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# Identification and potential application of quorum quenching bacterial endophytes from South African medicinal plants


By

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# DECLARATION BY CANDIDATE

I, Masego Patience Mokhasi declare that the thesis/dissertation, which I hereby submit for the degree MSc: Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:

DATE:

# DEDICATION

This study is dedicated to my parents, Ontefetse Gordon Mokhasi and Kedinametse Constance Mokhasi for their prayers, eternal love, steadfast support, and encouragement.

*'' Nothing in life is worthwhile unless you take risks. Fall forward. Every failed experiment is one step closer to success.''*

Denzel Washington

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# ABSTRACT

Medicinal plants harbour diverse endophytic bacteria, which produce potentially therapeutic secondary metabolites. This study aimed to identify bacterial endophytes from South African medicinal plants that may disrupt pathogenic bacterial biofilms through quorum quenching (QQ) mechanism. Bacterial endophytes were isolated from six medicinal plants: *Artemisia afra, Alpinia galangal, Aloe vera, Bulbine alooides, Mondia whitei* and *Tulbaghia violacea*. Isolated endophytes were screened for quorum quenching activity. Those without QQ activity were screened for quorum sensing (QS) activity. QQ active isolates were then identified through Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) and 16S rRNA. The identified isolates were then assessed for their antibiofilm properties. A total of 34 endophytic bacteria were isolated from medicinal plants. Analyses by MALDI-TOF mass spectrometry identified *Bacillus* (33.3%) as the dominant genus, followed by *Pseudomonas* (29.2%), *Acidovorax* (12.5%), *Micrococcus* (4.2%), *Raoultella* (4.2%) and 12.5% unidentified species. Analyses by the 16S rRNA sequence revealed a 70.83% similarity to MALDI-TOF results. Of the twenty-four isolates screened for QQ activity, fourteen isolates (58.3%) showed potent AHL signal molecule inhibition. Isolation and amplification of the gene (*aii*A) showed that the occurrence of AHL lactonase in cell-free lysate and sequence alignment demonstrated that AiiA constitutes "HXHXDH" zinc-binding motif preserved in a few groups of metalloenzymes. Effects of QQ isolates against biofilm-forming pathogens: *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus, Serratia marcescens,* and *Bacillus subtilis* depicted different effects where whole-cell isolates displayed biofilm inhibition range of 10% - 84%. Whereas, cell-free lysate isolates resulted in the reduction of cell attachment and restraint of biofilm arrangement with a 0.074% -38% and 1.63% - 64% decrease respectively. Visualization of biofilm under Confocal Laser Scanning Microscopy (CLMS) indicated a reduction in complete biomass development in treated pathogens. This study shows that South African medicinal plants harbour bacterial endophytes, which may serve as potential QQ candidates for the design of treatment against bacterial infections through evidence-based approaches.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AHL | : Acylhomoserine Lactone |
| AI-2 | : Autoinducer 2 |
| AIP | : Autoinducing Peptide |
| ATCC | : American Type Culture Collection |
| CLSM | : Confocal Laser Scanning Microscopy |
| DNA | : Deoxyribonucleic Acid |
| DSF | : Diffusible Signal Factor |
| EID | : Emerging Infectious Diseases |
| EPS | : Exopolymeric Substance |
| HIV | : Human Immunodeficiency Virus |
| IPUF | : Indigenous Plant Use Forum |
| ISR | : Induced Systemic Resistance |
| LB | : Luria-Bertani broth |
| MALDI-TOF | : Matrix Assisted Laser Desorption Ionization- Time of flight |
| MICs | : Minimum Inhibitory Concentrations |
| NCBI | : National Center for Biotechnology Information |
| PCR | : Polymerase Chain Reaction |
| PPB | : Potassium Phosphate Buffer |
| PQS | : *Pseudomonas* Quinolone Signal |
| QQ | : Quorum quenching |
| QS | : Quorum sensing |
| QSS | : Quorum sensing systems |
| rRNA | : Ribosomal ribonucleic acid |
| TSA | : Tryptone Soy Agar |
| TSB | : Tryptone Soy Broth |
| UV/Vis | : Ultraviolet and visible absorption |

# CHAPTER 1

# INTRODUCTION

## 1. BACKGROUND

Excessive use of antibiotics has led to a widespread bacterial resistance (Ventola, 2015). Hence, the identification of novel antipathogenic agents is critical (Williams, 2002; Livermore, 2004). The slow rate of finding new antibiotics is outpaced by the spread of drug resistance (Nathan, 2004).

Currently, antibiotics kill bacteria or prevent their proliferation by disrupting critical metabolic processes. This strategy imposes a selection pressure that contributes to the occurrence of antibiotic-resistant strains (Dong *et al.,* 2007). Therefore, any systematic technique or strategy that can combat bacterial disease without imposing selective pressure or directly killing of pathogens will be sufficient for infection control. Likewise, it will aid reduce or suspend the event of antibiotic resistance in microbial networks (Dong *et al*., 2007).

Microbial networks refers to biofilms, in which bacteria adhere and stick to the surfaces and construct societies embedded in a self-emitted exopolymeric substance (EPS) made up of DNA, proteins, and polysaccharides (Bzdrenga *et al.,* 2016). Biofilms offer bacteria security against ecological pressures (UV, desiccation, antimicrobial compounds and against clearance by the immune system) and therefore adds to difficulty in management of chronic diseases/infections (Ganesh & Rai, 2018; Bzdrenga *et al.,* 2016). Hence, bacterial biofilms render conventional antibiotics ineffective (De Kievit & Iglewski, 2000).

The maintenance of bacterial biofilms depends on quorum sensing (QS) whereby the bacterial community produces, detects and responds in a populace density manner to small diffusible signal molecules called autoinducers (Mellbye *et al*., 2016; Waters & Bassler, 2005; Ganesh & Rai, 2018)). When the density of bacterial population increases, so does the concentration of QS signals, promoting a synchronized expression of virulence genes (Whitehead *et al*., 2001; Schuster *et al.,* 2013). Interference or distortion of bacterial chemical cell-cell communication (quorum sensing) is well accepted as quorum quenching (QQ). This QQ appears to be a promising alternative treatment strategy to control bacterial infections (Hentzer & Givskov, 2003; Kusari *et al*., 2014). QQ systems have a potential for application, with a wide variety of microorganisms, owing to the fact that pathogens can communicate and act communally in

regulating infection-related characteristics, including the manifestation of virulence genes and the construction of biofilms (Dong *et al.,* 2007).

Numerous studies on QQ phenomena have been reported, however, limited research has been piloted on the QQ strategies from endophytic bacteria found in medicinal plants (LaSarre & Federle, 2013; Jahangir *et al.,* 2012). Bacterial endophytes exist in the innermost tissues of many plants for a large portion of its life cycle without causing damage to the host (Passari *et al.,* 2015).

Endophytes have potential to contribute to the evolutionary sustainability of various secondary metabolites, offering resistance against ailments as well as a potential source of sustenance (Strobel *et al.,* 2004). Pimentel *et al.,* (2011) proposed that endophytes possess merged hereditary data from host plants, which incite their versatility and improve their protection systems across pathogens and insects.

Although there are reports on new endophytic bacteria with unique chemical compounds, there is paucity of published data available on the biodiversity of endophytic bacteria linked with traditional medicinal plants from exclusive surroundings (Nimnoi *et al.,* 2010; Qin *et al.,* 2008). South Africa alone retains a rich variety of medicinal plants as well as information on their utilization. Documented literature shows approximately 24 000 taxa present in the region with only ~ 3 000 species utilized as pharmaceuticals while 350 are generally utilized in customary South African herbal medication (Van Wyk *et al.,* 2009; Abdalla & McGaw, 2018). For these reasons, this investigation aimed to explore the diverse bacterial endophytes as potential for secondary metabolites with antipathogenic or QQ properties. Furthermore, this research aimed to identify possible suitable methods to degenerate or inhibit the biofilm formation and ultimately combat possible bacterial infections.

## 1.1 PROBLEM STATEMENT

Emerging infectious diseases (EID) contribute as leading causes of death with approximately 700 000 people dying annually worldwide (WHO, 2020). This is further exacerbated by the failure of antibiotics due to high levels of antimicrobial resistance. (Garcia-Contreras *et al.,* 2013). The exponential rise of antibiotic-resistant microorganisms severely limits the available options for treating bacterial infections (Chen *et al.,* 2013). Thus, unique strategies to combat bacterial infections are vital as alternatives to the current antibiotics.

## 1.2 RATIONALE

The enormous proliferation in infections worldwide emphasizes failure to deal with these medical problems. Hence, there is a need to search for novel and useful compounds/enzymes that can contribute and provide sufficient relief to human health (Muzzamal *et al.*, 2012). Natural products, particularly from endophytic microbes may have the potential to inhibit or kill a wide variety of harmful pathogens including phytopathogens and other microbial pathogens that affect both humans and animals (Muzzamal *et al.*, 2012).

Unlike common antibiotics, quorum quenching has been proposed as a great treatment alternative to combat bacterial infections. This is mainly due to the fact that quorum quenching approach strictly alleviate the virulence factors of pathogenic bacterial strains instead of killing them and thus does not result in the occurrence of selection pressure (Clatworthy *et al.*, 2007).

## 1.3 HYPOTHESIS

South African medicinal plants possess endophytic bacteria with putative QQ enzymes/genes that have an inhibitory effect on the biofilm-forming pathogens.

## 1.4 STATEMENT OF PURPOSE

The purpose of conducting this study was to isolate endophytic bacteria from South African medicinal plants that possess QQ properties and to disrupt biofilm-forming bacteria by interrupting the QS systems of selected pathogens.

## 1.5 RESEARCH QUESTIONS

This study sought to address the following questions:

- Do South African medicinal plants harbour bacterial endophytes?
- Which bacterial endophytes possess compounds/enzymes that inhibit/ reduces microbial growth?
- Do medicinal plant-associated bacterial endophytes produce bioactive compounds/enzymes that have quorum quenching activity?
- What specific QQ genes associated with QQ activity against pathogens do bacterial endophytes possess?
- What effect do endophytic bacteria bioactive compounds/enzymes have on biofilm formation?

## 1.6 OBJECTIVES

To address the above research questions, the following objectives were set:

- To conduct a literature search and collect medicinal plants (n = 6) of interest based on their ethnobotanical history.
- To use plate-based extraction methods to isolate and extract endophytic bacteria from selected medicinal plants.
- To use qualitative agar assays to screen for QQ potential of isolated endophytic bacterial strains.
- To use specialised techniques: MALDI-TOF and 16S rRNA to identify and to characterize the endophytic bacteria.
- To use PCR to determine the type of QQ genes present in bacteria capable of QQ.
- To perform biofilm assays to determine the effects of QQ compounds on biofilm-forming pathogens.

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# CHAPTER 2

# LITERATURE REVIEW

## 2. INTRODUCTION

The study of Ayurveda, Chinese, Thai and Unani folk medicine lead to the discovery of biologically effective compounds and lead structures, thus resulting in the advancement of altered derivatives with improved activity or diminished poisonous quality (Patwardhan *et al.,* 2004). Plant endophytic microbes have been acknowledged as potential source of new biomolecules and chemicals having a latent function in horticulture, curative, and nourishment industry (Pimentel *et al.,* 2011). Additionally, investigating the biodiversity of endophytic strains for new metabolites prompts for the discovery of new medications for the management of infections (Vijayalakshmi *et al.,* 2016).

In search for new biomolecules against bacterial infections, six therapeutic plants were selected (based on their medicinal use) for this study namely; *Tulbaghia violacea, Bulbine alooides, Aloe vera, Mondia whiteii, Artemisia afra* and *Alpinia galangal* that have noteworthy restorative and trade significance. This chapter provides a succinct overview of the relevant literature on bioprospecting the naturally occurring bacterial endophytes for therapeutic and antivirulence properties.

## 2.1. A BRIEF BACKGROUND ON THE MEDICINAL PLANTS OF INTEREST

The six medicinal plants described were selected based on a standard criteria such as (i) plants must have obtained unique traits or features for their sustenance or constitute rare biology, (ii) plants must have an ethnobotanical history, and (iii) plants must have been growing since existence, or indigenous species. Some of the plants selected for this study are endemic to a particular area/region hence they were chosen. Lastly, plants should acclimatise to the area of important biodiversity or specific geographic locality; this was the case for *Alpinia galangal* (Kusari & Spiteller, 2012).

### 2.1.1 *Artemisia afra*

*Artemisia afra* is a standout amongst the highly prevalent and utilized herbal medicines in Southern Africa (Figure 2.1A). *A. afra* is a lasting timbered bush, reaching 2m in height with a verdant, bushy and furrowed stalk (Van Wyk *et al.,* 1997). Its leaves are of a delicate surface, dull green on the adaxial side, achieving a length of 8cm and a breadth of 4cm at maturity. The plant has an identifiable scent with fragrant and sharp notes. While, after wounding there is

detectable the sweet scent (Hilliard, 1977). *A. afra* is utilised in treating a wide variety of illnesses. These include conditions affecting the respiratory, gastric, renal, cardiac, neuromuscular and endocrine systems (Thring & Weitz, 2006). Thus, this infers *A. afra* to possess antiviral, anti-bacterial and anti-inflammatory actions. The use of A. afra in the relief of both influenza and the common cold could be indicative of potential antiviral activity (Thring & Weitz, 2006)

*A. afra* is found in the highland locales of Uganda, Tanzania, Kenya and as far as Ethiopia. Likewise, in Southern Africa, the species are common, for example, in Zimbabwe, South Africa and Namibia. Within South Africa, it develops in the Northern regions, including Limpopo and Gauteng. The plant is also found in eastern parts of South Africa particularly in the KwaZulu-Natal region from the drift to the Drakensberg. *A. afra* is additionally found in the nations of Lesotho and Swaziland which are contained within South African borders. It is also indigenous to the South Western region of the country extending towards the Western Cape in the South. (Liu *et al.,* 2009).

The common names depict this herb might be portrayed to the far-reaching use by various tribal communities (Watt & Breyer-Brandwijk, 1932). It is identified as "Umhlonyane", "Mhlonyane", "Lanyana", "Lengana", "African wornwood" and "Wilde als" in Xhosa, IsiZulu, Sotho, Setswana, English and Afrikaans dialect respectively (Liu *et al.,* 2009).

### 2.1.2 *Alpinia galangal*

*Alpinia galangal*, otherwise called greater galangal in English and Kulanjan in Hindi (Chouni & Paul, 2018). It is vastly utilised as Ayurveda and Siddha drug in the treatment of different illnesses, including diabetes mellitus, microbial infections, aggravations, rheumatic torments, chest torment, and dyspepsia, fever, liver disease, kidney sickness, cancer, and even HIV (Figure 2.1B) (Chouni & Paul, 2018).

This plant is not indigenous to South Africa. *A.galanga*l is distributed in the Himalaya and Southern area of the Western Ghats in India (De-Pooter *et al.,* 1985). Usually developed in Konkan and North Kanara (Kiuchi *et al.,* 2002). *A. galangal* is known as a more noteworthy galangal. Its foundations' stocks are tuberous and to some degree sweet-smell. The leaves are prolonged lanceolate, exceptional, glabrous, and green above, paler underneath (Chouni & Paul, 2018).

### 2.1.3 *Bulbine alooides*

*Bulbine alooides* belongs to the *Asphodelaceae* family (Figure 2.1C). The leaves of the plants are succulent and soft. The plant has an aloe-like appearance, however, the leaves are thornless and fleshy green. The plant is native to South Africa, and found in Gauteng, Limpopo, Eastern Cape, KwaZulu-Natal and the Western Cape provinces (Van Wyk *et al.,* 1997). The plant is used management of different ailments including rheumatism, syphilis, wound, rashes, diarrhoea, urinary tract infections and venereal disease (Van Wyk *et al.,* 1997).

### 2.1.4 *Tulbaghia violacea*

This plant is generally known as "wild garlic", “wilde knoffel”, “isihaqa” and “itswele lomlambo” in English, Afrikaans, Zulu and Xhosa respectively (Figure 2.1D). It is indigenous to South Africa, particularly in the Eastern Cape, Limpopo and KwaZulu-Natal provinces as well as in the neighbouring country Zimbabwe (Maoela, 2005; Belewa *et al.,* 2011). This proliferative bulbous plant with drawn-out contracted tied-like leaves and pinkish cylindrical blooms grow up to 30 cm long. It has been overused for a considerable length of time by the indigenous people as a ‘’fix-all’’ cure (Bungu *et al.,* 2006). The leaves are utilized as snake anti-agents and as vegetables for consumption (Street, 2008).

### 2.1.5 *Aloe vera*

*Aloe vera* has been renowned and utilized, for a considerable length of time, for its health, skincare, restorative and beauty properties (Figure 2.1E) (Surjushe *et al.,* 2008). *Aloe barbadensis miller* is the known vegetal name of *Aloe vera.* It forms part of the *Asphodelaceae (Liliaceae)* family unit and is a hedge plant or arboreal, annual, xerophytic, succulent, pea-green shaded plant. It develops predominantly in dry locales of Africa, Asia, Europe and America (Surjushe *et al.,* 2008).

The Egyptians utilized *A. vera* in the treatment of wounds, burnings, and diseases for the absolute first time. The Greeks, Spanish and African individuals utilized it for different techniques for a few purposes (Hashemi *et al.,* 2015). A portion of the pharmacological activities ascribed to the *A. vera* plant incorporates anti-inflammatory, anti-arthritis, antibacterial, anti-fungal, and hypoglycemic impacts just to give some examples (Hashemi *et al.,* 2015).

### 2.1.6 *Mondia whitei*

*Mondia whitei* is a well-known curative plant that forms part of the *Apocynaceae* family (Figure 2.1F). The class *Mondia skeels* comprises two species, *M. whitei* and *M. ecornuta* (N.E.Br) (Aremu *et al.,* 2011). *M. whitei* is usually recognised as white ginger, tonic root or "umondi" or "mundi" (in Zulu, from which the conventional name was determined). *M. whitei* is a pervasive African species. *M. whitei* has been utilized for its therapeutic, ethnic and nutritive properties by the African people for generations. Across different gatherings of individuals, for example, individuals of Uganda, Nigeria, and Benin. *M. whitei* is identified as a mellow diuretic, to relieve stomach torments, mitigate queasiness concerning the treatment of fever, bilharzia and sexual dysfunctions (Oketch-Rabah, 2012). The vast majority of the plant material gathered from the wild and this has aided altogether to its helpful status (Aremu *et al.,* 2011).

## 2.2 BACTERIAL ENDOPHYTES

The unearthing and rigorous investigation of plant allied microbes known as endophytes have led to the possibility of searching for probable advantages of these life forms. Endophytes exist in a mutualistic association with their host plant for a minimum of half its life cycle (Ryan *et al.,* 2008; Kandel *et al.,* 2017; Alvin *et al.,* 2014). Although they are soil-borne, these bacteria can infiltrate plant roots, and certain strains can travel to aerial sites such as xylem, bark, and leaves. Bacterial densities in aboveground tissues are typically lower when compared to root colonizing populations.

The word ''endophyte'' was stemmed from the Greek words ''endon'' which suggests inside and "phyton'' which means plant (Kandel *et al.,* 2017). All known plant species that exist are a possible host for a minimum of one if not more endophytes; this reality suggests endophytes as omnipresent organisms (Guerin, 1898; Redecker *et al.,* 2000; Strobel *et al.,* 2004).


**Figure 2. 1:** Images of medicinal plants of interest. [A] *Artemisia afra,* [B]*Alpinia galangal,* [C]*Bulbine alooides,* [D] *Tulbaghia violacea,* [E] *Aloe vera* and [F] *Mondia whitei.* (Photos were taken by Mokhasi M.P.).

### 2.2.1 BENEFITS/ROLES PLAYED BY BACTERIAL ENDOPHYTES

Endophytes produce an assortment of biologically active secondary metabolites that offers the potential for medical, farming, and trade exploitation (Strobel & Daisy, 2003; Staniek *et al.,* 2008; Aly *et al.,* 2010). Endophytes provide host plants with tolerance against several ecological stresses such as herbivory, high temperatures, high salt levels, diseases, and drought (Figure 2.2.) (Arnold *et al.,* 2003).

#### 2.2.1.1 Biological Activities

Endophytes' bioactive natural products include metabolites with antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-inflammatory, immunosuppressive drugs and many other related compounds (Anjum & Chandra, 2015). This is entirely due to inhabiting unique biological niches (higher plants) and unfamiliar environments (Strobel & Daisy, 2003). Diverse classes recorded to display various biological activities include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, and lignans, etc (Anjum & Chandra, 2015).

Since endophytes can co-exist within the same locale as their pathogenic mates, their competitiveness may be relatively greater. However, little is known about QQ endophytic bacteria that occupy medicinal plants. Endophytic biodiversity may help identify new effective drugs against human, plant and animal infections (Ryan *et al.,* 2008). For this reason, this study aimed to search for endophytic bacteria associated with medicinal plants for their bioactivities.

#### 2.2.1.2 Essential Compounds

The close-knit connection amongst host plants and endophytes is through the activity of compounds made by the microbes and host cells (Brader *et al.,* 2014). Many present a variety of fitness improvements and employ several favourable effects on host plant health. These include stimulation of growth, nitrogen fixation and resistance to drought, herbivores, and parasites (Chen *et al.,* 1995; Kirchhorf *et al.,* 1997).

#### 2.2.1.3 Plant disease tolerance and resistance

A study conducted by Miliute *et al.* (2015) recorded different impacts of endophytic microorganisms on plant welfare and development. This includes assisting with nutrient accessibility and take-up, improving stress tolerance and providing ailment resistance. Plant development promoting capacity of endophytes may be due to several mechanisms. These may include the manufacture of plant growth hormones, relations that change internal plant hormone manufacture or activity that will build the availability of nutrients, for example, nitrogen and phosphorus (Glick, 2012).

Endophytes promoting the resistance of plants to infection is due to their ability to manufacture an assortment of compounds. For example, antimicrobial or chitinase enzymes can restrain the development of plant infectious agents and function as biocontrol operators (Brader *et al.*, 2014; Christina *et al.*, 2013). Endophytes have conjointly been displayed to incite a dormant infection mechanism of defense, called induced systemic resistance (ISR), which presents an enriched standard of immunity to a wide range of pathogens (Pieterse *et al.*, 2014).

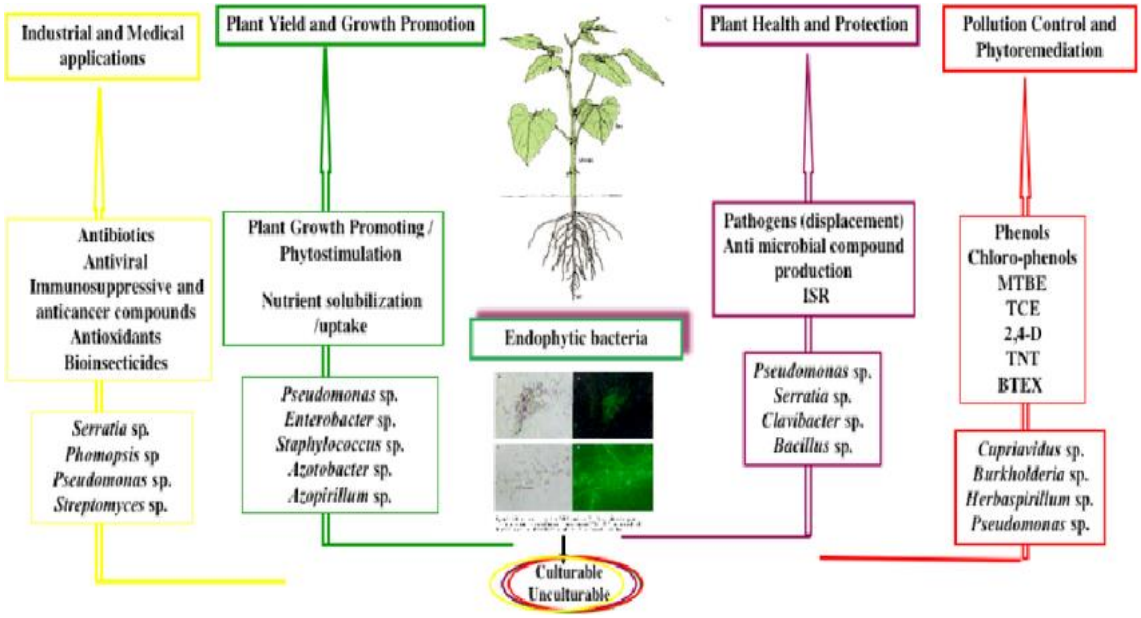


**Figure 2. 2:** A Schematic diagram summarising the benefits and roles of endophytic bacteria (Ryan et al., 2008).

## 2.3 INTRODUCTION TO QUORUM SENSING (QS)

QS Systems are detected in Gram-negative and Gram-positive bacterial species. Following the production of signal molecules, transferred extracellularly and their concentration builds relatively to the population density of bacteria (Figure 2.2).

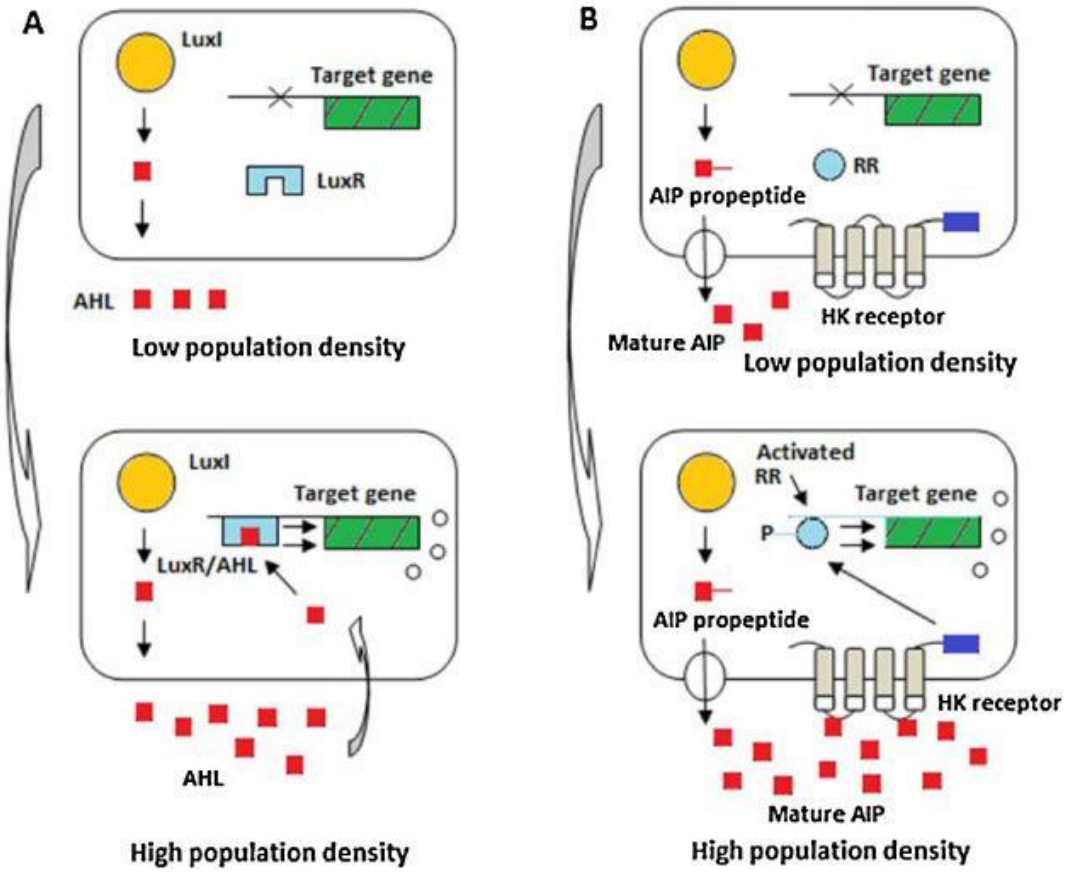


**Figure 2. 3:** Schematic diagram depicting the quorum sensing system of [A] Gram-negative bacteria and [B] Gram-positive bacteria. When the population density is low, signal molecules (AHLs and AIPs) are present in very low concentration and that results in the receptors being degraded and therefore the target gene not expressed (Siddiqui *et al.*, 2015; Rutherford & Bassler, 2012). However, when the population density is high and the signal molecule concentration reaches a specific threshold concentration, signalling molecules bind to receptors to make a complex resulting in an activated receptor, which then allows the target gene expression to occur.

