Phyto beneficial properties of some endophytic bacteria isolate from of Thorn apple (*Datura stramonium*) from Adama, Oromia Regional State of Ethiopia

Thummim Mohammed

A Thesis Submitted to the Department of Applied Biology

College of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the degree of master's in Biology Specialization in Applied Microbiology

School of Post Graduate Studies

Adama Science and Technology University

April 2025

Adama, Ethiopia

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DECLARATION

I hereby declare that this Master Thesis paper entitled "**Phyto beneficial properties of some endophytic bacteria isolate from of Thorn apple (*Datura stramonium*) from Adama, Oromia Regional State of Ethiopia**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

Thummim Mohammed Abdulahi		
Name of student	Signature	Date

RECOMMENDATION OF ADVISORS/ SUPERVISORS

I, the major advisor/supervisor of this research thesis, hereby certify that I have closely advised/supervised the student while developing this thesis and read the draft thesis/ entitled "**Phyto beneficial properties of some endophytic bacteria isolate from of Thorn apple (*Datura stramonium*) from Adama, Oromia Regional State of Ethiopia**" prepared under my guidance by Thummim Mohammed submitted in partial fulfillment of the requirements for the degree of Masters of Science in Applied Microbiology. Therefore, I recommend the submission of the thesis to the department for further review and evaluation.

Seid Mohammed (PhD) Assoc prof		
Major Advisor	Signature	Date

APPROVAL PAGE FOR THESIS PAPER

I/we hereby certify that the recommendations and suggestions provided by the thesis review committee were duly integrated into the final thesis entitled " **Phyto beneficial properties of some endophytic bacteria isolate from of Thorn apple (*Datura stramonium*) from Adama, Oromia Regional State of Ethiopia** in Ethiopia"

Seid Mohammed (PhD) Assoc prof		
Major Advisor	Signature	Date

APPROVAL OF BOARD OF REVIEWERS

We, the undersigned, members of the Board of Reviewers of the thesis open defense by Thummim Mohammed have read and evaluated the thesis entitled "**Phyto beneficial properties of some endophytic bacteria isolate from of Thorn apple (*Datura stramonium*) from Adama, Oromia Regional State of Ethiopia**" and assessed the understanding of the candidate about the thesis research. This is, therefore, to certify that the thesis research is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Microbiology and we recommend the implementation of the thesis.

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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and College Graduate Committee (CGC).

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LIST OF ACRONYMS AND ABBREVIATIONS

AA	Acetic acid
ACC	1-aminocyclopropane-1-caroxylate
ATTC	American-Type Culture Collections
CDW	Cell dry weight
CPT	Camptothecin
ET	Ethylene
FA	Formic acid
FISH	Fluorescence insitu hybridization
GC	Gas Chromatography
IAA	Indole-3-acetic acid
ISR	Induced Systemic Resistance
JA	Jasmonic acid
LA	Lactic acid
LSV	Logarithmic score value
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization- Time of Flight Mass Spectroscopy
MEGA	Molecular Evolutionary Genetic Analyzer
MS	Mass Spectrometry
NCBI	National Center for Biotechnology Information
OD	Optical density
PGPB	Plant Growth Promoting Bacteria
Rpm	Revolutions per Minute
SA	Salicylic acid
UV	Ultraviolet

ABSTRACT

The global demand for sustainable agriculture has spurred interest in endophytic bacteria as biofertilizers and biocontrol agents. Datura stramonium, a medicinal plant abundant in Ethiopia, harbors diverse endophytes with potential Phyto-beneficial properties, yet their characterization remains limited. This study aims to screen and characterize endophytic bacterial isolates from the internal parts of Datura stramonium for their growth-promoting and biocontrol traits. Endophytic bacteria were isolated from surface-sterilized Datura stramonium tissues collected in Adama, Ethiopia. Isolates underwent morphological, microscopic, and biochemical characterization. Beneficial traits were screened via phosphate and zinc solubilization and antagonistic activity against test pathogens. Protein profiles were analyzed using MALDI-TOF, and selected isolates were identified by 16S rRNA sequencing. Growth responses to temperature, pH, salt concentration, carbon, and nitrogen sources were evaluated. Secondary metabolites were extracted using ethyl acetate, analyzed by GC-MS and TLC, and tested for antimicrobial activity. Isolates included Pseudomonas otitidis, Enterococcus faecium, Enterococcus faecalis, Klebsiella oxytoca, Bacillus cereus, Pseudomonas aeruginosa, Enterobacter hormaechei, and Paenibacillus thiaminolyticus, identified via protein profiles and sequencing. Enterococcus sp. EBIRDS-8, isolated from roots with 98.0% similarity to Enterococcus faecalis, was the most frequent and exhibited optimal growth under specific carbon, nitrogen, temperature, pH, and salt conditions. This isolate solubilized phosphate and zinc and produced secondary metabolites with inhibitory effects against pathogens, suggesting its dual role in growth promotion and biocontrol. Ethyl acetate extracts from other isolates also showed antimicrobial activity, though efficacy varied. Endophytic bacteria from Datura stramonium, particularly Enterococcus sp. EBIRDS-8, demonstrate significant plant growth-promoting and pathogen-suppressing capabilities, driven by their ability to solubilize nutrients and produce bioactive metabolites. These findings highlight their potential as sustainable agricultural tools. Field trials should validate the efficacy of Enterococcus sp. EBIRDS-8 and other isolates under natural conditions. Molecular studies and optimization of metabolite production could enhance their application, while agricultural policies in Ethiopia should support the integration of such biofertilizers to reduce chemical inputs.

Key words: Endophytic bacteria, *Datura stramonium*, Phosphate solubilization, Zinc solubilization, Secondary metabolites, *Enterococcus* sp. EBIRDS-8

CHAPTER ONE

1. INTRODUCTION

1.1. Background study

Datura stramonium (DS) is an annual plant that is native to North, Central, and South America as well as Europe, Asia, and Africa. It is a member of the Solanaceae family and may be found all over the world (Bayih, 2014). It grows up to 5 feet tall as a bush with freely branching, upright leaves, and an unpleasant smell.

Endophytes are possessed by all or most plants, and in most cases, they are transmitted through seeds, initiating the promotion of growth and plant health immediately upon seed germination (Johnston-Monje & Raizada, 2011). Internal plant tissues are inhabited by any microorganism, commonly of fungal or bacterial origin, termed an endophyte, and this occurs without inducing disease (Kandel et al., 2017). Alternatively, some endophytes may be sourced from the soil, providing similar benefits to plants (Verma & White, 2018). The roles played by endophytic microbes in plants, enhancing nutrients acquired by plants (Hossain et al., 2017), defending plants from pathogens and insects (Kumar & Verma, 2018), increasing stress tolerance in plants, influencing plant development (Irizarry & White, 2018), and inhibiting weed growth and are crucial to plant growth and function (White et al., 2018). The specific mechanisms through which various functions in plants are fulfilled by endophytic microbes are likely to differ depending on the microbe and plant. Recently, there have been studies that focus on the use of microorganisms to enhance plant nutrition in the soil and control plant phytopathogen without the use of synthetic fertilizers, pesticides, or herbicides. These bacterial species are also associated with nitrogen fixation by forming symbiosis (Parniske, 2018). Research has shown that some endophytes also colonize agronomic crops and play a role in providing and enhancing nutrient availability and biological control mechanisms against pathogens and insect pests. These bacterial communities can be also employed for crop management by increasing the quality of soil fertility (Rani et al., 2019). Endophytic bacteria are not limited to a single function but have multiple plant-growth-promoting and biocontrol traits that can be released simultaneously. For example, endophytic *P. polymyxa* is able to fix nitrogen, solubilize phosphorous, synthesize phytohormones, and display bio-control properties against pathogenic fungi under biotic and abiotic stresses (Khan et al., 2020).

Endophytic bacteria use various physical, molecular, and biochemical mechanisms to perform and display various growth and biocontrol traits (Dudeja et al., 2021). The ability to exhibit most of the plant growth-promoting and biological control traits qualifies the specific endophytic bacteria to be a reliable agent in plant growth, reproduction, and protection; therefore, such bacteria can be researched further and formulated for commercial purposes (Chen et al., 2022). Most of the beneficial endophytic bacteria absorb important nutrients such as copper, iron, zinc, and magnesium. Certain minerals can be used by the plant to aid in its growth. They can solubilize minerals such as P, Zn and K. Research has demonstrated that *D. Stramonium* growth aided by the solubilization of P and Zn by *C. freundii* CAMRI-5 and *E. kobei* EAMRI-2 (Ebu et al., 2023). The penetration of endophytic bacteria into the plant roots allows plants to extract these metals from the microbes (White et al., 2019). On the other hand, inoculating plants with endophytic bacteria could inhibit disease symptoms initiated by disease-causing organisms such as insects, nematodes, fungi bacteria, and viruses (Medison et al., 2022; Salehi-Sardoei et al., 2024).

Endophytic bacterial species that promote plant growth were not exhaustively reported in Ethiopia. As a result, the present studies intended to screen and identify these Endophytic bacteria isolate obtained from *D. stramonium*, a well-known medicinal plant in Ethiopia.

1.2. Statement of the problem

The core issue is the limited research on the diversity of endophytic bacteria in the leaf, root, and stem of *Datura stramonium* (Ethiopian thorn apple). This gap hinders our understanding of the bacterial species present, their distribution, and their ecological roles, especially in Ethiopia's distinct environmental conditions. Addressing this is vital for unlocking potential agricultural and environmental benefits. Studying these endophytes matters because they could enhance plant growth, improve nutrient uptake, and offer natural disease resistance. This research could reduce dependence on synthetic chemicals like fertilizers and pesticides, which harm the environment, while also improving soil fertility and providing natural phytopathogen control through antimicrobial properties or enhanced plant defenses. The broader impact lies in advancing sustainable agriculture in Ethiopia and beyond. By filling this knowledge gap, the research could lead to innovative, eco-friendly solutions for challenges like soil degradation, chemical overuse, and crop disease management.

1.3. Significance of the study

This work is significant because it addresses the previously uncharted domain of the variety of endophytic bacterial isolates within distinct plant sections of Thorn apple (*D. stramonium*) in Ethiopia. Understanding the precise bacterial species found in this plant's leaf, root, and stem is critical for deciphering the complexities of plant-microbe interactions in Ethiopia's unique ecosystem. This study's findings made major contributions to the understanding of endophytic bacteria, providing insights into their distribution, abundance, and potential roles. Furthermore, the study's findings paved the door for exploiting the beneficial properties of these endophytic bacteria for Ethiopian-specific agricultural and environmental applications. Finally, the value of this study stems from its ability to increase our understanding of microbial dynamics in *D. stramonium*, hence promoting options for sustainable agricultural practices and environmental protection in the region.

1.4. Objective of the study

1.4.1. General objective

- To screen and characterize endophytic bacterial isolates from the internal part of Thorn apple (*Datura stramonium*)

1.4.2. Specific Objectives

- To isolate and conduct biochemical tests and protein profiles using MALDI TOF MS analysis and 16 S rRNA gene sequencing for the diversity study of endophytic bacterial isolates that were obtained from the leaf, root, and stem parts of Thorn apple (*D. stramonium*).
- To determine the phosphate (P) and zinc (Zn) solubilization efficiency of these endophytic bacterial isolates
- To carry out optimization against these specific diversities of endophytic bacterial isolates based on their efficiency of P and Zn solubilization.
- To assess the antibacterial activity
- To analyze the chemical composition by using GC-MS.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Description of *Datura stramonium*

Datura stramonium, also known as thorn apple, jimsonweed, devil's snare, or devil's trumpet, is a poisonous flowering plant from the nightshade family Solanaceae (Karimzadeh & Rabiei, 2020). It belongs to the genus *Datura* and the tribe *Daturae*. It is thought to have originated in Central America and has since spread to many other parts of the world (Shonle & Bergelson, 2000). It grows aggressively as an invasive weed in temperate and tropical areas. In traditional medicine,

Datura stramonium is widely used to cure a variety of diseases. It has also been used as a psychedelic, producing powerful, sacred, or esoteric visions of the anticholinergic/ antimuscarinic, deliriant variety (Baker, 2023). Ingestion of *D. stramonium* can cause anticholinergic syndrome, which is characterized by profound and long-lasting disorientation or delirium and can be fatal (Gaire & Subedi, 2013; Soulaidopoulos et al., 2017). The psychedelic effects are related to tropane alkaloids found in the plant, which make it extremely dangerous (El Bazaoui et al., 2011).

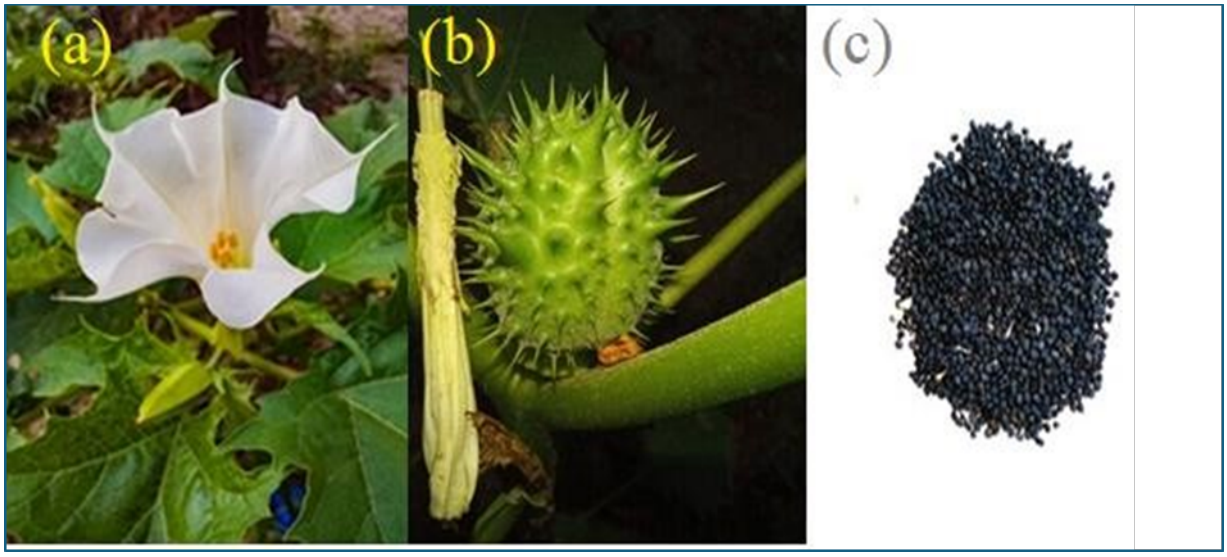


Figure 1. Thorn apple (*D.stramonium*) a) represented flower b) represent fruits c)seeds part of *D. stramonium* (Giesen et al., 2007)

Datura stramonium (Figure 1) is a tall, annual herb that stands upright and branches freely, reaching heights of 60–150 cm (2–5 ft) (Giesen et al., 2007; Stace, 1997). Its root is long,

dense, fibrous, and white-colored. The sturdy and erect stem is leafy and smooth, with hues ranging from pale yellow green to reddish-purple. The stalk constantly divides into branches, each of which produces a leaf and a single, erect bloom. The leaves measure around 8 to 20 cm (3 to 8 in) in length and are smooth, serrated, soft, and irregularly undulated. The upper surface is deeper green, while the underside is pale green. The leaves have a bitter and unpleasant smell, which is retained even in herb extracts after drying (Gibson, 1964; Giesen et al., 2007; Robertson & Roberts, 2003).

Datura stramonium often flowers over the summer months (Jiménez-Lobato et al., 2018). The fragrant trumpet-shaped flowers range in color from white to creamy or violet and are 6 to 9 cm (2+1⁄2- 3+1⁄2 in) in length. These blooms appear on short stems that grow from the edges of the leaves or the spots where the branches fork. The elongated, tubular calyx is inflated at the base and steeply angled, with five-pointed teeth on top. The half-unfolded white, funnel-shaped corolla has pronounced ribs. The blooms bloom largely at night and provide a pleasant aroma, enticing nocturnal insects to feed. The seed capsule, which resembles an egg in appearance, measures 3 to 8 cm (1 to 3 in) in diameter and can be spiny or smooth. When the capsule reaches maturity, it separates into four chambers, each bearing a large number of little black seeds**.**

2.2 Traditional Medicinal value

Datura stramonium, commonly known as Jimsonweed or thorn apple, has been valued in traditional medicine across various cultures for its potent bioactive properties. In Ethiopia and other regions, it has been traditionally used to treat ailments such as asthma, rheumatism, and skin infections due to its alkaloid content, including atropine and scopolamine, which exhibit antispasmodic and analgesic effects (Tesfaye & Berhanu, 2018). Indigenous communities have also employed its leaves and seeds in rituals and as a sedative, though its high toxicity necessitates careful preparation to avoid adverse effects (Moshi et al., 2019). Despite its medicinal significance, the plant's traditional use is often overshadowed by its reputation as a poisonous weed, highlighting a dual perception in ethnobotanical contexts (Singh & Kumar, 2020). These traditional applications underscore the cultural importance of *D. stramonium* and suggest potential for further pharmacological exploration.

In addition to its medicinal applications, *Datura stramonium* holds significant spiritual and cultural value in traditional practices across various societies. In some African communities, including parts of Ethiopia, the plant has been used in shamanistic rituals to induce visions

or altered states of consciousness, attributed to its psychoactive alkaloids like hyoscyamine (Abebe & Chala, 2021). Healers have historically prepared decoctions from its roots to treat chronic pain and inflammation, valuing its potency despite the risks of overdose (Khan & Patel, 2017). Furthermore, its use as a protective charm against evil spirits reflects its embedded role in folklore and traditional belief systems (Oluwole et al., 2022). This multifaceted traditional value highlights *D. stramonium* as more than a mere weed, positioning it as a culturally significant species warranting deeper ethnobotanical and scientific investigation.

2.3 Natural defenses

Datura stramonium's chemical responses are a natural defense mechanism against dangers (Gómez- Reino et al., 2003). These hazards include biotic factors like herbivores, diseases, viruses, fungi, and oomycetes, as well as abiotic variables like drought, light, temperature, and nutrient deficiency. *D. stramonium* adapts to these environments using protein activity connected to domains. For example, it generates terpenoids to battle herbivores in various locations and responds to abiotic stress. Abiotic responses are controlled by protein kinase regulatory subunits, which are not only over-represented and enlarged but also favorably selected. These characteristics demonstrate photochemical divergence, highlighting the plant's overall flexibility (Feigin et al., 2021).

Furthermore, terpenoids mediate plant defensive responses by promoting terpene metabolite activity. This function protects the plant from herbivore harm by reducing taste receptor sensitivity in particular insects that come into touch with it. Furthermore, terpenoids attract carnivores, who then target the same herbivores. Positively chosen and enlarged proteins in *D. stramonium* contain genes related to this immunological response. Overall, these chemicals affect the central nervous systems of the species that consume them, discouraging herbivorous behaviour. Terpenoids can promote plant-to-plant communication, which could benefit a community-wide response to threats. *D. stramonium* employs leaf trichomes as another defensive trait to ward off herbivore (Jameel et al., 2024). Understanding the plant's physiology is critical for understanding defensive mechanisms, especially as an annual plant with few options for biomass recovery after destruction and no regrowth meristems. As a result, even modest attacks might cause leaf harm. To compensate, they have a huge starting size for redundancy, increased longevity, and the capacity to metabolize when harmed. The evolution of these features differs from usual defense mechanisms in that *D. stramonium*

combines resistance and growth at the same time, rather than relying primarily on either (Bello-bedoy & Núñez-Farfán, 2011).

2.3 Diversity of Endophytic Bacteria

Endophytic bacteria species are found in all types of vascular plants. They can be found in monocotyledonous and dicotyledonous plants (Afzal et al., 2019; Ryan et al., 2008). The presences of endophytic bacteria in algae, ferns, and bryophytes have previously been documented. Endophytic bacteria are found in several types of plants, from sea grasses to towering trees. These community of bacterial species can be associated with lichens like Usnea longissima (Q. Wang et al., 2022). Several attempts have been made to ascertain the overall number of endophytic bacteria. However, the number of endophytic bacterial species may fluctuate due to the development of new techniques and methodologies for identifying this wide array of microorganisms. Prior research on the diversity of endophytic populations has shown that various plant hosts can harbor a similar collection of endophytic bacteria. Furthermore, a single plant species can support several endophytic genera and species, and the size of the endophytic community can be controlled by parameters such as tissue, plant variety, and season of isolation (Miliute et al., 2015).

It has been proven that certain bacterial such as *Actinobacteria, Bacteroidetes, Firmicutes*, and *Proteobacteria* have identified based on 16S rDNA sequences. These bacterial genera associated with plant growth promotion. These are a total of 23 distinct phyla of bacteria, of which they account for about 96% endophytic bacterial species which sequenced, belonged to prokaryotes (Pr et al., 2015). Proteobacteria account for more than 50% of the database's sequences. *Gamma- proteobacteria* isolates are most usually found as endophytes in this phylum, which includes genera such as *Acinetobacter, Enterobacter, Pantoea, Pseudomonas, Serratia,* and *Stenotrophomonas*. Endophytic microorganisms such as *Microbacterium, Staphylococcus (Firmicutes), Streptomyces, Arthrobacter* (from Actinobacteria), *Paenibacillus, Mycobacterium*, and *Bacillus* are among *γ-proteobacteria* group is the most diverse in crops (Miliute et al., 2015; Pr et al., 2015).

