Molecular Characterization of Endophytic Bacteria from Some Medicinal Plants and Their Potential Application in Agriculture and Industry

Muktar Ahmed Adem

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Sciences

Presented in Partial Fulfillment of the Requirement for the Degree of Masters in Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

June, 2022

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Declaration

I hereby declare that this Master Thesis entitled “Molecular Characterization of Endophytic Bacteria from Some Medicinal Plants and Their Potential Application in Agriculture and Industry” 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.

Name of Student	Signature	Date

Recommendation

We the advisors of this thesis hereby certify that we have read the revised version of this thesis entitled “Molecular Characterization of Endophytic Bacteria from Some Medicinal Plants and Their Potential Application in Agriculture and Industry” prepared under our guidance by Muktar Ahmed submitted in partial fulfillment of the requirements for the degree of Masters of Science in Bioterchnology, Therefore we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

Major advisor	Signature	Date
Co-advisor	Signature	Date

Approval Page

We the advisors of the thesis entitled "Molecular Characterization of Endophytic Bacteria from Some Medicinal Plants and Their Potential Application in Agriculture and Industry" and developed by Muktar Ahmed here by certify that the recommendations and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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Co-advisor	Signature	Date

We the undersigned members of the board of examiners of the thesis by Muktar Ahmed have read and evaluated the thesis entitled "Molecular Characterization of Endophytic Bacteria from Some Medicinal Plants and Their Potential Application in Agriculture and Industry" and examined the candidate during open defense. This is therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Masters of Science in Biotechnology.

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List of Acronyms and Abbreviations

ABIS ..... Advanced Bacterial Identification Software

ACC ..... 1-aminocyclopropane 1-carboxylic acid

AIMs ..... Agriculturally Important Microbes

ASTU ..... Adama Science and Technology University

ATCC ..... American Type Culture Collection

BCG ..... Bromocresol green

BLAST ..... Basic Local Alignment Search Tool

BM ..... Biomass

CI ..... Clearing Index

CMC ..... Carboxymethycellulose

DF ..... Dworkin Foster

eDT ..... extended Direct Transfer

EPS ..... Exo-Polysaccharides

ET ..... Ethylene

HCCA ..... α-cyano-4-hydroxycinnamic acid

IAA ..... indole-3-acetic acid

ISR ..... Induced Systemic Resistance

IVET ..... In Vivo Expression Technology

JA ..... Jasmonic Acid

LB ..... Luria Bertani

LSV ..... Logarithmic Score Value

MALDI-TOF MS ..... Matrix Assisted Laser Desorption Ionization- Time of Flight Mass Spectroscopy

MAMPs ..... Microbe Associated Molecular Pattern

MEGA ..... Molecular Evolutionary Genetic Analyzer

MHA ..... Mueller-Hinton Agar

MOMPs ..... Major Outer Membrane Proteins

MR ..... Methyl Red

NA ..... Nutrient Agar

NCBI ..... National Center for Biotechnology Information

OD ..... Optical Density

PAMPs ..... Pathogen Associated Molecular Pattern

PBS ..... Phosphate Buffered Saline

PGP ..... Plant growth promotion

PGPR ..... Plant Growth Promoting Rhizobacteria

PMF ..... Peptide Mass Fingerprint

PSAM ..... Pectinase Screening Agar Medium

RDP ..... Ribosomal Database Project

Rpm ..... Rotations per Minute

SA ..... Salicylic Acid

SIM media ..... Sulfide Indole Motility media

STEM ..... Science Technology Engineering and Mathematics

VP ..... Voges Proskauer

Abstract

Medicinal plants are known to harbor diverse species of endophytic bacteria which are known for their secretion of beneficial secondary metabolites like enzymes and antimicrobial compounds. Medicinal plants of Artemisia annua, Ocimum lamiifolium, and Moringa oleifera are widely used in Ethiopia for medicinal, culinary, and nutritional applications. The current study aimed to isolate the endophytic bacteria of these medicinal plants and identify the strains using biochemical tests and 16S rRNA molecular sequencing and evaluate the ability of the isolates for important plant growth promotion features as well as their ability to produce industrially important exo-enzymes. In this study, 45 endophytic bacteria were isolated from the three medicinal plants, 15 from each medicinal plant's leaf, stem, and root parts. Preliminary tests of Gram's staining and Endospore staining were done. Biochemical tests including catalase test, citrate utilization test, H2S production, motility, methyl-red, voges-proskauer, indole, and urease tests were conducted for each of the isolates. Enzymatic activities of the endophytic bacteria such as the amylase, cellulase, pectinase, and protease producing ability were checked for each isolate. The plant growth-promoting abilities of the isolates like phosphate solubilization, zinc solubilization, and ACC deaminase production were checked. The secondary metabolites of isolates were extracted using ethyl acetate and the antibacterial activities of the extracts were evaluated. In this study, MALDI-TOF MS analysis of the isolated endophytic bacteria revealed the species diversity of the endophytic bacteria isolated from the medicinal plants. These endophytic bacterial isolates were found to be Bacillus cereus, Bacillus subtilis, Citrobacter freundii, Enterobacter asburiae, Enterobacter cloacae, Enterobacter kobei, Enterobacter ludwigii, Enterococcus faecium, and Pseudomonas monteilli. Bacillus and Enterobacter species were isolated in the recent study at the highest frequency. The most frequently isolated bacterial species were sent for 16S rRNA gene sequencing and found to be Enterobacter species with 99.47% sequence similarity to Enterobacter cloacae. This result was the same as the result obtained from MALDI-TOF MS analysis. Since this isolate was obtained from root part of Artemisia annua, its name is designated as Enterobacter sp. RPAAI8. This isolate was found to be capable of using certain cheap and cost-effective agro-wastes for its growth. The isolates were found to be excellent pectinase and protease producers which suggested the possibility of the isolates to be used in industrial applications. The isolates were also able to exhibit plant growth promoting attributes by solubilizing insoluble phosphate and zinc as well as ACC deaminase production. The ethyl acetate extract of the isolates showed inhibitory activity against test organisms.

Key words: *Endophytic bacteria, PGP, MALDI-TOF MS, 16S rRNA, Antimicrobial, Enzymes*

CHAPTER ONE

1. Introduction

1.1. Background of the study

Active research on the plant endosphere as a habitat for nonpathogenic bacteria started in the 1990s, triggered by the increasing number of reports on the beneficial effects of plant growth-promoting rhizobacteria (PGPR) (Compant *et al.*, 2021). According to Reinhold-Hurek and Hurek (1998) endophytic bacteria are a subclass of plant growth promoting rhizobacteria (PGPR). These are in fact a specialized group of rhizobacteria that have acquired the ability to invade their plant host (Compant *et al.*, 2012). Chanway *et al.*, (2000) and Hardoim *et al.*, (2008) stated in a report that endophytic bacteria share all the important traits consistent with the host plant growth promotion found in rhizobacteria. However, the beneficial effects provided by the endophytic bacteria to host plants are usually greater than those provided by many rhizospheric bacteria. Hence they have been used to enhance plant growth either directly or indirectly (Abod *et al.*, 2019). Such effects may exacerbate when plants are challenged by stress conditions.

According to Ryan *et al.*, (2008), the increasing interest in studying microbial communities in the environment together with the development of molecular, cultivation-independent tools (like the DNA fingerprinting tools, 16S rRNA, gene-based denaturating gradient gel electrophoresis or terminal restriction fragment length polymorphism analysis) to study their community structure also triggered interest to research on endosphere microbiota. It has been found that there are about 300,000 plant species thought to be host for endophytic diversity of bacterial strains, which can exist within plant tissue cells and are unique in their adaptations to specific chemical environment of plant cells. The diversity of endophytic bacterial isolates that are able to produce and share certain genetic based plant specific compounds makes them to be a source of noble natural products (Rosenblueth & Martínez-Romero, 2006; G. A. Strobel, 2003).

Endophytes are associated with agronomic crops, prairie plants and medicinal plants by residing within the specific chemical environment these plants provide. For instance, a 16S rRNA gene sequence result has revealed bacterial cells such as *Cellulomonas, Clavibacter, Curtobacterium*, and *Microbacterium* from three prairies and three agronomic crops (Zinniel *et al.*, 2002). Furthermore, using amplified ribosomal DNA restriction analysis (ARDRA) and 16S rRNA-sequencing endophytic bacterial cells such as *Achromobacter, Agrobacterium, Bordetella, Chryseobacterium Cupriavidus, Flavobacterium* genera, *Ochrobactrum, Pseudoxanthomonas* and *Stenotrophomonas* genera were isolated from maize (*Zea mays* L.) plant (Youseif, 2018). Diversity of endophytic bacterial members are also detected from leaves, stems and root parts of *Stellera chamaejasme* L. which is a medicinal plant. The members of the endophytic bacteria isolated included *Actinobacteria* (14.1%), *Bacteroidetes* (2.1%), *Chloroflexi* (1.9%), *Cyanobacteria* (1.7%), *Firmicutes* (36.5%), and *Proteobacteria* (43.2%) (Jin *et al.*, 2014).

These potential endophytic bacteria are able to produce different chemical compounds that can be used for plant growth promotion, like 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole 3 acetic acid (IAA), and siderophores. It has been reported that endophytic bacterial isolates are capable of phosphate solubilization and nitrogen fixation (Jasim *et al.*, 2014). It has been similarly reported that endophytic bacterial isolates are capable of producing different enzymes. For instance, it has been reported by Nxumalo *et al.*, (2020), that *Pseudomonas sp*. produce amylase, cellulase, esterase and proteinase. These capacities of the endophytic bacteria to produce industrially important enzymes have attracted the attention of many researchers to search for novel enzyme sources for industrial application.

Based on these facts, this research aimed to isolate endophytic bacteria from three traditional medicinal plants to identify their potential as plant growth promoting bacteria in order to understand their ability to replace chemical fertilizers in the future. This study also aimed to bio-prospect the ability of these bacteria to produce industrially important enzymes and secondary metabolites with different bioactivities.

Diversity of interesting endophytic bacterial isolates with diverse roles in plant physiology can be expected from endophytes that live in medicinal plant tissues such as *Artemisia annua* (Chikugn), *Moringa oleifera* (Shiferaw) and *Ocimum lamiifolium* (Damakese). It is also true

that growth promoting properties of endophytic bacteria from these three plants has not yet been well studied in Ethiopia. So studies on isolation and characterization of endophytic bacteria from *Artemisia annua* (Chikugn), *Moringa olifera* (Shiferaw) and *Ocimum lamiifolium* (Damakese) will be found to be very significant and a novel study in Ethiopia. In fact, there are many reports on the use of endophytic bacterial isolates for improving the productivity, disease resistance, and protection of plants against pests, bioremediation and an inherent capability of plant growth promotion (PGP) (A. Kumar *et al.*, 2019). Therefore, the present study was designed to isolate and identify potential endophytic bacterial isolates biochemically and molecularly that can offer an eco-friendly help to compl the chemical fertilizers, and to be used as biofertilizers and biopesticides in developing a sustainable, safe and effective agricultural system. This study is also designed to add to the existing knowledge of bacterial endophytes, their taxonomy and their potential to the scientific community.

1.2. Statement of the problem

Agricultural advancements, particularly green revolution, enforced the use of chemical fertilizers and pesticides for greater production and higher yield (Rakshit *et al.*, 2015). However, persistent chemo inputs into soil over the years have rendered a catastrophic effect on the soil micro-biota as well as on human health. Singh (2018) believes that this phenomenon is quite evident from the fact that many agricultural lands have been rendered completely unproductive, while there is a significant upsurge of certain deadly diseases such as cancer, diabetes, hypertension etc. due to a constant exposure to the chemical compounds. Degradation rates of the chemical fertilizers are quite negligible owing to their complex structures. This leads to their bioaccumulation and bio-magnification thereby resulting in a loss in specific biodiversity and additionally, severe groundwater contamination.

Apart from the abovementioned concerns, an exponentially increasing human population along with a persistent caveat of abiotic stress is demanding the utilization of other eco-friendly alternatives chiefly to ensure food security (Keswani *et al.*, 2014). The absence of detailed knowledge about endophytic bacteria and their potentials in our country has caused these agriculturally important microbes to be neglected and the use of potentially harmful agro-chemicals to persist.

1.3. Significance of the study

Microbes, particularly bacteria, have an inherent capability of plant growth promotion (PGP) and bioremediation. Bacterial genera, in particular, have been used as plant growth promoting agents since the 1970s with a variety of reports justifying their capability of phosphate solubilization, zinc solubilization, nitrogen fixation, biological control of plant diseases and other PGP attributes (Rakshit *et al.*, 2015). The current shift in agricultural practices has thereby gradually incorporated minimal use of agrochemicals in field practices while simultaneously employing bacterial microbiota as bio-inoculant, either singly or in consortia. Taking into concern the impressive role of biopesticides and biofertilizers in the promotion of sustainable agriculture, which basically include, target-specificity, environmental safety, and biodegradability, it is believed copious researches are necessary for promoting the use of these microbes. And there are in fact several researches on these microbes being carried out by scientists and researchers worldwide. In spite of the fact that many researches are being carried out worldwide, when we come to Ethiopia the use of these green inputs is significantly less. Therefore, this study significantly adds to the existing knowledge of bacterial endophytes, their taxonomy and their potential to be used as biofertilizers and biopesticides in developing a sustainable, safe and effective agriculture system in addition with their potential use in forestry and other fields, we believe also this research could serve as an eye opener for the potentials of the endophytic bacteria to produce different important enzymes and secondary metabolites.

1.4. Objectives of the study

1.4.1. General objective

- To characterize endophytic bacterial isolates from medicinal plants of *Artemisia annua* (Chikugn), *Moringa oleifera* (Shiferaw) and *Ocimum lamiifolium* (Damakese) for their potential application in agriculture and industry.

1.4.2. Specific objectives

- To screen endophytic bacterial isolates with potential plant growth promoting abilities from their natural hosts (Plant parts)

- To evaluate cultural condition and suitable carbon source for optimum growth of screened potential endophytic bacterial isolates
- To perform enzymatic activities for screened endophytic bacterial isolates
- To perform biochemical and molecular characterization for screened pure isolates of endophytic bacteria

1.5 Research questions

This research tried to address the following research questions:

- Which plants have potential endophytic bacteria in them?
- What species of bacteria are these endophytic bacteria?
- What is the benefit of these endophytic bacteria for the plant they are occupying?

1.6 Scope (limitation and delimitation of the study)

This study concentrated only on the isolation, chemical and molecular characterization of potential endophytic bacteria from the Roots, Stems and Leaves of *Artemisia annua* (Chikugn), *Moringa oleifera* (Shiferaw) and *Ocimum lamiifolium* (Damakese). This research didn't test the plant growth promoting (PGP) performance of the isolated endophytic bacteria *in vivo* as bio-inoculant, either singly or in consortia. Nor was this research aimed to quantitatively assess the enzymatic activities of the isolated endophytic bacteria but focused only on the qualitative assessment of their enzymatic activities.

CHAPTER TWO

2. Literature Review

2.1. Plants and Associated Endophytes

Endophytes are defined as microorganisms, mainly fungi and bacteria that are associated with host plant tissues without causing any antagonistic effects against the plants and promote plant growth by secreting various bioactive substances (Strobel *et al.*, 2004). As reported by Kusari *et al.*, (2012), the term endophyte (endo-inside, phyte-plant) was introduced by De Bary in 1866 and the first endophyte was isolated from *Lolium temulentum* by Freeman in 1904. Thrall *et al.*, (2007), elucidates that endophytes are involved in the synthesis of siderophores, in the production of plant hormones, in nitrogen fixation, solubilization of immobilized phosphorus, in nutrient cycling, production of volatile organic compounds, pathogenic resistance and stress tolerance. Usually plant roots help the plant tissues to grow by absorbing water and nutrients and in return discharging a rich source of organic acids, amino acids and sugars into the soil, which induce microorganisms to colonize the soil area around the plant roots. In addition, seeds during germination also release low molecular weight organic compounds into the surroundings thereby attracting the microbes in the rhizosphere and rhizoplane.

Many endophytes are found in the rhizosphere and only some selected microorganisms occupy the internal root tissues and reach the xylem (Rosenblueth & Martínez-Romero, 2004). At this stage the microorganisms get differentiated into pathogenic, associative, symbiotic, or neutralistic adaptation within the plant, and plant beneficial microorganisms become endophytic (Hayat *et al.*, 2010). Moreover, Mano & Morisaki, (2008) state that endophytes can enter plants through lenticles, wounds (which occur at emerging site of lateral root and root tips), through vascular system or xylem vessels or germinating radicles, secondary roots, stomata or through foliar damage and also through seeds and leaves. Inside seeds, they are found to be present in the embryonic stage through the endosperm. Later, when the seeds germinate, the endophytes are released into the external environment and invade the internal tissues of the growing plants (Johnston-Monje & Raizada, 2011). By this mode, endophytes

are transferred from generation to generation. The endophytes entering the plants through various routes can be localized at a single point or may spread to the whole plant (G. Strobel *et al.*, 2004).

The organic compounds of plants act as chemo-attractants and facilitate the movement of bacteria by flagellum-mediated chemotaxis (Buschart *et al.*, 2012). Flavanoids are an example of plant secondary metabolites that act like chemo-attractants for colonization of bacteria (Bhattacharyya & Jha, 2012; Chamam *et al.*, 2013; Santi *et al.*, 2013). As reported by *Pin et al.*, (2002), plants growing in low phosphate environment release organic acids like malic acid to attract the microbes. Surette *et al.*, (2003), says that, similarly the products of microbes produced during their metabolism influences the plant colonization.

It was reported by Chang *et al.*, (2009), that endophytes have lipopolysaccharides on their surface and plant derived isoflavanoids recognize these lipopolysaccharide. Endophytes such as *Azospirillum brasilense* move towards the extract of seeds like cereals with strong adhesion due to its protein called Major Outer Membrane Proteins (MOMPs) present in their glycosylated flagellum as reported by Lugtenberg & Kamilova, (2009), whereas Reinhold-Hurek *et al.*, (2006), stated that *Pseudomonas* species movement comprises of the usage of type IV pili in colonizing the plants. Similarly, *Pseudomonas fluorescensuses* use secretion system such as SSIII for their entry into plant roots (Preston, 2007) and *Azoarcus* endophyte uses type IV pili and twitching motility for their colonization (Dulla *et al.*, 2012).

2.2. Colonization of plants by endophytic bacteria

It is evident that endophytic bacteria can be considered a subset of rhizospheric bacteria even though, compared to rhizobacteria, endophytic growth has an added advantage over rhizospheric growth (Marquez-Santacruz *et al.*, 2010; Misko & Germida, 2002). Living within plant tissues allows endophytic bacteria to be in close contact with the plant host to readily exert a direct beneficial effect in return for consistent supply of nutrients. In fact, *Compant et al.*, (2010) stated that endophytic bacteria represent a class of specialized Rhizobacteria that have acquired the ability to invade plant roots after establishing a rhizospheric population.

Batteries of different bacterial traits determine the colonization of plant host by the endophytic bacteria. These traits are collectively referred to as colonization traits and regulate the entire

process of plant colonization. The colonization process involves complex communication between the two partners (De Weert *et al.*, 2002; Rosenblueth & Martínez-Romero, 2006). The process usually starts from the roots, and requires recognition of specific compounds in the root exudates by the endophytic bacteria. Plants produce these root exudates to interact with the beneficial bacteria for their own ecological advantage (Compant *et al.,* 2005). Moreover, it has been reported by Hallmann *et al.*, (1997) that endophytic bacteria colonize the plant interior in a sequence of events similar to rhizosphere colonization by rhizobacteria. However, says Compant *et al.,* (2010), endophytic colonization involves a suite of environmental and genetic factors that allow a bacterium to enter plant endosphere. Although, endophytic bacteria usually enter the plants through the root zone, the aerial parts of the plants, including stems, leaves, flowers and cotyledons, may also be used (Zinniel *et al.,* 2002). Once inside the roots, endophytic bacteria can now systemically infect the adjacent plant tissues.

Vadassery & Oelmüller, (2009), explain that the plants defense mechanism differentiates between the beneficial microbes and pathogens invading the plant. Increase in an intracellular calcium ion by a plant cell can activate such a defense gene mechanism during pathogenic entry and establish the beneficial interaction for endophytes through a casual balance. In addition, plants can sense the entry of pathogen by cell wall integrity (Hématy *et al.*, 2009). A further defense mechanism explained by Martínez-Viveros *et al.*, (2010), is pathogen-associated (or microbe-associated) molecular pattern (PAMPs/MAMPs) defense response of the plants that recognizes the pathogens and which acts as a true filter in selecting the beneficial microbes. Quorum sensing is yet another technique that differentiates endophytic bacteria from pathogens where communication process is made by pathogenic bacteria by producing signal molecules called auto inducers to track the density of population *(Hardoim et al.,* 2008).

After establishing themselves in the rhizosphere and rhizoplane, Hallmann, (2001), says bacterial endophytes are known to make their way inside the plant root and colonize themselves with sub-populations ranging from 10^5-10^7 cfu/g fresh weight. This involves bacterial adhesion to cell surface structures, which is mediated by polysaccharides, pili and bacterial adhesins (Hori & Matsumoto, 2010). According to Böhm *et al.,* (2007) once on the

root surface, the bacteria might reach the root entry sites, like lateral root emergence and wounds, using type IV pili mediated twitching motility or other distinct colonization site and colonization pattern preferences which every endophytic bacterium has and once these bacteria have established themselves on the root surfaces, they start to penetrate into the root interior using specialized mechanisms (Zachow *et al.*, 2010).

The process of penetration into the host plant is achieved by one of the two ways which are called passive or active. Passive penetration can occur at cracks present at root emergence areas, root tips, or those created by deleterious organisms. Active penetration is achieved by the competent endophytic bacteria by means of dedicated machinery of attachment and proliferation (Hardoim *et al.*, 2008). This involves presence of lipopolysaccharides, flagella, pili, twitching motility, and quorum sensing, which can affect endophytic colonization and bacterial movement inside the host plants (Böhm *et al.*, 2007). In addition, Compant *et al.* (2005), stated that the secretion of cell-wall degrading enzymes, mainly pectinases and cellulases are known to be involved in bacterial penetration and spreading within the plant.

Although not experimentally proven, it has been proposed by Elbeltagy *et al.* (2000), that endophytic bacteria produce low levels of cell-wall degrading enzymes, as compared to phytopathogens that produce deleteriously high levels of these enzymes, and can thus avoid triggering plant defense systems. Furthermore, another way proposed by Zinniel *et al.* (2002), which shows how endophytic bacteria avoid being detected as a pathogen by the plant is by maintaining low cell densities (2-6 log cfu/gfw) as compared to pathogenic bacteria (7-10 log cfu/gfw). And hence, according to Hardoim *et al.*, (2008), the endophytic presence of bacteria is determined by chance factors and bacterial genetic determinants that enable bacterial-plant crosstalk, leading to an active endophytic colonization process. The plant host also plays a critical role in selecting an endophytic partner, where secretion of specific root exudates and a selective plant defense response are considered important factors in the selection of specific endophytes (Rosenblueth & Martínez-Romero, 2006).

2.3. Endophytic bacteria in different plant compartments

Plants host diverse microbiota in different compartments and tissues, i.e. vegetative organs like roots, stems and leaves and also reproductive/disseminating organs (flowers, fruits/seeds).

Bacterial densities inside plant tissues typically range from 10^5 to 10^7 of cultivable cells per gram of root to 10^3-10^4 in leaves and stems. In flowers, fruits and seeds typically 10^2-10^3 cells per gram tissue are found. These numbers pinpoint to the soil environment as a major reservoir of potential endophytes (Compant *et al.*, 2010). However, as reported by Liu *et al.* (2017), the plant immune system may control the abundance of endophytes and maintain the most 'plant-favorable' bacterial density in the different organs. Even though, pathogens can overcome plant defense and can therefore reach higher cell numbers than non-pathogenic strains (Brader *et al.*, 2017). High bacterial cell density can be detrimental for the host organs. For instance, high cell density is known to induce quorum sensing (i.e. cell-density dependent) regulated processes such as virulence and pathogenicity but are also important for beneficial functions (Braeken *et al.*, 2008; Hartmann *et al.*, 2014). Seeds usually show low bacterial numbers and do not provide suitable conditions for microbial growth. Mitter *et al.* (2017), explained when seeds germinate, the bacterial cell density increases, seed-derived endophytes colonize the different plant tissues of the emerging plant.

According to Compant *et al.* (2010) and Brader *et al.* (2017), the occurrence, abundance and activities of individual bacterial taxa depend on the micro-environment provided by the plant compartment, the plant genotype and physiology as well as the surrounding environment (Afzal *et al.*, 2019; Barret *et al.*, 2015; Hardoim *et al.*, 2015; Turner *et al.*, 2013). Hounsome *et al.* (2008), states that roots, stems, leaves, flowers, fruits and seeds show different chemical conditions, in terms of organic acids, carbohydrates, vitamins, sugars, but also hormones, amino acids, fatty acids, flavonoids, glucosinolates, as well as phenolic compounds, pH and water, which are essential for plant growth, development, stress adaptation and defense. Each chemical environment enables the growth of specific microorganisms showing appropriate metabolic activities to colonize and inhabit plant organs and tissues, leading to different microbial assemblages as seen below in figure 1 (Compant *et al.*, 2010; Vorholt, 2012).

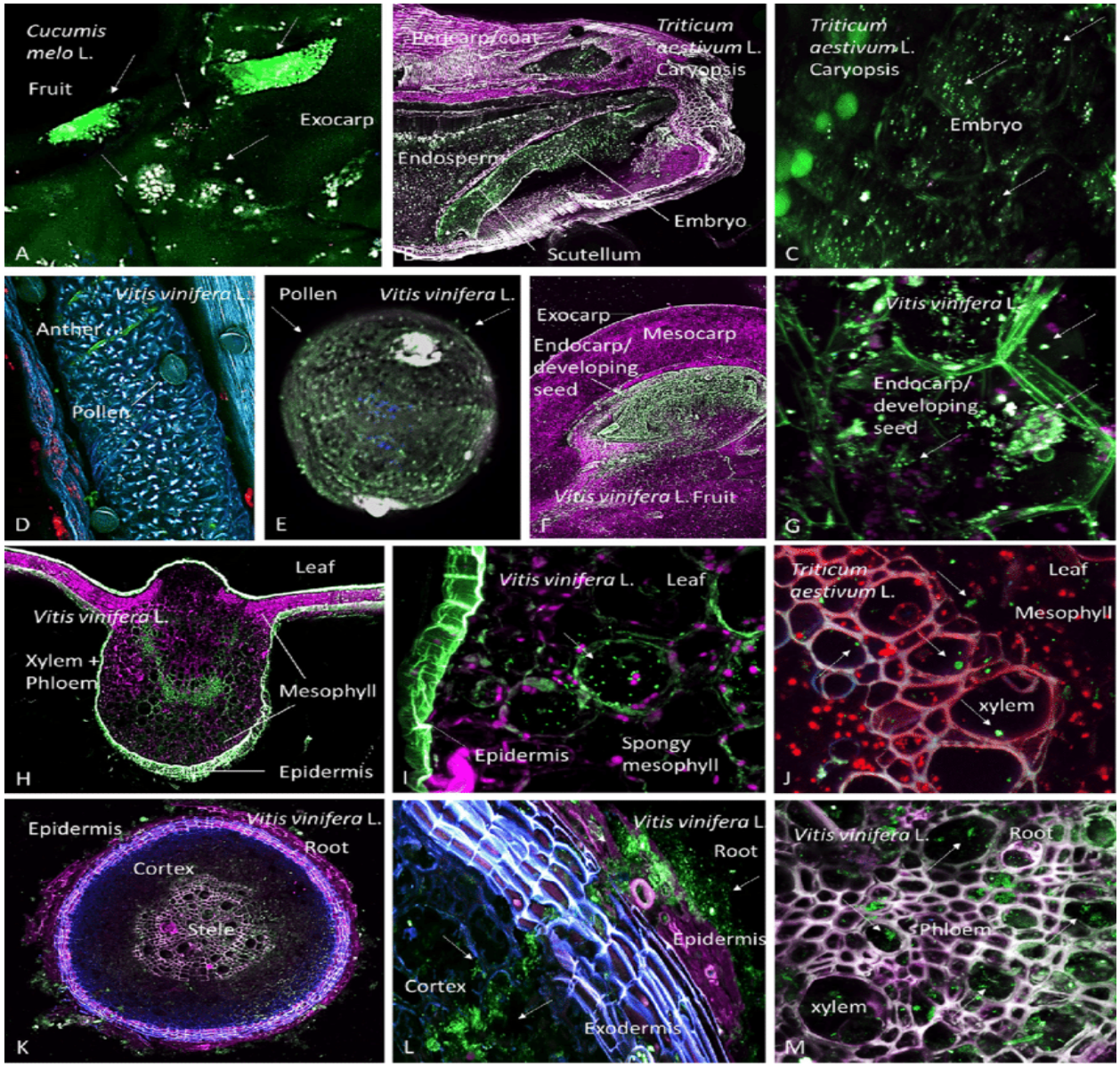


Figure 1: Confocal microscopy pictures of reproductive/disseminating or vegetative plant organs and bacteria.

(A) *Cucumis melo* L. fruit. (B) and (C) Caryopsis of *Triticum aestivum* L. (D) Anther of *Vitis vinifera* L. (E) Pollen of *Vitis vinifera* L. (F) and (G) Developing fruit and seed of *Vitis vinifera* L. (H) and (I) Leaf of *Vitis vinifera* L. (J) Leaf of *Triticum aestivum* L. (K), (L) and (M) Root of *Vitis vinifera* L. /Transversal section (A, H-M), longitudinal section (B and C, F and G), surface (D, E). Bacteria (arrows) appear as green, orange, white (A) by fluorescence in situ hybridization using general and specific probes targeting bacterial taxa or green (C, E, G, I, J, L, M) due to general staining with Syto9 ® . Scale of a bacterium: 1-2 µm. (Compant *et al.*, 2021)

2.4. Plant tissues and bacterial microbiota composition

In comparison to other plant organs, root bacterial microbiota often (but not always) contain the highest diversity of microorganisms (Amend *et al.*, 2019). Mostly found taxa are *Acidobacteria, Verrucomicrobia, Bacteroidetes, Proteobacteria, Planctomycetes* and *Actinobacteria* and most of them can be also found in the rhizosphere. However, a subpopulation of rhizosphere microbiota can enter roots. The most abundant phyla often found were, for instance, in grapevine roots, *Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, Planctomycetes, Chloroflexi, Firmicutes* and *Gemmatimonatedes* (Samad *et al.*, 2017). For maize, *Proteobacteria, Firmicutes,* and *Bacteroidetes* have been also found as predominant phyla inside roots (Correa-Galeote *et al.*, 2018). Deveau *et al.* (2018), states that different plant species usually host different microbiota composition, but also the plant genotype and developmental stage affect diversity and shape community structure. Furthermore, the soil environment, its history, as well as biotic or abiotic stresses lead to a shift in the diversity and abundance of the different taxa inhabiting roots. The presence of different microorganisms like fungi or mycorrhizae-like fungi and microbial interactions can additionally influence the composition of root endophytic bacteria.

Above-ground organ microbiota such as in stems, fruits or seeds have been shown to be influenced by several factors, including the soil environment (Afzal *et al.*, 2019). Interestingly, Harrison & Griffin, (2020) recently reported that variation in endophyte assemblages between below and aboveground tissues varied with the host growth habit. They demonstrated that in woody plants stems hosted the richest endophyte communities; where as in *graminoids* roots had the richest communities. Inside leaves, different endophytic assemblages have been found depending on external factors, such as climate and microclimate, as well as plant species and genotypes. *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* have been isolated from maize seeds.

2.5. Diversity of endophytic bacteria

Finding an endophyte-free plant is a rare exception in the natural environment and moreover bacterial endophytes have been found in every plant species that has been studied (Partida-Martínez and Heil, 2011). In fact, Timmusk *et al.* (2011), believes that a plant without the

associated beneficial bacteria would be less fit to deal with phytopathogens and be more susceptible to the stress conditions. The type of endophytic diversity present in a plant can depend on several factors. Apart from bacterial competence to colonize plants as endophytes, the host plant and environmental factors can effectively influence the endophytic diversity of a particular plant. Host plant age, genotype, geographical location, and even the tissue being analyzed can determine the type of endophytic bacteria it harbors. Moreover, Shi *et al.* (2014), explains that host plant growth stages can also determine the endophytic diversity of a plant, where plant stages enriched in nutrient availability tend to have increased bacterial diversity.