### 2.3.1 QS system of Gram-negative bacteria

In Gram-negative bacteria (Figure 2.3A), autoinducer (AI) particles are comprised of minimal synthetic groups like acyl-homoserine lactones (AHLs), alkylquinolones, α-hydroxyketones and diffusible signal factor (fatty acid compounds). It consists of an AHL synthase (LuxI-type family protein) as well as an AHL receptor (LuxR-type family translation controller) (Umesha & Shivakumar, 2013). When a particular signal molecule quorum is attained, the QS regulated targets are expressed. Analogous LuxI/LuxR framework has been recognized in numerous Gram-negative bacteria, each equipped for creating particular AHLs. AHL facilitated QS was initially depicted in *Vibrio fischeri*, a bioluminescent bacterium whereby the LuxI and LuxR proteins regulated the outflow of a luciferase (LuxCDABE operon) (Fuqua *et al.*, 1994). LuxI

is in charge of the creation of the AHL signalling particle 3-oxo-C6HSL. LuxR is the receiver of 3-oxo-C6HSL and the transcription activator of Lux-regulated genes (Miller & Bassler, 2001).

When the 3-oxo-C6HSL is made, it can uninhibitedly be diffused into the ecological unit and cumulate with expanding cell densities. Once the accumulation of 3-oxo-C6HSL achieves its limit, it will bind with the LuxR protein to create the LuxR-HSL complex and afterward initiate the transcription of LuxCDABE (Umesha & Shivakumar, 2013). In *Pseudomonas aeruginosa* and *Serratia marcescens*, these signalling frameworks control the manifestation of virulence factors (Umesha & Shivakumar, 2013). *Pseudomonas aeruginosa* has two schemes analogous to LuxI/LuxR. LasI/LasR has been indicated to regulate biofilm development and the creation of extracellular enzymes, and the conversion of another quorum-sensing system, RhII/RhIR, including an extra degree of control through AHL signalling (Umesha & Shivakumar, 2013).

### 2.3.2 QS systems of Gram-positive bacteria

Gram-positive microbes commonly utilize modified oligopeptides as signals and "two-part" type layer bound sensor histidine kinases as receptors (Figure 2.3B). A phosphorylation cascade, influencing a DNA-restricting transcriptional regulatory protein, called a response regulator (Waters & Bassler, 2005; Miller & Bassler, 2001). Alike to the processes of Gram-negative microbes utilizing LuxI/R QSS, every Gram-positive bacterium utilizes a signal not the same as that utilized by other microorganisms and the related receptors are extremely sensitive to the signal structures (Waters & Bassler, 2005). In this manner, as in LuxI/R frameworks, peptide QS circuits are comprehended to present intraspecific correspondence. Peptide signals are not diffusible over the film, henceforth signal discharge is mediated by devoted oligopeptide exporters (Hawver *et al.,* 2016).

Most peptide QS signals are separated from bigger precursor peptides, which at that point are adjusted to contain lactone and thiolactone rings, lanthionines and isoprenyl gatherings (Ansaldi *et al.,* 2002; Nakayama *et al.,* 2001).

### 2.3.3 Other QS systems

QSS within other bacteria that do not make their signal particles is noted, however, they react to those created by others. For example, (1) *Escherichia coli*, which has SdiA, an analogue of LuxR (Kalia, 2013), and (2) *Burkholderia cepacia*, whereby in cystic fibrosis patients observe and react to QS indicators delivered by *Pseudomonas aeruginosa* (Riedel *et al.,* 2001).

Generally, QSS provides access to ecological specialties and an upgraded capacity to battle against its rivals (Venturi, 2006). For example, QS activities in *Candida albicans*, a eukaryotic contagious pathogen produces farnesol, which is crucial for the alteration of yeast cells to mycelium shape (Hornby *et al.,* 2001). Thus making this process vital for its destructive demeanour (Oh *et al.,* 2001).

### 2.3.4 QS and biofilm formation

Bacteria are unicellular microorganisms that can proliferate, divide, sense and adapt to environmental signals independently (Solano *et al.,* 2014). Despite being autonomous, bacteria achieve a cooperative manner when a coordinated effort with other parties to undertake cooperative functions like bioluminescence production, biofilm formation, and exoenzyme secretion is required (Solano *et al.,* 2014). This coordination occurs through a mechanism of intracellular chemical communication called quorum sensing (QS) (Solano *et al.,* 2014). Some bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Serratia marcescens,* and *Proteus mirabilis* are capable of forming biofilms and responsible for many bacterial infections

Biofilms refer to microbial groups that adhere to surfaces or interphase and embedded in a self-secreted exopolymeric substance (Ganesh & Rai, 2018). Inside the biofilm, microorganisms are shielded from ecological pressure, for example, drought, attack by the immune system, protozoa ingestion, and antimicrobials (Solano *et al.,* 2014; Ganesh & Rai, 2018). Biofilm formation/development is directed by various physical, chemical and natural processes. Microbial biofilm progresses through five sequential stages, including preliminary reversible attachment, irreversible attachment, and maturation phase I, maturation period II and scattering (Sauer *et al.,* 2002). A schematic diagram depicting the different stages of microbial biofilm development is portrayed in Figure 2.4 below.

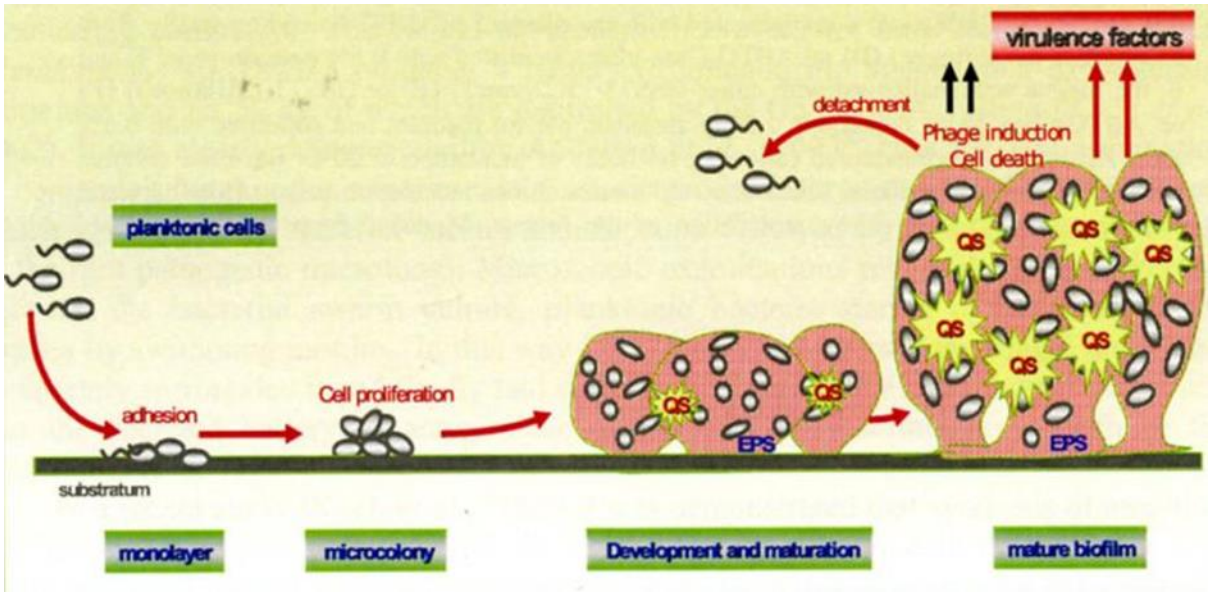


**Figure 2. 4:** Schematic diagram depicting different stages of how biofilm formation occurs. Planktonic cells are attached reversibly to the surface and then become irreversibly attached through several forces, which at that point prompts the arrangement of a colony of bacterial cells superficially. With the help of quorum sensing and other signalling occasions, the development and stabilization of biofilms happen. From there on, organisms inside the biofilm scatter by the arrival of surface microscopic organisms dwelling on the highest point of biofilm structure for colonization to another surface (Gupta *et al.,* 2016).

Biofilm formation takes place in five stages (Figure 2.4). In the first stage, planktonic cells attach to the exterior through physical forces or bacterial adjuncts such as pili or flagella (Maric & Vranes, 2007). Few features like surface practicality, temperature and pressure can regulate bacterial appendage extensively (Gupta *et al.,* 2016). In the second phase, a portion of the reversibly affixed cells restrain and become permanently attached when the attraction forces are much superior to those that are repulsive (Garrett *et al.,* 2008). The third stage of biofilm development is the maturation stage I phase. In this stage, microbial cells start by communicating between themselves by the creation of autoinducer signals that results in the manifestation of biofilm distinct genes (Davies *et al.,* 1998). During this stage, microorganisms exude a matrix of extracellular polysaccharide substances (EPS) to keep the biofilm network stable (Gupta *et al.,* 2016).

In the fourth stage of development, the magnitude of the microcolony escalates and its thickness (Gupta *et al.,* 2016). Microcolonies in biofilm constitute varied microbial societies. These multispecies microbes work in a comparatively complex and synchronised way (Gupta

*et al.,* 2016). Their adjacent vicinity improves substrate trade, circulation of metabolic products and the elimination of waste products (Davey & O'toole, 2000).

The last phase of biofilm formation is the scattering which is the detaching of the biofilm and returns to its motile structure and immobilized cells (Hall-Stoodley *et al.,* 2004). During this phase, biofilms spread and colonize to new surfaces. Additionally, the microbial network inside the biofilm constructs distinctive saccharolytic chemicals that disturb the biofilm stabilizing polysaccharides and consequently discharges external bacteria dwelling on top of the biofilm structure for additional colonization on new surfaces (Gupta *et al.,* 2016).

## 2.4 INTRODUCTION TO QUORUM QUENCHING (QQ)

### 2.4.1 Quorum quenching strategies

QQ methodologies include the procedure of QS interference, which incorporates (I) lessening the activeness of AHL related receiver protein or AHL synthase (II) restraining the generation of QS indicator molecules, (III) degradation of the AHL, and (IV) simulating the signal molecules by incorporating complexes as homologues of signal molecules. Among the unique conceivable outcomes, enzymatic demotion of QS signal molecules (AHLs) has been valued and researched thoroughly (Kalia, 2013). Additionally, QQ has proven to be a target for both QS signal synthase and sensor or response regulator. These methodologies can link to accomplish restraint of AHLs and AIPs mediated QS in Gram-positive and Gram-negative bacteria respectively (Lade *et al.,* 2014).

As reported in the literature, the signal molecule AHL is a key factor in bacterial interaction. The degradation of this molecule obstructs its ample accumulation in the adjacent vicinity of the bacterial cell, which leads to an interruption of the communication system. This is the main proposed QQ strategy related to this study due to the blocking of QS by the signal molecule degradation, thereby presenting as a promising alternative strategy to diminish or reduce bacterial virulence (Medina-Martinez *et al.,* 2007).

### 2.4.2 Quorum quenching strategies using bacterial endophytes

Limited studies have been conducted on QQ strategies with endophytic bacteria. Endophytes are abundant and exist in almost all plant species and contribute to the evolutionary sustainability of various secondary metabolites that provide resistance against illnesses and subsistence (Strobel *et al.,* 2004). The compounds or enzymes produced by endophytic bacteria can disrupt the QS system of pathogens as presented below in Figure 2.5.

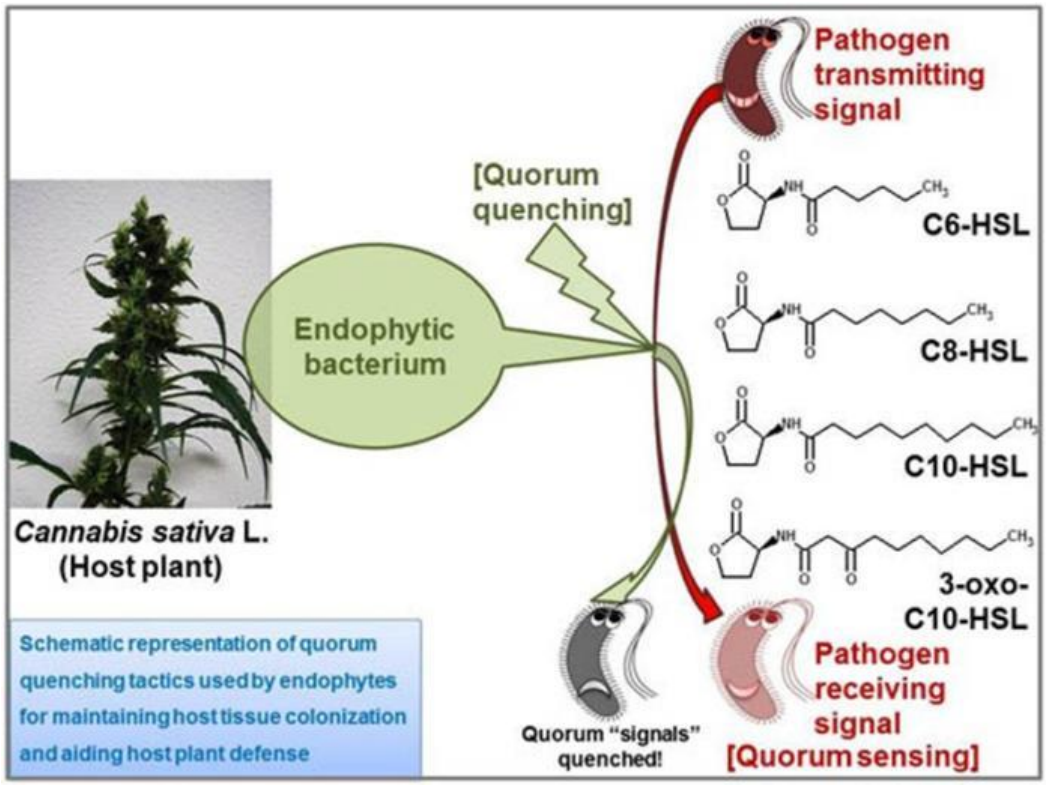


**Figure 2. 5:** A schematic diagram illustrating quorum-quenching tactics used by endophytic bacteria. Bacterial endophytes produce enzymes/compounds that impede/disrupt the communication among pathogens resulting in them not being able to recognize each other or even communicate ultimately not being able to colonize the host plant and causing an intended infection (Kusari *et al.*, 2014).

### 2.4.3 Quorum quenching enzymes

Various enzymes concerned with AHL degradation or alteration have been reported as (1) lactones (Uroz *et al.*, 2008), (2) amidases (otherwise called amidohydrolases or acylases) (Lin *et al.*, 2003) and (3) oxidoreductases (Utari *et al.*, 2017). These enzymes are present in bacteria, archaea, and eukaryotes. Few bacteria are equipped for splitting their own AHL signals, for example, *Agrobacterium* and *Pseudomonas* (Figure 2.6) (Grandclement *et al.*, 2016).

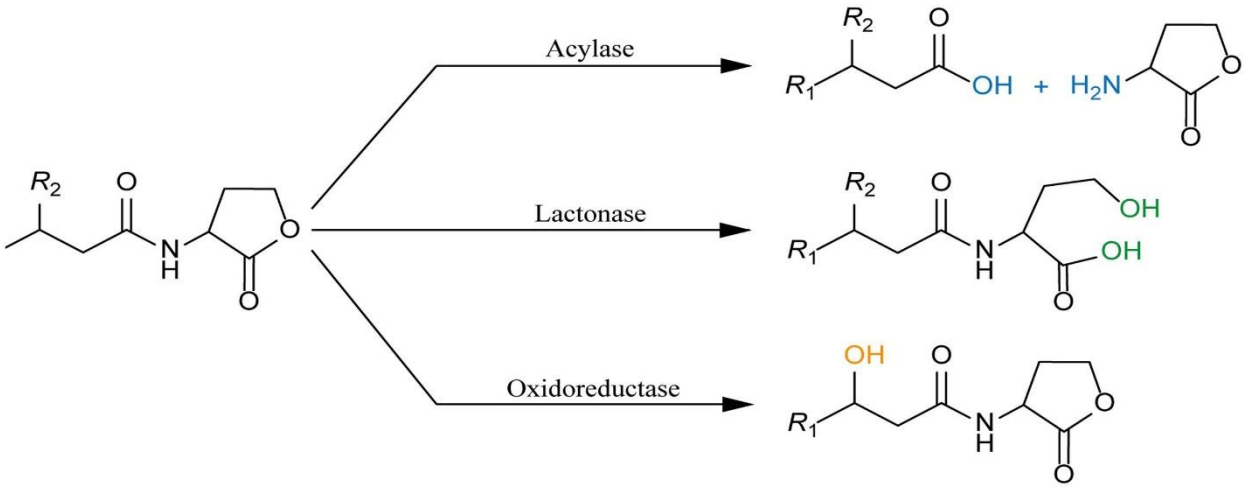


**Figure 2. 6**: Schematic outline of various AHL deactivating enzymes. The three classes of enzymes are arranged as QQ proteins/enzymes. (I) Acylases otherwise called amidases or amidohydrolases that hydrolyses the amide bond between acyl chain and homoserine lactone ring (in blue), (II) Lactonases that open homoserine lactone ring (in green) and (III) Oxidoreductases that changes the AHLs by oxidizing or diminishing the acyl chain without debasing the AHLs (in orange) (Utari *et al.*, 2017).

Different QS signals can be naturally degraded; these are comprehensive of 3-OH-PAME implicated in the control of microbial virulence. For example, *Ralstonia*, the DSF created by *Xanthomonas* (Grandclement *et al.*, 2016) and the PQS delivered by *Pseudomonas* (Pustelny *et al.*, 2009). Likewise, *Diketopiperazines* can be degraded by microbial actions in a conventional way, i.e. despite their QS signal action (Grandclement *et al.*, 2016).

The development and sequence of amino acid of AHL degrading enzymes vary, particularly for the families of four lactonases that are distinguished and thus include; the phosphotriesterase-like lactonases, the paraoxonases, metallo-β-lactonase like lactonases and the α/β-hydrolase crease lactonases (Grandclement *et al.*, 2016). The metallo-β-lactamase-like lactonases feature a selective overlap like *Aii*A and *Aii*B from *Bacillus thuringiensis* and *Agrobacterium tumefaciens* respectively (Liu *et al.*, 2005).

Endophytes discharge antimicrobials as hydrolytic enzymes which act as quorum sensing inhibitors (QSIs) to prevent the colonization of plant pathogens or rather to keep different pathogens from causing infection to the host plant (Alvin *et al.*, 2014). Some of the QQ enzymes isolated from bacterial endophytes with quorum quenching properties are reported in Table 2.1 below. These enzymes were not isolated from bacterial endophytes found in South African medicinal plants, but rather from medicinal plants in general.

**Table 2. 1:** QQ enzymes from endophytic bacteria.

| Endophytic bacteria | Source | QQ enzymes | References |
|---|---|---|---|
| *Bacillus cereus* | | AHL lactonase | Dong *et al.*, 2002 |
| *Bacillus mycoides* | *Lolium perenne* L. | AHL lactonase | Dong *et al.*, 2002 |
| *Pseudomonas aeruginosa* | *Zingiber officinale* | AHL acylase | Huang *et al.*, 2003 |
| *Ralstonia* strain XJ12b | *Pterocarpus santalinus* L. | AHL acylase | Lin *et al.*, 2003 |

### 2.4.4 Discussion of QQ endophytic bacteria against human pathogens

There is limited information about QQ endophytic bacteria against human pathogens, few studies, have reported on several bacterial endophytes with quorum quenching activities against well-known human pathogens (see Table 2.2 below).

**Table 2. 2:** Examples of QQ bacterial endophytes against human pathogens.

| Endophytic bacteria | Human pathogen | References |
|---|---|---|
| *Burkholderia glumae* | *Pseudomonas aeruginosa* | Smith *et al.*, 2003 |
| *Bacillus firmus* strain PT18 | *Pseudomonas aeruginosa* | Rajesh & Rai, 2014 |
| *Enterobacter asburiae* strain PT39 | *Pseudomonas aeruginosa* | Rajesh & Rai, 2014 |
| *Geobacillus kaustophilus* strain HTA426 | *Acinetobacter baumannii* | Paluch *et al.*, 2020 |

### 2.4.5 Quorum quenching strategies and resistance

QQ has gained a lot of interest as a potential remedial option to control both the discharge of virulence factors and the development of biofilm. However, the strategy does not bring about the killing of the microbes, hence a milder selection pressure (Alanis, 2005). Ongoing research revealed the impact of QS interruption on bacterial development is subject to the cultivation media utilized (for example supplement rich or not) (Diggle *et al.*, 2007), and may subsequently introduce a selection pressure, much milder than a biocide procedure, and select for resistant microorganisms (Defoirdt *et al.*, 2010; García -Contreras *et al.*, 2013).

QS-interrupted variation studies have shown that bacterial resistance to QS may emerge. Mutations expanding the efflux of C-30, a productive QQ furanone, and compensatory mutations appear as mechanisms to conquer QS interruptions (García -Contreras *et al.,* 2015; Maeda *et al.,* 2012). According to Sandoz *et al.,* (2007) microorganisms that halt QS-controlled factors are "social-con artists". Such QS-resistant mutants meddle with QQ attempts, yet recent studies indicate that QQ obstruction would spread gradually, as these mutants were observed to be less fit than their partners (Tay & Yew, 2013; Gerdt & Blackwell, 2014).

The development of resistance to QQ methodologies strongly depend on the actual technique utilized. The utilization of QQ enzymes is potentially the least resistant of all QQ systems since enzymes can act remotely and do not have to penetrate bacterial cells. Putative resistance mechanisms of QQ enzymes have been investigated and suggests that microorganisms may develop resistance for an expanded creation of the autoinducer molecule (AHL) to balance the hydrolysis by QQ catalysts (Defoirdt *et al.*, 2010; García -Contreras *et al.,* 2013). This could be circumvented by expanding the complete enzymatic movement in nature.

Another obstruction situation would comprise alterations to the chemical structure of the autoinducer. This chance is decreased by the fact that QQ proteins/enzymes are naturally broad spectrum and can be designed for change specificity. Another mechanism would consist of the selection of altered LuxR receptors with a huge liking for the autoinducer or with improved reaction to AHL accordingly. Hence, the QQ enzymes would not be functional enough at these low convergences of autoinducers (Collins *et al.,* 2005; Hawkins *et al.,* 2007). For this situation, enzyme manufacturing may offer answers to produce catalysts with higher attraction for AHLs (Hawkins *et al.,* 2007).

### 2.4.6 Different methodologies used to determine quorum quenching activity

There are three main groups of basic methods for bacterial QQ screening activity, namely: residual AHL measurement, plate inhibition assay and minimum medium assay (Paluch *et al.,* 2020).

AHL measurement by the qualitative and quantitative AI measurement is performed by direct or indirect techniques, for which a biosensor is required. Most of the techniques used are based

on the detection of signalling molecules that have functional groups that respond to specific chemicals giving colour reaction measured (colorimetric) or have bioluminescence ability. This method presents several limitations including, only molecules with a concentration of above 0.6 nM can be detected, therefore, not suitable for $C_{10}$-HSL detection (Patel *et al.,* 2016).

Minimum medium assay is a rapid technique for the separation of bacteria with the QQ ability from natural resources. This method is based on the utilization of AHLs by bacteria as the only source of essential elements and energy (Tang *et al.,* 2013). The method supplements AHLs as the source of nitrogen and carbon, to isolate bacteria with the ability to degrade such molecules because the growth of bacteria unable to resolve them cannot survive in such conditions (Chan *et al.,* 2009). This method also contains a few limitations. Researchers have found that the bacterial species, despite having AHL-degrading activity, are unable to grow in medium lacking in nutrients and other crucial components (Chan *et al.,* 2009; Uroz *et al.,* 2009). Therefore, this method is only a preliminary screening technique and it does not allow for the determination of which QQ molecules these bacteria use and how.

The plate diffusion assay is a method employed for the identification of QQ-producing bacteria. The principle of this method is that QS-inhibiting molecules produced by tested bacterial strains can infiltrate through the solid medium, and to detect them a biosensor strain is required (McLean *et al.,* 2004). This method is time-consuming, laborious, and the only parameter measured is inhibition zones that can be inaccurate hence a strong QQ activity is needed to observe them (Liu *et al.,* 2010).

## 2.5 RESEARCH GAP

The rapid and prevalent occurrence of antibiotic resistance in many microbial pathogens continues to emphasise the importance to explore new revenues to prevent and manage the progression of infectious diseases. Therefore, the importance of screening for endophytic bacteria for their functional role, appeals as a promising approach for alternative antipathogenic agents to overcome the increasing threats of drug resistance against human and plant pathogens (Tan & Zou, 2001). Insights on bacterial QS interactions present alternative opportunity to gain control in microbe-microbe and pathogen-host interactions. Bacterial endophytes have been hypothesized to produce enzymes that could potentially be able to interfere with QS systems of many microbial pathogens. Therefore, suggesting that QQ could potentially be an innate host defence mechanism. However, there have not been sufficient reports specifically on

bacterial endophytes enzymes isolated from traditional medicinal plants, thus against this background, the present study was conducted.

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# CHAPTER 3

# MATERIALS & METHODS

## 3. INTRODUCTION

The main objective of the work reported in this chapter was to evaluate the diversity of endophytic bacteria isolated from plant leaves of 6 medicinal plants of interest, namely; *Mondia whitei, Artemisia afra, Aloe vera, Bulbine alooides, Alpinia galangal,* and *Tulbaghia violaceae.* Thereafter to further screen, the bacterial isolates for their potential quorum quenching activities and assess their antibiofilm properties against major biofilm-forming pathogens, namely; *Staphylococcus aureus, Serratia marcescens, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Proteus mirabilis* that threaten many people's health and animals' health. The following methodologies were carried out to achieve the overall aim/objective of this study.

### 3.1 Bacterial strains and chemicals

AHL biosensor strain *Chromobacterium violaceum* CV026, does not produce violacein and detects short-chained AHLs ($C_4$-$C_8$) and AHL biomonitor strain (*Chromobacterium violaceum* ATCC 12472, produces violacein) was kindly donated to our laboratory by Gina from Centre for Microbial Ecology and Genomics (CMEG), University of Pretoria. *Chromobacterium* strains were grown in Luria-Bertani (LB) broth at 30 ℃ in rotary shaker incubator for 24 hours and stored in glycerol stocks at -75 °C. *Bacillus cereus* ATCC 14579 was used as a positive control strain for AHL-degrading activity. Six test organisms (*Staphylococcus aureus* ATCC 25923, *Serratia marcescens* ATCC 13880, *Bacillus subtilis* ATCC 6633D-5, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC BAA-47 and *Proteus mirabilis* ATCC 33583) were purchased from American Type Culture Collection, USA and used for biofilm studies.

### 3.2 Medicinal plant collection and surface sterilization

Six medicinal plants (n = 6, to limit the scope of research) of interest (Table 3.1.) were collected within the surroundings of the University of Pretoria, Hatfield campus, South Africa with the assistance of Mr. Jason Sampson (Department of Plant Science, University of Pretoria, SA). The collected plants were submitted to the plant herbarium repository for voucher specimens in the Plant Sciences Department, University of Pretoria, Hatfield campus, South Africa. The plants were chosen on basis of their reported medicinal use.

Before use, the plant leaves were surface sterilized using sterile distilled water, followed by 70% ethanol (v/v) for approximately 2 minutes, thereafter washed with sterile distilled water again. The leaves were disinfected by immersion in 0.5% sodium hypochlorite for 5 minutes, followed by rinsing with sterile distilled water (Sinha *et al.,* 2015). Furthermore, the samples were rinsed three times with de-ionized water to eliminate any possible contaminants present (Sinha *et al.*, 2015; Preveena & Bhore, 2013).

