ENDOPHYTIC BACTERIAL ANALYSIS IN SELECTED MEDICINAL PLANTS: *Ocimum sanctum* L. and *Ocimum basilicum* L.

DISSERTATION

Submitted to the University of Calicut in partial fulfilment of the requirement for the award of degree of Master of Science in Botany

Submitted by

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ABSTRACT

Endophytes, non-pathogenic microorganisms residing within plant tissues, are increasingly recognized for their role in enhancing plant health and producing bioactive compounds. This study focuses on the endophytic exploration of *Ocimum sanctum* (Holy Basil) and *Ocimum basilicum* (Sweet Basil), prominent medicinal plants. The objective is to assess the diversity and functionality of endophytes in these species, addressing concerns of interspecies adulteration due to their similar morphologies. Endophytes were isolated from leaf extracts using culture-dependent methods, followed by morphological and molecular identification techniques such as 16S rRNA sequencing and phylogenetic analysis. The findings reveal a diverse community of endophytic bacteria with biotechnological potential, highlighting their symbiotic interactions and potential applications in agriculture and medicine, including organic biofertilizers, biopesticides, and pharmaceuticals. This research underscores the importance of understanding endophytic diversity in medicinal plants for quality assurance in traditional medicine and drug discovery, offering insights into combating adulteration and exploring the therapeutic potential of *O. sanctum* and *O. basilicum.*

Keywords: *Ocimum sanctum, Ocimum basilicum*, Endophytic bacteria, 16S Rrna

INTRODUCTION

The quality and quantity of medicinal plants are highly impacted by environmental factors, including soil conditions, temperature, light levels, moisture content, and the kind and existence of soil fauna (Namdar *et al.*, 2019). It is becoming more widely acknowledged that the relationships that medicinal plants have with particular bacterial endophytes can also have a significant impact on those plants (Ek-Ramos *et al.*, 2019). Long-term, symbiotic partnerships between endophytes and host plants have the potential to promote plant development and are particularly beneficial in agricultural fields (Compant *et al*., 2010) Novel and safe bioactive combinations are still needed more and more to offer comfort and relief in all aspects of human life. In order to address the multitude of newly emerging diseases that are a consequence of contemporary living and declining environmental health, scientists are compelled to explore innovative and promising bioactive components. Medicinal plants are a vital source of phytocompounds, which are necessary for preserving health and serve as a haven for a variety of endophytic microorganisms (Petrini *et al*., 1992).

De Bary initially used the term endophyte (Greek endon, within; phyton, plant) in 1866.Endophytes are microorganisms, such as fungi or bacteria, that live their entire lives inside the tissues of living plants without producing harmful symptoms (Tan & Zou, 2001; Gunatilaka, 2006). The capable but little-studied source of phyto-constituents, endophytic bacteria, were selected from a variety of plant species' leaves, stems, roots, flowers, and seeds. Researchers are focusing more on endophytes—microorganisms that colonize healthy plant tissues—in an effort to find new bioactive compounds with medicinal value and negligible environmental impact (Kobayashi & Palumbo, 2000)This is because these organisms have the capacity to synthesize exclusively unique compounds and produce metabolites that were previously known from their host plants (Petrini, 1991; Chanway, 1996).Endophytic bacteria are recognized for their ability to create secondary metabolites, which hold numerous potential uses in the pharmaceutical sector.Endophytic microorganisms are a possible source of new chemicals, including antibiotic, antiviral, anticancer, and mycotic compounds, because some of them may create the same bioactive compounds as the plant (Christina *et al* ; 2013).Plants having a long history of ethnobotanical usage as traditional medicines have a rich endophytic microbial richness.

Endophytes have been shown to be an important source of pharmaceutical bioactive compounds for a long time. This is because many endophytes have been exposed to produce novel bioactive metabolites, including drugs that are immunosuppressive, antiviral, antibacterial, antifungal, antitumor, antioxidant, and anti-inflammatory. The production of a wide range of natural products, including alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans, and others, is well known about endophytes. They have also been shown to display a wide range of biological activity. Significantly, the secondary metabolites that endophytes create improve host plant fitness in a number of ways and have positive impacts on nitrogen fixation (Kirchhorf *et al* ; 1997, Reinhold-Hurek *et al ;* 1998), growth (Sturz *et al* ; 1997)and tolerance to herbivory, drought, parasitism (Chen *et al* ; 1995, Sturz & Matheson; 1996), and other stresses.For a variety of valid reasons, including their characterisation, research on population dynamics and diversity, the use of microbial inoculants to enhance plant growth and health, and as sources of novel physiologically active secondary metabolites, endophytes are typically isolated (Schulz *et al* ; 1996, Schulz & Boyle; 2005).

Ocimum sanctum and *Ocimum basilicum* leaves, the primary plant portion with medicinal potential, were used to isolate endophytes. These endophytes were first identified by 16S rRNA gene analysis and then, for a better resolution, by whole genome sequencing.

Adulteration is the process of partially or completely replacing the original crude drug with another substance that is either free of or has inferior therapeutic and chemical properties; or it is the addition of low-quality, spoiled, or spurious drugs; or it is the substitution of an entirely different drug that is identical to the original drug substituted with the goal of increasing profits (Kokate *et al*., 2007). Adulteration can also be characterized as combining or replacing the original drug material with hazardous compounds or drugs that do not meet regulatory requirements or with other phony, inferior, faulty, spoilt, or useless components of the same or different plant. If a medicine contains any amount of unclean, putrid, or degraded material, it is considered contaminated (Anonymous, 2003). An 1820 book on adulterations in food and culinary materials, written two centuries ago, provides evidence that this technique is centuries old.Adulteration has caused a loss in trust in herbal remedies (Dubey *et al*., 2004).

The biggest problem with the advertising of herbal products is adulteration in market samples. A large number of researchers have verified and checked adulterations (Tewari *et al;*1991, Vasudevan *et al ;*1983, Bisset *et al ;*1984, Sunita ; 1992, Uniyal *et al ;* 1993, Sarin *et al ;* 1996,

Gupta, 2003). Almost without exception, it is discovered that complaints of unpleasant events or side effects are caused by the presence of an unintentional herb rather than the intended herb (De Smet *et al* ;1992). Medicinal plant merchants have found scientific ways to produce adulterations of such high quality that it is very impossible to trace these adulterations without microscopic, phytochemical, and physicochemical investigation (Afaq ;1999). Medicinal plants are a valuable source for both modern and traditional medicine, including Ayurvedic, Chinese, homeopathic, and Unani. There is real evidence to support the benefits of herbal treatment. According to current estimates for 2013, Germany and France combined account for 45% of the $23 billion worldwide retail market. Approximately 80% of India's rural population relies on traditional medical practises and/or herbal remedies. As a matter of fact, over 70% of modern "synthetic" medications come from plants. The use of herbal remedies and medicinal plants increased as they became more popular with regular people. One of the most prevalent frauds in the sale of herbal raw materials is herbal adulteration (Mitra *et al* ; 2007, Shah *et al* ; 2010).