Not only nutrient availability but Peñuelas *et al.* (2012), states the climatic conditions can also influence the endophytic colonizers of a plant species. Peñuelas *et al.* (2012) observed that changes in climate significantly altered the abundance and composition of endophytic bacteria within the leaf tissues. Another important factor affecting the observable endophytic diversity of a plant is the method used to study these bacteria. The spectrum of bacteria recovered from a plant can depend on the nature, concentration and even length of the treatment time for a sterilizing agent used to recover bacteria.

The nature of plant host species is yet another factor that affects the endophytic bacterial diversity of a host plant. Different plant species growing in the same soil can have distinctly different endophytic diversity (Ding and Melcher, 2016). Germida *et al.* (1998) reported that canola and wheat plants grown in the same field had very different spectrum of bacterial species as endophytes. This observation was supported by Ding *et al.* (2013), who identified the host species being the most important determinant in selecting its endophytic community, followed by the sampling dates and the sampling locations. In fact, different cultivars of a plant species grown in the same soil can also differ in their endophytic diversity, as reported by Granér *et al.* (2003), for four different cultivars of *Brassica napus* having different endophytic bacterial inhabitants. Thus, the host plant species strongly governs the type of endophytic bacteria colonizing it.

More interestingly, the type of soil used to grow a plant can also determine its endophytic community. Thus, the same plant cultivar growing in different agricultural soils can have very different endophytic bacteria. Afzal *et al.*, (2019) mentions that an observation made by Song *et al.* (1999), states that there are significantly different endophytic bacterial diversity for a

peanut cultivar grown in different fields. Moreover, Rashid *et al.* (2012), isolated different types of endophytic bacteria by growing one cultivar of tomato in 15 different agricultural soils. Collectively, these reports indicate that occurrence of different endophytes is due to the diverse nature of soil samples.

The difference in endophytic community can also result by the preference imposed by the plant host in response to soil and stress conditions. Siciliano *et al.* (2001) reported that plants growing in petroleum hydrocarbon contaminated soil recruit particular endophytic bacteria which possess the potential contaminant-degrading genes. Moreover, the genes encoding for nitro-aromatic compound degradation were more prevalent in endophytic strains selected by the plants than within rhizospheric or soil microbial communities. Similarly, Granér *et al.* (2003) reported that wilt resistant cultivar of oilseed rape contained a higher proportion of endophytic bacteria antagonistic to the wilt causing *Verticillium longisporum* than the susceptible cultivar. Presence of phytopathogens in plants has been considered an important factor in the restructuring of endophytic bacterial communities. This was noticed by Bogas *et al.* (2015) by their work on the restructured endophytic communities of asymptomatic and symptomatic *Paullinia cupana* plants challenged by *Colletotrichum spp*. Hence, the selection of endophytic bacterial communities is a dynamic process that is tightly controlled by the host plant, where bacteria that favor plant host in a particular situation are preferred by the plant over other bacterial endophytes.

Endophytic bacterial diversity has been reported for a number of plant species. According to Miliute *et al.* (2015) and Santoyo *et al.* (2016), generally, *Proteobacteria* is the most predominant phylum frequently isolated from plants, including the classes α-, β-, and γ-*Proteobacteria*, where γ-*Proteobacteria* is the most diverse and dominant. Members of *Actinobacteria, Bacteroidetes,* and *Firmicutes* are also among the classes most commonly found as endophytes. Chaturvedi and Singh (2016), state other classes such as *Acidobacteria, Planctomycetes* and *Verrucomicrobia* are less commonly found. However, predominance of these phyla can vary with the type of host plant species. Hallmann *et al.* (1997) reported that among the most commonly isolated bacterial genera are *Bacillus, Burkholderia, Microbacterium, Micrococcus, Pantoea, Pseudomonas* and *Stenotrophomonas*, where *Bacillus* and *Pseudomonas* are the predominant genera.

2.6. Mechanisms of host plant growth promotion

Endophytic bacteria have been shown to pass on several beneficial effects on their plant host directly or indirectly. They can benefit plants directly by assisting plants in getting nutrients, and improve plant growth by modulating growth related hormones, which can help plants grow better under normal and stressed conditions (Ma *et al.*, 2016). It was shown by Tsegaye *et al.* (2020), that the plant growth promoting ability of endophytic bacteria goes beyond the production of enzymes that assist in degrading some hard to breakdown organic materials into sizes that can be assimilated by the plants. The endophytic bacteria also promote the growth of host plants they are occupying with supplying limiting inorganic nutrients by solubilizing, mobilizing and converting the insoluble nutrients into forms that can be directly used by the plants.

The accomplishment of the task of growth promotion is done either directly or indirectly. The direct method simply entails the provision of different important compounds that are synthesized by the endophytic bacteria itself or by assisting the bacteria in assimilating nutrients from the environment. The indirect method can be viewed as the defense force that protects the host plant from phyto-pathogens and attacks from harmful pests (Lodewyckx *et al.*, 2010). Miliute *et al.* (2015), explains endophytic bacteria improve plant growth indirectly by discouraging phytopathogens using mechanisms like antibiotic and lytic enzyme production, nutrient unavailability for the pathogens, and priming plant defense mechanisms and thereby protecting the plants from future attacks by pathogens. These beneficial processes are discussed below and summarized in Figure 2.

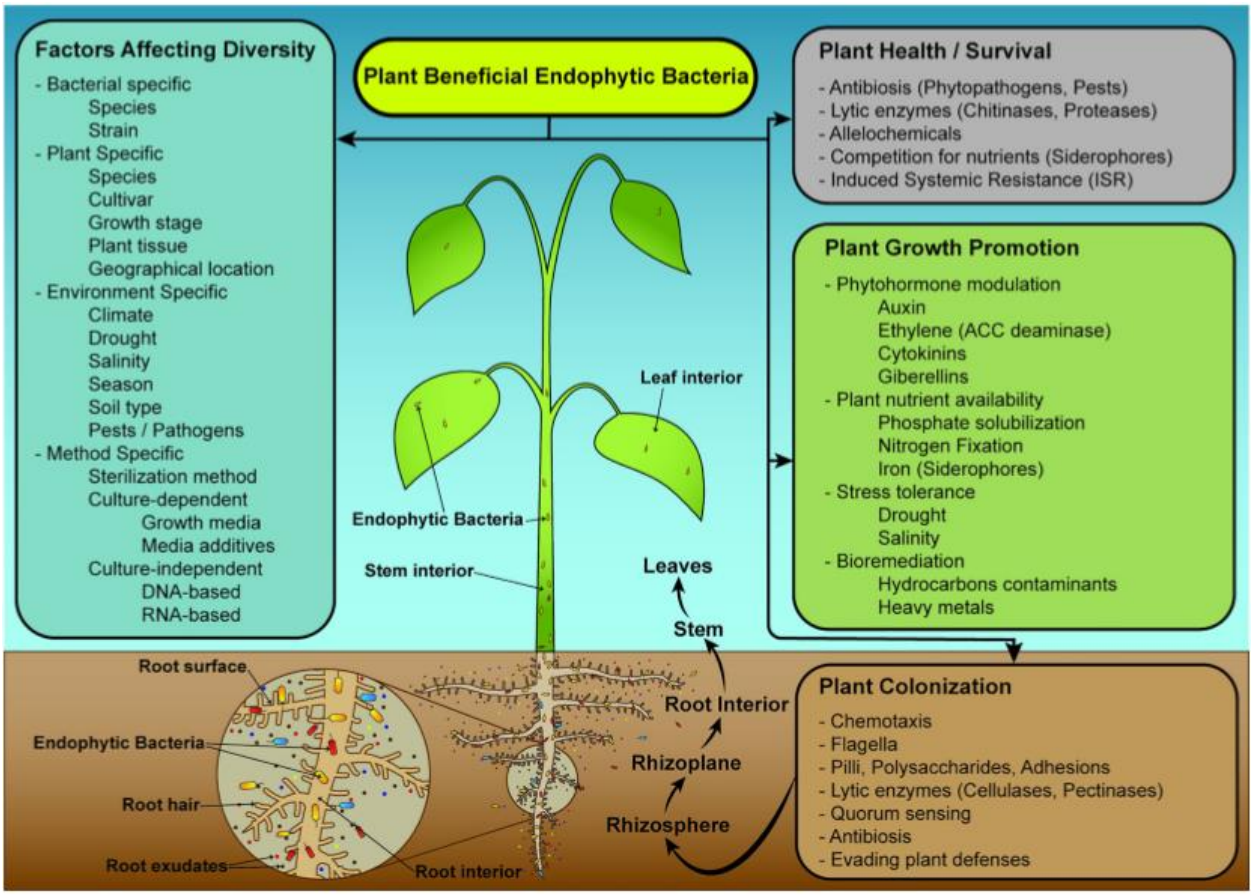


Figure 2: Mechanisms of plant growth promotion, colonization, and factors affecting diversity of endophytic bacteria in host plant (Afzal *et al.*, 2019).

2.6.1. Nutrient acquisition

Soils usually lack a sufficient quantity of one or more of the nutrient compounds necessary for plant growth. The endophytic bacteria can help their host plants in getting increased amounts of limiting plant nutrients, which include nitrogen, iron, phosphorus and zinc (Glick 2012). These mechanisms are discussed below.

2.6.2. Phosphorus availability

Phosphorous is a major micronutrient crucial for enzymatic reactions responsible for many plant physiological processes (Ahemad 2015). Although present in ample quantities, most of the soil phosphorus is insoluble, and therefore cannot support the plant growth due to its unavailability. Moreover, Ezawa *et al.* (2002), states that almost 75% of phosphorus applied as

fertilizer forms complexes with soil and becomes unavailable for the plants. It has been stated by Nautiyal *et al.,* (2000), that endophytic bacteria employ an array of mechanisms to increase the availability of phosphorus for plants which mainly include solubilizing precipitated phosphates, using mechanisms like acidification, chelation, ion exchange and production of organic acids. They can also increase phosphorus availability in the soil by secreting acid phosphatase that can mineralize organic phosphorus. Moreover, endophytic bacteria can prevent phosphate adsorption and fixation under phosphate-limiting conditions by assimilating solubilized phosphorus. Thus, these bacteria can act as a sink to provide phosphorus to the plants when they need it (Nautiyal *et al.*, 2000).

One common feature found in endophytic bacteria is phosphate solubilization. For instance, around 59-100% of endophytic populations from cactus, strawberry, sunflower, soybean and other legumes were mineral phosphate solubilizers (Afzal *et al.,* 2019). Puente (2009), examined the role of phosphate solubilizing endophytic bacteria by growing bacteria-free cacti on mineral phosphate supplemented with either endophytes or nutrients, and compared them with plants grown under sterile conditions. The inoculated plants grew well without nutrient addition, and their growth was comparable to fertilized plants, whereas the bacteria free unfertilized cacti failed to grow. This indicated that endophytic bacteria provided the developing plantlets with the limiting nutrient.

2.6.3. Zinc availability

A certain micronutrient crucial for optimum plant growth and development is Zinc. Although zinc is one of the imposing micronutrients its presence is sufficient in small amounts for the proper growth and development of plants. In plants, Zinc is used for carbohydrate and auxin metabolism and it acts as an anti-oxidant (Kamran *et al.,* 2017). It is well recognized that zinc metal cannot be degraded easily and is persistent in the environment for long periods of time. The usage of Zn containing fertilizers for extended amount of years coupled with its low melting temperature causes contamination to the environment, soil and fresh water bodies due to its divalent cation. It had been confirmed that the excessive presence of Zn in soil has had adverse effects but on the other hand Zn deficiency also results in impaired plant growth. The deficiency of zinc in crops is on the one hand because of scarcity of total Zn in soils and at the other is also due to the low solubility of Zn found in the soils. Normally, zinc can be found in

soil in forms such as franklinite ($ZnFe_2O_4$), hopeite ($Zn_3(PO_4)_2.H_2O$), smithsonite ($ZnCO_3$), sphalerite (ZnS), wellemite (Zn_2SiO_4) and zincite (ZnO). All of the forms of zinc mentioned above are insoluble forms of Zn and cannot be utilized by plants. Low availability of Zn affects the crop growth, lowers Zn level in seeds and grains henceforth affecting the nutritional quality that ultimately leads to Zn deficiency in consumers (Rehman *et al.*, 2021).

To overcome the Zn deficiency in soil for plant growth, normally chemical fertilizers are applied which obviously are causing environmental problems which we hope to annul in the future. Therefore, the question becomes what alternative could be of use? Answer, well soil microorganisms having Zn solubilization capacity. Certain bacteria including *Pseudomonas spp, Bacillus spp., Burkholderia cenocepacia* have been reported to transform insoluble forms of Zn to soluble form for enhanced availability and uptake by plants. Zn solubilizing bacteria can solubilize Zn through various mechanisms like acidification, production of siderophores and oxidoreductive systems on cell membranes (Saravanan *et al.*, 2011). These bacteria produce organic acids in soil which sequester the zinc cations and can also chelate zinc and enhance zinc solubility and uptake (Ashok *et al.*, 2019).

2.6.4. Control of ethylene levels

Ethylene is an important plant hormone that controls the plant response to abiotic and biotic stresses. It can control different developmental and physiological processes like root initiation, leaf senescence, root nodulation, abscission, cell elongation, fruit ripening and auxin transport (Sun *et al.,* 2016). Biotic and abiotic stresses result in an increased ethylene production in plants that leads to inhibition of root elongation, development of lateral roots and formation of root hair. Endophytic bacteria produce an enzyme called 1-aminocyclopropane-1carboxylate (ACC) deaminase that can hydrolyze ACC, which is a precursor of plant hormone ethylene. ACC degrading bacteria can bind to plant roots and cleave the exuded ACC into α-ketobutyrate and ammonia, and use it as a nitrogen source. Thus, hydrolysis of ACC can alleviate plant stress, thereby improving plant growth under stress conditions.

A number of plant growth promoting endophytic bacteria have been reported to have ACC deaminase activity (Nikolic *et al.*, 2011; Rashid *et al.*, 2012; Zhang *et al.*, 2011). In fact, the ability of these bacteria to benefit plant growth can be related to ACC deaminase production.

This was demonstrated by Sun *et al.* (2009) who mutated the ACC deaminase gene of the canola growth promoting *B. phytofirmans* PsJN and observed that the mutant was no longer able to promote canola root growth. However, introduction of wild-type ACC deaminase gene restored the growth promoting ability of the mutant *B.phytofirmans* PsJN, indicating the crucial role this enzyme plays in growth promotion of host plants.

2.6.5. Indirect growth promotion by suppression of phytopathogens

Endophytic bacteria enhance host plant growth indirectly by discouraging the growth of phytopathogens and plant pests. They can produce substances that can antagonize phytopathogens, like antibiotics, toxins, siderophores, hydrolytic enzymes and antimicrobial volatile organic compounds (Sheoran *et al.,* 2015). Lodewyckx *et al.* (2010), explains that both bacterial and fungal pathogens can be targeted by endophytic bacteria. Bacteria belonging to *Actinobacteria*, *Bacillus*, *Enterobacteor*, *Paenibacillus*, *Pseudomonas* and *Serratia* are the most commonly reported genera for their antimicrobial activity against phytopathogens. Endophytic bacteria have been demonstrated to successfully suppress fungal disease in plants like black pepper, potato and wheat (Aravind *et al.*, 2009; Coombs *et al.*, 2004; Sessitsch *et al.*, 2004). The antimicrobial activities against fungi can result from the production of fungal cell-wall targeting enzymes chitinase, proteases and glucanases. Antimicrobial activity against bacterial pathogens has been reported by Deng *et al.* (2011) and Dong et al (1994), for *Bacillus subtilis* BSn5 that targeted plant pathogen *Erwinia carotovora* subsp. *carotovora*. However, the exact mechanism of action against the pathogen is not known. Moreover, an endophytic bacteria *Pantoea vagans* C9-1 has been commercialized as a bacterial biocontrol agent for the fire blight. Activity against nematodes was reported by Aravind *et al.* (2009) for suppression of phytopathogenic burrowing nematode (*Radopholus similis* Thorne) by endophytic bacteria *Bacillus megaterium* BP17 and *Curtobacterium luteum* TC10. Endophytic bacteria that are active against plant pests have also been discovered, where genetically modified endophytic *Pseudomonas fluorescens* expressing *Bacillus thuringiensis* toxin and *Serratia marcescens* chitinase effectively targeted *Eldana saccharina* (Sugarcane Borer) larvae (Downing *et al.,* 2000),.

Pieterse *et al.* (2012) states that plant beneficial bacteria can also use a mechanism called induced systemic resistance (ISR) to protect their host plants from phytopathogens. ISR

induced by endophytic bacteria can protect host against fungal, bacterial and viral pathogens. The ISR primes plant defense mechanisms, thereby protecting unexposed plant parts against future pathogenic attacks by microbes and herbivorous insects. Endophytic bacteria can initiate ISR using salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) mediated pathways, which are usually a network of interconnected signaling pathways involved in ISR induction. Endophytic bacteria of genus *Bacillus, Pseudomonas* and *Serratia* have been shown to protect plant hosts using defense priming by ISR. The bacteria must also be able to overcome these host defense responses in order to colonize the host plant (Ma *et al.*, 2016).

ISR may involve both SA and JA/ET pathways. Niu *et al.* (2011) showed that *Bacillus cereus* AR156 triggered both SA and JA/ET signaling pathways in Arabidopsis to induce ISR, which led to an additive effect of plant protection. Similarly, Conn *et al.* (2008) indicated that *A. thaliana* plants inoculated with endophytic *Actinobacteria* showed up-regulation of both defense pathways, thereby protecting against subsequent infection by phytopathogenic bacteria *Erwinia carotovora* and fungus *Fusarium oxysporum*. However, the primed defense pathways differed for the two pathogen types. The resistance to *E. carotovora* was by JA/ET pathway, while resistance towards *F. oxysporum* was by SA pathway. Thus, the same bacterium was able to prime two different pathways to confer resistance to two different pathogens (Conn *et al.*, 2008).

2.6.6. Bacterial genes expressed in the endosphere

Endophytic bacteria colonize the plant endosphere and confer growth benefits to their host by using a wide variety of traits. Some of these traits have been confirmed by Yousaf *et al.* (2011) and Zhao *et al.* (2016), by using techniques like gene deletion/ disruption and complementation, by Rediers *et al.* (2003), using real-time PCR, by Roncato-Maccari *et al.*, (2003) using in vivo expression technology (IVET), using *gusA* fusion reporter system, while Cho *et al.*, (2007), used gene introduction, and Sheibani-Tezerji *et al.*, (2015) used RNA-seq based whole transcriptome profiling.

Sessitsch *et al.* (2005) and Sheibani-Tezerji *et al.* (2015) stated that most of these studies have been done on *B. phytofirmans* strain PsJN, a model endophytic bacterium, with the ability to competently colonize (both rhizosphere and endosphere) and promote growth of a variety of

different plant hosts, including *Arabidopsis thaliana*, grape, maize, potato, switch-grass, tomato, and wheat. Moreover, strain PsJN can also increase tolerance of host plants to abiotic stresses such as chilling and drought (Ait Barka *et al.*, 2006; Naveed *et al.*, 2014), and biotic stresses like inhibiting growth of fungal phytopathogens (Ait Barka *et al.*, 2006; V. K. Sharma & Nowak, 1998). Sheibani-Tezerji *et al.* (2015), reported that the strain PsJN have been shown to acquire IAA degradation, ACC deaminase, and quorum sensing to colonize host plants and produce beneficial effects. Moreover, in planta gene expression profiling revealed that, during its growth inside host plants, the bacterium expresses a number of different traits related to cellular homeostasis, cell redox homeostasis, energy production, general metabolism (amino acids, lipids, nucleotides, sugars), and transcription regulation. The same study revealed that the bacterium expresses enzymes related to oxidative stress when host plants are challenged with drought stress. Another study by Zhao *et al.* (2016), on strain PsJN indicated that the bacterium expresses traits involving iron uptake and storage. Nevertheless, more work is needed to elucidate the importance of different genes required by strain PsJN for its endophytic success. Other bacteria have also been studied for their endophytic gene expression and lifestyle. Collectively, these studies indicate that plants recruit those bacteria which benefit them in a certain niche or a situation. For example, plants growing in hydrocarbon contaminated areas prefer hydrocarbon degrading endophytic partners that actively express these traits during in planta growth (Yousaf *et al.*, 2011). Furthermore, these bacteria also readily express traits that allow active penetration and systemic colonization of host plants and allow competitive advantage over other plant endosphere dwelling bacteria.

2.7. Agro-waste usage as a source of carbon for the growth of microorganisms

Many Agro-Industrial wastes are being used as cost effective and highly effective sources of carbon and nitrogen for the growth and maintenance of important microorganisms. The abundance of agricultural wastes if properly utilized is in one hand a great fortune but in the other a source of highly damaging green-house gases and waste. Various agricultural and industrial wastes have been formulated as carbon and nitrogen sources for various strains of significant bacteria capable of producing various secondary metabolites. In particular, the synthetic medium, which contains simple carbon sources, nitrogen sources, and mineral salts,

has been altered by the addition of novel carbon sources. After that non-synthetic mediums were created using a variety of agricultural wastes (peanut, potato, corn, wheat, rice, and other by-products). Mineral salts ($MgSO_4$, K_2HPO_4) and yeast extract mixed with agricultural waste hydrolysate made up the majority of the non-synthetic growing media. The majority of the time when compared to the full synthetic medium, the media augmented with agricultural waste as a carbon source yielded higher concentrations of secondary metabolites and more biomass (Siddeeg and Tahoon, 2020).

Siddeeg and Tahoon, (2020) elucidate that the employed material and the applied pretreatment have an impact on the growth and generation of essential secondary metabolites. To reduce the inhibitory effects of various by-products (acetic acid, phenolic compounds, furfural, hydroxymethylfurfural, etc.) on microbial growth, resulted after acid hydrolysis of lignocellulosic materials, detoxification and enzymatic hydrolyzes can be employed. In addition, it was shown that enzymatic hydrolysation produces more secondary metabolites such as bioflocculants and polymers like glycan from microbes when compared to acid hydrolysates. Another method reported by Patil and Salunkhe, (2010), is the extraction of the carbon sources from the plant materials by soaking them in hot water under agitation. The only experiment describing this technology was the use of flowers of forest tree, *Madhuca latifolia L.*, to produce an exopolysaccharide bioflocculant of *Azotobacter indicus* ATCC 9540. Water extract of dry flower material (dry flower soaked in hot water for 2 hours under agitation (220 rpm) at 29 °C) was used for the strain culture yielding 6.10 g l^{-1} of polymer, higher to that obtained with sucrose and mannitol (Patil and Salunkhe, 2010).

2.8. Bio-active compounds

Medicinal plants are well known for their ability to produce different phyto-compounds which are of huge importance in medicinal as well as the industrial field. These compounds besides their importance as a novel source of phyto-compounds which are essential for maintaining good health they also create a comfortable environment for the plant dwelling endophytic bacteria. The endophytic bacteria which live in medicinal plants are reputable producers of important bio-active compounds that are known to have been secreted by their host plant and actively synthesize unique compounds of their own. Researchers have been investigating the potentials of these bacteria in hopes of finding novel compounds (Nxumalo *et al.*, 2020). In

agricultural settings, endophytic bacteria that help the host plant endure varied environmental stresses and combat bacterial and fungal infections are being used in the hopes of minimizing the use of chemical fertilizers in the future.

Some novel antibacterial and antifungal compounds have been screened from endophytic bacterial isolates of traditional medicinal plants. Due to their ability to generate antibiotics, these endophytic bacteria are considered as one of the potential sources of antibiotics. And in our research a simple extraction method was used to extract the secondary metabolites of the isolated endophytic bacteria and the anti-bacterial activity of the crude extract was tested against four bacteria including the bacteria from which the compound was extracted from.

2.9. Identification of bacteria isolated from environmental sources

Emerson *et al.* (2008), states that scientists have been designing classification schemes with the purpose of methodically identifying microbial species since the earliest knowledge of their existence. The effort that started in that era has also continued to our day and age. In this era of biotechnology the identification of microorganisms isolated from any source is of profound interest because by doing so it enables the researcher to determine what shall be done with the microorganism at hand. Based on the identity of the organism a researcher or clinician or any other microbiologist may classify the microorganism as harmful or beneficial, taking into consideration here is that, every microorganism deemed beneficial may cause harm if not properly handled and every microorganism may become beneficial if handled properly. But again to do just that identification must be done. There are two types of microbial identification systems, the first is the culture dependent identification system where a microorganism is grown in an axenic condition meaning that it is taken out of its natural habitat and is grown in a preset condition without any other microbes or contaminants in the culture setting. The other method is to detect microorganisms in their natural environment when nothing is predetermined and the existence of other bacteria is inevitable (Emerson *et al.*, 2008).

Bacterial identification techniques can be divided into two types. The first is genotyping procedures based on the molecular contents of the organism, primarily DNA. The second is the phenotypic technique, which is based on the physiological characteristics, chemical or

elemental composition, and metabolic characteristics of the organism. Recently the identification of bacteria based on their phenotype mainly their protein constituents is being explored in hopes of building better tools for the day to day activities of microbiologists and ease the identification process (Emerson *et al.*, 2008).

For decades, biochemical approaches have been utilized in small and large-scale laboratories to identify bacteria, and they are still used today. Since bacteria do not have enough physiological and morphological characteristics to define their differences several approaches have been established to differentiate their enzymatic or metabolic reactions, which has aided in the differentiation and identification to the genus or species level. But the natures of the bacteria and tests have made the identification process take from hours and sometimes to days (Rave *et al.,* 2018). And after the biochemical test results come out the user have to identify the genus or species of the bacteria from catalogues of defined sets of results either manually or using automated software. Of course there are some conventional biochemical identification kits developed like API strips, which made biochemical identification of bacteria a little easier and may only take a few hours to analyze and give results but the kits restricted the user to use only a set of predefined tests (Sorescu & Stoica, 2021).

Sorescu and Stoica (2021), recently presented an original online Automated Bacterial Identification Software (ABIS) which was freely available on the world wide web since 2007 for use by students, researchers, biologists, veterinary and human clinicians. The ABIS online software which was created by the authors by comparing to commercially available, standardized identification systems of API strips and apiweb™ bioMerieux software scored , overall, average identification percent as being 91.8% which was better than the 90.4% for apiweb™ bioMerieux software. The ABIS online identification tool presents 40-50 tests and requires at least 8 tests to identify a certain bacterial strain. The ABIS contains the morphological and biochemical characters of 16 reference strains and 123 wild isolates in 13 data bases covering areas of *Enterobacteriaceae, Pasteurellaceae, Campylobacteraceae, Bacillaceae, Lactobacillaceae, Staphylococcaceae, Streptococcaceae, Clostridium, Vibrio* and *Aeromonas, Listeriaceae, Neisseriaceae, Chromobacterium, Corynebacterium,* and Non-fermenters (Sorescu & Stoica, 2021).

Siqueira and Ro (2005), state that there is an advantage to using biochemical identification for unknown bacterial species, which is the nature of cultivation approaches being broad range that makes it possible to identify a great variety of microbial species in a sample, including those that are not being sought after. Another advantage of cultivation approaches is that they enable the physiology and pathogenicity of a certain bacteria to be studied, determine the antimicrobial susceptibilities of the microorganisms and also in some clinical settings allow the identification of a particular disease-causing microorganism fairly easily and cheaply following certain schemes. However Siqueira and Ro (2005), also state the disadvantages of biochemical identification approaches like being too costly, being insensitive to some particularly fastidious anaerobic bacteria and taking several days to weeks to identify them, for other relatively fast growing bacteria still taking several hours to days, depending on the experience of the person handling the identification process difference in sensitivity occurs which in turn affects the result. All of the above mentioned cons stressed the importance of development and application of better tools of identification compatible with environmental microbiological needs.

The appearance of new proteomics tools that investigate the biomolecules of microorganisms primarily based on mass spectrometry offers an excellent complement to the classical and molecular bacterial classification. The analytical technique of Mass spectrometry in which chemical compounds are ionized into charged molecules and the ratio of their mass to charge (m/z) is measured was discovered in the early 1900s. Though MS application was mainly restricted to the chemical sciences the discovery of Electron Spray Ionization (ESI) and Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) in the 1980s has enabled the application of MS in the identification of microorganisms by analyzing their large biological molecules like proteins (Emerson *et al.*, 2008). Emerson *et al.* (2008), states that in 1996 a Scientist by the name Holland with his colleagues published an article on the first use of MALDI-TOF MS for the rapid identification of whole bacteria. The output of the spectral data was then compared with archived reference spectra or by co-analysis with cultures of known bacteria.

Neelja *et al.* (2015), explains the principle and methodology behind MALDI-TOF MS bacterial identification. First the sample to be analyzed by MALDI MS is prepared by mixing

or coating with solution of an energy-absorbent, organic compound like α-cyano-4-hydroxycinnamic acid (HCCA), which is simply called matrix (Toubal *et al.*, 2018). When the matrix crystallizes on drying, the sample trapped within the matrix also co-crystallizes. Then the sample is taken to the MALDI-TOF analyzer where a laser beam shoots the target samples ionizing the matrix and the sample within in an automated mode, and also desorbing the samples from the target, generating singly protonated ions from analytes in the sample. The protonated ions are then accelerated at a fixed potential, where based on their mass-to charge ratio (m/z) are separated from each other. The charged analytes are then detected and measured using time of flight (TOF) analyzers. During MALDI-TOF analysis, the m/z ratio of an ion is measured by determining the time required for an ion to travel the length of the flight tube. To increase the length of the flight tube, and also correct small differences in energy among the ions few TOF analyzers incorporate an ion mirror at the rear end of the flight tube, which serves to reflect back ions through the flight tube to a detector. Finally a characteristic mass spectrum called peptide mass fingerprint (PMF) is generated for analytes on the TOF information. Identification of microbes is done by comparing the PMF with the PMFs contained in the database or simply the MS spectrum of unknown microbial isolates is compared with the MS spectra of known microbial isolates contained in the database (Neelja *et al.*, 2015).

CHAPTER THREE

3. Materials and Methods

3.1 Description of the study area

The samples of the plant materials were collected from different residential gardens in Adama city and Adama Science and Technology University. Adama is located at 08°32′29″N 39°16′08″E in east Shoa zone, Oromia Regional State, 99 km south east of Addis Ababa, the capital city of Ethiopia. The town is found in area of average altitude about 1712 m above sea level and has annual rain fall of 7600 mm and mean annual monthly temperature of 21°C. The climate of the area is hot or arid climatic type.

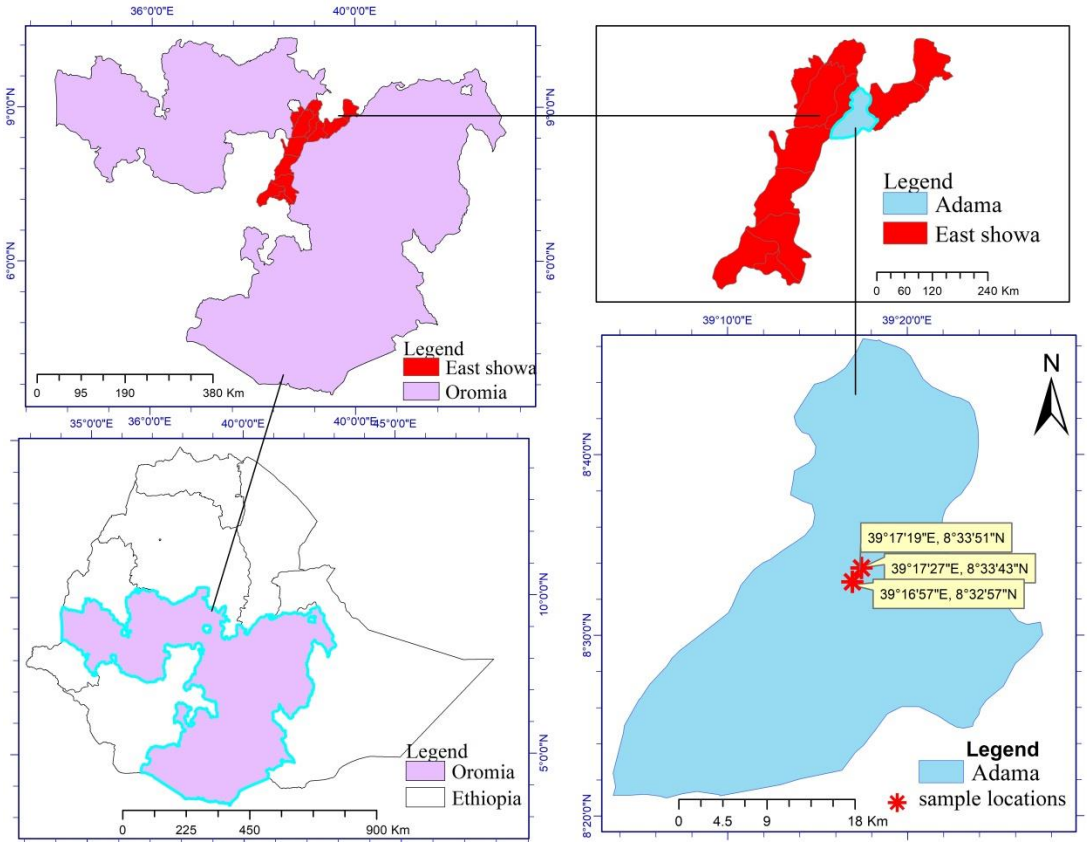


Figure 3: Map of plant samples collection sites

3.2. Sample collection

The medicinal plant samples from which the endophytic bacteria to be isolated were collected from different areas of Adama city. *Artermisia annua* plant samples were collected from Wondogenet research center and grown in the STEM center found in the Adama Science and Technology University compound. *Moringa oleifera* plant sample was collected from Adama Science and Technology University as well. Lastly, the *Ocimum lamiifolium* plant samples were collected from different gardens in different villages found in Adama city locally known as Cherka cherk and Ganda Gara. The plants were uprooted and extra soil present on the roots was removed gently and then the plant parts were cut into pieces and put into sterile plastic bags. Plant parts such as leaves, roots, and stems were considered for the isolation of endophytic bacteria. The sample bags were transported in an ice box to the biotechnology laboratory found at ASTU and stored at 4^{o}C in a refrigerator until isolation was conducted.

