, the plates were flooded with Lugol's iodine solution. The appearance of clear zones **(Figure 12)** around all colonies indicated the ability of all isolates to hydrolyze starch. The enzymatic indexdefined as the ratio of the diameter of the clear zone to that of the colonywas calculated as follows: 5 for S10, 1.6 for OZnS3, 1.1 for

OCdS3, 1.4 for S23, 2 for S22, 2.57 for BZnS5, 3 for BPbS2, and 2 for BCrS4, as shown in **Table V .** Compared to our study, in which all nine tested strains exhibited amylolytic activity on Gause's starch medium with enzymatic index (EI) values ranging from 1.1 to 5 the highest being recorded for strain S10 (EI = 5) the study by **Dos Santos et al (2024)** reported an average EI of 2.99 ,with strain MJ29 reaching a maximum of 4.36 Although both studies highlight notable amylase production, our strain S10 surpassed the most efficient strain in the other study.Other studies have also reported significant amylolytic activity insomestrains**(Al-Agamy et al., 2021;Sahu et al., 2024)**.

Figure 12: Amylolytic activityof four actinobacteria strains on Gause's medium

3.3. Screening for L-Tyrosinase activity

Tyrosinase production was evidenced by the formation of a brown halo around the colonies of our isolates, primarily attributed to melanin synthesis.This observation is consistent with findings reported in the scientific literature regarding tyrosinase production by various *Streptomyces* species **(Georgousakiet al., 2020; Rudrappa et al., 2022; Malekpour et al., 2024).**

3.4.Screening for pectinolytic activity

Pectinolytic activity was assessed using MP7 medium, where pectin served as the sole carbon source. Following incubation at 30 °C for 7 days, no clear zones were observed around the colonies of the tested strains, However, previous studies have reported that some actinobacteria strains can hydrolyze pectin, highlighting the variability of enzymatic potential among different isolates **(Dos Santos et al., 2024; Alshehri et al., 2024).**

3.5.Screening for L-asparaginase activity

L-Asparaginase activity was investigated by cultivating the isolates on an agar medium containing L-asparagine as the sole substrate, followed by incubation at 30 °C for 7 days. A positive enzymatic activity is indicated by a color change of the medium to pink around the bacterial colonies, due to the release of ammonia from the hydrolysis of L-asparagine **(Shukla and Mandal, 2013**).However, no such color change was observed for the

eight tested isolates, suggesting the absence of detectable L-Asparaginase activity under the experimental conditions.It is nevertheless important to note that several previous studies have highlighted the ability of actinobacteria strains to produce L-asparaginase as well as L-tyrosinase, which is recognized for its therapeutic potential, particularly in the treatment of specific cancers **(Morales-Gonzalez et al, 2018; El-Naggar et al, 2024).**

3.6.Screening for gelatinase activity

The studied actinobacterial strains were cultivated on gelatin-enriched agar medium and incubated at 30 °C for 7 days. The detection of gelatinolytic activity is based on the appearance of clear zones around the colonies, indicating the enzymatic hydrolysis of gelatin (**Kumar et al., 2012**). However, no such zones were observed for the tested strains, suggesting an absence of detectable gelatinase activity under the experimental conditions. Nevertheless, it is worth noting that other studies have reported the existence of actinobacteria strains with the ability to degrade gelatin **(Souagui et al., 2019; Souagui et al., 2023; Smati et al., 2025)**.