One of the dicotyledon families is Lamiaceae, or Labiatae. There are 3200 species and 200 genera in this family. Ocimum is a genus in the Lamiaceae family. According to some accounts, the genus contains about 50 and 60 species (Suchorska *et al* ;1966), however, some sources put the total nearly as 150. The genus is collectively known as basil plants (Simon *et al* ;1990). *Ocimum basilicum* and *Ocimum sanctum* are the two species of the genus that are most extensively researched and spread.

"*Ozo*" is the Greek word for "to smell."*Ocimum* derives its genus name from it (McIntosh *et al* ; 1853). The Latin term *basilisk*, which means "king," is the source of the English word *basilicum*. The French refer to it as "Herbe Royale," which highlights its favorable attributes (Muenscher *et al* ; 1978).An additional designation for it is "The king of herbs".Sweet Basil is the colloquial name for *O.basilicum*. It is found in tropical and hotter areas of the Indo-Pakistan Subcontinent, having originated in the warmer Indo-Malayan regions.

O. sanctum, often known as the "Queen of Herbs," is native to tropical parts of Asia and has been cultivated in India for over 3,000 years (Gupta *et al* ; 2002, Devi ; 2001). It is referred to as Tulsi in India and is regarded as the most sacred plant. Meaning "The incomparable one" is Tulsi. It contains a vast array of Ayurvedic medicinal significance, particularly in the Eastern regions.

The present study collectively focuses to improve the understanding of how adulteration affects medicinal plants at a microbiological level and to enhance the safety, efficacy, and authenticity of medicinal plant products. The study also aims to enhance the understanding of the complex interaction between endophytic bacteria and the medicinal plants.

OBJECTIVES

- To isolate and identify the types of endophytic bacteria present within adulterant medicinal plants. This involves characterizing the bacterial communities using molecular techniques.
- To perform genetic and molecular analysis of the endophytic bacterial community to understand their genetic diversity, functional capabilities.

REVIEW OF LITERATURE

Endophytic bacteria, residing within plant tissues without causing harm, are pivotal for enhancing the health and productivity of medicinal plants. Research by Wu *et al*. (2021) underscores their role in bolstering plant resilience against diseases and environmental stresses, thereby promoting overall growth and development. These bacteria also influence the synthesis of secondary metabolites with therapeutic qualities, making them valuable in agriculture and medicine. The genetic diversity of endophytic bacteria, coupled with the health of their host plants and local ecological conditions, shapes their dispersal and population dynamics. Leveraging these bacteria holds promise for increasing medicinal plant productivity, improving product quality, and advancing sustainable agricultural practices.

Duhan *et al*. (2020) conducted a study on "**Isolation, identification, and characterization of endophytic bacteria from the medicinal plant *Tinospora cordifolia***". Their research focused on the diverse array of beneficial microorganisms hosted by medicinal plants, with particular emphasis on endophytic bacteria, known for their production of bioactive compounds crucial for applications in industry, agriculture, and medicine. Employing morphological, biochemical, and molecular analyses, the study isolated and characterized thirty-eight distinct endophytic bacterial strains from leaf and stem explants of *T. cordifolia*. Molecular identification using 16S rDNA sequencing revealed twenty bacterial species from leaf explants and eighteen from stem explants, highlighting both parts of *T. cordifolia* as significant reservoirs of bacterial diversity. This comprehensive characterization underscores the potential of *T. cordifolia* as a source of diverse bacterial endophytes with implications for enhancing plant health and productivity, and advancing biotechnological applications in sustainable agriculture and pharmaceutical development.

Anjum and Chandra (2015) conducted an analysis titled "**Endophytic bacteria: Optimization of isolation procedures from various medicinal plants and their preliminary characterization**". The study aimed to enhance the isolation process of endophytic bacteria from *Catharanthus roseus*, *Ocimum sanctum*, *Mentha arvensis*, and *Stevia rebaudiana* by optimizing surface sterilization using different concentrations and durations of ethanol, sodium hypochlorite, and mercuric chloride. The isolated endophytes were then characterized through biochemical and microscopic analyses.

Rhoden S A *et al* (2015) worked on ***'"Phylogenetic analysis of endophytic bacterial isolates from leaves of the medicinal plant Trichilia elegans A. Juss. (Meliaceae)"***.Many types of organisms, including bacteria and fungi, can reside inside plants, mostly in the leaves and other aerial portions, without harming the plant. These bacteria, known as endophytes, generate a wide range of chemicals with potential applications in medicine and agriculture. The Meliaceae family includes *Trichilia elegans* A. Juss., which is widely distributed throughout South America. Phytochemical investigations of endophyte isolates of these plants have demonstrated biological activity.Thus, the purpose of this work was to confirm, by partial sequencing of 16S rRNA and phylogenetic analysis, the variety of bacterial endophytes from T. elegans. Sterilization was done with 5% sodium hypochlorite. Staphylococcus, Bacillus, Microbacterium, Pseudomonas, and Pantoea were the genera that were identified after 16S rRNA sequencing and phylogenetic analysis. An improved knowledge of *T. elegans'* microbiome would highlight the importance of conducting endophyte studies with an eye toward their potential uses in biotechnological fields related to agriculture, medicine, and the environment. These findings will yield significant insights into the diversity of endophytic bacteria living in medicinal plants.

Kumar (2014) reviewed "**Adulteration and substitution in endangered ASU medicinal plants of India**". Medicinal plants are crucial sources of alternative medicine in the Ayurvedic, Siddha, and Unani systems of the Indian System of Medicines (ISM), gaining popularity due to increasing awareness of synthetic medication side effects. However, adulteration and substitution of endangered ASU herbs pose significant challenges in herbal and pharmaceutical industries. These issues stem from deforestation, species extinction, and misidentification of rare and endangered herbal plants. Future research should focus on robust pharmacognostic and phytochemical analysis methods to ensure the precise identification, quality control, and assurance of ASU herbal medications.

Bhore S J, Preveena J & Kandasamy K I (2013) ***"Isolation and identification of bacterial endophytes from pharmaceutical agarwood-producing Aquilaria species".*** Aim of the study was to isolate and characterize endophytic bacteria linked to seven Malaysian varieties of *Aquilaria* that produce agarwood. Seven *Aquilaria* species' botanical samples were gathered, and samples of surface-sterilized tissue were used to extract endophytic bacteria. The 16S rRNA gene fragments were amplified by the PCR method, and the 16S rRNA gene sequence similarity based method was used to identify endophytic bacterial isolates (EBIs). Analysis of 77 cultivable EBIs and 16S rRNA gene sequences revealed that 18 distinct endophytic bacterial

species are linked to (seven) *Aquilaria* species. *Bacillus pumilus* accounted for the diversity (36.4%) of the isolates from 77 EBIs.