3.3. Isolation and screening of endophytic bacterial isolates

Isolation of endophytic bacteria: The different plant parts were cut with a sterile blade (leaves into 0.5 x 0.3 cm pieces, while roots and stems into 1 cm-length bars). The plant segments were washed under running tap water and surface sterilized by sequential immersion in 70% (v/v) ethanol for 1 min and sodium hypochlorite solution (4%) for 3 min as performed by Suryanarayanan *et al.* (1998). The surface sterilized plant samples were washed thrice using sterilized water to remove surface sterilizing agents. Following the procedure of Shweta *et al.* (2013) these segments were incubated on Nutrient agar (NA) (Glucose 10 g/L; NaCl 5 g/L; peptone 10 g/L; yeast extract 10 g/L; Agar 18 g/L at pH 7.0-7.2) plates amended with ketoconazole (20µg/ml) for 10 days at 25 ± 2^{o}C. The sterilized water taken from the third and final wash was also incubated using spread culture method as a positive control. A similar procedure without surface sterilization for few segments were done and used as a negative control to check for surface contamination.

3.4. Physiological and morphological characterization of isolated endophytic bacteria

Morphological characterizations were performed for the endophytic bacterial isolates according to the procedures by Boone and Castenholz, (2001). Colony feature such as colony color, elevation, margin, surface and spore formation were recorded for the endophytic bacteria after 48 hours of growth on nutrient agar medium. Microscopic examination was also performed to identify their spore formation, morphological type as rod, cocci, comma and spiral in shape.

3.4.1. Gram's staining procedures

Gram's staining was performed on the endophytic bacteria to differentiate between gram positive and gram negative species. Bacterial colonies were smeared on a drop of water on a clean glass slide then were left to air dry and heat fixed. Then the slides were flooded with crystal violate dye for 1 min followed by rinsing under tap water. Then the slides were flooded with gram's iodine for 1 min as a mordant and again rinsed under tap water. Thirdly, the slides were rinsed with 70% (v/v) ethanol for 15 sec and then rinsed with tap water. Lastly, the slides were flooded with safranin as a counter stain for 1 min and rinsed with tap water. Next the slides were allowed to dry and were viewed under microscope x100 with oil immersion (Woodland, 2004).

3.4.2. Endospore staining procedures

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 characterization of endophytic bacteria

Different biochemical tests were carried out to differentiate among the endophytic bacterial isolates. The biochemical tests such as catalase test, citrate utilization test, H_2S production test, indole production test, methyl red test, motility test, urease test and VOGES-PROSKAUER test were carried out. Depending on the test results, the bacteria could be categorized to the different genera they belong to.

3.5.1. Catalase test

For catalase test, fresh 18-24 hours old pure bacterial colonies were used. A 3-4 ml of 3% (v/v) H_2O_2 were poured into sterile test tubes and then pure endophytic bacterial colonies taken with the stick side of cotton swab were immersed fully into the test tubes. Then formation of bubbles due to conversion of H_2O_2 into H_2O and O_2 was checked for in the test tubes (Woodland, 2004). Slide method was also used for catalase test. Briefly a loopful of the isolated bacteria were smeared on a clean microscope slide and 2-3 drops of 3% (v/v) H_2O_2 was added directly on the smear, the formation of visible bubbles indicates positive result (Elmanama, 2009).

3.5.2. 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 35^0 C for 24-72 hrs. The bacterial colonies that can produce urease and convert the urea into CO_2, H_2O and NH_4 will convert the slant colors from yellow to bright pink (Vashist *et al.*, 2013).

3.5.3. Citrate utilization test

Simmons Citrate agar (Bromo thymol blue 0.08 g/L; $MgSO_4$ 0.2 g/L; (NH_4) $2HPO_4$ 1 g/L and agar 15 g/L) a medium containing citrate as the sole carbon source and ammonium salts as the sole nitrogen source was used to test for the ability of the endophytic bacteria to use organic

compounds other than sugars as their sole source of carbon. Fresh 18-24 hours old pure endophytic bacterial colonies were streaked on the slants and the test tubes were incubated at 37^{o}C for 24-48 hrs. If the bacteria metabolize citrate the color of the Simmons Citrate agar slants will change from green to deep blue (Woodward & Bartel, 2005).

3.5.4. Methyl red and Voges–Proskauer 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 37^{o}C. 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. H_2S production, motility and indole production (SIM) test

In order to find out the isolated organism's ability to reduce sulfur, produce indole and for motility, SIM medium was used. SIM medium is a differential medium that tests for the three characteristics simultaneously. For this experiment, SIM medium (Beef extract 3 g/L; $Na_2S_2O_3$ 0.025 g/L; Peptic digest of animal tissue 30 g/L; Peptonized iron 0.2 g/L and Agar 3 g/L) was prepared and was poured into test tubes. After solidifying the test tubes were inoculated by stabbing in the center to half an inch. Then the test tubes were incubated for 24 hours at 37^{o}C. After 24 hours the test tubes were checked for the formation of a black precipitate (H_2S production) and fuzzy area surrounding the stab line (motility) and then 2-3 drops of kovac's indole reagent was added to the tubes and the formation of red band was checked (Vashist *et al.*, 2013).

3.6. Tests for enzymatic activities of the isolated endophytic bacteria

The enzymatic activities of the isolated bacteria were tested. The tests included amylase test, cellulase test, pectinase test and protease test.

3.6.1. Extracellular amylase test

Extracellular amylase production was investigated by starch agar plate method. For the preparation of starch agar plate NA medium (Glucose 10 g/L; NaCl 5 g/L; Peptone 10 g/L; Yeast extract 10 g/L and Agar 18 g/L at pH 7.0-7.2) was supplemented with 10 g/L (w/v) of starch. The starch and the NA medium were autoclaved separately and mixed together post sterilization. The starch agar was poured onto sterile petri dishes and fresh bacterial colonies were inoculated with loop on the center of the agar plates once they solidified. Then after the plates were incubated for 48 hours at 37^{o}C they were flooded with Gram's Iodine for visualization of clearing zone (Vashist *et al.,* 2013).

3.6.2. Cellulase test

The extracellular cellulase production ability of the bacterial isolates was tested on carboxymethylcellulose (CMC) agar plates. For the preparation of the CMC agar medium, NA (Glucose 10 g/L; NaCl 5 g/L; Peptone 10 g/L; Yeast extract 10 g/L; Agar 18 g/L at pH 7.0-7.2) was supplemented with 1 g/L (w/v) of carboxymethylcellulose (CMC). The NA and CMC were autoclaved separately and mixed together post sterilization. The CMC medium was poured into sterile Petri dishes and fresh bacterial colonies were inoculated with loop at the center of the agar plates once they solidified. Then, after the plates were incubated for 48 hours at 37^{o}C the plates were flooded with 1% (w/v) Congo red solution for the visualization of clearing zones (Nxumalo *et al.*, 2020).

3.6.3. Pectinase test

The pectinase producing ability of the bacterial isolates was evaluated on Pectinase screening agar medium (PSAM) ($FeSO_4.7H_2O$, 0.01 g/L; K_2HPO_4, 1.8 g/L; $MgSO_4.7H_2O$, 0.2 g/L; NaCl, 0.1 g/L; NH_4Cl, 4.0 g/L; Pectin 10 g/L and Agar, 15 g/l) agar plate. The medium was prepared and autoclaved at a temperature 121^{o}C for 15 minutes. After autoclaving the medium was poured into sterile Petri dishes and solidified. Fresh colonies of the isolated bacteria were streaked at the center of the agar plates. Finally, the plates were incubated at 30^{o}C for four

days and at the end of the incubation period they were flooded with 50mM Potassium iodide-Iodine solution to visualize the clearing zones (Oumer & Dawit, 2018).

3.6.4. Protease test

For the evaluation of protease producing ability of the isolated bacteria, Skim milk agar plates were prepared. Briefly NA (g/L) (Glucose, 10 g/L; NaCl, 5 g/L; Peptone, 10 g/L; Yeast extract, 10 g/L; Agar, 18 g/L and pH 7.0-7.2) supplemented with 1% skim milk (w/v) was prepared with the addition of 0.0015% (w/v) Bromocresol green (BCG) dye for better visualization of the clearing zones. The medium was autoclaved with a temperature of 121^{o}C for 15min. After autoclaving, the medium was poured into sterile petri dishes and solidified. After that the plates were inoculated with the isolated bacteria and incubated at 37^{o}C for 48 h. A zone of proteolysis was detected on the agar plates after two days of incubation (Vijayaraghavan *et al.*, 2013).

3.7. Characterization of Plant growth promoting abilities of the isolated endophytic bacteria

3.7.1. Phosphate solubilization test

The phosphate solubilizing ability of the isolated endophytic bacteria was tested by spot inoculating on Pikovskaya medium ($Ca_3(PO)_4$, 5 g/L; $FeSO_4.7H_2O$, 0.002 g/L; Glucose, 10 g/L; $MgSO_4.7H_2O$, 0.1 g/L; NaCl, 0.2 g/L; $(NH_4)_2SO_4$, 0.5 g/L; KCl, 0.2 g/L; Yeast extract, 0.5 g/L; $MnSO_4.H_2O$, 0.002 g/L and Agar, 15 g/L at pH 7). The medium was dissolved by boiling and shaking gently, and then was sterilized by autoclaving at 121^{o}C for 15 min. The medium was poured into sterile Petri dishes and after solidifying was inoculated by fresh colonies of the isolated endophytic bacteria. The plates were incubated at 30^{o}C for 7 days and zone of phosphate solubilization was observed (Pande *et al.*, 2017).

3.7.2. Zinc solubilization test

The zinc solubilization ability of the endophytic bacteria was examined on minimal salts media. The minimal salts medium (Dextrose, 10 g/L; KCl, 0.2 g/L; K_2HPO_4, 0.1 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $(NH_4)_2SO_4$, 1 g/L and Agar 15 g/L at pH 7-7.2) was prepared and

insoluble zinc 1 g/L was added in the form of zinc oxide (ZnO). The medium was boiled to dissolve and was sterilized at 121^{o}C for 15 min. The sterilized medium was poured into sterile petri dishes and after solidifying the plates were spot inoculated with fresh colonies of the isolated bacterial colonies. The plates were then incubated at 30^{o}C for 14 days and examined for halo zones after incubation (Kamran *et al.*, 2017).

3.7.3. ACC deaminase production

The ACC deaminase production ability of the isolated bacteria was tested on Dworkin Foster (DF) salts minimal medium. DF salts minimal medium consisted of (Citric acid, 2 g/L; $(Cu)_2SO_4$, 50 µg/L; $FeSO_4.7H_2O$, 0.1 g/L; Gluconic acid, 2 g/L; Glucose, 2 g/L; H_3BO_3, 10 µg/L; KH_2PO_4, 4 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $MnSO_4$, 10 µg/L; MoO_3, 10 µg/L; Na_2HPO_4, 6 g/L and $ZnSO_4$, 70 µg/L) with addition of 2% (w/v) $(NH_4)_2SO_4$. The media was boiled to dissolve and autoclaved at 121^{o}C for 15 min. Then the medium was poured into sterile petri dishes and solidified. Then the agar plates were streaked with fresh bacterial colonies and incubated at 28^{o}C for 3 days. Bacterial growth on the media after 3 days was considered a positive result (Jasim *et al.*, 2013).

3.8. Identification of endophytic bacteria using MALDI-TOF MS

The isolated endophytic bacteria were investigated using the Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) which analyzes the protein or peptide constituents of a sample microorganism and enables identification of the microorganisms up to genus and species level. 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. The bacteria were grown overnight before analyzing. The extended direct transfer (eDT) procedure per the manufacturers instruction was used to analyze the bacteria (Daltonics, 2012). The extended direct transfer method uses 70% formic acid to lyse the cell membrane of the bacteria for simple extraction of the microbial proteins or peptides and makes for easier characterization of the samples. The target plate which contains 94 wells was cleaned thoroughly and well dried before starting the procedure. Then a distinct colony from the overnight grown bacteria was smeared directly on the wells as a thin film and left to dry. Then the spot was overlaid with 1µl of 70% (v/v) formic acid and

allowed to dry at room temperature. Then immediately the spot was overlaid with 1 µl of α-cyano-4-hydroxycinnamic acid (HCCA) solution and allowed to dry at room temperature. Then after the samples dried the target plate was placed inside the MALDI biotyper and analyzed. The samples are to be said adequately identified to the species level when the logarithmic score value (LSV) is greater than or equal to 1.9 (Toubal *et al.*, 2018).

3.9. Optimization of growth conditions for isolated endophytic bacteria

Cultural conditions were optimized for three selected endophytic bacterial isolates (CAMRI5, RPAAI8, and PGORI13) that were selected based on their high enzymatic and PGP activities. Temperature, pH, Carbon source and Nitrogen source preferences of the endophytic isolates were tested. The ability of agricultural waste materials to be used as cheap carbon sources for the three isolates was also investigated. All of the results are presented as Mean ± Standard Deviation (mean ± SD) of triplicates of OD_{600nm} and Biomass (BM) as cell dry weight (CDW g/l) measurements. One way analysis of variance (ANOVA) was applied to ascertain significant differences between the different growth conditions. Differences were considered to be significant when $P < 0.05$. Letters a, b and c are used to show statistically significant differences. If two points in a graph have the same letter it means that there is no significant difference between the two treatments. The method used to determine the optimum growth conditions was one factor at a time completely randomized method and taking triplicate measurements.

3.9.1. Optimization of growth temperature

To find out the optimum growth temperature of the isolated endophytic bacteria the isolates were all cultured on the same media, with the same volume of media and for the same amount of time. Luria Bertani (LB) broth (Glucose, 2 g/L; NaCl, 5 g/L; Tryptone, 10 g/L and Yeast extract, 10 g/L at pH 7.00) was the medium of choice for temperature optimization. A 250 ml of volume for each isolate was prepared and 200 µl of 24 hours old fresh inoculum of the isolated bacteria were incubated on 6 different temperatures of 25, 30, 35, 37, 40, and 45^{o}C for the same amount of time, 48 hours with 130 rpm. After 48 hours the optical density and bio mass of the culture was taken in triplicates and recorded. The generated data was analyzed using the Microsoft office excel software.

3.9.2. Optimization of pH

To find out the optimum pH range suitable for the growth of the isolated endophytic bacteria, the isolates were all cultured on the same medium, with the same volume of medium for the duration. LB broth (Glucose, 2 g/L; NaCl, 5 g/L; Tryptone, 10 g/L and Yeast extract, 10 g/L at pH 7.00) was the medium of choice for pH optimization and 250 ml of volume for each isolate was prepared. A 200 µl of 24 hours old fresh inoculum of each of the isolated bacteria was inoculated into 4 different flasks with 4 pH ranges of 6.5, 7, 7.5, and 8 and were incubated for 48 hours with 130 rpm. After 48 hours the optical density and bio mass of the culture were taken in triplicates and recorded. The generated data was analyzed using the Microsoft office excel software.

3.9.3. Suitable Nitrogen source optimization

To find out the most suitable nitrogen source for the growth of the isolated bacteria four nitrogen sources, two organic and two inorganic nitrogen sources were used. The organic nitrogen sources used were Yeast Extract and Peptone while the inorganic nitrogen sources were $(NH_4)_2SO_4$ and KNO_3. Minimal salts medium ($CaCl_2.H_2O$, 0.02 g/L; $FeSO_4.7H_2O$, 0.03 g/L; Glucose 2 g/L; K_2HPO_4, 1.73 g/L; KH_2PO_4, 0.68 g/L; $MgSO_4.7H_2O$, 0.1 g/L; and NaCl, 4 g/L) was prepared and the four different nitrogen sources were added 0.6% (w/v) to the media. After autoclaving, the media were transferred into 250 ml Erlenmeyer flasks and inoculated with 200 µl of 24 hours old fresh inoculum of the isolated bacteria. Then the flasks were incubated for 48 hours with agitation of 130 rpm. After 48 hours the optical density and biomass of the culture were taken in triplicates and recorded. The generated data was analyzed using Microsoft office excel software.

3.9.4. Suitable carbon source optimization

To find out the most suitable carbon source for the growth of the isolated bacteria four carbon containing organic compounds were used. Minimal salts medium (K_2HPO_4, 1.73 g/L; KH_2PO_4, 0.68 g/L; $MgSO_4.7H_2O$, 0.1 g/L; NaCl, 4 g/L; $FeSO_4.7H_2O$, 0.03 g/L; NH_4NO_3, 1 g/L and $CaCl_2.H_2O$, 0.02 g/L) was prepared. The carbon sources Glucose, Fructose, Sucrose and Mannitol were added 2% (w/v) each to the prepared minimal salts medium. After autoclaving

250 ml medium was poured into 500 ml Erlenmeyer flasks and 200 µl of 24 hours old fresh inoculum of the isolated bacteria were inoculated into the medium. Then the flasks were incubated for 48 hours under agitation of 130 rpm. After 48 hours the optical density and bio mass of the cultured media were taken in triplicates and recorded. The generated data was analyzed using the Microsoft office excel software.

3.9.5. Agro-waste as carbon source

To find out the preference of carbon sources and the ability of the isolated bacteria to utilize cheap agro-wastes as non-commercial carbon sources Teff straw, Corn cob and Vegetable waste were used as substitutes of commercial carbon sources. The carbon source from the three materials was prepared by drying and grinding the materials into fine powder using a grinder. Then the ground waste materials were sieved by 355 µm size sieves. Then following the procedure of Patil and Salunkhe (2010) 100 g of each agro-waste materials were soaked in 200 ml of hot distilled water (95^{o}C) for 2 hours under agitation of 200 rpm. After that the extract was filtered with muslin cloth and centrifuged at 3000 rpm for 5 min to get rid of any suspensions. Then 20 ml l^{-1} of the extract was added as a carbon source onto a carbon free liquid media (K_2HPO_4, 0.3 g/L; KH_2PO_4, 0.3 g/L; $MgSO_4.7H_2O$, 0.3 g/L; NaCl, 0.05 g/L; $FeCl_3.6H_2O$, 0.006 g/L; $CaCl_2.H_2O$, 0.05 g/L and Yeast extract, 3 g/L at pH 7.00). After autoclaving the media were transferred into 250 ml Erlenmeyer flasks and inoculated with 200 µl of 24 hours old fresh inoculum of the isolated bacteria (Patil & Salunkhe, 2010). Then the inoculums were incubated for 48 hours with agitation of 130 rpm. After 48 hours the optical density and the biomass of the cultured media were taken in triplicates and recorded.

3.10. Extraction of secondary metabolites from the isolated bacteria for antimicrobial assay

Simple solvent extraction of the secondary metabolites from selected isolates was carried out in order to analyze the extract's potential as a source of anti-microbial compounds. Following the methods of Syed and Rekha, (2019), the isolate selected for extraction was cultured in LB broth on a shaker incubator for 5 days at 130rpm at 30^{o}C. Then the culture was harvested and centrifuged at 4000 rpm for 30 min to remove the bacterial cells. The supernatant was collected and filtered using Whatman's No.1 filter paper. Then Ethyl acetate was added 1:1

ratio into the supernatant and was shaken vigorously for one hour before being left overnight at 4^{o}C. Then separatory funnel was used to separate the solvent phase containing the extracted secondary metabolites. After separation, the extracted secondary metabolites were concentrated using rotary evaporator at 40^{o}C and rpm of 90. The crude extracts were then re-suspended using methanol. The extracts were then stored at 4^{o}C until further use (Nxumalo *et al.*, 2020).

3.11. Evaluation of antimicrobial activities of ethyl acetate extract

The crude extract was prepared into two concentrations (0.8 g/ml and 0.4 g/ml) for the antibacterial activity analysis. Agar well diffusion method was used to evaluate the antibacterial activity of the extracted secondary metabolites from the isolates. Four bacterial strains *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and the endophytic isolate from which the metabolite was extracted from (RPAAI8 *Enterobacter cloacae)* were spread on Mueller-Hinton agar plates and 6mm wells were made using sterile cork borer. 50µl of the crude extracts and both the positive and negative controls were added in the wells. Ciprofloxacin 20 µg/ml was used as positive control and methanol was used as a negative control. The plates were incubated at 37^{o}C for 24 hrs. After incubation the inhibition zones were measured using a ruler and recorded (Nxumalo *et al.*, 2020).

3.12. Identification of isolated endophytic bacteria by 16S rRNA sequencing

The molecular identification of the isolated bacteria was also performed. Based on better performance such as collected 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 RPAAI-8 isolate was selected and sent for 16S rRNA gene sequence for identification. The genetic materials of the bacterial isolates were 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 isolates of endophytic bacteria were 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 hand book, 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.

CHAPTER FOUR

4. Results and Discussions

4.1. Isolation of endophytic bacteria from medicinal plant samples

After the plant parts were surface sterilized and incubated on NA for 10-15 days the colonies of endophytic bacteria emerged on the NA plates except for the negative control plates, which indicates that the grown bacteria were in fact endophytic (Figure 4). Several types of colonies were observed on the plates with different color, margin or shapes (Figure 4a and b). Tsegaye *et al.*, (2018) employed the same method to ensure the sterility of the medium and isolate pure endophytic bacterial cells from their natural habitat. The colony morphology of the isolated endophytic bacteria included various shapes, with flat and raised elevation, margin and different colors (Boone & Castenholz, 2001).

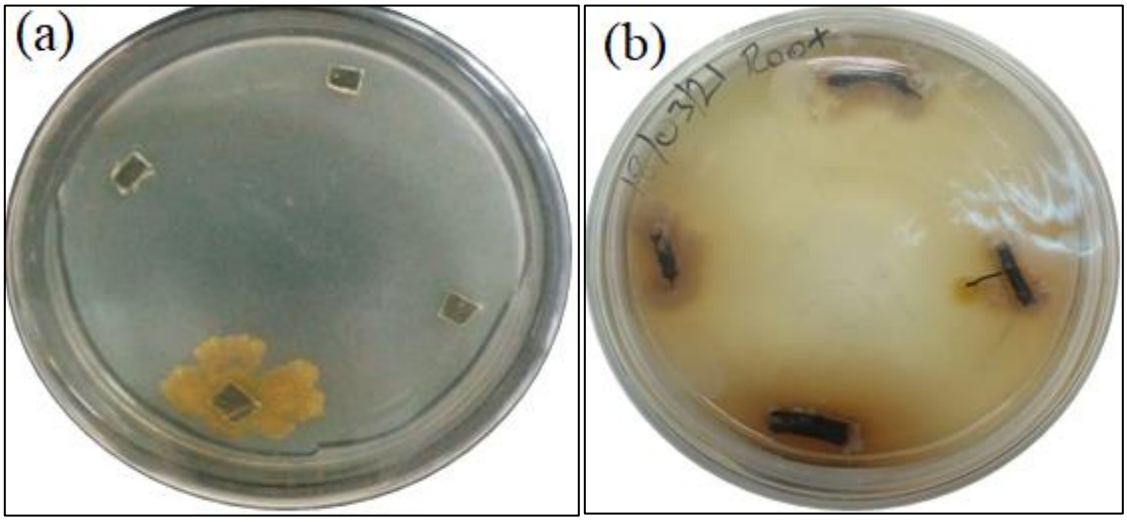


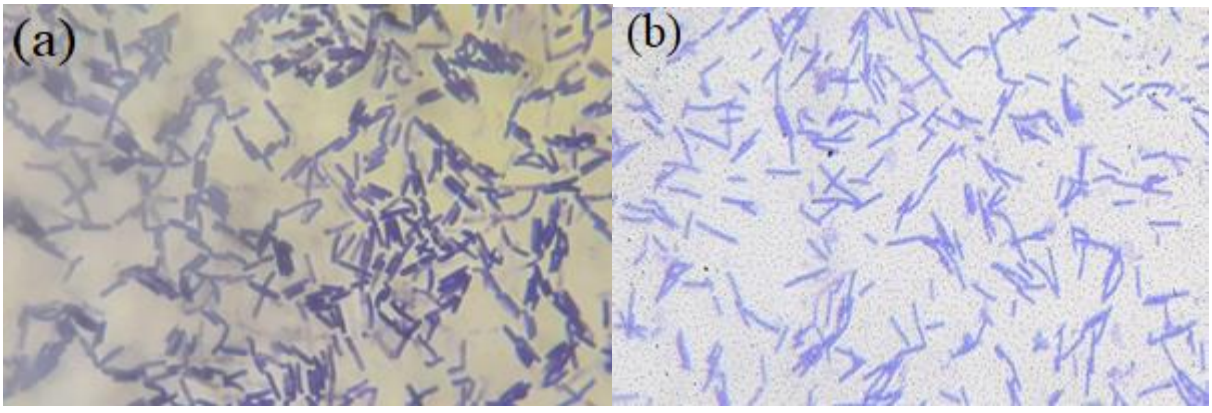
Figure 4: (a) Endophytic bacteria emerge from leaf parts of *Ocimum lamiifolium*. (b) Endophytic bacteria emerge from root parts of *Artemisia annua*

4.2. Morphological characterization of the endophytic bacterial isolates

Pure cultures of endophytic bacterial isolates with distinct morphological and biochemical features were obtained in this study (Figure 5). From the total of 45 bacteria isolated from the three medicinal plants,, 26 (58%) were gram positive and 19 (42%) were gram negative. The

endospore forming characteristics of the isolated bacteria was also recorded and it was found that 23 (51%) were endospore forming bacteria, whereas 22 (49%) were non-endospore formers. In line with study Ntabo *et al.* (2018), have observed more number of gram positive endophytic bacteria isolated from mangrove plants and higher number of endospore formers than non-formers.

As indicated in Figure 5a-f, different bacterial cells with distinct feature were obtained from *Artemisia annua* (Chikugn); *Moringa oleifera* (Shiferaw) and *Ocimum lamiifolium* (Damakese). The types of cells frequently isolated were those isolated from the root parts of *Ocimum lamiifolium* and they were identified to be *Bacillus cereus* (Figure 5a), then followed by *Bacillus subtilis* (Figure 5b), *Citrobacter freundii* (Figure 5c), *Pseudomonas monteilii* (Figure 5d), *Enterococcus faecium* (Figure 5e), and *Enterobacter cloacae* (Figure 5f). As indicated in Figure 5g, the isolate obtained from *Ocimum lamiifolium* (Damakese) found to be endospore former and identified as *Bacillus cereus* using MALDI-TOF analysis. Certain endophytic bacterial isolates were isolated from germinated maize kernels and characterized using 16S rRNA (Tomaz *et al.*, 2007). These isolates were found to be including species of *Bacillus* sp., *Frigoribacterium* sp., *Microbacterium* sp., *Paenibacillus* sp., *Pantoea* sp., and *Sphinomonas* sp (Tomaz et al., 2007). In other study, the 16S rRNA sequencing results showed that endophytic bacterial isolates such as *Bacillus sp., Corynebacterium sp., Chryseobacterium sp., Curtobacterium sp.* and *Staphylococcus sp.* were isolated from Rambutan Fruits (*Nephelium lappaceum* L.) Cultivar Binjai and predicted to be employed as a plant growth-promoting bacterial cells (Suhandono *et al.*, 2016).



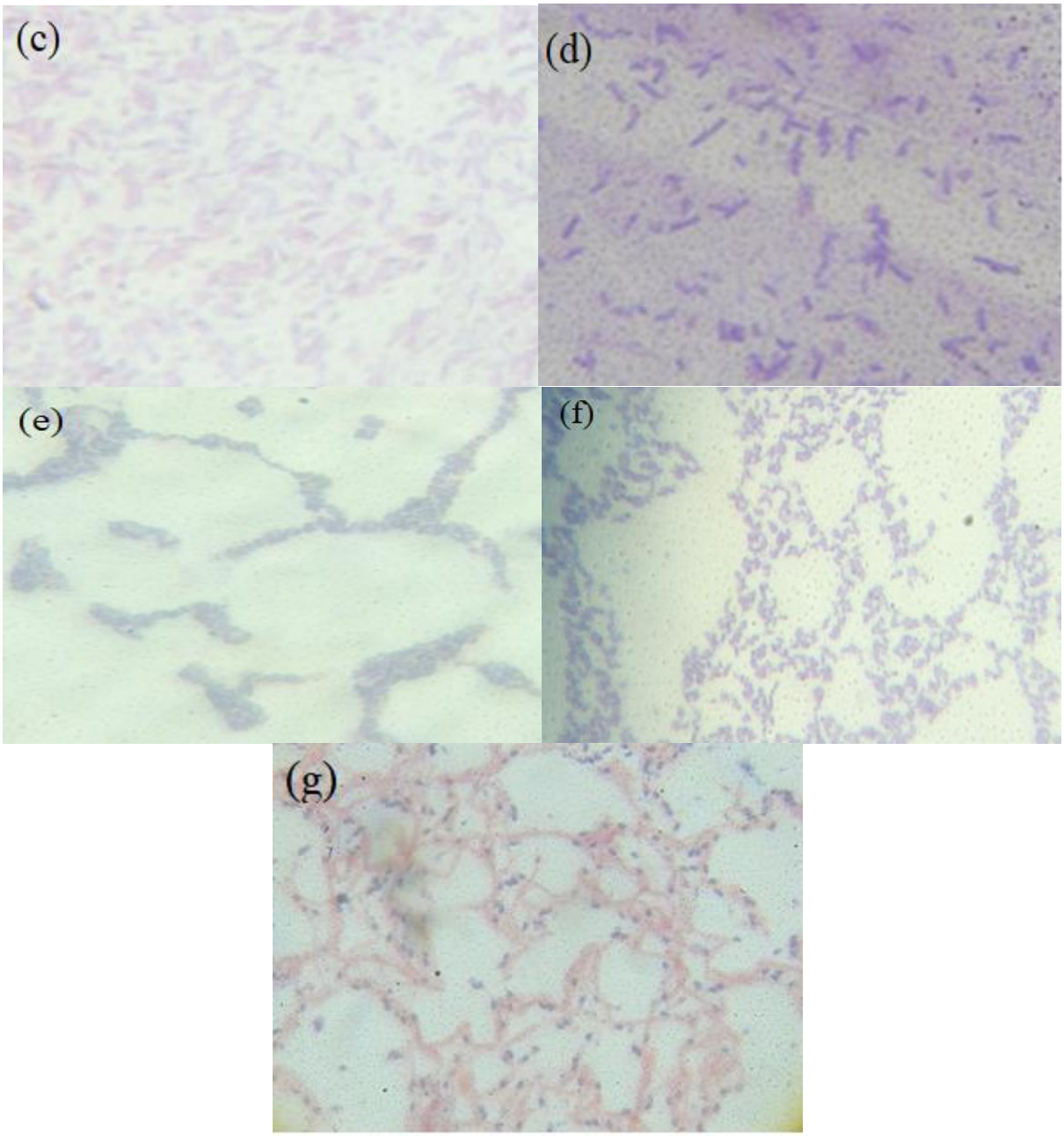


Figure 5: Gram's staining and endospore staining of isolated bacteria

(a) Isolate obtained from root part of *Ocimum* and found to be *Bacillus cereus* with gram positive long rods after being characterized using MALDI-TOF. (b) Gram positive rod shaped isolate obtained from stem part of *Ocimum* and recognized to be *Bacillus subtilis* after it was characterized by using MALDI-TOF. (c) Gram negative and rod shaped isolate found to be *Citrobacter freundii* that is isolated from root part of *Moringa*. (d) Long rod shaped gram

negative isolate obtained from root part of *Ocimum* and found to be *Pseudomonas monteilii* after MALDI-TOF characterization (e) Gram positive cocci isolate that is recoginzed as *Enterococcus faecium* by MALDI-TOF and was isolated from root part of *Artemisia*. (f) Bacterial isolate that is acquired from root part of *Artemisia* and found to be *Enterobacter cloacae* with short rod cellular morphology (g) Endospore forming bacteria that was identified to be Bacillus cereus by MALDI-TOF analysis.