3.7. Screening for protease activity

Proteolytic activity reflects the ability of some microorganisms to degrade proteins into peptides or amino acids through the action of specific enzymes known as proteases. These enzymes play a crucial role in various fundamental biological processes, such as nutrient assimilation, cellular differentiation, and microbial competition. In the present study, the isolates BCrS4, S10, S22, and S23 were cultured on nutrient agar medium supplemented with skimmed milk and incubated at 30 °C for 7 days. The appearance of translucent halos around the colonies confirmed proteolytic activity, indicating enzymatic degradation of milk proteins illustrated in figure 13. The enzymatic index was 3.4 for BCrS4, 1.6 for S10, 1.5 for S22, and 2.14 for S23, as showed in Table IV, highlighting the significant proteolytic potential of these strains under simple culture conditions. These results are consistent with those reported by **Amfar et al. (2021),** in which the ATIS61 isolate exhibited a maximum protease activity of 0.115 U/mL on the eighth day of incubation at 30 °C, confirming that some strains achieve optimal enzymatic activity under similar conditions. Furthermore, the study (**Majithiya et al.,2024)** on *Nocardiopsisdassonvillei* VCS-4, although focused on industrial optimization, also reported high protease production after 7 days of incubation at pH 8, which aligns with the conditions used in our experiment. These comparisons underscore the ability of our actinobacterial strains to efficiently produce proteolytic enzymes without the need for complex industrial setups.

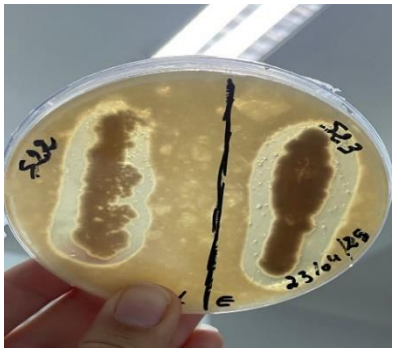

Figure 13: Proteolytic activity of strains S10, BCrS4, S22 and S23.

Table V: Enzymatic index recorded for proteolytic, amylolytic, and cellulolytic activities of different studied Strains.

Strains / activities	**S10**	**BCrS4**	**OZnS3**	**OCdS3**	**S23**	**S22**	**BZnS4**	**BZnS5**	**BPbS2**
Protease	1.6	3.4	/	/	2.14	1.5	/	/	/
Amylolytic	5	2	1.6	1.1	1.4	2	ND	2.57	3
Cellulolytic	/	2.4	6.2	3	3	4	3.6	3	4

ND: not detected, / : not do it

Enzymatic activities such as L-asparaginase and tyrosinase are of particular interest due to their therapeutic potential, especially in cancer treatment. Their exploitation could therefore contribute to the development of innovative biopharmaceutical agents for medical applications.

4. Antioxidant activities

4.1. Determination of DPPH

The table VI presents the average DPPH radical inhibition (percentage) values for different extracts, with strains BCrS4 and S22 inoculated on Mincer medium and strains OZnS3 and BCrS4 inoculated on SCA medium.

Table VI: The mean inhibition values of DPPH radical by different extracts, standard (± SD)

Extracts	% DPPH Inhibition
SOZnS3	87,63±1,11
MS22	89,63±0,43
SBCrS4 (on SCA)	86,32±0,82
MBCrS4 (on Mincer)	81,13±14,21

The SOZnS3, MS22, SBCrS4, and MBCrS4 extracts demonstrated notable antioxidant activity, as assessed by the DPPH assay, with respective inhibition percentages of 87.63%, 89.63%, 86.32%, and 81.13%. MS22 extract exhibited the highest activity (89.63%) with a low standard deviation (±0.44), indicating excellent result stability. Similarly, as shown in Table VI (Annex III), SOZnS3 (87.63% ±1.12) and SBCrS4 (86.32% ±0.82) extracts showed high and consistent antioxidant performance. In contrast, although MBCrS4 extract displayed a relatively high inhibition level (81.13%), its higher variability (±14.21) suggests lower reproducibility. These findings highlight the strong antioxidant potential of these extracts, particularly MS22 and SOZnS3, which emerge as promising candidates for applications requiring stable and efficient antioxidant properties.In a study reporting *Microbispora*sp., a marine actinobacterium, exhibited significant antioxidant activity, with DPPH scavenging and total antioxidant capacities reaching 75.2% and 78.5%, respectively, at a concentration of 100 µg/mL **(George et al., 2022).**In comparison, the antioxidant activities observed in MS22 and SOZnS3 extracts (89.63% and 87.63%) are noticeably higher, indicating a stronger free radical scavenging ability. Moreover, actinomycetes isolated from termite nests demonstrated moderate antioxidant activity, with IC50 values ranging from 76.64 to 126.22 µg/mL (**Fathoni et al., 2024)**. While these findings confirm the antioxidant potential of actinobacteria from unique ecological niches, the inhibition percentages recorded in our study particularly for MS22 and OZnS3 (89.63% and 87.63%) reflect higher efficacy, especially in light of the low variability and high reproducibility of our results. However, evaluation of IC_{50} values against the DPPH radical would enable a
more comprehensive discussion of our results. Indeed, due to limitations in the volumes of our crude extracts, this study could not be completed.