**Table 3. 1:** Medicinal plants of interest used for endophytic bacteria extractions.

| Plant name | Family | Reported medicinal uses | Specimen voucher no: |
|---|---|---|---|
| *Aloe vera* | *Asphodelaceae* | External burn treatment, constipation, skincare, an anti-inflammatory agent (Manvitha & Bidya, 2014). | PRU 125465 |
| *Bulbine alooides* | *Asphodelaceae* | Used in the treatment of HIV and AIDS (Klos *et al.,* 2009). | PRU 125466 |
| *Artemisia afra* | *Asteraceae* | Used for the treatment of colds, headaches, chills, dyspepsia, loss of cravings, bladder and kidney issues (Thring & Weitz, 2006). | PRU 125463 |
| *Alpinia galangal* | *Zingiberaceae* | Used for the treatment of diabetes mellitus, microbial infections, inflammations, chest pains, rheumatic pains and fever (Chouni & Paul, 2018). | PRU 125464 |
| *Tulbaghia violacea* | *Amaryllidaceae* | Used as a snake anti-agent, for the treatment of flu, fevers, skin diseases and asthma (Street, 2008). | PRU 125461 |
| *Mondia whitei* | *Apocynaceae* | Used as an aphrodisiac, treatment of sexual weaknesses, prevent premature ejaculation and increase sperm production, also used as an anti-depressant (Oketch-Rabah, 2012). | PRU 125462 |

### 3.3 Extraction and isolation of endophytic bacteria from medicinal plants

The sterilized plant leaves were removed using a sterilized razor blade, cut into pieces (3mm long), then placed onto prepared sterile Tryptone Soy Agar (TSA) plates (30g Tryptone Soy Broth (TSB) (Oxoid, England) and 15g Agar No. 2 bacteriological (Neogen, United Kingdom) per litre) and negative control plates (last rinse water) was included. Plates were incubated at

30 °C for 1-7 days until the growth of endophytic bacteria were observed on experimental plates and none on the negative control plate (El-Deeb *et al.,* 2013).

Some fragments of leaves were homogenized in 5 ml of 0.9% saline solution. Tissue extracts were serially diluted in 0.9% saline solution and plated in triplicates to recover any bacterial endophytes. The tissue extracts were then plated onto TSA (30g TSB and 15g Agar) plates and incubated at 30 °C until growth was observed. After the incubation period of 7 days, bacteria were recovered from agar plates and selected at random, purified and grouped according to phenotypic and morphological characteristics. A certain number of those that form part of each group were selected for further studies and analysis (El-Deeb *et al.,* 2013).

## 3.4 SCREENING FOR QQ POTENTIAL OF THE ISOLATED ENDOPHYTIC BACTERIA

### 3.4.1 Screening of QQ potential using a cross-feeding assay

To assess whether the endophytic bacterial isolates produce AHLs, a cross-feed assay was employed. This assay is dependent on the induction of the purple pigment production called violacein by the *Chromobacterium violaceum* CV026 after diffusion of the AHLs into the medium produced by the endophytic bacterial strains (Shang *et al.,* 2014). Biosensor strain *Chromobacterium violaceum* CV026 and biomonitor strain *Chromobacterium violaceum* ATCC 12472 and endophytic bacterial isolates were parallel-streaked as homogenous lines on LB agar medium (Figure 3.1). After incubation at 30º C for 24 hours, no appearance of purple pigment in CV026 could potentially reveal the degradation of natural AHLs by bacterial isolates and/or also indicate the presence of long-chained AHLs thus no violacein will be present as CV026 only commonly detects short-chained AHLs ($C_4$-$C_8$) molecules. The appearance of purple pigment in CV026 indicated no degradation of natural AHLs as well as the production of AHLs by the isolates. The bacterial isolates that showed stable and strong degradation activity of natural AHLs produced by ATCC 12472 were selected for further studies (Ha *et al.,* 2018). This experiment was done in triplicates.

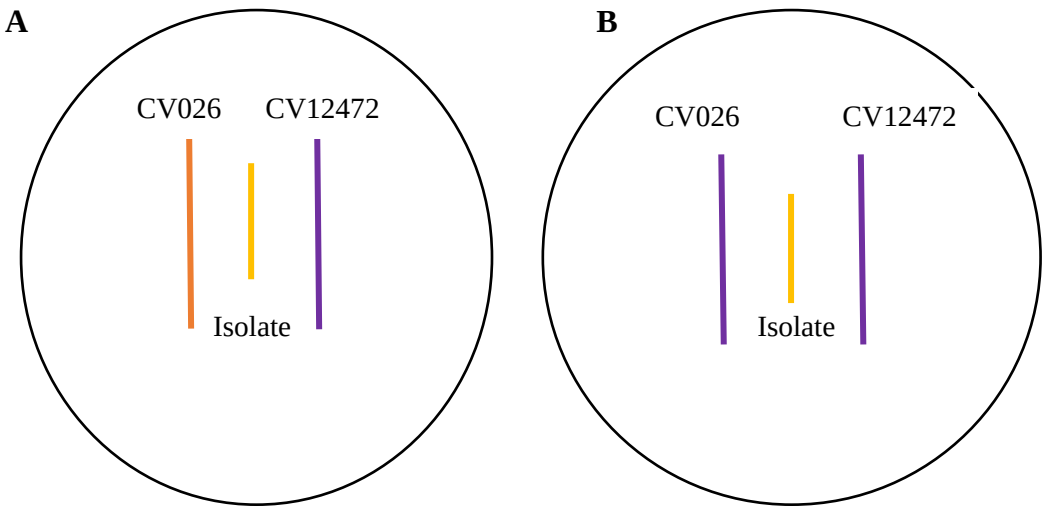


**Figure 3. 1:** Images depicting different results of QQ bioassay for natural degrading quorum quenching bacterial endophyte isolates. [A] No appearance of purple pigment in CV026 indicates the quorum sensing inhibition of bacterial isolates, [B] The appearance of purple pigmentation on CV026 indicates quorum sensing.

### 3.4.2 Cross-streak assay of non-quorum quenching isolates

To validate whether these isolates displayed an AHL-based QS regulation system, bacterial cultures were cross-streaked with the AHL biosensor CV026 (does not produce violacein, purple pigment) without exogenous AHLs (Figure 3.2). For AHLs detection, *Bacillus cereus* ATCC 14579 and *Chromobacterium violaceum* ATCC 12472 served as positive and negative controls, respectively (Chang *et al.*, 2012). This experiment was done in triplicates.

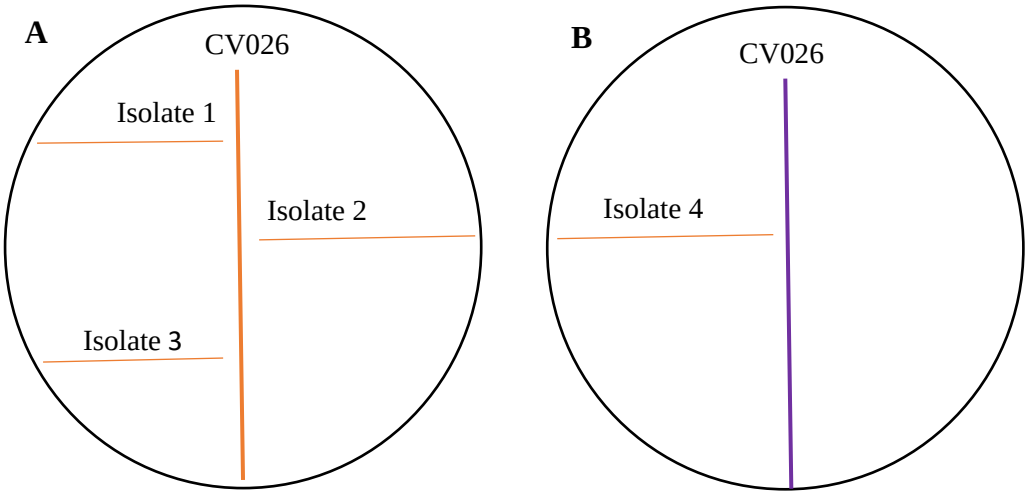

**Figure 3. 2:** Schematic diagram depicting cross-streak assay of *Chromobacterium violaceum* CV026 against different bacterial endophytic isolates to assess the quorum sensing activity of isolates. [A] No production of violacein observed on CV026, therefore no quorum sensing and [B] production of violacein observed on CV026, therefore there is quorum sensing.

## 3.5 PREPARATION OF CELL-FREE LYSATE

Cell-free lysates of bacterial endophytes were prepared according to the method described by Rajesh & Rai (2014) with minor modifications. Briefly, the endophytic bacterial isolates were developed in LB broth (25g of Luria Bertani broth (per litre)), incubated at 30°C with shaking for 48 hours followed by harvesting by centrifugation at 12,000 rpm for 10 minutes. The cell pellets were collected and suspended in 10 ml of potassium phosphate buffer (PPB, 100 mM; pH 7.0) and ground using a sonicator at 4°C for 45 -60 seconds to get cell-free lysate and centrifuged. The supernatant was sifted through 0.22 µm filter and the filtrate was collected as cell-free lysate which was stored at −20°C until use. All assays were performed with assay control (PPB) and positive control (cell-free lysate of *Bacillus cereus*).

## 3.6 IDENTIFICATION OF ENDOPHYTIC BACTERIA

### 3.6.1 Identification of bacterial endophytes with MALDI-TOF

Precisely 24 hours after incubation at 30°C in an incubator, single colonies of each isolate were picked from nutrient agar (NA) plates using a sterile toothpick. A thin smear of the single bacterial colony was made on the MALDI-target plate. The plate was left to air dry and 1 µl of matrix added over the dried smear and left to air dry. Samples were registered on MALDI-System and the MALDI-target plate loaded onto the Bruker Daltonik MALDI Biotyper (Bruker, USA) and ran the system to obtain results (Figure 3.3) (Wieser *et al.*, 2012). Measurements were performed in a linear positive ion mode, using a nitrogen laser (337 nm) at 50 Hz frequency. The acceleration voltage was 20 kV with delay acquisition and mass range of 3000-20 000 *m/z*.

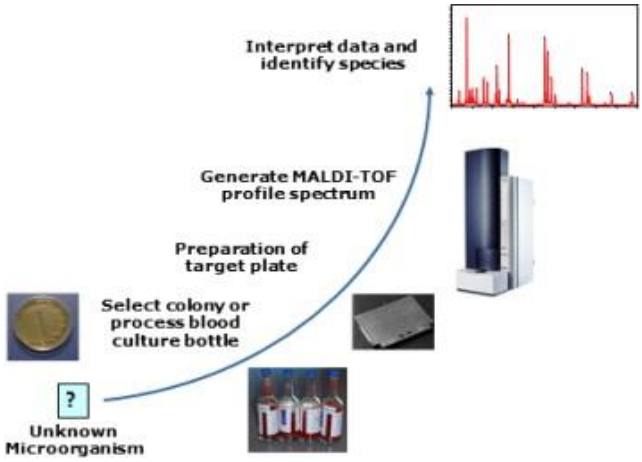


**Figure 3. 3:** Microbial identification using Bruker Daltonik MALDI Biotyper. (Image adapted from Murray, 2012). Unknown microorganisms are selected from the agar plate as single colonies and placed onto a MALDI target plate in duplicates. A profile spectrum on the MALDI software is generated and the system ran to generate data that can be used to identify unknown microorganisms.

### 3.6.2 DNA Extractions

Further identification of bacterial endophytic isolates, genomic-DNA was extracted from 24 hours old cultures of respective bacteria using the GeneJet genomic DNA purification kit (Thermo Fisher Scientific, USA) according to manufactures instructions. Briefly, about 2 ml of the 24 hours old bacterial culture was centrifuged for 10 minutes at 8000 rpm and the supernatant discarded. Pellets were resuspended in the digestion solution followed by the addition of Proteinase K and mixed thoroughly by a vortex. Sample mixtures were incubated at 56°C for approximately 30 minutes while mixing with vortex occasionally until the cells were completely lysed. RNase A solution was added to the solution and mixed thoroughly at room temperature followed by the addition of lysis solution and mixed thoroughly once again by a vortex. About 400 µl of 50% ethanol was added to the samples and mixed by vortex then transferred the prepared lysate to the GeneJet Genomic DNA purification column inserted in a collection tube. Samples were centrifuged for a minute at 8000 rpm and the supernatant discarded and replaced the collection tube with a clean one. Samples were thereafter washed using wash buffer 1 and centrifuged and discarded supernatant and washed again with wash buffer 2 and centrifuged once again and discarded supernatant and replaced the collection tube with sterile 1.5 ml or 2 ml tube and placed the purification column in them and added elution buffer then incubated for 2 minutes at room temperature before centrifugation at maximum speed for 1 minute. Samples were stored at -20°C until use.

### 3.6.3 PCR amplification

Preparative PCR amplification of 16S rRNA reactions were performed using the Phusion High Fidelity PCR kit (Thermo Fisher Scientific, USA) in volumes up to 25 µl containing, 0.5 µl of bacterial universal primers 27F (5`-GAGTTTGATCACTGGCTCAG-3`) and 1492R (5`-TACGGCTACCTTGTTACGACTT-3`) (Byers *et al.,* 1998), 2 µl of DNA template, 9 µl of molecular grade water and 10 µl of 2x Phusion master mix. (Thermo Fischer Scientific, USA).The thermocycling conditions using Bio-Rad T100 thermocycler (Bio-Rad Laboratories, USA) were maintained with an initial denaturation at 98 °C for 10 seconds, 30 amplification cycles of 98°C for 5 seconds, 60°C for 15 seconds, 72°C for 45 seconds and final polymerization step of 72°C for 1 minute. The PCR product was resolved in 1% agarose gel.

### 3.6.4 PCR purification and sequencing

PCR products were purified using the ZymoResearch DNA recovery kit (Zymo Research, USA). The sequencing reactions were prepared using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA) and then sequenced on an ABI 377 Automated Capillary DNA Sequencer (Applied Biosystems, USA). For the identification of the isolated endophytic bacteria, the 16S rRNA locus sequences were compared to those in the nucleotide database, GenBank (http://www.ncbi.nlm.nih.gov), using BLASTn comparisons.

## 3.7 PCR AMPLIFICATION OF *AII*A HOMOLOGUE GENE

The bacterial strains which identified positive for AHLs degradation bioassay were affirmed for AHL-lactonase activity by amplification of *aii*A homologue gene. Genomic DNA was amplified by using the forward *aii*A F (5`ATGGGATCCATGACAGTAAAGAAGCTTTAT-3`) and reverse *aii*A R2 (5`GTCGAATTCCTCAACAAGATACTCCTAATG-3) primers (Nusrat *et al.,* 2011). The thermal cycling conditions were maintained as initial denaturation at 98°C for 10 seconds, 30 cycles of 98°C (5 seconds), 60°C (15 seconds), 72°C (45 seconds) and final primer extension at 72°C for 1 minute. The PCR amplicons were gel purified and sequenced. The sequence was submitted to the GenBank database for verification (Dong *et al.,* 2002). The *aii*A was isolated, amplified and sequenced simply because it is the first protein identified that is capable of enzymatic inactivation of *N*-acylhomoserine lactones (Dong *et al.,* 2000).

## 3.8 EFFECT OF QUORUM QUENCHING ACTIVITY ON BIOFILM FORMING BACTERIA

### 3.8.1 Biofilm assay

The impact on biofilm by cell-free lysate of endophytic bacteria was tested utilizing static microtiter plate examination of Cady *et al.,* (2012) and performed in triplicates. Biofilm forming bacteria (*Staphylococcus aureus, Serratia marcescens, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Proteus mirabilis*) were grown in LB agar for 24 hours at 37°C and single colonies of each were dissolved in sterile distilled water. The dissolved cultures were resuspended in Luria Bertani broth to standardize the pathogen ($OD_{600nm}$=0.1) absorbance was read using Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, USA). Then 100 µl of sterile LB broth were transferred into sterile 96 well microtitre plates followed by 100 µl of freshly inoculated culture alongside 0.22 µm filter disinfected 100 µl of cell-free lysate was transferred into 96-well microtiter plate. The assay controls had no enzyme treatment (PPB). The plate was incubated at 37°C for 24 hours without shaking. The contents of each well were removed, the plates were washed thrice with sterile distilled water to remove loosely attached cells and non-adherent cells. Plates were thereafter dried in the oven for 45 minutes at 60°C and then stained with 100 µl of 0.1% crystal violet for 15 minutes incubated at room temperature. Afterward, plates were washed completely with distilled water multiple times. Ethanol (95%) was utilized to expel crystal violet from the biofilm and absorbance was estimated at 585 nm with SpectraMax Paradigm Multi-Mode Microplate Detection Platform (O`Toole, 2011). The experiment was performed in triplicates by inoculating samples in 3 wells containing the same cell-free lysates of bacterial endophytes. Positive control (Cell-free lysate of *B. cereus*) and negative control (Potassium phosphate buffer) was included. The average of the absorbance from 3 wells of each endophytic bacterial isolate treated pathogen was used to calculate the percentage biofilm inhibition which was reported as results (Bazargani & Rohloff, 2016).

$$\text{Percentage biofilm inhibition} = \frac{\text{OD negative control} - \text{OD experimental}}{\text{OD negative control}} \text{ X } 100$$

### 3.8.2 Pre-formed biofilm

The effect on preformed biofilm by cell-free lysate of endophytic bacteria was tested using a static microtiter plate assay of Cady *et al.* (2012) and performed in triplicates. Briefly, Biofilm forming bacteria *(Staphylococcus aureus, Serratia marcescens, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa*, and *Proteus mirabilis*) were grown in LB agar for 18 hours at 37°C and *Serratia marcescens* at 30°C and single colonies of each were dissolved in sterile distilled water. The dissolved culture was resuspended in Luria Bertani broth to standardize the pathogen ($OD_{600nm}$=0.1). Then 100 µl of sterile LB broth was transferred into 96 well microtitre plates followed by 100 µl of freshly inoculated culture and incubated at their respective temperatures for 8 hours to allow the biofilm to mature. After incubation, 0.22 µm filter-sterilized 100 µl of cell-free lysate was inoculated into the 96-well microtiter plate containing the matured biofilm. The assay controls were maintained without any enzyme treatment (PPB). The plate was incubated at 37°C for 24 hours without shaking. Planktonic cells were removed, the plates were washed thrice with sterile distilled water and dried in the oven for 45 minutes at 60°C. Thereafter, stained with 100 µl of 0.1% crystal violet for 15 minutes incubated at room temperature and then washed thoroughly with sterile distilled water 3 times. Ethanol (95%) was used to remove crystal violet from the biofilm and absorbance was measured at 585 nm with SpectraMax Paradigm Multi-Mode Microplate Detection Platform (Bazargani & Rohloff, 2016). The experiment was performed in triplicates by inoculating samples in 3 wells containing the same cell-free lysates of bacterial endophytes. Positive control (Cell-free lysate of *B. cereus*) and negative control (Potassium phosphate buffer) was included. The average of the absorbance from 3 wells of each endophytic bacterial isolate treated pathogen was used to calculate the percentage biofilm inhibition which was reported as results.

## 3.9 CONFOCAL LASER SCANNING MICROSCOPY (CLSM) ANALYSIS OF BIOFILMS

Precisely 24 hours post-exposure of biofilms to QQ potential bacterial endophytes, the biofilms of the test organisms (mentioned earlier) were washed thrice with sterile distilled water. Biofilm cell viability was determined by live/dead staining (Thermofisher, USA). The plates were then incubated for 15 minutes at 25°C in the dark/covered. The biofilm images were acquired using a confocal laser scanning microscope (Carl Zeiss AG, Germany) (Cerca *et al.*, 2012).

### 3.10 STATISTICAL ANALYSIS

One sample test and a t-test for equal means were used to analyse the significant effect of QQ treatments on biofilm formation. Statistical analysis was performed using PAST 3.22 software.

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# CHAPTER 4

# RESULTS & DISCUSSION

## 4. INTRODUCTION

Higher plants have a differentiated network of endophytic microorganisms and a symbiotic relationship. Bacteria receive nutrient rich environment, while they offer host plant defense against pathogens, drought and other harmful external stimuli (Rajesh & Rai, 2014). Bacterial endophytes associated with medicinal plants have been under-investigated, and this area remains a rich and unexploited reservoir of secondary metabolites with quorum quenching potential. In this chapter, the results of the isolated bacterial endophytes identified using MALDI-TOF and 16S rRNA screened for their QQ potential, QS activity, AHL lactonase activity, and antibiofilm activities are succinctly discussed.

## 4.1 ISOLATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA FROM PLANT LEAVES

Results obtained in this study revealed that medicinal plants (*Artemisia afra, Alpinia galangal, Aloe vera, Bulbine alooides, Mondia whitei* and *Tulbaghia violacea*) harbour an abundance of culturable endophytic bacteria. Thirty-four (34) endophytic bacteria were recovered from the leaves of six medicinal plants. The frequency of endophytic isolates was found to differ among the medicinal plants. The majority of the isolates were from *Aloe vera* 29.41% (10/34), followed by *Bulbine alooides* and *Mondia whitei* with an equal isolate percentage of 17.65% (6/34), and then followed by *Artemisia afra* plant 14.71% (5/34) and *Alpinia galangal* plant with 11.76% (4/34) isolates (Figure 4.1). The plant with the least isolates was *Tulbaghia violacea* with only 8.82% (3/34) isolates (Figure 4.1). Of the 34 isolates, 10 (29.41%) had overgrowth, thus were discarded. Only a subset (24/34) isolates were investigated as these were able to grow well on the laboratory isolation media. The full diversity and richness of endophytes in each plant, therefore, could have been greater. The absence of a bacterial colony on control plates (last rinse water) confirmed that isolates obtained are endophytes (Etiminani & Harighi, 2018).

A sum of 71% (24 isolates) of the isolates was subjected to morphological characterization. Nearly 62.5% (15/24) and 37.5% (9/24) of isolates were Gram-negative and positive respectively (Appendix I).

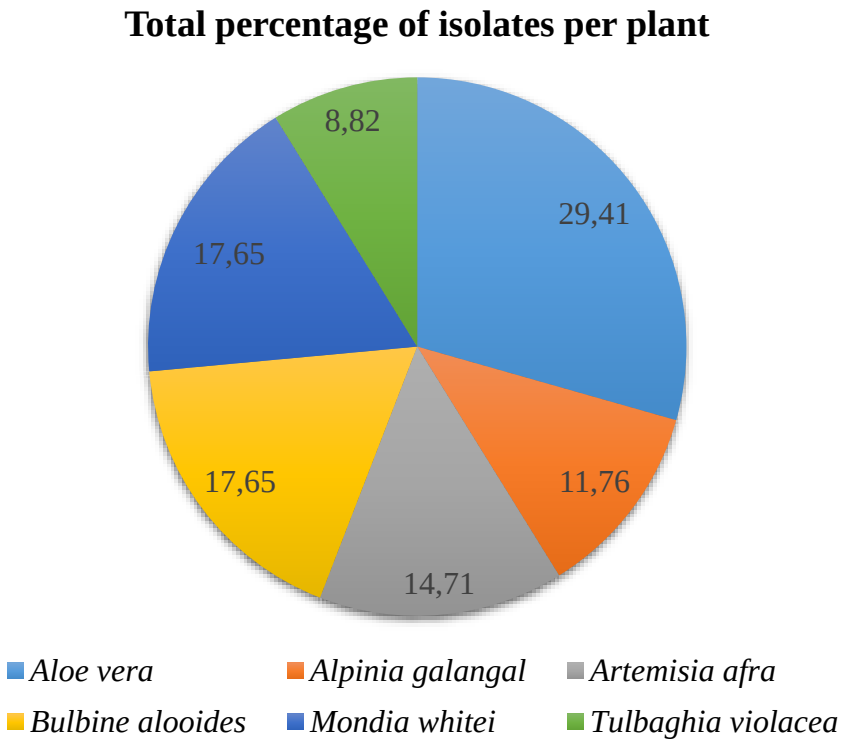


**Figure 4. 1:** Pie chart depicting the total percentage of isolates recovered per plant.

Based on literature, the majority of endophytes are soil-borne (Wang et al., 2013). Our data, suggests abundance of endophytic bacteria found in medicinal plant leaves and possibly in other plant parts. As reported by Kandel *et al.,* (2017), soil make-up and host plant genotype play a vital role in the enrolment of bacterial endophytes by the host plant. Some endophytic strains move progressively to raised parts, for instance, xylem, bark, leaves, etc. However, there is an enormous bacterial populace density decrease conversely with those that cautiously colonize the roots (Compant *et al.,* 2010). Additionally, bacterial quorum sensing compounds are likely associated with correspondence with the plant root and consequent colonization process (Zúñiga *et al.,* 2013).

A few reports demonstrate that bacterial endophytes help to give supplements as plant-development promoters and initiate resilience/obstruction against biotic and abiotic stress conditions (Ryan *et al.,* 2008). There have not been any reports on the five test plants except for *A. vera* used in this study as a source of bacterial endophytes, hence this work is the first to report of the presence of bacterial endophytes in *Artemisia afra, Alpinia galangal, Bulbine alooides, Mondia whitei* and *Tulbaghia violacea.*

## 4.2. BACTERIAL IDENTIFICATION

### 4.2.1 MALDI-TOF identification

Twenty-four (24) bacterial isolates were subjected to MALDI-TOF analysis for identification purposes. The most dominant genus observed was *Bacillus* with a match of above 30%, followed by *Pseudomonas* with a match of above 25% and then followed by *Acidovorax* with a match of 12.5%. *Micrococcus* and *Raoultella* being the least genus observed with a match of 4.2% each. (Figure 4.2).

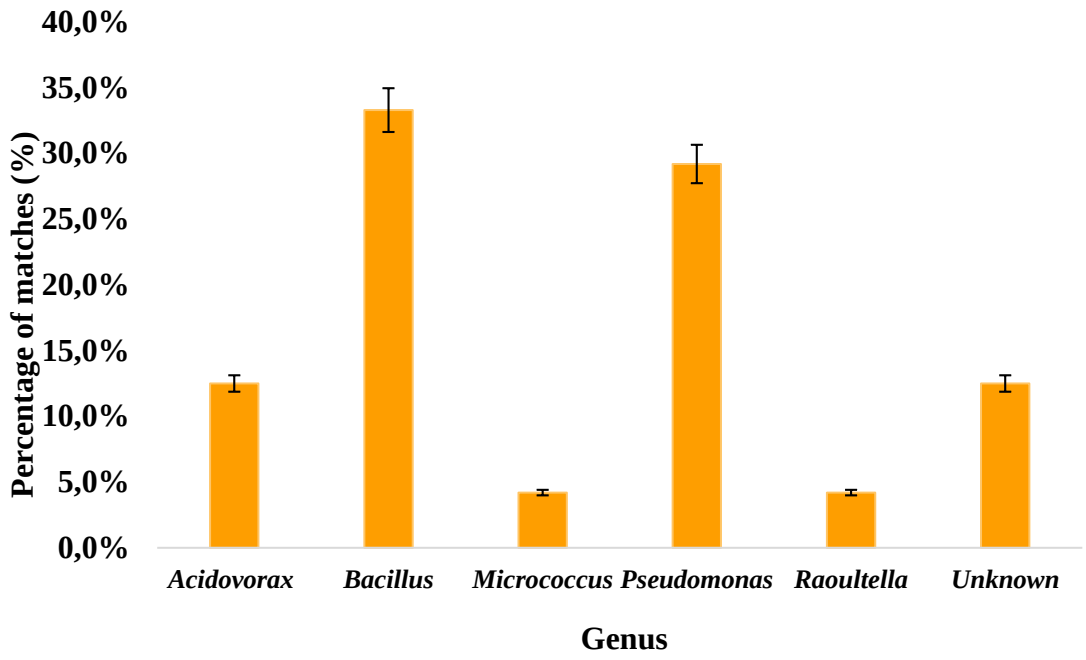


**Figure 4. 2:** Column graph depicting the most dominant bacterial genus observed by MALDI-TOF system.

The twenty-four (24) bacterial endophyte spectra were stored in the node of projects taxonomy tree linked to the MALDI BioTyper taxonomy tree database to identify the bacterial endophytes isolated. The Dendrogram generated from the 24 registered isolates spectra pointed out distinctive clusters (Figure 4.3). The 24 bacterial endophytes spectra constituted the first cluster with a distinct spectrum of one single strain (*A. temperans*). A distance level of 400 separated the *A. temperans* spectra. Whereas, sub-clustering reduced the maximal distance level to 150. A second cluster regrouped two *P. plecoglossicida* spectra. Three strains of *P. plecoglossicida* were distantly related to others, namely; *P. plecoglossicida –AV6, P. plecoglossicida-AV2,* and *P. plecoglossicida-AV9.* The whole of the spectra of Bacillus, unknown-BA3, and unknown-

BA1 made up another cluster with a maximum distance level of 350. All of the spectra of *P. fluorescens*, *P. aeruginosa*, and *R. terrigena* also made up another cluster with a maximum distance level of 600. *B. cereus-MW3*, *B. cereus-MW2* and *B. cereus-MW1* also made up another cluster with a maximum distance level of 150. The ten remaining strains/isolates united were in one cluster group assembling the spectra of other isolates. The clusters of all 24 isolates were not fully resolved and thus the results were inconclusive, which led to 16S rRNA sequencing to confirm results observed on MALDI-TOF. The 16S rRNA data largely agreed with the MALDI-TOF data.

