

# Isolation, Phenotypic Diversity, and Biochemical Characterization of Cultivable Endophytic Microorganisms Associated with *Avicennia marina* from the Mangrove Ecosystem of Navsari, Gujarat

Sweta Patel<sup>1</sup>, Nafisa Patel<sup>2</sup>

<sup>1</sup>Student, Naran Lala College of Professional & Applied Sciences, Navsari, Gujarat, India

<sup>2</sup>Associate Professor & Head of the Department (Microbiology), Naran Lala College of Professional & Applied Sciences, Navsari, Gujarat, India

## Abstract

Endophytic microorganisms residing within mangrove plants endure extreme abiotic stresses, making them valuable candidates for bioprospecting. This study evaluated the phenotypic diversity, biochemical characteristics, and metabolic capabilities of cultivable endophytic microorganisms associated with *Avicennia marina* from the mangrove ecosystem of Navsari, Gujarat, India. A total of 43 morphologically distinct isolates were obtained across three sampling events. From these, 15 representative isolates (14 bacterial and 1 fungal) were selected for detailed characterization. Stem tissues yielded the highest recovery of bacterial endophytes (57.1%), followed by root and leaf-tip tissues (21.4% each). Phenotypic profiling demonstrated a predominance of Gram-positive, rod-shaped bacteria (64.3%). Biochemical assays revealed universal catalase, oxidase, and nitrate reductase activities across all bacterial isolates, indicating robust aerobic respiratory metabolism. Differential substrate utilization highlighted distinct functional capabilities, including gelatinase activity and acetoin production in isolate ISOLATE 7, urease activity in ISOLATE 11, citrate utilization in ISOLATE 15, and broad carbohydrate fermentation with gas production in ISOLATE 6. These findings demonstrate substantial metabolic versatility among *A. marina* endophytes, highlighting their potential application as sources of novel extracellular enzymes and bioactive secondary metabolites.

**Keywords:** *Avicennia marina*; mangrove endophytes; metabolic diversity; biochemical characterization; hydrolytic enzymes; microbial bioprospecting.

## 1. Introduction

Mangrove ecosystems represent highly dynamic intertidal biomes situated along tropical and subtropical coastlines, functioning as critical biogeochemical buffers at the land–sea interface [1]. Occupying an estimated global area between 137,000 km<sup>2</sup> and 180,000 km<sup>2</sup> across more than 110 maritime nations, these intertidal forests comprise approximately 25% of the world's sheltered coastal margins [1, 2]. Evolving since the Late Cretaceous period, true mangrove species have developed complex

morphological, anatomical, and physiological mechanisms to thrive under extreme coastal stresses, including fluctuating salinity gradients, waterlogged anaerobic sediments, periodic tidal submergence, and high ultraviolet exposure [3, 4]. Specialized evolutionary adaptations - such as aerial respiratory roots (pneumatophores and stilt roots), foliar salt-secreting structures, cellular osmotic regulation, and viviparous seedling reproduction - allow these woody halophytes to colonize coastal environments uninhabitable by terrestrial flora [5]. Collectively, mangrove biomes provide crucial ecosystem services, including shoreline stabilization, wave energy dissipation, blue carbon sequestration, estuarine sediment entrapment, and habitat provisioning for estuarine biodiversity [1, 6].

Along the western coastline of India, the state of Gujarat possesses the country's second-largest mangrove extent, covering over 1,100 km<sup>2</sup>. In the South Gujarat maritime belt, which encompasses coastal stretches across Navsari, Surat, Valsad, and Bharuch districts - estuarine networks formed by major rivers such as the Narmada, Tapi, Purna, Ambika, and Auranga transport substantial alluvial silt, forming expansive mudflats highly conducive to mangrove establishment. Although the regional flora includes true mangrove species like *Rhizophora mucronata*, *Ceriops tagal*, *Sonneratia apetala*, *Bruguiera gymnorhiza*, and *Excoecaria agallocha*, the grey mangrove - *Avicennia marina*, serves as the dominant pioneer halophyte across the intertidal mudflats of Navsari. Taxonomically categorized under the phylum Tracheophyta, order Lamiales, and family Acanthaceae, *A. marina* exhibits a global distribution ranging from the eastern coast of Africa to the Indo-Pacific region [7, 8, 9].

Morphologically, *A. marina* typically develops as a multi-stemmed shrub or medium-sized tree growing 3 to 10 meters in height [10, 11]. The species displays marked ecophysiological plasticity and is grouped into three distinct allopatric varieties: *A. marina* var. *australasica*, var. *eucalyptifolia*, and var. *marina* [7]. Its survival in hypersaline and hypoxic mudflats is facilitated by smooth grey bark, thick sclerophyllous leaves containing specialized salt-excreting foliar glands, and an extensive shallow root network bearing thousands of vertical pencil pneumatophores that project up to 20 cm above the sediment surface for atmospheric gas exchange [12]. Dispersed by both hydrochory (water-mediated) and anemochory (wind-assisted) mechanisms, *A. marina* effectively tolerates hyper-saline conditions, thermal fluctuations, sediment heavy metal loads, and prolonged tidal inundation [3, 7].

The survival and ecophysiological performance of *A. marina* under continuous abiotic stress are significantly supported by mutualistic interactions with endophytic microorganisms - bacteria and fungi that inhabit internal host tissues (roots, stems, leaves, pneumatophores, and seeds) without causing pathogenetic damage [6, 13, 14, 15]. These micro-environments foster specialized endophyte communities through nutrient exchange and physiological communication [6]. Cultivable and molecular diversity assessments demonstrate that bacterial endophytes colonizing *A. marina* are predominantly composed of the phyla Proteobacteria (Pseudomonadota), Bacillota, and Actinomycetota - including core genera such as *Bacillus*, *Cytobacillus*, *Mixta*, *Pseudomonas*, *Halomonas*, and *Streptomyces* alongside fungal endophytes belonging to *Aspergillus*, *Penicillium*, *Cladosporium*, and *Fusarium* [16, 17, 18, 19].