4.2. Determination of total phenolic content (TPC)

The table VIIbelow presents the mean total phenolic content (TPC) expressed as mg GAE/ml ± SD, of different actinobacteria extracts: MBCrS4 and MS22(on Mincer medium), and SOZnS3 and SBCrS4(on SCA medium).

Table VII: Total phenolic content (TPC) of actinobacterial extracts expressed as mg GAE/ml ± SD.

Extracts	**Mean TPC (mg GAE/ml) ± SD**
MS22	20,02±0,001
SOZnS3	17,49±0,03
SBCrS4 (on SCA)	10,12±0,001
MBCrS4 (on Mincer)	20,03±0,001

Among the tested extracts, MBCrS4 and MS22 exhibited the highest polyphenol contents, nearing 20 mg GAE/ml, indicating a significant abundance of phenolic compounds with antioxidant properties. SOZnS3 followed with a content of 17.49 mg GAE/ml as illustrated in **Table VII** above also reflecting a substantial concentration of these molecules. However, SBCrS4display more moderate levels, around 10.13 mg GAE/ml, indicating that the Mincer medium is more suitable for phenolic compound production by the BCrS4 strain. According to **Almuhayawi et al., (2021),** the phenolic compound contents were higher than those obtained in our study, reaching up to 59.8 mg GAE/ml. In comparison, the extracts MBCrS4 and MS22 showed values around 20 mg GAE/mL, which nonetheless remains significant. This indicates that our strains are less rich in phenolic compounds, yet still exhibit a good antioxidant potential. Furthermore, in the study conducted by **Rammali et al., (2022),** the results were expressed in mg GAE/mg of extract, with a reported maximum value of 0.64 mg GAE/mg. The authors also reported strong antioxidant activity, reaching 35.79% measured by ABTS method. Our results are consistent with these findings, as the extracts with the highest phenolic content (MBCrS4 and MS22) also exhibited the strongest antioxidant activity. This supports the idea

that *Streptomyces* strains like those used in both cited studies have a good capacity to produce phenolic compounds, particularly when cultivated on Mincer medium **(Helmi,2025)**.The obtained results show that the least concentrated SOZnS3 extract (32 mg/ml) among those tested, exerts strong antioxidant activity against the DPPH radical (87% inhibition) equivalent to the MBCrS4 (53 mg/ml) and MS22 (46 mg/ml) extracts. The SOZnS3 extract exhibited also the lowest phenolic content (17 mgAGE/ml) compared with the other two extracts (with 20 mgAGE/ml). A correlation between phenolic content and free radical scavenging activity against DPPH, as well as the determination of IC50 values, would be essential to draw reliable conclusions.