The results observed for the newly obtained isolates from the different plant parts (leaves, stems and roots) of the medicinal plants (*Artemisia annua*, *Moringa oleifera* and *Ocimum lamiifolium*) suggest the use of these microbial inputs for plant promotion. This is because these bacterial isolates may supply certain nutrients for plant growth. They may also be able to enhance plant hormone production. In line with our suggestion, it was reported that endophytic bacterial cells when used as bioinoculants promote plant growth and yield, suppress certain pathogenic bacterial cells, can be used to remove certain chemical contaminants, as well as for phosphate and zinc solubilization, and contribute for nitrogen assimilation to diversity of plants (Rosenblueth & Martínez-Romero, 2006).

4.3. Biochemical characterization of isolated endophytic bacteria

Out of the 45 isolates 21 were found to be *Bacillus*, 16 isolates were *Enterobacter*. In the recent study 3 isolates were found to be *Enterococcus*, 2 were *Citrobacter* genera. *Pseudomonas*, (1) *Lysinibacillus* (1) and *Paenibacillus* (1) genera were also isolated from the plants. The full biochemical tests and the isolates identified are found in the appendices section.

When using ABIS for identification purposes the number and types of tests are not limited by a pattern and may vary according to laboratory availabilities (Sorescu and Stoica 2021). But in order for the ABIS to identify your isolates a minimum of 8 tests are required. In agreement with the current study Hussain et al. (2013), reported that ABIS online bacterial identification tool could be used for identification of bacterial strains isolated from water samples. Biochemical tests performed to identify bacterial isolates from waste water effluents failed to reach up-to the species level for identification of a certain genus *Raoutella sp* (Rave *et al.* 2018). This particular mishap shows one of the shortcomings of biochemical identification of

environmental bacteria and where it arises from. The usage of biochemical tests to identify environmental microorganisms is becoming obsolete, mainly due to the emergence of new technologies and tools that are time saving, accurate, easy, and not laborious and with the ability to produce highly reproducible results.

The decisive difficulty of using phenotypic characteristic to identify bacterial species is that the identification relies on the occurrence of a divergence or convergence (Siqueira and Ro 2005). Divergence occurs in some species lineages, genetically identical, but with time given has evolved to produce different phenotypic characteristics. Convergences occur in different species lineages, genetically different, but with time have evolved to produce a similar phenotypic behavior. In both cases the phenotypic identification will result in a wrong identification. All of the above mentioned cons stress the importance of development and application of better tools of identification compatible with environmental microbiological needs (Siqueira and Ro 2005).

4.4. Enzymatic activities for newly isolated endophytic bacterial cells

In this study the isolates showed high levels of enzymatic activities. This made it evident that endophytic bacteria are equipped with various enzymes (Figure 7a, b, c and d). The isolates which showed higher clearing zones were identified by measuring the zone of clearance around the colonies and they were purified and maintained for further characterization of their plant growth promoting abilities and identification by 16S rRNA. Four enzymatic tests were carried out in order to determine the enzymatic activities of the isolated bacteria.

For Amylase (starch hydrolysis) test (Figure 7c), the formation of clearing zones around bacterial colonies was examined on starch agar media. In this study 11 (24.4%) of the bacterial isolates were found to be extracellular amylase producers (Figure 7c). These amylase producers were recognized to be *Bacillus subtilis* (BAARI1, BAARI4), *Bacillus cereus* (BGORI9, BAMRI7) *Enterobacter asburiae* (EAARI3), *Enterobacter cloacae* (EAASI3, EEARI2, EAARI6, RPAAI8), *Enterococcus faecium* (EAMSI3), and *Enterobacter ludwigii* (EAARI5).

Cellulase test was carried out on CMC agar media and the formation of clearing zones around the colonies was observed. In this study 23 (51.11%) of the isolated bacteria were able to

produce cellulose degrading enzyme (Figure 7d). Among the 23 cellulase producing bacteria *Bacillus* and *Enterobacter* were the most frequently isolated genera each with 10 cellulase producing bacteria to their respective genera (Appendix 7). Seven strains for *Bacillus cereus* (BGOSI7, BGORI9, BAASI5, BAMSI4, BAMRI4, BAMRI6, BAMRI7) were the highest number of cellulase producers, with 5 *Enterobacter cloacae* (EGOSI8, EGORI12, EAARI2, EAARI6, RPAAI8), 3 *Bacillus subtilis* (BGORI4, BAARI1, BAARI4), 2 *Enterobacter kobei* (EAARI7, EAMRI2), 2 *Enterobacter ludwigii* (EGOSI4, EAARI5), 1 *Enterobacter asburiae* (EAARI3), 1 *Enterococcus faecium* (EAMSI3), 1 *Citrobacter freundii* (CAMRI5) and 1 *Pseudomonas monteilii* (PGORI13) joining the group.

A total of 31 (68.8%) of the isolated bacteria were found to be pectinase producers (Figure 7a). Largest pectinase producers from the isolates were EAARI3 *Enterobacter asburiae* strain and EAARI5 *Enterobacter ludwigii* strain showing maximum zone of clearance with 28mm and clearing index (CI) of 6.6.

Of the isolated bacteria 19 (42.2%) were protease enzyme producers (Figure 7b). The largest protease producer from the isolates was BGORI8 *Bacillus cereus* strain showing maximum zone of clearance of 23mm and CI of 4.83 (Figure 7b).

The clearing index (CI) of the isolates as confirmed by the clearing zone around the colonies was calculated as the ratio of the total diameter (colony + halo zone) to colony diameter. CI= Colony diameter + Halo zone diameter/ Colony diameter

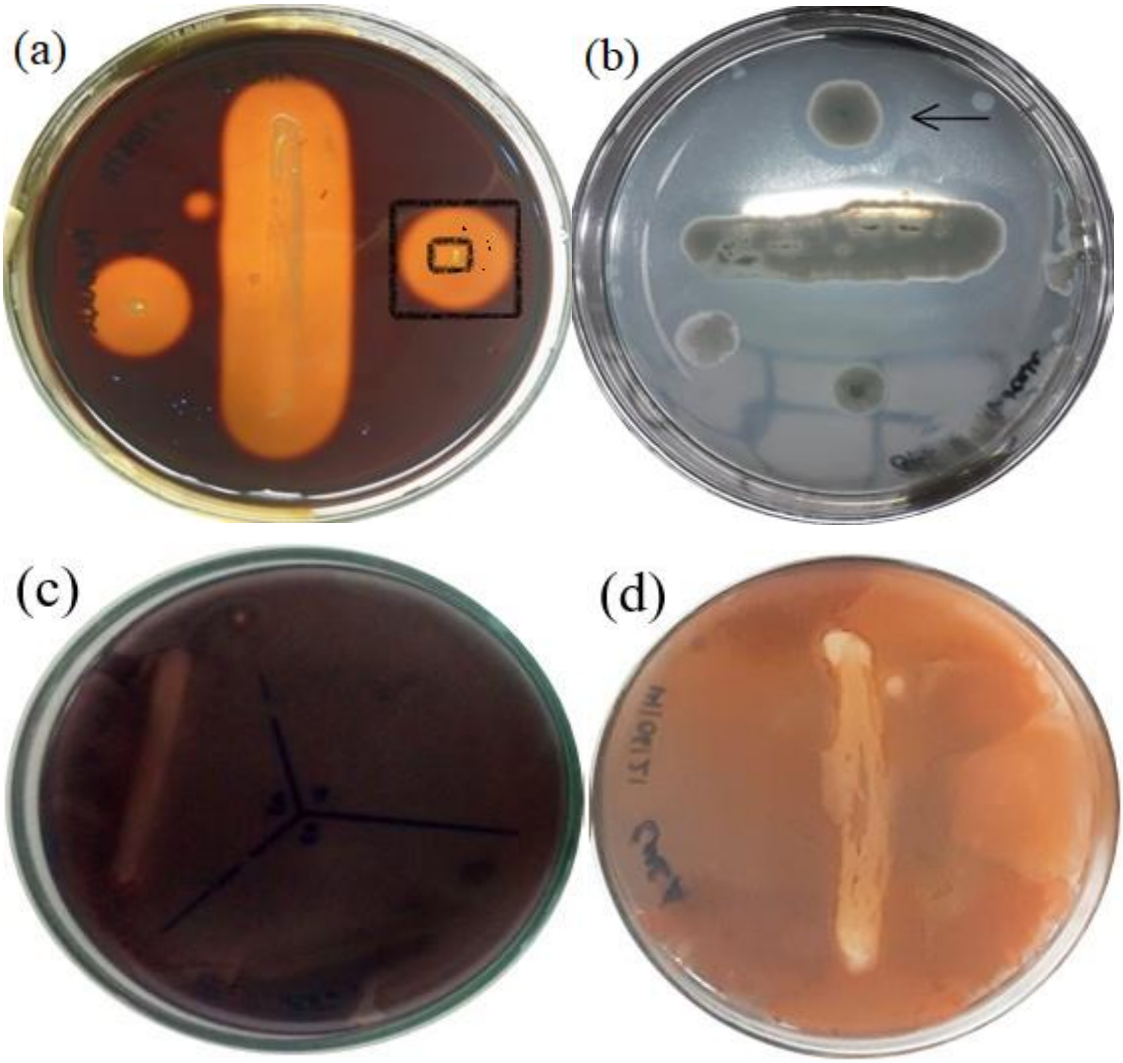


Figure 6: Enzyme production by isolated endophytic bacterial species

(a) Pectinase producing bacetria on Pectin agar plate for isolate BAMRI3 that is isolated from root part of *Moringa*. (b) Isolate PGORI13 that is obtained from root part of *Ocimum* and found to be positive for Protease when grown on skim milk agar plate. (c) Amylase producing ability of isolate RPAAI8 obtained from root part of *Artemisia* (d) Cellulase producing ability of isolate PGORI13 obtained from root part of *Ocimum*.

It was stated by Oumer and Dawit (2018) that the diameter of the clear zone is directly proportional to the enzymatic activity, hence the isolate with biggest clearing zone possess high enzymatic quantity and the isolate with the lowest clearing zone possess low enzymatic quantity. The clearing index of the isolates was calculated for pectinase and protease enzymes (Appendix 7 and 8).

It has been reported before about the enzymatic activities of endophytic bacterial isolates of Mangrove plants from Kenya (Ntabo et al. 2018). The isolates showed outstanding abilities of Pectinase, Protease, Amylase, Cellulase and Chitinase enzyme production with 69%, 95%, 88%, 92% and 92% of the isolates showing production of the enzymes, respectively. The isolates reported belonged to the genera *Bacillus, Streptomyces, Myroides,* and *Staphylococcus.* Meanwhile a research carried out in South Africa by Nxumalo *et al.* (2020) on the endophytic bacterial isolates of Anredera cordifolia CIX1 overall showed very good production of amylase and cellulase enzymes while also showing mediocre production of proteinase and esterase enzymes.

4.5. Plant growth promoting characteristics of the isolated endophytic bacteria

In this study the direct plant growth promoting ability of the isolated bacteria was evaluated by testing the phosphate and zinc solubilizing ability of the isolates. The ability of the isolates to synthesize the important stress reducing ACC-deaminase enzyme was also tested.

4.5.1. Phosphate solubilizing ability of the endophytic bacteria

The results obtained from this study showed that out of the 45 endophytic bacterial isolates 27 (60%) of them were able to solubilize the insoluble phosphate added in Pikovskaya's agar in the form of tri-calcium phosphate (Figure 7). In line with this study Passari *et al.*, (2016) have reported that out of 52 isolates, 44 (86%) isolates were found to be positive for phosphate solubilization by forming a clear halo zone on Pikovskaya's medium that was supplied with tri-calcium phosphate. The isolate PGORI13 (*Pseudomonas monteilli*) strain showed the highest clearing zone (24 mm) with 5.8 solubilization index. Isolate BGOSI7 and BAMLI1 which were identified as *Bacillus cereus* strains showed the lowest clearing zone with 7mm and solubilization index of 2.4. Passari *et al.* (2016) have reported in their study that endophytic bacterial isolate BPSAC 6 strain was found to be phosphate solubilizing bacteria with very high phosphate solubilization index (8.4) which is much higher than our isolates solubilization index. Full data of the isolates with phosphate solubilizing ability is included in the appendices section.

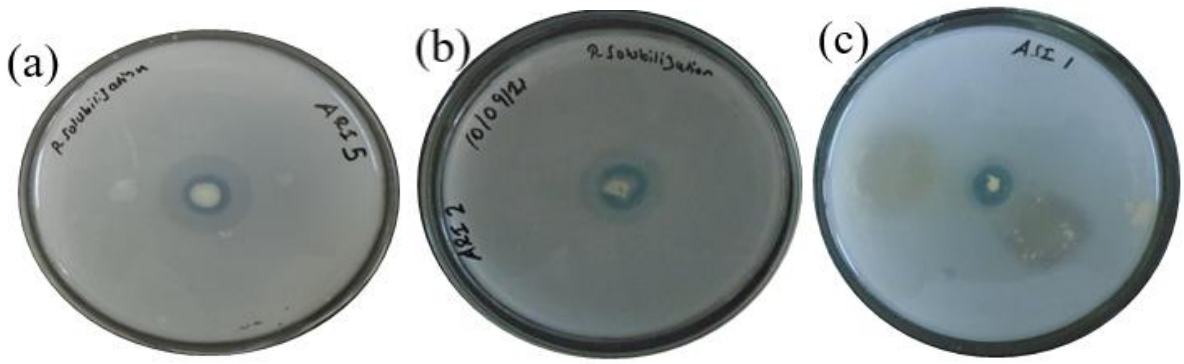


Figure 7: Phosphate solubilizing ability of isolated endophytic bacteria

(a) Phosphate solubilizing ability of *Citrobacter freundii* strain CAMRI5 isolated from root part of *Moringa oleifera*. Initially named as ARI-5 (b) Phosphate solubilizing ability of *Enterobacter kobei* strain EAMRI2 isolated from root part of *Moringa oleifera*. Initially named as ARI-2 (c) Phosphate solubilizing ability of *Bacillus cereus* strain BAMSI1 isolated from stem part of *Moringa oleifera*. Initially named as ASI-1

The high phosphate solubilizing ability of the *Pseudomonas* strain mentioned above is in agreement with a result reported by Tsegaye *et al.* (2020), who stated that *Pseudomonas stutzeri* primarily solubilized insoluble phosphate found in Pikovskaya's medium. The diversity of phosphate solubilizing bacteria is very wide, but Kalayu, (2019) says that among the various genera of phosphate solubilizing microorganisms *Bacillus*, *Pseudomonas* and *Rhizobium* are the most notable. The author further explained that microorganisms employ different mechanisms to solubilize phosphate such as lowering of soil pH which restrains the formation of calcium phosphates and rock phosphates which are insoluble forms of P found in soil, chelation of cations by producing organic and inorganic acids that compete for adsorption sites in the soil with phosphates and lastly but not least by mineralization of organic phosphates that are gained from animals and plant remains.

4.5.2. Zinc solubilizing ability of the endophytic bacteria

Of the isolated endophytic bacteria, 21 (46.6%) isolates were able to solubilize the insoluble zinc in the medium with halo zones around the colonies. The isolate RPAAI8 (*Enterobacter cloacae*) strain showed the highest halo zone (25 mm) with 5.16 solubilization index. EGOSI4

(*Enterobacter ludwigii*) and BGOSI6 (*Bacillus endophyticus*) isolates showed minimum zone of clearance (8 mm) with 2.33 solubilization index.

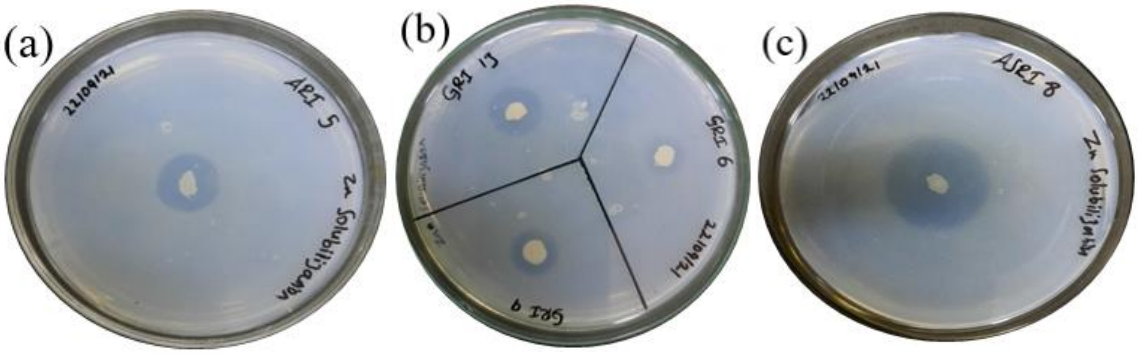


Figure 8: Zinc solubilizing ability of isolated endophytic bacteria

(a) Zn solubilizing ability of *Citrobacter freundii* strain isolate CAMRI-5 isolated from root part of *Moringa oleifera* (b) Zn solubilizing ability of Isolate PGORI-13 *Pseudomonas monteilli* strain, BGORI-9 *Bacillus cereus* strain and BGORI-6 *Bacillus endophyticus* strain. All were isolated from root part of *Ocimum lamiifolium*. (c) Zn solubilization due to inoculation by RPAAI8 isolate that was obtained from root part of *Artemisia annua* it was identified as *Enterobacter clocae* using MALDI-TOF.

Zinc solubilizing ability of soil dwelling bacteria have extensively been studied and reported by researchers all over the world but there are significantly much lower reporting's on the role of endophytic bacteria in zinc solubilization. Bacterial species such as *Acinetobacter, Bacillus, Burkholderia, Enterococcus, Pantoea agglomerans, Pseudomonas* and *Serratia* have previously been identified as zinc solubilizing species in the soil environment (Ashok *et al.*, 2019; Eshaghi *et al*., 2019; Kamran *et al.*, 2017; Rehman *et al.*, 2021; Poonam Sharma, 2014). Ashok *et al.* (2019) have explained that Zn-solubilizing bacteria employ different mechanisms to solubilize the insoluble zinc found in soil, some examples being chelation, release of organic acids, exchange reactions and acidification. Acidification is the most preferred method of zinc solubilizing bacteria (Ashok *et al.*, 2019). To our understanding the zinc solubilizing ability of endophytic origin bacterial species of *Enterobacter* has not been reported before in major studies. The recent 16S rRNA characterized isolate, *Enterobacter* might have been employed to alleviate zinc toxicity in *Artemisia annua* which is a similar prognosis for what

Islam and his co-authors reported for the role of *Proteus mirabilis* that prevents oxidative stress in maize (*Zea mays*) plants (Islam *et al.*, 2014).

4.5.3. ACC deaminase producing ability of the endophytic bacteria

The endophytic bacteria were also screened for the production of ACC deaminase on Dworkin-Foster (DF) salts minimal media amended with 0.2% ammonium sulphate. Here the growth of bacteria on the plates after incubation was considered as a positive result. 36 (80%) of the isolates were positive for ACC deaminase production. Out of the 36 ACC deaminase producing isolates 14 (39%) of isolates were *Bacillus cereus* strains, 9 (25%) were *Enterobacter cloacae*, 3 (8%) were *Bacillus subtilis*, 2 (5.5%) were *Enterobacter kobei*, 2 (5.5%) *Enterobacter asburiae*, 2 (5.5%) *Enterobacter ludwigii*, 2 (5.5%) *Citrobacter freundii*, 1 (3%) was *Enterococcus faecium* and 1 (3%) *Pseudomonas monteilli*. Aswathy *et al.* (2014), explains that ACC deaminase is an enzyme that is able to reduce the inhibitory effect the plant hormone ethylene has on plants during stressful conditions. The enzyme as the name suggests is capable of catalyzing the 1-aminocyclopropane-1-carboxylic acid (ACC) which is the immediate precursor of ethylene into α-ketobutyrate and ammonia and reduce the inhibitory effects of elevated levels of the plant hormone ethylene.

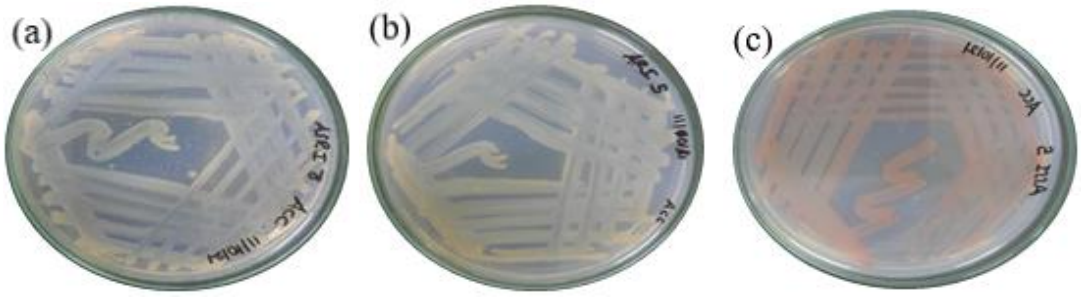


Figure 9: Growth on DF salts minimal media amended with Ammonium sulphate showing ACC deaminase producing ability of the isolate

(a) RPAAI8 Isolate (*Enterobacter cloacae*) strain that was isolated from root part of *Artemisia annua* (b) Isolate CAMRI5 *Citrobacter freundii* strain isolated from root part of *Moringa oleifera* (c) Isolate BAASI5 *Bacillus cereus* strain isolated from stem part of *Artemisia annua*

Aswathy *et al.* (2014), reported that out of four endophytic bacterial isolates of ginger (*Zingeber officinale*) *Pseudomonas* strain ZoB2 was able to produce ACC-deaminase as growth was observed on DF- salts minimal medium amended with 0.2% ammonium sulphate.

Jasim *et al.* (2013) had previously reported that they were able to isolate and characterize the plant growth promoting ability of endophytic bacteria from *Piper nigrum* and the isolates PnB10 and PnB11 which were identified as *Klebsiella sp.* and *Enterobacter sp.*, respectively were excellent growth promoters and were able to produce ACC, IAA and Phosphate solubilization.

4.6. Identification of Isolated endophytic bacteria by MALDI-TOF MS

The MALDI-TOF MS analysis of the isolated bacteria carried out at NAHDIC showed the diversity of the endophytic bacteria occupying the medicinal plants they were isolated from. 45 of the endophytic bacterial isolates were prepared for analysis by the extended direct transfer (eDT) method. The analysis was done twice and only five isolates were unable to be identified by the MALDI-TOF MS. As described by Neelja *et al.* (2015), MALDI based identifications are very accurate and misidentifying of microorganisms occur very rarely. They also explained that the inability of MALDI to identify some bacteria may well be because of the simple fact that the organism has not been included in the previous databases rather than a methodological error. *Bacillus spp*. dominated the quantity of isolates from the endosphere. From the 40 successfully identified isolates 20 (50%) were of genera *Bacillus*, 14 (35%) were *Enterobacter*, 3 (7.5%) were *Enterococcus*, 2 (5%) were *Citrobacter* and 1 (2.5%) was of *Pseudomonas* genera. *Enterobacter* species were the most diverse with 4 different species of *Enterobacter cloacae* (7), *Enterobacter asburiae* (3), *Enterobacter ludwigii* (2) and *Enterobacter kobei* (2). But the 22 *Bacillus* genera only belonged to the *Bacillus cereus* and *Bacillus subtilis* species. 3 isolates of *Enterococcus faecium*, 2 isolates of *Citrobacter freundii* and 1 *Pseudomonas monteilli* were found to be residing inside the medicinal plants.

Toubal *et al.* (2018), tried to isolate endophytic bacteria from Great Nettle (*Urtica dioica L.*) grown in Algeria and identify the isolates using MALDI-TOF. The report showed that they were able to isolate 57 endophytic bacterial isolates and identify 49 of them adequately by MALDI. The isolates from Great Nettle showed dominance by *Bacillaceae* represented essentially by four species namely *B. pumulis, B. anthracis, B. megaterium* and *B. cereus.* The rest of the isolates were species of the *Enterobacteriaceae* (*Escherichia coli, Pantoea agglomerans* and *Enterobacter amnigenus*), *Paenibacilliaceae* (*Paenibacillus lautus and*

Paenibacillus glucanolyticus) and less frequently families of *Staphylococcaceae* and *Enterococcaceae* were found with with *S. cohnii* and *E. faecium* respectively. Even though the gold standards in bacterial identification are 16S rRNA and 18S rRNA sequencing, the high cost involved for each identification and the requirement of trained personnel and powerful interpretation softwares makes them not suitable for casual application (Neelja *et al.*, 2015).

Table 1: MALDI-TOF MS analysis for isolated endophytic bacterial species these obtained from *Artemisia annua*

S.N.	Sample Id.	Species	LSV
1	BAALI1	*Bacillus subtilis*	1.9
2	EAALI2*	*Enterococcus faecium*	2.27
3	EAASI1	*Enterobacter asburiae*	2.07
4	EAASI4	*Enterobacter asburiae*	2.22
5	BAASI5	*Bacillus cereus*	1.73
6	BAARI1	*Bacillus subtilis*	2.16
7	EAARI2	*Enterobacter cloacae*	2.18
8	EAARI3	*Enterobacter asburiae*	2.09
9	BAARI4	*Bacillus subtilis*	2.11
10	EAARI5	*Enterobacter ludwigii*	1.79
11	EAARI6	*Enterobacter cloacae*	2.1
12	EAARI7	*Enterobacter kobei*	1.81
13	RPAAI8	*Enterobacter cloacae*	2.09

Table 2: MALDI-TOF MS analysis for isolated endophytic bacterial spcies these obtained from *Ocimum lamiifolium*

S.N.	Sample Id.	Species	LSV
1	BGOSI2	*Bacillus cereus*	1.94
2	BGOSI3	*Bacillus subtilis*	2.07
3	EGOSI4	*Enterobacter ludwigii*	2.06
4	BGOSI7	*Bacillus cereus*	2.12
5	EGOSI8	*Enterobacter cloacae*	1.73
6	BGORI4	*Bacillus subtilis*	2.21
7	BGORI7	*Bacillus cereus*	1.89
8	BGORI8	*Bacillus cereus*	1.81
9	BGORI9	*Bacillus cereus*	2.06
10	BGORI11	*Bacillus cereus*	2.12
11	EGORI12	*Enterobacter cloacae*	2.2

12	PGORI13	*Pseudomonas monteilli*	2.02

Table 3: MALDI-TOF MS analysis for isolated endophytic bacterial species these obtained from *Moringa oleifera*

S.N.	Sample Id.	Species	LSV
1	Sample Id.	Species	LSV
2	BAMLI1	*Bacillus cereus*	2.03
3	CAMLI2	*Citrobacter freundii*	2.18
4	BAMSI1	*Bacillus cereus*	1.82
5	EAMSI2	*Enterobacter cloacae*	2.26
6	EAMSI3*	*Enterococcus faecium*	2.03
7	BAMSI4	*Bacillus cereus*	2.03
8	EAMSI5	*Enterobacter cloacae*	2.22
9	EAMRI1*	*Enterococcus faecium*	2.26
10	EAMRI2	*Enterobacter kobei*	1.92
11	BAMRI3	*Bacillus subtilis*	1.81
12	BAMRI4	*Bacillus cereus*	1.81
13	CAMRI5	*Citrobacter freundii*	2.11
14	BAMRI6	*Bacillus cereus*	1.85
15	BAMRI7	*Bacillus cereus*	2.08
16	BAMRI8	*Bacillus cereus*	2.02

Key words: BGOSI/BGORI indicates *Bacillus* Ganda-gara Ocimum Stem Isolate, EGOSI/EGORI indicate *Enterobacter* Ganda-gara *Ocimum* Stem/Root Isolate, PGORI indicates *Pseudomonas* Ganda-gara *Ocimum* Root Isolate, BAMLI/BAMSI/BAMRI indicate *Bacillus* ASTU *Moringa* Leaf/Stem/Root Isolate, CAMLI/CAMRI indicate *Citrobacter* ASTU *Moringa* Leaf/Root Isolate, EAMSI/EAMRI indicate *Enterobacter* ASTU *Moringa* Stem/Root Isolate, BAALI/BAASI/BAARI indicate *Bacillus* ASTU *Artemisia* Leaf/Stem/Root Isolate, EAALI/EAASI/EAARI indicate *Enterobacter* ASTU *Artemisia* Leaf/Stem/Root Isolate, The * sign indicates *Enterococcus* genus, LSV stands for Logarithmic Score Value

4.7. Optimization of Growth conditions for the isolated bacteria

4.7.1. Optimization of pH

As shown in Figure 17a, out of the three isolates RPAAI8 isolate showed the highest (1.818667±0.010693) OD_{600nm} measurement at pH 6.5. However, the maximum CDW (0.0167±0.0011 g/l) was recovered from PGORI13 isolate that was obtained from root part of

Ocimum lamiifolium at pH 7.5 (Figure 17a). Isolate RPAAI8 similar to the OD_{600nm} measurement showed the highest CDW (g/l) production at pH 6.5 with 0.005367±0.001026 g/ml when compared with the other pH ranges. Out of the pH ranges, the highest OD_{600nm} measurement for PGORI13 was also recorded at pH 7.5 with 1.685333±0.011015 (Figure 17a). The minimum growth by OD_{600nm} as well as CDW (g/l) measurement was taken for CAMRI5 at pH 7.5 with 1.206337±0.000574 and 0.0008±0.000265 g/ml, respectively (Figure 17a). The highest growth seen for CAMRI5 was at pH 8 with OD_{600nm} and CDW (g/l) measurement of 1.522333±0.004726 and 0.005933±0.002401 g/ml, respectively. As indicated in Figure 17a, the variations were observed ($p<0.05$) for the recently isolated endophytic bacterial cells in terms of CDW (g/l).

Chi *et al.* (2018), reported that isolate LX3 of *Enterobacter cloacae* strain was able to grow in the pH range of 5-9 but optimum pH for growth of the strain was at pH7 which in our case is reduced by 0.5 and the optimum pH for the *Enterobacter cloacae* strain of the endophytic origin was recorded at 6.5. Maalej *et al.* (2014) have reported that for EPS producing *Pseudomonas sp*. optimum pH for growth ranges from 7-8 with high cell density at pH 7.3. Wang *et al.* (2021) have reported that *Citrobacter freundii* has pH tolerance ranging from pH 5-10 and was strongly inhibited at pH 4 but did not survive at pH 3. Bokhari *et al.* (2019) previously reported optimum growth pH for *Citrobacter freundii* strain IG1 was 7.

4.7.2. Optimization of growth Temperature

It was found out that all of the selected isolates growth and CDW (g/l) production was at its peak at 30^{o}C (Figure 10). At 25^{o}C the second highest growth and CDW (g/l) production by the selected isolates was observed. The growth and CDW (g/l) production of the isolates was at its lowest at 45^{o}C. Maximum OD_{600nm} measurement as well as CDW (g/l) measurement from the three isolates was obtained for PGORI13 at 30^{o}C with 1.645±0.053777 and 0.0068±0.000361 g/ml, respectively (Figure 10b). The minimum growth from the three isolates by OD_{600nm} measurement as well as CDW (g/l) measurement was obtained at 45^{o}C for PGORI13 with 0.0961±0.002707 and 0.0008±0.0001 g/ml, respectively. All of the optimization experiments following this one were carried out at the optimal growth temperature of the isolates which is 30^{o}C.