Endophytic bacteria are often collected from plant tissues that have been surface sterilized and are grown in a nutrient-rich medium (Papik et al., 2020). Various endophytes have recently been documented using culture-independent approaches such as whole-genome sequencing, 16S rRNA gene sequencing, and the internal transcribed spacer regions ITS1 and ITS2 of endophytic communities (Li et al., 2020). Endophytic bacteria are typically

enumerated and characterized using classic culture-based methods, which are dependent on the isolation medium and incubation conditions. Culture-independent approaches based on 16S rRNA, on the other hand, can identify uncultivable bacterial invaders as well as those that are rare or grow slowly, causing traditional culture-based protocols to miss them.

For several plant species, endophytic bacterial diversity has been reported. Proteobacteria, which includes the classes *α-, β-, and γ-Proteobacteria*, is usually the most common phylum recovered from plants; among these, *γ-Proteobacteria* is the most diverse and dominant (Santoyo et al., 2016). Furthermore, endophytes such as members of the *Actinobacteria, Bacteroidetes*, and *Firmicutes* are commonly encountered (Reinhold-Hurek & Hurek, 2011). Conversely, less often occurring classes include *Acidobacteria, Planctomycetes, and Verrucomicrobia* (Santoyo et al., 2016). However, the host plant species may have an impact on the prevalence of these phyla (Ding & Melcher, 2016). *Bacillus* and *Pseudomonas* are the most prevalent genera of bacteria isolated, with *Burkholderia, Microbacterium, Micrococcus, Pantoea*, and *Stenotrophomonas* being the other genera (Chaturvedi et al., 2016).

2.4 Beneficial roles of endophytic bacteria

Endophytes connected with ethnomedicinal plants can help to produce natural products with unique bioactivities. Endophytic bacteria are exciting research subjects due to their propensity to develop new and interesting bioactive secondary metabolites with agricultural, industrial, and medicinal applications (Fadiji & Babalola, 2020). They play essential roles in many parts of life, including their influence on host plants, the environment, and human existence. Endophytic bacteria create bioactive substances that plants can use to defend against infections or stimulate plant development, and particular endophytes can help with drug discovery. The bioactivities and structures of natural compounds generated by endophytic bacteria that protect against various diseases are diverse. This provides significant opportunities for endophytes to create secondary metabolites with agricultural, industrial, and medical applications. For example, endophytic bacteria isolated from the climbing shrub Miquelia dentate Bedd. (Icacianaceae) produces camptothecin, an anti-cancer alkaloid. Camptothecin (CPT) based medications remain of interest to scientists worldwide, with more CPT analogs emerging as prospective chemotherapeutic agents (S. S. Khan et al., 2020).

2.4.1 Endophytic bacteria as antimicrobial agents

Antimicrobial activity refers to the inhibition or destruction of disease-causing bacteria. For this goal, a variety of antimicrobial compounds are used, including antiviral, antibacterial, and antifungal characteristics Currently, bacterial infections contribute significantly to human and animal mortality rates, as well as the frequency of chronic diseases. Antibiotics have long been used as the primary treatment for bacterial illnesses due to their potency and cost-effectiveness Nonetheless, multiple studies have clearly shown that widespread antibiotic use has resulted in the evolution of multidrug-resistant bacterial species. The uncontrolled use of antibiotics has resulted in so-called "super-bacteria," which are resistant to all antibiotics. As a result, efforts have been focused on discovering new and promising strains of endophytic bacteria with antibacterial properties. Endophytic bacteria are ideal for their efficacy and lack of side effects (Amaning Danquah et al., 2022; Biharee et al., 2020). Bacillus sp. CY22, an endophytic bacterium isolated from *Platycodon grandiflorum*, has been shown to have antimicrobial properties against the fungal plant pathogens *Pythium ultimum*, *Rhizoctonia solani, Phytophthora capsici*, and *F. oxysporum* (Cho et al., 2003). Endophytic bacteria have been recognized in recent years for developing a variety of antimicrobial compounds with a novel mechanism of action due to their non-detrimental and close association with plants. Various secondary metabolites obtained from endophytic bacteria have found applications in medicine (Jasim et al., 2015; G. Strobel et al., 2004; G. A. Strobel, 2003).

2.4.2 Rhizosphere colonization by the endophytic bacteria

Endophytic bacteria compete for space and nutrients when colonizing the rhizosphere (Raaijmakers et al., 2002). Only bacteria, whether beneficial or pathogenic, that can compete to colonize a plant's rhizosphere will live in this environment and influence plant growth and development (Haas & Keel, 2003). Motility and polysaccharide synthesis are important bacterial features in plant rhizosphere colonization, as indicated by studies on endophytic *Alcaligenes faecalis* and *Azospirillum brasilense* (Santoyo et al., 2016).

Bacterial populations in the rhizosphere range from $10^{\wedge 7}$ to $10^{\wedge 9}$ cfu/g of soil, while those on the rhizoplane range from $10^{\wedge 5}$ to $10^{\wedge 7}$ cfu/g of fresh weight (Benizri et al., 2001). Bacterial detection techniques based on gfp/gusA tagged strains, immunomarkers, and fluorescence in situ hybridization (FISH) have indicated that bacterial cells populate the rhizosphere after being introduced into the soil (Gamalero et al., 2003). The bacterial cells

then connect to the root surfaces, forming a string of cells (Hansen et al., 1997). The bacteria can then colonize the entire root surface as well as some rhizodermal cells, forming micro-colonies or biofilms (Benizri et al., 2001). Rhizoplane colonization has been investigated in plants grown in vitro and in real soils (Compant et al., 2010).

The bacteria must successfully colonize the plant's rhizosphere and rhizoplane to have a positive impact on the host plant (Compant et al., 2005). While colonizing the host plant, they must also contend with competition from other rhizosphere members (Whipps, 2001). Moreover, the bacteria do not equally invade the host plant's root system. (Gamalero et al., 2003), for example, reported differences in *P. fluorescens strain A6RI* distribution and density based on root zone during colonization of tomato plants. Numerous factors influencing the process of root colonization by bacteria lead to this uneven colonization of plant roots by bacteria. (Compant et al., 2010) identified these parameters as root exudation patterns, bacterial adhesion, and motility, quorum sensing, bacterial growth rate, bacterial growth decreases by creating antagonistic chemicals, and effective nutrient acquisition. The bacteria also need to metabolically adjust to the variety of nutrients present in the plant root exudates for them to be successful. The competence with which *Pseudomonas putida KT2440* colonized the corn rhizosphere was shown by (Matillaet al., 2007) in their gene expression investigation, wherein the bacterial genes related to metabolism and oxidative stress were shown to be unregulated.

2.4.3 Root colonization by the endophytic bacteria

Bacterial endophytes are known to enter plant roots on their own after being established in the rhizosphere and rhizoplane. Once inside, they colonize the root with subpopulations that range in size from 10^5 to 10^7 cfu/g of fresh weight (Hallmann & Berg, 2006; Jeger & Spence, 2001). This process entails the mediation of bacterial adherence to cell surface structures, which is made possible by pili, bacterial adhesins, and polysaccharides (Hori & Matsumoto, 2010). Once on the root surface, the bacteria may use type IV pili-mediated twitching motility to reach the root entry sites, such as wounds and lateral root emergence. This characteristic is crucial since it was shown in the diazotrophic endophyte *Azoarcus* sp BH72 colonizing rice roots; mutants with impaired pilus retraction colonized the root surface at lower rates than the wild-type bacteria (Böhm et al., 2007). However, every endophytic bacterium has a unique colonization pattern and prefers a particular colonization site (Zachow et al., 2010). By specific processes, these bacteria begin to infiltrate the interior of the roots once they have established themselves on the surface.

Both passive and active methods of infiltration into the host are possible. Cracks caused by harmful organisms, those found at root emerging locations, or cracks at the terminals of roots can all be sites of passive penetration (Pr et al., 2015). Competent endophytic bacteria use specialized machinery for adhesion and growth to accomplish active penetration. This process involves the presence of lipopolysaccharides, flagella, pili, twitching motility, and quorum sensing, all of which might have an impact on endophytic colonization and bacterial movement inside the host plants (Suárez-Moreno et al., 2012). Furthermore, it is known that the production of enzymes that break down cell walls, primarily pectinases and cellulases, contributes to bacterial infiltration and growth inside plants (Compant et al., 2010). It has been suggested though not supported by experiments—that endophytic bacteria produce fewer enzymes that break down cell walls than phytopathogens, which produce unnecessarily excessive amounts of these enzymes and can therefore avoid inciting plant defense mechanisms (Elbeltagy et al., 2000). Additionally, maintaining lower cell densities (2–6 log cfu/gfw) than harmful bacteria (7–10 log cfu/gfw) is another method by which endophytic bacteria evade being identified by the plant as a pathogen (Zinniel et al., 2002). Therefore, random events and bacterial genetic determinants that permit bacterial-plant interaction dictate the endophytic presence of bacteria and result in an active endophytic colonization process (Pr et al., 2015). The emission of particular root exudates and a selective plant defence response are considered significant factors in the selection of endophytes. The plant host also plays a crucial role in choosing an endophytic partner (Rosenblueth & Martínez-Romero, 2006).

2.4.4 Mechanisms of host plant growth promotion

Endophytic bacteria have been shown to have several positive effects on their plant host, either directly or indirectly. These bacteria can directly benefit plants by improving their ability to absorb nutrients and by influencing hormones linked to plant growth. This helps plants grow more robustly in both normal and stressful environments (Ma et al., 2016). By inhibiting phytopathogens through the production of lytic enzymes and antibiotics, endophytic bacteria indirectly promote plant growth by depriving the pathogens of nutrients and enhancing plant defense mechanisms, which protect the plants from probable future pathogen attacks (Miliute et al., 2015).

Soil usually lacks enough levels of one or more critical nutrient components needed for plant growth. Endophytic bacteria can help host plants acquire higher concentrations of these scarce plant nutrients, like phosphorus, nitrogen, and iron. These plant growth-promoting

bacteria (PGPB) have also been employed to remove and replace the use of certain harmful chemicals in agriculture, horticulture, and silviculture, and hence reported for cleaning up the environment (Glick, 2012).

Endophytic bacteria can boost the nitrogen concentration for the host plant's growth. These bacteria can provide fixed atmospheric nitrogen to their host plants by expressing nitrogenase activity. In such as away, these plants have been used to promote the growth of maize cultivars (Zea mays). These endophytic bacterial genera are predominantly including *Burkholderia, Brevundimonas, Enterobacter, Herbaspirillum, Pseudomonas, , Pantoea, Rhanella,* and *Rhizobium* (Montañez et al., 2012). All N_2-fixing bacteria have a highly conserved gene, which can produce nitrogenase upon its expression, and there is convincing evidence that lateral gene transfer occurred (Ivleva et al., 2016). Under controlled conditions, nitrogen-fixing bacteria *like Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Azoarcus* sp BH72*, Azospirillum brasilense, Burkholderia* spp*.,* and others have been shown to increase host plant biomass through N_2 fixation (Bhattacharjee et al., 2008). When it comes to helping plants adapt to soil settings where nitrogen is scarce, associative nitrogen-fixing endophytes function better than rhizosphere microbes, consequently fostering plant health and growth. These bacteria can convert N_2 to ammonia (Hurek & Reinhold-Hurek, 2003). The same authors further reported *Azoarcus* sp. strain BH72 as a model species for nitrogen-fixing.

Plants living in soils with low nitrogen levels may benefit from endophytic nitrogen-fixing bacteria that speed up the nitrogen fixation and accumulation process. Regarding nitrogen-fixing efficiency, endophytic bacteria fall short of Rhizobium, which is linked with root nodules. It was also reported that *P. aeruginosa* PM389 is among the employed diazotrophic endophytic bacterium, which can colonize and promote the growth of *Pennisetum glaucum (L.)* (Gupta et al., 2013). On the other hand, *Gluconacetobacter diazotrophicus* are quite good for the promotion of the growth of *Pinus flexilis (limber pine*) and *Picea engelmannii* by fixing nitrogen gas. On the other hand, *Gluconacetobacter diazotrophicus* is good for promoting the growth of *Pinus flexilis (limber pine)* and *Picea engelmannii* by fixing nitrogen gas. It has been determined that certain strains of *G. diazotrophicus* coexist well with sugarcane and pine trees (Carrell & Frank, 2014). Similarly, it has been reported that *Paenibacillus* strain P22 has been used for nitrogen-fixation and employed for the growth of Poplar Plants following metabolic signature (Scherling et al., 2009).

The focus here is on phosphorus, another crucial micronutrient, which is highly required for enzymatic activities involved in numerous physiological processes in plants due to certain Phosphate-solubilizing bacteria species. Even while soil phosphorus is abundant, most of it is insoluble and cannot sustain plant growth (Ahemad, 2015). Furthermore, around 75% of phosphorus is added as fertilizer complexes with soil, making it available to plants growth due to arbuscular mycorrhizal symbiosis, Thus, phosphorus is the most important `currency' in the symbiosis processes (Ezawa et al., 2002). By using mechanisms like acidification, chelation, ion exchange, and the synthesis of organic acids, endophytic bacteria can solubilize precipitated phosphates and increase the availability of phosphorus for plants (Nautiyal et al., 2000). By secreting acid phosphatase, which can mineralize organic phosphorus, they can also increase the amount of phosphorus that is available in the soil (Van Der Heijden et al., 2008). In an endophytic bacterium, the phosphate solubilization characteristic is frequently reported. By cultivating bacteria-free cacti on mineral phosphate supplemented with either endophytes or nutrients and comparing them with plants grown under sterile conditions, investigated the role of phosphate- solubilizing endophytic bacteria. For example, 59–100% of endophytic populations from cactus, strawberry, sunflower, soybean, and other legumes were found to be mineral phosphate solubilizers (Palaniappan et al., 2010).

By producing growth-regulating phytohormones, endophytic bacteria can improve the nutrition uptake and metabolism of host plants. Endophytic colonization produced improved plant biomass and nutrient uptake, according to recent research exploring the possible significance of plant hormones secreted by endophytic bacteria (Ismail et al., 2021). There are generally five different kinds of plant hormones: indole-3-acetic acid (IAA), ethylene, gibberellins, cytokinins, and abscisic acid (Fahad et al., 2015). Of these, ethylene and IAA are thought to be the most important hormones in plant-bacterial interactions (Afzal et al., 2019; Shi et al., 2014).

An important auxin for plants, IAA engages in many physiological processes in plants. These include the induction of plant defense mechanisms, control of plant development, and cell-cell communication. In addition, IAA promotes adventitious and lateral root growth, regulates stress resistance, influences photosynthesis and metabolite biosynthesis, and mediates responses to stimuli. IAA can also control the synthesis of other hormones found in plants, such as ethylene (Glick, 2012).

One keyway that endophytic bacteria can promote plant growth is by altering the IAA pools in plants. The generation of IAA by endophytic bacteria has the potential to enhance the biomass and surface area of plant roots, as well as stimulate the growth of lateral roots in the host plant. found that IAA was created by endophytic bacteria that were separated from terrestrial orchids (Afzal et al., 2019; Dias et al., 2009). They discovered that the bacterial culture supernatant increased the length and quantity of growing roots in kidney beans, stimulating the production of roots. This finding raises the possibility that bacterial IAA has a function in the root system development of plants. More concrete proof of the function of bacterial IAA in advantageous plant effects was reported, which was employed for the growth of *P. putida* GR12-2. If there is lacking IAA synthesis, plant growth promotion is limited, especially for plant root growth and lateral root formation (Patten & Glick, 2002).

Higher concentrations of bacterial IAA synthesis might stunt growth, whilst lower amounts can promote plant root growth. In an extension of research, which examined the impact of IAA concentrations on plant development, mung bean plants grown from a mutant of *P. putida* GR12- 2, which was designed to overproduce IAA, had much shorter roots than uninoculated control plants (Patten & Glick, 2002). This is in line with the high IAA production (Rashid et al., 2012), which has been linked to elevated ethylene biosynthesis (Woodward & Bartel, 2005), and hence for production of a plant stress hormone. The synthesis of IAA is not the only way that endophytic bacteria can promote the growth of their host plants (HERLINA et al., 2017). On the other hand, the opposite process, the breakdown of IAA, can also play a crucial role in promoting plant growth. The plant growth-promoting and IAA-degrading *P. putida* 1290 neutralized the inhibitory effects of exogenous IAA on radish root elongation (Leveau & Lindow, 2005).

Furthermore, compared to the wild-type strain, it has been demonstrated that a mutant *Burkholderia phytofirmans PsJN* lacking in IAA mineralization has been unable to lessen the inhibitory effects of exogenous IAA against the roots of A. thaliana (Zúñiga et al., 2013). Still, a key factor in choosing helpful bacteria for plants is the generation of IAA by the bacteria. Additionally, as bacterial IAA production often benefits plants with low levels of endogenous IAA, plant IAA levels can decide whether bacterial IAA stimulates or suppresses plant growth (Glick, 2012).

One essential plant hormone that controls how a plant reacts to biotic and abiotic stressors is ethylene. According to (L. Sun et al., 2016) it controls several physiological and

developmental processes, including fruit ripening, root initiation, leaf senescence, root nodulation, abscission, cell elongation, and auxin transport. The biotic and abiotic stressors that cause plants to produce more ethylene restrict the growth of lateral roots, root hair creation, and root elongation (Afzal et al., 2019).

The plant hormone ethylene is derived from 1-aminocyclopropane-1-carboxylate (ACC), which can be hydrolyzed by an enzyme generated by endophytic bacteria. When bacteria with ACC deaminase activity cling to plant roots, they can convert secreted ACC to ammonia and *α- ketobutyrate* (Y. Sun et al., 2009), which they can use as a source of nitrogen. Accordingly, plant stress can be reduced by the hydrolysis of ACC, which will promote plant development in stressful situations (Santoyo et al., 2016). It has been discovered that ACC deaminase activity is present in several endophytic bacteria that are known to stimulate plant development (Rashid et al., 2012). The ACC deaminase that these bacteria produce is what gives them the potential to stimulate plant development. This was demonstrated by Sun et al. (2009), who genetically modified the canola-promoting *B. phytofirmans PsJN's* ACC deaminase gene and found that the mutant was no longer able to promote canola root growth. Reintroducing the wild-type ACC deaminase gene, however, allowed the mutant *B. phytofirmans PsJN* to once again be capable of boosting plant development, highlighting the vital function that this enzyme plays in doing so (Afzal et al., 2019).

Several studies have shown that a wide variety of endophytic bacteria that are helpful to plants can produce gibberellins and cytokinins. It has been reported that the effects of *Azospirillum lipoferum* on maize plants treats with an inhibitor of gibberellin production and exposed to either drought stress or well-watered circumstances (Cohen et al., 2009). The endophytic bacterium's production of gibberellin was essential in reducing plant stress. Similarly, it has been used a cucumber cotyledon greening bioassay to identify cytokinin-like substances in the broth extracts of two endophytic bacteria isolated from *Gynura procumbens. P. polymaxa* and *P. resinovorans* were discovered as these two bacteria (Bhore et al., 2010). To fully understand how bacterial gibberellins and cytokinin's promote plant growth, more investigation is required (Afzal et al., 2019).

By inhibiting phytopathogens and plant pests, endophytic bacteria indirectly promote the growth of the host plant. They can create substances to counteract phytopathogens, including toxins, hydrolytic enzymes, antibiotics, and volatile organic compounds with antibacterial properties. Endophytic bacteria can target both bacterial and fungal diseases. When it comes

to their ability to combat phytopathogens, *Actinobacteria, Bacillus, Enterobacter, Paenibacillus, Pseudomonas*, and *Serratia* are the most frequently mentioned genera (Saharan & Nehra, 2011). Endophytic bacteria are successful in suppressing fungal disease in plants such as wheat, potatoes, and black pepper. The synthesis of enzymes that target the fungal cell wall, including glucanases, proteases, and chitinase, can have antimicrobial effects on fungus (Panicker & Sayyed, 2022; M. Singh et al., 2020). It has been revealed *that B. subtilis* BSn5 possesses antibacterial properties against bacterial infections (Deng et al., 2011). A bacterial bio-control agent for fire blight called *Pantoea vagans* C9-1 has been put on the market (Haq et al., 2024). It has been observed that endophytic bacteria, such as *Curtobacterium luteum* TC10 and *Bacillus megaterium* BP17, reduce phytopathogenic burrowing nematodes (Saad et al., 2022). It has been demonstrated that *Eldana saccharina* larvae are efficiently targeted by genetically altered endophytic *Pseudomonas fluorescens* that expresses the toxin from *Bacillus thuringiensis* and the chitinase from *Serratia marcescens*. Induced systemic resistance, or ISR, is a technique that plant-beneficial bacteria can use to shield host plants from phytopathogens (Yu et al., 2022).