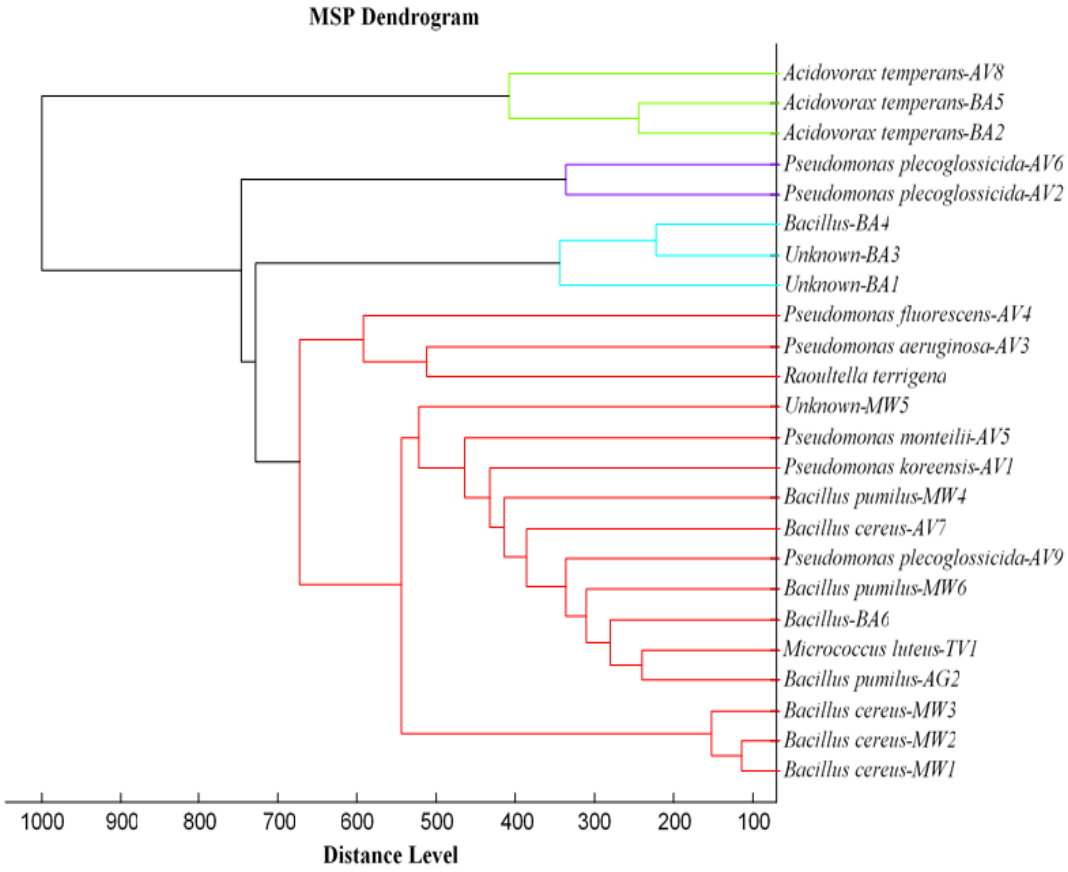


**Figure 4. 3:** Hierarchical cluster Dendrogram of 24 bacterial endophyte isolates.

Literature documents *Bacillus* and *Pseudomonas* as the most dominant genera in naturally occurring endophytic samples (Radhakrishnan *et al.*, 2017). With reference to Figure 4.2, our data agrees with the current literature standing as we found *Bacillus* and *Pseudomonas* to be the most commonly occurring. Both genera are well known for their antagonistic activities against many plant pathogens and thus contribute to the overall health and growth of their plant

hosts (Radhakrishnan *et al.*, 2017). *Bacillus* and *Pseudomonas* species are responsible for shielding their host plants from the attack of phytopathogens. For example, *Bacillus* species account as one of the most plenteous metabolites creating Gram-positive bacterial endophyte found in assorted environments (Reinhold-Hurek & Hurek, 2011; Frank *et al.*, 2017). This substantiates why *Bacillus* and *Pseudomonas* were the most common genera isolated in our study.

The potential clarification for the pervasive presence of *Bacillus* species and some *Pseudomonas* species in plant tissues is the assorted variety of the constructive outcomes on plant development and wellness they have displayed, by animating the host phenylpropanoid pathway or by delivering a few connected metabolites to the plants' digestion (Brader *et al.*, 2014; Haider *et al.*, 2016).

*Micrococcus* and *Raoultella* were the least genera observed. This could be because of the ecological conditions, for example, soil nourishment, moisture, temperature, and host genotype and age impact leaf bacterial communities of the test plants (Wagner *et al.*, 2016). *Micrococcus* is a known endophyte found mostly in wheat and potato plants (Barros *et al.*, 2010). Hence, these outcomes may mirror the presence of a particular bacterial network in these restorative plants or it might potentially mirror the restrictions of plant section confinement techniques utilized in this work. The segregation strategy utilized in this investigation possibly supports quickly developing bacterial species over slow-growing and less competitive bacterial species.

Akinsanya *et al.*, (2015), isolated various quantities of endophytes in various plants from different areas. Consequently, this is apparent that there is an incredible decent variety of endophytes in different plant tissues. Sturz *et al.* (1997) noticed that the endophytic populace of microbes is diverse in various plant hosts. In our examination, we have isolated *Acidovorax* sp., *Bacillus* sp., *Pseudomonas* sp., *Micrococcus* sp. and *Raoultella* sp. from plant leaf tissues (Figure 4.2). This shows that endophytic networks in the six therapeutic plants were diverse and the degree of decent variety may fluctuate fundamentally between plant species even from a similar area.

### 4.2.2 16S rRNA identification

To validate the MALDI-TOF results, the isolates were subjected to identification by amplifying the genomic DNA followed by 16S rRNA gene sequencing. The isolates were tentatively identified by BLAST analysis of obtained 16S rRNA sequence. Sequence analysis 16S rRNA of all isolates were successfully identified and the results are tabulated below. The comparability levels of sequences acquired were in the range 94-98% for *Bacillus* sp. and *Pseudomonas* sp. Nonetheless, 94% comparability for the isolate Ba 6 (*B. subtilis*) and Av 3 (*P. aeruginosa*) (Table 4.1) requires further examination.

**Table 4. 1:** Molecular identification of bacterial endophytes from six medicinal plants of interest based on blastN queries on NCBI.

| Isolate Code | Identified name | % Identity | Accession number |
|---|---|---|---|
| ***Ba 1*** | *Bacillus sp.* strain 7M1 | 98.55 | KY008747.1 |
| ***Ba 2*** | Uncultured *Acidovorax* sp. clone G13-M-7-E04 | 94.34 | FJ192061.1 |
| ***Ba 3*** | *Bacillus subtilis* strain 07-2 | 97.28 | EU596480.1 |
| ***Ba 4*** | *Bacillus subtilis* SY | 96.29 | JQ410791.1 |
| ***Ba 5*** | *Acidovorax sp.* strain 8160P6 | 97.94 | MK757665.1 |
| ***Ba 6*** | *Bacillus subtilis* strain Fito F264 | 94.64 | MG836682.1 |
| ***Aa 1*** | *Klebsiella* sp.5 | 89.90 | KC508809.1 |
| ***Ag 2*** | *Bacillus sp.* hb11 | 97.52 | KF863811.1 |
| ***Tv 1*** | *Micrococcus luteus* strain BMC3N12 2 | 96.48 | MG996880.1 |
| ***Av 1*** | *Rangifer tarandus greenlandicus* Isolate BAT5 | 90.32 | AF096441.1 |
| ***Av 2*** | Bacterium enrichment culture Clone AC01 GCFRUDD03FLFIE | 91.64 | JF688176.1 |
| ***Av 3*** | *Pseudomonas aeruginosa* strain jb9 | 94.71 | MF426269.1 |
| ***Av 4*** | *Pseudomonas azotoformans* strain P31 BA1H | 96.55 | MK883069.1 |
| ***Av 5*** | *Pseudomonas sp.* strain MY720 | 97.37 | MK680062.1 |
| ***Av 6*** | Bacterium strain BS0279 | 96.03 | MK823467.1 |
| ***Av 7*** | *Bacillus cereus* strain vpp1 | 97.05 | KM596528.1 |
| ***Av 8*** | Uncultured *Acidovorax sp.* clone DVBSD D315 | 98.21 | KF464896.1 |
| ***Av 9*** | *Pseudomonas sp.* strain TGRB5 | 95.44 | MH810307.1 |
| ***Mw 1*** | *Bacillus cereus* strain LJ4 | 97.14 | KF515654.1 |
| ***Mw 2*** | *Bacillus cereus* strain MJHN10 | 98.16 | MF693121.1 |
| ***Mw 3*** | *Bacillus cereus* isolate BRL02-31 | 98.22 | DQ339663.1 |
| ***Mw 4*** | *Bacillus sp.* MF3 | 97.95 | KF679677.1 |
| ***Mw 5*** | *Bacillus sp.* PR13 | 98.83 | JN208180.1 |
| ***Mw 6*** | *Bacillus pumilus* strain AMST.CvLe1 | 97.16 | MF615209.1 |

The microbial variety analysis uncovered the diverse composition of endophytic networks from the leaves of the six restorative plants. Findings distinguished 24 culturable endophytic bacteria isolates having a place within 5 distinct genera (Figure 4.2). The analysis of the 16S rRNA has uncovered the most pervasive endophytic bacterial genus in *A. vera* to be *Pseudomonas* (44.4%; 4/9 isolates). These outcomes correspond with Akinsanya *et al.*, (2015) in which *Pseudomonas* was recognized as one of the most pervasive endophytic microscopic organisms isolated from *A. vera* plant tissues. Idu *et al.*, (2010) reported *Bacillus* as the predominant species found in *M. whitei*. These results are in agreement with those obtained in this study which shows that the most predominant genus recognized from *M. whitei* was *Bacillus* (100%; 6/6 isolates). *Bacillus* was also the most predominant genus distinguished in *B. alooides* plant (66.67%; 4/6 isolates). The three strains of *Acidovorax* have been recently reported to be closely identified with the *Pseudomonas* genus (Willems *et al.,* 1990). A few reports (Berg *et al.,* 2005; Ren *et al.,* 2013; Saidi *et al.,* 2013) have similarly archived the presence of microorganisms belonging to *Bacillus* and *Pseudomonas* genus inside different parts of different plants as endophytic bacteria. A portion of the strains recognized in this investigation is associated with plant growth promotion and or biocontrol movement (Rosenblueth & Martinez-Romero, 2006).

## 4.3 QUORUM QUENCHING ACTIVITIES

Bacterial endophytes were subjected to a cross-feeding assay to screen for their QQ activity. The results (Figure 4.4) depict the different categories of QQ activities of the 24 isolates evaluated against the biosensor strain and the biomonitor strain. Of which 58.33% (14/24) endophytic bacterial strains showed some degree of QQ activity, whereas, 41.67% (10/24) showed no QQ activity. Of the active QQ isolates only 21.43 % (3/14) isolates showed strong/ effective QQ activity, 42.86 % (6/14) showed moderate QQ activity and only 35.71% (5/14) showed 2$^{nd}$ class moderate QQ activity.

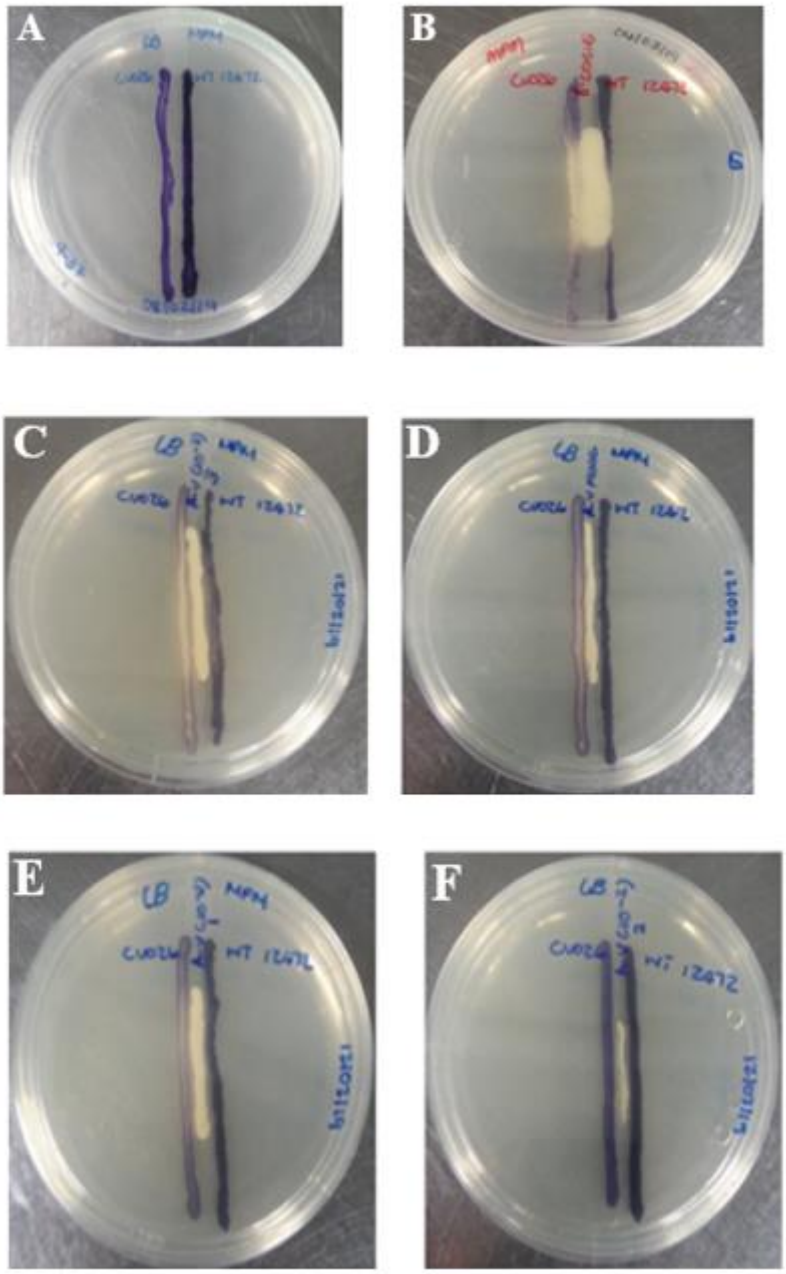


**Figure 4. 4:** Agar plate cross-feeding assay for screening of QQ bacterial endophyte isolate strains between *Chromobacterium violaceum* CV026 and *Chromobacterium violaceum* ATCC 12742**. [A]** Test plate, **[B]** Positive control plate (*Bacillus cereus* isolate), **[C]** Effective QQ activity, **[D]** Moderate QQ activity, **[E]** 2nd class moderate QQ activity and **[F]** Ineffective/ no QQ activity. Evidence for successful QQ activity is indicated by the opaque colour of CV026 and evidence for the production of AHL is indicated by the purple colouration of the biosensor strain CV026.

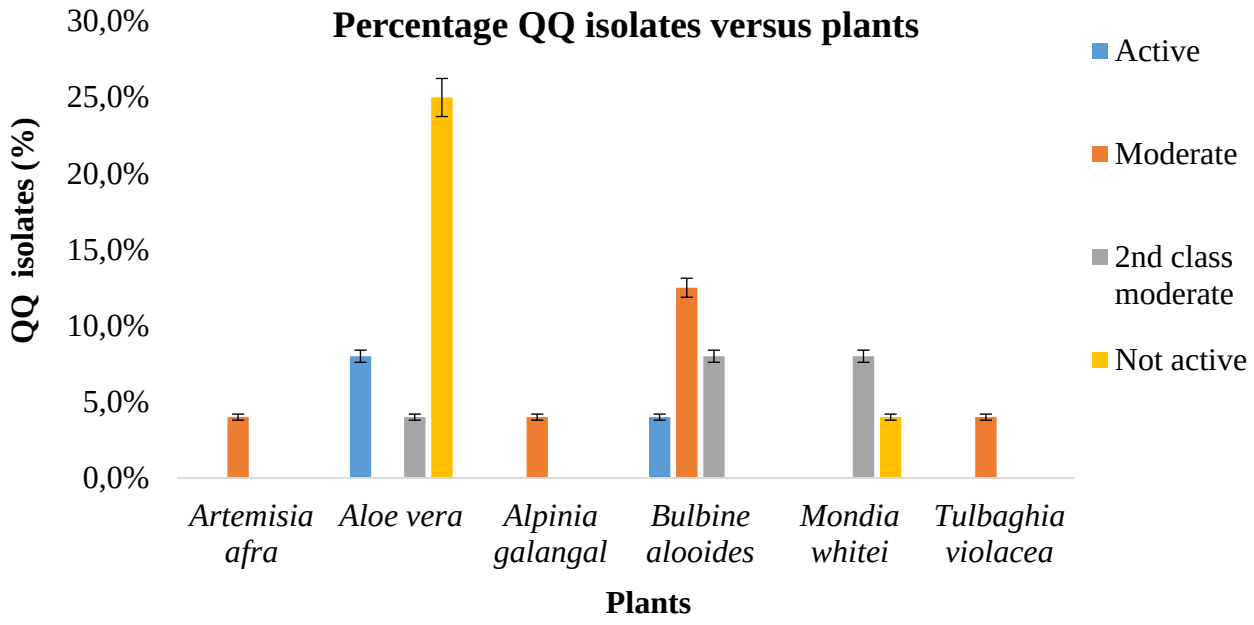


**Figure 4. 5:** Colum graph depicting % QQ isolates from the 6 medicinal plants of interest.

Eight percent of isolates that had potent quorum quenching activity were obtained from *Aloe vera*. While 25% of total isolates displayed no quorum quenching activity (Figure 4.5). This could imply that some of the therapeutic properties observed in *A. vera* plant are potentially due to the plant metabolites, while the endophytic bacteria from the plant may have been able to utilize some of these plant metabolites, hence the QQ potential. *Bulbine alooides* and *Mondia whitei* plants had an equal percentage (8%) of isolates that showed second-class moderate QQ activity as compared to other plants.

Our analysis did not reveal sufficient detection of AHL molecules. The test used in this study to detect AHL inhibition might not be sensitive enough to detect low levels of AHL molecules produced by the biomonitor strain. Positive QQ action results suggest that the degree of the signal molecule production or binding to receptor molecules was extremely low and the isolates had the option to hinder the biosensor strain from getting signal particles and keep it from producing violacein which is an attribute induced by quorum sensing (Bosgelmez-Tinaz *et al.*, 2005).

As revealed in literature, by disturbing the QS signal of pathogens enzymatically or non-enzymatically this can confine the pathogens from perceiving one another, accordingly, they are not ready to bestow their expected infection on the host since they cannot form a quorum and express harmful genes (Kusari *et al.*, 2014).

Around 2/3 of the tested isolates displayed some degree of QQ activity, which could also potentially be as a result of metabolism-dependent alkalization of the growth medium employed and subsequent non-enzymatic AHL ring-opening might also elicit the same effects observed as would certain growth-modulatory compounds and secondary metabolites.

### 4.4 QUORUM SENSING ACTIVITY

All the isolates that did not display QQ activity against *C. violaceum* CV026 were evaluated for QS activity using a T- streak assay to assess if they are responsible for QS activity or rather inactivating it (Figure 4.6).

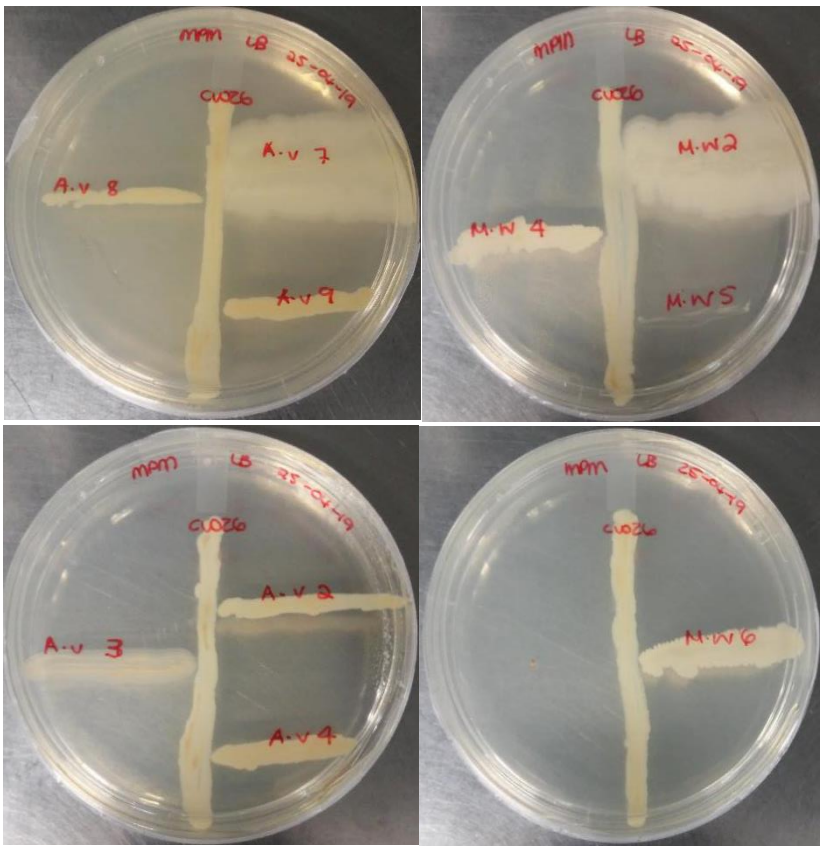


**Figure 4. 6:** Cross-streak assay for assessment of QS activity of none quorum quenching bacterial endophytes against *Chromobacterium violaceum* CV026.

Based on the results obtained above (Figure 4.6), none of the isolates produced any AHL molecules to be picked up by *C. violaceum* CV026. This means that none of the isolates were neither quorum quenchers nor quorum sensors suggesting that there could be some form of the

neutral relationship existing between the biosensor strain and the isolates. Further studies are needed to understand QQ and QS response activities.

### 4.5 AMPLIFICATION OF *AII*A HOMOLOGUE GENE

PCR amplification of *aii*A homologue gene for AHL lactonase activity was resolved by extracting genomic DNA, amplicons of around 800 bp utilizing 1% agarose gel electrophoresis. The past reports demonstrated the detection of around 800 bp as AHL lactonase with specific primers for *aii*A gene (Rajesh & Rai, 2014). Amplification (Figure 4.7) and sequencing of AHL lactonase from genomic DNA was achieved as distinctive evidence of AHL degrading enzymes present in the cell-free lysate of endophytic bacteria. Dong & Zhang, (2005); Sakr *et al.,* (2013) recently detailed the presence of *aii*A gene in numerous microorganisms and *B. weihenstephanensis,* respectively. Sequence analysis (Figure 4.8) of our AHL lactonase uncovered the presence of a few exceptionally moderated areas reported as lactonases from different species. The presence of motif "HXHXDH" accepted to play a role in metal binding and conserved in practically all metalloenzymes (Thomas *et al.,* 2005).

Two out of the fourteen isolates (Mw 1 and Mw 3) possessed the *aii*A gene. The two isolates consist of the *aii*A gene encoding AHL-Lactonase of the *Bacillus* group bacteria. The highest identity of these isolates with related species stored in GenBank was as follows; Mw 1 recognized to *Bacillus cereus* strain, LJ4 and Mw 3 distinguished as *Bacillus cereus* strain BRL02-31 (Table 4.1). In this study, two QQ bacteria were distinguished as *B. cereus,* which is a lactonase producing bacteria that forms part of the metallo beta-lactamase group that has been previously revealed (Khoiri *et al.,* 2017). As expected, most of the *Bacillus* species possess this gene, however, that was not observed even in those that were still identified as *Bacillus cereus* or *Bacillus subtilis* with different strain numbers. Therefore, for the isolates without *aii*A gene detection could be due to other mechanisms such as inactivating a different AHL-lactonase coded by a different gene or another enzyme i.e. AHL-acylase or AHL-oxidoreductase (Lin *et al.,* 2003). This therefore implies the *aii*A gene does not act as the key quorum-sensing signal molecule in those isolates that did not possess the gene. Also, variations in the *aii*A gene sequence i.e. preventing optimal primer binding or just non-optimal binding conditions as some templates are GC-rich and amplify less well in the absence of DMSO than others could account for the absence of *aii*A gene observed (Mammedov *et al.,* 2008).