Sawai *et al.* (1977) stated that the optimal growth temperature of *Citrobacter freundii* to be between 30^{o}C and 37^{o}C. Uddin *et al.* (2008) have reported that the bacterial fish pathogen *Citrobacter freundii* showed its peak growth at 36.1±0.4^{o}C, meanwhile, in this experiment peak OD_{600nm} and CDW (g/l) measurement for endophytic isolate CAMRI5 *Citrobacter freundii* strain was at 30^{o}C followed by 35^{o}C. The authors have also reported that peak growth temperature of two strains of *Pseudomonad sp.* was at 24.09±0.1 and 29.2±0.5^{o}C, respectively. And specifically, *pseudomonas fluorescens* whose peak growth occurred at 29.9±0.2 and 28.5±0.3ºC is closer to the endophytic *Pseudomonas monteilii* strain isolate PGORI13 with peak growth temperature at 30^{o}C. Similar to these observations was the observation made by Maalej *et al.* (2014), when working on the exo-polysaccharide (EPS) production of *Pseudomonas stutzeri* AS22. The maximum growth of *Pseudomonas stutzeri* AS22 as well as EPS production was achieved at 30^{o}C and elevated temperature of 37^{o}C reduced the cellular growth and EPS production by nearly half. Chi *et al.* (2018), reported that *Enterobacter* Isolate LX3 exhibited the ability to grow at temperature ranges of 20^{o}C-45^{o}C but the maximum growth temperature of the *E. cloacae* strain was reported to be 30^{o}C, which is the same as the recent isolate *Enterobacter cloacae* (RPAAI8) that was obtained from *Artemisia annua*.

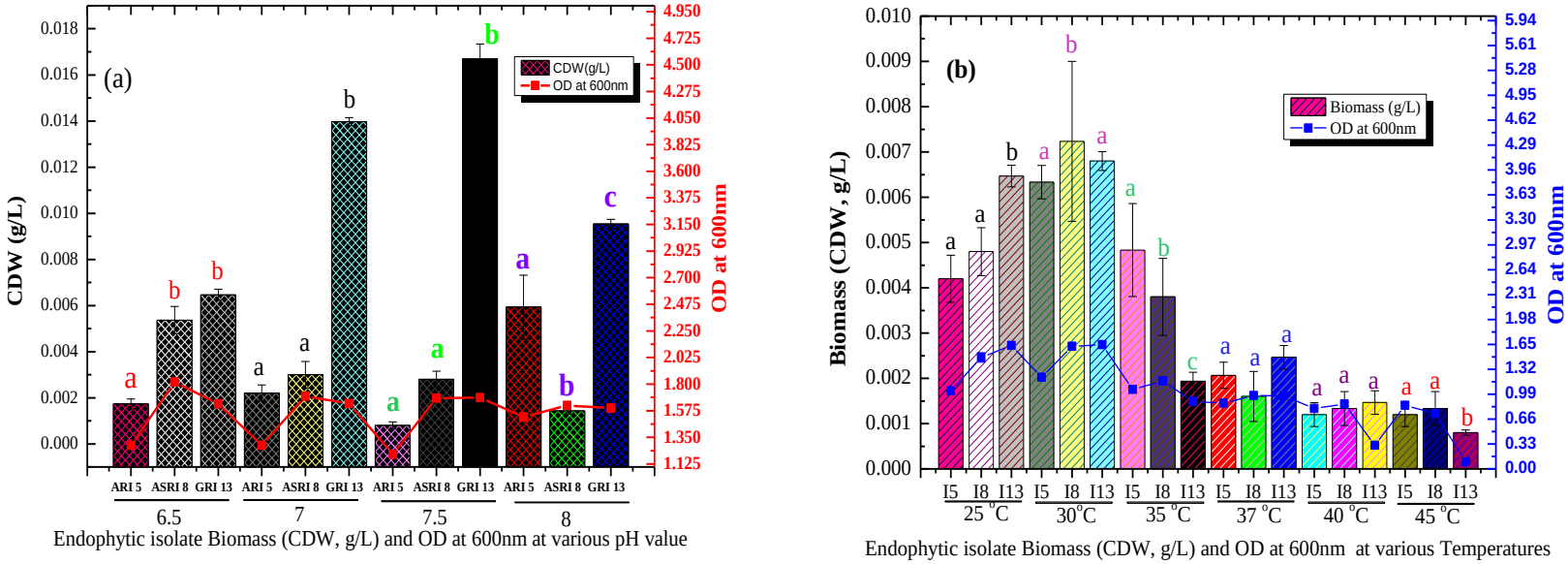


Figure 10: pH and Temperature optimization for selected endophytic bacterial isolates

(a) Effect of pH on growth and BM production of isolates CAMRI5, RPAAI8 and PGORI13 (b) Effect of temperature on the growth and biomass production of isolates CAMRI5, RPAAI8 and PGORI13 Key Words: **ARI5** indicates isolate CAMRI5, **ASRI8** indicates isolate RPAAI8, **GRI13** indicates isolate PGORI 13, **I5** indicates isolate CAMRI5, **I8** indicates isolate RPAAI8, **I13** indicates isolate PGORI13

4.7.3. Carbon source and Nitrogen source optimization

All three isolates exhibited maximum growth as well as CDW (g/l) production when glucose was used as the sole C source (Figure 18, 19 and 20). The Highest OD_{600nm} and CDW measurement taken for isolate RPAAI8, CAMRI5 and PGORI13 were (0.980333333±0.001528), (0.003666667±0.000251661), (0.944666667±0.001528), (0.0039±0.0001) (0.891666667±0.000577) and (0.003733333±0.00011547), respectively when grown with glucose as sole C source. The least preferred sole carbon source by all of the isolates was sucrose exhibiting the lowest OD_{600nm} and CDW (g/l) measurements.

Madhava and Armugam (2010), reported that *Pseudomonas aeruginosa* MTCC 5210 showed maximum growth with glucose compared to other carbon sources with reduced lag phase and extended log phase, where as in sucrose a reduction in growth OD_{600nm} was observed. Similarly Maalej *et al.* (2014), reported that *Pseudomonas stutzeri* AS22 showed maximum growth as well as EPS production when starch (not used in our experiment) was used as sole carbon source but glucose showed better growth compared to the other carbon sources.

Bacterial utilization of carbon sources differs on the precondition of presence of other carbon sources or not. In the case of sole carbon source presence in growth media bacteria grow well on glucose supplemented media over the other carbon sources. That maybe because one, they have no other carbon source option and two, glucose being a monomer is easily catalyzed for cellular functions such as growth and cell division. But in order to really understand the microbial choices of carbon sources a study should include multiple carbon sources to compare between (Beisel & Afroz, 2016).

All of the isolates showed maximum OD_{600nm} and CDW (g/l) measurement with peptone as sole N source except isolate RPAAI8 *Enterobacter cloacae* strain which showed maximum CDW (g/l) production with yeast extract as sole N source (Figure 11, 12, 13). RPAAI8, PGORI13 and CAMRI5 showed maximum OD_{600nm} measurement of 1.179666667 ± 0.008622, 1.126 ± 0.011269 and 1.093333333 ± 0.034196, respectively. OD_{600nm} measurement for nitrogen source utilization by isolate CAMRI5 and PGORI13 showed no significant difference between the two inorganic N sources, but there was significant difference ($p<0.05$) for the two organic N sources, with peptone taking the lead preference as N source compared

to yeast extract. OD_{600nm} measuremnt for isolate RPAAI8 showed that there was significant difference ($p<0.05$) in three of the N sources peptone, yeast extract and $(NH_4)_2SO_4$ being the primary, secondary and tertiary preferences of N sources, respectively.

Suman and Tanuja (2021), reported that they investigated the best nitrogen source for the optimal growth of *Enterobacter cloacae* strain DDT-1 and found out that peptone and yeast extract were the most preferred nitrogen sources by the bacteria. Similarly, Singh *et al.* (2011) reported that when evaluating the usage of $(NH_4)_2SO_4$, peptone and yeast extract as N sources by *Pseudomonas putida* SKG-1, the bacteria grew better when peptone was used as sole N source compared to other N sources, for growth of the strain.

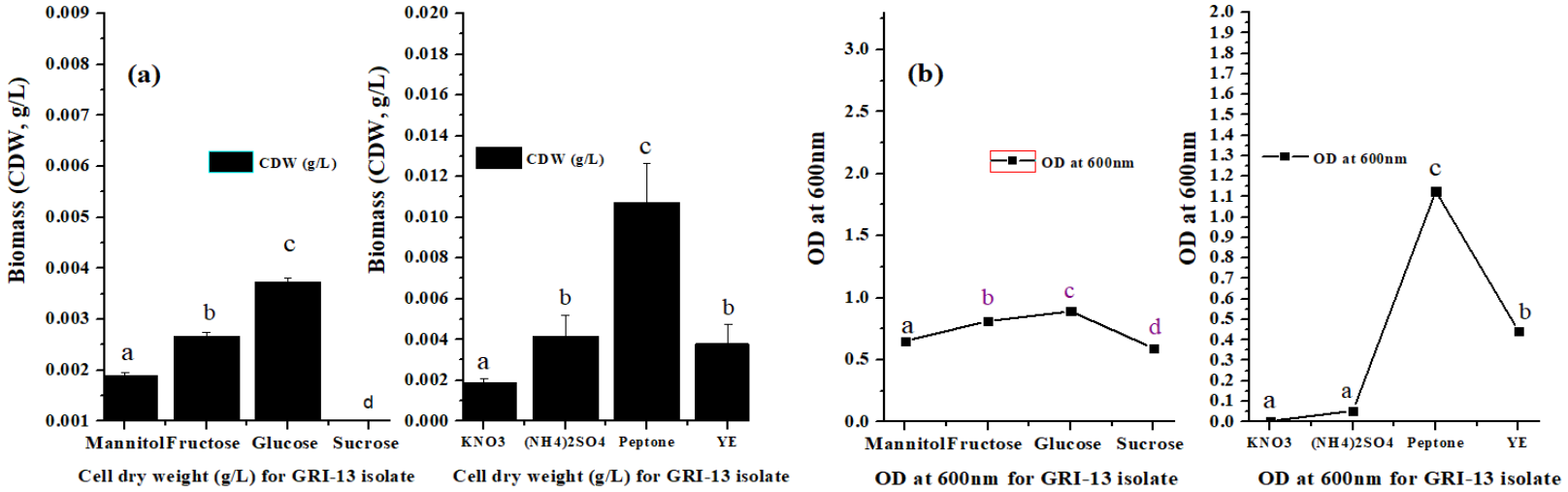


Figure 11: Effect of C and N sources on PGORI13 Isolate a) CDW b) OD_{600nm}

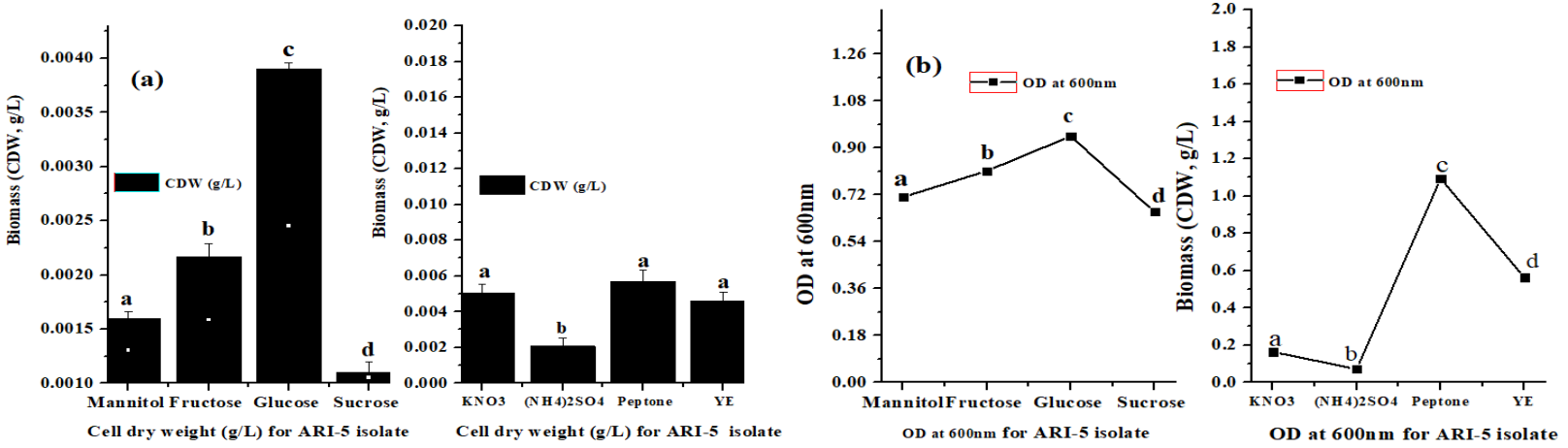


Figure 12: Effect of C and N sources on CAMRI5 Isolate a) CDW b) OD600nm

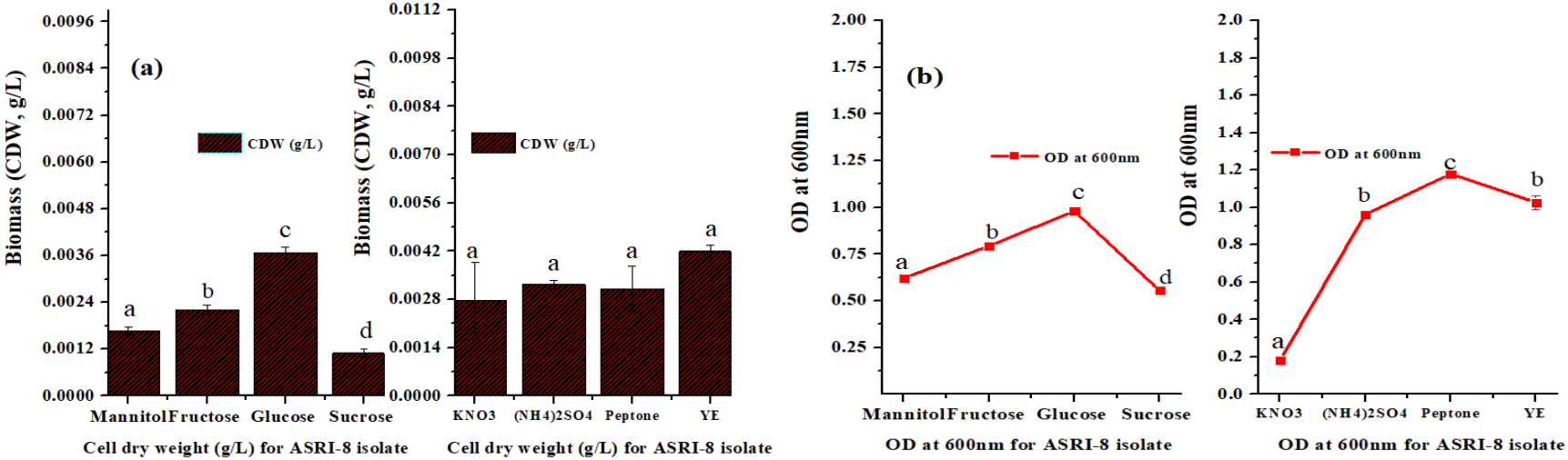


Figure 13: Effect of C and N sources on RPAAI8 Isolate a) CDW b) OD600nm

Key Words: GRI13 indicates isolate PGORI13, ARI5 indicates isolate CAMRI5, ASRI8 indicates isolate RPAAI8

4.7.5. Cost effective Agro-waste as a sole source of carbon

In this study, better growth was observed for RPAAI8 (Figure 21a) and PGORI 13 (Figure 21c) when corn cob was supplied as a sole carbon source, with maximum OD_{600nm} and CDW (g/l) measurements of (1.162333±0.004726), (0.005767±0.000231), and (1.321667±0.014012), (0.0057±0.0002), respectively. Teff bran extract showed maximum OD_{600nm} and CDW (g/l) measurements with (0.896667±0.002887) and (0.003967±0.000208), respectively for isolate CAMRI5 *Citrobacter freundii* strain that was isolated from root part of *Moringa oleifera*. Vegetable waste showed minimum OD_{600nm} and CDW (g/l) measurement for all strains isolated endophytic bacteria. The OD_{600nm} and CDW (g/l) measurement obtained when corn cob was used as sole carbon source for the three isolates is comparable to the OD_{600nm} and CDW (g/l) measurement obtained with LB medium when testing temperature and pH conditions. No significant variations ($p>0.005$) were observed between RPAAI-8 & PGORI-13 ($p=0.775$), CAMRI-5 & RPAAI-8 ($p=0.569$), and RPAAI-8 PGORI-13 ($p=0.396$) when grown on corn cob, and vegetable waste. As indicated in Figure 21, variation ($p<0.05$) was detected among teff bran, corn cob and vegetable waste when these were used as a sole carbon sources for a single strain.

In the current study, less biomass was collected from CAMRI-5, RPAAI-8 and PGORI-13 when vegetable wastes had been used as a sole carbon source. The isolated endophytic bacterial cells may be incapable of breaking down lipids which are a chemical constitute for vegetable waste. Similarly, it was found that agricultural wastes such as fruit and vegetable wastes could be used to support the growth of *Trichoderma harzianum* and. *Trichoderma viride* which are plant growth promoting species (Abd Elsallam *et al.*, 2021). Our isolates CAMRI-5, RPAAI-8 and PGORI-13 were found to utilize teff bran as a sole carbon source. This could be the first report for these isolates to grow on teff bran. These isolates were identified as *Citrobacter freundii*, *Enterobacter cloacae*, and *Pseudomonas monteilii* by MALDI TOF-MS.

Variation ($p<0.05$) was not observed between RPAAI-8 and PGORI-13 ($P=0.775$) in terms of CDW production at 95% confident interval. This could be due to the availability of array of hydrolytic enzymes among these various isolates. The enzymatic test (Appendix 7 and 8)

indicated that both RPAAI-8 (*Enterobacter cloacae* strain) and PGORI-13 (*Pseudomonas monteilii* strain) were positive for cellulase and pectinase tests. The presence of cellulase enzyme might be useful for the degradation of cellulose parts of corn cob (Li *et al.*, 2012). The same authors stated that *Candida tropicalis* is able to produce Xylitol after degradation of corn cob hemicellulose hydrolysate in a two-stage fed-batch fermentation process. In line with this study, it was confirmed that *Pseudomonas chlororaphis* subsp., *P. chlororaphis* subsp. *chlororaphis* RP-4, and *P. fluorescens* RS-1 were positive for cellulase production (Shahid *et al.*, 2021). It was stated that *Citrobacter freundii* strain was used for cellulose degradation by Lakhundi *et al.* (2016), which is a similar finding to our study and the *Citrobacter freundii* CAMRI-5 isolate that was obtained from the root part of *Moringa*.

Patil and Salunkhe (2010) have reported that the water extract of flower of *M. latifolia* L. showed higher biomass production of *Azotobacter indicus* ATCC 9540 as well as the EPS produced by the strain. They further elaborated that flower extract mainly contained sucrose as a principal component of its sugars. They also investigated the effect of other commercially available carbon sources and found out that the strain preferred the disaccharide sucrose followed by mannitol compared to other monosaccharaides like glucose or fructose, hence explaining the flower extract's high biomass yield. Vegetable wastes of tomato and carrot residues supported biomass production of the extremophiles *Haloterrigena hispanica* type strain FP1^{T} and *Halobacillus alkaliphilus* type strain FP5^{T} (Paola *et al.*, 2011).

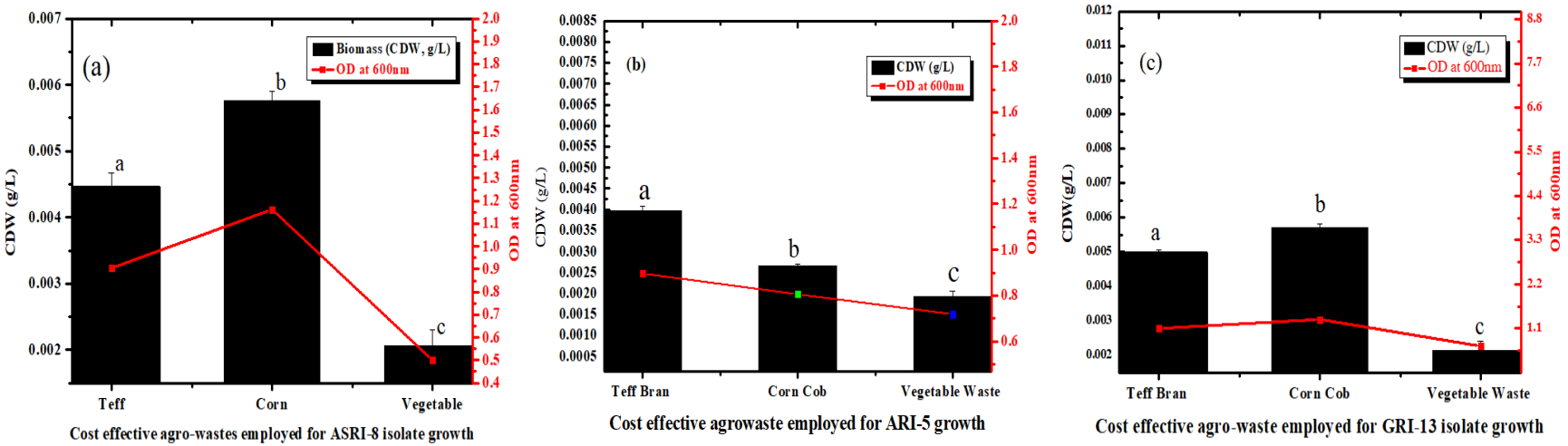


Figure 14: Evaluation of cost effective carbon sources (Teff bran, corn cob and Vegetable waste) against endophytic bacterial isolates obtained from different plant source. (a) Endopytic bacterial isolate (RPAAI-8) that is isolated from root part of *Artemisia* and found to be *Entrobacter cloacacae* after it had been characterized using MALDI TOF. (b) CAMRI-5 was obtained from root part of *Moringa* and identified as *Citrobacter freundii* with the maximum CDW for Teff bran. (c) Maximum growth of PGORI-13 that is isolated from *Ocimum* root and identified as *Pseudomonas monteilii* with long rod-shaped gram negative isolate. Data represented mean ±SD of three replicates (n=3) of CDW (g/ml) and OD_{600nm} for cost effective agro-wastes. According to one way ANOVA, there is significant variation at 95% confidence interval by using LSD test ($P>0.05$) with the different letters on line and bar for endophytic bacterial isolates that utilize cost effective agro-wastes.

Many articles regarding the usage of agro wastes such corn-cob, wheat straw or peanut hull as carbon source are written but mainly in the context of production of Exo-polysaccarides (EPS) especially bio-flocculants from bacteria. In regards to these applications Siddeeg and Tahoon (2020), have remarked that medium composed of agricultural wastes mainly corn-cob as sole carbon sources produced higher amounts of EPS and bacterial biomass when compared with the synthetic medium. Although the physico-chemical characteristics of teff bran lacks the appropriate data and the vegetable waste used also needs its own characterization of the physico-chemical constituents there are plenty of literatures explaining the physico-chemical properties of corn cobs. The nutritional analysis of corn cob assessed by different researchers indicates that corn cobs have crude protein ranging from 17-39 g/kg of dry matter, cellulose ranging from 345-355 g/kg of dry matter, hemicelluloses 135-392 g/kg of dry matter, also corn-cobs are 4-21% water soluble (Kanengoni *et al.*, 2015). Not just these corn cobs have fixed carbon content of 23% by weight percentage; elemental components such as carbon (44.4), hydrogen (5.6), nitrogen (0.43) and sulfur (1.3) by weight percentage are present in corn cobs. Metallic elemental components such as Al (0.31), K (1.53), Si (0.44), Na (1.32), Ca (0.11), Mg (0.42) and Fe (0.06) are present by weight percentage as well (Anukam *et al.*, 2017).

In the current study, certain enzymes such as amylase, cellulase, pectinase, and protease were detected for the endophytic bacterial isolates. Gross amount of these enzymes can be harvested at small scale and industrial level using these cost effective agro-wastes as the sole carbon sources as it was evident in our study. In line with study, it was recognized that maximum amount of xylanase enzymes were produced from *Bacillus pumilus* M1 and *Bacillus pumilus* M2 using 3% (w/v) corn cobs as a sole carbon source in a liquid medium (Yaşinok *et al.,* 2008). As indicated here in our study, considerable amount of CDW (g/L) were harvested for certain selected endophytic bacterial isolates when teff bran and corn cob had been employed as a sole carbon source. These cost effective carbon sources may be used to induce enzyme production which is a similar estimation to the finding. Certain endophytic bacterial isolates such as *Actinobacillus capsulatus, Enterobacter Cloacae ss disolvens, Enterobacter cloacae, Pseudomonas fluorescence* biotype G, *Pseudomonas fluorescens, Pantoae agglomerans* and *Staphylococcus hyicus were* screened by using Biolog Gene III micro plates that was filled with 71 different carbon sources (Tsegaye *et al.*, 2020).

4.8. Evaluation of antimicrobial activities of ethyl acetate extracts of the isolate RPAAI8

The extraction yielded 0.8g/500ml of crude extract. Syed and Rekha (2019), extracted secondary metabolites from fresh water *Actinomycetes* and got the same amount of crude metabolite extract as the current study. Nxumalo *et al.* (2020), on the other hand extracted the secondary metabolites from endophytic bacterial strain Isolate 1 (*P. aeruginosa* CP043328.1) and got 0.6mg/500ml of crude extract. They reported that the extracted metabolites were examined by well diffusion method for antibacterial activity against 5 bacterial strains and showed inhibition zones.

The extract exhibited inhibitory activity against all the test organisms (Table 4). Highest antibacterial activity was recorded against the *Bacillus subtilis* strain with 21±1.69 mm zone of inhibition from the crude extract with concentration of 0.8 g/ml (Figure 15).

The isolate RPAAI8 *Enterobacter cloacae* strain from which the crude metabolite was extracted from showed high levels of resistance as it covered the inhibition zone completely after being inhibited for about 10-12 hours. Sugiyama (2015), explains that Antibiotic-producing microorganisms are able to protect themselves from the fatal effect of their own product, this is called self-resistance. This trait of antibiotic-producing microorganisms is very important in understanding the similarity and the difference between drug resistance mechanism and self-resistance mechanism which in turn is significant in the development of novel therapeutic drugs.

Syed and Rekha (2019) evaluated the crude extract's antibacterial activity against *Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Proteus vulgaris*, and *Salmonella typhi* where they found the extract showing zones of inhibition against all the test bacteria. Nxumalo *et al.* (2020) reported that crude metabolite extracts of endophytic bacteria Isolate 1 strain *Pseudomonas aeruginosa* CP043328.1 demonstrated significant inhibitory ability against *E. coli*, *P. mirabilis*, *B. cereus* and *S. aureus*. And the extract was mostly effective on *S. aureus* with 31±1.25mm inhibition zone diameter.

Table 4: Inhibition zone of extracted secondary metabolite

Zone of inhibition of ethyl acetate extracts (mm)			Positive control (mm)
Test strains	**0.8 g/ml**	**0.4 g/ml**	**20 µg/ml**
B. subtilis	21±1.69	13±1.41	40±0.94
S. aureus	18±1.41	16±1.24	39±1.24
E. coli	20±1.41	13±0.94	35±1.24
E. cloacae	25±1.24	20±1.69	40±0.94

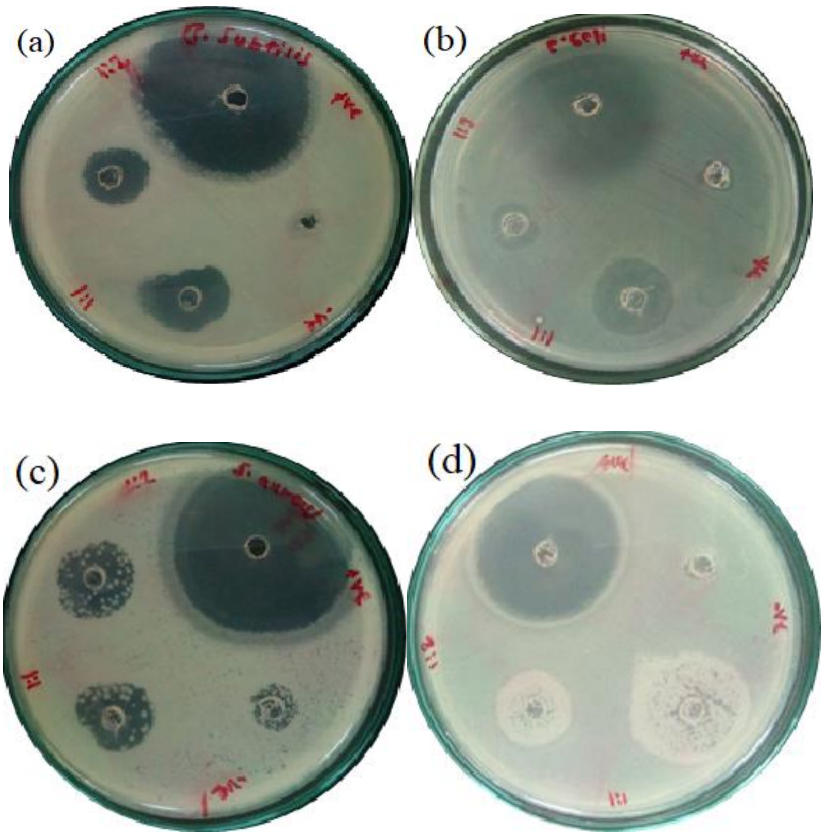


Figure 15: Inhibition zone of ethyl-acetate extracts A) against *Bacillus subtilis* B) against *Escherichia coli* C) against *Staphylococcus aureus* D) against isolate RPAAI8 *Enterobacter cloacae* strain

4.9. 16S rRNA sequencing and phylogenetic tree construction for endophytic isolate RPAAI8

The partial 16S rRNA gene sequence of one of the recent endophytic bacterial isolate that was obtained from root part of *Artemisia annua* was found to be 1451 bp in sequence length. This newly obtained isolate was found to be *Enterobacter* species with 99.73% sequence similarity with *Enterobacter cloacae subsp. dissolvens* LMG 2683^{T} (Z96079) and 99.3% sequence similarity with *Enterobacter sichuanensis* WCHECl1597^{T} (POVL01000141). RPAAI-8 isolate was also closest to *Enterobacter ludwigii* EN-119 ^{T} (JTLO01000001) with 99.11% sequence similarity. The same isolate was also identified as *Enterobacter cloacae* by using MALDI-TOF analysis. The GC composition of 16S rRNA gene sequence for the recent endophytic bacterial isolate and other closest strains are varied as indicated in Table 5 which indicated that the RPAAI-8 might be new endophytic bacterial isolate. Based on this, its name was designated as *Enterobacter* species isolate that is obtained from root part of *Artemisia annua* ASTU sample (*Enterobacter* sp RPAAI-8). In previous study, certain endophytic actinobacteria isolated from forest ecosystems was suggested for plant promotion, and promising antimicrobial potential against pathogenic microorganisms (Priyanka Sharma & Thakur, 2020).

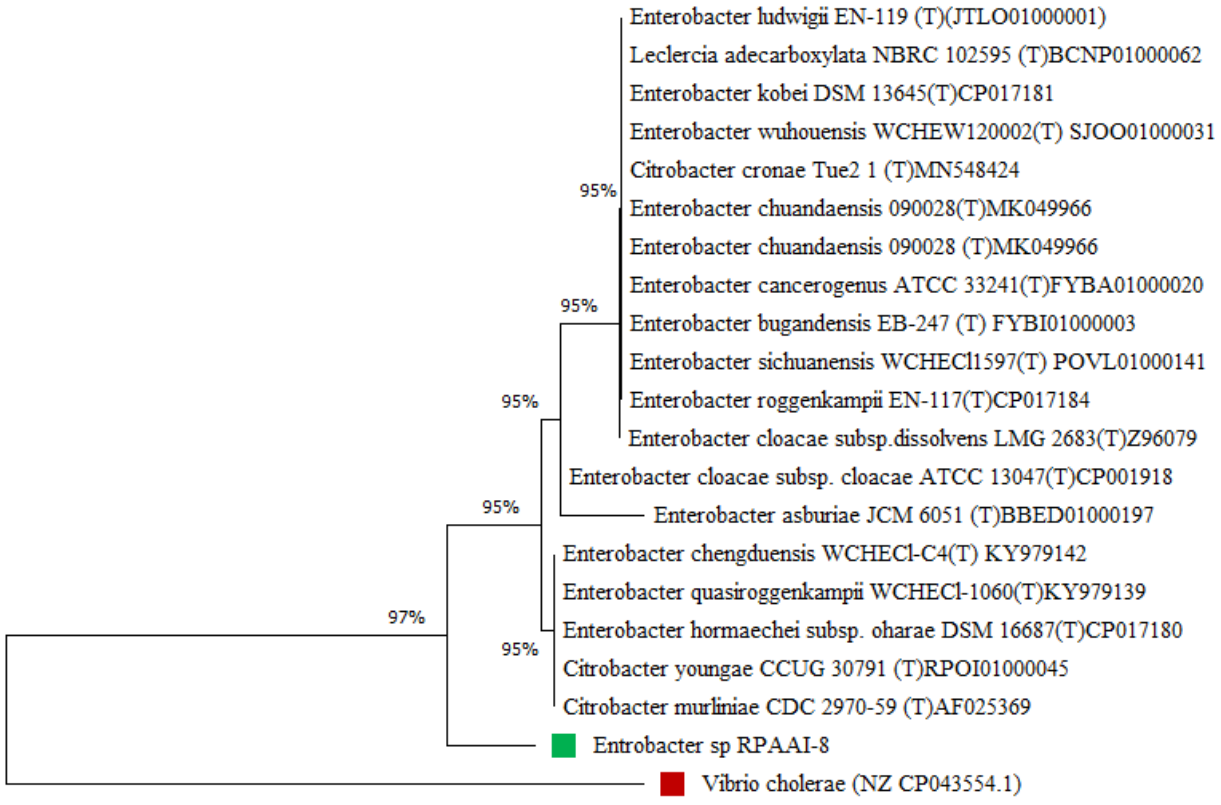


Figure 16: Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones *et al.*, 2018). The tree with the highest log likelihood (-7460.39) is shown. The percentage of trees in which the associated taxa clustered together is shown next to 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 a JTT model, and then selecting the topology with superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.00% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 amino acid sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 394 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (S. Kumar *et al.*, 2016).