Endophytic bacteria-induced ISR can defend hosts from bacterial, viral, and fungal infections. Endophytic bacteria have shown that ethylene (ET), salicylic acid (SA), and jasmonic acid (JA) mediated pathways can initiate ISR (Pieterse et al., 2012). It has been demonstrated by ISR that bacteria belonging to the genera *Bacillus, Pseudomonas*, and *Serratia* use defense priming to shield their plant hosts. Overcoming these host defense mechanisms is also necessary to promote bacterial colonization of the host plant. ISR could entail JA/ET and SA pathways (Navarro et al., 2006). In *Arabidopsis, B. cereus* AR156 activated the JA/ET and SA signaling pathways to cause ISR, which resulted in an additional layer of plant protection. After being injected into *A. thaliana* plants, endophytic Actinobacteria demonstrated up-regulation of both defensive mechanisms, preventing subsequent infection by the fungus *F. oxysporum* and the phytopathogenic bacteria *Erwinia carotovora*. For the two pathogen types, however, there were differences in the primed defense mechanisms. The JA/ET route mediated resistance to *E. carotovora*, whereas the SA pathway mediated resistance to *F. oxysporum*. As a result, the same bacteria might prime two distinct pathways to give resistance to two distinct infections (Afzal et al., 2019; Conn et al., 2008).

2.5 The host-specificity of endophytic bacteria that promote growth

The genotype of the plant host may have an impact on endophytic bacteria's capacity to stimulate plant development. Bacteria that stimulated the development of Solanum nigrum were shown to be very particular to the host, according to (Long et al., 2008), as they could not stimulate the growth of Nicotiana attenuate, a non-host plant. Nevertheless Kim et al., (2012) have observed that the genotype of the plant influences *B. phytofirmans PsJN's* ability to promote switchgrass development. *B. phytofirmans PsJN*, which was first isolated from onion roots, shows that many endophytic bacteria can have a wide range of hosts. It can promote the growth of a variety of plants, including Arabidopsis thaliana, grape, maize, potato, switch grass, tomato, and wheat (Sheibani-Tezerji et al., 2015). Furthermore, the bacterial genotype can have a major impact on the growth-promoting actions of bacterial endophytes on host plants. The capacity of several *B. phytofirmans* strains to promote the development of a single potato cultivar differed significantly (Afzal et al., 2019; Trognitz et al., 2008). Similarly, certain *Salmonella enterica* strains, which are endophytic, showed distinct colonization patterns in alfalfa roots and hypocotyls. This bacterial colonization, which associated with genetic components employed to promote plant growth (Dong et al., 1994).

There are several examples of endophytic bacteria promoting growth in non-host plants. From nickel-accumulating *Alyssum serpyllifolium*, (Ma et al., 2011) discovered Nickel-resistant *Pseudomonas sp.* A3R3, which under metal stress aided in the development of the non-host *Brassica juncea* and the host plant. Similar to this was showed that in heavy metal-contaminated soils, the development of both the non-host *Brassica napus* and the host *Elsholtzia splendens* was greatly boosted by the copper-resistant *Burkholderia* sp. GL12 and *B. megaterium* JL35 (L. Sun et al., 2016). It was demonstrated that endophytic bacterial isolates from crop plants that could block the wilt pathogen *Ralstonia solanacearum* (Thomas & Upreti, 2014) could also lessen the impact of the disease on a tomato plant that was not the host. Moreover, under gnotobiotic circumstances, endophytic bacteria isolated from several agricultural soils utilizing tomato plants were able to stimulate canola development (Rashid et al., 2012). Endophytic bacteria that were specifically isolated from the rhizosphere of Cannabis sativa utilizing canola had similar results (Afzal et al., 2015).

CHAPTER THREE

3.MATERIALS AND METHODS

3.1. Description of the study area

The study was carried out at Adama, Oromia region which is located around 92.2 kilometers southeast of Addis Ababa, Ethiopia. Adama is situated at an elevation of 1712 meters above sea level and coordinates are 8.54°N 39.27°E. The city is situated between the Great Rift Valley to the east and the foot of an escarpment to the west as indicated in Figure 2. It has an annual temperature of 22°C and 400–800 mm of rainfall. The overall area of the city is 9,616,399.5 m^2 (OWWDS, 2012).

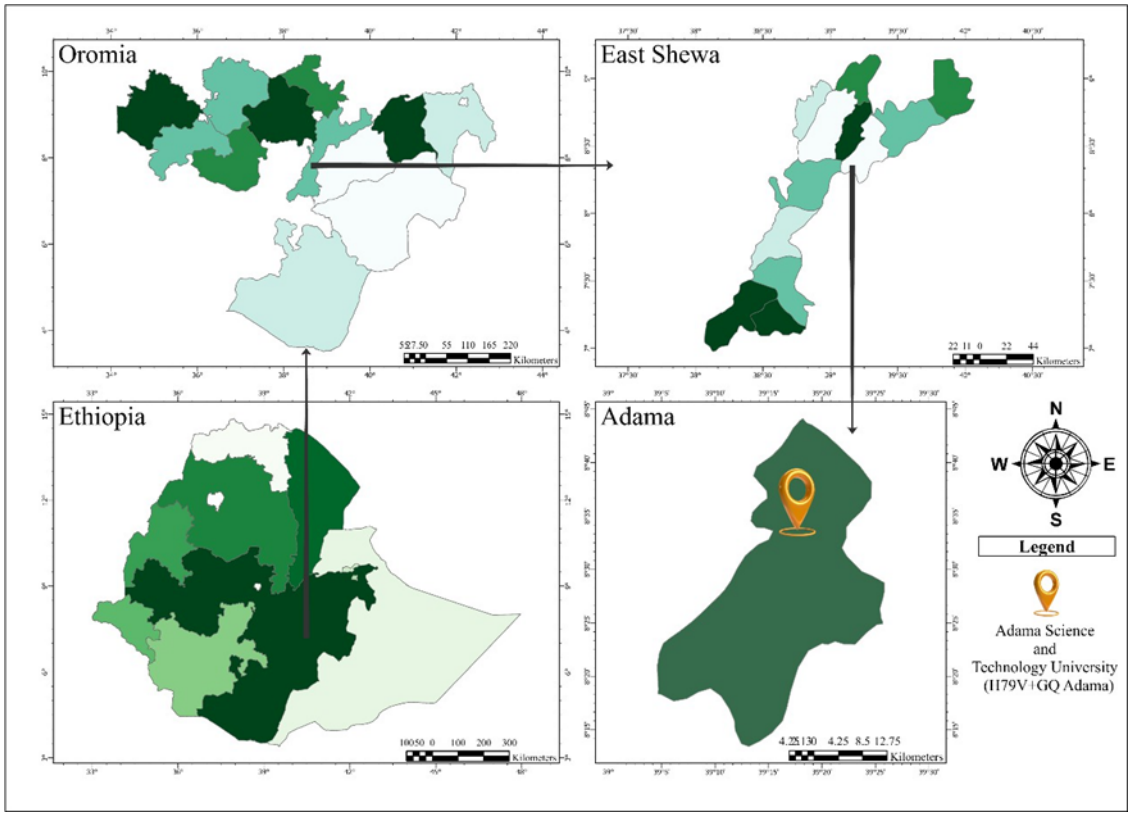


Figure 2. Location of the study area

3.2 Plant samples collection

Healthy, mature *Datura stramonium* specimens were collected from multiple sites across Adama, Oromia, Ethiopia, including Adama Science and Technology University. Leaves, stems, and roots were aseptically harvested from field-grown plants and authenticated by botanist Dr. Lemessa Kumesa to confirm their identity. The samples were then stored in

sterile containers and transported in a sterile ice box to the laboratory, accompanied by detailed records of geographical coordinates and environmental conditions.

3.3. Endophytic bacteria isolation

To prepare the tissues for the isolation of bacterial endophytes, they were first thoroughly rinsed with sterile distilled water to remove adhering soil particles and debris. The tissues were then surface sterilized by submerging them in 70% ethanol for 1-2 minutes to disrupt and remove surface bacteria, followed by immersion in a 3% sodium hypochlorite solution for 3 minutes to further sterilize the surface (Schulz et al., 1993). The concentration of the hypochlorite solution was adjusted based on the fragility of the tissue: delicate tissues like leaves were treated with a 1% solution, while sturdier tissues like roots were treated with a 5% solution (Hallmann et al., 2006). To remove any remaining traces of sodium hypochlorite, the plant tissues underwent five consecutive washes with sterile distilled water. This step was critical to ensure the complete removal of residual chemicals that might interfere with bacterial isolation. Sterility control was performed to confirm the effectiveness of the surface sterilization procedure. A small piece of each sterilized tissue type (leaf, stem, and root) was pressed onto a nutrient agar plate (HiMedia Laboratories, India) and incubated at 28°C for 48 hours. The absence of bacterial growth on the agar plate confirmed that the surface sterilization was successful. For the isolation of endophytes, the sterilized plant tissues were aseptically cut into small segments using a sterile scalpel approximately 1 cm segments for stems, roots, and leaves. These segments were then placed onto nutrient agar plates (HiMedia Laboratories, India) and incubated in an inverted position at 28°C for 2-5 days. Colony development was monitored daily, and the number, size, and morphology of bacterial colonies were recorded. Plates showing distinct bacterial colonies, with no evidence of fungal or environmental contamination, were selected for further analysis. From each plate, up to five distinct colonies, based on morphology (color, size, texture, and shape), were aseptically picked using a sterile inoculating loop and streaked onto fresh nutrient agar plates to obtain pure cultures. This selection ensured a representative sample of bacterial diversity within the plant tissues. The plates were incubated for an additional 24-48 hours at 28°C to confirm the purity of the isolated bacterial strains.

3.4. Morphological and microscopic characterization

3.4.1. Gram staining

To determine whether the isolates were Gram-positive or Gram-negative bacteria, a process of microscopic inspection using slide preparations and Gram staining techniques was utilized. A single, purified colony was taken using a loop from the plate and placed in the center of a clean slide. A smear was prepared from an overnight viable culture. The slide was heat-fixed, and crystal violet was applied for 1 min. The slide was gently washed with tap water and covered with gram's iodine for 1 min before being washed off by tilting the slide. The smear was decolorized with 95% ethyl alcohol for 15 sec and then rinsed with tap water. The smear was then covered with safranin for one minute and washed with tap water. Finally, the smear was blotted and left to dry in the air before being examined under the oil immersion objective (Wilmotte & Herdman, 2001).

3.4.2. Endospore staining

Endospore staining procedures were performed on the endophytic bacteria to determine their endospore producing ability. Bacterial colonies from slightly older cultures were smeared on a drop of water on a clean slide. Then the smears were heat fixed. Blotting papers were cut to fit the size of the slides and placed on the slides. The slides were flooded with malachite green (0.5% w/v) solution and were subjected to the heat of a flame for 5 min with the addition of ample amount of malachite green solution as needed. After that the blotting papers were removed and the slides were rinsed with tap water. Then the slides were flooded with safranin for 30 sec and rinsed with tap water afterwards. Finally, the slides were allowed to dry and were viewed under microscope x100 with oil immersion (Elmanama, 2009).

3.5. Biochemical tests

Various biochemical tests were performed for the bacterial isolates. Some of these biochemical tests were included catalase, citrate utilization, Indole, Methyl red, Urease, Voges-Proskaure test and other tests.

3.5.1. Catalase test

Three drops of 3% H2O2 (v/v) solution were added to EBIDS cultures grown on a nutrient broth medium. The bubble formation was indicated as a positive reaction, while no bubble formation was considered a negative reaction for the catalase test (Lemenh et al., 2021).

3.5.2. Citrate utilization test

The citrate utilization test is performed on Simmons citrate agar bacterial isolates were inoculated on simmon citrate agar and incubated at 28-30 °C for 48h. A negative citrate utilization test is indicated by the lack of growth and color change in the tube. A positive citrate result is indicated by growth and a blue color change (Lemenh et al., 2021).

3.5.3. Indole test

This test was used to identify bacterial isolates that can break down tryptophan to indole. Sterilized tubes containing tryptophan broth (4ml) were inoculated with a loopful of the isolates separately and incubated at 28-30°C for 24-28 h. At the end of the incubation period 0.5 ml of Kovac's reagent was added. The presence/absence of a ring was used to judge a positive/negative test for indole production ability (Dawodu & Akanbi, 2021).

3.5.4. The MR-VP test

The MR-VP test was carried out in order to determine which fermentation pathway is used by the bacteria to utilize glucose. MR-VP broth medium (Dextrose 5 g/L; Monopotassium phosphate 5 g/L; Pancreatic digest of casein 3.5 g/L; Peptic digest of animal tissue 3.5 g/L) was prepared and inoculated with bacterial isolates. The inoculated broths were incubated for 48 hours at 37oC. Then 10 ml of each of the incubated culture were poured into clean test tubes to test for the production of acetoin (VP test). To the first tubes 2-3 drops of methyl red solution was added and the formation of red color was checked. To the second tubes 3-4 drops of the mixture of α-naphthol and KOH solution was added and the solutions were shook together vigorously and set aside for about one hr. After an hour the test tubes were checked for a change in color from yellow to brownish-red to pink (Vashist et al., 2013).

3.5.5. Urease test

For urease test urea agar slants were prepared using urea agar base (Dextrose 1 g/L; disodium phosphate 1.2 g/L; monopotassium phosphate 0.8 g/L; NaCl 5 g/L; Peptone 1 g/L; Phenol red 0.012 g/L and agar 15 g/L) with addition of 40% (w/v) urea which was added to the agar base after sterilization and cooling down. Fresh 18-24 hours old pure endophytic bacterial colonies were streaked on the slants and incubated at 350 C for 24-72 hrs. The bacterial colonies that can produce urease and convert the urea into CO2, H2O and NH4 will convert the slant colors from yellow to bright pink (Vashist et al., 2013).

3.5.6. Voges-Proskaure test

It was carried out following the method (Vaughn et al., 1939) with little modification Voges Proskauer test (VP) broth prepared in two sets was inoculated with a loopful of the EBIDS and incubated for 48 h at 30 °C. α-naphthol solution (5 percent solution in 70% ethyl alcohol) (v/v) was added and shaken gently for 15 min. This is the extension of the Methyl red test and identifies the organism having the potential to produce butylene as a product. Acetoin is the in-between product of this reaction that is identified by using alpha-naphthol and 40% KOH. Acetoin oxidized to diacetyl in the existence of KOH. In the presence of alpha-naphthol, Diacetyl reacted with the guanidine component of peptone and produced a red color indicating a positive.

3.6. Screening for beneficial trials

3.6.1. Phosphate Solubilization Assay

The phosphate-solubilizing abilities of these diverse endophytic bacterial isolates were evaluated by inoculating them on Pikovskaya medium (Glucose 10.0 g/L, $Ca_3(PO_4)_2$ 5.0 g/L, $(NH_4)_2SO_4$ 0.5 g/L, Nacl 0.2 g/L, $MgSO_4 \cdot 7H_2O$ 0.1 g/L, KCl 0.2 g/L, Yeast Extract 0.5 g/L, $MnSO_4 \cdot H_2O$ 0.002g/L and FeSO4·7H2O 0.002 g/L). The medium was autoclaved at 121°C for 15 min, then poured into sterile plates, which were inoculated with the freshly isolated endophytic bacteria. Using a sterile inoculating loop, a small amount of each bacterial isolate was aseptically placed onto the center of Pikovskaya's agar plate. The plates were incubated at 28-30 °C for 5-7 d. After incubation, each plate was examined for clear halo zones around the bacterial colonies. These clear zones indicated phosphate solubilization, as the bacteria produced organic acids that solubilized the tricalcium phosphate in the medium. The diameter of both the colony and the clear zone around it was measured using a ruler. Measurements for each isolate were recorded. The plates were incubated at 30 °C for 7 d, during which zones of phosphate solubilization were observed and recorded. The results were extrapolated using a standard curve drawn with potassium dihydrogen phosphate (Pande et al., 2017).

P solubilization= Diameter (colony + halozone)/Diameter Colony.

3.6.2. Zinc Solubilization Assay

The zinc solubilization ability of these diverse endophytic bacterial isolates was examined using a minimal salt medium (Dextrose 1g/L, Ammonium sulphate 1g/L, Dipotassium

phosphate 7g/L, Monopotassium phosphate 2 g/L, Sodium citrate 0.5 g/L, Magnesium sulfate 0.1g/L, Agar 15 g/L and Distilled water 1L). The MSM was prepared with the addition of insoluble zinc oxide (ZnO) at a concentration of 1 g/L. The medium was sterilized at 121°C for 15 min, then poured into sterile Petri dishes and inoculated with fresh pure isolates. The diameter of clear halos around the bacterial growth was recorded after a 14-d incubation period at 30 °C. The solubilization index (SI) of the zinc-solubilizing bacteria was calculated as the sum of the colony diameter and the clear halo diameter divided by the colony diameter, according to the method of Gandhi et al. (2014).

Zn solubilization= Diameter (colony+halozone)/Diameter Colony

3.7. Antagonist activity of the isolated bacterial endophytes

The isolated endophytes were further analyzed for their potential antimicrobial activity against the human pathogenic bacteria *Escherichia coli* 700728^T (ATCC), *Staphylococcus aureus* 6538^T (ATCC) and *Streptococcus pyogenes* 19615^T (ATCC). The pathogens were purchased from Adama Public Health Research and Referral Laboratory Center and maintained and preserved as per laboratory guidelines. Each isolated endophytic bacterial strain was inoculated in nutrient broth and incubated at 28-30°C for 24-48 h to achieve an adequate bacterial cell density. Following incubation, the broth culture was centrifuged at 10,000 rpm for 10 minutes to separate the bacterial cells. The supernatant was collected and filtered through a 0.22 µm membrane filter to obtain a cell-free extract containing metabolites produced by the endophytic bacteria. Pathogenic bacteria, such as *E. coli* 700728^T (ATCC), *S. aureus* 6538^T (ATCC) and *S. pyogenes* 19615^T (ATCC) were selected for testing. Each pathogenic strain was inoculated into nutrient broth and incubated at 37°C for 18-24 h. The bacterial concentration of each pathogenic culture was adjusted to match the 0.5 McFarland standard (approximately 10^8 CFU/mL) by diluting with sterile saline as necessary. Using a sterile cotton swab, the standardized pathogenic bacterial suspension was evenly spread across the surface of a Mueller-Hinton agar plate to create a uniform bacterial lawn. The agar plate was allowed to dry for 5 min to set the bacterial layer. Filter paper disks (6 mm) were sterilized and soaked in the prepared endophytic bacterial extracts for a few minutes to allow absorption. Using sterile forceps, each soaked disk was placed onto the inoculated Mueller-Hinton agar plate, along with a control disk soaked in sterile distilled water to serve as a negative control. The plates were then incubated at 37°C for 18-24 h under aerobic conditions. After incubation, the plates were examined for clear zones of inhibition around each disk, indicating antibacterial activity. The diameter of each inhibition

zone was measured in millimeters using a ruler and the measurements were recorded, including those for the control disks.

3.8 Identification of endophytic bacterial isolates based on protein profile using MALDI-TOF MS

Pure isolates of *Endophytic bacteria isolates obtained from D. stramonium* (EBIDS) were identified following the methods of (Toubal et al., 2018). MALDI TOF-MS identification of the endophytic bacterial isolates was carried out at the National Animal Health Diagnostic and Investigation Center (NAHDIC) found in Sebeta. Briefly, all isolates were initially purified and screened based on their ability to antibacterial activity against human pathogen before identification using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Classification of these isolates was performed using the direct transfer method as described in the MALDI Biotyper 3.1 User Manual (Bruker Daltonics Inc.). Representative single colonies of the potentially diverse endophytic bacterial isolates were smeared as a thin film directly onto a spot on the MALDI target plate using a tooth applicator. The MALDI-TOF MS target plate was then overlaid with 1 µl of 70% (v/v) formic acid and allowed to dry at room temperature. Immediately after, the spot was overlaid with 1 µl of matrix solution consisting of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile (HCCA) solution and allowed to dry at room temperature. The resulting spectra were compared with reference spectra using the Biotyper 3.1 software (Bruker MALDI Biotyper, UK). Identification score cutoff values were applied to each measurement according to the manufacturer's instructions. Isolates with a score of ≥2.0 for a given species were considered adequately identified to the species level as prescribed by Drareni et al. (2018). *E. coli* 25922^{T} (ATCC) was used as a standard for calibration and quality control.