Janardhan B S & Vijayan K (2012) researched about ***"Types of endophytic bacteria associated with traditional medicinal plant Lantana camara Linn"***. The *Lantana camara* plant (Verbenaceae family) has long been used in conventional herbal therapies to treat skin irritations, leprosy, and scabies externally, as well as as an antiseptic for wounds. This plant's leaves have adulticidal properties against a variety of mosquito species. The primary goal of this investigation was to separate and identify the Endophytic Bacteria (EB) connected to *L. camara*. EB were isolated from surface-sterilized tissue samples using lantana camara twigs, leaves, and fruit samples. Endophytic bacterial isolates (EBIs) were identified by applying the 16S rRNA gene sequence similarity approach to identify the amplified 16S rRNA gene fragments. Fifty cultivable EBIs were examined, and based on an examination of their 16S rRNA gene sequences, it appears that 40 different kinds of EB are connected to *L. camara*. The majority of EBIs (24%) belonged to the *Bacillus* genus.

Bhagat J *et al* (2011) researched on ***"Molecular and functional characterization of endophytic fungi from traditional medicinal plants". In*** this work, 63 endophytic fungal isolates were obtained from two traditional medicinal plants—*Sapindus detergens* and *Ocimum sanctum*—that were grown in various parts of Amritsar, India. It was done to functionally characterize the fungi in order to determine their capacity to create antibacterial and anticancer agents. By sequencing the amplified ITSI-5.8-ITSII region of rDNA, sixteen strains were described at the molecular level. The endophytic fungus were divided into several clades by the phylogenetic tree. The Pleosporales order's fungal endophytes (*Alternaria sp*., *Phoma sojicola*, and *Exserohilum sp*.) produced a variety of biomolecules, such as cytotoxic activity opposed to various human cancer cell lines of the lung, ovary, breast, prostrate, and colon. These biomolecules demonstrated their functional versatility.

Tiwari R *et al* (2010) conducted a study on ***"Endophytic Bacteria from Ocimum sanctum and Their Yield Enhancing Capabilities".*** Beneficial microorganisms called endophytes live inside plants' intercellular structures. To discuss the ecological significance of endophytes, it is crucial to consider how they interact with their host plants and what role they play there. From healthy *Ocimum sanctum* leaves, four endophytic bacteria—OS-9, OS-10, OS-11, and OS-12—were identified. According to strain OS-11's molecular characterisation, it is closely linked to the *Bacillus subtilis* type strain.

METHODOLOGY

1. Collection of plant material

For the isolation of endophytic bacteria, the medicinal plants *Ocimum sanctum* and *Ocimum basilicum* were collected from the herbal garden of Kottakal Arya Vaidya Sala, Kottakal, Malappuram. Samples were placed in clean plastic bags, brought to the laboratory and used for further experimental purpose.

2. Isolation and purification of bacterial endophytes

The first and essential step in isolating endophytes is surface sterilization, which eliminates all surface microorganisms. Usually, oxidant or general sterilizing agents are applied to plant tissues for a particular period of time, followed by three to five sterile rinses. The most widely utilized isolation techniques are vacuum or pressure extraction, macerating the plant tissue and streaking it over nutrient agar, as well as surface sterilization of the plant tissue and plating small sterilized segments onto nutritional agar. In theoretical terms, the sterilizing chemical ought to eradicate all microbes present on the plant's surface while leaving the host tissue and endophytic microorganisms unaffected. However, this is difficult to accomplish since the circumstances needed to eliminate the final surface microbe may already be harmful for some endophytic microbes, and the agent may eventually invade the plant tissue (Zhao S *et al*;2016). The following procedures are typically involved in the isolation and purification of endophytic bacteria from plant tissue:

2.1. Pre-treatment

To get rid of soil particles that stuck to the leaves of each plant, each leaf was given a separate tap water wash. This served as pre-treatment for the majority of the microbial surface epiphytes.

2.2. Surface sterilization

The Fresh leaves collected were washed in running tap water for 15 minutes and then washed in Tween 20 (1 drop in 200 mL sterile distilled water [SDW]) for 1 minute and finally rinsed three times with SDW in the laminar air flow cabinet.Frequently used sterilizing agents are sodium hypochlorite: 1-5% for 2-10 minutes (Gardner JM *et al* ;1982) ethanol: 70-95% for 30seconds -4minutes (Dong Z *et al*;1994) hydrogen peroxide (McInroy JA *et al* ;1994) and

mercuric chloride 0.05-0.2% for 2-5 minutes (Maroof A *et al*; 2012). 1% and 5% sodium hypochlorite, 70% ethanol (alcohol) and Nucleus Free Water were employed as the surface sterilization agent for the current study's surface sterilization procedure after a thorough review of the literature. The treatment duration and mix of these ingredients vary.

2.3. Test for effectiveness of surface sterilization

The isolated microorganisms are the only ones classified as endophytes till the total surface sterilization of the plant tissue is verified. By imprinting surface-sterilized plant tissue onto nutrient media (Pleban S *et al*; 1995, Schulz B *et al*; 1998), cultivating aliquots of water from the final rinse onto nutrient media (McInroy JA *et al* ;1994), and dipping the surface-sterilized explants into nutrient broth (Gagne S *et al*; 1987), the surface sterilization process was validated. They were incubated as the test sample's control as well.

2.4. Media for isolating endophytic bacteria

Selecting the right growing medium is essential since it has a direct impact on the amount and type of endophytic bacteria that may be extracted from leaf tissue. Endophytic bacteria were isolated using nutrient agar medium. An antifungal medication called nystatin was added to the media used for isolating endophytic bacteria at a concentration of 30 µg/mL in order to reduce fungal development, as nutrient agar lacks any component that can inhibit the growth of endophytic fungus.

2.5. Isolation, purification, and subculture of endophytic bacteria

Following the surface-sterilization the leaves, the leaves were grinded using the mortar and pestle and made to a paste. The PBS buffer is added while grinding to avoid the dehydration of leaves. Then 70 µl of extract is spreaded on the nutrient agar medium that had been treated with antifungal agents with the help of a L-rod. Plates containing plant tissues are sealed with parafilm tape and cultured at 28±2°C to extract as many bacterial endophyte colonies as possible. For 48 hours, the observation was made. Following a 24-hour period from the bacterial cultures, distinct morphological colonies were chosen and subsequently streaked to get bacterial isolates. After subculturing each of the chosen isolates in nutrient agar slants, all of the purified endophytes were stored at 4°C until they were needed again.

