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Phylogenetic diversity of culturable bacterial endophytes associated with *Avicennia marina* from coastal mangroves of the Eastern Province, Saudi Arabia

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Abstract. *Alanazi AA, Hammami I, Alshammari A, Almahasheer H. 2026. Phylogenetic diversity of culturable bacterial endophytes associated with* Avicennia marina *from coastal mangroves of the Eastern Province, Saudi Arabia. Biodiversitas 27 (2): d270201. https://doi.org/10.13057/biodiv/d270201.* Mangrove ecosystems host diverse endophytic bacterial communities that contribute to plant survival under saline and tidally fluctuating environments. This study investigates the phylogenetic diversity and in vitro functional traits of culturable bacterial endophytes associated with *Avicennia marina* collected from coastal mangroves of the Eastern Province, Saudi Arabia. A total of sixteen bacterial isolates were recovered from leaf tissues and identified based on 16S rRNA gene sequencing, revealing dominance of the genus *Bacillus* (*B. safensis, B. subtilis, B. amyloliquefaciens, B. megaterium,* and *B. inaquosorum*), along with *Brevibacterium halotolerans*, expanding current knowledge of region- and host-associated mangrove endophytes functional screening demonstrated variable hydrolytic enzyme activities, including protease, amylase, cellulase, and chitinase production. Qualitative dye-decolorization assays showed that several isolates were capable of transforming lignin-mimicking dyes under laboratory conditions. In vitro antibacterial activity against selected human pathogens was observed in a subset of isolates, with minimum inhibitory concentrations ranging from 0.312 to 5 mg/mL. Scanning electron microscopy revealed morphological alterations in treated bacterial cells, supporting the inhibitory effects observed in the antimicrobial assay. Overall, this study provides baseline data on the culturable endophytic bacterial diversity associated with *A. marina* in the Arabian Gulf region and highlights preliminary functional traits that warrant further genomic and in planta validation.

Keywords: Antimicrobial activity, Arabian Gulf, *Avicennia marina*, *Brevibacterium halotolerans*, endophytic *Bacillus*

INTRODUCTION

Mangrove ecosystems are specialized coastal environments occurring along tropical and subtropical shorelines, characterized by high salinity, tidal inundation, anoxic sediments, and elevated organic matter (Almahasheer 2018; Shahimin et al. 2025). These extreme conditions select for resilient microbial communities that are essential for nutrient cycling, plant adaptation, and overall ecosystem functioning (Anton et al. 2020; Cadamuro et al. 2021; Alghrably et al. 2024). Among these communities, endophytic bacteria, which inhabit plant tissues without causing disease, represent a key yet understudied component of mangrove microbiomes (Yuan et al. 2023). By residing within internal tissues, endophytes can establish mutualistic interactions with their hosts, promoting survival under environmental stress and contributing to the maintenance of biodiversity in these ecosystems. Their presence also reflects a fine-scale adaptation of microbial communities to highly variable and often extreme mangrove conditions.

Endophytic bacteria support plant health and stress tolerance through multiple mechanisms, including phytohormone production, nutrient mobilization, nitrogen fixation, and siderophore-mediated iron acquisition (Gamalero and Glick 2011; Pattnaik et al. 2021; Nysanth et al. 2023). In mangroves, *Avicennia marina*-associated endophytes have been shown to enhance salinity tolerance, mitigate oxidative stress, and facilitate survival under hypoxic and fluctuating tidal conditions, reflecting long-term co-adaptation between host and microbes (Martin et al. 2019; Alghamdi et al. 2024; Savitri and Askitosari 2024). These relationships highlight the importance of microbial diversity itself, as different bacterial lineages may confer distinct functional advantages, influencing the range of metabolic and stress-tolerance capabilities within the host. Such diversity is critical for ecosystem resilience, particularly in regions where environmental conditions are highly dynamic.

From a biotechnological perspective, mangrove endophytic bacteria are recognized as rich sources of extracellular enzymes and bioactive metabolites with potential applications in agriculture, environmental remediation, and medicine (Escalas et al. 2019; Mayanglambam et al. 2020; Cárdenas-Moreno et al. 2023). Many isolates display antimicrobial activity against human and plant pathogens, and enzymatic capabilities for degrading hydrocarbons and synthetic dyes through laccases, peroxidases, and oxygenases (Mnif et al. 2015; Singh and Dubey 2018; Dewiyanti et al. 2022). These

functional traits are often lineage-specific, emphasizing the need for phylogenetically informed studies that link taxonomic identity to functional potential (Alghamdi et al. 2025). Understanding these links can guide the selection of strains for targeted applications and enhance sustainable use of microbial resources.

Despite their ecological and applied significance, the bacterial diversity of mangrove endophytes in the Arabian Gulf remains poorly characterized. Most studies in Saudi Arabia have focused on sediment-associated or rhizospheric microbes, while endophytic bacteria residing within internal plant tissues remain largely unexplored in both phylogenetic and taxonomic contexts. The extreme environmental conditions of the region, including hypersalinity, high temperatures, and limited freshwater input, likely select for distinct evolutionary lineages with unique adaptive traits. Consequently, the absence of systematic, culture-based investigations represents a regional and taxonomic knowledge gap, limiting our understanding of microbial diversity and its functional implications in mangrove ecosystems. Filling this gap is critical not only for biodiversity studies but also for identifying locally adapted strains with potential for biotechnological exploitation.