3.9.Identification of Potent endophytic bacterium by 16S rRNA sequencing

The molecular identification of the isolated bacteria was also performed Odisha, India, Heredity Life Sciences P.L.C. Based on better endophytes performers, antibacterial activity of the isolated bacterial such as collected Cell dry weight (CDW (g/L)), growth condition, Zn and P-solubilization, and its ethyl-acetate crude extracts that had shown better zone of inhibition against certain bacterial pathogens in this study, the EBIRDS-8 isolate was selected and sent for 16S rRNA gene sequence for identification. Genomic DNA was extracted and were sent to Odisha, India, Heredity Life Sciences P.L.C. laboratory for the

16S rRNA sequencing. The extraction of DNA for the isolated bacterial sample was carried out using the QIAGEN DNA and RNA isolation kit following the procedures of the manufacturer. Briefly pure isolate of endophytic bacterium was taken by plastic loops and added into properly labeled 2 ml eppendorf tubes containing about 0.6ml of PBS. Then the tubes were spun 20,000 × g for 5 min in a centrifuge. After the liquid was decanted 180 µl of enzymatic lysis buffer was added to the tubes and vortexed 10-20 sec. After that the tubes were incubated at 37^{o}C for 30 min. After the incubation, consecutively 25 µl of proteinase K was added to the tube and then 200 µl of buffer AL was added to the tubes and vortexed briefly. After vortexing the tubes were incubated at 56^{o}C for 30 min. After incubation 200 µl of 100% ethanol was added to the tubes and vortexed briefly. Using a micropipette the entire contents of the tube were transferred into labeled spin column. Then the columns were centrifuged at 10,000 × g for 1 min. After centrifugation the columns were removed from the collection tube and were placed into new collection tubes in addition with 500 µl of buffer AW1 and centrifuged again at 10,000 × g for 1 min. Then the columns were removed from the collection tubes and placed in a new collection tube in addition with AW2 and centrifuged at 20,000 × g for 3 min. Finally, the tubes were carefully removed from the centrifuge and the columns were transferred to a 1.5 ml tube and 200 µl of buffer AE was added to the column.

Then the column was let to stand at room temperature for 1 min prior to centrifugation at 10,000× g for 1 min. The column was discarded and the DNA was stored appropriately at -20^{o}C. (QIAgen DNeasy handbook, 2006). After the genetic material samples were received, they were double extracted following the manufacturers instruction using Quick-DNA™ Fungal/Bacterial Miniprep Kit Catalog No. D6005 from Zymo Research. Its quality was evaluated on 1.0% (w/v) agarose gel, a single band of high-molecular weight DNA has been observed.

The concentration of each DNA sample was measured by Nanodrop (Biotech instruments, USA). DNA was stored at -80^{o}C for further use. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 to 2.0 is generally accepted as pure for DNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Fragment of 16S rRNA gene was amplified by 27F and 1492R primers.

A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the ‗nr' database of NCBI GenBank database. Based on maximum identity score sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA11.

3.10. Culture condition and effect of various physical parameters on the growth of Diversities of endophytic bacterial isolates

3.10.1.Effect of pH value

The effects of pH on the growth of diverse endophytic bacterial isolates were evaluated using MSM supplemented with 1% (w/v) of the main carbon source at various pH levels (5.5, 6.5, 7.0, 7.5, and 8.0). The cultures were incubated at 30°C in a shaker incubator set at 150 rpm for 10 d. After the incubation period, OD_{600nm} and biomass (mg/l) were recorded in triplicate.

3.10.2.Effect of growth temperature

The effect of temperature on the ability of the selected diverse endophytic bacterial isolates to promote the growth of *D. stramonium* was determined using Mineral Salt Medium (composition per litre: NH_4NO_3 1.0 g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 0.2 g, KCl 0.15 g, $CaCl_2.2H_2O$ 0.1 g, $FeSO_4.6H_2O$ 0.001 g, $ZnSO_4.7H_2O$ 0.001 g, $MnSO_4$ 0.001 g) as described by Sivan et al. (2006), supplemented with 0.1% (w/v) EBIDS as the sole carbon source. The pH of the medium was adjusted to 7, and it was inoculated with a culture aged 18 to 24 h. The cultures were incubated in a shaker incubator at 150 rpm at various temperatures (25, 30, 32, 35, and 37 °C) for 10 d. After the incubation period, OD_{600nm} and biomass (mg/l) were recorded in triplicate.

3.10.3.Effect of carbon source

Four carbon-containing organic compounds were used to determine the most effective carbon source for the growth of the isolated bacteria. A minimal salts medium was prepared with 1.73 g/L of K_2HPO_4, 0.68 g/L of KH_2PO_4, 0.1 g/L of $MgSO_4.7H_2O$, 4 g/L of NaCl, 0.03 g/L of $FeSO_4.7H_2O$, 1 g/L of NH_4NO_3, and 0.02 g/L of $CaCl_2.H_2O$. To this medium, 2%

(w/v) of each carbon source—glucose, fructose, sucrose, and mannitol was added. After autoclaving, 500 ml Erlenmeyer flasks were filled with 250 ml of the medium, and 200 µl of freshly isolated bacteria, incubated for 24 h, was added. The flasks were then incubated at 130 rpm for 48 h. After the incubation period, the OD_{600nm} and biomass of the cultivated media were measured. The collected data were analyzed using Microsoft Excel.

3.10.4.Effect of Nitrogen Source

Four nitrogen sources two organic and two inorganics were used to determine the optimal nitrogen source for the growth of the isolated bacteria. The inorganic nitrogen sources were $(NH_4)_{2SO4}$ and KNO_3, while the organic sources were yeast extract and peptone. These nitrogen sources were added to the minimal salts medium (comprising 0.02 g/L $CaCl_2.H_2O$, 0.03 g/L $FeSO_4.7H_2O$, 2 g/L glucose, 1.73 g/L K_2HPO_4, 0.68 g/L KH_2PO_4, 0.1 g/L $MgSO_4.7H_2O$, and 4g/L NaCl) at a concentration of 0.6% (w/v). After autoclaving, 200 µl of the 24-h-old inoculum of the newly isolated bacteria was added to 250 ml Erlenmeyer flasks containing the media. The flasks were then incubated for 48 h with agitation at 130 rpm. Following the incubation period, the OD_{600nm} and biomass of the cultures were measured in triplicate and recorded. The resulting data were analyzed using Microsoft Excel.

3.10.5.Effect of salt concentration

The effect of varying salt concentrations on the growth of diverse endophytic bacterial isolates with superior P and Zn solubilization abilities was examined. This study considered different salt concentrations (0.1, 0.3, 0.5, and 1M) to optimize conditions for the newly isolated EBIs from *D. stramonium*. Their growth efficiency was evaluated by measuring biomass. The results were recorded for these isolates. Nutrient Agar containing the specified salt concentrations was used to assess the growth efficiency of these endophytic bacterial isolates. The pH and temperature were adjusted to optimal conditions for these EBIDS. The isolates were incubated in a shaker incubator at 150 rpm for 10 to 15 d. After the incubation period, OD_{600nm} and biomass (mg/l) were recorded in triplicate.

3.11. Secondary metabolite crude extracts preparation

Secondary metabolite crude extract was prepared by modifying the method of (Lodi, et. al., 2023). Bacterial endophyte EBIDS-11 that possess significant antibacterial activity from preliminary test were selected and cultured in 1 l Luria broth (LB) and incubated at 28 °C for 7 d on shaking incubator at 150 r/m. After incubation the broth was centrifuged at 10000 r/m for 10 min and supernatant was collected then equal volume of ethyl acetate was added and

shaken for 30 min in an extracting flask to extract secondary metabolites. Later, the organic phase of the liquid collected and evaporated using rotating evaporator. Then, the dry sample of crude extract with secondary metabolites was collected and stored at room temperature until further analysis.

3.12.Antimicrobial assay of secondary metabolite crude extract of EBIRDS-8

The antibacterial activity was tested by the agar diffusion method against certain strains such as *S. Pyogenes* 19615^T (ATCC), *S. aureus* 25923^T (ATCC) and Gram-negative bacteria (*E. coli* 25922^T (ATCC) and *P. aeruginosa* 27853^T (ATCC). The tested strains were inoculated onto medium. Then, pure colonies were collected and resuspended in a test tube containing 5 ml of NaCl 0.9% (w/v) to obtain a suspension with a 25% transmittance at λ = 580 nm or equivalent to a concentration of 1 x 10^8 CFU/mL of bacteria (of 0.5 Mc Farland). The same treatment was done in any kind of bacteria test. The extract was weighed 100 mg and dissolved in 2 ml DMSO.

Secondary metabolite crude extract 0.1g/2 ml stock solution was prepared and subjected to serial dilution to the concentrations 100, 50, 25, and 12.5 µg/ml using dimethyl sulfoxide (DMSO) Thus, this study obtained the 7.5% test concentration and sterilized discs or paper discs that were spilled with 10 µl extract and then put on the surface of the Mueller-Hinton agar media. This media had been previously swabbed with the suspension of the tested bacteria (1 x 10^8 CFU/mL). Then Petri was incubated in the incubator for 18 h at a temperature of 37ºC. Antibacterial activity was observed by measuring the inhibition diameter of the discs using a caliper. The positive control used in this research was 30 µg/disc Ciproflaxcin. Meanwhile, the negative control used in this research was the dimethyl sulfoxide (DMSO).

3.13. Gas chromatography–Mass spectrophotometry

The crude sample was sent for size- and polarity-based GC-MS analysis. The chemicals found in metabolites were identified using GC-MS. The GC-MS analysis was performed using a PerkinElmer GC Clarus 500 system and a gas chromatograph connected to a mass spectrometer. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used in the electron impact mode. An injection volume of 2 µL was employed, and helium gas (99.99%) was used as a carrier gas at a continuous flow rate of 1°mL/min. The injector temperature was kept constant at 250°C, the ion-source temperature was kept

constant at 200°C, and the oven temperature was programmed to begin at 110°C, then increased by 10 °C/min to 200°C, and then increased by 5 °C/min to 280°C, finishing with a 9-min isothermal at 280 °C. Fragments ranging in size from 45 to 450 kDa were used in the mass spectra. The total GC–MS run time was 36 min, with a solvent delay of 0 to 2 min. The relative percentage quantity of each component was determined by comparing the average peak area of each component to the total areas.

3.14. Profiles of Thin Layer Chromatography (TLC) of Ethyl Acetate Extracts of Endophytes Bacteria

Foe TLC determinations, commercially available or lab-prepared silica gel-coated TLC plate were employed. The ethyl acetate extract was dissolved in a small volume of a suitable solvent, such as hexane. Using a capillary tube, small and concentrated spots of the extract was applied onto the baseline (1–2 cm from the bottom edge) of the TLC plate. A mobile phase (hexane: ethyl acetate) suitable for separating the compounds in the extract was prepared. The TLC plate was placed into a developing chamber containing the mobile phase, ensuring that the solvent level is below the applied spots. The mobile phase was allowed to ascend the plate by capillary action until it nearly reaches the top. After development, the plate was removed and allowed it to air-dry. Finally, the spots were visualized under UV light at 254 nm or 365 nm. The RF was calculated as following methods.

Rf = Distance traveled by compound/ Distance traveled by solvent.

3.15. Data Analysis

The experimental results were analyzed using the STATISTICAL 25.0 program, employing variance analysis of ANOVA (IBM SPSS version 20.0). The data obtained were expressed as mean ± standard deviation (SD), and differences were considered significant at $p < 0.05$. One-way ANOVA was followed by Tukey's test at a 95.0% confidence level. Statistical analyses quantified the relative abundance of endophytic bacteria in different plant parts. Correlation and regression analyses were utilized to explore relationships between bacterial diversity, environmental factors, and plant health indicators.

CHAPTER FOUR

4. RESULTS AND DISCUSSIONS

4.1. Endophytic bacteria isolation from the plant part of *D. stramonium*

From the total of 50 bacteria isolated twenty-five cultivable bacterial isolates, chosen based on their morphological diversity on Nutrient Agar, were obtained from various parts of *D. stramonium* plants. The majorities of these isolates, displaying distinct morphological characteristics on Nutrient Agar, were found in the roots (40%), followed by, stems (20%), and leaves (10%) (Figure 3).

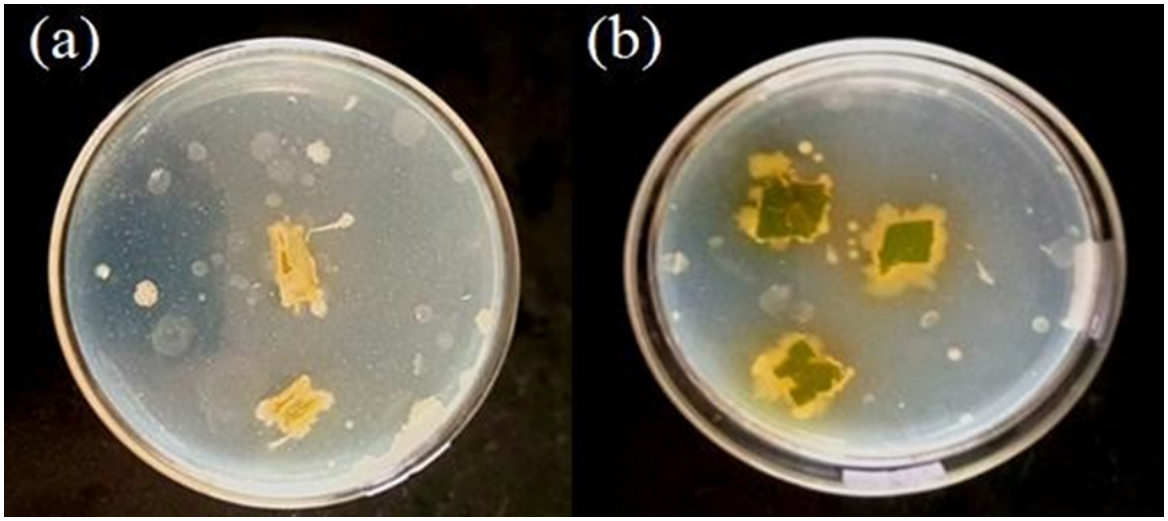


Figure 3: a) Endophytic bacteria emerge from Stem parts of D. Stramonium (EBIRDS-8). b) Endophytic bacteria emerge from root parts of D. Stramonium (EBILDS-12)

4.2. Morphological characterization of the endophytic bacterial isolates

In this study, pure cultures of endophytic bacteria with distinct morphological and biochemical traits were successfully isolated (Figure 4). From MALDI TOF identification out of the 15 bacterial isolates from *D. stramonium* medicinal plant, 5 were Gram-positive, and 8 were Gram-negative (Figure 4). Additionally, 2 isolates were endospore formers, while 11 were non-endospore formers. Different bacterial cells were isolated from *D. stramonium* (thorn apple), as shown in Figure 4.

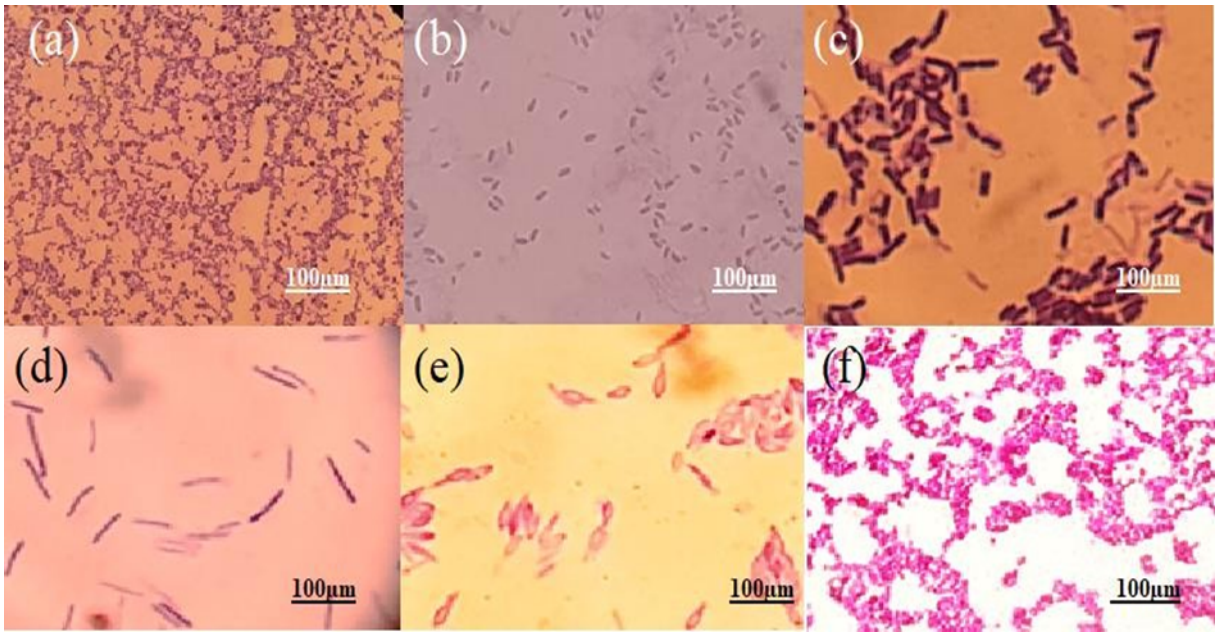


Figure 4: Gram staining of isolated bacteria a) Gram positive cocci- which *Enterococcus sp.* EBIRDS-8cocci shape bacteria b) Short rod-shaped which is Gram positive that designated as EBIRDS-13 c) Gram positive isolates that designated as EBISDS- 6 (d) Gram positive rod-shaped that designated as EBIRDS-14 (e) Gram negative and designated as EBILDS-10 (f) Gram-negative that obtained from the stem part of *D. stramonium* and designated as EBISDS-1

4.3. Biochemical test

Biochemical tests of the bacterial isolates from *Datura Stramonium* indicated in Tabel 1. The majority of them are rod-shaped, citrate positive, catalase positive, Gram negative, urease positive, and Voges-proskaure positive. Few of them are endospore formers (Table 1).

Table 1. Morphological and biochemical characterization endophytic isolates

Isolates	Gram staining	Shape	Endospore staining	Catalase test	Citrate test	Indole test	Methylred test	Urase test	Voges-proskaure test	Genera
EBISDS-1	-ve	rod	-ve	+ve	+ve	-ve	-ve	+ve	+ve	*Enterobacter*
EBILDS-3	+ve	rod	-ve	+ve	+ve	+ve	-ve	+ve	+ve	*Corynebacterium*
EBIRDS-4	+ve	rod	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Bacillus*
EBILDS-5	-ve	rod	-ve	+ve	+ve	+ve	-ve	+ve	+ve	*Enterobacter*
EBISDS-6	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	-ve	*Pseudomonas*
EBILDS-7	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	-ve	*Pseudomonas*
EBIRDS-8	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	-ve	*Pseudomonas*
EBILDS-9	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	-ve	*Pseudomonas*
EBILDS-10	+ve	rod	+ve	+ve	+ve	+ve	+ve	+ve	-ve	*Bacillus*
EBISDS-11	+ve	cocci	-ve	-ve	-ve	-ve	+ve	-ve	-ve	*Enterococcus*
EBILDS-12	-ve	rod	-ve	+ve	+ve	+ve	-ve	+ve	+ve	*Klebsiella*
EBIRDS-13	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	-ve	*Pseudomonas*
EBIRDS-14	+ve	rod	-ve	-ve	-ve	-ve	+ve	-ve	+ve	*Lactobacillus*

4.4. Screening of Beneficial traits

4.4.1. Phosphate Solubilization Assay

The results obtained from this study showed that out of the 15 endophytic bacterial isolates, all of them were able to solubilize the insoluble phosphate added in Pikovskaya‘s agar in the form of tri-calcium phosphate (Figure 5, Table 4). The data presented below in the table provides valuable insights into the phosphate solubilizing capabilities of various isolates. The formation of halo zones around colonies is a clear indicator of phosphate solubilization, which is further calculated using the solubilization index (SI) (Table 4). The solubilization index is a critical parameter, as it allows for the comparison of different isolates based on their ability to solubilize phosphate. A higher SI indicates a greater ability to solubilize phosphate, which is desirable for agricultural applications where phosphate availability is a limiting factor for plant growth (Kalayu, 2019). Phosphate solubilization by endophytic bacteria is a significant mechanism that enhances plant growth by making phosphorus more available to plants (Table 2). Endophytic bacteria, which live within plant tissues, can solubilize inorganic phosphate through the production of organic acids, such as gluconic acid, which convert insoluble phosphate compounds into forms that plants can absorb and utilize.