3. DNA Extraction and Amplification of 16S rDNA

3.1. DNA Isolation protocol

Bacterial DNA isolation was carried out using column based DNA extraction (XploregenTM Bacterial Extraction kit, Cat. no. XPBAD22-50). It carries the following steps:

1. Add 1 ml of XBA 1 to the beaded vial.
2. Add the sample in the beaded vial containing XBA 1.
3. Horizontal vortex the vial at maximum speed for 10 minutes.
4. Add 300 µl of XBA 2 to the vial.
5. Horizontal vortex the vial at maximum speed for 7 minutes.
1. 6.Centrifuge the tube at 10,000 rpm for 2 minutes at room temperature (RT).
6. Transfer 950 µl of supernatant to a sterile 2 ml vial.
7. Add 200 µl of XBA 3 solution and vortex for 5 seconds.
8. Centrifuge at 10,000 rpm for 2 minutes.
2. 10 Transfer 800 µl Supernatant to a clean sterile 2ml vial.
3. 11. Add 700 µl XBA 4 solution to the supernatant and vortex for 5 Seconds.
4. 12. Transler 700 µl of Lysate to the spin column (Do not discard the residual lysate) and Centrifuge at 10,000 rpm to 2 minutes.
5. 13. Discard the flow through.
6. 14. Repeat the above (12 and 13) steps to collect all the Lysate. (Ensure the entire lysate is processed via the spin column).
7. 15. Add 600 µl of XBA 5 to the spin column and Centrifuge at 10,000 rpm for 2 minutes.
8. 16. Discard the flow through.
9. 17. Add 600 µl of XBA 6 to the spin column Centrifuge at 10,000 rpm for 2 minutes and discard the flow through.
10. 18. Centrifuge the empty spin column for 5 minutes at 10,000 rpm.
11. 19. Place the spin column into a sterile 1.5 ml vial and incubate for 2 minutes.
12. 20. Add 30 µl XBA 7 to the center area of spin column and centrifuge for 5 minutes at 10,000 rpm.
13. 21. Place the spin column to a new sterile 1.5 ml vial. Discard the spin column and store both elution tubes for further processing.

3.2. DNA Quantity analysis

In many procedures where it is necessary to know how much DNA is present, such as when performing PCR techniques, DNA quantification is an essential step (Linacero, R., Rueda, J., Vázquez, A.M. (1998). DNA is isolated and then drops of 1X dsDNA HS buffer are added for the concentration analysis. The DNA concentration is measured using a qubit fluorometer.

3.3. POLYMERASE CHAIN REACTION

Polymerase Chain Reaction was carried out using TaKaRa Ex Taq Hot Start Version (RR006A) and was performed in PCR VERITI THERMO SCIENTIFIC. Total 50µl of general reaction mixture is required for the process. PCR was done in a solution containing 5 µL buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCI), 4 µL 2.5 mM dNTPs, 0.2-1 µM 10-50 Pmol of each primer (Primer 1&2): (As most PCR products amplified with TaKaRa Ex Taq HS have one A at the 3'-termini, the obtained PCR products can be directly cloned into a T-vector.), 0.25 µL 5 U/µL of TaKaRa Ex Taq HS. Add Taq DNA polymerase, 4µL 2.5mM dNTP mixture, up to 50µL sterile purified water, and 2 of previously extracted sample DNA (10-20 ng/µl), under required PCR conditions.

The PCR conditions was set as follows:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 sec	1 cycle
Denaturation	98°C	10 sec	32 cycle
Annealing	50°C	30 sec	32 cycle
Extension	72°C	45 sec	32 cycle
Final Extension	72°C	7 min	1 cycle
	10°C		

3.3. Agarose Gel Electrophoresis

After amplification PCR, PCR products and 100bp ladder were loaded in 2% agarose gel for electrophoresis at 150 V. Band was observed at 1500bp.

3.4. PCR product purification

PCR product (The amplified 16S genes) was purified using ExoSap-IT Reagent.An extremely effective technique for PCR purification is enzymatic cleansing. ExoSAP-IT reagent can be added straight to the PCR product, saving PCR amplicons and removing the need for additional processing while also reducing the likelihood of cross-contamination.

4. DNA Sequencing and Phylogenetic Analysis

4.1. Sanger sequencing

The Bigdye x Terminator V3.1 kit was utilized for the sequencing, and sample DNA was used as a template for a polymerase chain reaction (PCR). In the PCR procedure, a combination of chain termination bases (ddNTPs) and normal bases (dNTPs) is utilized. This will result in DNA fragments with varying lengths. The Applied Biosystems 3730 Genetic Analyzer was used to perform capillary electrophoresis, which separates the DNA fragments based on size.A laser is used to excite these fluorescently labeled bases at the end of each fragment.In the sequence, shorter fragments appear first, then progressively longer fragments. By measuring the fluorescence of the base that ends each length of the segment, a chromatograph is constructed that shows which base is present at each location along the DNA fragment.

4.2. Phylogenetic Analysis

Following sequencing, the samples' FASTA sequences were examined using the NCBI's Nucleotide Blast software (www.ncbi.nlm.nih.gov).

Chemicals used

Alcohol, Sodium hypochlorite, Nucleus Free Water, PBS buffer, XBA 1 buffer, XBA 2 buffer, XBA 3 buffer, XBA 4 buffer, XBA 5 buffer, XBA 6 buffer, XBA 7 buffer, 1X dsDNA HS buffer, TaKaRa Ex Taq Hot Start Version (RR006A) kit, Agarose gel, ExoSap-IT Reagent, Bigdye x Terminator V3.1 kit.

Equipments used

Autoclave, Laminar air flow, sterile plate, beaker with several measurements, Inoculation loop, L-rod, Micropipette, mortar & pestle, Burner, sterile cotton, Incubator, vortex apparatus, microcentrifuge tubes, centrifuge machine, qubit fluorometer, PCR VERITI THERMO SCIENTIFIC, Agarose Gel Electrophoresis, Applied Biosystems 3730 Genetic Analyser.

RESULT

Bacterial endophytes were isolated using fresh plant material (leaf tissue) from the medicinal plants *Ocimum sanctum* and *Ocimum basilicum*. An essential step in eliminating epiphytic microorganisms from sample explants was surface sterilization. Our investigation found this stage to be satisfactory because the control plate did not exhibit any development **Fig 1**.

Adequate number of bacterial growth in the form of colonies was observed in the plate in which we spreaded the leaf extract on nutrient agar media as shown in **Fig 2** and these isolates were considered as bacterial endophytes of *Ocimum sanctum* and *Ocimum basilicum* plants as no growth was existed on control plate.

The colonies were sub cultured by streak plate method **Fig 3** for 1 week and then from that the pure culture was obtained. From the pure culture the bacterial species was isolated . Following the species isolation of two plants DNA extraction is carried out by column based DNA extraction (XploregenTM Bacterial Extraction kit, Cat. no. XPBAD22-50). So the bacterial DNA was isolated from the two plants *Ocimum sanctum* and *Ocimum basilicum* then DNA quantity analysis was done to check the concentration and purity of the DNA we extracted from two plants and it was founded to be 16.6 and 12.6 respectively.

DNA amplification was done using Polymerised Chain Reaction (PCR) using TaKaRa Ex Taq Hot Start Version (RR006A) and was performed in PCR VERITI THERMO SCIENTIFIC under the PCR condition as mentioned in **Table 1**. The PCR product (the amplified 16s genes) along with 100bp ladder were loaded in 2% agarose gel for electrophoresis at 150 V. Hence a band was observed at 1500 bp as shown in **Fig 4**. PCR product (The amplified 16S genes) was purified using ExoSap-IT Reagent so all the unwanted short primers, dNTPs, enzymes, short-failed PCR products, and salts are removed from PCR reactions.