To address this gap, the present study investigates bacterial endophytes isolated from *A. marina* collected from the Eastern Province of Saudi Arabia, with primary focus on phylogenetic diversity assessed through culture-based isolation and 16S rRNA gene sequencing. The study characterizes the taxonomic composition and phylogenetic relationships of isolates to identify dominant lineages and previously underrepresented taxa. Subsequently, the functional potential of the endophytic community is evaluated via screening for extracellular hydrolytic enzymes, dye decolorization, and antibacterial activity against selected human pathogens. By integrating phylogenetic and functional analyses, this study provides novel insights into the diversity of *A. marina*-associated bacterial endophytes in the Arabian Gulf and highlights their preliminary biotechnological potential, contributing to regional biodiversity knowledge in line with Saudi Vision 2030. Collectively, these findings advance our understanding of how mangrove endophytes support host resilience and ecosystem sustainability, while also identifying promising microbial resources for biotechnology, bioremediation, and sustainable agriculture in extreme coastal environments.

MATERIALS AND METHODS

Experimental design overview

All experiments were conducted using three independent biological replicates, each consisting of three technical replicates, unless otherwise stated. Replication applied to endophyte isolation, enzyme assays, dye decolorization assays, antibacterial screening, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination, tomato colonization experiments, and Scanning Electron Microscopy (SEM) observations. Positive and negative controls were included in all assays: Augmentin (30 µg/mL) as the antibacterial positive control and sterile Nutrient Broth (NB) or uninoculated media as negative controls. Sterility checks were routinely performed throughout the study to ensure the endophytic origin of the isolates (Khalil et al. 2017).

This study employed a culture dependent isolation strategy; therefore, the results represent only the culturable fraction of the endophytic bacterial community, while non-culturable or fastidious taxa may remain underrepresented.

For MIC and MBC assays, MIC was defined as the lowest concentration of Cell-Free Supernatant (CFS) inhibiting visible bacterial growth, while MBC was defined as the lowest concentration yielding no colony formation after 24 h incubation on nutrient agar. For molecular identification, isolates showing ≥98.7% 16S rRNA gene sequence similarity to reference strains were considered conspecific at the species level.

Plant sample collection

Leaf and root samples of *A. marina* were collected in February 2024 from natural mangrove stands on Tarout Island, Eastern Province, Saudi Arabia (26°33′41.4″N, 50°05′11.4″E). Sampling focused on *A. marina* due to its dominance in Arabian Gulf mangrove forests and its documented tolerance to extreme salinity and temperature conditions. A total of 150 healthy, asymptomatic leaves were randomly collected from multiple trees across the sampling site to capture spatial heterogeneity. Roots were collected for comparative purposes; however, no culturable bacterial endophytes were recovered from root tissues under the applied isolation conditions (Nurunnabi et al. 2020).

Isolation of mangrove endophytic bacteria

Plant tissues were thoroughly washed with running tap water and surface-sterilized using 70% ethanol for 1 min, followed by 2% sodium hypochlorite for 3 min, and rinsed three times with sterile distilled water. The effectiveness of surface sterilization was verified by imprinting sterilized tissues and plating the final rinse water onto Nutrient Agar (NA), followed by incubation at 37°C for 24 h.

Endophytic bacteria were isolated using cutting and tissue grinding methods, plated onto NA, and purified through repeated subculturing. Pure cultures were maintained on NA at 4°C for short-term storage and preserved in nutrient broth supplemented with glycerol (20%, v/v) at -80°C for long-term storage (Ghribi et al. 2016). From the 150 leaf samples, 16 morphologically distinct bacterial endophytes were obtained and selected for further analysis.

Assessment of endophytic colonization in tomato plants

Surface-sterilized seeds of *Solanum lycopersicum* were sown in sterile soil and maintained under controlled greenhouse conditions (25 ± 2°C, 16/8 h light/dark photoperiod). At 15 days post-germination, seedlings were inoculated by soil drenching with bacterial suspensions adjusted to 10^8 CFU/mL, while control plants received sterile distilled water.

Endophytic colonization was assessed at 14-, 21-, and 45-days post-inoculation. Plant tissues were surface-sterilized, and the final rinse water was plated to confirm sterility. Tissues were homogenized, serially diluted, and plated onto NA for CFU quantification (Frikha-Gargouri et al. 2017). Tomato was selected as a heterologous host to assess host plasticity and general endophytic competence of the isolates, rather than to demonstrate direct agricultural application.

Morphological and biochemical characterization of endophytic bacterial isolates

Colony morphology was examined on NA after 24-48 h incubation. Gram staining and standard biochemical tests (oxidase, catalase, indole, and urease) were performed following classical microbiological protocols and Bergey's Manual. Phenotypic characteristics were used to support molecular identification and were compared with reference profiles using the DSMZ Bacterial Diversity Metadatabase (Tegegn et al. 2025).

Qualitative screening for lignin-mimicking dye decolorization

The dye decolorization potential of endophytic isolates was evaluated using three lignin-mimicking dyes: Methylene Blue (MB), Remazol Brilliant Blue (RBB), and Methyl Red (MR). Isolates were inoculated onto NA plates (pH 7.0) supplemented with 0.05% (w/v) of each dye and incubated at 37°C for 24-48 h (Das et al. 2023).

Clear halo formation around colonies was interpreted as enzymatic dye degradation, while uniform coloration changes were used to distinguish adsorption from degradation. Halo and colony diameters were measured using a digital caliper, and the enzymatic index (H/C ratio) was calculated. Uninoculated dye-containing plates served as negative controls. All assays were conducted in triplicate (Ghribi et al. 2016).

Molecular characterization of bacterial isolates

Genomic DNA was extracted using the Promega Genomic DNA Purification Kit. The near full-length 16S rRNA gene (~1,400 bp) was amplified using primers 27F and 1492R. PCR conditions: initial denaturation at 92°C for 5 min, denaturation at 95°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 1 min, final extension at 72°C for 7 min. PCR products were visualized on 1% agarose gels, and high-quality amplicons were sequenced. Sequences were trimmed in BioEdit, yielding ~1,300-1,400 bp. BLASTn searches were conducted against GenBank. Phylogenetic analyses were performed in MEGA 11 after alignment with MAFFT. Maximum likelihood trees were constructed using the HKY+F+G4 model (ModelFinder) with 10,000 ultrafast bootstrap replicates. Isolates with ≥98.7% similarity to type strains were assigned at the species level; others were assigned to the genus level (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017; Hoang et al. 2018; Minh et al. 2020; Tamura et al. 2021; Hong et al. 2024).