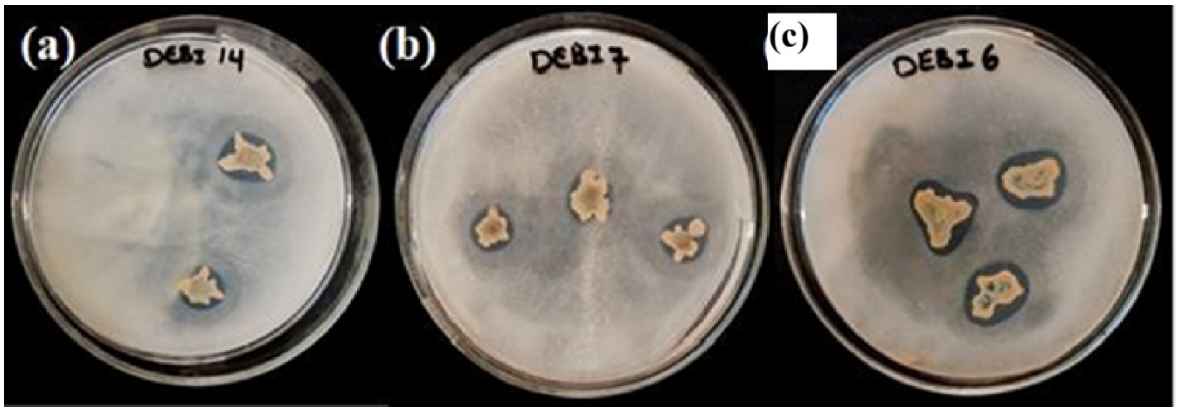


Figure 5: a) phosphate Solubilization against EBILDS-14. b) phosphate Solubilization against EBILDS-7 (1.8) c) phosphate Solubilization against EBISDS-6

Several studies have demonstrated the effectiveness of endophytic bacteria in solubilizing phosphate and promoting plant growth. For instance, endophytic *Pseudomonas* isolates have been shown to produce gluconic acid and solubilize phosphate, thereby enhancing the growth of *Pisum sativum L.* plants under phosphate-limiting conditions (Oteino et al., 2015). Similarly, endophytic bacteria isolated from banana tree roots exhibited significant

phosphate solubilization capabilities, particularly from tricalcium phosphate, and demonstrated acid phosphatase activity, which further aids in phosphate solubilization (Matos et al., 2017). In wheat cultivars, a consortium of endophytic and rhizospheric phosphate-solubilizing bacteria improved phosphorus using efficiency, leading to increased root and shoot biomass, grain yield, and overall plant growth in phosphorus-deficient soils (Emami et al., 2020). Additionally, endophytic bacteria from *Phyllanthus amarus*, identified as *Acinetobacter* sp. and *Bacillus* sp., showed positive plant growth-promoting traits and enhanced plant vigor under salt stress conditions (Joe et al., 2016).

Table 2. Phosphate screening

S.N.	Isolates	Colony diameter(mm)	Halo zone diameter(mm)	Solubilization index
1	*K. oxytoca* EBISDS-1	7	14	2
2	EBILDS-2	8	16	2
3	*Klebsiella* sp. EBILDS-3	8	11	1.37
4	*Pseudomonas* sp. EBIRDS-4	7	14	2
5	*Pseudomonas* sp. EBILDS- 5	8	11	1.37
6	*Paenibacillus* sp. EBISDS- 6	8	16	2
7	*Pseudomonas* sp. EBILDS-7	7	10	1.4
8	*E. Faecium* EBIRDS-8	8	15	1.8
9	*E. hormaechei* EBILDS-9	7	12	1.7
10	*E. faecium* EBILDS-10	7	12	1.7
11	*B. cereus* EBISDS-11	7	12	1.7
12	*P. aeruginosa* EBILDS-12	7	12	1.7
13	*P. otitidis* EBIRDS-13	8	15	1.8
14	*B. cereus* EBIRDS-14	7	13	1.8
15	EBILDS-15	8	16	2

4.4.2. Zinc Solubilization Assay

Among all the 25 isolates of endophytic bacteria, ten i.e., *Pseudomonas* sp. EBIRDS-8, *E. hormaechei* EBILDS-9, *K. oxytoca* EBISDS-1, *B. cereus* EBISDS-11, *B. cereus* EBIRDS-14, and *Paenibacillus* sp. EBISDS- 6 showed Zinc solubilization zones on minimal salt medium (Table 3). A maximum zone of 1.8 mm was observed for *P. otitidis* EBIRDS-13. Recent studies have demonstrated that *P. otitidis* EBIRDS-13 can effectively solubilize various insoluble zinc salts, such as zinc oxide (ZnO) and zinc carbonate ($ZnCO_3$). For instance a strain designated as ZSB13 from contaminated soil showed significant solubilization capabilities with a clear halo zone of 11 mm on agar plates containing ZnO. In liquid media, this strain released up to 18.2 ppm of zinc from ZnO. Additionally, molecular characterization confirmed ZSB13 as *P. oleovorans*, indicating its potential role as a biofertilizer due to its high resistance to zinc (Rehman et al., 2021). Another study highlighted those various strains of *Pseudomonas,* including *P. protegens*, and *P. aeruginosa*, exhibit strong zinc solubilization abilities, suggesting that these bacteria could be utilized in agricultural waste to improve soil fertility and crop yields. *K. oxytoca* EBISDS-1: has demonstrated a strong capacity to solubilize zinc oxide and zinc carbonate through organic acid production, significantly reducing the pH and increasing soluble zinc concentrations in culture media. *B. cereus* EBISDS-11, and *B. cereus* EBIRDS-14in vitro assays showed that these species could create substantial halo zones around colonies on agar plates containing insoluble zinc sources, indicating effective solubilization. Quantitative assays revealed up to 14.5 mg/kg of soluble zinc after incubation. *E. hormaechei* EBILDS-9 has been noted for its ability to solubilize various zinc compounds effectively, with research indicating that it can release significant amounts of soluble zinc into the medium during growth. *Paenibacillus* sp. EBISDS- 6has been characterized for its robust solubilization potential, particularly in acidic environments where it produces organic acids that facilitate the dissolution of insoluble zinc salts (Saravanan et al., 2004). The ability of *B. cereus* EBISDS-11, *E. hormaechei* EBILDS-9, *K. oxytoca* EBISDS-1, *Enterococcus* sp. EBIRDS-8, *P. otitidis* EBILDS 9, and *Paenibacillus* sp. EBISDS-6 to solubilize zinc presents a promising strategy for improving soil fertility and addressing micronutrient deficiencies in crops. By utilizing these bacteria as biofertilizers, agricultural practices can potentially reduce reliance on chemical fertilizers while enhancing plant growth and yield through increased availability of essential nutrients like zinc.

Table 3. Zinc screening

S.N.	Isolates	Colony diameter(mm)	Halo zone diameter(mm)	Solubilization index
1	*K. oxytoca* EBISDS-1	7	14	2
2	EBILDS-2	8	16	2
3	*Klebsiella* sp. EBILDS-3	8	11	1.37
4	*Pseudomonas* sp. EBIRDS-4	7	14	2
5	*Pseudomonas* sp. EBILDS-5	8	11	1.37
6	*Paenibacillus* sp. EBISDS-6	8	16	2
7	*Pseudomonas* sp. EBILDS-7	7	10	1.4
8	*E. Faecium* EBIRDS-8	8	15	1.8
9	*E. hormaechei* EBILDS-9	7	12	1.7
10	*E. faecium* EBILDS-10	7	12	1.7
11	*B. cereus* EBISDS-11	7	12	1.7
12	*P. aeruginosa* EBILDS-12	7	12	1.7
13	*P. otitidis* EBIRDS-13	8	15	1.8
14	*B. cereus* EBIRDS-14	7	13	1.8
15	EBILDS-15	8	16	2

4.5. Antagonist activity of the screened Beneficial traits

The test endophytes showed inhibitory activity against the pathogenic bacterial strains. The present study showed that *Enterococcus* sp. EBIRDS-8, *P. aeruginosa* EBILDS-12 and

others (Figure 6) can inhibit the growth of these human pathogenic bacterial strains (Table 4), suggesting that these microorganisms may serve as a resource for new antibiotics. The antimicrobial properties of the recent isolates could minimize the challenging antibiotic resistance.

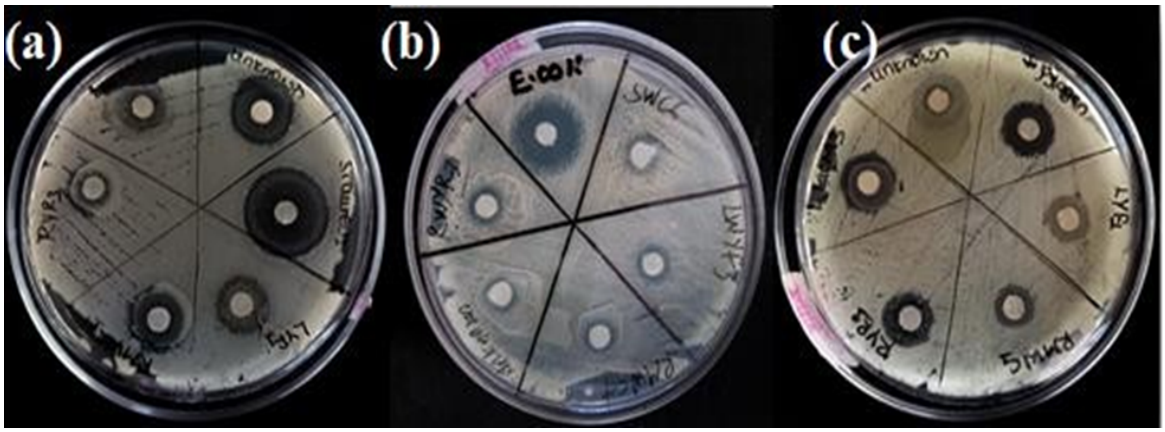


Figure 6: a) Isolated endophytic bacterial species antagonist activity against *S. aureus* 6538^T (ATCC) b) Screened EBIs with antagonist activity against pathogenic *E. coli* 700728^T (ATCC) (c) Isolated endophytic bacterial species antagonist activity against Pathogen *S. pyogenes* 19615^T (ATCC).

The identified strains, especially those belonging to the *Pseudomonas*, are known for their antimicrobial activity against various pathogens. The natural product obtained from the root part of *Codonopsis pilosula*-associated endophytic bacteria possess the ability to inhibit the growth of *E. coli, S. aureus, B. subtilis, Salmonella typhi*, and *P. aeruginosa* and the fungi *Candida albicans* and *Aspergillus niger* (Lodi, et al. 2023). Endophytic bacteria showed antibacterial properties against human pathogenic bacteria. Several studies have isolated endophytic bacteria from medicinal plants and evaluated their ability to inhibit the growth of human pathogens. One study isolated endophytic bacterium from the medicinal plants such as *Tinospora cordifolia, Catharanthus roseus, Tectona hamiltoniana*, and *Boscia variabilis*. They found that one endophyte from *C. roseus* and two from *B. variabilis* exhibited antibacterial activity against human pathogenic bacteria like *E. coli, Salmonella typhi, P. aeruginosa* and *S. aureus* (Myo et al., 2020).

Table 4. Antibacterial activity of the isolated bacterial strains against the human pathogenic bacteria (Zone diameter in mm)

Endophytes	Test Strain			
	E. coli 700728^T (ATCC)	*S. aureus* 6538^T (ATCC)	*S. pyogenes* 19615^T (ATCC).	Positive Control (Ciproflaxcin)
***Klebsiella* sp. EBILDS-3**	**13.3±1.52**	**9.67±0.57**	**12.3±2.08**	**22.5±0.5**
***Enterococcus* sp. EBIRDS-8**	**12±1**	**12.6±1.52**	**13±1**	**22.8±0.76**
***B. cereus* EBISDS-11**	**13±1**	**11±1.73**	**13.3±1.52**	**24.5±0.5**
***P. aeruginosa* EBILDS-12**	**11.67±1.52**	**11.67±1.52**	**11.3±1.52**	**24.8±0.28**
EBIRDS-20	**12.3±1.52**	**12±1**	**12.6±1.15**	**22.5±0.5**

Another study isolated endophytic bacteria from the weed *Ageratum conyzoides L.* and reported that they effectively inhibited the growth of human pathogens (Boonman et al., 2023). The same authors further illustrated those bacterial pathogens such as *Escherichia coli, Shigella flexneri*, and *Salmonella enterica ser.* Typhi inhibited by *Ageratum conyzoides L.* extracts which suggested containing bioactive compounds. This study suggested to produce specific bioactive compounds and used to affect the growth of certain pathogens as indicated in the Table 4 with various inhibition zones. As indicated in the Table 4, the lowest inhibition zone exhibited against *S. aureus* 6538^T (ATCC) with 9.67±0.57 mm due to *Klebsiella* sp. EBILDS-3 whereas the highest detected against *E. coli* 700728^T (ATCC) and *S. pyogenes* 19615^T (ATCC) with 13.3±1.52 mm zone of inhibition by *Klebsiella* sp. EBILDS-3 and *B. cereus* EBISDS-11. These inhibitions could be due to specific secondary metabolites (Table 7) which produced when these endophytic bacteria isolates were provided with suitable culture growth such as pH, temperatures, carbon sources, salt concentration, nitrogen sources and others (Figure 8a, b,&c). In line with this study, various culture conditions used to affect the growth of various bacterial species and hence their natural products and other efficiency (Aydamo et al., 2024; Kiros et al., 2023; Mohammed et al., 2019, 2020; Mohammed & Ray, 2022; Nedi et al., 2024).

4.6. Protein profile-based identification of EBIDS suing MALDI TOF MS

A total of 13 bacterial isolates were analyzed using MALDI-TOF mass spectrometry for species identification. Four isolates (EBILDS-9, EBIRDS-13, EBISDS-6, EBIRDS-8) were identified as *Pseudomonas otitidis*, Two isolates (EBIRDS-14, EBISDS-11) were identified as Enterococcus species (*E. faecium and E. faecalis*), Two isolates (EBILDS-5, EBILDS-12) were identified as *Klebsiella oxytoca* , Two isolates (EBILDS-3, EBIRDS-4) were identified as *Bacillus cereus*, One isolate each was identified as *Pseudomonas aeruginosa* (EBILDS-7), *Enterobacter hormaechei* (EBISDS-1), and *Paenibacillus thiaminolyticus* (EBILDS-10)..

The MALDI-TOF MS analysis provided reliable identifications for all 13 bacterial isolates, with the majority (61.5%) achieving high-confidence species-level identification. This demonstrates the effectiveness of MALDI-TOF MS as a rapid and accurate method for bacterial identification in environmental samples. The range of score values (1.72 to 2.29) indicate varying levels of confidence in the identifications. Factors that can influence these scores include the quality and purity of the bacterial cultures, the robustness of the reference database used for comparison, and the inherent variability in protein expression among different strains of the same species. The results reveal a diverse array of bacterial species in the samples, including members of the genera *Pseudomonas, Enterococcus, Klebsiella, Bacillus, Enterobacter,* and *Paenibacillus*. Previous study showed that *Artemisia annua, Moringa oleifera*, and *Ocimum lamiifolium* harboured endophytic bacterial isolates such as *B. cereus BAMRI-7*, *B. subtilis BGORI-4*, *Citrobacter freundii CAMLI- 2, Enterococcus asburiae EAASI-4, E. cloacae EAMSI-2, E. faecium EAMRI-1, E.ludwigii EGOSI-4, E. kobei EAMRI-2*, and *P. monteilli PGORI-13* based on protein profiles using MALDI-TOF MS (Ebu et al., 2023). In line with this study it was reported that *Bacillus, Escherichia, Enterobacter, Enterococcus Paenibacillus, Pantoea*, and *Staphylococcus* were obtained from the leaves, root and stem part of *Urtica dioica L.* (Toubal et al., 2022). The same authors further stated that the majority of these bacteria were isolated from Tlemcen which makes this type of region the richest in terms of endophytic bacteria isolates compared to region like Dellys. Bacterial species such as *Alcaligenes faecalis, B. subtilis, E. aerogenes, K. pneumoniae, Pantoea dispersa, P. stutzeri*, and *Stenotrophomonas maltophilia* were identified from *Curcuma zedoaria* based on protein profiles and suggested for plant growth promotion (Sulistiyani & Lisdiyanti, 2018).

This diversity suggests a complex microbial community in the sampled environment. The predominance of *P. otitidis* (4 out of 13 isolates) is noteworthy. While *P. otitidis* was originally isolated from clinical samples (Clark et al., 2006), its presence in environmental samples suggests potential ecological roles that required further investigation. Identifying known opportunistic pathogens such as *P. aeruginosa, Klebsiella oxytoca*, and *E. faecium* highlights the importance of these isolates for plant growth promotion even though they posepotential human health risk similar to the previous study (Tomulescu et al. 2021).

Table 5 Identification of endophytic bacterial isolates based on protein profile using MALDI-TOF MS

S.N.	Sample's Id	Genus	Most likely closest species	LSV	Most likely identified strains or isolates
1	EBISDS-1	*Klebsiella*	*oxytoca*	2.12	*K. oxytoca* EBISDS-1
2	EBILDS-3	*Klebsiella*	oxytoca	1.99	*Klebsiella* sp. EBILDS-3
3	EBIRDS-4	*Pseudomonas*	*otitidis*	1.86	*Pseudomonas* sp. EBIRDS-4
4	EBILDS- 5	*Pseudomonas*	*otitidis*	1.73	*Pseudomonas* sp. EBILDS- 5
5	EBISDS- 6	*Paenibacillus*	*thiaminolyticus*	1.72	*Paenibacillus* sp. EBISDS- 6
6	EBILDS-7	*Pseudomonas*	*otitidis*	1.93	*Pseudomonas* sp. EBILDS-7
7	EBIRDS-8	*Enterococcus*	*faecium*	1.84	*Enterococcus* sp. EBIRDS-8
8	EBILDS-9	*Enterobacter*	*hormaechei*	2.04	*E. hormaechei* EBILDS-9
9	EBILDS-10	*Enterococcus*	*faecium*	2.29	*E. faecium* EBILDS-10
10	EBISDS-11	*Bacillus*	*cereus*	2.20	*B. cereus* EBISDS-11
11	EBILDS-12	*Pseudomonas*	*aeruginosa*	2.00	*P. aeruginosa* EBILDS-12
12	EBIRDS-13	*Pseudomonas*	*otitidis*	2.06	*P. otitidis* EBIRDS-13
13	EBIRDS-14	*Bacillus*	*cereus*	2.15	*B. cereus* EBIRDS-14

Key note: LSV indicated Logarithmic score value

The MALDI-TOF score values, which indicate the reliability of the identification, ranged from 1.72 to 2.29. According to standard MALDI-TOF interpretation guidelines Scores ≥ 2.00 are considered high-confidence identifications at the species level, Scores between 1.70 and 1.99 are considered reliable for genus identification, and probable species identification Scores < 1.70 are considered unreliable identifications Based on these criteria 8 isolates had high- confidence species-level identifications (score ≥ 2.00), 5 isolates had reliable genus-level identifications with probable species identification (1.70 ≤ score < 2.00) No isolates had unreliable identifications. The highest score was observed for *Enterococcus faecium* (EBILDS-10) with a score of 2.29, while the lowest score was for *Paenibacillus* sp. (EBISDS- 6) with a score of 1.72.

The MALDI-TOF MS analysis provided reliable identifications for all 13 bacterial isolates, with the majority achieving high-confidence species-level identification. This demonstrates the effectiveness of MALDI-TOF MS as a rapid and accurate method for bacterial identification in environmental samples. The range of score values (1.72 to 2.29) indicate varying levels of confidence in the identifications. Factors that can influence these scores include the quality and purity of the bacterial cultures, the robustness of the reference database used for comparison, and the inherent variability in protein expression among different strains of the same species.

4.7. Phylogenetic tree for newly isolated Enterococcus sp. EBIRDS-8

The partial 16S rRNA gene sequence of one of the recent endophytic bacterial isolate that was obtained from root part of *D. stramonium* was found to contain 1451 bp in sequence length (Figure 7). This newly obtained isolate was found to be *Enterococcus* sp. with 98% sequence similarity with *Enterococcus fecalis* subsp. dissolvens MZ474974.1 and 97% sequence similarity with *E. fecalis* WW12 (Figure 5). This isolate was designated as (*Enterococcus* sp. EBIRDS-8). The recent isolate was also aligned with *E. avium* ZALY IQ11^{T} (OM021444), *E. hermanniensis* HBUAS64184^{T} (OP358137), *E. gallinarum* MG4621^{T} (ON631304), *E. hirae* CIFRI.BTL-42^{T} (PP754729), *E.durans* AK-IAUK5^{T} (MT032372), *E. faecium* BSE14^{T} (PQ416063), and *Enterobacter* sp. RPAAI-8^{T} (ON640911) (Figure 5).