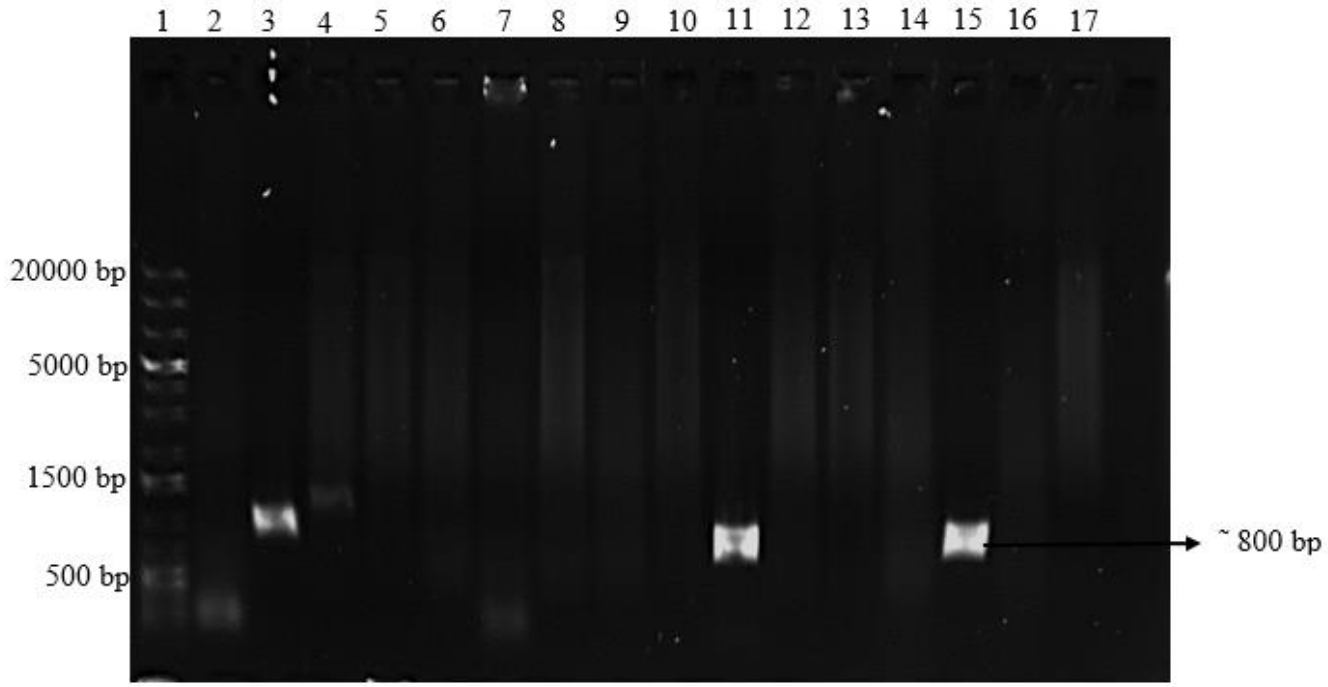


**Figure 4. 7:** Amplicons of aiiA homologue gene (800 bp) from endophytic bacterial isolates. **Lane 1**-1kb plus GeneRuler ladder, **Lane 2**- PCR control (without DNA template), **Lane 3**- Positive control (*B. cereus*), **Lane 4**- Av 6, **Lane 5**- Ba 4, **Lane 6**- Aa 1, **Lane 7**- Ag 2, **Lane 8**- Ba 2, **Lane 9**- Ba 3, **Lane 10**- Av 5, **Lane 11**- Mw 1, **Lane 12**- Ba 6, **Lane 13**- Ba 5, **Lane 14**- Ba 1, **Lane 15**- Mw 3, **Lane 16**- Tv 1 and **Lane 17**- Av 1

### 4.5.1 Multiple sequence alignment

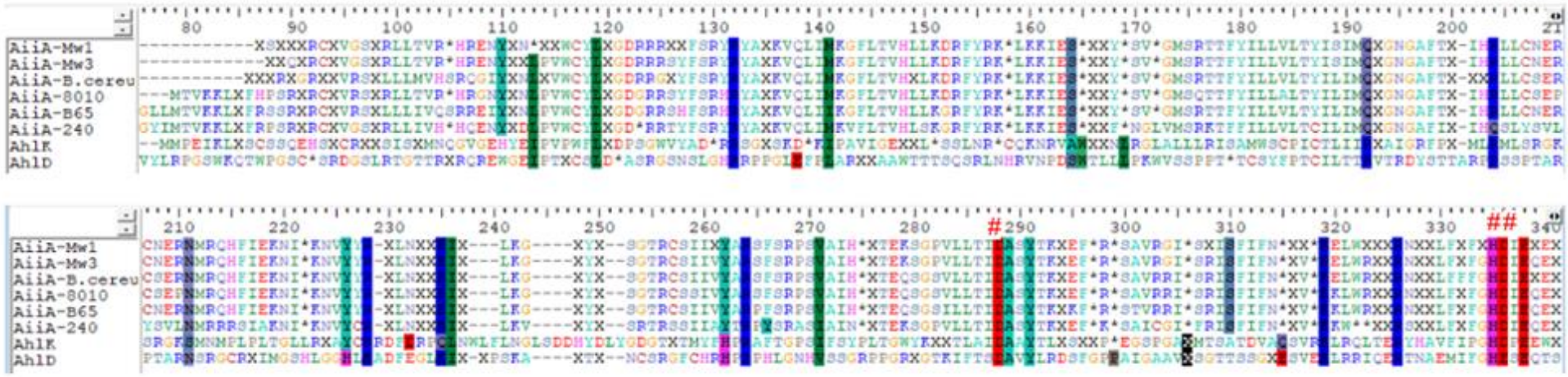


**Figure 4. 8:** Multiple alignments of the amino acid sequence of lactonase from *Bacillus cereus* strain FORC-086 (AiiA-Mw1) and *Bacillus cereus* (AiiA-Mw3) with other known sequences. AHL lactonase of AiiA-Mw1 and AiiA-Mw3 was aligned with *Klebsiella pneumoniae* (AhlK, accession number: AY222324.1), *Bacillus thuringiensis* serovar kurstaki strain 8010 (AiiA-8010, accession number: AY943832.1), *Bacillus weihenstephanensis* strain B65 (AiiA-B65, accession number: KC823046.1) and *Arthrobacter sp*. IBN110 (AhlD, accession number: AF525800.1). ClustalW was used for sequence alignment and Bio-Edit was used to identify identical residues and conserved residues. Metal ligands in the dinuclear zinc form of AHL lactonase were designated with a red hashtag.

## 4.6 BIOFILM ASSAY

### 4.6.1 Cell attachment

Cell-free lysates of QQ endophytic bacteria were assessed for the antibiofilm activity as potential effect of the enzyme/metabolite on the total biomass during cell attachment. The findings revealed a significant percentage of biofilm inhibition against *Staphylococcus aureus, Bacillus subtilis,* and *Proteus mirabilis* when the crystal violet staining technique was used.

**Table 4. 2:** Percentage (%) biofilm inhibition of biofilm-forming pathogens by cell-free lysates of endophytic bacteria during the cell attachment stage.

| | **Percentage (%) biofilm inhibition Biofilm forming pathogens** | | | | | |
|---|---|---|---|---|---|---|
| **Isolates** | *P. aeruginosa* | *E.coli* | *B. subitlis* | *S. aureus* | *P. mirabilis* | *S. marcescens* |
| *B. cereus* | -31.95 | **6.25** | -42.09 | **25.80** | **1.17** | **22.62** |
| *Assay control* | -50.95 | -60.20 | -30.35 | -15.20 | -55.20 | -20.30 |
| *Ba 1* | -9.11 | **8.67** | -18.87 | -100.40 | -68.65 | **0.94** |
| *Ba 2* | **21.88** | **12.21** | -86.84 | -98.24 | -1.78 | **2.26** |
| *Ba 3* | **35.96** | **41.53** | -43.55 | -70.52 | -24.20 | -35.74 |
| *Ba 4* | -46.96 | **24.40** | -43.20 | -90.20 | -94.09 | -29.87 |
| *Ba 5* | **1.5** | **23.80** | -98.45 | -86.85 | -64.64 | **31.31** |
| *Ba 6* | **35.5** | **21.50** | -6.42 | -73.55 | -49.44 | **15.24** |
| *Av 1* | -2.86 | **9.29** | -49.98 | -100 | -14.49 | -124.79 |
| *Av 5* | -14.74 | -10.95 | -114 | -46.09 | -20.09 | **21.66** |
| *Av 6* | -0.92 | **2.15** | -56.14 | **3.62** | **6.70** | **33.13** |
| *Mw 1* | -45.11 | **13.45** | -7.98 | **10.58** | -4.16 | **21.94** |
| *Mw 3* | **0.35** | -13.53 | -15.82 | **0.074** | **14.56** | **31.23** |
| *Aa 1* | **28.61** | **21.49** | -79.26 | **2.10** | **8.60** | **38.03** |
| *Ag 2* | -114.88 | **20.68** | -70.54 | -44.38 | -1.57 | **30.62** |
| *Tv1* | **16.73** | **14.86** | -32.65 | -10.04 | **2.71** | **17.61** |
| **P-value** | **0.1967** | **0.1571** | **0.0017**** | **0.0000**** | **0.0149*** | **0.7389** |

- *Negative values represent the enhancement or encouragement of cell-attachment. Bold values indicate biofilm inhibition/ restriction of cell-attachment. Assay control is a pathogen culture without isolate treatment. ** p<0.01, indicates that the treatments are highly significant *p<0.05, indicates the treatments are significant.*

The biofilm inhibition studies conducted using a cell-free lysate of quorum quenching bacterial isolates against *P. aeruginosa* cell attachment had a percentage inhibition of ≤ 35% while *E. coli* had a biofilm inhibition of ≤ 41% (Table 4.2). For *B. subtilis* there was no inhibition observed (Table 4.2), however, QQ isolates were rather promoting the attachment of cells to the polystyrene surface of the microtitre plate thus inducing the development of biofilm.

*P. mirabilis* shows only ≤ 15% biofilm inhibition, suggesting resistance against most of the endophytic bacterial cell-free lysates (Table 4.2). *S. marcescens* had a percentage biofilm inhibition ≤ 38% (Table 4.2). The endophytic bacterial cell-free lysates were effective against *S. marcescens* as compared to *P. mirabilis, S. aureus, E. coli, B. subtilis* and *P. aeruginosa*. In the case of *S. aureus* (Table 4.2), the majority of the cell-free lysates induced biofilm development; however, a small number was able to inhibit the biofilm formation. The inhibition observed was slightly less and only a ≤ 10.58% biofilm inhibition. *S. aureus* is a profoundly resistant pathogen which is additionally a multi-medicate resistant pathogen that is viewed as perilous to people and is answerable for some diseases that remain related to high morbidity and mortality both in clinics/hospitals and society (Rasigade *et al.*, 2014).

*P. aeruginosa* was resistant to half the potential quorum quencher strains as well as to the positive control (*B. cereus*) when assessed at the cell-attachment stage. This implies that most of the cell-free lysates will not be excellent quorum quenching agents' alternative to eradicate *P. aeruginosa* infections effectively as they would rather encourage the infection. According to literature, *P. aeruginosa* is notorious for its resistance to antibiotics and is consequently an especially treacherous pathogen (Yayan *et al.,* 2015). Based on the results obtained, *P. aeruginosa* was expected to display resistance to the cell-free lysates during the first stage of biofilm formation.

Similarly, *S. aureus, B. subtilis,* and *P. mirabilis* were highly resistant to the majority of the endophytic bacterial cell-free lysates. The cell-free lysates encourage the growth of *S. aureus, B. subtilis,* and *P. mirabilis* biofilm tremendously. Several factors that could potentially explain why *B. subtilis* biofilm was not inhibited by cell-free lysates include the cells of the supplement exhausted zones (slow-developing state) in the biofilm may prompt torpidity like

the stationary stage, which makes the microorganisms harsh toward the cell-free lysates of isolates since they separate rarely (Anderl *et al.*, 2003; Brown *et al.*, 1988). Several different investigations have likewise shown that biofilm cells experience a higher pace of mutation than their planktonic partners bringing about a 10-fold increment in the productivity of transfer of plasmid having resistance genes (Ma & Bryers, 2013).

On the other hand, *S. marcescens* and *E. coli* were the most sensitive to almost all of the cell-free lysates including the positive control. Thus, it can be concluded that most of these cell-free lysates have QQ potential against *S. marcescens* and *E.coli*, indicative as a potential for consideration in the development of alternative treatment at the cell-attachment stage.

In essence, one would expect that during cell-attachment majority of the pathogens' biofilm would be inhibited instead of being encouraged. However, that was not the case and thus these result do not agree with literature which shows that during cell-attachment (first stage of biofilm) mostly biofilms are unstable during this stage and can easily be eradicated (Paluch *et al.*, 2020).

### 4.6.2 Pre-formed biofilm with whole-cell

Whole-cell supernatant of QQ bacterial endophytes was used to determine the effect on the total biomass when the biofilm is pre-formed. This experiment was chosen to compare the results of cell-free lysates when the biofilm is already formed and to assess which of the two would be most effective against the biofilm-forming pathogens. The antibiofilm assay revealed a significant percentage of biofilm inhibition against *Serratia marcescens, Escherichia coli* and *Pseudomonas aeruginosa*.

**Table 4. 3:** Percentage (%) biofilm inhibition of biofilm-forming pathogens by whole-cell lysates of endophytic bacteria during the development stage.

| | **Percentage (%) biofilm inhibition Biofilm forming pathogens** | | | | | |
|---|---|---|---|---|---|---|
| **Isolates** | *P. aeruginosa* | *E.coli* | *B. subitlis* | *S. aureus* | *P. mirabilis* | *S. marcescens* |
| *Ciprofloxacin* | **86.74** | **22.71** | **33.58** | **14.84** | **28.12** | **23.25** |
| *Ba 1* | **45.62** | **84.79** | -99.64 | **52.45** | -89.62 | **59.19** |
| *Ba 2* | **4.35** | -0.80 | -2.82 | **29.79** | -14.1 | **53.05** |
| *Ba 3* | **51.92** | **68.48** | -60.14 | **31.25** | -85.10 | **59.59** |
| *Ba 4* | **55.19** | **48.91** | -53.55 | **39.35** | -116.66 | **56.83** |
| *Ba 5* | **31.73** | **15.33** | -29.43 | **12.98** | -0.05 | **60.16** |
| *Ba 6* | **49.46** | **32.78** | -10.34 | **44.69** | -26.46 | **52.60** |
| *Av 1* | **34.47** | **14.11** | **29.78** | -51.88 | **13.58** | -14.31 |
| *Av 5* | **53.9** | -23.63 | **26.79** | -43.90 | -5.59 | **32.11** |
| *Av 6* | **28.83** | **11.66** | -18.62 | -62.96 | **7.67** | **59.51** |
| *Mw 1* | **17.43** | **15.87** | **10.43** | -21.59 | **15.37** | **42.85** |
| *Mw 3* | **28.08** | **23.81** | **16.67** | **2.92** | **8.24** | **21.63** |
| *Aa 1* | **59.47** | **14.29** | -3.51 | -26.76 | **5.56** | **53.66** |
| *Ag 2* | **10.42** | **15.43** | -58.30 | -12.03 | -26.37 | **63.98** |
| *Tv1* | **22.20** | **6.62** | -0.13 | -30.24 | **8.86** | **38.82** |
| **P-value** | **0.0000**** | **0.0046**** | **0.1666** | **0.8856** | **0.1253** | **0.0000**** |

- Negative values represent the promotion of biofilm development. *Bold values indicate biofilm formation inhibition. The positive control used was 0.1 mg/ml of Ciprofloxacin. ** p<0.01, indicates that the treatments are highly significant *p<0.05, indicates the treatments are significant.*

*S. marcescens, P. aeruginosa* and *E. coli* were highly sensitive to whole-cell endophytic bacterial strains, including the positive control (Ciprofloxacin) inhibited growth of the pathogen. The majority of pre-formed biofilm inhibition percentage of *S. marcescens* and *P. aeruginosa* is above 30%. Particularly for *E.coli* pre-formed biofilm can be eradicated significantly by Ba 1 (*Bacillus* sp. strain 7M1) with over 80% inhibition followed by Ba 3 (*Bacillus subtilis* strain 07-2) with over 60% inhibition and Ba 4 with over 40% inhibition. On

the other hand, *P. aeruginosa* was sensitive to, Ba 1 (*Bacillus* sp. strain 7M1) with over 40% inhibition, Ba 3 (*Bacillus subtilis* strain 07-2), Ba 4 (*Bacillus subtilis* SY), Ba 6 ( *Bacillus subtilis* strain Fito F264) and Av 5 ( *Pseudomonas* sp. strain MY720) with over 50% inhibition and Aa 1 (*Klebsiella* sp.5) being the highest with 60% inhibition. From these results, these isolates appear to be potential treatment alternatives due to their ability to inhibit a pre-formed biofilm of these pathogens significantly.

*B. subtilis* and *P. mirabilis* appear to be extremely resistant to most of the endophytic bacterial lysates including Ba 1 (*Bacillus* sp. strain 7M1), Ba 2 (Uncultured *Acidovorax* sp. clone G13-M-7-E04), Ba 3 (*Bacillus subtilis* strain 07-2), Ba 4 (*Bacillus subtilis* SY), Ba 5 (*Acidovorax* sp. strain 8160P6), Ba 6 (*Bacillus subtilis* strain Fito F264) and Ag 2 (*Bacillus* sp. hb11) mostly (Table 4.3). However *B. subtilis* and *P. mirabilis* are significantly more sensitive to the positive control compared to *S. aureus, E.coli* and *S. marcescens*.

### 4.6.3 Pre-formed biofilm with cell-free lysate

Cell-free lysates of QQ endophytic bacteria were used for assessing the antibiofilm activity. Additionally, the effect of the enzymes on total biomass when the biofilm was determined. The antibiofilm assay using a microtitre plate revealed a significant percentage biofilm formation, enhancement for *Proteus mirabilis* and *Bacillus subtilis* by crystal violet staining.

*P. mirabilis* and *B. subtilis* were greatly resistant to the cell-free lysates when their biofilms were already pre-formed, hence the cell-free lysates were enhancing their biofilm formation instead of inhibiting it (Table 4.4). Meaning, it will be extremely difficult to eradicate *P. mirabilis* and *B. subtilis* infections when they have long been established. The majority of the isolates cell-free lysates promoted the biofilm growth of *P. mirabilis* and *B. subtilis* instead of inhibiting it. It can, therefore, be seen that most of these isolates are not suitable treatment alternatives to these pathogens. (Table 4.4). For *P. aeruginosa* more than half of the isolates enhance its biofilm growth greatly and those that inhibit it do so insignificantly meaning that this pathogen is still slightly resistant to most of the lysates and thus it will be difficult to eradicate it with those that do show some form of inhibition.

*S. marcescens* is also resistant to the isolates when its biofilm has already developed. Over half of the isolates, cell-free lysates were able to inhibit pre-formed biofilm of *E. coli*, however, the percentage inhibition was under 30%. Whereas, for *S. aureus*, a few of the isolates cell-free lysates can inhibit its biofilm even when it is already formed as some showed a percentage biofilm inhibition of approximately 50%.

**Table 4. 4:** Percentage (%) biofilm inhibition of biofilm-forming pathogens by cell-free lysates of endophytic bacteria during the development stage.

| | **Percentage (%) biofilm inhibition Biofilm forming pathogens** | | | | | |
|---|---|---|---|---|---|---|
| **Isolates** | *P. aeruginosa* | *E.coli* | *B. subitlis* | *S. aureus* | *P. mirabilis* | *S. marcescens* |
| *B. cereus* | -8.87 | -7.75 | -187.70 | -223.98 | -10.55 | **7.06** |
| *Assay control* | -12.81 | -30.20 | -10.20 | -20.50 | -20 | -15.20 |
| *Ba 1* | -150.40 | **21.06** | -168.50 | -6.95 | -99.02 | -0.69 |
| *Ba 2* | -59.37 | **8.83** | -31.97 | -37.29 | -13.79 | **11.26** |
| *Ba 3* | **20.61** | **23.93** | -100 | **50.74** | -62.64 | **1.63** |
| *Ba 4* | **8.87** | **21.59** | -193 | **64.19** | -120.20 | -9.49 |
| *Ba 5* | -122.52 | **19.60** | -165 | -139.20 | -91.17 | -13.26 |
| *Ba 6* | **7.00** | **3.34** | -151 | **49.82** | -46.52 | 1.72 |
| *Av 1* | **8.39** | -7.21 | -16.06 | -43.13 | -25.46 | **19.76** |
| *Av 5* | -12.87 | **7.46** | -95.40 | -216.04 | -46.95 | -16.54 |
| *Av 6* | -67.39 | -102.10 | -18.31 | -204.67 | -29.17 | **18.11** |
| *Mw 1* | -0.98 | -3.07 | -40.97 | **33.54** | -22.87 | **8.21** |
| *Mw 3* | **6.78** | -1.83 | -19.50 | **39.53** | -19.84 | **13.35** |
| *Aa 1* | -19.68 | **12.55** | -99.80 | **26.22** | -30.89 | **9.98** |
| *Ag 2* | -5.59 | **11.77** | -95.24 | -83.81 | -0.79 | **13.27** |
| *Tv1* | **22.06** | -30.02 | -16.09 | **16.87** | **5.02** | -3.39 |
| **P-value** | **0.0751** | **0.6430** | **0.0001**** | **0.1045** | **0.0005**** | **0.3458** |

- *Negative values represent the promotion of biofilm formation. Bold values indicate biofilm formation inhibition. Assay control is a pathogen culture without isolate treatment. ** p<0.01, indicates that the treatments are highly significant *p<0.05, indicates the treatments are significant.*

Inhibition of biofilm formation was conducted on the 14 isolates that showed QQ activity. The results showed different effects on the growth and development of a pre-formed biofilm as presented in Table 4.3- 4.4. The biofilm inhibition studies conducted with whole-cell lysates (Table 4.3).To the best of our knowledge, this work is a first of its kind to report on the biofilm inhibition effects of whole-cell lysates. The difference observed between the

same pathogens treated with whole-cell lysates and treated with cell-free lysates is highly significant. For instance, as seen in table 4.3 of *S. marcescens* and *P. mirabilis* during pre-formed biofilm inhibition with whole cell lysate, isolates were able to inhibit the biofilm development and growth of *S. marcescens* with a biofilm formation inhibition of ≤ 64% and only ≤ 16% for *P. mirabilis.*

During pre-formed biofilm inhibition of the same pathogens using cell-free lysates the biofilm inhibition was ≤ 20% for *S. marcescens* and ≤ 5% for *P. mirabilis.* These results show that the whole-cell lysates are strongly effective against these pathogens during the pre-formed biofilm stage especially against *S. marcescens.* In the case of *B. subtilis* and *S. aureus* during biofilm inhibition of pre-formed biofilm with whole-cell lysates a percentage biofilm inhibition of ≤ 30% and ≤ 53% respectively (Table 4.3). However, during biofilm inhibition of a pre-formed biofilm with cell-free lysates, for *B. subtilis* there was no inhibition observed and for *S. aureus* a biofilm inhibition of ≤ 65% was observed (Table 4.4), indicating that cell-free lysates outperformed whole-cell lysates in the case of inhibiting pre-formed biofilm of *S. aureus.*

In the case of *E. coli* and *P. aeruginosa* during biofilm inhibition analysis with whole-cell lysates, *E. coli* showed a biofilm inhibition of ≤ 85% whereas *P. aeruginosa* showed a biofilm inhibition of ≤ 60% (Table 4.3). However, during inhibition of pre-formed biofilm with cell-free lysates, *E. coli* showed a biofilm inhibition of ≤ 24% and *P. aeruginosa* showed a biofilm inhibition of ≤ 22% (Table 4.4). Based on these results it can be seen that whole cell lysate proved to be more effective against these biofilm-forming pathogens compared to their counterpart cell-free lysates. This could be because whole cell lysates are essentially supernatants of endophytic bacterial isolates, which potentially contain metabolites that are responsible for the effects observed against biofilm-forming pathogens. For example, supernatants of bacteria usually contain toxins that have been secreted by bacteria into the environment, including proteins and peptides, cell-wall degrading enzymes and other volatile compounds (Glick, 2005). Therefore, the results obtained in this study during inhibition of preformed biofilm agrees with literature as expected.

According to a study by Jamal *et al.,* (2018) inhibition of a fully matured biofilm is difficult. This therefore, supports or potentially explain why most of the cell-free lysates were not able to inhibit majority of the pre-formed biofilms. Therefore, the results obtained coincide with the literature.

Also, according to literature siderophores creation by endophytes improves plant development by adhering to accessible iron, going after this component with phytopathogens and shielding the host from their contagiousness (Sabate *et al.,* 2018). This could explain why whole-cell lysates were effective against biofilms of pathogens. In accordance with a report by Rewak-Soroczynska *et al.,* (2019) in order to inhibit an already pre-formed biofilm, it is crucial to use compounds that are capable of penetrating through the biofilm structure or that can disrupt it mechanically because if some compounds are not strong enough to inhibit the biofilm they would result in cellular death of the pathogen. The results obtained in this study of inhibition of pre-formed biofilm by whole-cell lysates therefore, correlate with literature and also could explain why reduction of bacterial growth does not necessarily imply quorum quenching.

### 4.7 CONFOCAL LASER SCANNING MICROSCOPY

The total biofilm inhibition visually confirmed reductions of *S. aureus* using confocal laser scanning microscopy (Figure 4.9). *E. coli* (Figure 4.10), *P. aeruginosa* (Figure 4.11), *P. mirabilis* (Figure 4.12) and *S. marcescens* (Figure 4.13) biofilms in the treated slides with cell-free lysates were observed. There was no inhibition on the biofilm of *B. subtilis* as the isolates were rather enhancing its growth instead of inhibiting it (Figure 4.14). In the case of untreated pathogen, more prevalent biofilms were observed, indicated by the green colour (frame A of all the figures), whereas in treated slides (B, C, D, and E) biofilm inhibition was observed, indicated by the red colour. These results, therefore, correlated with the crystal violet staining results (Table 4.2-4.4).

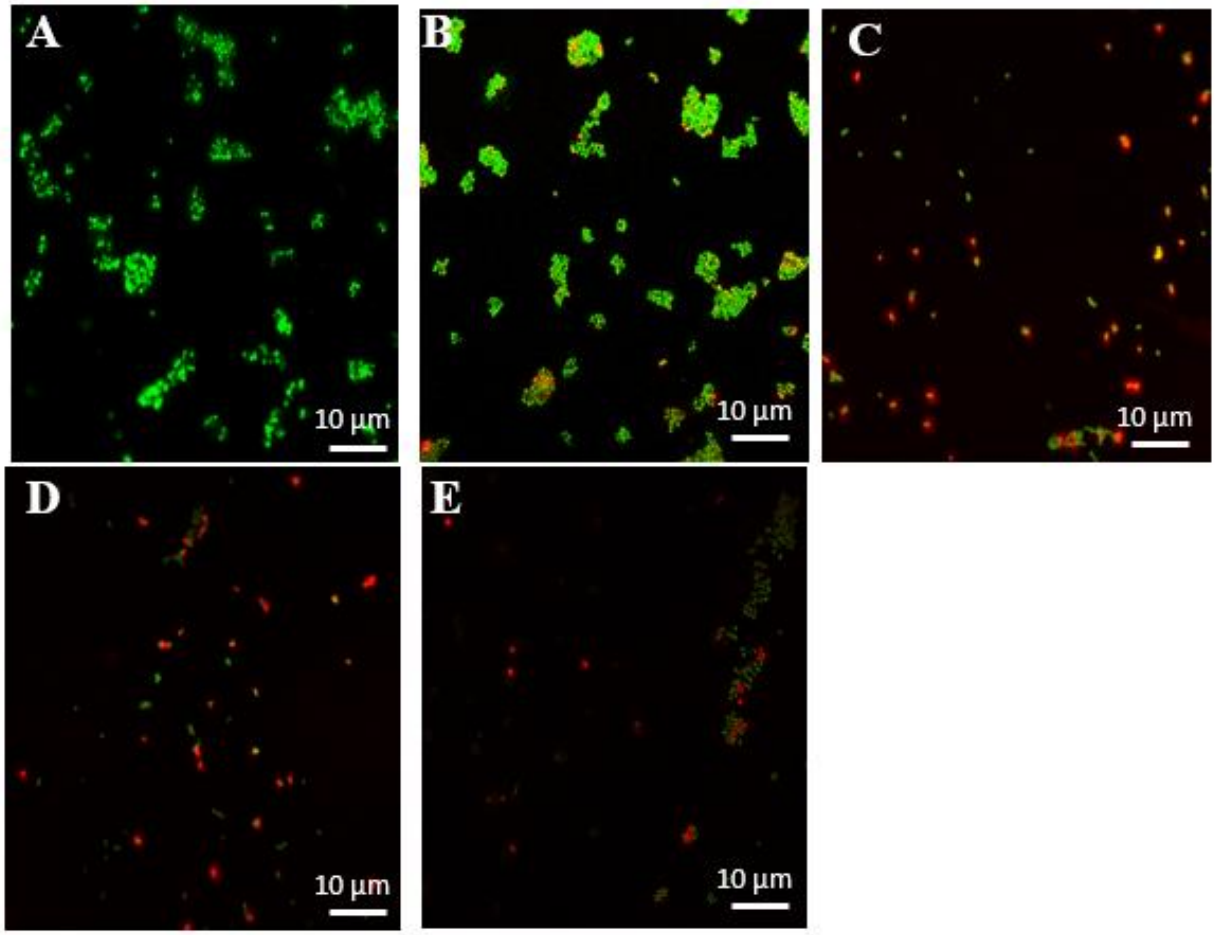


**Figure 4. 9:** Confocal laser scanning microscopy (CLSM) images of *Staphylococcus aureus* pre-formed biofilm when subjected to different treatments (100x magnification). **[A]** Assay control, **[B]** Positive control (cell-free lysate of *B.cereus*), **[C]** Biofilm treated with cell-free lysate of isolate Ba 4**, [D]** Biofilm treated with cell-free lysate of Ba 3 and **[E]** Biofilm treated with cell-free lysate of Ba 6.