Enzymatic profiling of mangrove endophytic *Bacillus* isolates

Extracellular hydrolytic enzymes (amylase, protease, cellulase, chitinase) were screened on specific substrate-containing plates. Clear zones indicated enzymatic activity; all enzyme assays were intentionally conducted as qualitative screenings to provide an initial assessment of functional diversity among isolates. Quantitative enzyme activity measurements were beyond the scope of this biodiversity-focused study and are suggested for future investigations. All assays were performed in triplicate (Hammami et al. 2013; Nursyirwani et al. 2020; Mamangkey et al. 2021).

Antibacterial activity

The antibacterial potential of the sixteen mangrove endophytic bacterial isolates was evaluated against 11 pathogenic bacterial strains (Rani et al. 2017), including both Gram-positive and Gram-negative bacteria. All assays were performed in three independent biological replicates, each with three technical replicates, to ensure reproducibility. Positive controls (Augmentin, 30 µg/mL) and negative controls (sterile NB) were included in all experiments. Sterility checks were routinely conducted to confirm the endophytic origin of isolates.

Cross-streak assay

Endophytic isolates were streaked as a single line on nutrient agar plates and incubated at 37°C for 24 h. Subsequently, pathogenic strains were streaked perpendicular to the endophyte streak. Growth inhibition along the intersection indicated antibacterial activity (Malash et al. 2022). The presence or absence of inhibition for each pathogen was recorded for all 16 isolates.

Agar well diffusion

Pathogenic bacteria were evenly spread on nutrient agar plates, and wells (6 mm diameter) were filled with 100 µL of Cell-Free Supernatant (CFS) from each endophytic isolate. CFS concentrations were standardized based on total protein content (mg/mL) to allow reproducible comparisons. Augmentin (30 µg/mL) served as a positive control, and sterile NB as a negative control. Plates were incubated at 37°C for 24 h, and inhibition zones were measured in millimeters (Islam et al. 2022).

MIC and MBC

MIC was determined using twofold serial dilutions of CFS in 96-well plates. Bacterial growth was monitored by measuring absorbance at 600 nm, and relative inhibition (%) was calculated using:

$$\text{Relative inhibition (\%)} = 100 \times \frac{(a-b)}{(c-b)}$$

Where: a represents the absorbance of CFS-treated cells, b represents the absorbance of the blank (sterile NB), and c represents the absorbance of the negative control (untreated bacterial suspension).

MBC was determined by plating aliquots from wells showing no visible growth onto nutrient agar and incubating at 37°C for 24 h. The lowest CFS concentration

that completely inhibited colony formation was recorded as MBC, while the lowest concentration preventing visible growth was recorded as MIC (Fokou et al. 2024).

All results were analyzed statistically, and inhibition patterns were compared across isolates and pathogens to identify the most potent endophytic strains. Scanning Electron Microscopy (SEM) was subsequently used to examine morphological changes in bacterial cells treated with CFS at MIC concentrations, providing insights into bactericidal mechanisms (Wang and Zeng 2022).

Scanning electron microscopy analysis of treated bacterial cells

Pathogens grown overnight, washed, and treated with isolate-specific MICs of CFS; controls received sterile NB. Cells fixed, dehydrated in graded ethanol, dried, gold-coated, and examined under SEM. Multiple fields were observed at magnifications of 5k× and 10k× to assess cell wall integrity, surface collapse, and cytoplasmic leakage (Wang and Zeng 2022).

Statistical analysis

All experiments included three biological replicates × three technical replicates. Data analyzed in IBM SPSS v25. One-way ANOVA was used for datasets meeting assumptions; Tukey HSD post hoc test identified significant differences. Data presented as mean ± SD; $p < 0.05$ considered significant. Non-parametric alternatives were considered when assumptions were violated (Katheng et al. 2025).

RESULTS AND DISCUSSION

Isolation, tissue specificity, and biodiversity of mangrove endophytic bacteria

Endophytic bacteria were isolated from surface-sterilized mangrove tissues of leaves, stems, and roots using two methods: Cutting and Grinding. No microbial growth was observed on imprint and rinse-plate controls, confirming successful surface sterilization. Only the Cutting method yielded viable colonies, producing sixteen isolates in total (Table 1), with fifteen from leaves and one from roots; no isolates were obtained from stems. Re-isolation experiments showed that all sixteen isolates successfully colonized tomato plants. Bacteria were first recovered from surface-sterilized roots at 14 days post-inoculation, and by day 21, they were detected in both roots and leaves. Colonization persisted up to 45 days, with no growth observed in control plants. Morphological observations revealed diversity among isolates grown on nutrient agar, with colony colors ranging from white to yellowish-brown and textures being mucoid, dry, or powdery, while margins were mainly smooth but occasionally undulate or irregular (Table 1). Microscopic examination showed that all isolates were Gram-positive, mostly short rods or cocci, with some coccobacillary and streptobacillary forms.

Morphological and biochemical characterization of endophytic bacterial isolates

Biochemical characterization of the sixteen mangrove-derived endophytic bacterial isolates showed that all were catalase- and oxidase-positive but negative for indole and urease activities (Table 1). Phylogenetic analysis based on 16S rRNA gene sequences revealed that eight isolates were affiliated with the genus *Bacillus*, including *B. safensis*, *B. licheniformis*, *B. subtilis*, *B. inaquosorum*, *B. mojavensis*, and *B. megaterium*, while one isolate was identified as *Brevibacterium halotolerans* (Figure 1).

