

Article

Biofilm and Quorum-Sensing Inhibition by Novel *Bacillus xiamenensis* MM07 Endophytic Isolate from *Paederia foetida* to Combat *Pseudomonas aeruginosa*

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Abstract

This study investigated the antibiofilm and anti-quorum-sensing (QS) potential of endophyte extracts isolated from medicinal plants and their validation against *Pseudomonas aeruginosa*. Endophytes were isolated from the plants using the serial dilution method, and the extracts produced by these endophytes were screened for antimicrobial and biofilm-inhibition activity using assays. The efficient extract was biochemically characterized, followed by validation of its secondary metabolite content. Furthermore, QS-regulatory gene expression levels and microscopy were used to confirm inhibition of biofilm formation. A total of 12 cultures, including 8 bacterial and 4 fungal, were isolated and screened, demonstrating efficient antimicrobial activity (zone of inhibition of 18.8 mm) and 64.3% antibiofilm activity. The efficient endophyte isolated from *Paederia foetida* was identified as *Bacillus xiamenensis* MM07 by 16S rRNA gene sequencing. MM07 extract analyses by biochemical and Fourier transform infrared methods revealed the presence of diverse biomolecules. A dose-dependent inhibition was observed, achieving up to 83.5, 60.3, 73.2, 82.7, 83.2, and 15.1 in biofilm formation and exopolysaccharide, violacein, pyocyanin, protease, and alginate production, along with 63.2% swimming ability at 30 µg/mL, respectively. Gas chromatography-mass spectrometry analyses validated the presence of major secondary metabolites, including 3,3-dimethyl-4-methylamino-butan-2-one, 6-amino-2-methyl-, 1-iodo-2-methylundecane, and hexadecanoic acid, with the potential to inhibit biofilm and QS activity. Quantitative real-time polymerase chain reaction of QS regulatory genes (*lasI*, *lasR*, *rhII*, and *rhIR*) and microscopy analysis confirmed the anti-QS properties, evidenced by a 40.3% decline in gene expression and biofilm inhibition by MM07 extract. These findings highlight the potential of novel *B. xiamenensis* MM07 endophyte from *P. foetida* as a sustainable source of biomolecules for combating biofilm-associated infections.



Academic Editor: Ligang Zhou

Received: 5 February 2026

Revised: 18 March 2026

Accepted: 20 March 2026

Published: 24 March 2026

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Keywords: antimicrobial agents; *Bacillus xiamenensis*; bioactive compounds; biofilm formation inhibition; *Paederia foetida*; *Pseudomonas aeruginosa*; quorum-sensing inhibition

1. Introduction

Microbial antibiotic resistance has emerged as a critical global health concern due to excessive antibiotic use and the formation of resilient biofilms, resulting in the effi-

cacy of antimicrobial treatments. Nearly 60% of all human infections are associated with biofilm-forming pathogens, resulting in widespread hospital-acquired infections [1,2]. Such infections frequently involve multidrug-resistant bacteria such as *Pseudomonas* and *Klebsiella*, leading to complex treatment due to the limited availability of effective antimicrobial therapies. Quorum sensing (QS), a cell-to-cell communication system, plays an essential role in biofilm formation and in regulating the production of virulence factors in such bacterial pathogens [3,4]. To combat bacterial pathogens, disrupting the QS-based communication system is recognized as a promising strategy by interfering with gene expressions involved in bacterial virulence and biofilm-associated infections, which are crucial for their maintenance [5,6]. Therefore, to address the growing threat of antimicrobial resistance, novel therapeutic approaches are urgently needed to prevent pathogenic infections without exacerbating the expansion of drug resistance. Plants, especially medicinal plant-based endophytes, are well known to produce a wide range of bioactive compounds that can be used to treat pathogens [7,8]. In ancient Indian and Chinese studies, the plants are recognized for their ethnobotanical relevance and broad traditional medicinal applications [9,10]. Recent findings revealed that the metabolism of these plants and their therapeutic properties are associated with living endophytes [8,11]. Endophytic bacteria or fungi residing symbiotically inside plant tissues without causing harm, including leaves, stems, flowers, and roots, are a fascinating and noteworthy research interest due to the broad diversity of medicinal plants and their ability to produce various therapeutic secondary metabolites with effective antimicrobial, anti-biofilm, and anti-QS properties [4]. Therefore, these plants are increasingly recognized as a source of unique endophytes having the potential to produce novel therapeutic biomolecules [12–14]. In mechanism, endophyte-derived biomolecules, including alkaloids, terpenoids, and polyketides, exhibited broad-spectrum antimicrobial properties via altering membrane permeability, inhibiting vital enzymatic processes, or targeting microbial cell walls [15]. Various studies have demonstrated the efficacy of endophytic bacterial and fungal extracts isolated from medicinal plants, including *Centella asiatica*, *Houttuynia cordata*, and *Paederia foetida*, in inhibiting pathogens [11,16–18]. *P. foetida*, commonly known as skunkvine, pilau maile, or Chinese fever vine, belongs to the *Rubiaceae* family and is widely recognized for its traditional medicine [19]. It is characterized by a prominent sulfurous odor of bruised leaves or stems, primarily due to sulfur compounds such as dimethyl disulfide in its essential oils. In Asia, this plant is native to the temperate and tropical regions and recognized as Gandha bhadulia or Gandha prasarini in India, with therapeutic potential as an anti-inflammatory, antinociceptive, and antimicrobial agent [11,20]. The phytochemical analysis of *P. foetida* revealed remarkable variations in bioactive composition and concentrations across plant parts and geographical regions [7]. A few recent findings demonstrate that endophytes, as microbial symbionts, are a rich, untapped resource for sustainable antimicrobial agents rather than conventional antibiotics [16,18]. Furthermore, the chemical profile diversity of endophytic extracts, associated with biomolecules such as aliphatic compounds, peptides, and phenolics, further extends their potential for broad pharmaceutical bioprospecting [21]. Therefore, the potential of endophytes to produce novel biomolecules, a critical area for the discovery of therapeutic antimicrobials, is particularly relevant for inhibiting multidrug-resistant pathogens [13,22]. This exceptional chemo-diversity, coupled with the potential for large-scale production via established fermentation systems, positions endophytic bacteria and fungi as a sustainable and scalable alternative to the direct extraction of bioactive compounds from medicinal plants. In Northeast India, endophytic bacteria, such as *Bacillus*, *Pseudomonas*, *Serratia*, and *Streptomyces*, isolated from ethnomedicinal plants have been shown to possess antimicrobial potential [23]. Previously, a limited study has demonstrated the antimicrobial potential of *P. foetida*-based endophytes and their characteristics, but their potential for QS inhibition