With the evolution within high-salinity, variable-temperature, and osmotic-gradient conditions, the enzymes and secondary metabolites synthesized by *A. marina* endophytes offer distinct bioprospecting advantages [16, 20]. These halotolerant biocatalysts maintain catalytic efficiency under severe industrial parameters, making them attractive targets for industrial enzymatic processes, coastal eco-remediation, bio-fertilizer development for saline agriculture, and bioactive drug discovery [16, 21]. Despite their potential, the metabolic capabilities of cultivable endophytic microbiomes associated with *A. marina* in specific estuarine ecosystems remain insufficiently characterized [22]. Therefore, this study aimed to

isolate, phenotypically characterize, and evaluate the hydrolytic enzyme-producing potential of cultivable bacterial and fungal endophytes isolated from *Avicennia marina* host tissues within the mangrove ecosystem of Navsari district, Gujarat, India.

## **2. Materials and Methodology**

### **2.1 Sample Collection and Pretreatment**

#### **2.1.1 Sample Collection**

Healthy, mature, and disease-free plants of *Avicennia marina* were selected from the natural mangrove ecosystem located in the Navsari district of South Gujarat, India (20.20009° N, 72.853193° E). Sampling was conducted during low tide under aseptic conditions to minimize environmental cross-contamination. The sampling site was chosen based on the natural abundance of *A. marina* and its ecological significance as a dominant mangrove species in the coastal region. Plant samples comprising leaves, stems, and roots along with their associated rhizospheric soil were collected aseptically during low tide using sterile stainless-steel scissors, scalpels, and spatulas to minimize external contamination. Approximately 10–20 g of rhizospheric soil tightly adhering to the root surface was collected into sterile polythene bags. Plant tissues were carefully excised without causing extensive damage to the host plants. Each sample was immediately transferred into sterile, pre-labelled polyethylene bags containing detailed information regarding sample identity, collection site, date, and sample type. To preserve the viability of the indigenous microbial communities and minimize changes in microbial composition during transportation, all samples were maintained under refrigerated conditions (approximately 4°C). [23, 24].

#### **2.1.2 Pretreatment and Surface Sterilization of Plant Samples**

The collected plant materials were subjected to a standardized surface sterilization protocol to eliminate epiphytic microorganisms while preserving the internal endophytic microbial population. This procedure is essential for ensuring that only microorganisms residing within plant tissues are isolated during subsequent culturing procedures.

Initially, all plant samples were washed thoroughly under running tap water for approximately 10–15 minutes to remove adhering soil particles, salt deposits, organic debris, and loosely attached microorganisms commonly present on mangrove surfaces. The samples were subsequently rinsed with sterile distilled water to eliminate any remaining particulate matter.

The cleaned plant materials were aseptically cut into small segments (approximately 0.5–1.0 cm in length) using sterile surgical blades inside a laminar airflow cabinet. Surface sterilization was then performed using a sequential chemical treatment protocol. The tissue segments were first immersed in 75% (v/v) ethanol for 2 minutes, which disrupts the cell membranes of surface-associated microorganisms and facilitates penetration of subsequent sterilizing agents. Following ethanol treatment, the samples were immediately transferred into 0.1% (w/v) mercuric chloride ( $\text{HgCl}_2$ ) solution for 1 minute to achieve effective elimination of persistent surface microbial contaminants. Subsequently, the samples were immersed in 2% (v/v) sodium hypochlorite ( $\text{NaOCl}$ ) solution for 1 minute as a secondary sterilization step to ensure complete removal of remaining epiphytic microorganisms.

After each sterilization step, the tissue segments were rinsed thoroughly with sterile distilled water. Following the final sodium hypochlorite treatment, the samples were washed five to six times with sterile distilled water to remove any residual sterilizing chemicals that could interfere with microbial growth during isolation.

## 2.2 Isolation and Characterization of Microorganisms

### 2.2.1 Isolation of Microorganisms

Following successful surface sterilization, the plant tissues were processed immediately for the isolation of endophytic microorganisms. Sterilized root and stem tissues were aseptically transferred into a sterile mortar containing a small volume (5–10 mL) of sterile distilled water and thoroughly macerated using a pre-sterilized mortar and pestle until a homogeneous tissue suspension was obtained. The homogenate was allowed to stand for approximately 5 minutes to facilitate the release of endophytic microorganisms from the internal plant tissues.

For the isolation of rhizospheric microorganisms, approximately 1 g of rhizospheric soil collected from the root zone of *Avicennia marina* was suspended in 9 mL of sterile distilled water to prepare the initial stock suspension ( $10^{-1}$  dilution). Serial ten-fold dilutions were prepared up to an appropriate dilution level ( $10^{-6}$ ) under aseptic conditions to reduce microbial load and facilitate the isolation of discrete colonies. Aliquots (100  $\mu$ L) of the tissue homogenate and serially diluted soil suspensions were inoculated separately onto different selective culture media using the spread plate technique. Three different culture media were employed for the selective isolation of different microbial groups.

Luria-Bertani (LB) agar was used for the isolation of endophytic bacteria because of its rich nutrient composition that supports the growth of a wide range of heterotrophic bacteria. Sabouraud Dextrose Agar (SDA) was employed for the isolation of endophytic fungi due to its acidic pH and high dextrose concentration, which favour fungal growth while suppressing bacterial contamination. Glucose Asparagine Agar (GAA) medium was used for the selective isolation of actinomycetes because it provides suitable nutritional conditions for the growth of filamentous actinobacteria.

All inoculated plates were incubated under aerobic conditions at  $28 \pm 2^\circ\text{C}$ . Bacterial cultures were monitored for 24–72 hours, whereas fungal and actinomycete cultures were observed periodically for 5–7 days, depending on their growth characteristics. The plates were examined daily for the appearance of microbial colonies exhibiting distinct morphological characteristics such as colony size, colour, pigmentation, texture, elevation, margin, opacity, and growth pattern. Morphologically distinct colonies were carefully selected and repeatedly sub-cultured onto freshly prepared respective media using the streak plate method until pure cultures were obtained. The purification process was repeated several times to ensure the isolation of single, uncontaminated microbial strains. Each purified isolate was assigned a unique laboratory identification code for subsequent characterization and experimental studies.