Qualitative screening for lignin-mimicking dye decolorization

The decolorization potential of sixteen mangrove-derived endophytic isolates was evaluated using three lignin-mimicking dyes: Methyl Red (MR), Methylene Blue (MB), and Remazol Brilliant Blue (RBB) (Figure 2). Fifteen isolates showed noticeable decolorization of MR within 48 h, whereas only five isolates degraded MB and two decolorized RBB (Table 1). This pattern reflects the increasing structural complexity and resistance of the dyes. *Bacillus subtilis* A4, *B. pumilus* A7, *B. megaterium* A9, and *B. amyloliquefaciens* A11 exhibited broad-spectrum activity, with *B. amyloliquefaciens* A11 achieving complete decolorization of all dyes tested.

The dye decolorization assay was conducted as a qualitative screening, and decolorization was assessed based on visible halo formation and color changes around the colonies. Quantitative enzymatic index (H/C ratio) values were therefore not calculated, as the objective of this assay was preliminary functional screening rather than comparative quantification.

Enzymatic profiling of mangrove endophytic *Bacillus* isolates

The enzymatic potential of sixteen mangrove-derived endophytic isolates was assessed using qualitative plate-based screening assays to detect extracellular enzyme production. Protease activity was detected in all isolates, indicating a conserved capacity for extracellular protein hydrolysis among mangrove-associated endophytes. Amylase activity was observed in fifteen isolates, while *Bacillus safensis* A26 showed no detectable starch-degrading activity.

Chitinase production was restricted to five isolates (*B. subtilis* A4, *B. amyloliquefaciens* A5 and A14, *Bacillus m.* A9, and *B. inaquosorum* A13), suggesting strain-specific specialization, whereas cellulase activity was detected in approximately half of the isolates, reflecting variability in cellulose-degrading potential. Enzymatic activity was evaluated based on clear zone formation around colonies without quantitative comparison of enzyme intensity (Figure 3 and Table 1).

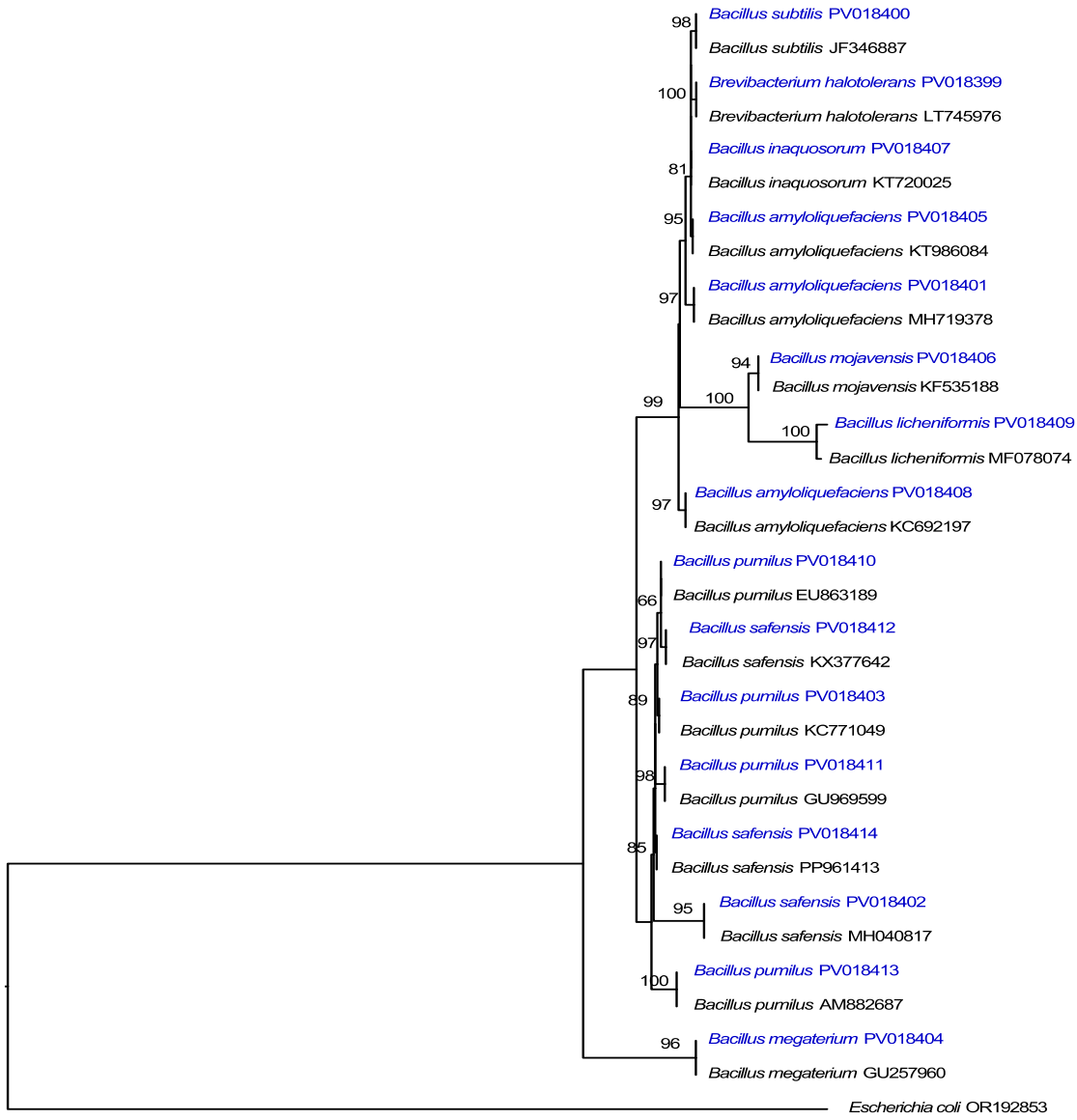

Figure 1. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences of mangrove endophytic bacterial isolates. Bootstrap values (1000 replicates) are shown at nodes. *Escherichia coli* (OR192853) was used as an outgroup. Isolates obtained in this study are highlighted

Screening of antibacterial activity of mangrove endophytic bacteria

The antibacterial activity of sixteen mangrove-derived *Bacillus* isolates was initially screened using the cross-streak method. Thirteen isolates showed observable antagonistic activity against the tested Gram-positive and Gram-negative pathogens. From these, five isolates demonstrating the strongest and most reproducible inhibition patterns were selected for quantitative evaluation using the agar well diffusion assay.