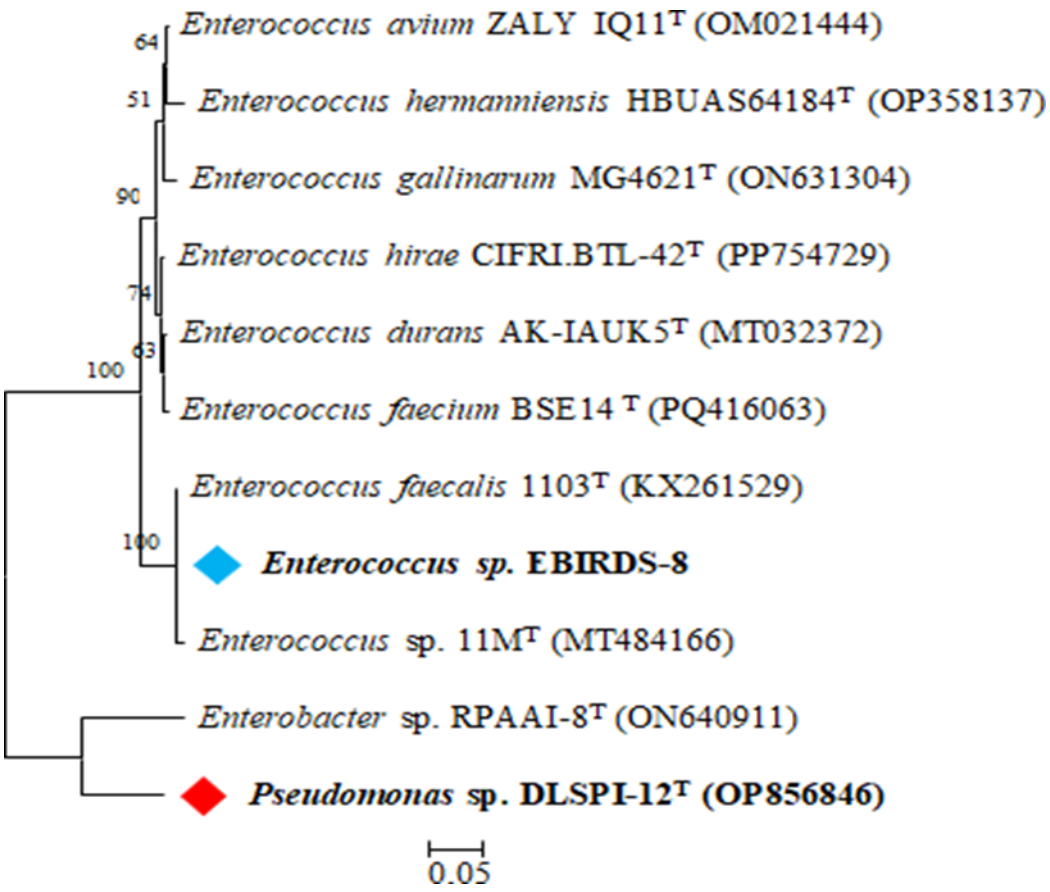


Figure 7: Phylogenic tree using Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and Jukes-Cantor model (Jukes, 1969). The tree with the highest log likelihood (-4794.76) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Jukes-Cantor model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1537 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Felsenstein, 1985; Tamura et al., 2021)

However, the recent isolate had less sequence similarity with *Pseudomonas* sp. DLSPI-12^T (OP856846) (Figure 7). Our isolate showed the same clade with *Enterococcus faecalis* 1103^T (KX261529) and *Enterococcus* sp. 11M^T (MT484166) (Figure 7). Previous study showed that six members of the *Enterococcus* such as *E. durans, E. faecium, E. hirae, E. mundtii E. ratti,* and *E.villorum*, were identified using 16S rRNA gene sequence similarity (Naser et al.,

2005). These strains could also share sequence similarity with our recent isolate since they belonged to the same genus. Diversities of other Enterococci were also isolated and recognized based 16S rRNA gene against fecal samples. These species were mainly belonged to *E. faecalis, E. faecium*, and *E. casseliflavus* (Ryu et al., 2013). These endophytic bacterial isolates were also previously identified using 16S rRNA from *Artemisia annua, Moringa oleifera*, and *Ocimum lamiifolium,* medicinal plants (Ebu et al., 2023). The isolates were also identified as *Enterobacter* sp. RPAAI-8^T (ON640911) (Ebu et al., 2023). Our isolate also shared sequence similarity with *Enterobacter* sp. RPAAI-8^T (ON640911) as indicated in Figure 5.

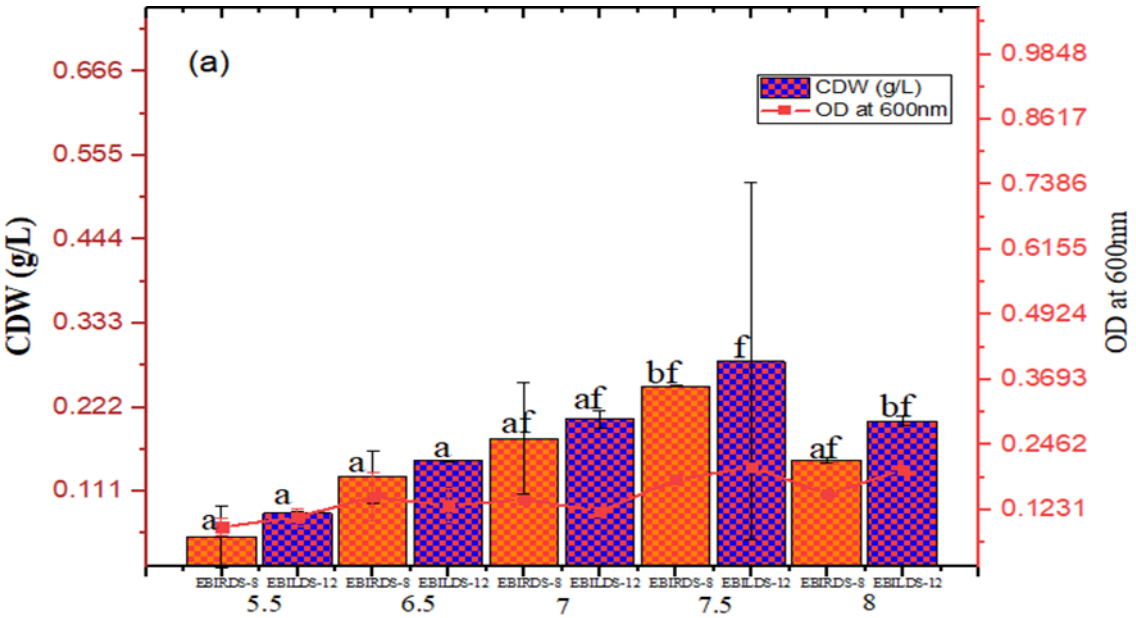
4.8. Effect of Physiochemical parameters on the growth of the isolates

4.8.1. Effect of pH value

Biomass, expressed as CDW (g/L), generally increases with increasing pH concentration. The highest biomass (0.2833±0.23 g/L) was observed at pH 8 for *P. aeruginosa* EBILDS-12 followed by 0.251±0.001 g/L for *Enterococcus* sp. EBIRDS-8. However, *Enterococcus* sp. EBIRDS-8 gave the lowest (0.0507±0.041 g/L) CDW (g/L) at pH5.5 followed by *P. aeruginosa* EBILDS-12 which gave 0.0817±0.0029 g/L (Figure 8a). The $OD_{600\ nm}$ follows a similar trend, with the highest (0.204±0.0077) OD_{600nm} values at pH7.5 against *P. aeruginosa* EBILDS-12 (Figure 8a). At pH 5.5 and 6.5, *Enterococcus* sp. EBIRDS- higher biomass and OD_{600nm} values compared to *P. aeruginosa* EBILDS-12 (Figure 8a). At pH 7.0, 7.5, and 8.0, *P. aeruginosa* EBILDS-12 exhibited higher biomass with OD_{600nm} values compared to *Enterococcus* sp. EBIRDS-8 (Figure 8a). This study examines the effects of pH on the biomass production of endophytic bacteria isolated from medicinal plants, specifically focusing on two treatments: *Pseudomonas sp*. EBILDS-12 and *Enterococcus* sp. EBIRDS-8 (Figure 8a). The results indicate a clear relationship between pH levels and biomass production, with the highest biomass and OD_{600nm} observed at pH 8.0 for both bacterial strains (Figure 8a). The observation that biomass, measured as cell dry weight (CDW), peaked at pH 8.0 aligns with existing literature emphasizing the preference of many *Pseudomonas* species for alkaline conditions. For instance, research by (Zhang et al., 2022) indicates that *Pseudomonas* strains exhibit enhanced growth and metabolic activity in alkaline environments, which supports our findings regarding *Pseudomonas* sp. EBILDS-7. The higher biomass and OD_{600nm} values at pH 8.0 suggest that these conditions may optimize nutrient availability and metabolic efficiency for *Pseudomonas* sp. (Figure 8a). The biomass

obtained from *Enterococcus* sp. EBIRDS-8 and *P. aeruginosa* EBILDS-12 varied and found to be determined by pH value, one of culture conditions. No significant variations detected between pH5.5 & pH6.5 (*P=0.142, 0.240*), pH5.5 & pH8 (*P=0.137, 0.295*), pH6.5 & pH7 (*P=0.406, 0.648*), pH6.5 & pH8 (*P=0.272, 0.427*), pH7 & pH8 (*P=0.970*), and others for these isolates. Variations in terms of CDW (g/L) obtained among pH5.5 & pH7.5 (*P=0.006, 0.018*), pH5.5 & pH8 (*P=0.030*), and pH5.5 & pH7 (*P=0.027*) for these tested isolates.

In contrast, the performance of *Enterococcus* sp. EBIRDS-8 at lower pH levels (5.5 and 6.5) indicates their adaptability to slightly acidic environments. The higher biomass and OD_{600nm} values for *Enterococcus* sp. EBIRDS-8 at these pH levels suggest that *Enterococcus* may thrive better under such conditions compared to *Pseudomonas* sp. This is consistent with findings from (Fernández et al., 2018), who noted that certain *E. faecium* can grow well in moderately acidic environments and in media of different pH, highlighting their ecological versatility.



Effect of various pH value sources against EBIRDS-8 and EBILDS-12 for CDW (g/L) and OD at 600nm

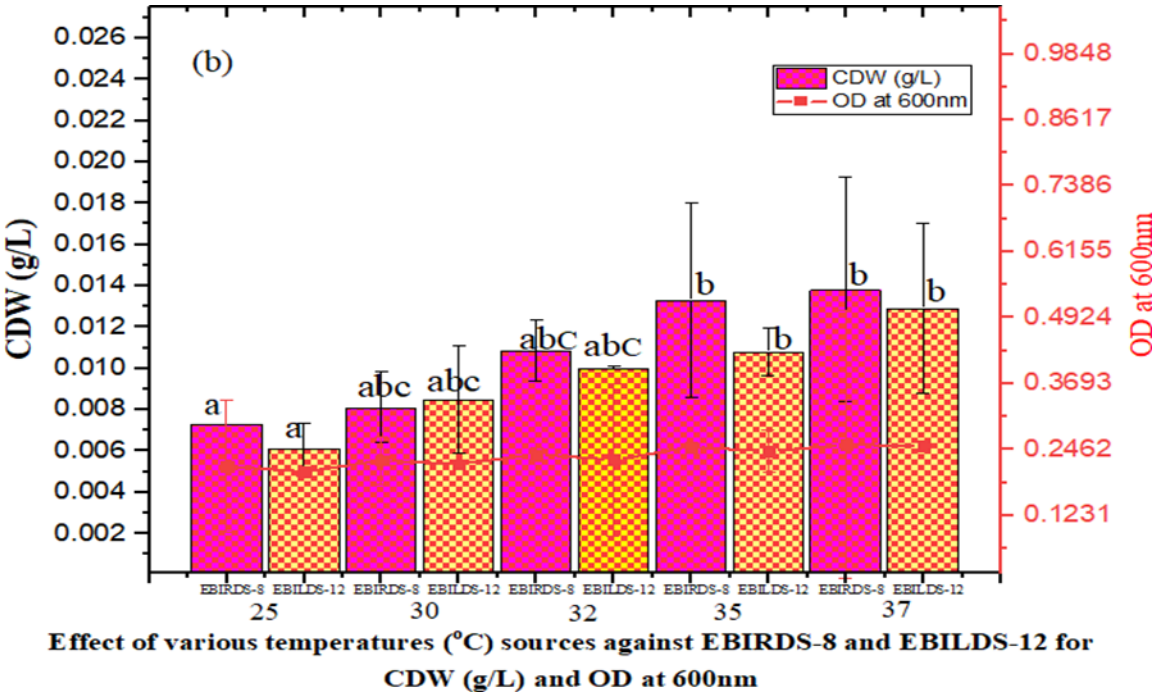


Figure 8 (a) Effect of various pH values against certain endophytic bacterial isolates in terms of CDW (g/L) and OD_{600nm} (b) Effect of various temperature range against certain endophytic bacterial isolates, which obtained from the root, leaf and stem part of *D. stramonium* for CDW (g/L) and OD_{600nm}. Data represented mean ± SD of three replicate (n = 3) for CDW (g/L) and OD_{600nm} with significant variation having different letter on column bar.

At pH levels of 7.0, 7.5, and 8.0, *P. aeruginosa* EBILDS-12 consistently outperformed *Enterococcus* sp. EBIRDS-8 in terms of biomass and OD_{600nm} values (Figure 8a). This trend reinforces the notion that *P. reactans* WL20-3 are better suited at high alkalinity and low temperature to conditions, which is further supported by studies indicating their metabolic advantages in such environments (L. Wang et al., 2023). The differential performance between these two genera across varying pH levels emphasizes their unique ecological niches and metabolic adaptations.

4.8.2. Effect of Temperature

The Figure 8b shows how temperature affects a specific measurement like CDW (g/L) and OD_{600nm}. As the temperature rises from 25 to 37°C, the CDW (g/L) increases with optimum biomass at 37 °C (Figure 8b), indicating a positive relationship (Figure 8b). The increase is especially noticeable between 35°C and 37°C, suggesting that higher temperatures have a stronger effect (Figure 8b). In this study, *Pseudomonas* sp EBILDS-12 gave the lowest CDW

(g/L) (0.0061±0.0012 g/L) with 0.2048± 0.0085 OD600nm (Figure 8b). However, Enterococcus sp. EBIRDS-8 produced the highest CDW (g/L) (0.0138±0.0066) (Figure 8b) at 37 ^{0}C with 0.255± 0.25 OD_{600nm}. Overall, these results highlight the significant role of temperature in influencing the biomass and OD of bacterial species, which collected from their natural habitat (Figure 8b). It was also stated that the temperatures and other culture conditions affected the growth rate of bacterial species with optimum biomass by increasing these culture conditions until they remained constant at concentrations above the optimum (Ebu et al., 2023; Kim et al., 2002).

This study examines the effects of temperature on the biomass production of endophytic bacteria, specifically *Pseudomonas* sp EBILDS-12 and *Enterococcus* sp. EBIRDS-8, isolated from medicinal plants. The results indicate that *Pseudomonas* sp. consistently exhibits greater biomass than *Enterococcus* across all tested temperatures, with a notable increase in biomass as the temperature rises from 25 to 37°C (Figure 8b). This trend highlights the significant role temperature plays in influencing the growth dynamics of these endophytic bacteria (Figure 8b). It was confirmed that there are no variations in CDW (g/L) between *Enterococcus* sp. EBIRDS-8 and *Pseudomonas* sp EBILDS-12 at 25 & 30^{o}C, 25 & 32^{o}C, 30 & 32^{o}C, 30 & 37^{o}C, 32 & 37^{o}C, and 35 & 37^{o}C with $P<0.05$. As it was observed in Figure 8b, variation in terms of CDW (g/L) was recorded at 25 & 35^{o}C ($P=0.018$) and 25 & 37^{o}C ($P=0.031, 0.025, 0.012$) against *Enterococcus* sp. EBIRDS-8, *Pseudomonas* sp EBILDS- 12 and *Enterococcus* sp. EBIRDS-8 & *Pseudomonas* sp EBILDS-12. As indicated in this study, the temperatures affect the growth of bacterial cells (Figure 8b). This shows that the highest and lowest temperature ranges might be suppressed the growth of certain bacterial species and result in insignificant CDW (g/L) and OD even though other culture conditions were optimized as similar study carried out by (Felip et al., 1996).

This study's positive correlation between temperature and biomass production aligns with existing literature. As temperatures increase, metabolic rates and enzymatic activities rise, enhancing growth and biomass accumulation in many microbial species. For example, a review by (Meena et al., 2023) discusses how higher temperatures can promote microbial growth by increasing enzymatic activity and nutrient uptake efficiency. The pronounced increase in biomass between 35 and 37°C suggests that *Pseudomonas stutzeri* and *Acinetobacter haemolyticus* may have adapted to thrive under warmer conditions, which is consistent with findings from previous studies indicating that many *Pseudomonas stutzeri* prefer higher temperatures for optimal growth (Parthipan et al., 2018; Y. Wang et al., 2021).

In contrast, *Enterococcus* species generally show less biomass production across all temperatures compared to Pseudomonas. This difference may be attributed to the distinct ecological niches and metabolic pathways of these two genera. *Enterococcus* species are often more resilient in fluctuating environments but may not exhibit the same level of growth enhancement at elevated temperatures as *Pseudomonas*.

4.8.3. Effect of Carbon Source

In the recent study, the lowest and highest Mean CDW (g/L) were found to be 0.0051 ±0.0053 and 0.012±0.0023 g/L against CDW (g/L) against mannitol (*P. aeruginosa* EBILDS-12) and Glucose (*Enterococcus* sp. EBIRDS-8), respectively (Figure 9a,). As illustrated in Figure 9a, significant variation detected among glucose & sucrose (*P= 0.022*) for *P. aeruginosa* EBILDS-12 & *Enterococcus* sp. EBIRDS-8), Glucose & sucrose *(P=0 .010* for *Enterococcus* sp. EBIRDS-8), Glucose & Mannitol (*P=0.028*) for, *Enterococcus* sp. EBIRDS-8, Fructose & Sucrose (*P=0.003*) for *Enterococcus* sp. EBIRDS-8 and *P. aeruginosa* EBILDS-12, fructose & sucrose (*P=0. 008*) for *P. aeruginosa* EBILDS-12, and mannitol and Fructose (*P=0.046*) for *P. aeruginosa* EBILDS-12 in terms of CDW (g/L). Glucose is often considered the most efficient carbon source for many bacteria, including endophytes (Figure 9a). Studies indicate that *P. aeruginosa* EBILDS-12 demonstrates a high growth rate and metabolic activity when utilizing glucose, which can be quickly assimilated and metabolized into energy. This rapid utilization leads to increased bacterial biomass production, making glucose a preferred choice for many bacterial species (Stoop & Pharr, 1993).

Fructose has been shown to support bacterial growth effectively, often yielding results which is comparable to glucose. However, its utilization may vary depending on the specific metabolic pathways of the bacteria. For instance, some strains of *Pseudomonas* can utilize fructose efficiently under certain conditions, potentially leading to competitive advantages in nutrient-rich environments (Pandey,2016). *Enterococcus* sp. EBIRDS-8 (0.009110± .0017800 g/L) and *P. aeruginosa* EBILDS-12 (0.013510±0.0018194 g/L) found to utilize fructose as a carbon source for their growth with CDW (g/L). When provided with fructose as a sole carbon source, the same isolates gave considerable amounts of OD_{600nm} with 0.1986±0.0015 to 0.22367±0.001 (Figure 9a). Variations were also documented among Fructose & Mannitol against *P. aeruginosa* EBILDS-12 (*P=0.046*) & *Enterococcus* sp. EBIRDS-8 (*P=0.011*), fructose & Sucrose (*P=0.003*) against *P. aeruginosa* EBILDS-12 &

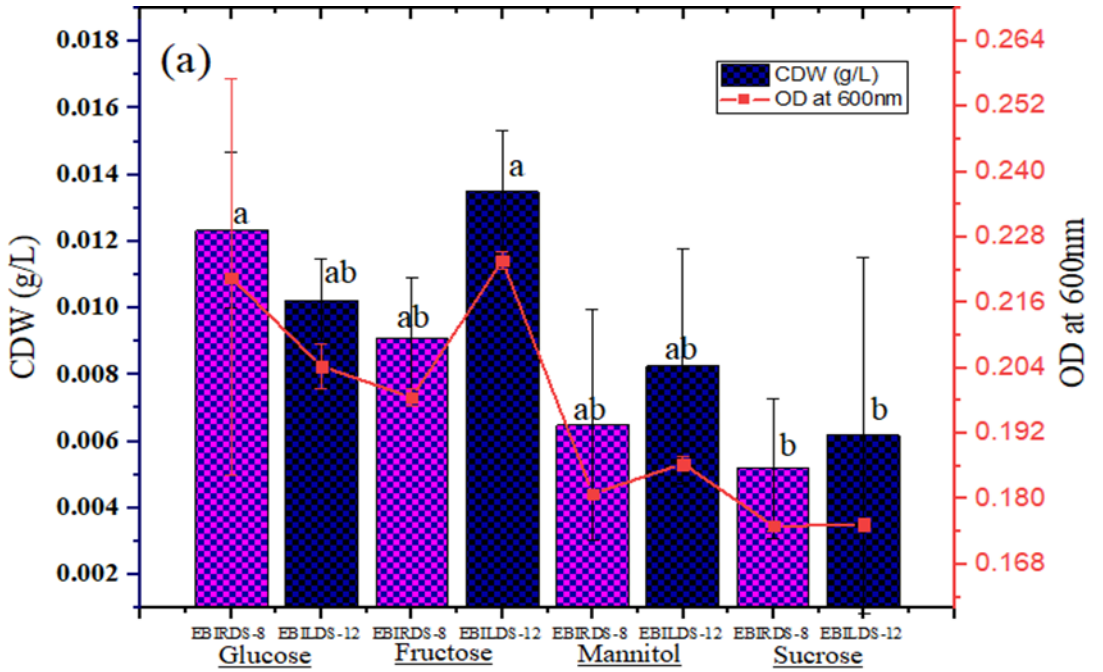
Enterococcus sp. EBIRDS-8, and fructose & Sucrose (*P=0.008*) against *P. aeruginosa* EBILDS-12.