DNA sequencing is done by Sanger sequencing. The Bigdye x Terminator V3.1 kit was utilized for the sequencing, and sample DNA was used as a template for a polymerase chain reaction (PCR). In the PCR procedure, a combination of chain termination bases (ddNTPs) and normal bases (dNTPs) is utilized. This will result in DNA fragments with varying lengths. The Applied Biosystems 3730 Genetic Analyzer was used to perform capillary electrophoresis, which separates the DNA fragments based on size.A laser is used to excite these fluorescently labeled bases at the end of each fragment.In the sequence, shorter fragments appear first, then

progressively longer fragments. By measuring the fluorescence of the base that ends each length of the segment, a chromatograph is constructed that shows which base is present at each location along the DNA fragment as illustrated in **Fig 5**.

The Sanger sequencing is done both forward and reverse direction for two plants also. The trimmed forward sequence of *Ocimum sanctum* is as follows,

(889 bp)

GTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGT
AACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTA
ATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTG
TCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC
AAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGC
TCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGCTTGCACCTTGACGGTACC
TAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGT
CTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACT
TGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGA
TGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAG
GAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGAGTGCTAAGTGTTAGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCA
TTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGT.

Trimmed reverse Sequence of *Ocimum sanctum* is as follows,

(872 bp)

GGCTGGCTCCATAAAGGTTACCTCACCGACTTCGGGTGTTGCAAACTCTCGTGGT
GTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATC
CGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAA
CTGAGAACAGATTTGTGGGATTGGCTAAACCTTGCGGTCTCGCAGCCCTTTGTTC

TGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGA
ATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCT
CACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCGAAGGG
AAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGC
GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCC
TTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGC
TGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGT
GGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTC
AGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCA
TTTCACCGCTACACGTGGAAATTCCACTCTCCTCTTCTGCACTCAAGTTTCCCAGT
TTCCAATGACCCTCCC CGGGTTGAGCCGGGGGCTTTCACATCAGACTT.

In the case of *Ocimum basilicum*, the trimmed forward sequence is,

(898 bp)

GCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGG
GCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCG
GCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGT
CACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATG
GACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTA
AAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGAC
GGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTT
AAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGG
AACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGAGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAA
CGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGTGGA.

The trimmed reverse sequence of *Ocimum basilicum* is,

(875 bp)

CGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGG
TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGAT
CCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGA
ACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTT
CTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACG
TCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTG
AATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT
CTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAG
GGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTC
GCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATT
CCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTA
GCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGG
CGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGGT
CAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGA
TTTCACCGCTACACGTGGAAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGT
TTCCAATGACCCTCCCCGGGTTGAGCCGGGGGCTTTCACATCAGACTTAA.

After the sequencing, the FASTA sequence of the sample1 and 2 was analyzed in the Blast programme of NCBI (www.ncbi.nlm.nih.gov) to check the similarity of the endophytic bacteria we have isolated with the sequence that's already uploaded in the NCBI. The BLAST result shows that,

Sample 1 (Ramatulasi): The bacterial endophyte isolated from *Ocimum sanctum* shows 100.00% similarity to *Bacillus Subtilis*.

Sample 4 (Tulasi): The bacterial endophyte isolated from *Ocimum basilicum* is 99.77 identical to *Bacillus Xiamenensis*.

Finally we have constructed a dendrogram for both the samples *Ocimum sanctum* and *Ocimum basilicum* respectively with the first 10 similar organisms which shows much resemblance in the sequence **Fig 6** & **7**.

The overall sequencing result is mentioned in the below :

Table 2 :

Sample	**Primer**	**Raw length** (bp)	**Trimmed length** (bp)	**Trim start**	**Trim end**	**Average QV score**
Ocimum sanctum	Forward	1186	889	28	917	50
Ocimum sanctum	Reverse	1190	972	19	891	52
Ocimum basilicum	Forward	1188	898	21	919	51
Ocimum basilicum	Reverse	1172	875	17	892	50

Fig 1: Control plate

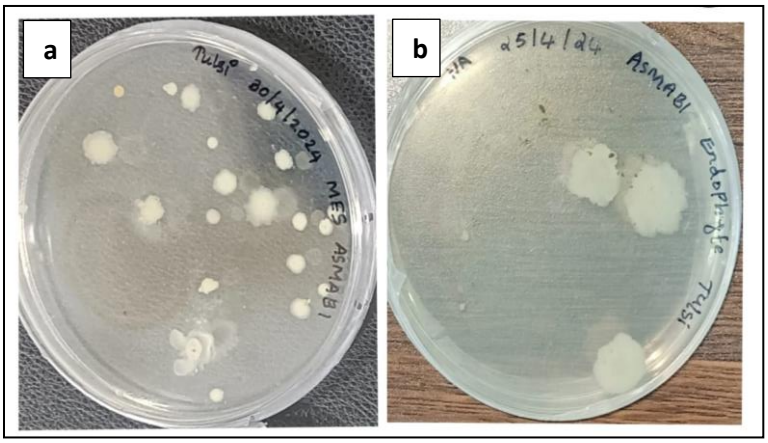

Fig 2: Multiple colony of bacterial endophytes grown in culture plate

a. *Ocimum basilicum* *b.Ocimum sanctum*

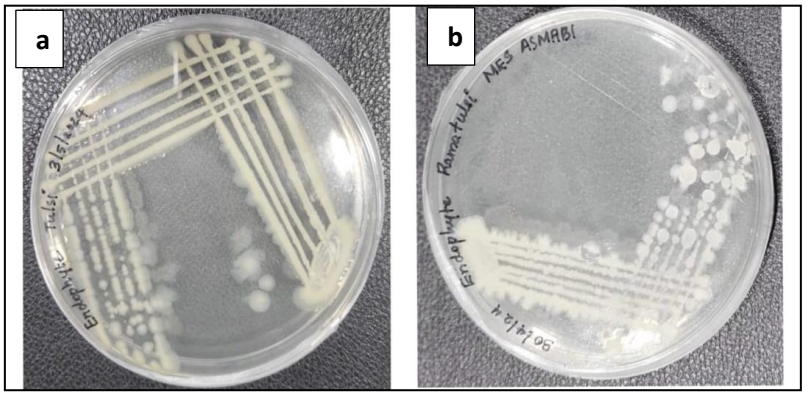

Fig 3: Streak plating for pure culture : a.) *Ocimum basilicum* b. *Ocimum sanctum*