5.Antibiofilm activity

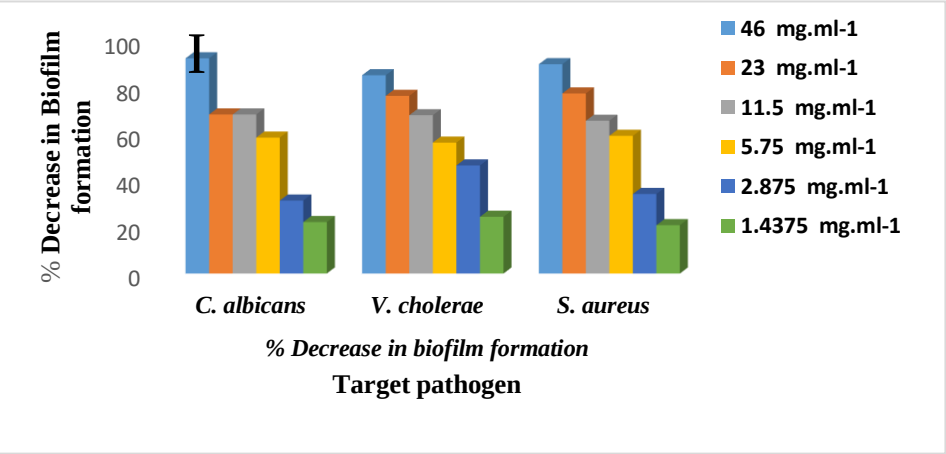

Figure 14: Percentage of biofilm inhibition by the crude MS22 extract against selected pathogens.

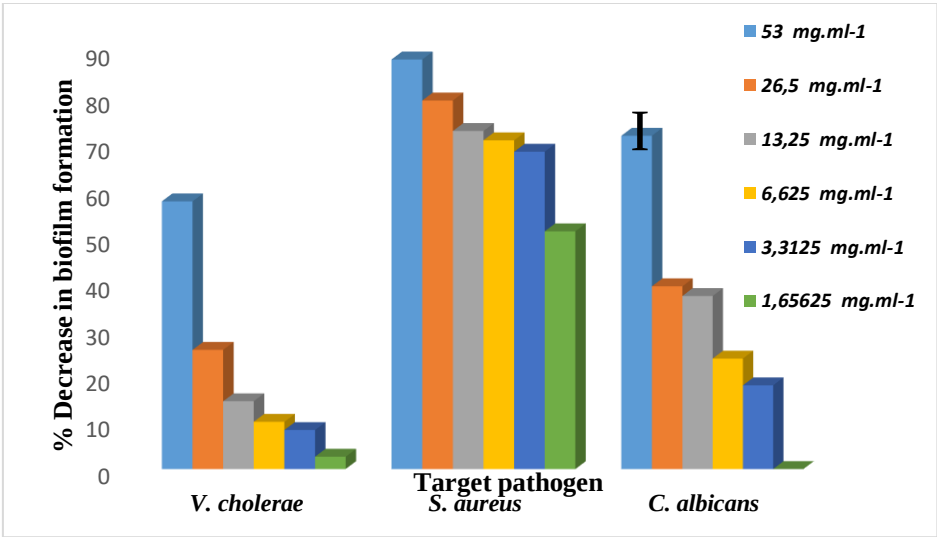

Figure 15: Percentage of biofilm inhibition by the crude MBCrS4 extract against selected pathogens.

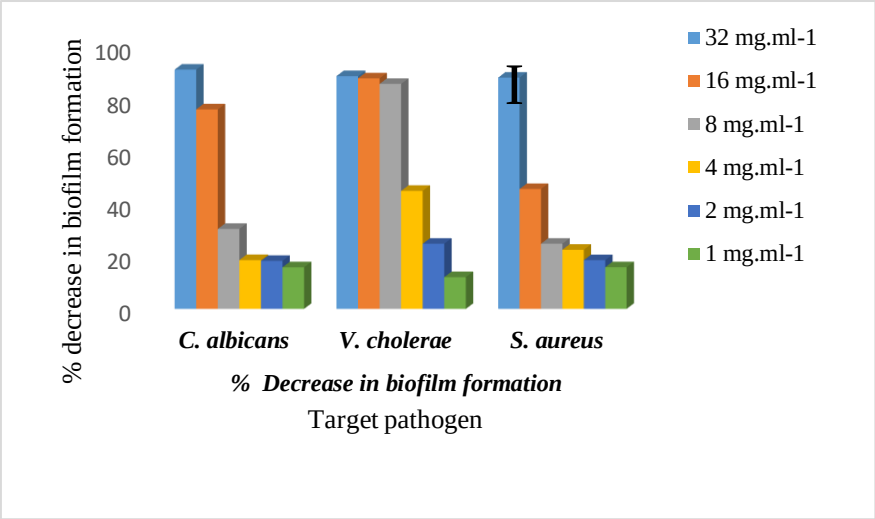

Figure 16: Percentage of biofilm inhibition by the crude SOZnS3extract against selected pathogens.