### 2.2.2 Maintenance and Preservation of Microbial Isolates

Pure isolates obtained from different plant tissues were maintained under appropriate laboratory conditions to preserve their viability and genetic stability throughout the course of the investigation. The cultures were incubated under optimum growth conditions and subsequently stored at  $4 \pm 2^\circ\text{C}$ .

For long-term preservation, actively growing bacterial cultures were inoculated into sterile appendorf containing 50% (v/v) sterile glycerol. The glycerol stocks were thoroughly mixed to ensure uniform cell distribution and stored at  $-80^\circ\text{C}$  until further use. (Gayathri et al., 2010).

## 2.3 Biochemical Characterization of Bacterial Isolates

The bacterial isolates were subjected to a series of conventional biochemical tests for preliminary phenotypic characterization. Pure, actively growing cultures were used for all biochemical assays. Fresh bacterial cultures were prepared on appropriate growth medium and incubated under aerobic conditions prior to testing. All biochemical tests were performed using standard microbiological procedures, with

appropriate positive and negative controls where applicable. The tests included indole production, methyl red, Voges–Proskauer, Simmons' citrate utilization, gelatin hydrolysis, phenylalanine deaminase, oxidase, catalase, nitrate reduction, and urease assays. The biochemical characteristics were evaluated based on the characteristic reactions produced by the respective isolates following incubation and reagent addition, as applicable [25, 26].

### **2.3.1 Indole Production Test**

Indole production was determined to assess the ability of the bacterial isolates to hydrolyse tryptophan through the action of the enzyme tryptophanase, resulting in the formation of indole. A loopful of a fresh bacterial culture was aseptically inoculated into sterile tryptone broth containing tryptophan and incubated at 35–37°C for 24–48 h. Following incubation, Kovac's reagent was carefully added to the inoculated broth and allowed to react without vigorous mixing. The formation of a characteristic red to cherry-red colour at the surface of the medium was considered indicative of indole production, whereas the absence of the characteristic colour development was recorded as a negative reaction. The test was performed according to standard biochemical characterization procedures.

### **2.3.2 Methyl Red Test**

The methyl red (MR) test was performed to determine the ability of the bacterial isolates to carry out mixed-acid fermentation of glucose, resulting in the production of stable acidic end products. Fresh bacterial cultures were inoculated into sterile MR-VP broth and incubated at 35–37°C for 24–48 h. After incubation, approximately 5 drops of methyl red indicator were added to the culture and mixed gently. A distinct red colouration indicated a positive reaction resulting from the accumulation of stable acidic fermentation products, whereas a yellow colouration indicated a negative reaction.

### **2.3.3 Voges–Proskauer Test**

The Voges–Proskauer (VP) test was employed to detect the production of acetoin, an intermediate metabolite associated with the 2,3-butanediol fermentation pathway. The bacterial isolates were inoculated into MR-VP broth and incubated at 35–37°C for 24–48 h. Following incubation, the culture was treated with  $\alpha$ -naphthol reagent followed by potassium hydroxide (KOH) according to the standard VP procedure. The inoculated tubes were gently agitated to facilitate aeration and subsequently allowed to stand for approximately 15–30 min. Development of a pink to red colouration was considered indicative of a positive VP reaction, while the absence of the characteristic colour development was recorded as negative.

### **2.3.4 Simmons' Citrate Utilization Test**

Citrate utilization was assessed using Simmons' citrate agar to determine the ability of the bacterial isolates to utilize citrate as the sole carbon source. A freshly grown bacterial culture was lightly streaked over the surface of a sterile Simmons' citrate agar slant using an aseptic inoculating loop. The inoculated slants were incubated at 35–37°C for 24–48 h. Utilization of citrate was determined based on visible bacterial growth accompanied by a change in the colour of the medium from green to blue, resulting from alkalinization of the medium during citrate metabolism. Absence of growth and retention of the original green colour were considered indicative of a negative reaction.

### **2.3.5 Gelatin Hydrolysis Test**

Gelatin hydrolysis was performed to determine the ability of bacterial isolates to produce extracellular gelatinase capable of hydrolysing gelatin into smaller peptides and amino acids. Fresh bacterial cultures were inoculated into nutrient gelatin medium by stabbing the medium with a sterile inoculating needle. The inoculated tubes were incubated at 35–37°C for 24–48 h. Following incubation, the tubes were cooled under conditions appropriate for assessing gelatin liquefaction. Persistent liquefaction of the medium

following cooling was interpreted as evidence of gelatin hydrolysis, whereas solidification of the medium indicated the absence of detectable gelatinase activity. The assay was conducted following standard biochemical characterization procedures.

### **2.3.6 Phenylalanine Deaminase Test**

Phenylalanine deaminase activity was determined to assess the ability of bacterial isolates to deaminate L-phenylalanine, producing phenylpyruvic acid. Each isolate was aseptically streaked onto the surface of a phenylalanine agar slant and incubated at 35–37°C for 18–24 h. After incubation, 10% ferric chloride solution was added to the inoculated surface of the medium. Development of a characteristic green colouration was considered a positive reaction, resulting from the formation of a ferric–phenylpyruvate complex, whereas absence of the green colouration was recorded as negative.

### **2.3.7 Oxidase Test**

The oxidase test was performed to determine the presence of cytochrome c oxidase in the bacterial isolates. A portion of a freshly grown bacterial colony was transferred aseptically onto an appropriate oxidase test surface using a sterile applicator. Oxidase reagent was subsequently applied to the bacterial growth. Development of a dark purple to blue colouration within the specified reaction period was considered indicative of a positive oxidase reaction. Absence of the characteristic colour change was recorded as a negative reaction. Fresh bacterial cultures were used to obtain reliable results and to minimize interference associated with prolonged culture age.

### **2.3.8 Catalase Test**

Catalase activity was determined by evaluating the ability of the bacterial isolates to decompose hydrogen peroxide into water and molecular oxygen. A portion of a freshly grown bacterial colony was transferred to a clean, dry glass slide, followed by the addition of 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Immediate formation of visible bubbles due to the liberation of oxygen was considered a positive catalase reaction, whereas the absence of bubble formation was recorded as negative. Fresh cultures were used for the assay to ensure reliable detection of catalase activity.