As indicated there is no significant variations were observed among fructose & Glucose (*P=0.203*) against *Enterococcus* sp. EBIRDS-8, fructose & Glucose (*P=0.648*) against *Enterococcus* sp. EBIRDS-8 & *P. aeruginosa* EBILDS-12, fructose & mannitol (*P= 0.296*) against *Enterococcus* sp. EBIRDS-8, and others. Similarly, in our previous study, it was further confirmed that fructose can be employed for cellular growth for certain bacterial species collected from different ecosystems; these reported for various purpose (Ebu et al., 2023; Kiros et al., 2023; Mohammed et al., 2019, 2022; Mohammed & Ray, 2022). The supplementation of fructose and other carbon sources in the minimal medium during the growth of endophytic bacterial isolates might increase the metabolites employed for fighting certain plant-borne pathogens. These suppressed the development of the plants and hence used for plant growth promotion. Similarly, it was reported that fructose metabolites (Durmus et al., 2012) have been found to increase the efficiency of various bioactive compounds, which employed to suppress the growth of *S. aureus* pathogen. The same authors further reported for the first time that fructose on the nanorough surfaces can decreases the number of planktonic *S. aureus* bacteria in the solution and biofilm formation after 24 h (Durmus et al., 2012).

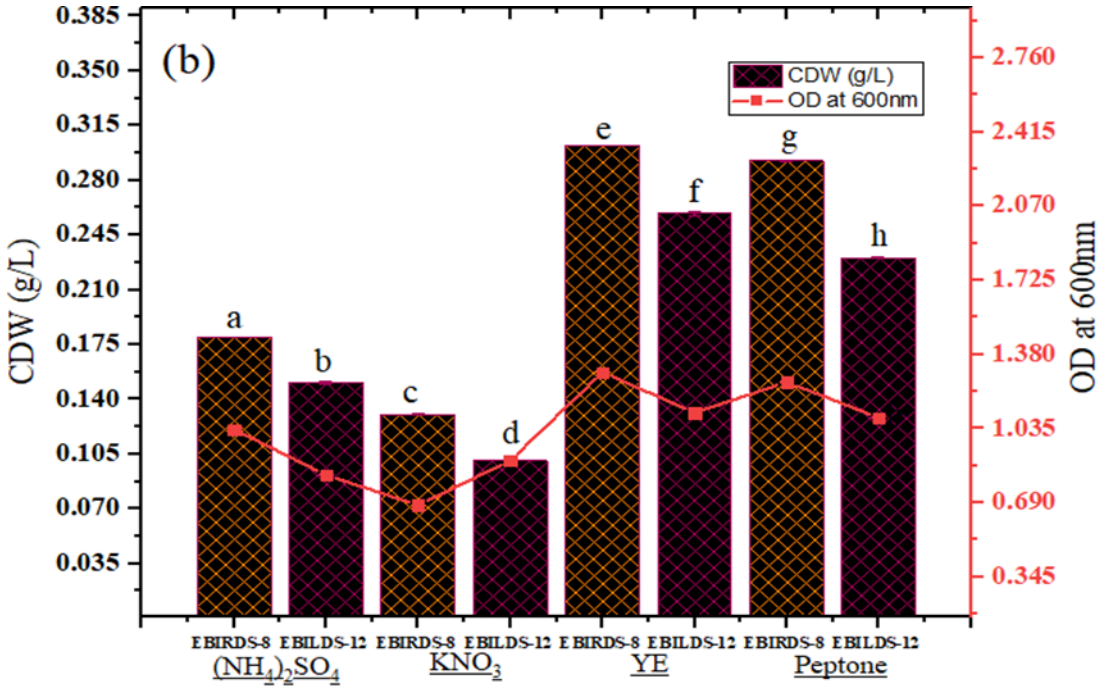
Mannitol, which is a sugar alcohol, can serve as a carbon source. However, it is generally less efficiently utilized when compared to glucose and fructose (Figure 9a). as indicated in Figure 9a, 0.0065±0.0035 and 0.008±0.0035 CDW (g/L) were recorded for *Enterococcus* sp. EBIRDS-8 and *P. aeruginosa* EBILDS-12, respectively. It was observed that 0.181±0.00 to 0.186 OD_{600nm} were documented against these EBIs. The previous study shown that mannitol can enhance osmotic balance and stress tolerance in bacteria but may not support optimal growth rates compared to more readily fermentable sugars like glucose or sucrose (Panigrahi et al., 2020). The metabolic pathways involved in mannitol catabolism are complex and may involve specific enzymes that are not universally present in all bacterial strains (Stoop & Pharr, 1993).

It has been stated that Sucrose, can also be effective for bacterial growth. CDW (g/L) and OD_{600nm} were obtained from *Enterococcus* sp. EBIRDS-8 (0.00518±0.0021, 0.175±0.001) and *P. aeruginosa* EBILDS-12 (0.0067±0.005, 0.175±0.001) (Figure 9a). However, unlike glucose, sucrose utilized at a slower rate. It requires invertases which can yield glucose and

fructose before it can be completely metabolized. Additional since sucrose is a double sugar, it is challenging to these EBI to easily break down and employ for their cellular growth, a similar report to the previous study (Mohammed et al., 2019). This process can limit the immediate availability of energy for bacterial growth, although sucrose still supports significant growth rates for endophytic bacteria like *E. faecium* (Franzino et al., 2022).



Effect of various carbon sources against EBIRDS-8 and EBILDS-12 for CDW (g/L) and OD at 600nm



Effect of various Nitrogen sources against EBIRDS-8 and EBILDS-12 for CDW (g/L) and OD at 600nm

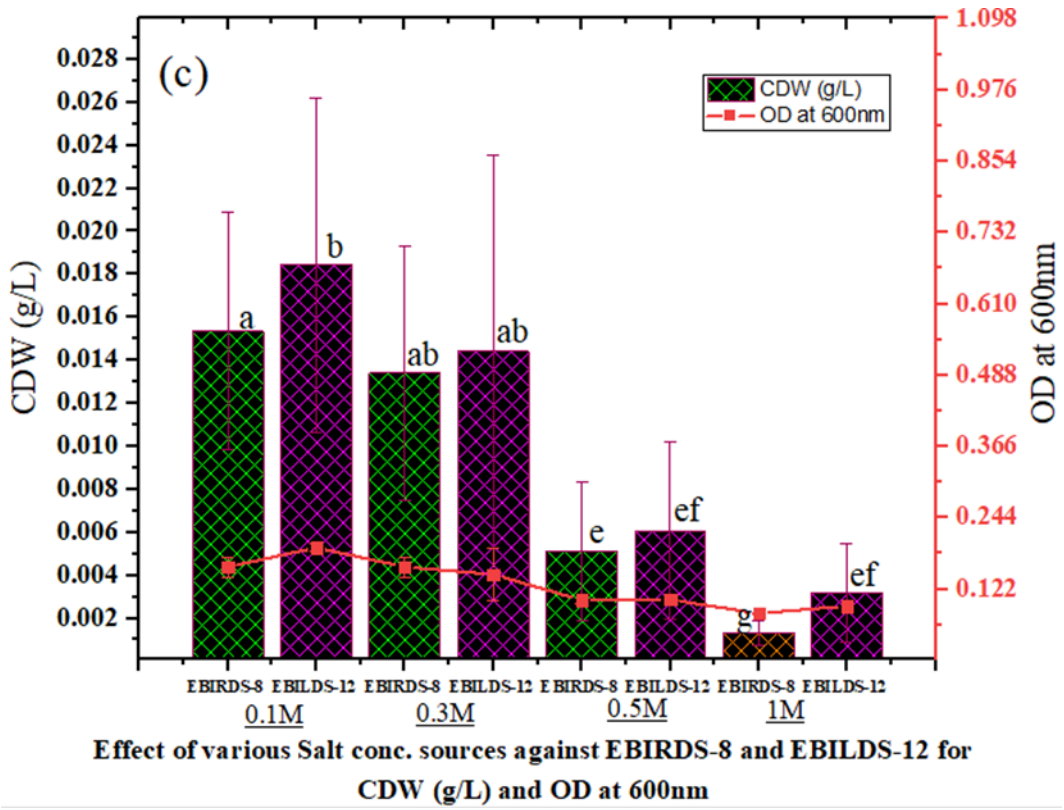


Figure 9: (a) CDW (g/L) and OD600nm obtained from various carbon sources against *Enterococcus* sp. EBIRDS-8 and *P. aeruginosa* EBILDS-12 (b)) CDW (g/L) and OD_{600nm} obtained from various nitrogen sources against *Enterococcus* sp. EBIRDS-8 and *P. aeruginosa* EBILDS-12. (c) CDW (g/L) and OD_{600nm} obtained from various Salt concentration against *Enterococcus* sp. EBIRDS-8 and *P. aeruginosa* EBILDS-12. Data represented mean ± SD of three replicate (n = 3) for CDW (g/L) and OD_{600nm} with significant variation having different letter on column bar.

4.8.4. Effect of nitrogen source

The biomass and optical density at 600nm (OD_{600nm}) for various nitrogen sources used for the growth of *Enterococcus* sp. EBIRDS-8 and *P. aeruginosa* EBILDS-12. Among the nitrogen sources tested, peptone (0.3022±0.00002 g/L) and yeast extract (0.292±0.0008) ,the highest biomass production for *Enterococcus* sp. EBIRDS-8, indicating their effectiveness in supporting microbial growth with 1.0867±0.0011 to 1.2967±0.0011 OD_{600nm} for the tested isolates (Figure 9b). However, KNO_3 and $(NH_4)_2SO_4$ showed lower biomass compared to peptone and yeast extract, suggesting they are less effective nitrogen sources for the microorganisms under the tested conditions (Figure 9b,). The OD_{600nm}, a proxy for cell density, correlated well with the biomass measurements, providing a consistent assessment of the growth patterns across the different nitrogen sources. The present study exhibited that

great significant variation *(P=0.000*) displayed among these nitrogen sources for *Enterococcus* sp. EBIRDS-8 and *P. aeruginosa* EBILDS-12 (Figure 9a).

Ammonium sulfate $(NH_4)_2SO_4$ (Figure 9b) is a highly soluble nitrogen source that provides ammonium ions, which are readily assimilated by many bacteria. Studies have shown that *P. aeruginosa* EBILDS-12 thrives in media containing ammonium sulfate, leading to increased biomass and enhanced production of secondary metabolites. Potassium nitrate (KNO_3) (Figure 9b) serves as another effective nitrogen source. It provides both potassium and nitrate ions, which can be beneficial for bacterial metabolism. Research indicates that *Enterococcus* sp. EBIRDS-8 exhibits considerable growth when cultured with KNO_3 (Figure 9b), although its utilization may not be as efficient as ammonium-based sources. The presence of potassium can also enhance the stability of cellular membranes and contribute to overall bacterial health under stress conditions (Murniasih et al., 2023). Yeast extract is rich in vitamins, amino acids, and other growth factors, making it an excellent organic nitrogen source for endophytic bacteria. *P. aeruginosa* EBILDS-12 and *Enterococcus* sp. EBIRDS-8 show significant growth enhancement when cultured with yeast extract.

4.8.5. Effect of Salt concentration

The biomass (CDW, g/L) and OD_{600nm} appear to vary with salt concentration. The lowest (0.00133±0.0005) and highest (0.01843±0.0077 g/L) CDW (g/L) were obtained at 0.1 and 1 M for *Enterococcus* sp. EBIRDS-8 and *P. aeruginosa* EBILDS-12, respectively (Figure 9a,). Considerable CDW (g/L) was also recorded from *Enterococcus* sp. EBIRDS-8 (0.0153±0.0055) at 0.1 M salt concentration as indicated in Figure 9c. The biomass and OD_{600nm} values at 0.1M might be the highest, while 1M could show a significant decrease, suggesting salt stress impacts microbial growth (Figure 9c). This study investigates the effects of salt concentration on the biomass production of endophytic bacteria, specifically *P. aeruginosa* EBILDS-12 and *Enterococcus* sp. EBIRDS-8. The results reveal a clear relationship between salt concentration and microbial growth, with lower concentrations (0.1 and 0.3 M) supporting higher biomass and OD_{600nm} values, while higher concentrations (0.5 and 1M) lead to a decline in these parameters. This pattern suggests that increasing salt concentration imposes stress on the microbial populations, inhibiting their growth.

No variations were detected among 0.1 & 0.3 M (*P=0.671*) for *Enterococcus* sp. EBIRDS-8,0.1 & 0.5M (*P=0.055*) for *Enterococcus* sp. EBIRDS-8 & *P. aeruginosa* EBILDS-12, 0.1 & 0.3M (*P=0.838*) for *Enterococcus* sp. EBIRDS-8 & *P. aeruginosa* EBILDS-12, 1 & 0.5

M (*P=0.668*) for *Enterococcus* sp. EBIRDS-8 & *P. aeruginosa* EBILDS-12, and others as exhibited. However, great signification variations were detected among 0.1 & 1M (Figure 9c) for *Enterococcus* sp. EBIRDS-8, 0.1 & 1M (*P=0.002*) for *Enterococcus* sp. EBIRDS-8 & *P. aeruginosa* EBILDS-12, 0.1 & 0.5 M *(P=0.014*) for *P. aeruginosa* EBILDS-12 and others. Similarly, variation had been reported for various PHA producing and polyethylene degrading bacterial isolates in terms of CDW (g/L) and OD_{600nm} (Mohammed et al., 2019, 2020; Nedi et al., 2024).

The observation that lower salt concentrations promote higher biomass production aligns with existing literature on microbial osmoregulation. Many microorganisms, including *Pseudomonas* and *Enterococcus* spp, have evolved mechanisms to cope with osmotic stress; however, excessive salt can disrupt cellular processes. Studies have shown that low to moderate salt concentrations can enhance microbial growth by providing essential ions without causing osmotic stress (Khan et al., 2022). The significant decrease in biomass and OD_{600nm} higher salt concentrations (0.5 and 1M) suggests that salt stress negatively impacts microbial growth. This phenomenon is well-documented in microbial ecology; high salinity can lead to plasmolysis, enzyme denaturation, and disruption of metabolic pathways (Zhang et al., 2021). For instance, a study by (Tiwari et al., 2023) highlighted that elevated salinity levels can inhibit the growth of *Pseudomonas* species by affecting nutrient uptake and metabolic efficiency. While both *P. aeruginosa* EBILDS-12 and *Enterococcus* sp. EBIRDS-8 show similar trends in response to varying salt concentrations, it is essential to consider their ecological adaptations. *Pseudomonas* species are known for their resilience in diverse environments, including saline conditions, which may explain their relatively higher biomass compared to *Enterococcus* at elevated salt levels. study showed that (Aqel et al., 2023) certain *P. aeruginosa* possess specific adaptations that allow them to thrive under osmotic stress, while *E. faecalis* (García-Solache & Rice, 2019) may be more sensitive to such conditions.

4.9. Antimicrobial assay of secondary metabolite crude extract of *Enterococcus* sp. EBIRDS-8

The evaluation of the antimicrobial activities of ethyl acetate extracts from isolated endophytic bacteria revealed significant antibacterial properties against various test strains (Figure 10). The study demonstrated that the zones of inhibition increased with higher concentrations of the ethyl acetate extract (Figure 10).

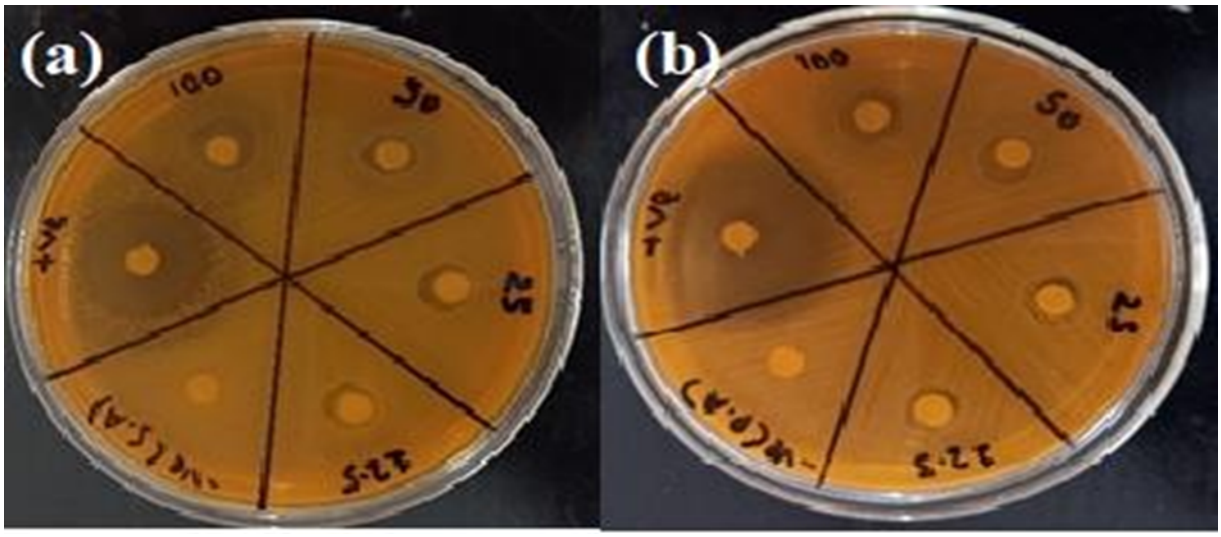


Figure 10: a) antibacterial Activity against *S. aureus* 25923^T (ATCC) b) antibacterial Activity against *P. aeruginosa* 27853^T (ATCC).

At the lowest concentration of 12.5 mg/ml, the inhibition zones ranged from 7.1 to 8.3 mm (Table 6), while at the highest concentration of 100 mg/ml, the zones expanded to 10.1 to 12.4 mm (Figure 10). Notably, the positive control (20 µg/ml) exhibited a zone of inhibition between 22.5 and 24.8 mm, surpassing even the highest test concentration. Among the tested strains, *P. aeruginosa* 27853^T (ATCC showed the strongest inhibition, followed by *S. aureus* 25923^T (ATCC), *S. pyogenes* 19615^T (ATCC), and *E. coli* 25922^T (ATCC), indicating that the ethyl acetate extract possesses potent antibacterial activity against these pathogens (Efendi et al., 2022).

Table 6: Zone of inhibition of ethyl acetate extraction from *Enterococcus* sp. EBIRDS-8

Zone of inhibition of ethyl acetate extract (mm)					
	Concentrations 1mg/ml				
Test Strains	12.5	25	50	100	Ciprofloxacin (mm) 20 µg/ml
E. coli 700728^T (ATCC)	7.16 ±0.28	8.1±0.2	9.3±0.28	10.1±0.2	22.5±0.5
S. pyogenes 19615^T (ATCC)	7.3±0.28	8.3±0.28	9.16±0.28	10.3±0.2	22.8±0.76
S. aureus 6538^T (ATCC)	7.3±0.2	8.16±0.28	9.2±0.34	10.23±0.25	24.5±0.5
P. aeruginosa 27853^T (ATCC).	8.03±0.46	10.26±0.25	11.26±0.25	12.2±0.25	24.8±0.28

The results indicate that ethyl acetate extracts from endophytic bacteria can effectively inhibit bacterial growth. The observed increase in inhibition zones suggests that higher concentrations enhance the bioactive compound's availability, which may disrupt bacterial cell functions or inhibit metabolic processes (Table 6). The strongest inhibition against *P. aeruginosa* aligns with previous findings that highlight this bacterium's susceptibility to various phytochemicals and natural extracts (Salem et al., 2022). The presence of phenolic compounds in these extracts likely contributes to their antibacterial activity, as phenolics are known for their ability to interfere with microbial cell wall synthesis and function (Hartanti et al., 2016). Moreover, while positive control showed superior inhibition, the results for ethyl acetate extracts are promising for developing natural antibacterial agents, particularly in an era of increasing antibiotic resistance. Future studies should focus on identifying specific bioactive compounds within these extracts and exploring their mechanisms of action to optimize their use in clinical and agricultural settings (Salem et al., 2022).