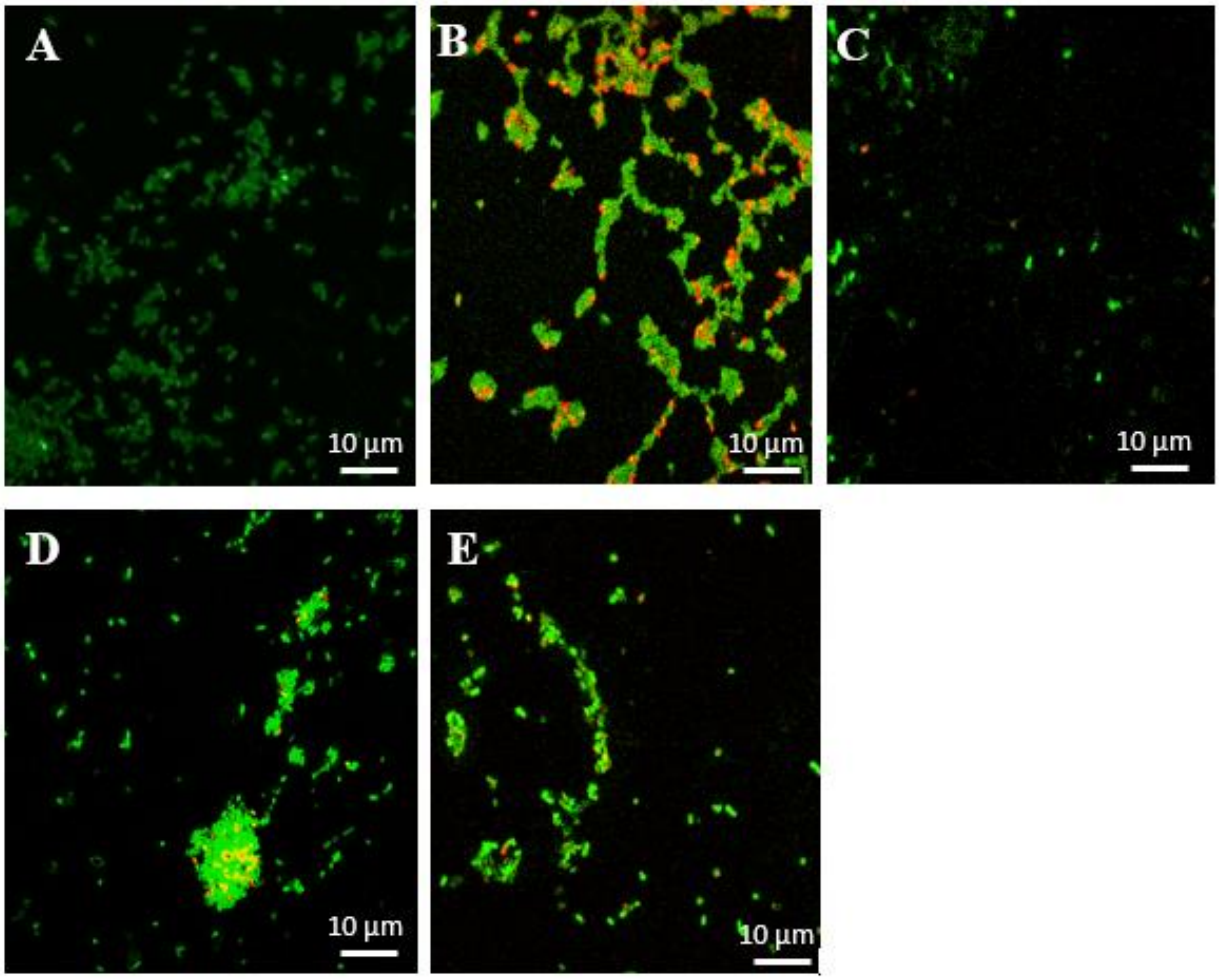


**Figure 4. 10:** Confocal laser scanning microscopy (CLSM) images of *Escherichia coli* pre-formed biofilm when subjected to different treatments (100x magnification). **[A]** Assay control, **[B]** Positive control (cell-free lysate of *B.cereus*), **[C]** Biofilm treated with cell-free lysate of isolate Ba 1, **[D]** Biofilm treated with cell-free lysate of Ba 3 and **[E]** Biofilm treated with cell-free lysate of Ba 4.

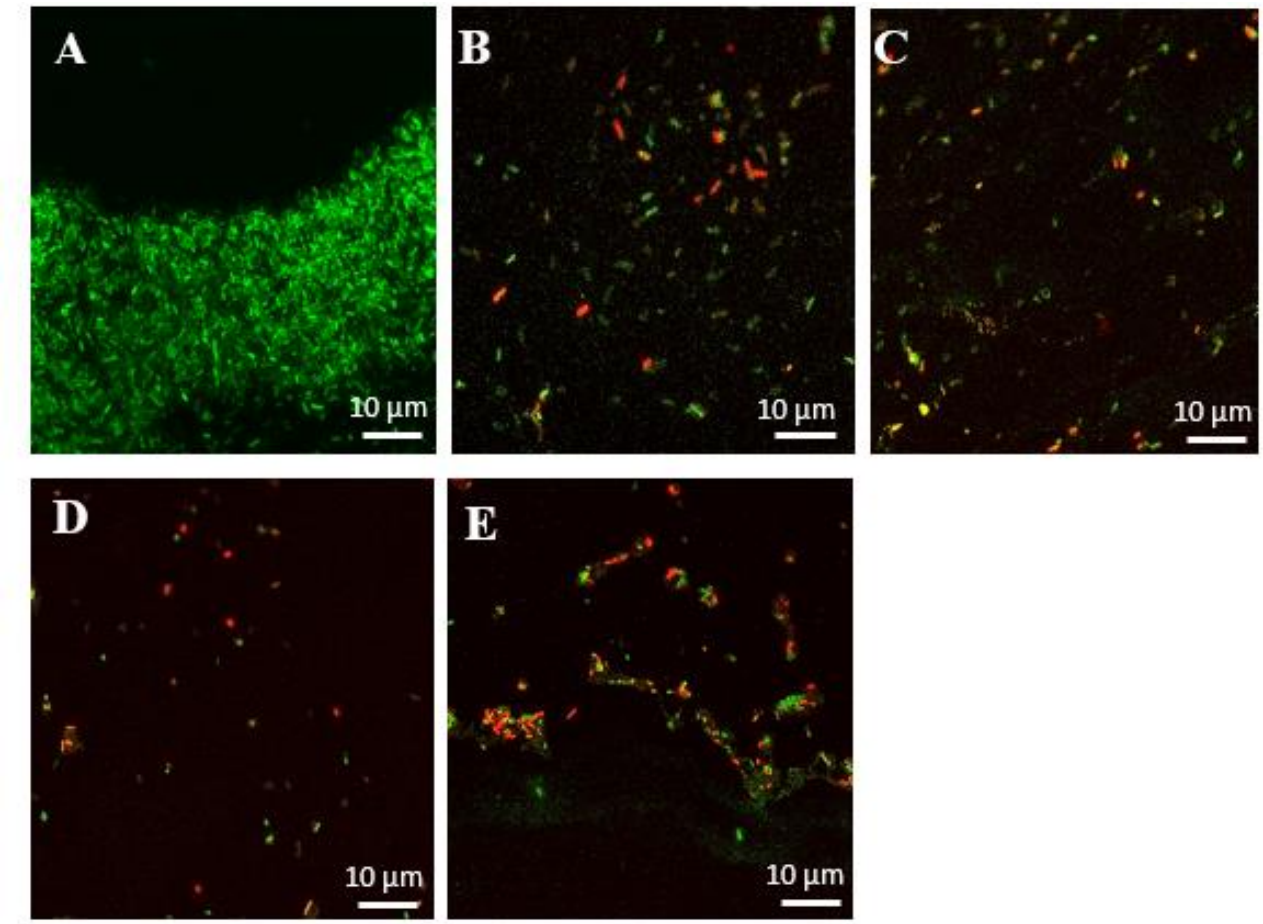


**Figure 4. 11:** Confocal laser scanning microscopy (CLSM) images of *Pseudomonas aeruginosa* pre-formed biofilm when subjected to different treatments (100x magnification). **[A]** Assay control, **[B]** Positive control (cell-free lysate of *B.cereus*), **[C]** Biofilm treated with cell-free lysate of isolate Tv 1, **[D]** Biofilm treated with cell-free lysate of Ba 3 and **[E]** Biofilm treated with cell-free lysate of Ba 4.

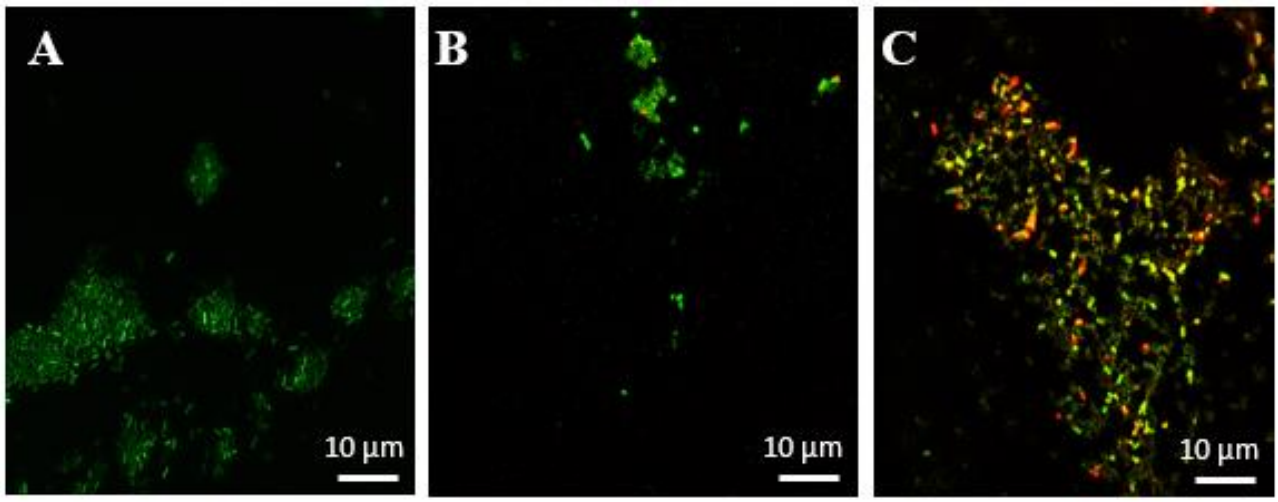


**Figure 4. 12:** Confocal laser scanning microscopy (CLSM) images of *Proteus mirabilis* pre-formed biofilm when subjected to different treatments (100x magnification). **[A]** Assay control, **[B]** Positive control (cell-free lysate of *B.cereus*) and **[C]** Biofilm treated with cell-free lysate of isolate Tv 1.

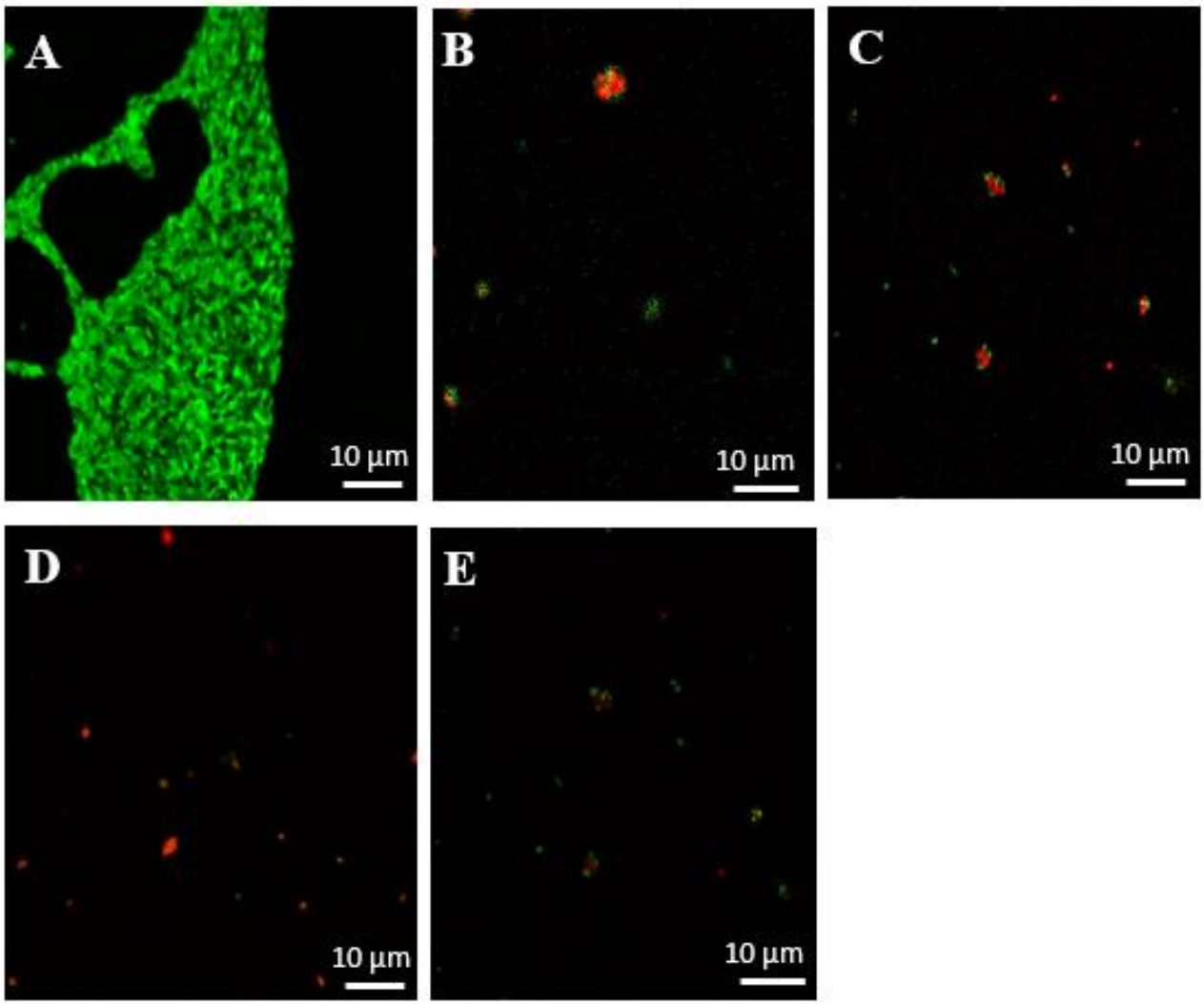


**Figure 4. 13:** Confocal laser scanning microscopy (CLSM) images of *Serratia marcescens* pre-formed biofilm when subjected to different treatments (100x magnification). **[A]** Assay control, **[B]** Positive control (cell-free lysate of *B.cereus*), **[C]** Biofilm treated with cell-free lysate of isolate Av 1, **[D]** Biofilm treated with cell-free lysate of Av 6 and **[E]** Biofilm treated with cell-free lysate of Mw 3.

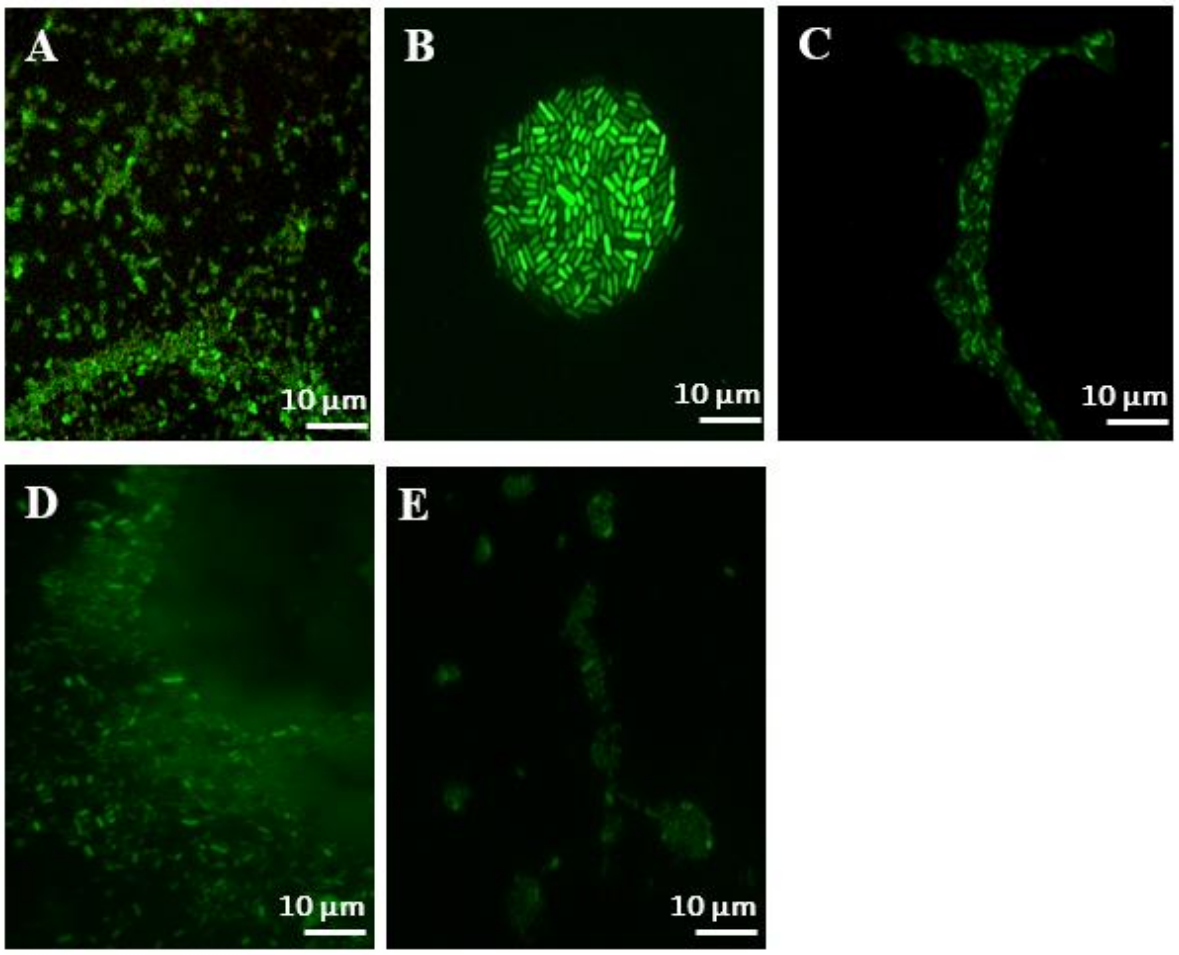


**Figure 4. 14:** Confocal laser scanning microscopy (CLSM) images of *Bacillus subtilis* pre-formed biofilm when subjected to different treatments (100x magnification). **[A]** Assay control, **[B]** Positive control (cell-free lysate of *B.cereus*), **[C]** Biofilm treated with cell-free lysate of isolate Ba 1, **[D]** Biofilm treated with cell-free lysate of Ba 4 and **[E]** Biofilm treated with cell-free lysate of Ba 5.

The confocal laser scanning microscopy study indicated a reduction in the development of absolute biomass in treated cells when contrasted with the assay (untreated) control. It demonstrates the capacity of cell-free lysate with the evacuation of biofilm attachment capacity in the five pathogens. The cell-free lysates successfully counteracted over 20% biofilm arrangement by reducing swimming and swarming motilities, however, they neglected to repress planktonic cells, as do antimicrobial operators. The results obtained agree with literature (Fuente-Nunez *et al.*, 2012).

*B. subtilis* was not inhibited by any cell-free lysates (Figure 4.14) but rather the cell-free lysates encouraged its attachment to the microtitre plate surface and promoted the development of biofilm. Restraint of biofilm arrangement because of high affinity to extracellular DNA, which plays a significant part in biofilm development may encourage the separation or interruption

of stable biofilms (Pompilio *et al.,* 2012). Obstruction with cell signalling prompts restraint of biofilm arrangement in *las/agr* quorum-sensing frameworks (Mookherjee *et al.,* 2017). Literature shows that QQ enzymes from bacterial endophytic cell-free lysates are liable for turning off signal transmissions by signal degradation, which might clarify the antibiofilm properties showed by those cell-free lysates that can restrain the biofilms of the pathogens (Borges & Simoes, 2019).

Generally, a significant number of the cell-free lysates are equipped for infiltrating through the biofilm network and repress quorum-sensing gene articulation, as an outcome hindrance of biofilm development results (Hentzer *et al.,* 2002), thus the results obtained in this study coincide with literature. Additionally, cell-free lysates, in general, evades the production of virulent situations as proved by the treatment of anti-toxins and mutations in light of changes in environmental conditions (Rajesh & Rai, 2014).

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# CHAPTER 5

# CONCLUSION & RECOMMENDATIONS

## 5. OVERVIEW

Most bacteria that cause infections are resistant to a minimum of one if not all the medication ordinarily used for treatment. Some organisms are highly resistant against all approved antibiotics as a result of the environmental antibiotic pressure activates the biological process mechanisms that select for resistant strains (Zhu *et al.*, 2011).

The ongoing investigation into QSS has introduced compounds with potential to disturb microbial capacity to communicate and subsequently impair or lessen their virulent ability. The benefit of the QQ approach is purported not to result in selection pressures that decide for resistance (Zhu *et al.*, 2011).

In-depth research has been done mainly on endophytic fungi; however, less has been done on indigenous medicinal South African plants, more so there have been no reports documented concerning endophytic bacteria with quorum quenching properties. Hence, this study aimed to isolate endophytic bacteria from South African medicinal plants that possess QQ properties and to disrupt QS systems of selected biofilm-forming pathogens.

## 5.1. ACHIEVEMENT OF OBJECTIVES

In this section, the objectives of the study are assessed together with the outcomes of the study.

- The first objective was to conduct a literature search and collect medicinal plants of interest. A thorough literature review assisted in the identification of six medicinal plant species (*Aloe vera, Alpinia galangal, Artemisia afra, Bulbine alooides, Mondia whitei* and *Tulbaghia violacea*) with excellent medicinal properties in South Africa to treat bacterial infections. Samples of these plants were collected around the University of Pretoria, Hatfield campus (Pretoria, South Africa). A literature review also guided us with the selection of bacteria commonly associated with biofilm formation and well-known bacterial infections. This objective was achieved.
- The second objective of this study was to use plate-based extraction methods for the isolation and extraction of endophytic bacterial strains. The objective was successfully achieved. The plant samples, mainly leaves, were used to extract potential endophytic bacteria, which resulted in thirty-four (34) bacterial endophytes extracted. Ten isolates were discarded.

- The third objective was to screen for the QQ potential of the isolated bacterial endophytes. In this study, endophytic bacterial isolates were evaluated as quorum sensing inhibitors (i.e. quorum-quenchers). The results showed that only 14 out of 24 isolates inhibited quorum sensing regulated violacein production in *C. violaceum* ATCC 12472 without interfering with its growth. Thus, this objective was successfully achieved. However, this objective could be improved by assessing AHL degradation studies of these isolated bacterial endophytes to confirm their QQ activity.
- The fourth objective was to use MALDI-TOF and 16S rRNA to identify the isolated strains. The twenty-four bacterial endophytes isolated were successfully identified by MALDI-TOF and confirmed by 16S rRNA, which revealed that *Bacillus* and *Pseudomonas* were the two dominant genera identified amongst the isolates. Interestingly, when comparing MALDI-TOF and 16S rRNA results, it can be confirmed that there was a 70.83% similarity in terms of the genera identified by both methods. Ideally, 16S rRNA is a much better option as isolates are identified to species level compared to MALDI-TOF whereby it is not reliable in terms of species-level identification.
- The fifth objective was to determine the QQ genes present in the strains capable of QQ. Of the 14 that showed QQ potential, only 2 endophytic bacterial isolates exhibited the *aii*A gene, which codes for a quorum quenching agent. The *aii*A encodes lactonase AiiA which serves as a QS inhibitory specialist (Yin *et al.*, 2011). Therefore, this objective was successfully achieved. However, it would be advisable to consider looking at other QQ genes such as those that codes for AHL acylase and AHL oxidoreductase which could account for the other 12 isolates that the *aii*A gene was not detected.
- Lastly, the objective of this study was to determine the effects of QQ enzymes/compounds on biofilm-forming pathogens, which was successfully achieved. Different results were obtained as some of the isolates were enhancing biofilm formation in one pathogen and inhibiting it in the other pathogen. Some QQ enzymes of the isolates, however, were extremely effective in inhibiting the pathogens' biofilm when compared to the positive control. Also, it was determined that *Bacillus cereus* used as a positive control in this study and many other studies might not necessarily be such good positive control, as it seems effective only against certain pathogens but not

all. Therefore, it would be advisable to rather look for strong QQ bacteria that could be used as a positive control.

## 5.2. CONTRIBUTION OF THE STUDY

Based on the study conducted, an overview of how QQ enzymes are excellent alternative treatment options in terms of treating bacterial infections was determined.

Initially, this study aimed to uncover whether South African medicinal plants harbour endophytic bacteria. Based on the results obtained, it was confirmed that the six medicinal plants used in this study do harbour different bacterial endophytes. These results, therefore, suggest that many of the medicinal properties observed in some of the medicinal plants used in this study could be attributable to the relationship they have with their bacterial endophytes which could potentially account for the therapeutic activities seen in the medicinal plants. Therefore, this study suggests that South African medicinal plants are indeed a hotspot for endophytic bacteria with QQ potential.

Secondly, this study wanted to find out which bacterial endophytes possessed compounds/ enzymes that inhibit/reduce bacterial growth. It was found that 14 isolates with QQ potential were able to reduce microbial growth and also inhibit biofilm formation in some pathogens without killing the microbes. This coincides with the principle of quorum quenching. Interestingly, it was also discovered that not necessarily all QQ enzymes could inhibit biofilms some were inducing the formation of biofilms in some pathogens. Therefore, not all but a few cell-free lysates (enzymes) do stand a chance as potential treatment alternatives against bacterial infections.

Additionally, the study sought to find out if the plant-associated bacterial endophytes produced compounds/enzymes that have quorum quenching activity. By assessing the AHL-lactonase activity of the 14 QQ isolates, it was revealed that only two bacterial endophytes detected the *aii*A gene, which codes for AHL-lactonase activity and therefore suggests the enzymatic degradation observed in the QQ activity of these two isolates. However, this is not sufficient to rule out AHL lactonase activity and thus more studies are required. Also, the study was not able to reveal what compound/enzyme could have constituted the QQ activity observed in the other 12 QQ isolates. Thus, this calls for more investigations/ research to be conducted in this regard.

## 5.3. RECOMMENDATIONS

Until recently, little has been accomplished worldwide inside the examination of the endophytic bacterium, and hardly any strains have been isolated, suggesting that the opportunity to search for promising strains and their novel natural products in a few specialties and environments is huge. Subsequently, from this examination, the accompanying recommendations for additional exploration are made:

- The investigation of large numbers of samples of a single plant species from different sources is highly recommended. This will allow the discovery of different or rather diverse endophytic bacterial strains of a certain plant species.
- Since endophytes from South African medicinal plants have not been studied in detail, it would be beneficial to the research world to screen them for the production of bioactive metabolites.
- Additional in-depth analysis of the bioactivities, as well as secondary metabolites of the endophytic bacterium, is highly suggested as this may result in the isolation of the latest drug candidates, particularly from those potential plants, because the endophytic compounds could also be responsible for the complete activity.
- AHL degradation assays to confirm the *aii*A homologue gene PCR amplification results.
- Furthermore, some studies have already shown that in medical treatment, the combinatorial use of antibiotics with anti-QS ways is another helpful side of QS inhibitors (LaSarre & Federle, 2013). The rationale for co-administration studies is to check if there'll be any synergistic activities discovered that may provide compelling proof that will cause new treatment choices for troublesome to eradicate microorganism infections.
- Consider growth curve analysis studies to support and/or verify whether the results obtained from biofilm assay studies are due to enzymatic degradation or inhibition of bacterial growth by antimicrobial compounds present in the lysates.
- Consider using fluorescence microscopy to assess the effect of QQ endophytic bacterial enzymes on biofilm-forming pathogens to allow for comparison with those obtained by confocal laser scanning microscopy and also to assess the effect of QQ enzymes on cell attachment microscopically.