These isolates exhibited pathogen-specific inhibitory responses: *Bacillus inaquosorum* A13 and *B. safensis* A24 were most effective against Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*), while *B. subtilis* A4, *B. amyloliquefaciens* A5, and *B. megaterium* A9 primarily inhibited the Gram-negative Pathogens *Klebsiella oxytoca*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Relative inhibition percentages confirmed that *S. aureus* was the most susceptible, followed by *S. pyogenes*, *K. oxytoca*, and *E. coli*, whereas *P. aeruginosa* was the least responsive (Table 2).

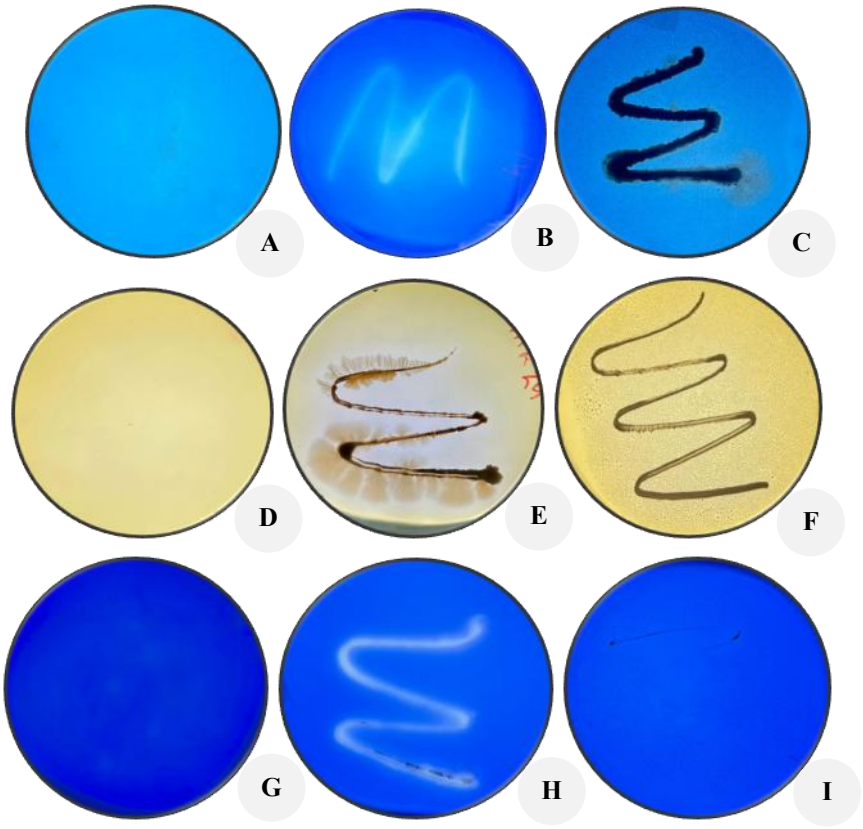

Figure 2. The decolorization potential of lignin-mimicking dyes by some mangrove endophytic bacterial Isolates**.** Decolorization of 0.05% dye-containing plates. A. Remazol brilliant blue - before incubation, B. Remazol brilliant blue - after incubation positive control, C. Remazol brilliant blue - after incubation negative control, D. Methyl red - before incubation, E. Methyl red - after incubation positive control, F. Methyl red - after incubation negative control, G. Methyl blue - before incubation, H. Methyl blue - after incubation positive control, I. Methyl blue - after incubation negative control, after 48 h incubation at 37°C and pH 7

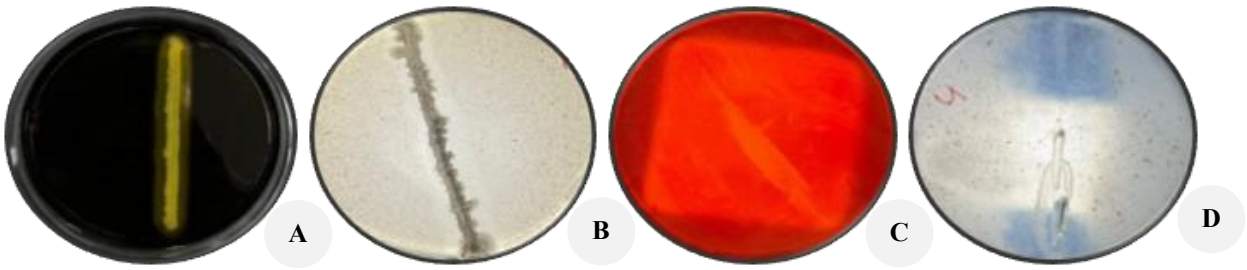

Figure 3. Enzymatic activities of mangrove leaf endophytic *Bacillus* isolates. A. Amylase, B. Protease, C. Cellulase, D. Chitinase

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC assays were performed to quantitatively validate the antibacterial activity observed during initial screening. Although thirteen isolates showed inhibition in the cross-streak assay, only five were included (Table 3) because these isolates produced clear and measurable inhibition zones suitable for quantitative MIC/MBC determination.