### **2.3.9 Nitrate Reduction Test**

The nitrate reduction test was performed to determine the ability of the bacterial isolates to reduce nitrate (NO<sub>3</sub><sup>-</sup>) to nitrite (NO<sub>2</sub><sup>-</sup>) or other reduced nitrogenous compounds. Each isolate was inoculated into sterile nitrate reduction broth and incubated at 35–37°C for 24–48 h. Following incubation, nitrate reduction reagents were added according to the standard procedure. Development of a red colour following the addition of the appropriate reagents indicated reduction of nitrate to nitrite. In reactions where no colour development was observed, further confirmation was performed using the prescribed secondary reagent to distinguish between unreduced nitrate and reduction beyond nitrite. The reaction was interpreted according to standard nitrate reduction procedures.

### **2.3.10 Urease Test**

Urease activity was determined to assess the ability of the bacterial isolates to hydrolyse urea through production of the enzyme urease. The isolates were inoculated onto urea-containing medium and incubated at 35–37°C for 24–48 h. Hydrolysis of urea results in the formation of ammonia, causing an increase in the pH of the medium. This alkaline shift was detected using phenol red as the pH indicator. Development of a pink to reddish-pink colouration of the medium was considered indicative of urease activity, whereas absence of the characteristic colour change was recorded as a negative reaction.

### **2.3.11 Carbohydrate Utilization Test**

Carbohydrate utilization by the bacterial isolates was determined using a basal carbohydrate fermentation

medium supplemented separately with glucose, lactose, mannose, maltose, xylose, and sucrose as the test carbohydrates. The assay was performed to determine the ability of the isolates to ferment individual carbohydrates with the production of acid and/or gas.

The carbohydrate fermentation medium was prepared according to the manufacturer's/standard formulation and dispensed into sterile fermentation tubes containing an inverted Durham tube for detection of gas production. Each carbohydrate was tested separately, and the medium was sterilized by the recommended method. Freshly grown bacterial cultures were aseptically inoculated into the respective carbohydrate-containing media using a sterile inoculating loop. An uninoculated tube of each carbohydrate medium was maintained as a negative control. The inoculated tubes were incubated at 35–37°C for 24–48 h under aerobic conditions.

Carbohydrate fermentation was assessed based on changes in the colour of the indicator present in the medium. Acid production, resulting from the fermentation of the respective carbohydrate, was recorded based on the characteristic colour change of the medium from its original colour. Gas production was determined by the formation and accumulation of a visible gas bubble within the inverted Durham tube. The absence of the characteristic acid reaction and gas accumulation was recorded as a negative reaction. The reactions were recorded separately as acid (+/–) and gas (+/–) for each carbohydrate tested.

The carbohydrate utilization profile of the isolates was determined using glucose, lactose, mannose, maltose, xylose, and sucrose. The resulting fermentation profiles were used as phenotypic characteristics for the preliminary differentiation and biochemical characterization of the bacterial isolates. The test was performed following standard procedures for carbohydrate fermentation and biochemical characterization of bacteria.

### 3. Results

#### 3.1. Sample Collection and Pretreatment

Healthy and mature tissues of *Avicennia marina*, including stems, along with rhizospheric soil samples collected from mangrove ecosystems of Navsari district, Gujarat, were processed for the isolation of endophytic microorganisms. The applied surface sterilization protocol was found to be effective, as no visible contamination was observed during the isolation process, indicating the successful removal of epiphytic microorganisms from the plant surface.

A total of three sample collections were conducted during the study period. Following isolation, a total of 43 morphologically distinct microbial isolates were recovered from the collected samples. The number of isolates varied among the sampling periods.

The first sampling, conducted on 1 October 2022, yielded the highest number of isolates, with 22 distinct microbial cultures successfully recovered. The second sampling carried out on 24 November 2023 resulted in the isolation of 15 microbial cultures. In the third sampling event, conducted on 3 March 2024, 6 isolates were obtained.

The isolates exhibited diverse colony morphologies with variations in colony size, texture, elevation, and margin characteristics, suggesting the presence of a diverse microbial community associated with *A. marina*. The purified isolates were maintained on suitable culture media and preserved for further morphological, biochemical, and molecular characterization.



**Figure 1: Sample Collection**

### 3.1.1 Isolation of Endophytic Microorganisms from *Avicennia marina*

Healthy stem and root tissues of *Avicennia marina* collected during three independent sampling events were processed for the isolation of cultivable endophytic microorganisms. Following surface sterilization and aseptic maceration, tissue homogenates were cultured on selective media for the recovery of bacterial, fungal, and actinomycete endophytes. After incubation, microbial colonies with distinct morphological characteristics appeared on the culture media at different time intervals. Individual colonies were repeatedly sub-cultured to obtain pure cultures, ensuring the elimination of mixed microbial populations and the establishment of morphologically uniform isolates.

A total of 43 endophytic microbial isolates were successfully recovered from the stem and root tissues of *Avicennia marina*. The isolates exhibited considerable diversity in their cultural and morphological characteristics, including variations in colony size, shape, colour, pigmentation, elevation, margin, surface texture, opacity, and growth pattern. Such phenotypic diversity indicates the presence of a heterogeneous cultivable endophytic microbial community naturally associated with *Avicennia marina*. The recovery of diverse microorganisms from healthy plant tissues further demonstrates the ecological richness of the mangrove endophytic microbiota and suggests the existence of microorganisms with different physiological and metabolic capabilities.

Following isolation, all forty-three isolates were purified through repeated streaking on fresh media to obtain pure cultures. The purified isolates were subsequently maintained under appropriate laboratory conditions for further investigation

A systematic selection strategy was adopted to identify representative isolates for detailed investigation. Initially, all recovered isolates were examined for their colony morphology and cultural characteristics. Isolates exhibiting distinct phenotypic features, stable growth during repeated sub-culturing, and well-defined colony characteristics were considered for further evaluation. Particular emphasis was placed on selecting isolates that represented the morphological diversity observed among the total collection, thereby minimizing redundancy arising from isolates displaying nearly identical colony characteristics.