As indicated in Figure 16, our isolate obtained from the root part of *Artemisia annua* (RPAAI-8) has shown common ancestry with other unrelated genus of bacterial cells such as

Citrobacter cronae Tue2_1^{T} (MN548424), *Citrobacter murliniae* CDC 2970-59^{T} (AF025369), *Citrobacter youngae* CCUG 30791^{T} (RPOI01000045), and *Leclercia adecarboxylata* NBRC 102595^{T} (BCNP01000062) (Figure 16, Table 5). The maximum likelihood (ML) phylogenetic tree based on 16S rRNA is presented in Figure 16.

Table 5: Sequence similarity between our isolate and other strains with their respective GC% and accession numbers

S.N.	Organism	Strain	Accession	GC%	Sequence similarity with newly isolates strain
1	*Enterobacter sp.*	*Enterobacter* sp.	ON640911	54.63	Current isolates
2	*Enterobacter cloacae subsp. dissolvens*	LMG 2683^{T}	Z96079	54.89	99.73
3	*Enterobacter sichuanensis*	WCHECl1597^{T}	POVL01000141	54.86	99.59
4	*Enterobacter kobei*	DSM 13645^{T}	CP017181	54.92	99.52
5	*Enterobacter chuandaensis*	090028^{T}	MK049966	55.06	99.25
6	*Enterobacter chengduensis*	WCHECl-C4^{T}	KY979142	55.12	99.38
7	*Leclercia adecarboxylata*	NBRC 102595T	BCNP01000062	54.72	99.32
8	*Enterobacter chuandaensis*	090028^{T}	MK049966	55.06	99.25
9	*Enterobacter cloacae subsp. cloacae*	ATCC 13047^{T}	CP001918	55.05	99.18
10	*Enterobacter ludwigii*	EN-119 ^{T}	JTLO01000001	54.99	99.11
11	*Enterobacter roggenkampii*	EN-117^{T}	CP017184	55.20	99.11
12	*Enterobacter cancerogenus*	ATCC 33241^{T}	FYBA01000020	54.86	98.97
13	*Citrobacter youngae*	CCUG 30791^{T}	RPOI01000045	54.99	98.97
14	*Enterobacter bugandensis*	EB-247^{T}	FYBI01000003	54.99	98.84
15	*Enterobacter quasiroggenkampii*	WCHECl-1060^{T}	KY979139	54.99	98.84

16	*Enterobacter huaxiensis*	090008^{T}	MK049964	98.83
17	*Citrobacter murliniae*	CDC 2970-59^{T}	AF025369	98.77
18	*Enterobacter hormaechei subsp. oharae*	DSM 16687^{T}	CP017180	98.77
19	*Citrobacter cronae*	Tue2_1(T)	MN548424	98.77
20	*Enterobacter asburiae*	JCM 6051(T)	BBED01000197	98.77

Similar to our study Macedo-Raygoza *et al*. (2019), identified a strain of endophytic bacteria isolated from banana (*Musa* spp.) using 16S rRNA sequencing. The amplified and sequenced bacterial 16S rRNA fragment was about 1200bp long and was 100% similar to *E. cloacae* KP7988817.1 strain. This result was also corroborated by MALDI-TOF MS identification result. Other study conducted by Kazerooni *et al*. (2020) revealed an endophytic isolate which was able to fight off Pythium damping-off disease of cucumber as being *Enterobacter cloacae* strain by 16S RNA sequencing.

5. Conclusions and Recommendations

5.1. Conclusions

In this research 45 endophytic bacterial isolates were isolated from the leaf, stem, and root parts of *Artemisia annua, Moringa oleifera,* and *Ocimum lamiifolium* plants successfully. The emerged endophytic bacteria were purified and identified by biochemical methods as well as MALDI-TOF MS as belonging to the genera *Bacillus, Citrobacter, Enterococcus, Enterobacter*, and *Pseudomonas*. The isolates were able to produce different industrially as well as agriculturally important lytic enzymes with 24.4%, 51.11%, 68.8%, and 42.2% of the isolates producing the enzymes amylase, cellulase, pectinase, and protease, respectively, showing promise of being used as novel sources of important enzymes. The isolates were found to be prolific phosphate and zinc solubilizers as demonstrated by the formation of clearing zones on agar plates containing insoluble forms of phosphate and zinc. 60% of the isolates were able to solubilize insoluble phosphate whereas 46.6% were able to solubilize insoluble zinc, showing the ability of the isolates to be used as bio-fertilizers to reduce the impact of chemical fertilizers added in phosphate and zinc-deficient agricultural fields by being used as bio-inoculants for plants. 80% of the isolates also showed the ability to produce the important plant stress alleviating enzyme 1-aminocyclopropane 1-carboxylic acid deaminase (ACC-deaminase) further strengthening the prospect of the isolates being used as bio-fertilizers. Isolate RPAAI-8 showed the extraordinary ability of production by being able to produce all of the four lytic enzymes and also solubilize both insoluble phosphate and zinc found in the agar plates. Evaluation of growth conditions for three representative isolates showed that temperature of 30^{o}C with pH range 6.5-7.5 and glucose and peptone as sole sources of carbon and nitrogen, respectively, were optimal growth conditions for the isolates. Ethyl acetate secondary metabolite extract of the hyper-producer isolate RPAAI-8 strain yielded in 0.8 g/500 ml of crude extracts. The crude extracts exhibited inhibitory activity against three test organisms *B. subtilis, E. coli,* and *S. aureus,* and the isolate RPAAI-8 itself showed resistance against its secondary metabolite. The BLAST analysis of 16S rRNA sequencing of the isolate showed 99.47% similarity to *Enterobacter cloacae* strains.

5.2. Recommendations

Based on the findings of the research the following recommendations are made:

- The potential of the endophytic bactria of these medicinal plants was not studied to its full right leaving a gap in the knowledge of the endosphere dwelling bacteria of the medicinal plants, so further investigation of the ability of the endophytic bacteria of these medicinal plants is necessary.
- The enzymatic activities of the endophytic bacterial isolates should be quantitatively assessed because they have exhibited very high levels enzyme production especially pectinase enzyme.
- The plant growth promoting abilities of the isolates grabs attention and this indicates there is the need to examine the ability of the isolates to be used as bio-fertilizers so the assessment of the ability of the isolates to be used as bio-inoculant should be investigated.
- The endosphere of medicinal plants should be explored more in hopes of finding novel sources of bioactive molecule producing microorganisms.

6. References

Abd Elsallam, E. M., El-moslamy, S. H., Abd El-al, A., & Zahran, H. F. (2021). Scaling-up production of cost-effective and eco-friendly bio-fertilizer and its application on Barley green fodder via IoT hydroponic system. *Journal of Genetic Engineering and Biotechnology*, *19*(97), 1–12. https://doi.org/https://doi.org/10.1186/s43141-021-00196-1

Abod, É., Laslo, É., Szentes, S., Lányi, S., & Mara, G. (2019). Plant Growth-Promoting Bacteria: Strategies to Improve Wheat Growth and Development Under Sustainable Agriculture. *Plant Growth Promoting Rhizobacteria for Agricultural Sustainability*, 1–17. https://doi.org/10.1007/978-981-13-7553-8_1

Afzal, I., Shinwari, Z. K., Sikandar, S., & Shahzad, S. (2019). Plant beneficial endophytic bacteria: Mechanisms, diversity, host range and genetic determinants. *Microbiological Research*, *221*(February), 36–49. https://doi.org/10.1016/j.micres.2019.02.001

Ahemad, M. (2015). Phosphate-solubilizing bacteria-assisted phytoremediation of metalliferous soils: a review. *3 Biotech*, *5*(2), 111–121. https://doi.org/10.1007/s13205-014-0206-0

Ait Barka, E., Nowak, J., & Clément, C. (2006). Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. *Applied and Environmental Microbiology*, *72*(11), 7246–7252. https://doi.org/10.1128/AEM.01047-06

Amend, A. S., Cobian, G. M., Laruson, A. J., Remple, K., Tucker, S. J., Poff, K. E., Antaky, C., Boraks, A., Jones, C. A., Kuehu, D., Lensing, B. R., Pejhanmehr, M., Richardson, D. T., & Riley, P. P. (2019). Phytobiomes are compositionally nested from the ground up. *PeerJ*, *2019*(3). https://doi.org/10.7717/peerj.6609

Anukam, A. I., Goso, B. P., Okoh, O. O., & Mamphweli, S. N. (2017). Studies on Characterization of Corn Cob for Application in a Gasification Process for Energy Production. *Journal of Chemistry*, *2017*, 1–9. https://doi.org/https://doi.org/10.1155/2017/6478389

Aravind, R., Kumar, A., Eapen, S. J., & Ramana, K. V. (2009). Endophytic bacterial flora in root and stem tissues of black pepper (*Piper nigrum* L.) genotype: isolation, identification and evaluation against *Phytophthora capsici*. *Letters in Applied Microbiology*, *48*(1), 58–64. https://doi.org/10.1111/j.1472-765X.2008.02486.x

Ashok, K., Savita, D., Pramod, L., Indra, B., & Srishti, P. (2019). Zinc-Solubilizing Bacteria: A Boon for Sustainable Agriculture. *Microorganisms for Sustainability*, *12*,. https://doi.org/https://doi.org/10.1007/978-981-13-6536-2_8

Aswathy, B. J., Joseph, A., John, C. J., & Radhakrishnan, J. M. E. K. (2014). *Isolation and characterization of plant growth promoting endophytic bacteria from the rhizome of Zingiber officinale*. 197–204. https://doi.org/10.1007/s13205-013-0143-3

Barret, M., Briand, M., Bonneau, S., Préveaux, A., Valière, S., Bouchez, O., Hunault, G.,

Simoneau, P., & Jacquesa, M. A. (2015). Emergence shapes the structure of the seed microbiota. *Applied and Environmental Microbiology*, *81*(4), 1257–1266. https://doi.org/10.1128/AEM.03722-14

Beisel, C. L., & Afroz, T. (2016). Rethinking the Hierarchy of Sugar Utilization in Bacteria. *Journal of Bacteriology*, *198*(3), 374–376. https://doi.org/10.1128/JB.00890-15.Editor

Bhattacharyya, P. N., & Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. *World Journal of Microbiology and Biotechnology*, *28*(4), 1327–1350. https://doi.org/10.1007/s11274-011-0979-9

Bogas, A. C., Ferreira, A. J., Araújo, W. L., Astolfi-Filho, S., Kitajima, E. W., Lacava, P. T., & Azevedo, J. L. (2015). Endophytic bacterial diversity in the phyllosphere of Amazon *Paullinia cupana* associated with asymptomatic and symptomatic anthracnose. *SpringerPlus*, *4*(1). https://doi.org/10.1186/s40064-015-1037-0

Böhm, M., Hurek, T., & Reinhold-Hurek, B. (2007). Twitching motility is essential for endophytic rice colonization by the N2-fixing endophyte *Azoarcus sp*. strain BH72. *Molecular Plant-Microbe Interactions*, *20*(5), 526–533. https://doi.org/10.1094/MPMI-20-5-0526

Bokhari, S. S., Qayyum, G., Rafi, U., & Qurashi, A. W. (2019). *Bacillus cereus* and *Citrobacter freundii* from *Lumbricus terrestris* Facilitate Vermicomposting and Improve Soil Characteristics. *Pakistan J. Zool.*, *51*(5), 1769–1776. https://doi.org/http://dx.doi.org/10.17582/journal.pjz/2019.51.5.1769.1776

Boone, D. R., & Castenholz, R. W. (2001). *Bergey's manual of systematic bacteriology: The Archaea and the Deeply Branching and Phototrophic Bacteria* (G. M. Garrity (ed.); Volume 1,). Springer International Publishing.

Brader, G., Compant, S., Vescio, K., Mitter, B., Trognitz, F., Ma, L. J., & Sessitsch, A. (2017). Ecology and Genomic Insights into Plant-Pathogenic and Plant-Nonpathogenic Endophytes. *Annual Review of Phytopathology*, *55*, 61–83. https://doi.org/10.1146/annurev-phyto-080516-035641

Braeken, K., Daniels, R., Ndayizeye, M., & Vanderleyden, J. (2008). *Quorum Sensing in Bacteria-Plant Interactions Quorum Sensing in Bacteria-Plant Interactions*. *January*. https://doi.org/10.1007/978-3-540-75575-3

Buschart, A., Sachs, S., Chen, X., Herglotz, J., Krause, A., & Reinhold-Hurek, B. (2012). Flagella mediate endophytic competence rather than act as MAMPs in rice-*Azoarcus sp*. strain BH72 interactions. *Molecular Plant-Microbe Interactions*, *25*(2), 191–199. https://doi.org/10.1094/MPMI-05-11-0138

Chamam, A., Sanguin, H., Bellvert, F., Meiffren, G., Comte, G., Wisniewski-Dyé, F., Bertrand, C., & Prigent-Combaret, C. (2013). Plant secondary metabolite profiling evidences strain-dependent effect in the *Azospirillum-Oryza* sativa association. *Phytochemistry*, *87*, 65–77. https://doi.org/10.1016/j.phytochem.2012.11.009

Chang, C., Damiani, I., Puppo, A., & Frendo, P. (2009). Redox changes during the legume-

rhizobium symbiosis. *Molecular Plant*, *2*(3), 370–377. https://doi.org/10.1093/mp/ssn090

Chanway, C. P., Shishido, M., Nairn, J., Jungwirth, S., Markham, J., Xiao, G., & Holl, F. B. (2000). Endophytic colonization and field responses of hybrid spruce seedlings after inoculation with plant growth-promoting rhizobacteria. *Forest Ecology and Management*, *133*(1–2), 81–88. https://doi.org/10.1016/S0378-1127(99)00300-X

Chaturvedi, H., & Singh, V. (2016). Potential of Bacterial Endophytes as Plant Growth Promoting Factors. *Journal of Plant Pathology & Microbiology*, *7*(9). https://doi.org/10.4172/2157-7471.1000376

Chi, Q., Tang, W., Liu, L., Meng, J., Dong, X., Chen, W., & Li, X. (2018). Isolation and Properties of *Enterobacter sp* . LX3 Capable of Producing Indoleacetic Acid. *Appl. Sci.*, *8*, 1–11. https://doi.org/10.3390/app8112108

Cho, H. S., Park, S. Y., Ryu, C. M., Kim, J. F., Kim, J. G., & Park, S. H. (2007). Interference of quorum sensing and virulence of the rice pathogen *Burkholderia glumae* by an engineered endophytic bacterium. *FEMS Microbiology Ecology*, *60*(1), 14–23. https://doi.org/10.1111/j.1574-6941.2007.00280.x

Compant, S., Cambon, M. C., Vacher, C., Mitter, B., Samad, A., & Sessitsch, A. (2021). The plant endosphere world – bacterial life within plants. *Environmental Microbiology*, *23*(4), 1812–1829. https://doi.org/10.1111/1462-2920.15240

Compant, S., Clément, C., & Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry*, *42*(5), 669–678. https://doi.org/10.1016/j.soilbio.2009.11.024

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C., & Barka, E. A. (2005). Endophytic colonization of *Vitis vinifera L*. by plant growth-promoting bacterium *Burkholderia sp.* strain PsJN. *Applied and Environmental Microbiology*, *71*(4), 1685–1693. https://doi.org/10.1128/AEM.71.4.1685-1693.2005

Compant, S., Sessitsch, A., & Mathieu, F. (2012). The 125th anniversary of the first postulation of the soil origin of endophytic bacteria - a tribute to M.L.V. Galippe. *Plant and Soil*, *356*(1–2), 299–301. https://doi.org/10.1007/s11104-012-1204-9

Conn, V.M., Walker, A.R. and Franco, C. M. M. (2008). in *Arabidopsis thaliana*. *Nature Communications*, *5*(May), 1–9. http://dx.doi.org/10.1038/ncomms5104

Coombs, J. T., Michelsen, P. P., & Franco, C. M. M. (2004). Evaluation of endophytic actinobacteria as antagonists of Gaeumannomyces graminis var. tritici in wheat. *Biological Control*, *29*(3), 359–366. https://doi.org/10.1016/j.biocontrol.2003.08.001

Correa-Galeote, D., Bedmar, E. J., & Arone, G. J. (2018). Maize endophytic bacterial diversity as affected by soil cultivation history. *Frontiers in Microbiology*, *9*(MAR), 1–9. https://doi.org/10.3389/fmicb.2018.00484

Daltonics, B. (2012). *MALDI Biotyper 3.1 User Manual*. *1*(May), 1–212.

De Weert, S., Vermeiren, H., Mulders, I. H. M., Kuiper, I., Hendrickx, N., Bloemberg, G. V., Vanderleyden, J., De Mot, R., & Lugtenberg, B. J. J. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens. Molecular Plant-Microbe Interactions*, *15*(11), 1173–1180. https://doi.org/10.1094/MPMI.2002.15.11.1173

Deng, Y., Zhu, Y., Wang, P., Zhu, L., Zheng, J., Li, R., Ruan, L., Peng, D., & Sun, M. (2011). Complete genome sequence of *Bacillus subtilis* BSn5, an endophytic bacterium of *Amorphophallus konjac* with antimicrobial activity for the plant pathogen *Erwinia carotovora* subsp. *carotovora*. *Journal of Bacteriology*, *193*(8), 2070–2071. https://doi.org/10.1128/JB.00129-11

Deveau, A., Bonito, G., Uehling, J., Paoletti, M., Becker, M., Bindschedler, S., Hacquard, S., Hervé, V., Labbé, J., Lastovetsky, O. A., Mieszkin, S., Millet, L. J., Vajna, B., Junier, P., Bonfante, P., Krom, B. P., Olsson, S., van Elsas, J. D., & Wick, L. Y. (2018). Bacterial-fungal interactions: Ecology, mechanisms and challenges. *FEMS Microbiology Reviews*, *42*(3), 335–352. https://doi.org/10.1093/femsre/fuy008

Ding, T., & Melcher, U. (2016). Influences of plant species, season and location on leaf endophytic bacterial communities of non-cultivated plants. *PLoS ONE*, *11*(3), 1–13. https://doi.org/10.1371/journal.pone.0150895

Ding, T., Palmer, M. W., & Melcher, U. (2013). Community terminal restriction fragment length polymorphisms reveal insights into the diversity and dynamics of leaf endophytic bacteria. *BMC Microbiology*, *13*(1), 1–11. https://doi.org/10.1186/1471-2180-13-1

Dong, Z., Canny, M. J., Mccully, M. E., Roboredo, M. R., Cabadilla, C. F., Ortega, E., Rodés, R., Canada, Z. D., & De, D. (1994). *Dong et al. 105 (4) 1139 (1994).pdf*. 1139–1147.

Downing, K. J., Leslie, G., & Thomson, J. A. (2000). Biocontrol of the sugarcane borer *Eldana saccharina* by expression of the *Bacillus thuringiensis* cry1Ac7 and *Serratia marcescens* chiA genes in sugarcane-associated bacteria. *Applied and Environmental Microbiology*, *66*(7), 2804–2810. https://doi.org/10.1128/AEM.66.7.2804-2810.2000

Dulla, G. F. J., Go, R. A., Stahl, D. A., & Davidson, S. K. (2012). *Verminephrobacter eiseniae* type IV pili and flagella are required to colonize earthworm nephridia. *ISME Journal*, *6*(6), 1166–1175. https://doi.org/10.1038/ismej.2011.183

Elbeltagy, A., Nishioka, K., Suzuki, H., Sato, T., Sato, Y. I., Morisaki, H., Mitsui, H., & Minamisawa, K. (2000). Isolation and characterization of endophytic bacteria from wild and traditionally cultivated rice varieties. *Soil Science and Plant Nutrition*, *46*(3), 617–629. https://doi.org/10.1080/00380768.2000.10409127

Elmanama, A. A. (2009). *G eneral M icrobiology*. Islamic University - Gaza.

Emerson, D., Agulto, L., Liu, H., & Liu, L. (2008). Identifying and Characterizing Bacteria in an Era of Genomics and Proteomics. *Bioscience*, *58*(10), 925–936. https://academic.oup.com/bioscience/article/58/10/925/245827

Eshaghi, E., Nosrati, R., Owlia, P., & Malboobi, M. A. (2019). *Zinc solubilization*

characteristics of efficient siderophore-producing soil bacteria. *11*(5), 419–430.

Ezawa, T., Smith, S. E., & Smith, F. A. (2002). P metabolism and transport in AM fungi. *Plant and Soil*, *244*(1–2), 221–230. https://doi.org/10.1023/A:1020258325010

Germida, J. J., Siciliano, S. D., De Freitas, J. R., & Seib, A. M. (1998). Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). *FEMS Microbiology Ecology*, *26*(1), 43–50. https://doi.org/10.1016/S0168-6496(98)00020-8

Glick, B. R. (2012). *Plant Growth-Promoting Bacteria : Mechanisms and Applications*. *2012*.

Granér, G., Persson, P., Meijer, J., & Alström, S. (2003). A study on microbial diversity in different cultivars of *Brassica napus* in relation to its wilt pathogen, *Verticillium longisporum. FEMS Microbiology Letters*, *224*(2), 269–276. https://doi.org/10.1016/S0378-1097(03)00449-X

Hallmann, J. (2001). Biotic Interactions in Plant–Pathogen Associations. In M.J. Jeger and N.J. Spence (Ed.), *Biotic Interactions in Plant–Pathogen Associations* (pp. 87–121). CABI Publishing.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., & Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology*, *43*(10), 895–914. https://doi.org/10.1139/m97-131

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., Döring, M., & Sessitsch, A. (2015). The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiology and Molecular Biology Reviews*, *79*(3), 293–320. https://doi.org/10.1128/mmbr.00050-14

Hardoim, P. R., van Overbeek, L. S., & Elsas, J. D. van. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology*, *16*(10), 463–471. https://doi.org/10.1016/j.tim.2008.07.008

Harrison, J. G., & Griffin, E. A. (2020). The diversity and distribution of endophytes across biomes, plant phylogeny and host tissues: how far have we come and where do we go from here? *Environmental Microbiology*, *22*(6), 2107–2123. https://doi.org/10.1111/1462-2920.14968

Hartmann, A., Rothballer, M., Hense, B. A., & Schröder, P. (2014). Bacterial quorum sensing compounds are important modulators of microbe-plant interactions. *Frontiers in Plant Science*, *5*(APR), 1–4. https://doi.org/10.3389/fpls.2014.00131

Hayat, R., Ali, S., Amara, U., Khalid, R., & Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: A review. *Annals of Microbiology*, *60*(4), 579–598. https://doi.org/10.1007/s13213-010-0117-1

Hématy, K., Cherk, C., & Somerville, S. (2009). Host-pathogen warfare at the plant cell wall. *Current Opinion in Plant Biology*, *12*(4), 406–413.

https://doi.org/10.1016/j.pbi.2009.06.007

Hori, K., & Matsumoto, S. (2010). Bacterial adhesion: From mechanism to control. *Biochemical Engineering Journal*, *48*(3), 424–434. https://doi.org/10.1016/j.bej.2009.11.014

Hounsome, N., Hounsome, B., Tomos, D., & Edwards-Jones, G. (2008). Plant metabolites and nutritional quality of vegetables. *Journal of Food Science*, *73*(4), 48–65. https://doi.org/10.1111/j.1750-3841.2008.00716.x

Hussain, T., Roohi, A., Munir, S., Ahmed, I., Edel-hermann, V., Kim, K. Y., Anees, M., Hussain, T., Roohi, A., Munir, S., Ahmed, I., Khan, J., Hussain, T., Roohi, A., Munir, S., Ahmed, I., & Khan, J. (2013). Biochemical characterization and identification of bacterial strains isolated from drinking water sources of Kohat, Pakistan. *African Journal of Microbiology Research, Academic Journal*, *7*(16), 1579–1590. https://doi.org/10.5897/AJMR12.2204

Islam, F., Yasmeen, T., Riaz, M., Saleem, M., Ali, S., & Hammad, S. (2014). Ecotoxicology and Environmental Safety *Proteus mirabilis* alleviates zinc toxicity by preventing oxidative stress in maize (*Zea mays*) plants. *Ecotoxicology and Environmental Safety*, *110*, 143–152. https://doi.org/10.1016/j.ecoenv.2014.08.020

Jasim, B., John, C., Mathew, J., & Radhakrishnan, E. K. (2013). Plant growth promoting potential of endophytic bacteria isolated from *Piper nigrum*. *Plant Growth Regul*, *71*, 1–11. https://doi.org/10.1007/s10725-013-9802-y

Jasim, B., Joseph, A. A., John, C. J., Mathew, J., & Radhakrishnan, E. K. (2014). Isolation and characterization of plant growth promoting endophytic bacteria from the rhizome of *Zingiber officinale*. *3 Biotech*, *4*(2), 197–204. https://doi.org/10.1007/s13205-013-0143-3

Jin, H., Yang, X. Y., Yan, Z. Q., Liu, Q., Li, X. Z., Chen, J. X., Zhang, D. H., Zeng, L. M., & Qin, B. (2014). Characterization of rhizosphere and endophytic bacterial communities from leaves, stems and roots of medicinal *Stellera chamaejasme* L. *Systematic and Applied Microbiology*, *37*(5), 376–385. https://doi.org/10.1016/j.syapm.2014.05.001

Johnston-Monje, D., & Raizada, M. N. (2011). Conservation and diversity of seed associated endophytes in *Zea* across boundaries of evolution, ethnography and ecology. *PLoS ONE*, *6*(6). https://doi.org/10.1371/journal.pone.0020396

Jones, D. T., Taylor, W. R., & Thornton, J. M. (2018). The rapid generation of mutation data matrices from protein sequences. *CABIOS*, *8*(3), 275–282. https://academic.oup.com/bioinformatics/article-abstract/8/3/275/193076

Kalayu, G. (2019). Phosphate Solubilizing Microorganisms : Promising Approach as Biofertilizers. *International Journal of Agronomy*, *2019*, 1–7. https://doi.org/https://doi.org/10.1155/2019/4917256

Kamran, S., Shahid, I., Baig, D. N., Rizwan, M., & Malik, K. A. (2017). *Contribution of Zinc Solubilizing Bacteria in Growth Promotion and Zinc Content of Wheat*. *8*(December). https://doi.org/10.3389/fmicb.2017.02593

Kanengoni, A. T., Chimonyo, M., Ndimba, B. K., & Dzama, K. (2015). Potential of Using Maize Cobs in Pig Diets — A Review. *Asian Australas. J. Anim. Sci.*, *28*(12), 1669–1679. https://doi.org/http://dx.doi.org/10.5713/ajas.15.0053

Kazerooni, E. A., Al-Shibli, H., Nasehi, A., & Al-Sadi, A. M. (2020). Endophytic *enterobacter cloacae* exhibits antagonistic activity against pythium damping-off of cucumber. *Ciencia Rural*, *50*(8), 1–7. https://doi.org/10.1590/0103-8478cr20191035

Keswani, C., Mishra, S., Sarma, B. K., Singh, S. P., & Singh, H. B. (2014). Unraveling the efficient applications of secondary metabolites of various *Trichoderma spp*. *Applied Microbiology and Biotechnology*, *98*(2), 533–544. https://doi.org/10.1007/s00253-013-5344-5

Kumar, A., Meena, M. L., Shivran, B. C., Pal, H., & Meena, B. L. (2019). Impact of bio-fertilizer on growth, yield and quality of onion (*Allium cepa* L.) cv. Pusa Red. *Plant Archives*, *19*(1), 772–776.

Kumar, S., Stecher, G., Tamura, K., & Medicine, E. (2016). MEGA7 : Molecular Evolutionary Genetics Analysis version 7 . 0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/doi:10.1093/molbev/msw054

Kusari, S., Hertweck, C., & Spiteller, M. (2012). Chemical ecology of endophytic fungi: Origins of secondary metabolites. *Chemistry and Biology*, *19*(7), 792–798. https://doi.org/10.1016/j.chembiol.2012.06.004

Lakhundi, S. S., Duedu, K. O., Cain, N., Nagy, R., Krakowiak, J., & French, C. E. (2016). *Citrobacter freundii* as a test platform for recombinant cellulose degradation systems. *Letters in Applied Microbiology*, *64*(1), 35–42. https://doi.org/10.1111/lam.12668

Li, M., Meng, X., & Du, F. (2012). Xylitol production by *Candida tropicalis* from corn cob hemicellulose hydrolysate in a two-stage fed-batch fermentation process. *J Chem Technol Biotechnol*, *87*, 387–392. https://doi.org/10.1002/jctb.2732

Liu, H., Carvalhais, L. C., Crawford, M., Singh, E., Dennis, P. G., Pieterse, C. M. J., & Schenk, P. M. (2017). Inner plant values: Diversity, colonization and benefits from endophytic bacteria. *Frontiers in Microbiology*, *8*(DEC), 1–17. https://doi.org/10.3389/fmicb.2017.02552

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E. R. B., Mezgeay, M., Lelie, D. Van Der, Edward, R. B., & Taghavi, S. (2010). Endophytic Bacteria and Their Potential Applications. *Critical Reviews in Plant Sciences*, *February 2013*, 37–41.

Lugtenberg, B., & Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology*, *63*, 541–556. https://doi.org/10.1146/annurev.micro.62.081307.162918

Ma, Y., Rajkumar, M., Zhang, C., & Freitas, H. (2016). Beneficial role of bacterial endophytes in heavy metal phytoremediation. *Journal of Environmental Management*, *174*, 14–25. https://doi.org/10.1016/j.jenvman.2016.02.047

Maalej, H., Hmidet, N., Boisset, C., Buon, L., Heyraud, A., & Nasri, M. (2014). Optimization

of exopolysaccharide production from *Pseudomonas stutzeri* AS22 and examination of its metal-binding abilities. *Journal of Applied Microbiology*, *118*, 356–367. https://doi.org/10.1111/jam.12688

Macedo-Raygoza, G. M., Valdez-Salas, B., Prado, F. M., Prieto, K. R., Yamaguchi, L. F., Kato, M. J., Canto-Canché, B. B., Carrillo-Beltrán, M., Di Mascio, P., White, J. F., & Beltrán-García, M. J. (2019). *Enterobacter cloacae*, an Endophyte That Establishes a Nutrient-Transfer Symbiosis With Banana Plants and Protects Against the Black Sigatoka Pathogen. *Frontiers in Microbiology*, *10*(3), 1–15. https://doi.org/10.3389/fmicb.2019.00804

Madhava, E. C., & Armugam, G. (2010). Condition Stabilization for *Pseudomonas aeruginosa* MTCC 5210 to Yield High Titers of Extracellular Anti-Microbial Secondary Metabolite Using Response Surface Methodolgy. *Current Research in Bacteriology*, *3*(4), 197–213.