4.10. Gas Chromatography -Mass Spectrophotometry Analysis

The chromatographic analysis of the ethyl acetate extracts of of endophytic bacterium from *D. stramonium* using gas chromatography-mass spectrometry is shown in Table 7. From this Table 7, it can be observed that one peak can have one, two or three compounds at the same

retention time. Peak 2 containing methyl hydrogen disulfide and hydrazine has the highest percentage (41%) and Peak 4 containing thiazole and butanoic acid has the lowest (0.032%) (Figure 11)

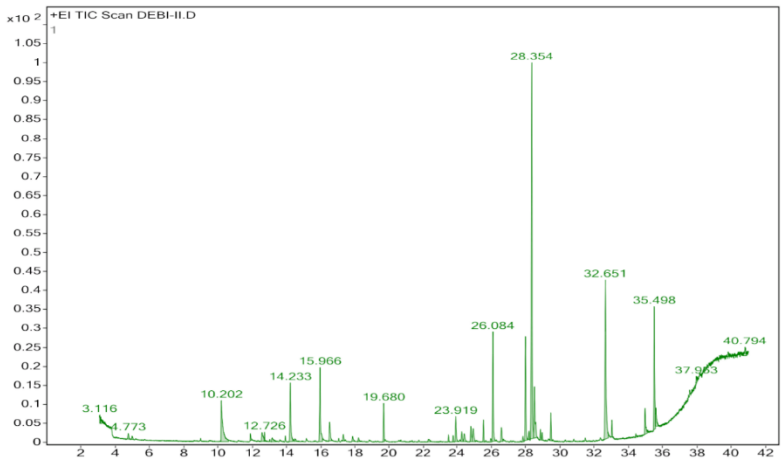


Figure 11: Gas chromatogram of isolated endophytic bacteria.

Table 7: Secondary metabolites of *Enterococcus* sp. EBIRDS-8 due to GCMS analysis

No	RT	Compound Name	Molecular formula	Composition (%)
1	10.202	Boc-4-methylproline	$C_{10}H_{17}NO_3$	6.45
2	12.576	Pyrido[1,2-a]azepine-6,7,8,9-tetracarboxylic acid, 10-(benzoyloxy)- 6,7-dihydro-, tetramethyl ester	$C_{25}H_{23}NO_{10}$	1.28
3	14.233	Phenylacetic acid	$C_8H_8O_2$	4.98
4	15.966	β-Phenylpropionic acid	$C_9H_{10}O_2$	6.18
5	16.52	3-Chloro-4-formylphenyl propanoate	$C_{10}H_9ClO_3$	1.8
6	19.68	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	2.12
7	23.919	(3R,8aS)-3-methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a] pyrazine-1,4- dione	$C_8H_{12}N_2O_2$	1.91
8	24.797	Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_7H_{10}N_2O_2$	1.51
9	24.929	Pentachlorophenol (PCP)	C_6HCl_5O	1.55
10	27.985	Cyclo(Pro-Val)	$C_{10}H_{16}N_2O_2$	7.42
11	28.354	Cyclo(leucylprolyl)	$C_{11}H_{18}N_2O_2$	29.6
12	29.463	trans-2-nonadecene	$C_{19}H_{38}$	1.76
13	32.651	2,5-PDMP	$C_{12}H_{22}N_2O_2$	15.45
14	34.961	Acrylamide	$C_{14}H_{16}N_2O_2$	2.43
15	35.498	Cyclo(Phenylalanylprolyl)	$C_{14}H_{16}N_2O_2$	8.59

In this study, metabolites such as , Acrylamide, Boc-4-methylproline, β-Phenylpropionic acid, 2,4-Di-tert-butylphenol, Pyrido[1,2-a]azepine-6,7,8,9-tetracarboxylic acid, 10-(benzoyloxy)-6,7-dihydro-,Phenylacetic acid, tetramethyl ester, Cyclo(Phenylalanylprolyl), Pentachlorophenol (PCP), Cyclo(leucylprolyl), Cyclo(Pro-Val), 2,5-PDMPtrans-2-nonadecene and others were predicted based on GCMS from *D. Stramonium*. However, in the previous study certain metabolites such as eugenol, 2-pentadecanone 6,10,14 trimethyl, pentadecanoic acid, pentadecanoic acid, 1 4-methyl- methyl ester, phytol, 9,12,15-octadecatrienoic acid, heptacosane, n-hexadecanoic, 6-octadecanoic acid, 9, 12 octadecanoic

acid, dodecanoic & tetradecanoic acids were predicted from *D. metel* L. using GC-MS analysis which is from the same genus (Hanif et al., 2022). The presence of these secondary metabolites, which obtained from *D. stramonium* might be employed for inhibiting certain bacterial and fungi pathogens. Similarly, it was hypothesized that secondary metabolites, these derived from *D. metel L.* (Hanif et al., 2022) used for inhibiting the growth of pathogenic fungi. Other study revealed that malonic acid, pentanedioic acid, butanoic acid, threitol, azelaic acid, galactonic acid, myo-inositol, octadecanoic acid, pseudo eridine, tetradecanoic acid, butanedioic acid, alanine, hexadecanoic acid, oleic acid, propanedioic acid, and L-methionine which mostly belonged to organic acid were derived from the same plant species, *D. stramonium* (Zhang et al., 2015).

4.11. Profiles of Thin Layer Chromatography (TLC) of Ethyl Acetate Extracts of Endophytes Bacteria

In this study, TLC chromatograms were recorded for *Enterococcus* sp. EBIRDS-8a&bwith the Rf value of 0.43 (43%) (Figure 12). The use of ethyl acetate has been widely applied in various research of antibiotics isolation. This is because antibiotics are generally semipolar and easily isolated with ethyl acetate (Riyanti & Radjasa, 2009). In the presence study, the metabolite which could have medicinal value have been separated and quantified as showed in Figure 11 with visible spots using TLC chromatograms, a similar work done by Binert-Kusztal et al., (2021). This spot (Figure 12) indicated pure metabolites which could be employed for inhibiting pathogenic bacteria and fungi.

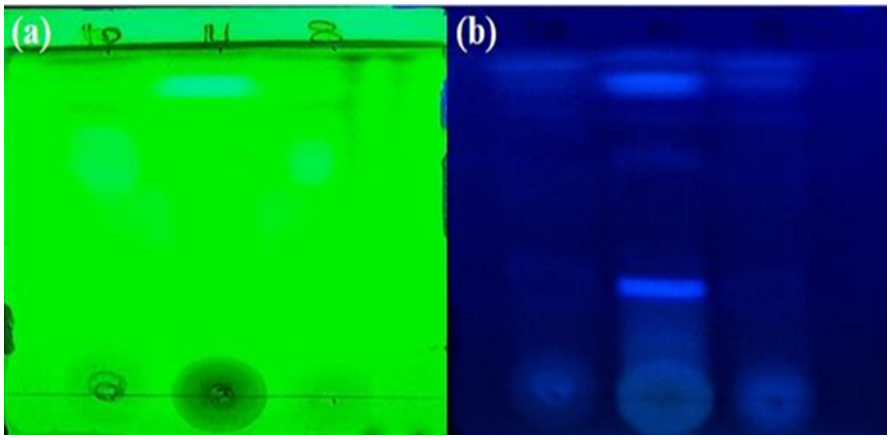


Figure 12: (a) The TLC chromatograms with visible spots under UV 254 nm of the metabolites obtained from *Enterococcus sp*. EBIRDS-8.

5. CONCLUSION AND RECOMMENDATIONS

In these studies, the following main findings were derived. Certain endophytic bacteria isolates obtained from various parts of *D. Stramonium* emerged on the medium in this study. Gram- positive and negative EBIs were noticed from *D. Stramonium*. Some of these isolates were also found to be endospore, catalase, citrate, indole, methyl red, urease, and Voges Proskauer positives & negatives. These EBIs were predicted as *B. cereus* EBIRDS-14, *E. hormaechei* EBILDS-9, *E. faecium* EBILDS-10, *K. oxytoca* EBISDS-1, *Klebsiella* sp. EBILDS-3 *Pseudomonas aeruginosa* (EBILDS-12), *Enterobacter hormaechei* (EBILDS-9), *Paenibacillus* sp. (EBISDS-6), *P. aeruginosa* EBILDS-12, *P. otitidis* EBIRDS-13. *Pseudomonas* sp. EBIRDS-4, *Pseudomonas* sp. EBIRDS-5, and others based on protein profiles using MALDI TOF MS analysis. EBI with better performers were not identified and predicted as *Enterococcus* sp. EBIRDS-8 based on 16S rRNA gene analysis. This isolate was highly similar to *Enterococcus fecalis* subsp. dissolvens MZ474974.1. Few of the recent EBIs (e.g. EBILDS-3, EBIRDS-8, EBISDS-11, and EBIRDS-20) were effective against killing certain pathogens with a 9.67±0.57 to 13.3±1.52 mm zone of inhibition. Phosphate and zinc solubilization assays were performed, and a few of them (e.g. EBISDS-1, EBILDS-2, EBIRDS-8, EBILDS-15, EBILDS-12 and others) showed considerable Solubilization index (1.37 to 2). Effect of various culture conditions such as pH, temperatures, carbon source, nitrogen sources, and salt concentration were conducted, and considerable amounts of OD_{600nm} and CDW (g/L) were obtained, which raged from against certain EBIs such as *Enterococcus* sp. EBIRDS-8. The highest CDW (g/L) and OD_{600nm} were obtained against nitrogen sources. Certain secondary metabolites suggested for inhibiting pathogens were identified as Acrylamide, Boc-4-methyl proline, Phenylacetic acid, β-Phenylpropionic acid, 2,4- Di-tert-butylphenol, Pentachlorophenol (PCP), Cyclo(Pro-Val), Hexahydropyrrolo [1,2-a]pyrazine-1,4-dione and others based on GC-MS analysis. These secondary metabolite extractions revealed promising antimicrobial activity, further demonstrating the isolates' potential to combat pathogenic organisms. These findings underscore endophytic bacteria's vital role in promoting sustainable agricultural practices and addressing microbial resistance challenges. Further research should focus on utilizing the isolates for agricultural & antimicrobial applications, identification of other EBIs which can employed for these applications based on whole genomic sequences, metagenomics, transcriptomics, metabolomics, and Lipidomics, and other tools.

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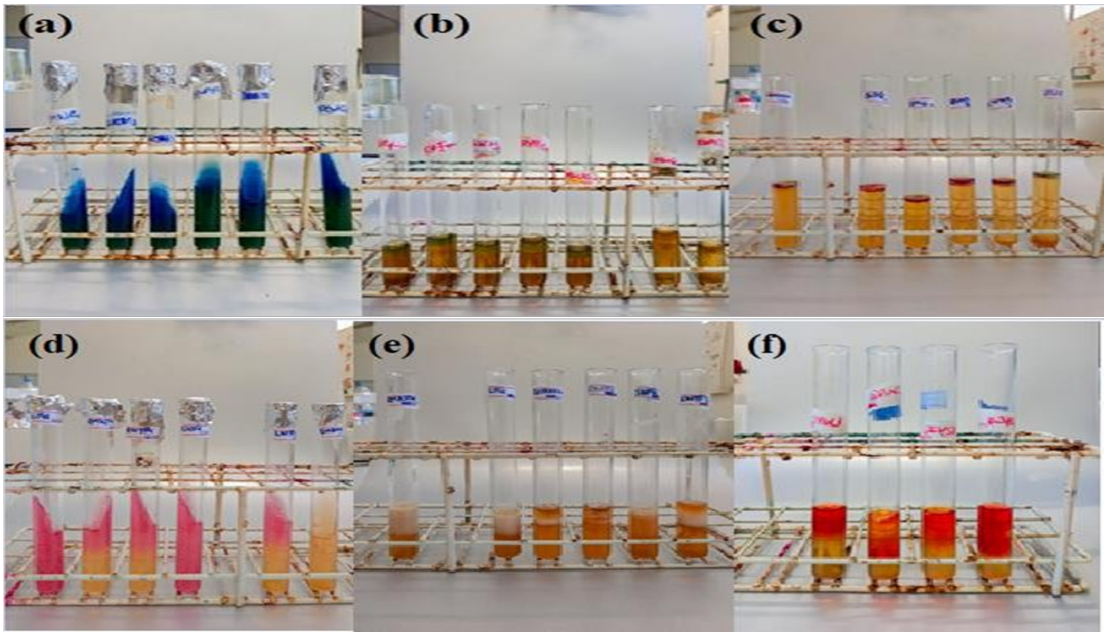
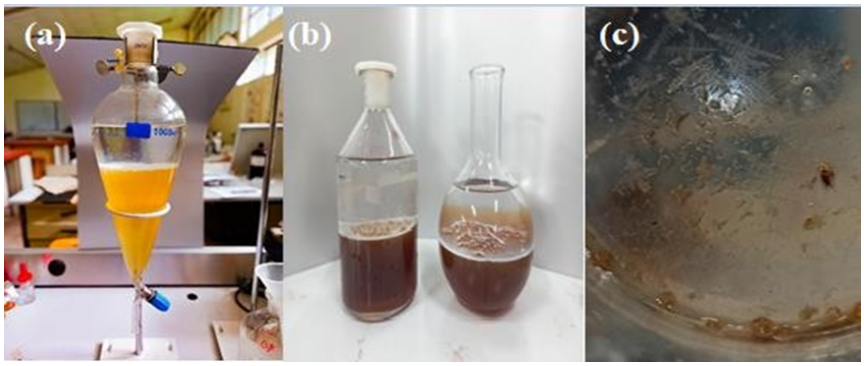
7. APPENDICES

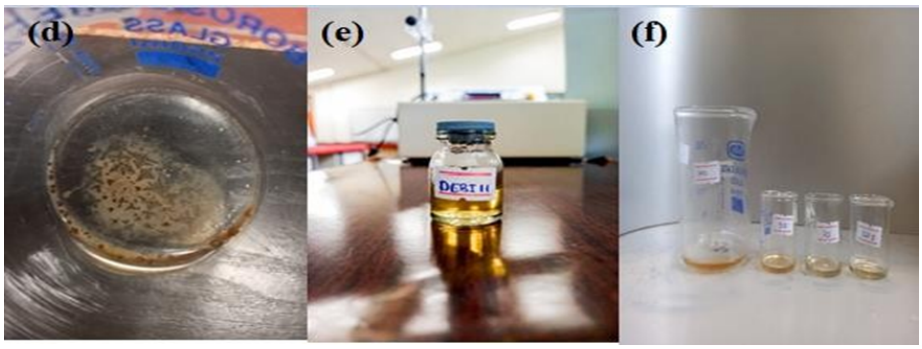
Appendix 1: Sample of the *D. stramonium*

Appendix 2: Biochemical test



Appendix 3: Crude extract against *D. stramonium*





Appendix 4: Morphological Characterization of Endophytic Bacteria

S.N.	Isolates	Shape	Surface	Color	Oduor
1	EBISDS-1	Round	Smooth	Off white	Odorless
2	EBILDS-2	Round	Smooth	Yellow	Oduor has
3	EBILDS-3	Round	Mucoid	Off white	Oduor has
4	EBIRDS-4	Filamentous	Shiny smooth	Yellow	Oduor has
5	EBILDS-5	Round	Smooth	Yellow	Oduor has
6	EBISDS-6	Round	Smooth	White	Oduor has
7	EBILDS-7	Filamentous	Shinny smooth	White Brown	Oduor has
8	EBIRDS-8	Irregular	Rough	Yellow	Oduor has
9	EBILDS-9	Filamentous	Shinny Smooth	Yellow	Oduor has
10	EBILDS-10	Round	Smooth	Yellow	Oduor has
11	EBISDS-11	Round	Smooth	Off white	Oduor has
12	EBILDS-12	Round	Shinny Smooth	Yellow	Oduor has
13	EBIRDS-13	Round	Shinny Smooth	Creamish White	Oduor has
14	EBIRDS-14	Round	Shinny Smooth	Creamish White	Oduor has
15	EBILDS-15	Irregular	Elevated	Off white	Oduor has
16	EBISDS-16	Rhazoid	Smooth	White Brown	Oduor has
17	EBILDS-17	Round	Smooth	Creamish White	Oduor has
18	EBIRDS-18	Round	Smooth	Yellow	Oduor has
19	EBISDS-19	Filamentous	Smooth	White Brown	Oduor has
20	EBIRDS-20	Rhazoid	Rough	Yellow	Oduor has
21	EBILDS-21	Round	Smooth	Yellow	Oduor has
22	EBILDS-22	Filamentous	Smooth	White Brown	Oduor has
23	EBIRDS-23	Irregular	Rough	White	Oduor has
24	EBISDS-24	Round	Smooth	White	Oduor has

25	EBILDS-25	Rhazoid	Rough	White Brown	Oduor has

Appendix 5: Mean, SD, Standard error, minimum and maximum value of Antagonist test

S.N.	EBIs against *S. aureus* 6538^T (ATCC)	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
1	EBILDS-3 against *S. aureus* 6538^T (ATCC)	3	11.33	1.53	.88	7.54	15.13	10.00	13.00
2	EBILDS-9 against *S. aureus* 6538^T (ATCC)	3	9.67	.578	.33	8.23	11.10	9.00	10.00
3	EBISDS-11against *S. aureus* 6538^T (ATCC)	3	10.0	2.00	1.151	5.03	14.97	8.00	12.00
4	EBIRDS-13 against *S. aureus* 6538^T (ATCC)	3	12.00	2.00	1.15	7.03	16.97	10.00	14.00
5	EBIRDS-20 against *S. aureus* 6538^T (ATCC)	3	11.00	1.73	1.00	6.69	15.30	10.00	13.00
6	Ciproflaxcin	3	20.00	.00	.00	20.00	20.00	20.00	20.00
7	Total	18	12.33	3.83	.91	10.43	14.24	8.00	20.00

Appendix 6: One-Way-ANOVA data effect of nitrogen source against some beneficial traits

CDW (g/L) for various Nitrogen sources for EBIRDS-8 and EBILDS-12

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) for EBIRDS-8 against $(NH_4)_2SO_4$	3	.180030	.0000520	.0000300	.179901	.180159	.1800	.1801
CDW (g/L) for EBILDS-12 against $(NH_4)_2SO_4$	3	.150520	.0009007	.0005200	.148283	.152757	.1500	.1516
CDW (g/L) for EBIRDS-8 against KNO_3	3	.130440	.0007621	.0004400	.128547	.132333	.1300	.1313
CDW (g/L) for EBIRLDS-12 against KNO_3	3	.100590	.0010219	.0005900	.098051	.103129	.1000	.1018
CDW (g/L) for EBIRDS-8 against Yeast Extract	3	.302200	.0003464	.0002000	.301339	.303061	.3020	.3026
CDW (g/L) for EBILDS-12 against Yeast Extract	3	.258780	.0013510	.0007800	.255424	.262136	.2580	.2603
CDW (g/L) for EBIRDS-8 against Peptone	3	.292450	.0007794	.0004500	.290514	.294386	.2920	.2934
CDW (g/L) for EBILDS-12 against Peptone	3	.230430	.0007448	.0004300	.228580	.232280	.2300	.2313
Total	24	.205680	.0728487	.0148702	.174919	.236441	.1000	.3026

LOCUS *Enterococcus* 1414 bp DNA linear BCT 09-JAN-2025

DEFINITION sp. EBIRDS-8.

ACCESSION

VERSION

KEYWORDS .

SOURCE *Enterococcus*

ORGANISM *Enterococcus*

Bacteria; *Bacillati; Bacillota; Bacilli; Lactobacillales; Enterococcaceae.*

REFERENCE 1 (bases 1 to 1414)

AUTHORS Thummim,M. and Ebu,S.M.

TITLE Diversity of endophytic bacteria isolates from leaf, root and steam part of Thorn apple (*Datura stramonium*) in Ethiopia

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1414)

AUTHORS Thummim,M. and Ebu,S.M.

TITLE Direct Submission

JOURNAL Submitted (09-JAN-2025) Department of Applied Biology, Adama Science and Technology University, Adama Gara Lugo, Adama City, Oromia +251, Ethiopia

COMMENT ##Assembly-Data-START##

Sequencing Technology:: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

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121 gtgctaatac cgcataacag tttatgccgc atggcataag agtgaaaggc gctttcgggt

181 gtcgctgatg gatggacccg cggtgcatta gctagttggt gaggtaacgg ctcaccaagg

241 ccacgatgca tagccgacct gagagggtga tcggccacac tgggactgag acacggccca

301 gactcctacg ggaggcagca gtagggaatc ttcggcaatg gacgaaagtc tgaccgagca

361 acgccgcgtg agtgaagaag gttttcggat cgtaaaactc tgttgttaga gaagaacaag

421 gacgttagta actgaacgtc ccctgacggt atctaaccag aaagccacgg ctaactacgt

481 gccagcagcc gcggtaatac gtaggtggca agcgttgtcc gggatttatt gggcgtaaag

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601 ttggaaactg ggagacttga gtgcagaaga ggagagtgga attccatgtg tagcggtgaa

661 atgcgtagat atatggagga acaccagtgg cgaaggcggc tctctggtct gtaactgacg

721 ctgaggctcg aaagcgtggg gagcaaacag gattagatac cctggtagtc cacgccgtaa

781 acgatgagtg ctaagtgttg gagggtttcc gcccttcagt gctgcagcaa acgcattaag

841 cactccgcct ggggagtacg accgcaaggt tgaaactcaa aggaattgac gggggcccgc

901 acaagcggtg gagcatgtgg tttaattcga agcaacgcga agaaccttac caggtcttga

961 catcctttga ccactctaga gatagagctt tcccttcggg gacaaagtga caggtggtgc

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