The MIC results demonstrated pathogen-dependent variability: the lowest MIC (0.312 mg/mL) was recorded for B. inaquosorum A13 and B. safensis A24 against *S. aureus* and *S. pyogenes*, respectively, whereas higher concentrations (1.25 mg/mL) were required for B. subtilis A4 and B. amyloliquefaciens A5 to inhibit *K. oxytoca* and *E. coli*. *Pseudomonas aeruginosa* showed the highest resistance with an MIC of 5 mg/mL for *B. megaterium* A9.

For the positive control (Augmentin), identical MIC and MBC values observed against *P. aeruginosa* reflect its well-documented intrinsic resistance to β-lactam/β-lactamase inhibitor combinations.

MBC/MIC ratios ranging from 0.52 to 1.0 indicate predominantly bactericidal behavior of the *Bacillus* cell-free supernatants. These findings confirm that the selected isolates possess strong and quantifiable antimicrobial potential, consistent with the qualitative screening while providing the required quantitative rigor requested by the reviewers.

Table 1. Morphological, biochemical, and enzymatic characterization and dye-decolorization potential of mangrove endophytic bacterial isolates from Tarout, Saudi Arabia

Isolate (species)	Morphological characteristics	Biochemical characteristics	Enzymatic properties	Dye decolorization	GenBank accession number
Brevibacterium halotolerans A2	Colonies yellow to brown, mucoid, smooth-edged, circular, medium size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase +, Protease +, Amylase +	MR -, RBB -, MB -	PV018399
Bacillus subtilis A4	Colonies pale yellow, mucoid, rough margins, circular, large size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase +, Cellulase +, Protease +, Amylase +	MR +, RBB +, MB -	PV018400
Bacillus amyloliquefaciens A5	Colonies white, mucoid, smooth margins, circular, small size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase +, Cellulase +, Protease +, Amylase +	MR +, RBB -, MB -	PV018401
Bacillus safensis A6	Colonies white, mucoid, smooth, concave, medium size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase -, Protease +, Amylase +	MR +, RBB -, MB +	PV018402
Bacillus pumilus A7	Colonies white, mucoid, smooth, circular, small size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase -, Protease +, Amylase +	MR +, RBB +, MB -	PV018403
Bacillus megaterium A9	Colonies yellow to brown, mucoid, smooth, circular, large size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase +, Cellulase +, Protease +, Amylase +	MR +, RBB +, MB -	PV018404
Bacillus amyloliquefaciens A11	Colonies pale yellow, non-mucoid, smooth, circular, large size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase +, Protease +, Amylase +	MR +, RBB +, MB +	PV018405
Bacillus mojavensis A12	Colonies yellow to brown, non-mucoid, rough, powdery, small size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase -, Protease +, Amylase +	MR -, RBB -, MB -	PV018406
Bacillus inaquosorum A13	Colonies white, mucoid, rough, circular, large size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase +, Cellulase +, Protease +, Amylase +	MR -, RBB -, MB -	PV018407
Bacillus amyloliquefaciens A14	Colonies white, mucoid, rough, circular, large size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase +, Cellulase +, Protease +, Amylase +	MR -, RBB -, MB -	PV018408
Bacillus licheniformis A18	Colonies pale yellow, non-mucoid, rough, powdery, small size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase +, Cellulase +, Protease +, Amylase +	MR +, RBB -, MB +	PV018409
Bacillus pumilus A20	Colonies white, mucoid, smooth, circular, small size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase -, Protease +, Amylase +	MR +, RBB -, MB -	PV018410
Bacillus pumilus A23	Colonies white, mucoid, smooth, circular, medium size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase -, Protease +, Amylase +	MR -, RBB -, MB -	PV018411
Bacillus safensis A24	Colonies white, mucoid, rough, concave, medium size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase -, Protease +, Amylase +	MR -, RBB +, MB -	PV018412
Bacillus safensis A26	Colonies white, mucoid, rough, concave, medium size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase -, Protease +, Amylase +	MR -, RBB -, MB -	PV018414
Bacillus pumilus A27	Colonies white, mucoid, smooth, concave, medium size	Urease -, Indole -, Oxidase +, Catalase +, Gram +	Chitinase -, Cellulase -, Protease +, Amylase +	MR +, RBB -, MB -	PV018413

Note: "+" denotes a positive reaction or enzymatic activity, "-" denotes a negative reaction or absence of activity. MR: Methyl Red, RBB: Remazol Brilliant Blue, MB: Methylene Blue. Colony size was visually estimated. Dye-decolorization and extracellular enzyme activities (protease, amylase, chitinase, and cellulase) were assessed qualitatively; quantitative enzymatic indices (H/C ratios) were not determined

Table 2. Relative percentage of inhibition of pathogenic bacteria by the CFS of m angrove-derived *Bacillus* isolates, as determined using the agar well diffusion assay

Bacterial strain (endophyte)	Pathogenic bacteria	Relative percentage inhibition (%) (Mean ± SD)
Bacillus subtilis A4	*Klebsiella oxytoca* 700324	70.89% ± 1.5
Bacillus amyloliquefaciens A5	*Escherichia coli* 25922	68.95% ± 1.2
Bacillus megaterium A9	*Pseudomonas aeruginosa* 27853	30.85% ± 1.0
Bacillus safensis A24	*Streptococcus pyogenes* 19615	71.49% ± 1.8
Bacillus inaquosorum A13	*Staphylococcus aureus* 29213	76.21% ± 1.5

Note: Values are expressed as mean ± SD of three independent replicates. Augmentin (30 µg) served as the positive control, while NB was used as the negative control

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Bacillus* CFS and MBC/MIC Ratios