Mano, H., & Morisaki, H. (2008). Endophytic bacteria in the rice plant. *Microbes and Environments*, *23*(2), 109–117. https://doi.org/10.1264/jsme2.23.109

Marquez-Santacruz, H. A., Hernandez-Leon, R., Orozco-Mosqueda, M. C., Velazquez-Sepulveda, I., & Santoyo, G. (2010). Diversity of bacterial endophytes in roots of Mexican husk tomato plants (*Physalis ixocarpa*) and their detection in the rhizosphere. *Genetics and Molecular Research : GMR*, *9*(4), 2372–2380. https://doi.org/10.4238/vol9-4gmr921

Martínez-Viveros, O., Jorquera, M. A., Crowley, D. E., Gajardo, G., & Mora, M. L. (2010). Mechanisms and practical considerations involved in plant growth promotion by Rhizobacteria. *Journal of Soil Science and Plant Nutrition*, *10*(3), 293–319. https://doi.org/10.4067/S0718-95162010000100006

Miliute, I., Buzaite, O., Baniulis, D., & Stanys, V. (2015). Bakterinių endofitų reikšmė žemės ūkio augalų atsparumui stresui: Apžvalga. *Zemdirbyste*, *102*(4), 465–478. https://doi.org/10.13080/z-a.2015.102.060

Misko, A. L., & Germida, J. J. (2002). Taxonomic and functional diversity of *pseudomonads* isolated from the roots of field-grown canola. *FEMS Microbiology Ecology*, *42*(3), 399–407. https://doi.org/10.1016/S0168-6496(02)00355-0

Mitter, B., Pfaffenbichler, N., Flavell, R., Compant, S., Antonielli, L., Petric, A., Berninger, T., Naveed, M., Sheibani-Tezerji, R., Maltzahn, G. von, & Sessitsch, A. (2017). A new approach to modify plant microbiomes and traits by introducing beneficial bacteria at flowering into progeny seeds. *Frontiers in Microbiology*, *8*(JAN), 1–10. https://doi.org/10.3389/fmicb.2017.00011

Nautiyal, C. S., Bhadauria, S., Kumar, P., Lal, H., Mondal, R., & Verma, D. (2000). Stress induced phosphate solubilization in bacteria isolated from alkaline soils. *FEMS Microbiology Letters*, *182*(2), 291–296. https://doi.org/10.1016/S0378-1097(99)00605-9

Naveed, M., Mitter, B., Reichenauer, T. G., Wieczorek, K., & Sessitsch, A. (2014). Increased drought stress resilience of maize through endophytic colonization by *Burkholderia*

phytofirmans PsJN and *Enterobacter sp.* FD17. *Environmental and Experimental Botany*, *97*, 30–39. https://doi.org/10.1016/j.envexpbot.2013.09.014

Neelja, S., Manish, K., Pawan, K. K., & Jugsharan, S. V. (2015). *MALDI-TOF mass spectrometry : an emerging technology for microbial identification and diagnosis*. *6*(August), 1–16. https://doi.org/10.3389/fmicb.2015.00791

Nikolic, B., Schwab, H., & Sessitsch, A. (2011). Metagenomic analysis of the 1-aminocyclopropane-1-carboxylate deaminase gene (acdS) operon of an uncultured bacterial endophyte colonizing *Solanum tuberosum* L. *Archives of Microbiology*, *193*(9), 665–676. https://doi.org/10.1007/s00203-011-0703-z

Niu, D. D., Liu, H. X., Jiang, C. H., Wang, Y. P., Wang, Q. Y., Jin, H. L., & Guo, J. H. (2011). The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. *Molecular Plant-Microbe Interactions*, *24*(5), 533–542. https://doi.org/10.1094/MPMI-09-10-0213

Ntabo, R. M., Nyamache, A. K., Lwande, W., Kabii, J., & Nonoh, J. (2018). *Enzymatic Activity of Endophytic Bacterial Isolates from Selected Mangrove Plants in Kenya*. 354–363. https://doi.org/10.2174/1874285801812010354

Nxumalo, C. I., Ngidi, L. S., Siyabonga, J., & Shandu, E. (2020). *Isolation of endophytic bacteria from the leaves of Anredera cordifolia CIX1 for metabolites and their biological activities*. 1–11.

Oumer, O. J., & Dawit, A. (2018). *Screening and Molecular Identification of Pectinase Producing Microbes from Coffee Pulp*. *2018*.

Pande, A., Pandey, P., Mehra, S., & Singh, M. (2017). Phenotypic and genotypic characterization of phosphate solubilizing bacteria and their efficiency on the growth of maize. *Journal of Genetic Engineering and Biotechnology*, *15*(2), 379–391. https://doi.org/10.1016/j.jgeb.2017.06.005

Paola, D. D., Gabriella, F., Gianluca, A., Giuseppina, T., Barbara, N., & Annarita, P. (2011). ReUse of Vegetable Wastes as Cheap Substrates for Extremophile Biomass Re-Use of Vegetable Wastes as Cheap Substrates for Extremophile Biomass Production. *Waste Biomass Valor*, *2*(January 2014), 103–111. https://doi.org/10.1007/s12649-011-9062-x

Partida-Martínez, L. P., & Heil, M. (2011). The microbe-free plant: Fact or artifact? *Frontiers in Plant Science*, *2*(DEC), 1–16. https://doi.org/10.3389/fpls.2011.00100

Passari, A. K., Mishra, V. K., Leo, V. V., Gupta, V. K., & Singh, B. P. (2016). Phytohormone production endowed with antagonistic potential and plant growth promoting abilities of culturable endophytic bacteria isolated from *Clerodendrum colebrookianum* Walp. *Microbiological Research*, *193*, 57–73. https://doi.org/https://doi.org/10.1016/j.micres.2016.09.006

Patil, S. V, & Salunkhe, R. B. (2010). Bioflocculant Exopolysaccharide Production by *Azotobacter indicus* Using Flower Extract of *Madhuca latifolia* L. *Appl Biochem*

Biotechnol, *162*, 1095–1108. https://doi.org/10.1007/s12010-009-8820-8

Peñuelas, J., Rico, L., Ogaya, R., Jump, A. S., & Terradas, J. (2012). Summer season and long-term drought increase the richness of bacteria and fungi in the foliar phyllosphere of *Quercus ilex* in a mixed Mediterranean forest. *Plant Biology*, *14*(4), 565–575. https://doi.org/10.1111/j.1438-8677.2011.00532.x

Pieterse, C. M. J., Van Der Does, D., Zamioudis, C., Leon-Reyes, A., & Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology*, *28*, 489–521. https://doi.org/10.1146/annurev-cellbio-092910-154055

Pin, M. A., Magalhaes, J. V, Alves, V. M. C., & Kochian, L. V. (2002). *PP 2002-129-1194.pdf*. *129*(July), 1194–1206. https://doi.org/10.1104/pp.002295.volved

Preston, G. M. (2007). Metropolitan Microbes: Type III Secretion in Multihost Symbionts. *Cell Host and Microbe*, *2*(5), 291–294. https://doi.org/10.1016/j.chom.2007.10.004

Puente, M. E. (2009). *This file was created by scanning the printed publication . Text errors identified by the software have been corrected : however some errors may remain . Endophytic bacteria in cacti seeds can improve the development of cactus seedlings*. *66*, 402–408.

Rakshit, A., Singh, H. B., & Sen, A. (2015). Nutrient use efficiency: From basics to advances. *Nutrient Use Efficiency: From Basics to Advance*, *December*, 1–417. https://doi.org/10.1007/978-81-322-2169-2

Rashid, S., Charles, T. C., & Glick, B. R. (2012). Isolation and characterization of new plant growth-promoting bacterial endophytes. *Applied Soil Ecology*, *61*, 217–224. https://doi.org/10.1016/j.apsoil.2011.09.011

Rave, A. F. G., Kuss, A. V, Peil, G. H. S., Ladeira, S. R., Villarreal, J. P. V, & Nascente, P. S. (2018). Biochemical identification techniques and antibiotic susceptibility profile of lipolytic ambiental bacteria from effluents. *Brazilian Journal of Biology*, *79*(4), 555–565. https://doi.org/https://doi.org/10.1590/1519-6984.05616

Rediers, H., Bonnecarrère, V., Rainey, P. B., Hamonts, K., Vanderleyden, J., & De Mot, R. (2003). Development and Application of a dapB-Based in Vivo Expression Technology System to Study Colonization of Rice by the Endophytic Nitrogen-Fixing Bacterium Pseudomonas stutzeri A15. *Applied and Environmental Microbiology*, *69*(11), 6864–6874. https://doi.org/10.1128/AEM.69.11.6864-6874.2003

Rehman, H. F., Ashraf, A., Muzammil, S., Siddique, M. H., & Ali, T. (2021). *Assessment of zinc solubilization potential of zinc-resistant Pseudomonas oleovorans strain ZSB13 isolated from contaminated soil*. *83*, 1–7. https://doi.org/doi.org/10.1590/1519-6984.240015

Reinhold-Hurek, B., & Hurek, T. (1998). Life in grasses: Diazotrophic endophytes. *Trends in Microbiology*, *6*(4), 139–144. https://doi.org/10.1016/S0966-842X(98)01229-3

Reinhold-Hurek, B., Maes, T., Gemmer, S., Van Montagu, M., & Hurek, T. (2006). An endoglucanase is involved in infection of rice roots by the not-cellulose-metabolizing endophyte Azoarcus Sp. strain BH72. *Molecular Plant-Microbe Interactions*, *19*(2), 181–188. https://doi.org/10.1094/MPMI-19-0181

Roncato-Maccari, L. D. B., Ramos, H. J. O., Pedrosa, F. O., Alquini, Y., Chubatsu, L. S., Yates, M. G., Rigo, L. U., Steffens, M. B. R., & Souza, E. M. (2003). Endophytic Herbaspirillum seropedicae expresses nif genes in gramineous plants. *FEMS Microbiology Ecology*, *45*(1), 39–47. https://doi.org/10.1016/S0168-6496(03)00108-9

Rosenblueth, M., & Martínez-Romero, E. (2004). Rhizobium etli maize populations and their competitivenes for roof colonization. *Archives of Microbiology*, *181*(5), 337–344. https://doi.org/10.1007/s00203-004-0661-9

Rosenblueth, M., & Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions*, *19*(8), 827–837. https://doi.org/10.1094/MPMI-19-0827

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., & Dowling, D. N. (2008). Bacterial endophytes: Recent developments and applications. *FEMS Microbiology Letters*, *278*(1), 1–9. https://doi.org/10.1111/j.1574-6968.2007.00918.x

Samad, A., Trognitz, F., Compant, S., Antonielli, L., & Sessitsch, A. (2017). Shared and host-specific microbiome diversity and functioning of grapevine and accompanying weed plants. *Environmental Microbiology*, *19*(4), 1407–1424. https://doi.org/10.1111/1462-2920.13618

Santi, C., Bogusz, D., & Franche, C. (2013). Biological nitrogen fixation in non-legume plants. *Annals of Botany*, *111*(5), 743–767. https://doi.org/10.1093/aob/mct048

Santoyo, G., Moreno-Hagelsieb, G., del Carmen Orozco-Mosqueda, M., & Glick, B. R. (2016). Plant growth-promoting bacterial endophytes. *Microbiological Research*, *183*, 92–99. https://doi.org/10.1016/j.micres.2015.11.008

Saravanan, V. S., Kumar, M. R., & Sa, T. M. (2011). Bacteria in Agrobiology: Plant Nutrient Management. *Bacteria in Agrobiology: Plant Nutrient Management*. https://doi.org/10.1007/978-3-642-21061-7

Sawai, T., Nakajima, S., Morohoshi, S. Y., & Sciences, P. (1977). Thermolabile Repression of Cephalosporinase Synthesis in Citrobacter freundii. *Microbiol . Immunol.*, *21*(11), 631–638.

Sessitsch, A., Coenye, T., Sturz, A. V., Vandamme, P., Barka, E. A., Salles, J. F., Van Elsas, J. D., Faure, D., Reiter, B., Glick, B. R., Wang-Pruski, G., & Nowak, J. (2005). Burkholderia phytofirmans sp. nov., a novel plant-associated bacterium with plant-beneficial properties. *International Journal of Systematic and Evolutionary Microbiology*, *55*(3), 1187–1192. https://doi.org/10.1099/ijs.0.63149-0

Sessitsch, A., Reiter, B., & Berg, G. (2004). Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. *Canadian*

Journal of Microbiology, *50*(4), 239–249. https://doi.org/10.1139/w03-118

Shahid, I., Han, J., Hardie, D., Noreen, D., Kauser, B., Malik, A., Borchers, C. H., & Mehnaz, S. (2021). Profiling of antimicrobial metabolites of plant growth promoting Pseudomonas spp . isolated from different plant hosts. *3 Biotech*, *11*(48), 1–14. https://doi.org/10.1007/s13205-020-02585-8

Sharma, Poonam. (2014). *Assessment of Zinc solubilization by Endophytic Bacteria in Legume Assessment of Zinc solubilization by Endophytic Bacteria in Legume Rhizosphere Sabhjeet Kaur Navprabhjot Kaur*. *June*.

Sharma, Priyanka, & Thakur, D. (2020). Antimicrobial biosynthetic potential and diversity of culturable soil actinobacteria from forest ecosystems of Northeast India. *Scientific Reports*, *10*(4104), 1–18. https://doi.org/10.1038/s41598-020-60968-6

Sharma, V. K., & Nowak, J. (1998). Enhancement of verticillium wilt resistance in tomato transplants by in vitro co-culture of seedlings with a plant growth promoting rhizobacterium (Pseudomonas sp. strain PsJN). *Canadian Journal of Microbiology*, *44*(6), 528–536. https://doi.org/10.1139/cjm-44-6-528

Sheibani-Tezerji, R., Rattei, T., Sessitsch, A., Trognitz, F., & Mitter, B. (2015). Transcriptome profiling of the endophyte burkholderia phytofirmans psjn indicates sensing of the plant environment and drought stress. *MBio*, *6*(5), 1–11. https://doi.org/10.1128/mBio.00621-15

Sheoran, N., Valiya Nadakkakath, A., Munjal, V., Kundu, A., Subaharan, K., Venugopal, V., Rajamma, S., Eapen, S. J., & Kumar, A. (2015). Genetic analysis of plant endophytic Pseudomonas putida BP25 and chemo-profiling of its antimicrobial volatile organic compounds. *Microbiological Research*, *173*, 66–78. https://doi.org/10.1016/j.micres.2015.02.001

Shi, Y., Yang, H., Zhang, T., Sun, J., & Lou, K. (2014). Illumina-based analysis of endophytic bacterial diversity and space-time dynamics in sugar beet on the north slope of Tianshan mountain. *Applied Microbiology and Biotechnology*, *98*(14), 6375–6385. https://doi.org/10.1007/s00253-014-5720-9

Siciliano, S. D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., Ouellette, D., Roy, R., Whyte, L. G., Banks, M. K., Schwab, P., Lee, K., & Greer, C. W. (2001). Selection of Specific Endophytic Bacterial Genotypes by Plants in Response to Soil Contamination. *Applied and Environmental Microbiology*, *67*(6), 2469–2475. https://doi.org/10.1128/AEM.67.6.2469-2475.2001

Siddeeg, S. M., & Tahoon, M. A. (2020). *Agro-industrial waste materials and wastewater as growth media for microbial bioflocculants production : a review Agro-industrial waste materials and wastewater as growth media for microbial bio fl occulants production : a review*.

Singh, S. (2018). Endophytic Bacteria: an Essential Requirement of Phyto Nutrition. *Nutrition & Food Science International Journal*, *5*(2), 1–5.

https://doi.org/10.19080/nfsij.2018.05.555657

Singh, S. K., Singh, S. K., Tripathi, V. R., Khare, S. K., & Garg, S. K. (2011). Comparative one-factor-at-a-time , response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic Pseudomonas putida SKG-1 isolate. *Microbial Cell Factories*, *10*(114), 1–13.

Siqueira, J. F., & Ro, I. N. (2005). Exploiting Molecular Methods to Explore Endodontic Infections : Part 1 — Current Molecular Technologies for Microbiological Diagnosis. *Journal of Endodentics*, *31*(6), 411–423. https://doi.org/http://dx.doi.org/10.1097/01.don.0000157989.44949.26.

Sorescu, I., & Stoica, C. (2021). Online Advanced Bacterial Identification Software, an Original Tool for Phenotypic Bacterial Identification. *Rom Biotechnol Lett*, *26*(6), 3047–3053. https://doi.org/10.25083/rbl/26.6/3047-3053

Strobel, G. A. (2003). Endophytes as sources of bioactive products. *Microbes and Infection*, *5*(6), 535–544. https://doi.org/10.1016/S1286-4579(03)00073-X

Strobel, G., Daisy, B., Castillo, U., & Harper, J. (2004). Natural Products from Endophytic Microorganisms. *Journal of Natural Products*, *67*(2), 257–268. https://doi.org/10.1021/np030397v

Sugiyama, M. (2015). Structural biological study of self-resistance determinants in antibiotic-producing actinomycetes. *The Journal of Antibiotics*, *68*(February), 543–550. https://doi.org/10.1038/ja.2015.32

Suhandono, S., Kusumawardhani, M. K., & Aditiawati, P. (2016). Isolation and Molecular Identification of Endophytic Bacteria From Rambutan Fruits (Nephelium lappaceum L ... Isolation and Molecular Identi fi cation of Endophytic Bacteria From. *HAYATI Journal of Biosciences*, *February*, 1–6. https://doi.org/10.1016/j.hjb.2016.01.005

Suman, S., & Tanuja. (2021). Isolation and Characterization of a Bacterial Strain Capable of Degrading DDTs Under Aerobic Conditions and Its Use in Bioremediation of Contaminated Soil. *Microbiology Insights*, *14*, 1–7. https://doi.org/10.1177/11786361211024289

Sun, L., Wang, X., & Li, Y. (2016). Increased plant growth and copper uptake of host and non-host plants by metal-resistant and plant growth-promoting endophytic bacteria. *International Journal of Phytoremediation*, *18*(5), 494–501. https://doi.org/10.1080/15226514.2015.1115962

Sun, Y., Cheng, Z., & Glick, B. R. (2009). The presence of a 1-aminocyclopropane-1-carboxylate (ACC) deaminase deletion mutation alters the physiology of the endophytic plant growth-promoting bacterium Burkholderia phytofirmans PsJN. *FEMS Microbiology Letters*, *296*(1), 131–136. https://doi.org/10.1111/j.1574-6968.2009.01625.x

Surette, M. A., Sturz, A. V., Lada, R. R., & Nowak, J. (2003). Bacterial endophytes in processing carrots (Daucus carota L. var. sativus): Their localization, population density, biodiversity and their effects on plant growth. *Plant and Soil*, *253*(2), 381–390.

https://doi.org/10.1023/A:1024835208421

Suryanarayanan, T. S., Kumaresan, V., & Johnson, J. A. (1998). Foliar fungal endophytes from two species of the mangrove Rhizophora . *Canadian Journal of Microbiology*, *44*(10), 1003–1006. https://doi.org/10.1139/w98-087

Syed, Z. H., & Rekha, G. (2019). ANTIMICROBIAL ACTIVITY OF ETHYL ACETATE EXTRACT OF ANTIBIOTIC ISOLATED FROM FRESH WATER ACTINOMYCETES. *INTERNATIONAL JOURNAL OF RESEARCH CULTURE SOCIETY*, *3*(6(A)), 56–61. https://www.researchgate.net/publication/334731932

Thrall, P. H., Hochberg, M. E., Burdon, J. J., & Bever, J. D. (2007). Coevolution of symbiotic mutualists and parasites in a community context. *Trends in Ecology and Evolution*, *22*(3), 120–126. https://doi.org/10.1016/j.tree.2006.11.007

Timmusk, S., Paalme, V., Pavlicek, T., Bergquist, J., Vangala, A., Danilas, T., & Nevo, E. (2011). Bacterial distribution in the rhizosphere of wild barley under contrasting microclimates. *PLoS ONE*, *6*(3), 1–7. https://doi.org/10.1371/journal.pone.0017968

Tomaz, R., Ales, L., Marina, D., & Maja, R. (2007). Isolation of bacterial endophytes from germinated maize kernels. *Canadian Journal of Microbiology*, *52*, 802–808. https://doi.org/10.1139/W07-048

Toubal, S., Bouchenak, O., Elhaddad, D., Yahiaoui, K., Boumaza, S., & Arab, K. (2018). MALDI-TOF MS detection of endophytic bacteria associated with great nettle (urtica dioica l.), grown in Algeria. *Polish Journal of Microbiology*, *67*(1), 67–72. https://doi.org/10.5604/01.3001.0011.6145

Tsegaye, Z., Assefa, F., Tefera, G., Alemu, T., & Gizaw, B. (2018). *Plant Pathology & Microbiology Characterization and Identification of Tef (Eragrostis tef) Seed Endophytic Bacterial Species and Evaluate their Effect on Plant Growth Promotion*. *9*(4). https://doi.org/10.4172/2157-7471.1000438

Tsegaye, Z., Desta, A. F., Tenkegna, T. A., & Assefa, F. (2020). *SCREENING AND BIOCHEMICAL IDENTIFICATION OF TEF (Eragrostis tef Zucc .) Trotter) ENDOPHYTIC BACTERIAL SPECIES WITH PLANT- GROWTH-PROMOTING , BIOTIC AND ABIOTIC STRESS TOLERANCE*. *March*. https://doi.org/10.4038/jsc.v10i2.22

Turner, T. R., James, E. K., & Poole, P. S. (2013). The plant microbiome. *Genome Biology*, *14*(6), 1–10. https://doi.org/10.1186/gb-2013-14-6-209

Uddin, M. N., Al-Harbi, A. H., & Wakabayashi, H. (2008). Optimum Temperatures for the Peak Growth of Some Selected Bacterial Fish Pathogens. *Asian Fisheries Science*, *21*(February), 205–214. https://www.researchgate.net/publication/271846750

Vadassery, J., & Oelmüller, R. (2009). Calcium signaling in pathogenic and beneficial plant microbe interactions: what can we learn from the interaction between Piriformospora indica and Arabidopsis thaliana. *Plant Signaling & Behavior*, *4*(11), 1024–1027. https://doi.org/10.4161/psb.4.11.9800

Vashist, H., Sharma, D., & Gupta, A. (2013). A REVIEW ON COMMONLY USED BIOCHEMICAL TEST FOR BACTERIA. *Innovare Journal of Life Science*, *1*(1), 1–7. https://doi.org/10.1109/ICDM.2013.109

Vijayaraghavan, P., Gnana, S., & Vincent, P. (2013). *A simple method for the detection of protease activity on agar plates using bromocresolgreen dye*. *4*, 628–630.

Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nature Reviews Microbiology*, *10*(12), 828–840. https://doi.org/10.1038/nrmicro2910

Wang, Y., Elzenga, T., & Dirk, J. (2021). Effect of culture conditions on the performance of lignocellulose - degrading synthetic microbial consortia. *Applied Microbiology and Biotechnology*, *105*, 7981–7995. https://doi.org/10.1007/s00253-021-11591-6

Woodland, J. (2004). Chapter 5 Bacteriology. In J. Woodland (Ed.), *NWFHS Laboratory Procedures Manual* (2nd ed., Issue June, pp. 1–44). USFWS.

Woodward, A. W., & Bartel, B. (2005). Auxin: Regulation, action, and interaction. *Annals of Botany*, *95*(5), 707–735. https://doi.org/10.1093/aob/mci083

Yaşinok, A. E., Iffet, F., & Haberal, M. (2008). Isolation of Endopyhtic and Xylanolytic *Bacillus pumilus* Strains from *Zea mays* * *Zea mays* ' da n Endofitik ve Ksilanolitik *Bacillus pumilus* S uşlarının. *TARIM BİLİMLERİ DERGİSİ*, *14*(4), 374–380. https://doi.org/10.1501/Tarimbil

Yousaf, S., Afzal, M., Reichenauer, T. G., Brady, C. L., & Sessitsch, A. (2011). Hydrocarbon degradation, plant colonization and gene expression of alkane degradation genes by endophytic *Enterobacter ludwigii* strains. *Environmental Pollution*, *159*(10), 2675–2683. https://doi.org/10.1016/j.envpol.2011.05.031

Youseif, S. H. (2018). Annals of Agricultural Sciences Genetic diversity of plant growth promoting rhizobacteria and their e ff ects on the growth of maize plants under greenhouse conditions. *Annals of Agricultural Sciences*, *February*, 0–1. https://doi.org/10.1016/j.aoas.2018.04.002

Zachow, C., Fatehi, J., Cardinale, M., Tilcher, R., & Berg, G. (2010). Strain-specific colonization pattern of Rhizoctonia antagonists in the root system of sugar beet. *FEMS Microbiology Ecology*, *74*(1), 124–135. https://doi.org/10.1111/j.1574-6941.2010.00930.x

Zhang, Y. feng, He, L. yan, Chen, Z. jin, Wang, Q. ya, Qian, M., & Sheng, X. fang. (2011). Characterization of ACC deaminase-producing endophytic bacteria isolated from copper-tolerant plants and their potential in promoting the growth and copper accumulation of Brassica napus. *Chemosphere*, *83*(1), 57–62. https://doi.org/10.1016/j.chemosphere.2011.01.041

Zhao, S., Wei, H., Lin, C. Y., Zeng, Y., Tucker, M. P., Himmel, M. E., & Ding, S. Y. (2016). Burkholderia phytofirmans inoculation-induced changes on the shoot cell anatomy and iron accumulation reveal novel components of Arabidopsis-endophyte interaction that can benefit downstream biomass deconstruction. *Frontiers in Plant Science*, *7*(JAN2016),

12. https://doi.org/10.3389/fpls.2016.00024

Zinniel, D. K., Lambrecht, P., Harris, N. B., Feng, Z., Kuczmarski, D., Higley, P., Ishimaru, C. A., Arunakumari, A., Barletta, R. G., & Vidaver, A. K. (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Applied and Environmental Microbiology*, *68*(5), 2198–2208. https://doi.org/10.1128/AEM.68.5.2198-2208.2002

7. Appendices

Appendix 1: Biochemical characteristics and enzymatic activities of endophytic bacteria isolated from *Ocimum lamiifolium*

Plant Species	Bacterial Isolate code	Physiological and Biochemical characteristics											Enzymatic activities				Recommended Genera of Bacteria by ABIS
		Gram staining	Shape	Endospore	Urease	Citrate	Catalase	MR	VP	Motility	H_2S	Indole	Amylase	Cellulase	Pectinase	Protease	
Ocimum lamiifolium	LGOLI4#	-	R	+	+	+	+	+	-	-	+	-	-	-	-	-	*Lysinibacillus sphaericus*
	BGOSI2	+	R	+	+	+	+	+	-	-	+	+	-	-	+	+	*Bacillus niacini*
	BGOSI3	+	R	+	+	+	+	+	-	-	+	+	-	-	+	+	*Bacillus endophyticus*
	EGOSI4	-	R	-	+	+	+	+	-	-	+	-	-	+	-	+	*Leminorella grimonti*
	BGOSI6#	+	R	+	+	+	+	+	-	-	-	-	-	-	+	+	*Bacillus endophyticus*
	BGOSI7	+	R	+	+	+	+	+	-	-	+	+	-	+	+	+	*Lysinibacillus sphaericus*
	EGOSI8	-	R	-	-	+	+	+	-	-	+	-	-	+	+	+	*Leminorella grimontii*
	BGORI4	+	R	+	+	+	+	-	-	-	+	+	-	+	-	+	*Bacillus niacini*
	BGORI5#	-	R	+	+	+	+	+	-	-	+	+	-	-	-	-	*Bacillus niacini*
	BGORI7	+	R	+	+	+	+	+	-	-	-	+	-	-	+	-	*Bacillus niacini*
	BGORI8	+	R	+	+	+	+	+	-	-	+	+	-	-	+	+	*Bacillus endophyticus*
	BGORI9	+	R	+	-	+	+	+	-	-	+	+	+	+	-	+	*Bacillus cereus*
	BGORI11	+	R	+	+	+	+	+	-	-	+	+	-	-	-	+	*Bacillus niacini*
	EGORI12	-	R	+	-	+	+	+	-	-	+	-	-	+	-	-	*Enterobacter hormaechei*
	PGORI13	-	R	-	+	+	+	+	-	-	+	+	-	+	-	+	No matching

Key words: BGOSI/BGORI indicates *Bacillus* Ganda-gara Ocimum Stem Isolate, EGOSI/EGORI indicate *Enterobacter* Ganda-gara Ocimum Stem/Root Isolate, PGORI indicates *Pseudomonas* Ganda-gara Ocimum Root Isolate, # indicates isolates which couldn't be identified by MALDI-TOF MS and are Identified by biochemical method

Appendix 2: Biochemical characteristics and enzymatic activities of endophytic bacteria isolated from *Moringa oleifera*

Plant Species	Bacterial Isolate code	Physiological and Biochemical characteristics											Enzymatic activities				Recommended Genera of bacteria by ABIS
		Gram staining	Shape	Endospore	Urease test	Citrate test	Catalase test	MR test	VP test	Motility	H_2S	Indole test	Amylase test	Cellulase test	Pectinase test	Protease test	
Moringa oleifera	BAMLI1	+	R	+	+	+	+	+	+	-	-	-	-	-	+	-	*Bacillus mycoides*
	CAMLI2	-	R	-	+	+	+	+	-	-	-	-	-	-	+	-	*Citrobacter rodentium*
	BAMSI1	+	R	-	+	-	+	+	+	+	-	-	-	-	-	-	*Bacillus pumulis*
	EAMSI2	-	R	-	+	+	+	+	+	-	-	-	-	-	+	-	*Enterobacter hormaechei*
	EAMSI3*	+	C	-	+	+	-	+	+	-	-	-	+	+	+	-	No matching
	BAMSI4	+	R	+	+	+	+	+	+	-	-	-	-	+	+	-	*Bacillus endophyticus*
	EAMSI5	-	R	-	+	+	-	+	+	-	-	-	-	-	+	-	*Enterobacter hormaechei*
	EAMRI1*	+	C	-	+	-	+	+	+	-	-	-	-	-	+	-	No matching
	EAMRI2	-	R	-	+	+	+	+	+	-	+	+	-	+	-	+	*Raoultella (Klebsiella) ornithinolytica*
	BAMRI3	+	R	-	+	+	-	+	+	-	-	-	-	-	+	-	*Bacillus mycoides*
	BAMRI4	+	R	+	+	-	+	+	+	-	+	-	-	+	+	-	*Bacillus acidiceler*
	CAMRI5	-	R	-	-	+	+	+	+	-	+	-	-	+	+	+	*Leminorella grimontii*
	BAMRI6	+	R	+	+	+	+	+	+	-	-	-	-	+	+	-	*Bacillus licheniformis*
	BAMRI7	+	R	+	-	-	-	+	+	-	-	-	+	+	+	-	*Bacillus anthracis*
	BAMRI8	+	R	+	+	+	+	+	+	+	-	-	-	-	+	-	*Bacillus pumilus (possibility of B. safensis)*

Key words: BAMLI/BAMSI/BAMRI Bacillus ASTU Moringa Leaf/Stem/Root Isolate, CAMLI/CAMRI Citrobacter ASTU Moringa Leaf/Root Isolate, EAMSI/EAMRI Enterobacter ASTU Moringa Stem/Root Isolate, The * sign indicates Enterococcus genus

Appendix 3: Biochemical characteristics and enzymatic activities of endophytic bacteria isolated from *Artemisia annua*

Plant Species	Bacterial Isolate code	Physiological and Biochemical characteristics										Enzymatic activities				Recommended Genera of bacteria by ABIS
		Grams staining	Shape	Endospore test	Urease test	Citrate test	Catalase test	MR test	VP test	Motility	H_2S	Indole test	Amylase test	Cellulase test	Pectinase test	Protease test	
Artemisia annua	BAALI1	+	R	+	+	+	+	-	+	-	-	-	-	-	+	-	*Bacillus endophyticus*
	EAALI2*	+	R	-	+	+	+	+	+	-	-	-	-	-	+	+	*Enterococcus faecalis*
	EAASI1	-	R	-	-	+	+	-	+	-	-	-	-	-	+	-	*Enterobacter cancergenus*
	PAASI2#	+	R	-	+	+	+	+	+	+	-	-	-	-	+	+	*Paenibacillus pectinilyticus*
	BAASI3#	+	R	-	+	+	-	-	+	-	-	-	+	-	-	-	*Bacillus aeolius*
	EAASI4	-	R	-	+	+	+	-	+	+	-	-	-	-	+	-	*Enterobacter cloacae*
	BAASI5	+	R	+	-	+	+	-	+	-	-	-	-	+	+	-	*Bacillus endophyticus*
	BAARI1	+	R	+	+	+	-	-	+	-	-	-	+	+	-	-	*Bacillus aeolius*
	EAARI2	-	R	-	+	+	+	-	+	-	-	-	+	+	+	-	*Enterobacter cloacae*
	EAARI3	-	R	-	+	+	+	-	+	+	-	-	+	+	+	-	*Enterobacter cloacae*
	BAARI4	+	R	+	+	+	+	-	+	-	-	-	+	+	+	+	*Bacillus mycoides*
	EAARI5	-	R	-	+	+	+	+	+	-	-	-	+	+	+	+	*Enterobacter cloacae*
	EAARI6	-	R	-	+	+	+	+	+	-	-	-	+	+	+	+	*Raoultella planticola*
	EAARI7	-	R	-	+	+	-	-	+	-	-	-	-	+	-	+	*Klebsiella singaporensis*
	RPAAI8	-	R	-	+	+	+	-	+	-	-	-	+	+	+	+	*Enterobacter cloacae*