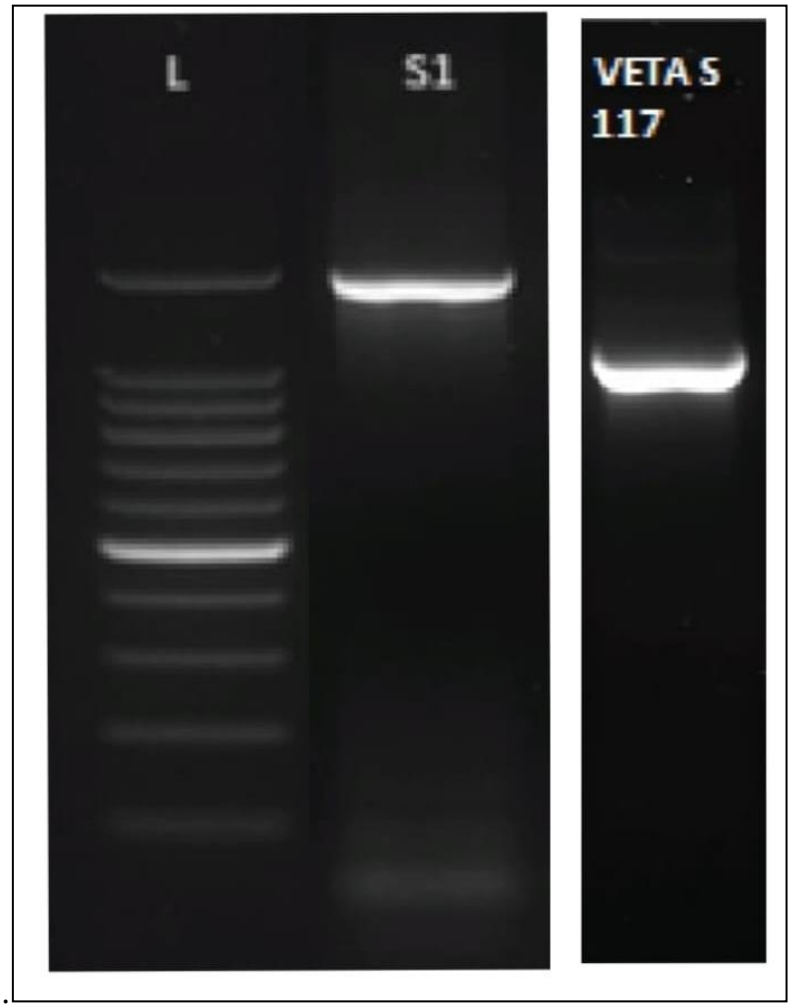

Fig 4: Agarose gel electrophoresis image of isolated gDNA (genomic DNA) of different bacterial isolates on 2% (w/v) agarose gel band appears to be 1500 bp.

VETAS & S: Endophytic bacterial isolates. **LAD**: Marker (100 base pair DNA ladder).

VETAS 117 -*Ocimum sanctum*, **S1-** *Ocimum basilicum.*

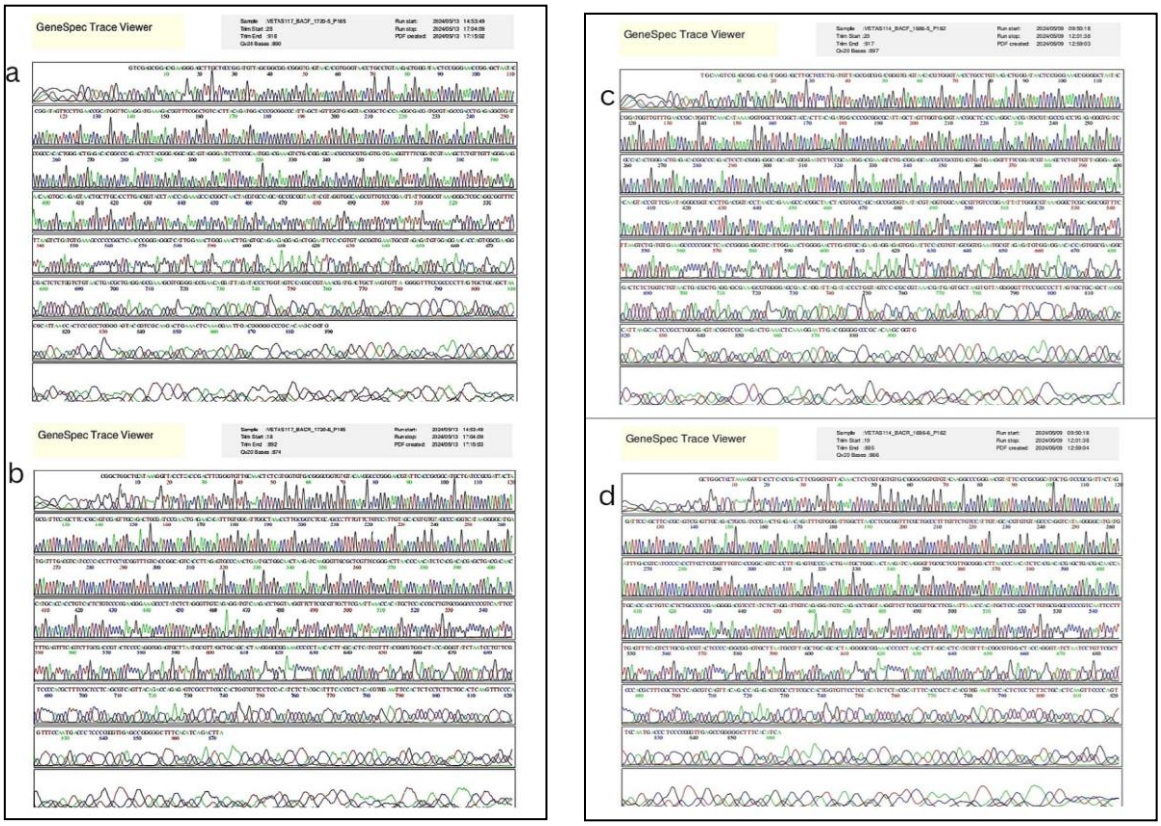

Fig 5: Chromatogram of DNA fragments isolated from *Ocimum sanctum* and *Ocimum basilicum* respectively.

a) Forward : *Ocimum sanctum*, b) Reverse : *Ocimum sanctum*.