The antibiofilm activity of the MS22, MBCrS4 and SOZnS3 extracts was evaluated against *S. aureus*, *V. cholerae*, and *C. albicans*. The results, presented in the figures below (**Figures**14, 15, and 16), demonstrate a dose-dependent inhibition of biofilm formation. The MS22 extract induced a reduction in biofilm formation ranging from 20% to 89% for *S. aureus*, 24% to 85% for *V. cholerae*, and 22% to 92% for *C. albicans*, depending on the concentration used. In turn, the MBCrS4 extract showed an inhibition ranging from 51% to 88% against *S. aureus*, from 2% to 57% against *V. cholerae*, and from 16% to 72% against *C. albicans*. As for the SOZnS3 extract, it induced an inhibition ranging from 16% to 88% against *S. aureus*, from 12% to 89% against *V. cholerae*, and from 16% to 91% against *C. albicans*. These findings indicate that all three extracts exhibit significant antibiofilm activity, with maximum efficacy observed at the highest concentrations tested. However, MS22 extract stands out for its stronger and pronounced activity, particularly against *C. albicans*. In the study conducted by **Wijaya et al.(2023),** isolates of *Streptomyces spp*. exhibited antibiofilm activities ranging from 6.06% to 94% against *S. aureus*. Similarly, in our study, the extracts showed inhibition levels ranging from 20% to 89% for MS22, 51% to 88% for MBCrS4, and 16% to 88% for SOZnS3 against *S. aureus*, demonstrating comparable performance. Similarly, **Prastya et al., (2023**) reported that *Streptomyces* spp. inhibited biofilm formation by up to 94% against *S. aureus*. Our extracts displayed similar activity, with inhibition reaching up to 89% with S22. Furthermore, **Agatha and Waturangi (2022)** reported that crude extracts and supernatants from Actinobacteria were capable of inhibiting biofilm formation at concentrations as low as 20 mg/mL, against various pathogens, including *S. aureus*, *E.coli*,and *C.albicans*.

IIThe persistent rise in microbial resistance underlines the urgent need for innovative antimicrobial compounds. Continued exploration of new bioactive molecules, especially from underexplored microbial sources, offers promising prospects for combating resistant pathogens.

Ten actinobacteria strains, isolated by Dr. SOUAGUI Samiha from soil and water samples in the Bejaia region, and their ethyl acetate crude extracts, were studied with the aim of screening and highlighting their biological activities.

The assessment of their bioactive potential demonstrated notable variability in activity, influenced by both the strain and the target organism tested. Among the five tested crude extracts**,** BCrS4 (particularly the Mincer-derived extract, (MBCrS4)) emerged as the most promising, exhibiting the broadest and strongest antimicrobial activity with inhibition zones up to 30 mm against*A. niger*, 28 mm against*Penicillium sp.*, and 24 mm against *C. albicans* and also towards the multidrug-resistant bacteria *E. coli* ST131 (15 mm). This strong bioactivity was complemented by a high polyphenol content (~20 mg GAE/mL) and a notable antioxidant capacity (81.13% DPPH inhibition). However, S22 crude extractexhibited the strongest antioxidant activity (89.63%) with good reproducibility along with a high total phenolic content (~20 mg GAE/ml). Regarding MIC values**,** The **S**OZnS3 extract was the most potent with a MIC value <0.125 mg/ml against *S. aureus*, followed by MS22 and SBCrS4, showing that all three extracts have strong antimicrobial activity at low concentrations. Regarding enzymes production**,**S10 recorded the highest amylolytic index (EI = 5)**,** while OZnS3showed the highest cellulolytic index (EI = 6.2)**,** confirming their strong degradative enzyme potential. Additionally, strain BCrS4 exhibited the highest proteolytic index (EI = 3.4) among all tested strains.