Based on these preliminary observations, 15 representative endophytic isolates were selected from the original collection of 43 isolates for detailed characterization. The selected isolates encompassed a broad range of colony morphologies and growth characteristics, making them suitable representatives of the cultivable endophytic microbial diversity associated with *Avicennia marina*.

| Sr. No. | Isolate Code | Source Tissue |
|---------|--------------|---------------|
| 1       | ISOLATE 1    | Root          |
| 2       | ISOLATE 2    | Stem          |
| 3       | ISOLATE 3    | Stem          |

|    |            |      |
|----|------------|------|
| 4  | ISOLATE 4  | Stem |
| 5  | ISOLATE 5  | Stem |
| 6  | ISOLATE 6  | Root |
| 7  | ISOLATE 7  | Stem |
| 8  | ISOLATE 8  | Stem |
| 9  | ISOLATE 9  | Root |
| 10 | ISOLATE 10 | Stem |
| 11 | ISOLATE 11 | Root |
| 12 | ISOLATE 12 | Stem |
| 13 | ISOLATE 13 | Root |
| 14 | ISOLATE 14 | Stem |
| 15 | ISOLATE 15 | Stem |

**Table 1 : Isolation of Microorganisms**

### 3.1.2 Morphological Characterization of Endophytes

A total of fifteen representative endophytic isolates (ISOLATE 1–ISOLATE 15), recovered from different tissues of *Avicennia marina* were subjected to preliminary morphological characterization based on Gram reaction and colony morphology. The isolates originated from the tip, stem and root tissues, indicating the successful colonization of multiple plant niches by diverse endophytic bacterial and fungal communities. Among the fifteen isolates, nine (64.3%) were Gram-positive, whereas five (35.7%) were Gram-negative and one is fungi. Gram-positive bacteria constituted the dominant group and were represented mainly by rod-shaped cells (ISOLATE 2, ISOLATE 4, ISOLATE 5, ISOLATE 7, ISOLATE 9, ISOLATE 13, ISOLATE 14, and ISOLATE 15), while two isolates (ISOLATE 1 and ISOLATE 11) were identified as Gram-positive cocci. The Gram-negative group consisted exclusively of rod-shaped bacteria (ISOLATE 3, ISOLATE 6, ISOLATE 10, and ISOLATE 12).

Colony morphology exhibited considerable variation among the isolates. Circular colonies were observed in four isolates (ISOLATE 2, ISOLATE 6, ISOLATE 7, and ISOLATE 15), whereas the remaining ten isolates produced irregularly shaped colonies. Colony margins were either entire or undulate, with entire margins recorded in seven isolates (ISOLATE 2, ISOLATE 6, ISOLATE 9, ISOLATE 10, ISOLATE 13, ISOLATE 14, and ISOLATE 15), while the remaining isolates displayed undulate margins. The majority of isolates exhibited a raised colony elevation, whereas only ISOLATE 2 and ISOLATE 15 formed convex colonies and ISOLATE 6 showed a flat elevation.

The colony surface characteristics also varied among the isolates. Dry colonies predominated and were observed in seven isolates (ISOLATE 1, ISOLATE 3, ISOLATE 4, ISOLATE 5, ISOLATE 9, ISOLATE 11, and ISOLATE 15). Moist colonies were produced by five isolates (ISOLATE 7, ISOLATE 10, ISOLATE 12, ISOLATE 13, and ISOLATE 14), whereas two isolates (ISOLATE 2 and ISOLATE 6) exhibited a viscous colony surface. Regarding colony opacity, opaque colonies were more prevalent and were recorded for ten isolates, while four isolates (ISOLATE 2, ISOLATE 6, ISOLATE 7, and ISOLATE 15) exhibited translucent colonies.

With respect to tissue distribution, bacterial endophytes were isolated predominantly from stem tissues (eight isolates), followed by root tissues (three isolates) and tip tissues (three isolates). The observed diversity in Gram reaction, cellular morphology, and colony characteristics suggests the presence of a

heterogeneous bacterial endophytic community associated with different tissues of *A. marina*, providing a morphological basis for subsequent biochemical, physiological, and molecular characterization.

| Isolate    | Origin | Gram reaction       | Shape     | Margin   | Elevation | Surface | Opacity     |
|------------|--------|---------------------|-----------|----------|-----------|---------|-------------|
| ISOLATE 1  | Tip    | Gram positive cocci | Circular  | Undulate | Raised    | Dry     | Opaque      |
| ISOLATE 2  | Tip    | Gram positive rod   | Circular  | Entire   | Convex    | Viscous | Translucent |
| ISOLATE 3  | Tip    | Gram negative rod   | Irregular | Undulate | Raised    | Dry     | Opaque      |
| ISOLATE 4  | Stem   | Gram positive rod   | Irregular | Undulate | Raised    | Dry     | Opaque      |
| ISOLATE 5  | Root   | Gram positive rod   | Irregular | Undulate | Raised    | Dry     | Opaque      |
| ISOLATE 6  | Root   | Gram negative rod   | Circular  | Entire   | Flat      | Viscous | Translucent |
| ISOLATE 7  | Stem   | Gram positive rod   | Circular  | Undulate | Raised    | Moist   | Opaque      |
| ISOLATE 9  | Stem   | Gram positive rod   | Irregular | Entire   | Raised    | Dry     | Opaque      |
| ISOLATE 10 | Stem   | Gram negative rod   | Irregular | Entire   | Raised    | Moist   | Opaque      |
| ISOLATE 11 | Stem   | Gram positive cocci | Circular  | Undulate | Raised    | Dry     | Opaque      |
| ISOLATE 12 | Stem   | Gram negative rod   | Irregular | Undulate | Raised    | Moist   | Opaque      |
| ISOLATE 13 | Stem   | Gram positive rod   | Irregular | Entire   | Raised    | Moist   | Opaque      |
| ISOLATE 14 | Stem   | Gram positive rod   | Irregular | Entire   | Raised    | Moist   | Opaque      |
| ISOLATE 15 | Root   | Gram positive rod   | Circular  | Entire   | Convex    | Dry     | Opaque      |

**Table 2: Gram Reaction and Morphology of isolates**

### 3.1.3 Biochemical Characterization of Endophytic Bacterial Isolates

The selected endophytic bacterial isolates were characterized using a series of enzymatic and biochemical assays to evaluate their physiological and metabolic properties. The results revealed considerable phenotypic diversity among the isolates while also demonstrating several common biochemical characteristics.