Key words: BAALI/BAASI/BAARI Bacillus ASTU Artemisia Leaf/Stem/Root Isolate, EAALI/EAASI/EAARI Enterobacter ASTU Artemisia Leaf/Stem/Root Isolate, The * sign indicates Enterococcus genus, The # sign indicates isolates which could not be identified by MALDI-TOF MS and were identified by biochemical methods

Appendix 4: Clearing index of pectinase producing isolates

Pectinase Producers	Bacterial Genera	Colony Size (mm)	Clear Zone (mm)	Clearing index
BGOSI3	*Bacillus subtilis*	5mm	14mm	3.8
BGOSI6#	*Bacillus endophyticus*	5mm	12mm	3.4
BGOSI7	*Bacillus cereus*	5mm	12mm	3.4
EGOSI8	*Enterobacter cloacae*	5mm	14mm	3.8
BGORI7	*Bacillus cereus*	5mm	14mm	3.8
BGORI8	*Bacillus cereus*	5mm	18mm	4.6
BAMLI1	*Bacillus cereus*	5mm	9mm	2.8
CAMLI2	*Citrobacter freundii*	5mm	18mm	4.6
EAMSI2	*Enterobacter cloacae*	5mm	18mm	4.6
EAMSI3*	*Enterococcus faecium*	5mm	16mm	4.2
BAMSI4	*Bacillus cereus*	5mm	16mm	4.2
EAMSI5	*Enterobacter cloacae*	5mm	18mm	4.6
EAMRI1*	*Enterococcus faecium*	5mm	14mm	3.8
BAMRI3	*Bacillus subtilis*	5mm	16mm	4.2
BAMRI4	*Bacillus cereus*	5mm	10mm	3
CAMRI5	*Citrobacter freundii*	5mm	12mm	3.4
BAMRI6	*Bacillus cereus*	5mm	20mm	5
BAMRI7	*Bacillus cereus*	5mm	15mm	4
BAMRI8	*Bacillus cereus*	5mm	20mm	5
BAALI1	*Bacillus subtilis*	5mm	18mm	4.6
EAALI2	*Enterococcus faecium*	5mm	18mm	4.6
EAASI1	*Enterobacter asburiae*	5mm	22mm	5.4
PAASI2#	*Paenibacillus pectinilyticus*	5mm	24mm	5.8
EAASI4	*Enterobacter asburiae*	5mm	22mm	5.4
BAASI5	*Bacillus cereus*	5mm	23mm	5.6
EAARI2	*Enterobacter cloacae*	5mm	26mm	6.2
EAARI3	*Enterobacter asburiae*	5mm	28mm	6.6
BAARI4	*Bacillus subtilis*	5mm	25mm	6
EAARI5	*Enterobacter ludwigii*	5mm	28mm	6.6
EAARI6	*Enterobacter cloacae*	5mm	24mm	5.8
RPAAI8	*Enterobacter cloacae*	5mm	23mm	5.6

Key words: **Key words**: **BGOSI/BGORI** indicates **B**acillus **G**anda-gara **O**cimum **S**tem **I**solate, **EGOSI/EGORI** indicate **E**nterobacter **G**anda-gara **O**cimum **S**tem/**R**oot **I**solate, **PGORI** indicates **P**seudomonas **G**anda-gara **O**cimum **R**oot **I**solate, **BAMLI**/**BAMSI**/**BAMRI** **B**acillus **A**STU **M**oringa **L**eaf/**S**tem/**R**oot **I**solate, **CAMLI**/**CAMRI** **C**itrobacter **A**STU **M**oringa **L**eaf/**R**oot **I**solate, **EAMSI**/**EAMRI** **E**nterobacter **A**STU **M**oringa **S**tem/**R**oot **I**solate, **BAALI**/**BAASI**/**BAARI** **B**acillus **A**STU

Artemisia **L**eaf/**S**tem/**R**oot **I**solate, **EAALI/EAASI/EAARI E**nterobacter **A**STU **A**rtemisia **L**eaf/**S**tem/**R**oot **I**solate, **PAASI**[#] **P**aenibacillus **A**STU **A**rtemisia **S**tem **I**solate, the * sign indicates Enterococcus genus, the # sign indicates isolates which couldn't be identified by MALDI-TOF MS and are Identified by biochemical method

Appendix 5: Clearing index of protease producing isolates

Protease producers	Bacterial Genera	Colony Size (mm)	Clear Zone (mm)	Clearing Index
BGOSI3	*Bacillus subtilis*	6mm	14mm	3.33
EGOSI4	*Enterobacter ludwigii*	6mm	10mm	2.66
BGOSI6#	*Bacillus endophyticus*	6mm	11mm	2.83
BGOSI7	*Bacillus cereus*	5mm	10mm	3
EGOSI8	*Enterobacter cloacae*	5mm	8mm	2.6
BGORI4	*Bacillus subtilis*	6mm	11mm	2.83
BGORI8	*Bacillus cereus*	6mm	23mm	4.83
BGORI9	*Bacillus cereus*	6mm	18mm	4
BGORI11	*Bacillus cereus*	5mm	12mm	3.4
PGORI13	*Pseudomonas monteilli*	6mm	20mm	4.33
EAMRI2	*Enterobacter kobei*	6mm	11mm	2.83
CAMRI5	*Citrobacter freundii*	6mm	15mm	3.5
EAALI2*	*Enterococcus faecium*	5mm	10mm	3
PAASI2#	*Paenibacillus pectinilyticus*	5mm	8mm	2.6
BAARI4	*Bacillus subtilis*	5mm	10mm	3
EAARI5	*Enterobacter ludwigii*	6mm	11mm	2.83
EAARI6	*Enterobacter cloacae*	6mm	14mm	3.33
EAARI7	*Enterobacter kobei*	5mm	18mm	4.6
RPAAI8	*Enterobacter cloacae*	6mm	13mm	3.16

Key words: **Key words**: **BGOSI/BGORI** indicates **B**acillus **G**anda-gara **O**cimum **S**tem **I**solate, **EGOSI/EGORI** indicate **E**nterobacter **G**anda-gara **O**cimum **S**tem/**R**oot **I**solate, **PGORI** indicates **P**seudomonas **G**anda-gara **O**cimum **R**oot **I**solate, **BAMLI/BAMSI/BAMRI** **B**acillus **A**STU **M**oringa **L**eaf/**S**tem/**R**oot **I**solate, **CAMLI/CAMRI** **C**itrobacter **A**STU **M**oringa **L**eaf/**R**oot **I**solate, **EAMSI/EAMRI** **E**nterobacter **A**STU **M**oringa **S**tem/**R**oot **I**solate, **BAALI/BAASI/BAARI** **B**acillus **A**STU **A**rtemisia **L**eaf/**S**tem/**R**oot **I**solate, **EAALI/EAASI/EAARI** **E**nterobacter **A**STU **A**rtemisia **L**eaf/**S**tem/**R**oot **I**solate, **PAASI#** **P**aenibacillus **A**STU **A**rtemisia **S**tem **I**solate, the * sign indicates Enterococcus genus, the # sign indicates isolates which couldn't be identified by MALDI-TOF MS and are Identified by biochemical method

Appendix 6: Phosphate solubilization index of the isolates

Phosphate Solubilizers	Bacterial Genera	Colony Size (mm)	Clear Zone (mm)	Phosphate Solubilization Index
LGOLI4[#]	*Lysinibacillus sphaericus*	6mm	14mm	3.33
BGOSI3	*Bacillus subtilis*	5mm	10mm	3
EGOSI4	*Enterobacter ludwigii*	6mm	22mm	4.66
BGOSI6[#]	*Bacillus endophyticus*	6mm	18mm	4
BGOSI7	*Bacillus cereus*	5mm	7mm	2.4
EGOSI8	*Enterobacter cloacae*	5mm	8mm	2.6
BGORI4	*Bacillus subtilis*	5mm	18mm	4.6
BGORI8	*Bacillus cereus*	6mm	22mm	4.66
BGORI9	*Bacillus cereus*	5mm	14mm	3.8
BGORI11	*Bacillus cereus*	6mm	20mm	4.33
EGORI12	*Enterobacter cloacae*	6mm	15mm	3.5
PGORI13	*Pseudomonas monteilli*	5mm	24mm	5.8
BAMLI1	*Bacillus cereus*	5mm	7mm	2.4
BAMSI1	*Bacillus cereus*	5mm	10mm	3
EAMRI2	*Enterobacter kobei*	6mm	24mm	5
BAMRI4	*Bacillus cereus*	5mm	8mm	2.6
CAMRI5	*Citrobacter freundii*	6mm	22mm	4.66
BAMRI6	*Bacillus cereus*	5mm	8mm	2.6
BAMRI8	*Bacillus cereus*	6mm	18mm	4
EAALI2[*]	*Enterococcus faecium*	6mm	12mm	3
PAASI2[#]	*Paenibacillus pectinilyticus*	6mm	12mm	3
BAASI3[#]	*Bacillus aeolius*	6mm	13mm	3.16
BAASI5	*Bacillus cereus*	6mm	10mm	2.66
BAARI1	*Bacillus subtilis*	6mm	14mm	3.33
BAARI4	*Bacillus subtilis*	8mm	12mm	2.5
EAARI7	*Enterobacter kobei*	6mm	13mm	3.16
RPAAI8	*Enterobacter cloacae*	8mm	13mm	2.62

Key words: Key words: BGOSI/BGORI indicates *Bacillus* Ganda-gara Ocimum Stem Isolate, EGOSI/EGORI indicate *Enterobacter* Ganda-gara Ocimum Stem/Root Isolate, PGORI indicates *Pseudomonas* Ganda-gara Ocimum Root Isolate, BAMLI/BAMSI/BAMRI *Bacillus* ASTU Moringa Leaf/Stem/Root Isolate, CAMLI/CAMRI *Citrobacter* ASTU Moringa Leaf/Root Isolate, EAMSI/EAMRI *Enterobacter* ASTU Moringa Stem/Root Isolate, BAALI/BAASI/BAARI *Bacillus* ASTU *Artemisia* Leaf/Stem/Root Isolate, EAALI/EAASI/EAARI *Enterobacter* ASTU *Artemisia* Leaf/Stem/Root Isolate, LGOLI Lysinibacillus Ganda-gara Ocimum Leaf Isolate, PAASI[#] Paenibacillus ASTU Artemisia Stem

Isolate, the * sign indicates Enterococcus genus, the # sign indicates isolates which couldn’t be identified by MALDI-TOF MS and are Identified by biochemical method

Appendix 7: Zinc solubilization index of the isolates

Zinc Solubilizers	Bacterial Genera	Colony Size (mm)	Clear Zone (mm)	Zinc Solubilization Index
LGOLI4#	*Lysinibacillus sphaericus*	6mm	8mm	2.33
BGOSI3	*Bacillus subtilis*	5mm	8mm	2.6
EGOSI4	*Enterobacter ludwigii*	6mm	8mm	2.33
BGOSI6#	*Bacillus endophyticus*	6mm	8mm	2.33
BGOSI7	*Bacillus cereus*	6mm	10mm	2.66
EGOSI8	*Enterobacter cloacae*	5mm	9mm	2.8
BGORI4	*Bacillus subtilis*	5mm	10mm	3
BGORI9	*Bacillus cereus*	6mm	11mm	2.83
EGORI12	*Enterobacter cloacae*	6mm	8mm	2.33
PGORI13	*Pseudomonas monteilli*	5mm	14mm	3.8
BAMSI1	*Bacillus cereus*	5mm	7mm	2.4
EAMSI2	*Enterobacter cloacae*	6mm	9mm	2.5
EAMSI3*	*Enterococcus faecium*	6mm	12mm	3
EAMRI2	*Enterobacter kobei*	6mm	10mm	2.66
CAMRI5	*Citrobacter freundii*	6mm	14mm	3.33
EAASI1	*Enterobacter asburiae*	5mm	14mm	3.8
EAASI4	*Enterobacter asburiae*	6mm	10mm	2.66
EAARI2	*Enterobacter cloacae*	5mm	8mm	2.6
EAARI3	*Enterobacter asburiae*	5mm	8mm	2.6
EAARI5	*Enterobacter ludwigii*	5mm	8mm	2.6
RPAAI8	*Enterobacter cloacae*	6mm	25mm	5.16

Key words: Key words: BGOSI/BGORI indicates Bacillus Ganda-gara Ocimum Stem Isolate, EGOSI/EGORI indicate Enterobacter Ganda-gara Ocimum Stem/Root Isolate, PGORI indicates Pseudomonas Ganda-gara Ocimum Root Isolate, BAMSI Bacillus ASTU Moringa Stem Isolate, CAMLI/CAMRI Citrobacter ASTU Moringa Leaf/Root Isolate, EAMSI/EAMRI Enterobacter ASTU Moringa Stem/Root Isolate, EAASI/EAARI Enterobacter ASTU Artemisia Stem/Root Isolate, LGOLI Lysinibacillus Ganda-gara Ocimum Leaf Isolate , The * sign indicates Enterococcus genus, the # sign indicates isolates which couldn't be identified by MALDI-TOF MS and are identified by biochemical method

Bruker MALDI Biotyper

Identification Results

Appendix 8: MALDI-TOF MS identification result

Run info:

Run identifier: 211101-1557-1011027865

Comment: Bacteria ID – samples from Adama (any bacteria)

Muktar

Operator: tof-user@MBT-WIN10

Run creation date/Time: 2021-11-01T16:20:28.830

Number of tests: 44

Type: Standard

BTS-QC passed

BTS-QC Position D8:0

Instrument ID: 8604832.05381

Server version 4.1.100 (PYTH) 174 2019-06-158_01-16-09

Result overview

Sample Name	Sample ID	Organisms (best match)	Score Value	Organisms (second best match)	Score value
A1 (-) (C)	30719 (Standard)	*Bacillus cereus*	2.02	*Bacillus cereus*	1.93
A2 (+++) (A)	30697 (Standard)	*Bacillus cereus*	2.03	*Bacillus mycoides*	1.73
A3 (+) (B)	30687 (Standard)	*Bacillus cereus*	1.89	*Bacillus cereus*	1.76
A4 (+) (B)	30712 (Standard)	*Enterobacter kobei*	1.81	*Enterobacter asburiae*	1.79
A5 (-) (C)	30693 (Standard)	*Bacillus cereus*	2.03	*Bacillus cereus*	1.91
A6 (+++) (A)	30683 (Standard)	*Bacillus cereus*	2.12	*Bacillus cereus*	1.91

Result overview table-continued on next page

Result overview table-continued from previous page

Sample Name	Sample ID	Organisms (best match)	Score Value	Organisms (second best match)	Score value
A7 (+) (B)	30716 (Standard)	*Bacillus subtilis*	1.81	*Bacillus subtilis*	1.79
A8 (+++) (A)	30706 (Standard)	*Bacillus subtilis*	2.16	*Bacillus subtilis*	2.10
A9 (+++) (B)	30708 (Standard)	*Enterobacter asburiae*	2.09	*Enterobacter ludwigii*	2.03
A10 (+) (B)	30707 (Standard)	*Enterobacter cloacae*	1.87	*Enterobacter bugandesis*	1.83
A11 (+++) (A)	30686 (Standard)	*Bacillus subtilis*	2.21	*Bacillus subtilis*	2.10
A12 (+) (B)	30699 (Standard)	*Bacillus subtilis*	1.90	*Bacillus subtilis*	1.88
B1 (+++) (A)	30718 (Standard)	*Citrobacter freundii*	2.11	*Citrobacter freundii*	1.98
B2 (-) (C)	30682 (Standard)	**No Organism Identification Possible**	1.48	**No Organism Identification Possible**	1.48
B3 (+++) (A)	30709 (Standard)	*Bacillus subtilis*	2.11	*Bacillus subtilis*	2.02
B4 (+++) (B)	30698 (Standard)	*Enterobacter cloacae*	2.22	*Enterobacter ludwigii*	2.18
B5 (+) (B)	30717 (Standard)	*Bacillus cereus*	1.81	**No Organism Identification Possible**	1.60
B6 (+++) (B)	30685 (Standard)	*Enterobacter asburiae*	2.21	*Enterobacter ludwigii*	2.14
B7 (+) (B)	30678 (Standard)	*Bacillus cereus*	1.94	*Bacillus cereus*	1.86
B8 (+++) (A)	30689 (Standard)	*Bacillus cereus*	2.06	*Bacillus cereus*	1.86

Result overview table-continued on next page

Result overview table-continued from previous page					
Sample Name	**Sample ID**	**Organisms (best match)**	**Score Value**	**Organisms (second best match)**	**Score value**
B9 (+++) (A)	30700 (Standard)	*Enterococcus faecium*	2.27	*Enterococcus faecium*	2.24
B10 (+++) (B)	30681 (Standard)	*Enterobacter ludwigii*	2.06	*Enterobacter kobei*	2.04
B11 (+) (A)	30701 (Standard)	*Enterobacter asburiae*	2.07	*Enterobacter kobei*	2.02
B12 (+) (B)	30705 (Standard)	*Bacillus cereus*	1.73	**No Organism Identification Possible**	1.56
C1 (+) (B)	30715 (Standard)	*Enterobacter kobei*	1.92	*Enterobacter asburiae*	1.87
C2 (+++) (A)	30692 (Standard)	*Baciilus cereus*	2.12	*Bacillus cereus*	1.94
C3 (+++) (A)	30680 (Standard)	*Bacillus subtilis*	2.07	*Bacillus subtilis*	2.04
C4 (+++) (A)	30713 (Standard)	*Enterobacter cloacae*	2.09	*Enterobacter ludwigii*	1.99
C5 (+) (B)	30694 (Standard)	*Bacillus cereus*	1.82	Bacillus cereus	1.75
C6 (+++) (A)	30679 (Standard)	*Bacillus cereus*	2.08	Bacillus cereus	2.04
C7 (+++) (A)	30696 (Standard)	*Enterococcus faecium*	2.03	Enterococcus faecium	1.99
C8 (+) (B)	30688 (Standard)	*Bacillus cereus*	1.81	**No Organism Identification Possible**	1.58
C9 (-) (C)	30702 (Standard)	**No Organism Identification Possible**	1.52	**No Organism Identification Possible**	1.49
C10 (+++) (B)	30711 (Standard)	*Enterobacter cloacae*	2.10	*Enterobacter ludwigii*	2.03
C11 (+++) (B)	30704 (Standard)	*Enterobacter asburiae*	2.22	*Enterobacter kobei*	1.75

C12 (+++) (B)	30695 (Standard)	*Enterobacter cloacae*	2.26	*Enterobacter asburiae*	2.10
D1 (+++) (A)	ALI2 D1 (Standard)	*Citrobacter freundii*	2.18	*Citrobacter freundii*	2.09
D2 (-) (C)	30703 (Standard)	**No Organism Identification Possible**	1.40	**No Organism Identification Possible**	1.40
D3 (+++) (A)	30690 (Standard)	*Pseudomonas monteilii*	2.02	*Psedudomonas putida*	1.97
D4 (+++) (A)	30684 (Standard)	*Enterobacter cloacae*	2.18	*Enterobacter ludwigii*	1.92
D5 (+++) (A)	30714 (Standard)	*Enterococcus faecium*	2.26	*Enterococcus faecium*	2.15
D6 (+) (B)	30710 (Standard)	*Enterobacter ludwigii*	1.79	*Enterobacter cloacae*	1.79
D7 (+) (B)	ARI6 D7 (Standard)	*Bacillus cereus*	1.85	*Bacillus cereus*	1.78
D8 (+++) (A)	BTS (BTS)	*Escherichia coli*	2.28	*Escherichia coli*	2.27

Key:

Range	Interpretation	Symbol	Colour
2.00-3.00	High confidence identification	+++	Green
1.70-1.99	Low confidence identification	+	Yellow
0.00-1.69	No organism identification possible	-	Red

Appendix 9: Effect of temperature on growth of isolated endophytic bacteria

Tem	Isolates	Biomass (CDW, g/L)						OD					
		R1	R2	R3	AV	SD	SEM	R1	R2	R3	AV	SD	SEM
25	CAMRI5	0.0051	0.0033	0.0042	0.0042	0.0009	0.00052	1.000692	1.100096	1.001988	1.034259	0.057021	0.032921
25	RPAAI 8	0.004	0.0058	0.0046	0.0048	0.000917	0.000529	1.600692	1.400492	1.426213	1.475799	0.108922	0.062886
25	PGORI 13	0.0068	0.006	0.0066	0.006467	0.000416	0.00024	1.699	1.608	1.597	1.634766	0.055885	0.032265
30	CAMRI 5	0.0056	0.0066	0.0068	0.006333	0.000643	0.000371	1.232	1.226	1.186	1.214667	0.025007	0.014438
30	RPAAI 8	0.073	0.075	0.069	0.072333	0.003055	0.001764	1.708	1.562	1.600692	1.623564	0.07564	0.043671
30	PGORI 13	0.0071	0.0064	0.0069	0.0068	0.000361	0.000208	1.707	1.611	1.617	1.645	0.053777	0.031048
35	CAMRI 5	0.0056	0.0028	0.0061	0.004833	0.001779	0.001027	1.102	1.022	1.032	1.052	0.043589	0.025166
35	RPAAI 8	0.0035	0.0054	0.0025	0.0038	0.001473	0.00085	1.208	1.188	1.102	1.166	0.056321	0.032517
35	PGORI 13	0.0016	0.0023	0.0019	0.001933	0.000351	0.000203	0.898	0.982	0.803	0.894333	0.089556	0.051705
37	CAMRI 5	0.0016	0.0026	0.002	0.002067	0.000503	0.000291	0.855	0.88	0.877	0.870667	0.01365	0.007881
37	RPAAI 8	0.001	0.0011	0.0027	0.0016	0.000954	0.000551	0.996	0.966	0.951	0.971	0.022913	0.013229
37	PGORI 13	0.0025	0.0029	0.002	0.002467	0.000451	0.00026	0.992	1.021	0.892	0.968452	0.067816	0.039154
40	CAMRI 5	0.0011	0.0008	0.0017	0.0012	0.000458	0.000265	0.8	0.799	0.803	0.800667	0.002082	0.001202
40	RPAAI 8	0.0007	0.0013	0.002	0.001333	0.000651	0.000376	0.899	0.859	0.819	0.859	0.04	0.023094
40	PGORI 13	0.0019	0.001	0.0015	0.001467	0.000451	0.00026	0.323	0.316	0.293	0.310667	0.015695	0.009062
45	CAMRI 5	0.0011	0.0008	0.0017	0.0012	0.000458	0.000265	0.833	0.85	0.839	0.840667	0.008622	0.004978
45	RPAAI 8	0.0007	0.0013	0.002	0.001333	0.000651	0.000376	0.759	0.716004	0.724054	0.733019	0.022857	0.013197
45	PGORI 13	0.0007	0.0009	0.0008	0.0008	0.0001	5.77E-05	0.093	0.098	0.097	0.0961	0.002707	0.001563

Appendix 10: Effect of pH on growth of isolated endophytic bacterial isolates

pH	Isolates	Biomass						OD					
		R1	R2	R3	AV	SD	SEM	R1	R2	R3	AV	SD	SEM
6.5	CAMRI 5	0.0019	0.0013	0.002	0.001733	0.000379	0.000219	1.286	1.272497	1.286249	1.281582	0.007869	0.004543
6.5	RPAAI 8	0.0045	0.0051	0.0065	0.005367	0.001026	0.000593	1.807	1.821	1.828	1.818667	0.010693	0.006173
6.5	PGORI 13	0.0068	0.006	0.0066	**0.006467**	0.000416	0.00024	1.699	1.608	1.597299	1.634766	0.055885	0.032265
7	CAMRI 5	0.0029	0.0018	0.0019	0.0022	0.000608	0.000351	1.29	1.275	1.284	1.283	0.00755	0.004359
7	RPAAI 8	0.0041	0.0027	0.0022	0.003	0.000985	0.000569	1.706	1.696	1.692	1.698	0.007211	0.004163
7	PGORI 13	0.0137	0.0143	0.0139	**0.013967**	0.000306	0.000176	1.628	1.64	1.639	1.635667	0.006658	0.003844
7.5	CAMRI 5	0.0007	0.0011	0.0006	0.0008	0.000265	0.000153	1.206011	1.207	1.206	1.206337	0.000574	0.000331
7.5	RPAAI 8	0.0031	0.0021	0.0032	0.0028	0.000608	0.000351	1.682	1.677	1.687	1.682	0.005	0.002887
7.5	PGORI 13	0.0167	0.0178	0.0156	0.0167	0.0011	0.000635	1.686	1.696	1.674	1.685333	0.011015	0.00636
8	CAMRI 5	0.0077	0.0032	0.0069	0.005933	0.002401	0.001386	1.526	1.517	1.524	1.522333	0.004726	0.002728
8	RPAAI 8	0.0012	0.0017	0.0014	0.001433	0.000252	0.000145	1.616	1.632	1.613	1.620333	0.010214	0.005897
8	PGORI 13	0.0092	0.0099	0.0095	0.009533	0.000351	0.000203	1.588	1.605	1.599	1.597333	0.008622	0.004978

Appendix 11: Effect of Carbon and Nitrogen sources on isolate CAMRI5

CS for CAMRI-5	Biomass (CDW, g/L)						OD at 600nm					
	R1	R2	R3	AV	SD	SEM	R1	R2	R3	AV	SD	SEM
Mannitol	0.0017	0.0015	0.0016	0.0016	1E-04	5.7735E-05	0.712	0.71	0.712	0.711333333	0.001155	0.000667
Fructose	0.0024	0.002	0.0021	0.002166667	0.000208167	0.000120185	0.813	0.809	0.812	0.811333333	0.002082	0.001202
Glucose	0.0038	0.0039	0.004	0.0039	0.0001	5.7735E-05	0.943	0.945	0.946	0.944666667	0.001528	0.000882
Sucrose	0.0013	0.001	0.001	0.0011	0.000173205	0.0001	0.655	0.653	0.653	0.653666667	0.001155	0.000667
NS for CAMRI-5	Biomass (CDW, g/L)						OD at 600nm					
	R1	R2	R3	AV	SD	SEM	R1	R2	R3	AV	SD	SEM
KNO3	0.0052	0.0042	0.0058	0.005066667	0.00080829	0.000466667	0.150	0.161	0.178	0.163713739	0.014337	0.008277
$(NH_4)_2SO_4$	0.0012	0.0026	0.0024	0.002066667	0.000757188	0.000437163	0.0548	0.078	0.082	0.071601697	0.014683	0.008477
Peptone	0.0053	0.0049	0.0069	0.0057	0.001058301	0.00061101	1.054	1.116	1.11	1.093333333	0.034196	0.019743
YE	0.0048	0.0053	0.0037	0.0046	0.000818535	0.000472582	0.567	0.564	0.559	0.563333333	0.004041	0.002333

Appendix 12: Effect of carbon and nitrogen sources on isolate RPAAI8

CS for RPAAI8	Biomass (CDW, g/L)						OD at 600nm					
	R1	R2	R3	AV	SD	SEM	R1	R2	R3	AV	SD	SEM
Mannitol	0.0017	0.0018	0.0015	0.001666667	0.000152753	8.81917E-05	0.623	0.624	0.62	0.622333333	0.002082	0.006973
Fructose	0.002	0.0022	0.0024	0.0022	0.0002	0.00011547	0.792	0.795	0.796	0.794333333	0.002082	0.005777
Glucose	0.0034	0.0039	0.0037	0.003666667	0.000251661	0.000145297	0.979	0.982	0.98	0.980333333	0.001528	0.002856
Sucrose	0.0013	0.001	0.001	0.0011	0.000173205	0.0001	0.557	0.554	0.554	0.555	0.001732	0.002758
NS for RPAAI8	Biomass (CDW, g/L)						OD at 600nm					
	R1	R2	R3	AV	SD	SEM	R1	R2	R3	AV	SD	SEM
KNO3	0.0049	0.0011	0.0023	0.002766667	0.001942507	0.001121507	0.192	0.169	0.183	0.181333333	0.01159	0.006692
(NH4)2SO4	0.0021	0.0057	0.0019	0.003233333	0.002138535	0.001234684	0.927	0.976	0.984	0.962333333	0.03086	0.017817
Peptone	0.0044	0.0027	0.0022	0.0031	0.001153256	0.000665833	1.178	1.172	1.189	1.179666667	0.008622	0.004978
Yeast extract	0.0016	0.0075	0.0035	0.0042	0.003011644	0.001738774	0.951	1.065	1.064	1.026666667	0.065531	0.037834

Appendix 13: Effect of carbon and nitrogen sources on isolate PGORI13

CS for PGORI13	Biomass (CDW, g/L)						OD at 600nm					
	R1	R2	R3	AV	SD	SEM	R1	R2	R3	AV	SD	SEM
Mannitol	0.0019	0.002	0.0018	0.0019	0.0001	5.7735E-05	0.651	0.653	0.651	0.651666667	0.001155	0.000667
Fructose	0.0026	0.0028	0.0026	0.002666667	0.00011547	6.66667E-05	0.811	0.813	0.812	0.812	0.001	0.000577
Glucose	0.0038	0.0036	0.0038	0.003733333	0.00011547	6.66667E-05	0.892	0.891	0.892	0.891666667	0.000577	0.000333
Sucrose	0.001	0.0009	0.001	0.000966667	5.7735E-05	3.33333E-05	0.593	0.592	0.593	0.592666667	0.000577	0.000333
NS for PGORI13	Biomass (CDW, g/L)						OD at 600nm					
	R1	R2	R3	AV	SD	SEM	R1	R2	R3	AV	SD	SEM
KNO3	0.0022	0.0019	0.0015	0.001866667	0.000351188	0.000202759	0.004	0.003	0.004	0.003666667	0.000577	0.000333
$(NH_4)_2SO_4$	0.006	0.0025	0.004	0.004166667	0.001755942	0.001013794	0.055	0.054	0.052	0.053666667	0.001528	0.000882
Peptone	0.0085	0.0092	0.0145	0.010733333	0.003280752	0.001894143	1.12	1.119	1.139	1.126	0.011269	0.006506
YE	0.0056	0.0033	0.0025	0.0038	0.001609348	0.000929157	0.423	0.445	0.466	0.444666667	0.021502	0.012414

Appendix 14: Cheap Agro-waste as carbon source for RPAAI8, CAMRI5 and PGORI13

Cheap Carbon source (RPAAI8)	Biomass (g/l)						OD at 600nm					
	Reading 1	Reading 2	Reading 3	AV	SD	SEM	Reading 1	Reading 2	Reading 3	AV	SD	SEM
Teff Bran	0.0045	0.0048	0.0041	0.004467	0.000351	0.000203	0.908	0.904	0.904	0.905333	0.002309	0.001333
Corn Cob	0.0055	0.0059	0.0059	0.005767	0.000231	0.000133	1.157	1.166	1.164	1.162333	0.004726	0.002728
Vegetable Waste	0.0022	0.0024	0.0016	0.002067	0.000416	0.00024	0.493	0.497	0.509	0.499667	0.008327	0.004807

Cheap Carbon source (CAMRI5)	Reading 1	Reading 2	Reading 3	AV	SD	SEM		Reading 1	Reading 2	Reading 3	AV	SD	SEM
Teff Bran	0.0042	0.0038	0.0039	0.003967	0.000208	0.00012		0.9	0.895	0.895	0.896667	0.002887	0.001667
Corn Cob	0.0027	0.0026	0.0027	0.002667	5.77E-05	3.33E-05		0.81	0.799	0.809	0.806	0.006083	0.003512
Vegetable Waste	0.0017	0.002	0.0021	0.001933	0.000208	0.00012		0.713	0.719	0.724	0.718667	0.005508	0.00318

Cheap Carbon source (PGORI13)	Reading 1	Reading 2	Reading 3	AV	SD	SEM		Reading 1	Reading 2	Reading 3	AV	SD	SEM
Teff Bran	0.0051	0.0049	0.005	0.005	0.0001	5.77E-05		1.103	1.093	1.098	1.098	0.005	0.002887
Corn Cob	0.0059	0.0055	0.0057	0.0057	0.0002	0.000115		1.333	1.306	1.326	1.321667	0.014012	0.00809
Vegetable Waste	0.0016	0.0023	0.0025	0.002133	0.000473	0.000273		0.639	0.655	0.672	0.655333	0.016503	0.009528