The antibiofilm activity evaluation, highlighted the MS22 extract with inhibition rates reaching up to 92% against*C. albicans* and 89% against *S. aureus*, followed closely by SOZnS3 and MBCrS4. These results identify S22 as the most potent strain**,** with strong antioxidant, antibiofilm, and enzymatic activities, while BCrS4remains the strongest antimicrobial strain. OZnS3, despite moderate antimicrobial inhibition zones against the tested pathogens, it exhibited excellent MIC values, high cellulases production, and interesting antibiofilm and antioxidant potential.

The present work opens up several perspectives for further investigation. First, it would be relevant to extract the active compounds produced by the strains OCdS3, BZnS4, BZnS5,

BPbS2, and S10. The minimum inhibitory concentrations (MICs) of the active extracts derived from these strains should be determined to better evaluate their antimicrobial potential. In addition, the antioxidant and antibiofilm activities of the extracts that have not yet been tested deserve thorough investigation. Testing concentrations below 0.2 mg/mL and < 0.125 mg/mL would allow assessment of their efficacy at low doses. The study of radical scavenging activity (DPPH assay) should also be complemented by the determination of IC_{50} values. To gain deeper insight into the spectrum of activity, the range of target microorganisms should be broadened to include strains that are multidrug-resistant to antibiotics. Furthermore, optimization of the production parameters of active compounds using Design of Experiments (DOE) approaches could enhance their yield and activity. Identifying the bioactive strains responsible for the observed biological effects and characterizing the produced biomolecules regarding their structure, chemical nature, and properties would provide a comprehensive understanding of their biotechnological potential.

Abstract

The evaluation of antimicrobial activity revealed significant antagonistic effects in several strains, particularly BZns4, BZns5, BCrS4, and BPbs2. Inhibition zones reached up to 49 mm against *S. aureus* for BZns4 and 39 mm against *E. coli* for BPbs2, demonstrating strong activity against both Gram-positive and Gram-negative bacteria, as well as pathogenic fungi. Notably, BZns5 exhibited a 35 mm inhibition zone against *C. albicans*, while BCrS4 inhibited *A. niger* (30 mm), *Pennicilliumsp.* (28 mm), and *C. albicans* (24 mm).Furthermore, the crude ethyl acetate extract of BCrS4 showed substantial antifungal activity against theaforementioned fungal strains. The minimum inhibitory concentration (MIC) values of ethyl acetate extracts from BCrS4, S22, and OZns3 were all found to be below 0.2 mg/mL, indicating strong antimicrobial potential.The enzymatic activity screening revealed diverse profiles among the strains. The predominant activities included cellulolytic activity, with a maximum enzymatic index (EI) of 6.2 observed in OZns3, followed by amylolytic activity (EI = 5) in strain S10, and proteolytic activity in BCrS4 (EI = 3.4).The crude ethyl acetate extracts were also assessed for antioxidant activity by measuring their total phenolic content and DPPH radical scavenging capacity. Results revealed high polyphenol contents ranging from 10.12 to 20.03 mg GAE/mL, with MBCrS4 showing the highest and SBCrS4 the lowest. Antioxidant activity, evaluated using the DPPH assay, ranged from 89.63% for S22 to 81.13% for BCrS4. the antibiofilm activity of the active extracts was evaluated against *S. aureus, V. cholerae*, and *C. albicans*, showing dose-dependent inhibition, reaching up to 92.20% against *C. albicans*.These findings highlight the strong potential of actinobacteria as a valuable source of bioactive molecules with applications in medical, environmental, and agricultural fields.

Keywords : Actinobacteria, antimicrobial activity, antibiofilm activity, antioxidant activity, enzymatic activity.