All isolates were positive for oxidase, catalase, and nitrate reductase activities, indicating the presence of cytochrome c oxidase and catalase enzymes as well as their ability to reduce nitrate. These observations suggest that the isolates possess active aerobic respiratory metabolism and are capable of utilizing nitrate as an alternative electron acceptor under appropriate conditions. In contrast, all isolates were negative for the indole production test, indicating the absence of detectable tryptophanase activity under the experimental conditions.

The Methyl Red (MR) and Voges–Proskauer (VP) tests demonstrated variability in glucose fermentation pathways among the isolates. Positive MR reactions were observed in isolates ISOLATE 1, ISOLATE 5, ISOLATE 6, ISOLATE 12, and ISOLATE 13, indicating the production of stable acidic end-products during glucose metabolism. In comparison, only isolate ISOLATE 7 produced a positive VP reaction, suggesting the formation of acetoin through the butanediol fermentation pathway, whereas the remaining isolates were VP negative.

The ability to utilize citrate as the sole carbon source was detected only in isolate ISOLATE 15, while all other isolates were unable to utilize citrate under the conditions employed. Gelatin hydrolysis was observed exclusively in isolate ISOLATE 7, indicating the production of extracellular gelatinase. Phenylalanine deaminase activity was detected in isolates ISOLATE 2, ISOLATE 4, and ISOLATE 6, whereas the remaining isolates did not exhibit this enzymatic activity. Urease production was recorded only in isolate ISOLATE 11, demonstrating its ability to hydrolyze urea into ammonia and carbon dioxide, while all other isolates were urease negative.

Overall, the biochemical characterization demonstrated distinct enzymatic profiles among the selected bacterial endophytes, providing useful phenotypic characteristics for their preliminary differentiation.

|                                 | ISO<br>LA<br>TE<br>1 | ISO<br>LA<br>TE<br>2 | ISO<br>LA<br>TE<br>3 | ISO<br>LA<br>TE<br>4 | ISO<br>LA<br>TE<br>5 | ISO<br>LA<br>TE<br>6 | ISO<br>LA<br>TE<br>7 | ISO<br>LA<br>TE<br>9 | ISO<br>LA<br>TE<br>10 | ISO<br>LA<br>TE<br>11 | ISO<br>LA<br>TE<br>12 | ISO<br>LA<br>TE<br>13 | ISO<br>LA<br>TE<br>14 | ISO<br>LA<br>TE<br>15 |
|---------------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Indo<br>le<br>test              | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                     | -                     | -                     | -                     | -                     | -                     |
| Met<br>hyl<br>red               | +                    | -                    | -                    | -                    | +                    | +                    | -                    | -                    | -                     | -                     | +                     | +                     | -                     | -                     |
| Vog<br>es-<br>Pros<br>kau<br>er | -                    | -                    | -                    | -                    | -                    | -                    | +                    | -                    | -                     | -                     | -                     | -                     | -                     | -                     |
| Sim<br>mon<br>'s<br>Citr<br>ate | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                     | -                     | -                     | -                     | -                     | +                     |

|                         |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|-------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gelatinase              | - | - | - | - | - | - | + | - | - | - | - | - | - | - |
| Phenylalanine deaminase | - | + | - | + | - | + | - | - | - | - | - | - | - | - |
| Oxidase                 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Catalase                | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Nitrate reductase       | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Urease                  | - | - | - | - | - | - | - | - | - | + | - | - | - | - |

**Table 3 : Biochemical Test of isolates**

### 3.1.4 Carbohydrate Fermentation Profile of Endophytic Bacterial Isolates

The carbohydrate fermentation assay was performed using six different carbohydrates, namely glucose, lactose, mannose, maltose, xylose, and sucrose, to evaluate the metabolic versatility of the selected endophytic bacterial isolates. Considerable variation was observed in the utilization of individual carbohydrates, reflecting the metabolic diversity among the isolates.

All fourteen isolates fermented glucose with acid production, confirming glucose as the primary carbohydrate utilized by the bacterial endophytes. Gas production during glucose fermentation was observed only in isolate ISOLATE 6, whereas the remaining isolates produced acid without detectable gas formation.

Lactose fermentation was comparatively limited among the isolates. Acid production was observed in isolates ISOLATE 3, ISOLATE 5, ISOLATE 9, and ISOLATE 13, while the remaining isolates were unable to ferment lactose. Gas production from lactose was detected only in isolate ISOLATE 6.

Mannose fermentation was more widely distributed, with acid production recorded in isolates ISOLATE 2, ISOLATE 3, ISOLATE 5, ISOLATE 6, ISOLATE 7, ISOLATE 9, ISOLATE 10, ISOLATE 11, ISOLATE 13, ISOLATE 14, and ISOLATE 15. In contrast, isolates ISOLATE 1, ISOLATE 4, and ISOLATE 12 did not ferment mannose. None of the mannose-positive isolates produced gas.

Maltose served as a fermentable substrate for the majority of the isolates. Acid production was observed in ISOLATE 1, ISOLATE 2, ISOLATE 3, ISOLATE 5, ISOLATE 6, ISOLATE 7, ISOLATE 9, ISOLATE 10, ISOLATE 11, ISOLATE 12, ISOLATE 13, and ISOLATE 15, whereas isolates ISOLATE 4 and

ISOLATE 14 did not ferment maltose. Similar to glucose and lactose, gas production during maltose fermentation was detected only in isolate ISOLATE 6.