c) Forward : *Ocimum basilicum*, d) Reverse : *Ocimum basilicum.*

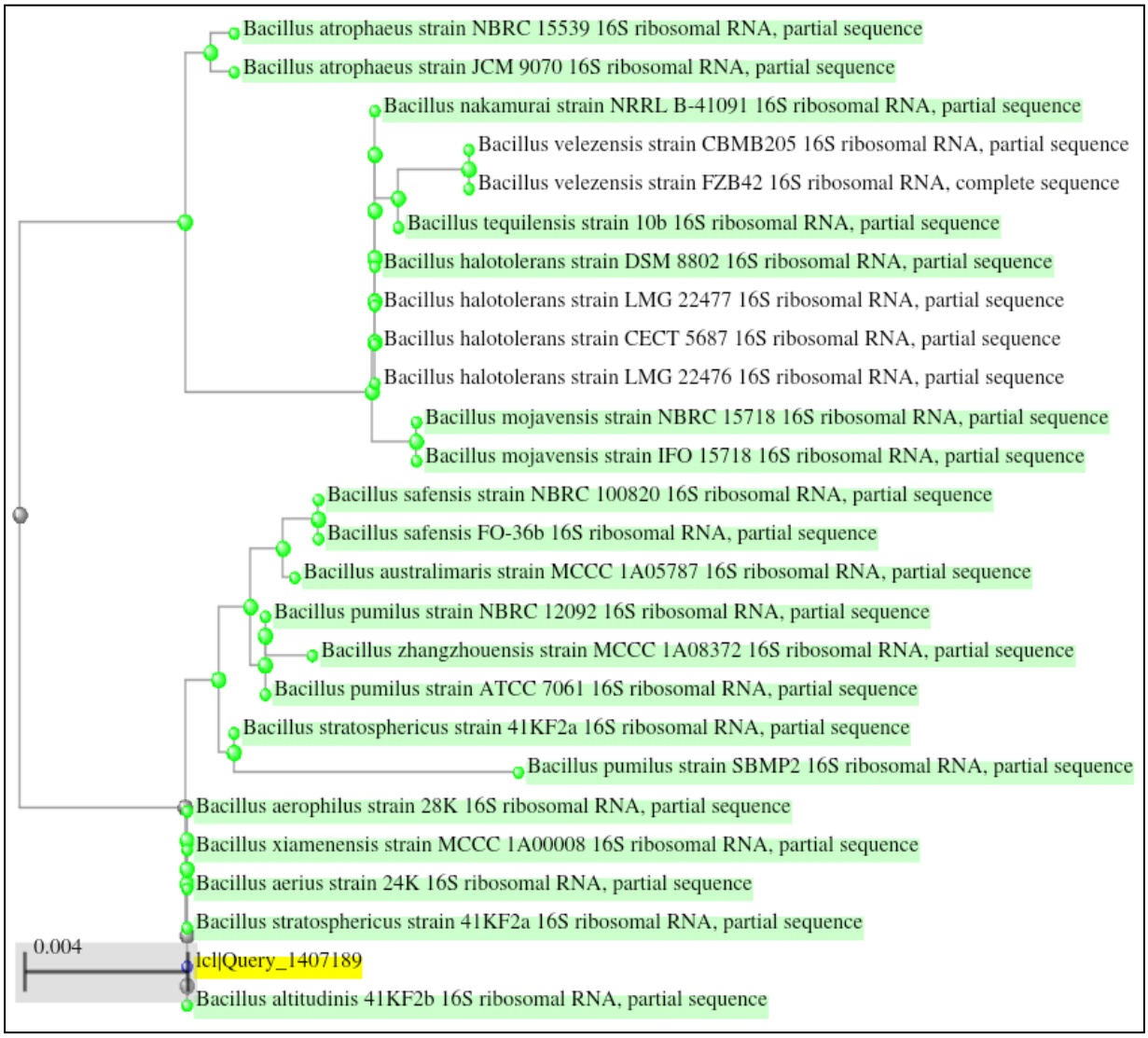

Fig 6: Dendrogram representing *Ocimum sanctum*. Phylogenetic tree retrieved from BLAST analysis showing the evolutionary relationship of *Bacillus Xiamenensis* with its closest BLAST hits based on multiple sequence alignment.

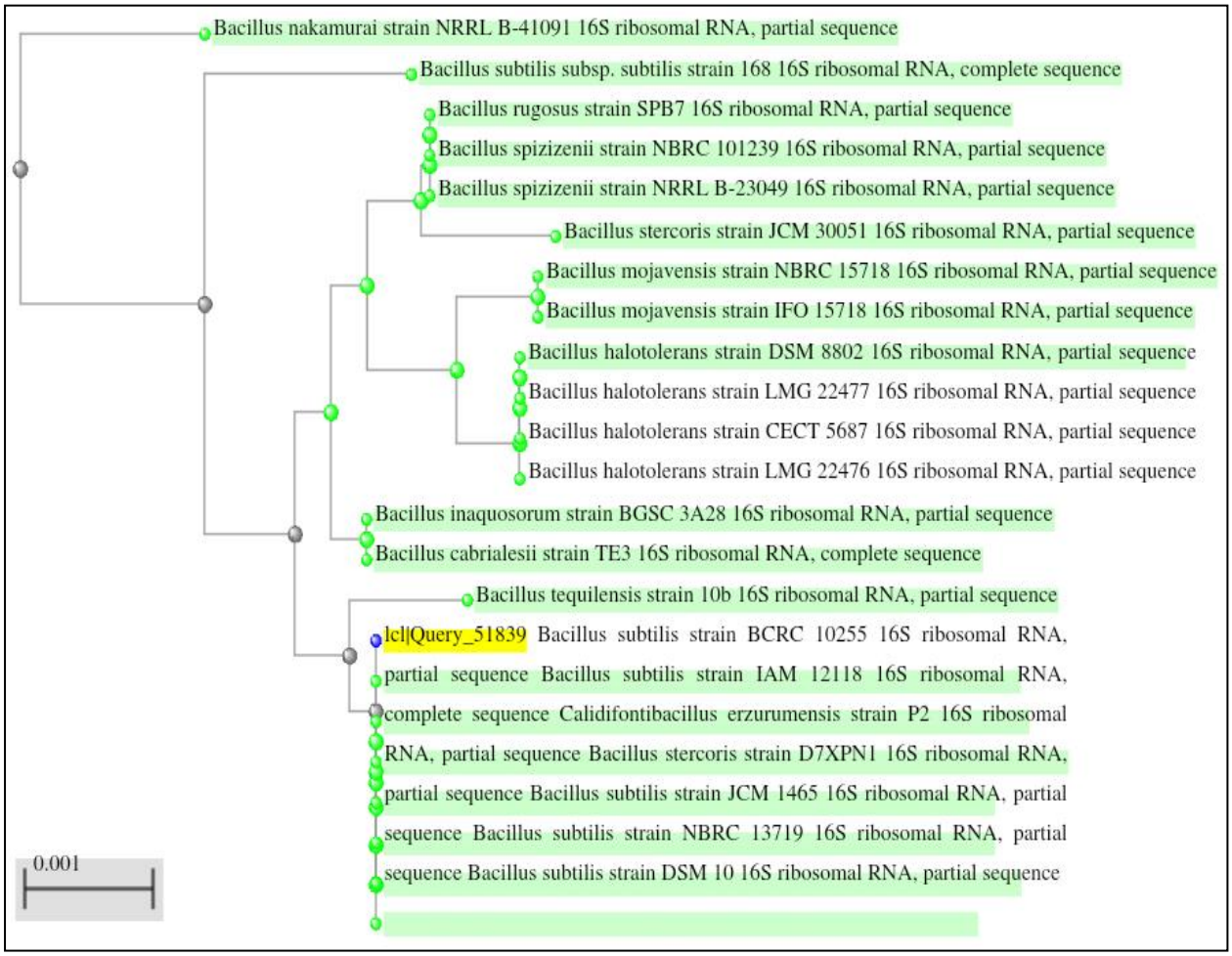

Fig 7: Dendrogram representing *Ocimum basilicum*. Phylogenetic tree retrieved from BLAST analysis showing the evolutionary relationship of *Bacillus Subtilis* with its closest BLAST hits based on multiple sequence alignment.