Résumé

Cette étude porte sur dix souches isolées d'échantillons de sols et d'eaux de la région de Béjaïa. L'étude macro et microrphologique a permis de mettre en évidence leur caractère mycélien. L'évaluation de l'activité antimicrobienne a révélé un effet antagoniste important chez plusieurs souches, en particulier BZnS4, BZnS5, BCrS4 et BPbS2, avec des zones d'inhibition atteignant 49mm à l'encontre de *S. aureus*pour BZnS4 **et** 39 mm contre*E. coli*pour BPbS2, démontrant une forte activité contre les bactéries Gram-positives et Gram-négatives, ainsi que contre les champignons pathogènes. Notamment, BZnS5 qui a montré 35 mm contre*C. albicans*,etBCrS4 ayant inhibé*A. niger*(30 mm),*Penicillium*sp. (28 mm) et*C. albicans*(24 mm)**.**Par ailleurs, l'extrait brut d'acétate d'éthyle de la souche BCrS4 a présenté, aussi bien, une activité antifongique importante à l'égardde *C. albicans*, *Aspergillusniger* et *Penicillium sp*. L'évaluation des concentrations minimales inhibitrices (CMIs) des extraits bruts d'acétate d'éthyles des souches BCrS4, S22 et OZnS3 a révélé des valeurs < 0,2 mg/mL .De plus, l'étude des activités enzymatiques des souches, a montré des profils variés.Les activités prédominantes incluent l'activité cellulolytique, avec un indice enzymatique maximal de 6,2 observé chez OZnS3, suivie de l'activité amylolytique (IE = 5) chez la souche S10, ainsi que l'activité protéolytique, où BCrS4 a présenté un indice enzymatique de 3,4. Les extraits bruts d'acétate d'éthyle des souches ont également fait objet de l'étude de leur activité antioxydante à travers l'analyse de leurs teneurs en composés phénoliques, et de l'activité antiradicalaire contre le DPPH. Les resultats ont montré une teneur élevée en polyphénols comprise entre 10,12% mg GAE/mL et 20,03mg GAE/mL, avec un maximum atteint par l'extrait MBCrS4 et un minimum par l'extrait SBCrS4,une activité antioxydante à l'égard du DPPH élevée allant de 89,63 % pour MS22 à 81,13% pour MBCrS4. L'activité antibiofilm des extraitsactifs a également été évaluée à l'encontre de (*S. aureus, V. cholerae et C. albicans*) et a présenté une inhibition dose-dépendante pouvant atteindre 92,20 % à l'égard de *C. albicans*. Ces résultats soulignent le fort potentiel desactinobactéries comme source de molécules actives pour applications dans divers domaines médical, environnemental et agricol.

Mots clés : Actinobactéries, activité antimicrobienne, activité antibiofilm, activité antioxydante, activité enzymatique.

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Appendices

Appendices I

Materials and analysis instruments used in the experiment

- Autoclave (advantage-lab)
- Analyticalbalance(aeadam)
- Bunsen burner
- Beakers
- Bottles (Iso,flasks)
- Erlenmeyer flasks
- Funnel
- Glass Pasteur pipette
- Graduatedcylinder
- Inoculatingloop
- Incubator(Binder , memmert)
- Magnetic stirrer(velpscientifica)
- Magnetic baraux
- Microplates
- Microplatereader(BioTek)
- Optical microscope(iscope ew10x/20)
- pHmeter (milwaukee)
- Petri dishes
- Test tubes
- spectrophotometer (UV mini 1240 ; SHIMADZU)
- Vortex mixer(VELP scientifica : ZX 3)
- Water bath (GFL)

Appendices II

Composition of culture media

I. Media for subculture

SCA Medium (StarchCasein Agar) (Kuster and Williams, 1964)

Starch ..... 10 g
Casein ..... 0.3 g
KNO_3 ..... 2 g
K_2HPO_4 ..... 2 g
NaCl ..... 2 g
$MgSO_4$ ..... $7H_2O$ 0.05 g
$CaCO_3$ ..... 0.02 g
$FeSO_4$ ..... $7H_2O$ 0.01 g
Agar ..... 18 g
Distilledwater ..... 1000 mL
pH ..... 7.2 ± 0.2

Mincer Medium

Starch ..... 10.0 g
Yeastextract ..... 4.0 g
Peptone ..... 4.0 g
Agar ..... 20.0 g
Distilledwater ..... 1000 mL
pH ..... 7.2 ± 0.2

Nutrient Agar (NA)