- Since some QQ enzymes had enhanced effects on biofilm formation, this aspect needs to be further investigated to determine the possible mechanisms involved in enhancing biofilm formation.
- Since QS is concerned in each gram-positive and gram-negative microorganism, so the utilization of quorum quenching enzymes/metabolites with broad-spectrum activity might offer a good strategy as a substitute for antimicrobials.
- Molecular docking to predict the binding conformation of small molecule ligands, this will assist in the future when considering drug design.
- Future directions during this field relating to pertinence, strategies of treatment and delivery, specificity, safety, and prices have to be compelled to be investigated. Considering that endophytic microorganism and derivative compounds indicated great potential as QSIs, they need additional attention to extend interest in using endophytic microorganism resources for advanced biofilm management and microorganism infection management.

## 5.4. REFERENCES

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# APPENDICES

**Appendix I:** Morphological characteristics of extracted bacterial endophytes.

| Isolate code | Colony shape & Size | Margin (Edge) | Elevation | Colour | Texture | Gram-stain |
|---|---|---|---|---|---|---|
| ***Ba 1*** | Irregular & Large | L | Um | W | Mucoid. Shiny & Smooth | Negative |
| ***Ba 2*** | Round & Small | E | F | GB | Smooth and shiny | Negative |
| ***Ba 3*** | Irregular & Large | L | Um | W | Mucoid, shiny and smooth | Negative |
| ***Ba 4*** | Round & Small | E | Co | GB | Smooth & Shiny | Positive |
| ***Ba 5*** | Round & Small | E | SR | C | Shiny & Smooth | Negative |
| ***Ba 6*** | Round & Small | E | SR | C | Smooth | Positive |
| ***Aa 1*** | Round & Small | Un | Um | C | Mucoid | Negative |
| ***Ag 2*** | Round & Small | E | F | C | Smooth | Negative |
| ***Tv 1*** | Punctiform & Small | E | Co | Y | Smooth | Positive |
| ***Av 1*** | Round & Small | E | F | C | Smooth & Shiny | Negative |
| ***Av2*** | Round & Small | E | SR | C | Smooth | Negative |
| ***Av3*** | Round & Small | E | SR | C | Mucoid | Negative |
| ***Av 4*** | Punctiform and small | E | Co | LC | Smooth | Negative |
| ***Av 5*** | Round & Small | E | F | CW | Smooth & dull | Positive |
| ***Av 6*** | Round & Small | E | SR | C | Glistening & Smooth | Positive |
| ***Av 7*** | Punctiform & Small | E | Co | GB | Smooth | Negative |
| ***Av 8*** | Round & Small | E | SR | W | Smooth & Shiny | Negative |
| ***Av 9*** | Round & Small | E | SR | C | Smooth & Shiny | Negative |
| ***Mw 1*** | Irregular & Large | Un | F | W | Matte & Smooth | Positive |
| ***Mw 2*** | Irregular & Large | Un | F | W | Matte & Smooth | Positive |
| ***Mw 3*** | Irregular & Large | Un | F | W | Matte & Smooth | Positive |
| ***Mw 4*** | Round & Small | E | F | W | Smooth | Negative |
| ***Mw 5*** | Round & Small | E | SR | C | Smooth & Shiny | Negative |
| ***Mw 6*** | Round & Small | E | SR | C | Smooth | Positive |

Index: **E**=Entire, **F**= Flat, **SR=** slightly raised, **L**=Lobate, **Un** =Undulate, **Um**=Umbonate, **W**= White, **C**=Cream, **Co**= Convex, **GB**= Golden-brown, **LC**= Light cream, **CW**= Cream-white, **Y**= Yellow.

**Appendix II:** Minimum Inhibitory Concentration (MICs) for endophytic bacterial isolates.

| Pathogen | Endophytic bacterial Isolates | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ba 1 | | | | | Ba 2 | | | | | Ba 3 | | | | |
| | Concentrations (%) | | | | | | | | | | | | | | |
| | 100 | 50 | 25 | 12.5 | 6.25 | 100 | 50 | 25 | 12.5 | 6.25 | 100 | 50 | 25 | 12.5 | 6.25 |
| *Bacillus subtilis* | + | + | + | + | - | - | - | - | - | - | + | + | + | - | - |
| *Escherichia coli* | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| *Pseudomonas aeruginosa* | + | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| *Proteus mirabilis* | + | - | - | - | - | - | - | - | - | - | + | - | - | - | - |
| *Serratia marcescens* | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + |
| *Staphylococcus aureus* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 1% DMSO | - | | | | | - | | | | | - | | | | |
| Ciproflaxacin (0.1 mg/ml) | + | | | | | + | | | | | + | | | | |

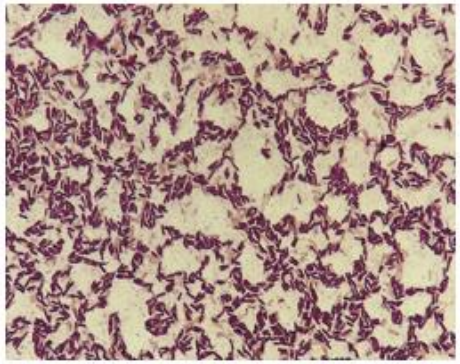
| Pathogen | Endophytic bacterial Isolates | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ba 4 | | | | | Ba 5 | | | | | Ba 6 | | | | |
| | Concentrations (%) | | | | | | | | | | | | | | |
| | 100 | 50 | 25 | 12.5 | 6.25 | 100 | 50 | 25 | 12.5 | 6.25 | 100 | 50 | 25 | 12.5 | 6.25 |
| *Bacillus subtilis* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Escherichia coli* | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| *Pseudomonas aeruginosa* | + | - | - | + | - | + | + | + | + | + | - | - | - | - | - |
| *Proteus mirabilis* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Serratia marcescens* | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + |
| *Staphylococcus aureus* | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - |
| 1% DMSO | - | | | | | - | | | | | - | | | | |
| Ciproflaxacin (0.1mg/ml) | + | | | | | + | | | | | + | | | | |

| Pathogen | Endophytic bacterial Isolates | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aa 1 | | | | | Ag 2 | | | | | Tv 1 | | | | |
| | Concentrations (%) | | | | | | | | | | | | | | |
| | 100 | 50 | 25 | 12.5 | 6.25 | 100 | 50 | 25 | 12.5 | 6.25 | 100 | 50 | 25 | 12.5 | 6.25 |
| *Bacillus subtilis* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Escherichia coli* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Pseudomonas aeruginosa* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Proteus mirabilis* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Serratia marcescens* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Staphylococcus aureus* | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - |
| 1% DMSO | | | - | | | | | - | | | | | - | | |
| Ciproflaxacin (0.1mg/ml) | | | + | | | | | + | | | | | + | | |

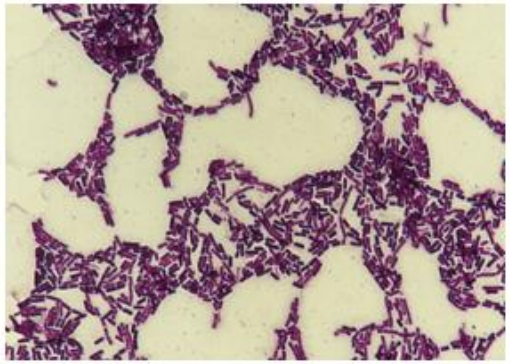
| Pathogen | Endophytic bacterial Isolates | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mw 1 | | | | | Mw 3 | | | | | Av 1 | | | | |
| | Concentrations (%) | | | | | | | | | | | | | | |
| | 100 | 50 | 25 | 12.5 | 6.25 | 100 | 50 | 25 | 12.5 | 6.25 | 100 | 50 | 25 | 12.5 | 6.25 |
| *Bacillus subtilis* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Escherichia coli* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Pseudomonas aeruginosa* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Proteus mirabilis* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Serratia marcescens* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Staphylococcus aureus* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 1% DMSO | | | - | | | | | - | | | | | - | | |
| Ciproflaxacin (0.1mg/ml) | | | + | | | | | + | | | | | + | | |

| Pathogen | Endophytic bacterial Isolates | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Av5 | | | | | Av 6 | | | | |
| | Concentrations (%) | | | | | | | | | |
| | 100 | 50 | 25 | 12.5 | 6.25 | 100 | 50 | 25 | 12.5 | 6.25 |
| *Bacillus subtilis* | - | - | - | - | - | - | - | - | - | - |
| *Escherichia coli* | - | - | - | - | - | - | - | - | - | - |
| *Pseudomonas aeruginosa* | - | - | - | - | - | - | - | - | - | - |
| *Proteus mirabilis* | - | - | - | - | - | - | - | - | - | - |
| *Serratia marcescens* | - | - | - | - | - | - | - | - | - | - |
| *Staphylococcus aureus* | - | - | - | - | - | - | - | - | - | - |
| 1% DMSO | | | - | | | | | - | | |
| Ciproflaxacin (0.1mg/ml) | | | + | | | | | + | | |

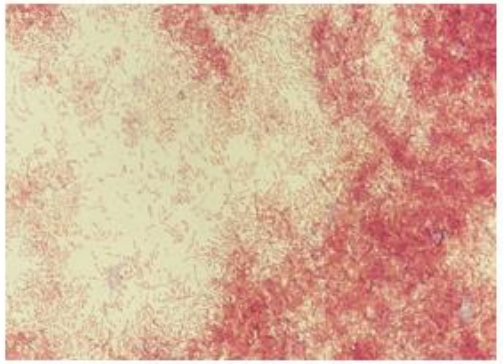
**Appendix III:** Gram-stain images of bacterial endophytic isolates.



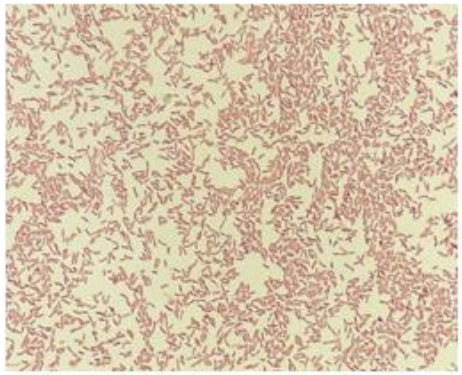
Ba 6 gram-positive



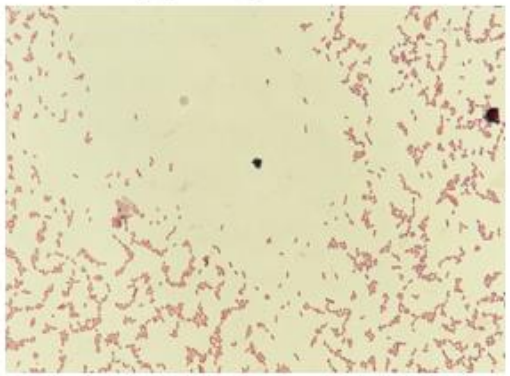
Mw 3 gram-positive



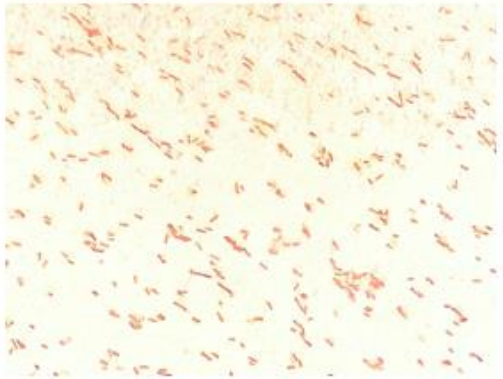
Ba 5 gram-negative



Av 1 gram-negative



Aa 1 gram-negative



Ba 1 gram-negative

**Appendix IV:** Biofilm cell attachment data.

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate1 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 23 | 0.3609 | 0.1576 | 0.2253 | 0.1669 | 0.1829 | 0.1828 | 0.1568 | 0.1045 | 0.1753 | 0.1734 | 0.2935 | 0.5349 | |
| | 0.276 | 0.2014 | 0.1941 | 0.1309 | 0.178 | 0.1305 | 0.2402 | 0.2492 | 0.2118 | 0.2688 | 0.2512 | 0.2622 | |
| | 0.2277 | 0.2096 | 0.2507 | 0.4277 | 0.1737 | 0.3876 | 0.3244 | 0.2072 | 0.1476 | 0.145 | 0.1746 | 0.167 | |
| | 0.3118 | 0.6073 | 0.5454 | 0.2483 | 0.2719 | 0.2754 | 0.1475 | 0.1804 | 0.3538 | 0.2881 | 0.3196 | 0.2918 | |
| | 0.0961 | 0.0888 | 0.0865 | 0.088 | 0.0947 | 0.0944 | 0.1366 | 0.1204 | 0.1063 | 0.1214 | 0.2059 | 0.1257 | |
| | | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| | 0.3609 | 0.1576 | 0.2253 | 0.1669 | 0.1829 | 0.1828 | 0.1568 | 0.1045 | 0.1753 | 0.1734 | 0.2935 | 0.5349 | |
| | 0.276 | 0.2014 | 0.1941 | 0.1309 | 0.178 | 0.1305 | 0.2402 | 0.2492 | 0.2118 | 0.2688 | 0.2512 | 0.2622 | |
| | 0.2277 | 0.2096 | 0.2507 | 0.4277 | 0.1737 | 0.3876 | 0.3244 | 0.2072 | 0.1476 | 0.145 | 0.1746 | 0.167 | |
| | 0.3118 | 0.6073 | 0.5454 | 0.2483 | 0.2719 | 0.2754 | 0.1475 | 0.1804 | 0.3538 | 0.2881 | 0.3196 | 0.2918 | |
| | 0.0961 | 0.0888 | 0.0865 | 0.088 | 0.0947 | 0.0944 | 0.1366 | 0.1204 | 0.1063 | 0.1214 | 0.2059 | 0.1257 | |
| | | | | | | | | | | | | | |
| Plate2 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 1.431 | 1.268 | 1.1687 | 0.9114 | 1.0734 | 0.867 | 1.8269 | 0.9813 | 1.0261 | 1.0055 | 0.6485 | 0.7631 | |
| | 0.5466 | 0.5072 | 0.4934 | 0.4596 | 0.4728 | 0.5046 | 0.3677 | 0.3637 | 0.3118 | 0.3699 | 0.4015 | 0.4381 | |
| | 0.298 | 0.2432 | 0.2568 | 0.277 | 0.2069 | 0.2564 | 0.2738 | 0.259 | 0.2344 | 0.2721 | 0.2638 | 0.2746 | |
| | 0.4139 | 0.3643 | 0.4172 | 0.3021 | 0.2578 | 0.3512 | 0.2574 | 0.2961 | 0.2745 | 0.1993 | 0.2004 | 0.2148 | |
| | 1.7506 | 0.9955 | 1.6053 | 0.1011 | 0.1104 | 0.1301 | 0.1197 | 0.0993 | 0.1206 | 0.1121 | 0.1085 | 0.1163 | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 1.431 | 1.268 | 1.1687 | 0.9114 | 1.0734 | 0.867 | 1.8269 | 0.9813 | 1.0261 | 1.0055 | 0.6485 | 0.7631 |
| | 0.5466 | 0.5072 | 0.4934 | 0.4596 | 0.4728 | 0.5046 | 0.3677 | 0.3637 | 0.3118 | 0.3699 | 0.4015 | 0.4381 |
| | 0.298 | 0.2432 | 0.2568 | 0.277 | 0.2069 | 0.2564 | 0.2738 | 0.259 | 0.2344 | 0.2721 | 0.2638 | 0.2746 |
| | 0.4139 | 0.3643 | 0.4172 | 0.3021 | 0.2578 | 0.3512 | 0.2574 | 0.2961 | 0.2745 | 0.1993 | 0.2004 | 0.2148 |
| | 1.7506 | 0.9955 | 1.6053 | 0.1011 | 0.1104 | 0.1301 | 0.1197 | 0.0993 | 0.1206 | 0.1121 | 0.1085 | 0.1163 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate3 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 0.2249 | 0.2815 | 0.2984 | 0.429 | 0.392 | 0.444 | 0.3117 | 0.3315 | 0.3288 | 0.3964 | 0.3481 | 0.225 | |
| | 0.3407 | 0.483 | 0.52 | 0.1994 | 0.2411 | 0.2801 | 0.3391 | 0.334 | 0.3423 | 1.0214 | 1.3539 | 1.247 | |
| | 0.3924 | 0.3242 | 0.3407 | 0.2504 | 0.2439 | 0.2368 | 0.2377 | 0.2659 | 0.2807 | 0.3792 | 0.4186 | 0.416 | |
| | 0.5861 | 0.3346 | 0.2339 | 0.2558 | 0.3582 | 0.2842 | 0.2513 | 0.2145 | 0.2114 | 0.2936 | 0.3513 | 0.3172 | |
| | 0.1408 | 0.1255 | 0.1492 | 0.1575 | 0.1629 | 0.1688 | 0.1636 | 0.158 | 0.1692 | 0.1527 | 0.1596 | 0.1551 | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 0.2249 | 0.2815 | 0.2984 | 0.429 | 0.392 | 0.444 | 0.3117 | 0.3315 | 0.3288 | 0.3964 | 0.3481 | 0.225 |
| | 0.3407 | 0.483 | 0.52 | 0.1994 | 0.2411 | 0.2801 | 0.3391 | 0.334 | 0.3423 | 1.0214 | 1.3539 | 1.247 |
| | 0.3924 | 0.3242 | 0.3407 | 0.2504 | 0.2439 | 0.2368 | 0.2377 | 0.2659 | 0.2807 | 0.3792 | 0.4186 | 0.416 |
| | 0.5861 | 0.3346 | 0.2339 | 0.2558 | 0.3582 | 0.2842 | 0.2513 | 0.2145 | 0.2114 | 0.2936 | 0.3513 | 0.3172 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.1408 | 0.1255 | 0.1492 | 0.1575 | 0.1629 | 0.1688 | 0.1636 | 0.158 | 0.1692 | 0.1527 | 0.1596 | 0.1551 | |
| Plate4 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 0.8722 | 0.7458 | 0.7122 | 0.7071 | 0.8087 | 0.7834 | 0.9824 | 1.1499 | 1.0605 | 1.2331 | 0.8756 | 0.9462 | |
| | 0.8584 | 0.5064 | 0.2511 | 0.6559 | 0.665 | 0.6728 | 0.6227 | 0.5987 | 0.5412 | 0.7104 | 0.5281 | 0.6044 | |
| | 0.679 | 0.438 | 0.456 | 0.5943 | 0.6077 | 0.6343 | 0.6086 | 0.5 | 0.509 | 0.4812 | 0.5075 | 0.469 | |
| | 0.5339 | 0.532 | 0.566 | 0.6273 | 0.6671 | 0.6435 | 0.7427 | 0.7996 | 0.8099 | 0.6135 | 0.6006 | 0.6061 | |
| | 0.1506 | 0.1474 | 0.1488 | 0.1556 | 0.1461 | 0.1479 | 0.148 | 0.1459 | 0.1548 | 0.1572 | 0.1487 | 0.1471 | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| | 0.8722 | 0.7458 | 0.7122 | 0.7071 | 0.8087 | 0.7834 | 0.9824 | 1.1499 | 1.0605 | 1.2331 | 0.8756 | 0.9462 | |
| | 0.8584 | 0.5064 | 0.2511 | 0.6559 | 0.665 | 0.6728 | 0.6227 | 0.5987 | 0.5412 | 0.7104 | 0.5281 | 0.6044 | |
| | 0.679 | 0.438 | 0.456 | 0.5943 | 0.6077 | 0.6343 | 0.6086 | 0.5 | 0.509 | 0.4812 | 0.5075 | 0.469 | |
| | 0.5339 | 0.532 | 0.566 | 0.6273 | 0.6671 | 0.6435 | 0.7427 | 0.7996 | 0.8099 | 0.6135 | 0.6006 | 0.6061 | |
| | 0.1506 | 0.1474 | 0.1488 | 0.1556 | 0.1461 | 0.1479 | 0.148 | 0.1459 | 0.1548 | 0.1572 | 0.1487 | 0.1471 | |
| Plate5 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 0.6508 | 0.6085 | 0.7351 | 0.4041 | 0.4005 | 0.3989 | 0.5128 | 0.4506 | 0.5053 | 0.677 | 0.8481 | 0.7703 | |
| | 0.6667 | 0.6684 | 0.6118 | 0.5923 | 0.5584 | 0.6166 | 0.4614 | 0.4344 | 0.4582 | 0.457 | 0.5244 | 0.4389 | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.4011 | 0.3584 | 0.344 | 0.4036 | 0.4121 | 0.4162 | 0.3421 | 0.3378 | 0.3306 | 0.3529 | 0.3656 | 0.3624 |
| | 0.3801 | 0.3746 | 0.4465 | 0.3994 | 0.3822 | 0.369 | 0.3866 | 0.3767 | 0.4193 | 0.3315 | 0.3899 | 0.4474 |
| | 0.1367 | 0.5846 | 0.8873 | 0.1685 | 0.1724 | 0.1409 | 0.1512 | 0.1404 | 0.14 | 0.1336 | 0.1398 | 0.1518 |
| | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 0.6508 | 0.6085 | 0.7351 | 0.4041 | 0.4005 | 0.3989 | 0.5128 | 0.4506 | 0.5053 | 0.677 | 0.8481 | 0.7703 |
| | 0.6667 | 0.6684 | 0.6118 | 0.5923 | 0.5584 | 0.6166 | 0.4614 | 0.4344 | 0.4582 | 0.457 | 0.5244 | 0.4389 |
| | 0.4011 | 0.3584 | 0.344 | 0.4036 | 0.4121 | 0.4162 | 0.3421 | 0.3378 | 0.3306 | 0.3529 | 0.3656 | 0.3624 |
| | 0.3801 | 0.3746 | 0.4465 | 0.3994 | 0.3822 | 0.369 | 0.3866 | 0.3767 | 0.4193 | 0.3315 | 0.3899 | 0.4474 |
| | 0.1367 | 0.5846 | 0.8873 | 0.1685 | 0.1724 | 0.1409 | 0.1512 | 0.1404 | 0.14 | 0.1336 | 0.1398 | 0.1518 |
| | | | | | | | | | | | | |
| Plate6 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 22.5 | 1.5737 | 1.1667 | 1.2025 | 1.2492 | 1.2508 | 1.2901 | 0.7149 | 0.8741 | 0.9355 | 1.0638 | 1.0512 | 1.1488 |
| | 1.1582 | 0.9184 | 1.2132 | 1.0503 | 1.1227 | 1.216 | 1.2474 | 1.3396 | 1.3292 | 1.5827 | 1.6153 | 1.592 |
| | 1.5458 | 1.3622 | 1.3164 | 1.2166 | 1.1959 | 1.324 | 1.5127 | 1.6377 | 1.7514 | 1.1114 | 1.0409 | 1.2374 |
| | 1.5122 | 0.8428 | 1.0694 | 1.2152 | 1.3433 | 1.1175 | 1.2487 | 1.6272 | 1.4414 | 1.3401 | 1.3107 | 1.3968 |
| | 0.8809 | 0.1421 | 1.0511 | 0.1412 | 0.1304 | 0.1534 | 0.1366 | 0.1397 | 0.1317 | 0.1355 | 0.1378 | 0.1381 |
| | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 1.5737 | 1.1667 | 1.2025 | 1.2492 | 1.2508 | 1.2901 | 0.7149 | 0.8741 | 0.9355 | 1.0638 | 1.0512 | 1.1488 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1.1582 | 0.9184 | 1.2132 | 1.0503 | 1.1227 | 1.216 | 1.2474 | 1.3396 | 1.3292 | 1.5827 | 1.6153 | 1.592 |
| | 1.5458 | 1.3622 | 1.3164 | 1.2166 | 1.1959 | 1.324 | 1.5127 | 1.6377 | 1.7514 | 1.1114 | 1.0409 | 1.2374 |
| | 1.5122 | 0.8428 | 1.0694 | 1.2152 | 1.3433 | 1.1175 | 1.2487 | 1.6272 | 1.4414 | 1.3401 | 1.3107 | 1.3968 |
| | 0.8809 | 0.1421 | 1.0511 | 0.1412 | 0.1304 | 0.1534 | 0.1366 | 0.1397 | 0.1317 | 0.1355 | 0.1378 | 0.1381 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate7 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 1.1406 | 0.7852 | 0.9488 | 0.9657 | 0.9306 | 0.9926 | 0.5539 | 0.7215 | 0.7264 | 0.8126 | 0.8329 | 0.9129 | |
| | 0.853 | 0.6726 | 0.8938 | 0.7993 | 0.9097 | 0.9861 | 0.9879 | 1.1006 | 1.0592 | 1.2284 | 1.3013 | 1.2769 | |
| | 1.1994 | 1.0419 | 1.0466 | 0.9838 | 1.0041 | 1.0925 | 1.2438 | 1.2565 | 1.3201 | 0.9186 | 0.8152 | 0.9829 | |
| | 1.1873 | 0.6654 | 0.8782 | 1.0174 | 1.0815 | 0.9574 | 1.0089 | 1.28 | 1.1002 | 1.0975 | 1.0703 | 1.1359 | |
| | 0.706 | 0.1182 | 0.8426 | 0.1181 | 0.1103 | 0.1347 | 0.1155 | 0.1192 | 0.116 | 0.1159 | 0.1154 | 0.1166 | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 1.1406 | 0.7852 | 0.9488 | 0.9657 | 0.9306 | 0.9926 | 0.5539 | 0.7215 | 0.7264 | 0.8126 | 0.8329 | 0.9129 |
| 0.853 | 0.6726 | 0.8938 | 0.7993 | 0.9097 | 0.9861 | 0.9879 | 1.1006 | 1.0592 | 1.2284 | 1.3013 | 1.2769 |
| 1.1994 | 1.0419 | 1.0466 | 0.9838 | 1.0041 | 1.0925 | 1.2438 | 1.2565 | 1.3201 | 0.9186 | 0.8152 | 0.9829 |
| 1.1873 | 0.6654 | 0.8782 | 1.0174 | 1.0815 | 0.9574 | 1.0089 | 1.28 | 1.1002 | 1.0975 | 1.0703 | 1.1359 |
| 0.706 | 0.1182 | 0.8426 | 0.1181 | 0.1103 | 0.1347 | 0.1155 | 0.1192 | 0.116 | 0.1159 | 0.1154 | 0.1166 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate8 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 0.6792 | 0.6 | 0.6441 | 0.3793 | 0.3508 | 0.3655 | 0.4947 | 0.4504 | 0.4922 | 0.6564 | 0.747 | 0.7026 | |
| | 0.6508 | 0.6992 | 0.622 | 0.5702 | 0.519 | 0.573 | 0.4275 | 0.4046 | 0.4185 | 0.368 | 0.4146 | 0.4139 | |
| | 0.362 | 0.3419 | 0.3355 | 0.3961 | 0.4017 | 0.4106 | 0.3552 | 0.3292 | 0.3302 | 0.3316 | 0.363 | 0.3507 | |
| | 0.3668 | 0.3625 | 0.4425 | 0.4 | 0.3753 | 0.3608 | 0.4187 | 0.4198 | 0.4154 | 0.3444 | 0.3766 | 0.3923 | |
| | 0.1195 | 0.5674 | 0.849 | 0.149 | 0.1551 | 0.1212 | 0.13 | 0.1228 | 0.1207 | 0.1186 | 0.1233 | 0.1294 | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 0.6792 | 0.6 | 0.6441 | 0.3793 | 0.3508 | 0.3655 | 0.4947 | 0.4504 | 0.4922 | 0.6564 | 0.747 | 0.7026 | |
| 0.6508 | 0.6992 | 0.622 | 0.5702 | 0.519 | 0.573 | 0.4275 | 0.4046 | 0.4185 | 0.368 | 0.4146 | 0.4139 | |
| 0.362 | 0.3419 | 0.3355 | 0.3961 | 0.4017 | 0.4106 | 0.3552 | 0.3292 | 0.3302 | 0.3316 | 0.363 | 0.3507 | |
| 0.3668 | 0.3625 | 0.4425 | 0.4 | 0.3753 | 0.3608 | 0.4187 | 0.4198 | 0.4154 | 0.3444 | 0.3766 | 0.3923 | |
| 0.1195 | 0.5674 | 0.849 | 0.149 | 0.1551 | 0.1212 | 0.13 | 0.1228 | 0.1207 | 0.1186 | 0.1233 | 0.1294 | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate9 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 0.7685 | 0.6793 | 0.638 | 0.6541 | 0.7136 | 0.7438 | 0.9428 | 1.0476 | 0.9233 | 0.9895 | 0.7233 | 0.7712 | |
| | 0.7365 | 0.4784 | 0.2117 | 0.608 | 0.5832 | 0.6042 | 0.58 | 0.5362 | 0.5251 | 0.5236 | 0.442 | 0.5488 | |
| | 0.602 | 0.3916 | 0.4443 | 0.5846 | 0.5379 | 0.5555 | 0.545 | 0.508 | 0.5168 | 0.5167 | 0.5369 | 0.515 | |
| | 0.5118 | 0.4947 | 0.4939 | 0.5952 | 0.58 | 0.6069 | 0.7061 | 0.7132 | 0.7338 | 0.5526 | 0.5349 | 0.5669 | |
| | 0.124 | 0.1256 | 0.1255 | 0.1295 | 0.1262 | 0.1258 | 0.1251 | 0.1235 | 0.1287 | 0.1355 | 0.1269 | 0.1253 | |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.7685 | 0.6793 | 0.638 | 0.6541 | 0.7136 | 0.7438 | 0.9428 | 1.0476 | 0.9233 | 0.9895 | 0.7233 | 0.7712 |
| | 0.7365 | 0.4784 | 0.2117 | 0.608 | 0.5832 | 0.6042 | 0.58 | 0.5362 | 0.5251 | 0.5236 | 0.442 | 0.5488 |
| | 0.602 | 0.3916 | 0.4443 | 0.5846 | 0.5379 | 0.5555 | 0.545 | 0.508 | 0.5168 | 0.5167 | 0.5369 | 0.515 |
| | 0.5118 | 0.4947 | 0.4939 | 0.5952 | 0.58 | 0.6069 | 0.7061 | 0.7132 | 0.7338 | 0.5526 | 0.5349 | 0.5669 |
| | 0.124 | 0.1256 | 0.1255 | 0.1295 | 0.1262 | 0.1258 | 0.1251 | 0.1235 | 0.1287 | 0.1355 | 0.1269 | 0.1253 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate10 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 0.1952 | 0.3002 | 0.2798 | 0.412 | 0.362 | 0.3813 | 0.2886 | 0.3155 | 0.3109 | 0.4288 | 0.3368 | 0.1959 | |
| | 0.2986 | 0.4115 | 0.4616 | 0.1962 | 0.232 | 0.2509 | 0.312 | 0.2993 | 0.318 | 0.9714 | 1.0673 | 1.204 | |
| | 0.3512 | 0.2897 | 0.2646 | 0.2194 | 0.2067 | 0.21 | 0.2215 | 0.2268 | 0.2561 | 0.3382 | 0.3371 | 0.3667 | |
| | 0.4476 | 0.2698 | 0.1998 | 0.2312 | 0.3367 | 0.2424 | 0.2184 | 0.2033 | 0.2001 | 0.2634 | 0.3054 | 0.2964 | |
| | 0.1285 | 0.1078 | 0.1245 | 0.1337 | 0.138 | 0.1375 | 0.132 | 0.1351 | 0.1421 | 0.1303 | 0.1366 | 0.1329 | |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.1952 | 0.3002 | 0.2798 | 0.412 | 0.362 | 0.3813 | 0.2886 | 0.3155 | 0.3109 | 0.4288 | 0.3368 | 0.1959 |
| | 0.2986 | 0.4115 | 0.4616 | 0.1962 | 0.232 | 0.2509 | 0.312 | 0.2993 | 0.318 | 0.9714 | 1.0673 | 1.204 |
| | 0.3512 | 0.2897 | 0.2646 | 0.2194 | 0.2067 | 0.21 | 0.2215 | 0.2268 | 0.2561 | 0.3382 | 0.3371 | 0.3667 |
| | 0.4476 | 0.2698 | 0.1998 | 0.2312 | 0.3367 | 0.2424 | 0.2184 | 0.2033 | 0.2001 | 0.2634 | 0.3054 | 0.2964 |
| | 0.1285 | 0.1078 | 0.1245 | 0.1337 | 0.138 | 0.1375 | 0.132 | 0.1351 | 0.1421 | 0.1303 | 0.1366 | 0.1329 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate11 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 1.2783 | 1.1232 | 1.1026 | 0.8704 | 1.0395 | 0.8983 | 1.765 | 1.1177 | 0.9864 | 0.902 | 0.6318 | 0.6754 | |
| | 0.4962 | 0.461 | 0.468 | 0.4416 | 0.4533 | 0.5039 | 0.3243 | 0.3341 | 0.2835 | 0.3429 | 0.3722 | 0.3672 | |
| | 0.2545 | 0.221 | 0.2297 | 0.2502 | 0.2054 | 0.2581 | 0.2621 | 0.249 | 0.248 | 0.2443 | 0.23 | 0.2269 | |
| | 0.3671 | 0.3252 | 0.3517 | 0.2399 | 0.2207 | 0.2847 | 0.237 | 0.2437 | 0.2374 | 0.1869 | 0.1818 | 0.1938 | |
| | 1.4415 | 0.8367 | 1.3621 | 0.0918 | 0.0974 | 0.1081 | 0.1063 | 0.09 | 0.1118 | 0.0968 | 0.097 | 0.1004 | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 1.2783 | 1.1232 | 1.1026 | 0.8704 | 1.0395 | 0.8983 | 1.765 | 1.1177 | 0.9864 | 0.902 | 0.6318 | 0.6754 | |
| 0.4962 | 0.461 | 0.468 | 0.4416 | 0.4533 | 0.5039 | 0.3243 | 0.3341 | 0.2835 | 0.3429 | 0.3722 | 0.3672 | |
| 0.2545 | 0.221 | 0.2297 | 0.2502 | 0.2054 | 0.2581 | 0.2621 | 0.249 | 0.248 | 0.2443 | 0.23 | 0.2269 | |
| 0.3671 | 0.3252 | 0.3517 | 0.2399 | 0.2207 | 0.2847 | 0.237 | 0.2437 | 0.2374 | 0.1869 | 0.1818 | 0.1938 | |
| 1.4415 | 0.8367 | 1.3621 | 0.0918 | 0.0974 | 0.1081 | 0.1063 | 0.09 | 0.1118 | 0.0968 | 0.097 | 0.1004 | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate12 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | | 1 |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 22.5 | 0.3422 | 0.1572 | 0.2158 | 0.1605 | 0.1861 | 0.1617 | 0.1563 | 0.0978 | 0.1738 | 0.1987 | 0.304 | 0.5309 | |
| | 0.2741 | 0.2036 | 0.1909 | 0.1264 | 0.1795 | 0.1315 | 0.2439 | 0.2526 | 0.1908 | 0.2708 | 0.2725 | 0.2528 | |
| | 0.2542 | 0.2038 | 0.2313 | 0.3445 | 0.1664 | 0.3176 | 0.3217 | 0.1867 | 0.1671 | 0.1492 | 0.1657 | 0.1595 | |
| | 0.4027 | 0.581 | 0.5426 | 0.2486 | 0.257 | 0.2536 | 0.1527 | 0.1878 | 0.2959 | 0.2604 | 0.2943 | 0.2743 | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.0878 | 0.0823 | 0.0762 | 0.0814 | 0.0894 | 0.0867 | 0.1277 | 0.1082 | 0.0952 | 0.1016 | 0.1755 | 0.118 |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 0.3422 | 0.1572 | 0.2158 | 0.1605 | 0.1861 | 0.1617 | 0.1563 | 0.0978 | 0.1738 | 0.1987 | 0.304 | 0.5309 |
| | 0.2741 | 0.2036 | 0.1909 | 0.1264 | 0.1795 | 0.1315 | 0.2439 | 0.2526 | 0.1908 | 0.2708 | 0.2725 | 0.2528 |
| | 0.2542 | 0.2038 | 0.2313 | 0.3445 | 0.1664 | 0.3176 | 0.3217 | 0.1867 | 0.1671 | 0.1492 | 0.1657 | 0.1595 |
| | 0.4027 | 0.581 | 0.5426 | 0.2486 | 0.257 | 0.2536 | 0.1527 | 0.1878 | 0.2959 | 0.2604 | 0.2943 | 0.2743 |
| | 0.0878 | 0.0823 | 0.0762 | 0.0814 | 0.0894 | 0.0867 | 0.1277 | 0.1082 | 0.0952 | 0.1016 | 0.1755 | 0.118 |