None of the isolates produced acid from xylose, indicating that this pentose sugar was not utilized under the conditions of the present study. Likewise, gas production from xylose was observed exclusively in isolate ISOLATE 6.

Sucrose fermentation was observed in most of the isolates. Acid production was recorded in ISOLATE 1, ISOLATE 2, ISOLATE 3, ISOLATE 5, ISOLATE 6, ISOLATE 7, ISOLATE 9, ISOLATE 10, ISOLATE 12, ISOLATE 13, ISOLATE 14, and ISOLATE 15, whereas ISOLATE 4 and ISOLATE 11 did not ferment sucrose. Gas production from sucrose was detected only in isolate ISOLATE 6.

Overall, the carbohydrate fermentation profiles demonstrated substantial metabolic variability among the endophytic bacterial isolates. Although all isolates shared the ability to utilize glucose, differences in the fermentation of lactose, mannose, maltose, and sucrose highlighted their physiological diversity. These phenotypic characteristics provide valuable information for the preliminary characterization and differentiation of the bacterial isolates and complement their subsequent molecular identification.

| Su<br>gar       |              | ISO<br>LA<br>TE<br>1 | ISO<br>LA<br>TE<br>2 | ISO<br>LA<br>TE<br>3 | ISO<br>LA<br>TE<br>4 | ISO<br>LA<br>TE<br>5 | ISO<br>LA<br>TE<br>6 | ISO<br>LA<br>TE<br>7 | ISO<br>LA<br>TE<br>9 | ISO<br>LA<br>TE<br>10 | ISO<br>LA<br>TE<br>11 | ISO<br>LA<br>TE<br>12 | ISO<br>LA<br>TE<br>13 | ISO<br>LA<br>TE<br>14 | ISO<br>LA<br>TE<br>15 |
|-----------------|--------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Glu<br>cos<br>e | A<br>ci<br>d | +                    | +                    | +                    | +                    | +                    | +                    | +                    | +                    | +                     | +                     | +                     | +                     | +                     | +                     |
|                 | G<br>a<br>s  | -                    | -                    | -                    | -                    | -                    | +                    | -                    | -                    | -                     | -                     | -                     | -                     | -                     | -                     |
| Lac<br>tos<br>e | A<br>ci<br>d | -                    | -                    | +                    | -                    | +                    | -                    | -                    | +                    | -                     | -                     | -                     | +                     | -                     | -                     |
|                 | G<br>a<br>s  | -                    | -                    | -                    | -                    | -                    | +                    | -                    | -                    | -                     | -                     | -                     | -                     | -                     | -                     |
| Ma<br>nno<br>se | A<br>ci<br>d | -                    | +                    | +                    | -                    | +                    | +                    | +                    | +                    | +                     | +                     | -                     | +                     | +                     | +                     |
|                 | G<br>a<br>s  | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                     | -                     | -                     | -                     | -                     | -                     |
| Ma<br>ltos<br>e | A<br>ci<br>d | +                    | +                    | +                    | -                    | +                    | +                    | +                    | +                    | +                     | +                     | +                     | +                     | -                     | +                     |

|                 |                  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|-----------------|------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|                 | G<br>a<br>s      | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| Xyl<br>ose      | A<br>c<br>i<br>d | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
|                 | G<br>a<br>s      | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| Suc<br>ros<br>e | A<br>c<br>i<br>d | + | + | + | - | + | + | + | + | + | - | + | + | + | + |
|                 | G<br>a<br>s      | - | - | - | - | - | + | - | - | - | - | - | - | - | - |

**Table 4 : Carbohydrate utilization test of isolates**

## Discussion

Mangrove ecosystems are highly dynamic intertidal environments characterized by fluctuating salinity, tidal inundation, waterlogging, and other environmental stresses. These conditions create selective pressures that influence the composition and functional characteristics of microorganisms associated with mangrove plants. Endophytic microorganisms residing within plant tissues are increasingly recognized as important components of plant-associated microbial communities and may contribute to nutrient acquisition, stress tolerance, and production of bioactive metabolites [27].

In the present study, 43 morphologically distinct cultivable endophytic microorganisms were recovered from *Avicennia marina* tissues collected from the mangrove ecosystem of Navsari, Gujarat. The recovery of multiple morphologically distinct isolates indicates considerable cultivable microbial diversity within the examined plant tissues. Similar diversity has been reported in previous investigations of *A. marina* endophytes. Soldan et al. (2019) reported diverse bacterial endophytes associated with *A. marina* propagules and demonstrated their potential importance in plant establishment and salt-stress conditions. These observations support the presence of diverse cultivable bacterial communities associated with *A. marina*, although the number and composition of recovered isolates may vary according to geographical location, plant tissue, culture medium, incubation conditions, and isolation strategy.

In the present study, 15 representative isolates were selected from the original collection of 43 isolates based on differences in colony morphology and growth characteristics. Among these, 14 were bacterial isolates and one was fungal. Bacterial endophytes were recovered predominantly from stem tissues, followed by root and tip tissues. Tissue-associated variation in microbial communities has also been reported in *A. marina*, suggesting that different plant compartments may provide distinct ecological niches for microorganisms [17]. However, the higher recovery from stem tissue in the present study should be interpreted cautiously because culture-dependent isolation represents only microorganisms capable of growing under the selected laboratory conditions.

The predominance of Gram-positive bacteria (64.3%) was an important observation in the present study. Gram-positive rod-shaped bacteria represented the major morphological group among the selected

isolates. Similar observations have been reported in studies of cultivable endophytes associated with *A. marina*. Soldan et al. (2019) reported bacterial endophytes belonging to several major bacterial groups, including Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes. More recent investigations have also demonstrated considerable bacterial diversity associated with *A. marina* tissues [17]. The relatively high representation of Gram-positive bacteria in the present study may reflect their ability to persist under the physicochemical conditions associated with mangrove environments; however, molecular identification would be necessary to establish their taxonomic composition.

Considerable variation was observed in colony morphology, including colony shape, margin, elevation, surface texture and opacity. Such phenotypic variation provides preliminary evidence of heterogeneity among the cultivable isolates. Morphological characterization is a useful initial approach for differentiating microbial isolates before biochemical and molecular identification [25]. However, colony morphology alone cannot be used for definitive taxonomic identification because different bacterial taxa may exhibit similar cultural characteristics.