Nutrient agar powder ..... 20 g
Distilledwater ..... 1000 mL
pH ..... 7.0± 0.2

II.Media for enzymatic tests

Gause's Agar

KNO_3 ..... 1.0 g
K_2HPO_4 ..... 0.5 g
$MgSO_4$ ..... 0.5 g
NaCl..... 0.5 g
$FeSO_4$ ..... 0.01 g
Starch..... 20.0 g
Agar ..... 30.0 g
Distilledwater ..... 1000 mL
pH ..... **7**.4 ± 0.2

Gelatin Nutrient Agar

Peptone ..... 5.0 g
Beefextract ..... 3.0 g
Gelatin..... 4.0 g
Agar 15g
Distilledwater ..... 1000 mL
pH ..... 7.0 ± 0.2

Cellulose Agar

Cellulose ..... 5.0 g
$NaNO_3$..... 1.0 g
K_2HPO_4 ..... 1.0 g
$MgSO_4$ ..... 0.015 g
Yeastextract..... 0.5 g
Agar ..... 15.0 g
Distilledwater ..... 1000 mL
pH ..... **7**.0 ± 0.2

Egg Yolk Agar (1%)

Peptone ..... 10.0 g
Yeastextract ..... 5.0 g
NaCl..... 1.0 g
Agar ..... 20.0 g
Egg yolk..... 100 mL
Distilledwater ..... 1000 mL
pH ..... **7.**0± 0.2

Sierra Medium Supplementedwith Tween 80

Peptone ..... 10.0 g
NaCl..... 5.0 g
$CaCl_2,2H_2O$ ..... 0.1 g
Agar ..... 18.0 g
Tween 80..... 10.0 mL
Distilledwater ..... 1000 mL
pH ..... 7.4 ± 0.2

Skim Milk Agar

Peptone ..... 10.0 g
NaCl..... 5.0 g
Yeastextract..... 3.0 g
Agar ..... 20.0 g
Skimmilk..... 100.0 g
Distilledwater ..... 1000 mL
PH..... 6.5–7.2± 0.2

Tyrosine Agar

Peptone ..... 5.0 g
Meat extract..... 3.0 g
L-tyrosine..... 5.0 g
Agar ..... 20.0 g
Distilled water ..... 1000 mL
pH ..... 7.0± 0.2

MP7 Agar

Glucose ..... 5.0 g
Pectin ..... 5.0 g
KH_2PO_4 ..... 4.0 g
Na_2HPO_4 ..... 6.0 g
Yeastextract..... 1.0 g
$(NH_4)_2SO_4$ ..... 2.0 g
$FeSO_4 \cdot 7H_2O$ ..... 0.001 g
$MgSO_4$ ..... 0.2 g
$CaCl_2$..... 0.001 g
H_3BO_3 ..... 0.00001 g
$MnSO_4$ ..... 0.00001 g
$ZnSO_4$..... $7H_2O$0.00007 g
$CuSO_4$ ..... $5H_2O$0.00005 g
MoO_3..... 0.00001 g
Agar ..... 15.0 g
Distille water..... 1000 mL
pH ..... 7.2–7.4± 0.2

III. Isolation media

EosinMethylene Blue (EMB) Agar Medium

EMB powder (dehydrated) ..... 36 g
Distilledwater ..... 1000 mL
pH ..... 7.1 ± 0.2

Chapman

Chapman powder (dehydrated) ..... 55 g
Distilled water ..... 1000 mL
pH ..... 7.4 ± 0.2

Solutions

Ethanol 96%

Ethanol 96% 100mL
Distilledwater 40,85mL

Congo Red Solution at 0.1%:

Congo red..... 0.1 g
Distilledwater ..... 100 mL

Lugo solution

Potassium iodide (KI) ..... 2 g
Iodine (I2) ..... 1 g
Distilled water ..... 100 mL

Physiological Water Solution

Sodium chloride ..... 9.0 g
Distilledwater ..... 1000 mL
pH ..... 7± 0.2

Sodium Chloride (NaCl) 1M Solution

Sodium chloride ..... 38.0 g
Distilledwater ..... 1000 mL