DISCUSSION

Contrary to popular belief, not all adulterations are deliberate misconduct. Our experience and knowledge have shown us that there is an inadvertent adulteration and substitution of the original, endangered herbal medications. Suppliers and national Vanders lack literacy and are unaware that their supply is fake and substituted. Primary causes include nomenclature misunderstanding, non-accessibility, and ignorance of true plant species. In addition to their improper scientific identification of both microscopic and macroscopic details, the scientific community and traditional physicians (Hakeem and Vad, manufacturers of medicinal plants-based ASU products) are also ignorant of this and have engaged in organoleptic malpractice, intentionally or unintentionally consuming spurious, adulterated, and substitute materials in the manufacturing of various herbal formulations from the production end with the sole intention of achieving annual targets or reaping financial benefits.

In order to maintain their quality and safety, efficacy as multidimensional innovative investigation, evolution with controlling or regular monitoring of microbial load germs, bacterial contamination, or microbial contamination as per detect of Compile standard parameters limits and Secretary, AYUSH, hopefully some genuine ASU herbal drug pharmacy and pharmaceutical industries follow high quality standards using modern techniques and instruments.

Ocimum sanctum and *Ocimum basilicum* have long been utilized in the Ayurvedic medical system to treat a wide range of illnesses (Joshi, 2013). However, advancements and the opening of several new research fields are brought about by recent molecular and genetic findings in medicinal plant research. Future studies on these two species have a lot of potential given their extensive medical applications.The goal of the current studies was to identify the bacterial endophytes found in the leaf tissues of *Ocimum basilicum* and *Ocimum sanctum* and to discuss the properties that are shown by the endophytes.It is readily absorbed that endophytic bacteria are present in almost all terrestrial and aquatic plants (Strobel and Daisy, 2003).Endophytes are known to inhabit intercellular gaps and create useful bioactive metabolites that are important for modern medicine, according to their microecology (Bacon and Hinton, 2002; Chanway, 1998; McCully, 2001).

It is possible to extract various species of bacterial endophytes from a single plant (Zinniel *et al*., 2002).Plant tissue type and endophyte location have an impact on the bacterial ecology and

colonization frequency.When looking for new biomolecules, endophytic bacteria are a valuable source of genetic material (Guimaraes *et al,* 2008).This study contained endophytic bacteria that were obtained from traditional medicinal herbs (*O. sanctum* and *O. basilicum*) from Arya Vaidya Sala, Kotakkal. Traditional medicine has utilized the plants in this study to treat a variety of ailments, such as cough, cold, respiratory conditions, malaria, anorexia, constipation, acidity, and gum pain (Arulmozhi *et al*, 2005; Sharma *et al*, 2010).

The bacterial endophyte isolated from *O. sanctum* and *O. basilicum* are *Bacillus Xiamenensis* and *Bacillus Subtilis* respectively. Numerous endophytic bacteria, including previously described strains from different genera, were discovered to be present in the plants including *Bacillus altitudinis, B. tequilensis, B.safensis, B. subtilis* (Saravanakumar *et al*, 2009; Singh *et al,* 2016; Nagendran *et al*, 2014). etc. The bacterial strain from *O.basilicum* was identified by means of the 16S rRNA sequence obtained after PCR amplification and sequencing. A comparison of the 16S rRNA sequences of the isolated strain with the sequences available in the GenBank database showed that the isolated bacteria had a 99.77% similarity to *Bacillus xiamenensis* and that of *O.sanctum* shows 100 % similarity to *Bacillus Subtilis*. A phylogenetic tree was constructed on the basis of these sequences along with those aligned from databases (Fig: 6 & 7). Dendrogram representing *Ocimum sanctum*, retrieved from BLAST analysis showing the evolutionary relationship of *Bacillus Xiamenensis* with its closest BLAST hits based on multiple sequence alignment and *Ocimum basilicum* retrieved from BLAST analysis showing the evolutionary relationship of *Bacillus Subtilis*.

In order to maintain plant health over the long term, endophytic *Bacillus* species are becoming increasingly significant strains that both stimulate plant development and act as biological control agents (Zhao *et al,* 2015). The antagonistic characteristics of *B. xiamenensis* and *B. pumilus* endophytic strains in preventing downy mildew infection in grapevine seedlings (Zhang *et al,* 2017). Later on, endophytic *B. subtilis* was discovered to possess antifungal activity and characteristics that promote plant growth, making it a promising candidate for bioinoculation (Haidar *et al,* 2018). Research on pepper and wheat has shown that *B. subtilis* can greatly raise the amount of chlorophyll in leaves. By accelerating the production of chlorophyll and hence raising the rate of photosynthesis in the leaf tissues, *B. subtilis* inoculants can increase the amount of nutrients available for plant growth (Tao SY, 2019). The ways in which B. subtilis stimulates plant development have been thoroughly investigated. For instance, it has been demonstrated that *B. subtilis* is capable of producing a wide range of physiologically

active enzymes that, to varied degrees, can control plant development and chlorophyll accumulation (Gale RT *et al*, 2017).

According to other research, *B. subtilis* can enhance the nutrients, texture, and microbial habitat of soil, all of which are factors that support plant growth (Wang LH *et al*, 2018). *B. subtilis* is a novel kind of bioinoculant that can enhance soil conditions, reduce pollutants, and is environmentally benign—all of which are consistent with the "simulative habitat cultivation" theory. *B. subtilis* therefore has a wide range of potential applications in the ecological cultivation of Chinese medicinal ingredients.

CONCLUSION

The research on *Ocimum sanctum* and *Ocimum basilicum* has revealed a rich diversity of bacterial endophytes that play pivotal roles in the health, growth, and disease resistance of these medicinal plants. Prominent species like *Bacillus xiamenensis* and *Bacillus subtilis* have been isolated, known for producing bioactive compounds such as lipopeptides, polyketides, and siderophores, which contribute significantly to the therapeutic properties of their host plants. Sanger sequencing of the 16S rRNA gene has enabled precise identification and characterization of these endophytic bacteria, highlighting *Bacillus subtilis* from *O. basilicum* and *Bacillus xiamenensis* from *O. sanctum* as dominant species. Moving forward, future research should explore the functional roles of these endophytes, including their interactions with host plants, mechanisms of plant growth promotion, and their potential applications in sustainable agriculture and medicine.

Future Directions

- Further investigation is needed to explore the specific mechanisms by which *Bacillus xiamenensis* and *Bacillus subtilis* enhance plant health and productivity in *Ocimum sanctum* and *Ocimum basilicum.*
- Research efforts should focus on optimizing the cultivation and application of these endophytic bacteria to harness their full potential in agricultural and medicinal contexts.
- Continued exploration of the bioactive compounds produced by these endophytes could lead to the development of novel therapeutic agents and agricultural bioproducts.
- Long-term studies are warranted to evaluate the ecological impacts and sustainability of using endophytic bacteria in agricultural practices.

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