Biochemical characterization further demonstrated considerable physiological variation among the bacterial isolates. All 14 bacterial isolates were positive for catalase, oxidase and nitrate reduction, whereas all isolates were negative for indole production. The positive catalase reaction indicates the ability of the isolates to decompose hydrogen peroxide, while oxidase positivity indicates detectable cytochrome oxidase activity. Nitrate reduction demonstrates the ability of the isolates to reduce nitrate under the experimental conditions. Similar biochemical profiling has been used for preliminary characterization of mangrove-associated bacterial isolates [25, 28].

The absence of detectable indole production in all isolates indicates that indole was not detected under the conditions employed for the assay. However, a negative indole test should not be interpreted as complete absence of indole-related metabolic pathways because metabolite production can depend on substrate availability and culture conditions.

Variation in Methyl Red and Voges–Proskauer reactions provided additional evidence of differences in carbohydrate metabolism among the isolates. Positive MR reactions were observed in ISOLATE 1, ISOLATE 5, ISOLATE 6, ISOLATE 12 and ISOLATE 13, indicating production of stable acidic end products from glucose metabolism. In contrast, only ISOLATE 7 showed a positive VP reaction, indicating detectable acetoin production. Such differences demonstrate that the bacterial isolates possess distinct metabolic pathways for glucose utilization.

The restricted occurrence of other biochemical characteristics was also noteworthy. Gelatin hydrolysis was detected only in ISOLATE 7, indicating detectable extracellular gelatinase activity. Extracellular hydrolytic enzymes may contribute to the degradation of complex organic substrates and nutrient acquisition. Mangrove-associated microorganisms have received considerable attention as potential sources of hydrolytic enzymes and other bioactive products because of their adaptation to challenging environmental conditions [20].

Phenylalanine deaminase activity was observed in ISOLATE 2, ISOLATE 4 and ISOLATE 6, indicating differences in amino-acid metabolism among the isolates. Similarly, urease activity was detected only in ISOLATE 11. Urease enables microorganisms to hydrolyse urea with the formation of ammonia and carbon dioxide. The occurrence of this activity in one isolate indicates functional differentiation among the bacterial endophytes. Nevertheless, the ecological significance of these activities within *A. marina* tissues would require further quantitative and plant-associated studies.

The carbohydrate fermentation profiles provided additional evidence of metabolic diversity. All 14 bacterial isolates fermented glucose with acid production, whereas lactose, mannose, maltose and sucrose showed isolate-specific patterns. None of the isolates produced acid from xylose under the experimental conditions. ISOLATE 6 was distinctive because it produced gas from glucose, lactose, maltose, xylose and sucrose. The variation in carbohydrate utilization demonstrates that the selected isolates possess different metabolic capabilities and provides useful phenotypic characteristics for their preliminary differentiation.

The observed metabolic diversity may be related to the complex ecological conditions of mangrove ecosystems. Mangrove-associated microorganisms interact with plant-derived compounds, organic matter and nutrients under fluctuating salinity, moisture and oxygen conditions. Microbial communities associated with *A. marina* have been shown to vary according to plant compartment and environmental conditions [17]. Such environmental heterogeneity may contribute to the selection of microorganisms with different physiological and metabolic capabilities.

Endophytic microorganisms may also contribute to plant stress adaptation through nutrient acquisition, production of plant-growth-promoting compounds, modulation of plant hormones and regulation of stress responses [27]. Soldan et al. (2019) demonstrated that bacterial endophytes isolated from *A. marina* could promote plant establishment and improve the performance of crop plants under saline conditions. Therefore, the cultivable endophytes recovered in the present study may represent potential candidates for subsequent functional investigations, although their plant-beneficial effects cannot be established solely from the biochemical results obtained here.

The present study represents a culture-dependent assessment and therefore does not capture the complete microbial diversity associated with *A. marina*. The 43 isolates represent microorganisms capable of growing under the selected culture conditions, while the 15 isolates were selected as representatives of observed phenotypic diversity. Molecular identification, particularly 16S rRNA gene sequencing for bacterial isolates, would provide greater taxonomic resolution and allow comparison with previously reported *A. marina* endophytes. Recent studies have demonstrated the value of molecular approaches for revealing the bacterial diversity associated with *A. marina* and identifying potentially novel or functionally important bacterial taxa [17].

Overall, the present findings demonstrate considerable phenotypic and biochemical diversity among cultivable endophytic microorganisms associated with *Avicennia marina* from the Navsari mangrove ecosystem. The variation in Gram reaction, colony morphology, biochemical characteristics and carbohydrate utilization provides a useful preliminary basis for further molecular and functional characterization. In particular, isolates such as ISOLATE 6, ISOLATE 7, ISOLATE 11 and ISOLATE 15, which displayed distinctive biochemical characteristics, may be considered for further investigation. Further molecular identification and functional assays will be necessary to establish their taxonomic identity, ecological significance and potential biotechnological applications.

## 5. Conclusion

This study successfully isolated and characterized 43 cultivable endophytic microorganisms from *Avicennia marina* in the Navsari mangrove ecosystem, establishing a high recovery rate predominantly from stem tissues (57.1%). Detailed phenotypic and biochemical evaluation of 15 representative isolates (ISOLATE 1–ISOLATE 15) revealed substantial physiological diversity. All bacterial isolates demonstrated universal catalase, oxidase, and nitrate reductase activities, reflecting active aerobic

respiration and metabolic adaptability under stress. Specific isolates exhibited distinct functional traits, including extracellular gelatinase production and acetoin synthesis (ISOLATE 7), urease activity (ISOLATE 11), citrate utilization (ISOLATE 15), and gas-producing carbohydrate fermentation (ISOLATE 6). The predominance of Gram-positive, metabolically versatile bacterial endophytes underscores the ecological richness of *A. marina* microbiomes, highlighting these strains as valuable targets for bioprospecting hydrolytic enzymes and plant-associated bioactive metabolites.

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