**Appendix V:** Biofilm development data.

| Plate1 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 21.5 | 1.0282 | 0.8786 | 0.9018 | 0.8721 | 0.7692 | 0.8339 | 0.9476 | 0.915 | 0.8813 | 0.9838 | 1.0327 | 1.0376 |
| | 1.1281 | 1.0052 | 1.0259 | 0.9095 | 0.9112 | 0.9206 | 0.7186 | 0.7629 | 0.7568 | 1.1055 | 1.0036 | 1.1418 |
| | 0.7644 | 0.7348 | 0.7849 | 0.8389 | 0.8198 | 0.9017 | 0.8152 | 0.77 | 0.8319 | 0.8859 | 0.7685 | 0.8566 |
| | 0.8018 | 0.8142 | 0.8031 | 0.9616 | 0.9437 | 0.9785 | 0.9364 | 0.9243 | 0.9288 | 0.8204 | 0.8147 | 0.9574 |
| | 0.0755 | 0.0969 | 0.0763 | 0.0826 | 0.0959 | 0.0834 | 0.0829 | 0.0932 | 0.141 | 0.1016 | 0.0832 | 0.1053 |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 1.0282 | 0.8786 | 0.9018 | 0.8721 | 0.7692 | 0.8339 | 0.9476 | 0.915 | 0.8813 | 0.9838 | 1.0327 | 1.0376 |
| | 1.1281 | 1.0052 | 1.0259 | 0.9095 | 0.9112 | 0.9206 | 0.7186 | 0.7629 | 0.7568 | 1.1055 | 1.0036 | 1.1418 |
| | 0.7644 | 0.7348 | 0.7849 | 0.8389 | 0.8198 | 0.9017 | 0.8152 | 0.77 | 0.8319 | 0.8859 | 0.7685 | 0.8566 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.8018 | 0.8142 | 0.8031 | 0.9616 | 0.9437 | 0.9785 | 0.9364 | 0.9243 | 0.9288 | 0.8204 | 0.8147 | 0.9574 |
| | 0.0755 | 0.0969 | 0.0763 | 0.0826 | 0.0959 | 0.0834 | 0.0829 | 0.0932 | 0.141 | 0.1016 | 0.0832 | 0.1053 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate2 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 21.5 | 2.6896 | 2.5958 | 2.6066 | 1.2143 | 0.8272 | 0.6378 | 0.554 | 0.3754 | 0.4052 | 0.5375 | 0.3973 | 0.5973 |
| | 2.4152 | 1.212 | 0.6953 | 0.7088 | 0.4684 | 0.3863 | 0.6526 | 0.4891 | 0.3984 | 0.8991 | 0.6477 | 0.3506 |
| | 1.2346 | 0.7399 | 0.8399 | 0.4244 | 0.3794 | 0.8939 | 0.4003 | 0.7062 | 0.4606 | 0.6991 | 0.7713 | 0.5417 |
| | 0.437 | 0.5004 | 0.8377 | 0.3925 | 0.5183 | 0.3997 | 0.6845 | 0.3455 | 0.6513 | 0.5735 | 0.6359 | 0.6163 |
| | 0.2649 | 0.2454 | 0.2362 | 0.2392 | 0.2594 | 0.2572 | 0.2578 | 0.2391 | 0.2382 | 0.24 | 0.2451 | 0.2577 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 2.6896 | 2.5958 | 2.6066 | 1.2143 | 0.8272 | 0.6378 | 0.554 | 0.3754 | 0.4052 | 0.5375 | 0.3973 | 0.5973 |
| | 2.4152 | 1.212 | 0.6953 | 0.7088 | 0.4684 | 0.3863 | 0.6526 | 0.4891 | 0.3984 | 0.8991 | 0.6477 | 0.3506 |
| | 1.2346 | 0.7399 | 0.8399 | 0.4244 | 0.3794 | 0.8939 | 0.4003 | 0.7062 | 0.4606 | 0.6991 | 0.7713 | 0.5417 |
| | 0.437 | 0.5004 | 0.8377 | 0.3925 | 0.5183 | 0.3997 | 0.6845 | 0.3455 | 0.6513 | 0.5735 | 0.6359 | 0.6163 |
| | 0.2649 | 0.2454 | 0.2362 | 0.2392 | 0.2594 | 0.2572 | 0.2578 | 0.2391 | 0.2382 | 0.24 | 0.2451 | 0.2577 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate3 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 21.5 | 1.0368 | 1.0656 | 0.9763 | 0.5111 | 0.4989 | 0.5031 | 0.767 | 0.7309 | 0.7952 | 0.9693 | 0.9657 | 1.4286 |
| | 1.0138 | 1.0135 | 1.0158 | 0.9644 | 0.9585 | 0.9543 | 0.4479 | 0.4392 | 0.4436 | 0.4286 | 0.6748 | 1.1369 |
| | 0.4207 | 0.4702 | 0.4658 | 0.543 | 0.527 | 0.5465 | 0.4601 | 0.4622 | 0.4476 | 0.4294 | 0.587 | 1.2743 |
| | 0.7262 | 0.7457 | 0.7667 | 0.4815 | 0.4126 | 0.437 | 0.3797 | 0.3781 | 0.3888 | 0.5094 | 0.6882 | 2.1009 |
| | 0.2497 | 0.2577 | 1.4147 | 0.2248 | 0.2216 | 0.2202 | 0.2189 | 0.2198 | 0.2166 | 0.2132 | 0.2151 | 0.2744 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 1.0368 | 1.0656 | 0.9763 | 0.5111 | 0.4989 | 0.5031 | 0.767 | 0.7309 | 0.7952 | 0.9693 | 0.9657 | 1.4286 |
| | 1.0138 | 1.0135 | 1.0158 | 0.9644 | 0.9585 | 0.9543 | 0.4479 | 0.4392 | 0.4436 | 0.4286 | 0.6748 | 1.1369 |
| | 0.4207 | 0.4702 | 0.4658 | 0.543 | 0.527 | 0.5465 | 0.4601 | 0.4622 | 0.4476 | 0.4294 | 0.587 | 1.2743 |
| | 0.7262 | 0.7457 | 0.7667 | 0.4815 | 0.4126 | 0.437 | 0.3797 | 0.3781 | 0.3888 | 0.5094 | 0.6882 | 2.1009 |
| | 0.2497 | 0.2577 | 1.4147 | 0.2248 | 0.2216 | 0.2202 | 0.2189 | 0.2198 | 0.2166 | 0.2132 | 0.2151 | 0.2744 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate4 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 21.5 | 1.4169 | 1.3264 | 1.2804 | 0.7636 | 0.7603 | 0.7765 | 1.1042 | 1.0608 | 1.1237 | 1.1103 | 1.4809 | 1.8604 |
| | 1.2754 | 1.3851 | 1.2044 | 0.956 | 1.0083 | 0.9978 | 0.8712 | 0.8255 | 0.8397 | 0.8063 | 0.7849 | 1.3797 |
| | 0.9448 | 0.8623 | 0.8045 | 0.8008 | 0.8309 | 0.8522 | 0.8504 | 0.784 | 0.7884 | 0.741 | 0.7324 | 1.173 |
| | 0.7751 | 0.6293 | 0.6331 | 0.5801 | 0.6483 | 0.6918 | 0.6975 | 0.6543 | 0.67 | 0.6875 | 0.667 | 0.8804 |
| | 0.3644 | 0.2982 | 0.3066 | 0.311 | 0.3406 | 0.3424 | 0.3197 | 0.2924 | 0.2794 | 0.2605 | 0.2559 | 0.2555 |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1.4169 | 1.3264 | 1.2804 | 0.7636 | 0.7603 | 0.7765 | 1.1042 | 1.0608 | 1.1237 | 1.1103 | 1.4809 | 1.8604 |
| | 1.2754 | 1.3851 | 1.2044 | 0.956 | 1.0083 | 0.9978 | 0.8712 | 0.8255 | 0.8397 | 0.8063 | 0.7849 | 1.3797 |
| | 0.9448 | 0.8623 | 0.8045 | 0.8008 | 0.8309 | 0.8522 | 0.8504 | 0.784 | 0.7884 | 0.741 | 0.7324 | 1.173 |
| | 0.7751 | 0.6293 | 0.6331 | 0.5801 | 0.6483 | 0.6918 | 0.6975 | 0.6543 | 0.67 | 0.6875 | 0.667 | 0.8804 |
| | 0.3644 | 0.2982 | 0.3066 | 0.311 | 0.3406 | 0.3424 | 0.3197 | 0.2924 | 0.2794 | 0.2605 | 0.2559 | 0.2555 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate5 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 21.5 | 1.3285 | 1.2017 | 1.1537 | 1.3483 | 1.3899 | 1.5166 | 1.205 | 1.1536 | 1.1914 | 1.3325 | 1.1757 | 1.1508 |
| | 1.2475 | 1.22 | 1.2846 | 1.5218 | 1.6656 | 1.6352 | 1.7979 | 1.585 | 1.6203 | 1.3962 | 1.4585 | 1.4637 |
| | 1.6775 | 1.8198 | 1.7342 | 1.5007 | 1.7032 | 1.6062 | 1.5448 | 1.563 | 1.6443 | 1.4104 | 1.3522 | 1.3184 |
| | 1.4047 | 1.3149 | 1.3979 | 1.8871 | 2.1132 | 2.0676 | 1.4901 | 1.5097 | 1.6671 | 1.6581 | 1.6585 | 1.712 |
| | 0.1001 | 0.0947 | 0.0954 | 0.0968 | 0.0884 | 0.0836 | 0.1596 | 0.0801 | 0.0849 | 0.0745 | 0.0793 | 0.0829 |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1.3285 | 1.2017 | 1.1537 | 1.3483 | 1.3899 | 1.5166 | 1.205 | 1.1536 | 1.1914 | 1.3325 | 1.1757 | 1.1508 |
| | 1.2475 | 1.22 | 1.2846 | 1.5218 | 1.6656 | 1.6352 | 1.7979 | 1.585 | 1.6203 | 1.3962 | 1.4585 | 1.4637 |
| | 1.6775 | 1.8198 | 1.7342 | 1.5007 | 1.7032 | 1.6062 | 1.5448 | 1.563 | 1.6443 | 1.4104 | 1.3522 | 1.3184 |
| | 1.4047 | 1.3149 | 1.3979 | 1.8871 | 2.1132 | 2.0676 | 1.4901 | 1.5097 | 1.6671 | 1.6581 | 1.6585 | 1.712 |
| | 0.1001 | 0.0947 | 0.0954 | 0.0968 | 0.0884 | 0.0836 | 0.1596 | 0.0801 | 0.0849 | 0.0745 | 0.0793 | 0.0829 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate6 | 1.3 | PlateFormat | Endpoint | Absorbance | Raw | FALSE | 1 | | | | | |
| Temperature(¡C) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 21.5 | 0.5919 | 0.2197 | 0.2761 | 0.4796 | 0.4783 | 0.4382 | 0.1611 | 0.171 | 0.1689 | 0.1648 | 0.0982 | 0.1012 |
| | 0.9033 | 0.8357 | 0.6939 | 0.1694 | 0.1869 | 0.154 | 0.6889 | 0.4043 | 0.3625 | 0.9576 | 0.9255 | 1.331 |
| | 0.6762 | 1.2528 | 1.1695 | 0.233 | 0.2289 | 0.2139 | 0.2129 | 0.1867 | 0.2153 | 0.2262 | 0.2627 | 0.2613 |
| | 0.5567 | 0.7162 | 0.5964 | 0.33 | 0.2533 | 0.2621 | 0.3576 | 0.3396 | 0.3197 | 1.3574 | 1.1801 | 0.7574 |
| | 0.1094 | 0.089 | 0.3505 | 0.0894 | 0.1087 | 0.0869 | 0.0859 | 0.0879 | 0.0939 | 0.0996 | 0.0902 | 0.087 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 0.5919 | 0.2197 | 0.2761 | 0.4796 | 0.4783 | 0.4382 | 0.1611 | 0.171 | 0.1689 | 0.1648 | 0.0982 | 0.1012 |
| 0.9033 | 0.8357 | 0.6939 | 0.1694 | 0.1869 | 0.154 | 0.6889 | 0.4043 | 0.3625 | 0.9576 | 0.9255 | 1.331 |
| 0.6762 | 1.2528 | 1.1695 | 0.233 | 0.2289 | 0.2139 | 0.2129 | 0.1867 | 0.2153 | 0.2262 | 0.2627 | 0.2613 |
| 0.5567 | 0.7162 | 0.5964 | 0.33 | 0.2533 | 0.2621 | 0.3576 | 0.3396 | 0.3197 | 1.3574 | 1.1801 | 0.7574 |
| 0.1094 | 0.089 | 0.3505 | 0.0894 | 0.1087 | 0.0869 | 0.0859 | 0.0879 | 0.0939 | 0.0996 | 0.0902 | 0.087 |

**Appendix VI:** Poster presented at IPUF (Indigenous Plant Use Forum) Conference held in Tshepise, July 2019. Same poster presented at BGM Research Day, October 2019. Got 3rd prize for this poster presentation.

# IDENTIFICATION OF QUORUM QUENCHING BACTERIAL ENDOPHYTES FROM SOUTH AFRICAN MEDICINAL PLANTS.

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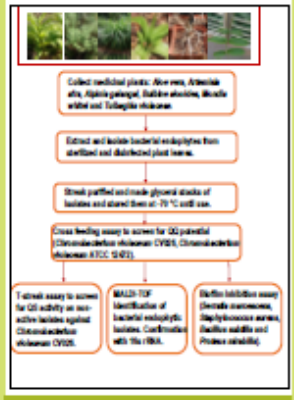
## INTRODUCTION

Infectious bacterial diseases are one of the leading cause of death, thus, the failure of antibiotics are unable to eradicate the biofilm-forming pathogens [1]. The formation of biofilms is strongly dependent on the chemical communication between groups of bacteria also known as quorum sensing (QS) [2]. Disrupting this bacterial communication is known as quorum quenching (QQ) which appears as a promising strategy and a potential to alleviate bacterial infections [3]. Endophytic bacteria are well known to provide their host plant with protection against many plant diseases and pathogens [4]. Thus the aim of this study was to isolate endophytic bacteria that may have quorum quenching potential and antibiofilm properties.

## MATERIALS & METHODS



## RESULTS & DISCUSSION

**Bacterial endophyte isolation & Extractions** A total of 34 bacterial endophyte isolates were recovered from plant leaves and homogenized plant tissues. 29.41% (10) isolates were discarded due to overgrowth and 70.59% (24) isolates were subjected to purification followed by storage in glycerol stocks at -70°C.

**Cross-feeding assay:** Endophytic bacterial isolates were used to block CV026 from picking up AHL molecules from *C. violaceum* 12472. QQ activity showed 58.3% (14) isolates with potential potent AHL signal molecules inhibition and 41.67% (10) had no QQ activity (Figure 1).

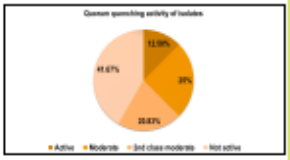


Figure 1: Pie chart depicting percentage QQ activity of bacterial endophytic isolates against *Chromobacterium violaceum* CV026 and *Chromobacterium violaceum* ATCC 12472.

**T-streak assay:** None of the 41.67% (10) isolates showed QS ability, thus indicating that they are neither quorum quenchers nor quorum sensors.

**MALDI-TOF identification:** A total of 21 isolates were identified through the use of MALDI-TOF, 3 isolates were unknown. Identification using MALDI-TOF showed the most dominant genus (Figure 2) as *Bacillus* (33.3%), followed by *Pseudomonas* (29.2%) and *Acidovorax* (12.5%) with the least being *Micrococcus* (4.2%), *Raoultella* (4.2%) and about 12.5% were unidentified.

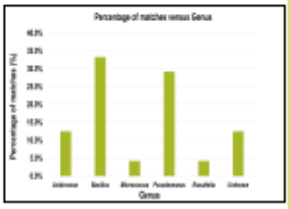


Figure 2: Colum graph depicting the most dominant genus identified through MALDI-TOF.

**Dendrograms:** The identified isolates from MALDI-TOF were subjected to hierarchical clustering to asses how similar or dissimilar the identified isolates were from each other. The clusters were not fully resolved and thus results of Dendrograms were inconclusive (Figure 3).

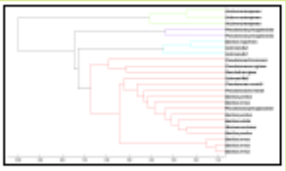


Figure 3: Hierarchical cluster Dendrograms of 24 endophytic bacterial isolates

**Identification confirmation by 16s rRNA:** Using extracted genomic DNA, PCR amplicons of approximately 1500 bp was detected by gel electrophoresis (Figure 4). PCR amplicon obtained was sequenced and compared to with 16s rRNA reference gene sequence available in the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov). 16s rRNA confirmed 87.5% isolates as MALDI-TOF.

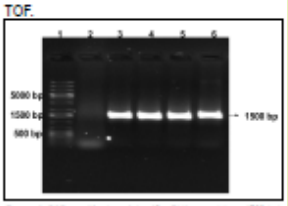


Figure 4: PCR amplification of the 16s rRNA gene (about 1500 bp) from endophytic bacterial isolates using specific primers 27F and 1492R. Lane 1: 1kb GeneRuler ladder, lane 2: Negative control, lane 3-6: PCR amplicons of isolates.

**Biofilm inhibition assay:** The potential quorum quenchers subjected to biofilm inhibition assays shows for *P. mirabilis*, the isolates had ≤15% inhibition and *S. marcescens*, isolates had ≤64% inhibition (Figure 5). For *B. subtilis*, isolates had ≤34% inhibition *S.aureus*, isolates had ≤52% inhibition (Figure 6 ).

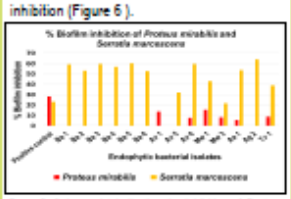


Figure 5: Column plot indicating the inhibition of *Proteus mirabilis* and *Serratia marcescens* biofilm development to the micro-well surfaces following exposure to various endophytic bacterial strains.

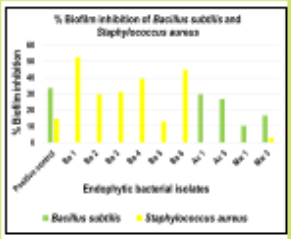


Figure 6: Column plot indicating the inhibition of *Bacillus subtilis* and *Staphylococcus aureus* biofilm development to the micro-well surfaces following exposure to various endophytic bacterial strains.

## CONCLUSION

This study therefore confirms that South African medicinal plants do harbor bacterial endophytes and suggests that bacterial endophytes with antibiofilm and/or antipathogenic activity may serve as potential candidates for use in drug development through evidence-based approaches.

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