Species name	Pathogenic Bacteria	Tested CFS			Positive control (Augmentin antibiotic)		
		MIC	MBC	R	MIC	MBC	R
Bacillus subtilis A4	*Klebsiella oxytoca*	1.25	2.5	0.5	2.5	5	0.5
Bacillus amyloliquefaciens A5	*Escherichia coli*	1.25	2.5	0.5	2.5	5	0.5
Bacillus megaterium A9	*Pseudomonas aeruginosa*	5	5	1	5	5	1
Bacillus safensis A24	*Streptococcus pyogenes*	0.312	0.6	0.52	2.5	2.5	1
Bacillus inaquosorum A13	*Staphylococcus aureus*	0.312	0.6	0.52	2.5	2.5	1

Affected cell imaging by Scanning Electron Microscope (SEM)

The SEM analysis was conducted to visualize the morphological alterations induced by *Bacillus* CFS on selected pathogenic bacteria at their respective MICs (Figure 4). Treated cells exhibited clear structural damage compared to untreated controls, including cell deformation, surface collapse, membrane disruption, and reduced cell integrity. These observations provide visual confirmation of the bactericidal activity demonstrated in the agar diffusion and MIC/MBC assays.

Discussion

Mangrove endophytic bacteria isolated from *A. marina* in the Eastern Province of Saudi Arabia exhibited considerable phylogenetic diversity, with *Bacillus* spp. forming the dominant taxonomic group and a minor representation of *B. halotolerans* (Alghamdi et al. 2023; Xu et al. 2023). The predominance of *Bacillus* is consistent with previous studies on mangrove-associated endophytes and reflects their ecological fitness under saline, nutrient-fluctuating, and oxidative stress conditions typical of intertidalintertidal environments. (Sengupta et al. 2020; Byregowda et al. 2022; Liu et al. 2022). Importantly, this study contributes to addressing the limited data available on endophytic bacterial diversity from Saudi Arabian mangroves, which remain underrepresented in global mangrove microbiome research (Almahasheer 2018; Alghamdi et al. 2024). A pronounced tissue-specific colonization pattern was observed, with successful isolation of culturable endophytic bacteria exclusively from leaf tissues, while no isolates were recovered from roots under the applied conditions. Leaves are metabolically active organs that provide diverse carbon sources and heterogeneous microhabitats, potentially supporting higher endophytic diversity compared to subterranean tissues (Sengupta et al. 2020; Byregowda et al. 2022). These findings highlight tissue specificity as a relevant biodiversity outcome rather than methodological limitation and align with the first objective of documenting culturable endophytic diversity across host tissues.

Morphological and biochemical characterization further supported the phylogenetic findings, as most isolates were Gram-positive, catalase- and oxidase positive, and spore-forming. These traits are commonly associated with bacterial persistence in saline and oxidative environments and reinforce the ecological adaptation of *Bacillus* spp. within mangrove tissues. The detection of *B. halotolerans*, although limited to a single isolate, suggests the presence of metabolically distinct minor taxa that may contribute to functional complementarity within the endophytic community (Castro et al. 2014; Liu et al. 2022; Mondal et al. 2024).

Functional assays revealed variability in extracellular enzymatic activities, including protease, amylase, cellulase, and chitinase production. Such enzymatic traits have been widely reported among plant-associated and mangrove endophytic bacteria and are commonly linked to nutrient cycling, organic matter turnover, and tissue colonization processes (Sengupta et al. 2020; Byregowda et al. 2022; Mondal et al. 2024). However, in the present study, these activities are interpreted as phenotypic indicators of functional diversity rather than definitive ecological roles, as in situ validation was beyond the scope of this work. Similarly, qualitative dye-decolorization assays demonstrated the ability of certain isolates, particularly *B. amyloliquefaciens* A11, to transform lignin-mimicking substrates under controlled conditions. Comparable dye-decolorization and xenobiotic transformation capacities have previously been documented for mangrove-derived and endophytic *Bacillus* species, reflecting metabolic versatility rather than confirmed environmental bioremediation capacity (Mnif et al. 2015; Singh and Dubey 2018; Dewiyanti et al. 2022).

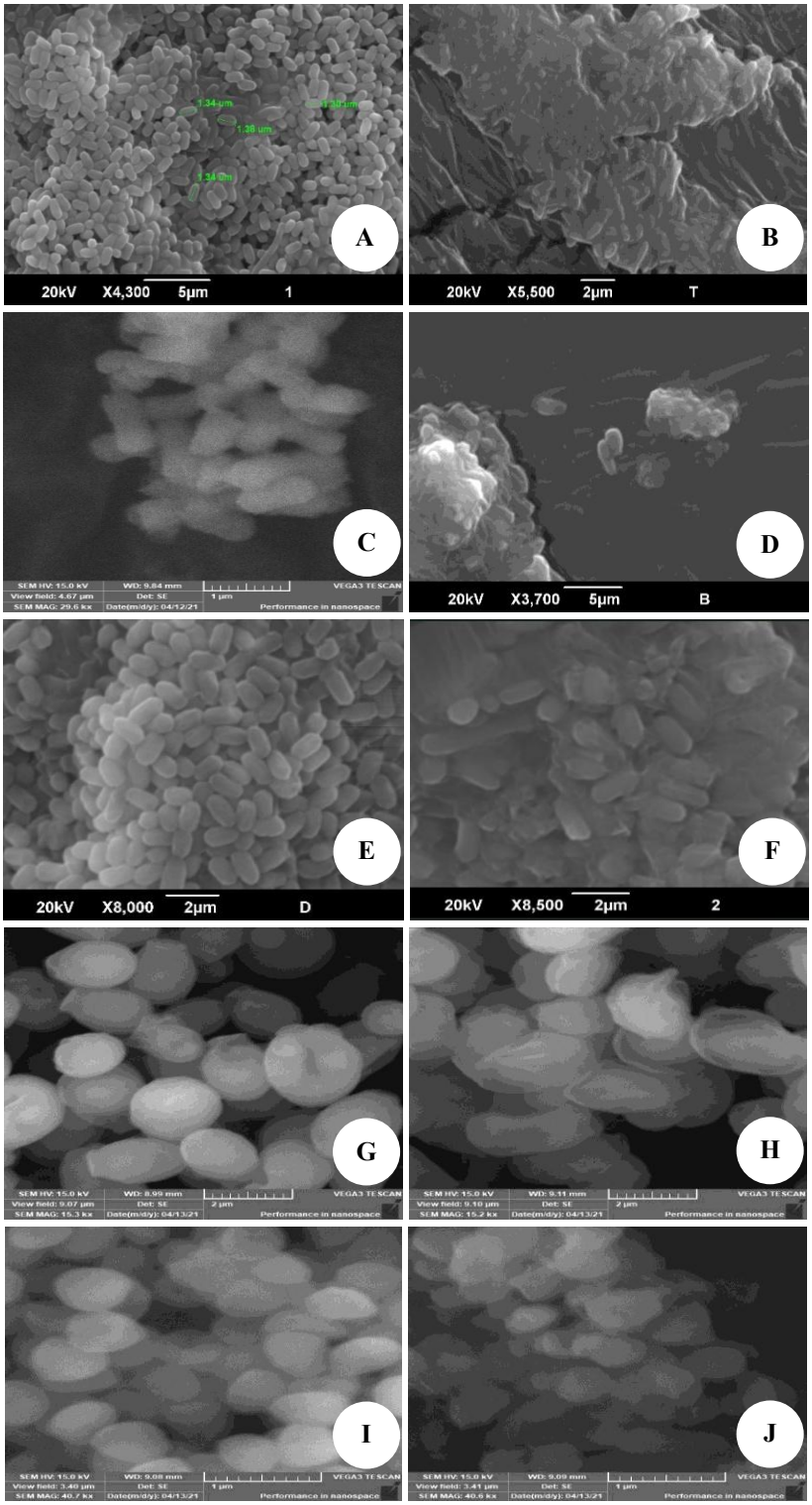

Figure 4. SEM micrographs showing morphological alterations of pathogenic bacteria before (Control) and after treatment (Treated) with CFS of mangrove-derived *Bacillus* isolates at their respective MICs: A-B. *E. coli* treated with *B. amyloliquefaciens* A5 (1.25 mg/mL), C-D. *Klebsiella oxytoca* treated with *B. subtilis* A4 (1.25 mg/mL), E-F. *Pseudomonas aeruginosa* treated with *B. megaterium* A9 (5 mg/mL), G-H. *Streptococcus pyogenes* treated with *B. safensis* A24 (0.312 mg/mL), I-J. *Staphylococcus aureus* treated with *B. inaquosorum* A13 (0.312 mg/mL). Scale bars are indicated on each micrograph

Antibacterial screening revealed strain- and pathogen-specific inhibitory patterns, with several *Bacillus* isolates exhibiting selective activity against Gram-positive or Gram-negative bacteria, a trend widely reported for plant-associated and mangrove-derived endophytic *Bacillus* spp. (Mnif et al. 2015; Rani et al. 2017; Singh and Dubey 2018). SEM observations supported these findings by revealing morphological alterations in treated bacterial cells, including cell deformation and surface disruption, which are commonly associated with antibacterial stress responses induced by bacterial metabolites (Islam et al. 2022; Wang and Zeng 2022). However, these observations are presented descriptively, without attribution to specific antimicrobial compounds. The absence of genomic or metabolomic analyses precludes mechanistic interpretation, and this conservative approach was intentionally maintained, consistent with biodiversity-focused studies relying on phenotypic screening (Mayanglambam et al. 2020).

Several limitations should be acknowledged. The culture-dependent methodology restricts the analysis to culturable taxa, potentially underrepresenting the full endophytic community, a limitation widely reported in mangrove and plant endophyte studies relying on cultivation-based approaches (dos Reis et al. 2022). Additionally, sampling was limited to a single geographic location and season, which may constrain broader ecological generalization, as spatial and temporal variability strongly influence mangrove-associated microbial communities (Behera et al. 2019; Navarro et al. 2025) Functional traits were assessed in vitro without genetic confirmation of associated biosynthetic pathways or in planta validation of colonization or biocontrol potential, a common constraint in preliminary biodiversity and functional screening studies (Escalas et al. 2019).

Overall, this study provides baseline insight into the phylogenetic structure and functional breadth of culturable endophytic bacteria associated with *A. marina* in Saudi Arabian mangroves. By emphasizing biodiversity, tissue specificity, and ecological interpretation over application-driven conclusions, the findings contribute to a more balanced understanding of mangrove endophyte communities in an understudied region.

In conclusion, this study provides baseline insight into the culturable endophytic bacterial diversity associated with *A. marina* in the Eastern Province of Saudi Arabia. The recovered community was dominated by *Bacillus* species, while the detection of *B. halotolerans* expands current knowledge of endophytic bacterial diversity within Arabian Gulf mangroves, an understudied region in global mangrove microbiome research. Variability in enzymatic activities, selective antimicrobial effects, and qualitative dye-decolorization capacities reflects phenotypic diversity and ecological adaptability of the isolated endophytes under saline and temperature-stressed coastal conditions. These functional traits represent ecological potential rather than confirmed applications and require further validation. The findings emphasize the biodiversity value of mangrove-associated endophytic bacteria and their potential contribution to host resilience and ecosystem stability. All isolates are preserved at -80 °C in the Microbiology Laboratory, College of Science, Imam Abdulrahman Bin Faisal University, and are available upon reasonable request for academic research to support future biodiversity, ecological, and comparative studies.

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