has not been evaluated [11,18]. Therefore, analyzing endophytes from *P. foetida* for their antimicrobial and anti-QS activity can be beneficial for developing novel therapeutics. In the present study, endophytic microorganisms from medicinal plants, including *C. asiatica*, *H. cordata*, and *P. foetida*, were isolated, and the extracts produced by these endophytes were screened for potential to inhibit microbial pathogens. Furthermore, the efficient endophytic isolate based on *P. foetida* was identified as *Bacillus xiamenensis* MM07 based on molecular characteristics, and its extract composition and properties were validated for inhibiting QS and biofilm formation through biochemical, microscopy, and QS-regulatory gene expression analyses.

2. Materials and Methods

2.1. Chemicals and Materials

Azocasein and furanone were purchased from Sigma-Aldrich, Darmstadt, Germany. Congo red dye and gentamycin were obtained from HiMedia, Mumbai, India. All other chemicals of analytical grade were obtained from commercial sources. Microbial cultures of *Bacillus cereus* MTCC 430, *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 40, *Klebsiella pneumoniae* MTCC 618, *P. aeruginosa* MTCC 2297, and *Staphylococcus aureus* MTCC 3160 were obtained from the Microbial Type Culture Collection, CSIR-IMTECH, Chandigarh, India. *Chromobacterium violaceum* ATCC 12472 and *P. aeruginosa* PAO1 (ATCC 15692) were procured from the American Type Culture Collection (ATCC). The cultivation of *C. violaceum* and *P. aeruginosa* was performed in Luria–Bertani (LB) broth at 28 °C and 37 °C for 24 h, respectively.

2.2. Screening of Plants for Endophytes Isolation

Plants of *H. cordata* and *P. foetida* were collected from Besseria village, Tezpur (26°39'42.4" N, 92°44'41.2" E), and *C. asiatica* from Tezpur University (26°42'00.1" N, 92°49'49.4" E), Assam (India) in April 2022. These plant samples were recovered using sterile gloves and transported to the laboratory in sealed bags. These samples were washed with tap water, followed by distilled water (3 times). After cutting, especially leaves and stems, the samples were sterilized by dipping in distilled water (1 min), 70.0% (v/v) ethanol (0.5 min), and then 2.5% (v/v) sodium hypochlorite (2 min). Furthermore, the samples were aseptically macerated in 1 mL of phosphate-buffered saline (PBS). The resulting homogenate was serially diluted and spread/plated onto nutrient agar (NA), then incubated at 28 °C for up to 72 h. The morphologically distinct bacterial colonies were isolated and selected for further assessment.

2.3. Biochemical Characterizations of Endophytes and Their Extract Preparation

The isolated endophyte's cellulase activity was evaluated using 1.5% (w/v) carboxymethyl cellulose agar plates by streaking and incubating plates for up to 48 h at 37 °C. After the desired incubation, the plates were flooded with 0.3% (w/v) Congo red solution for 10 min [24]. Similarly, pectinase activity was assessed on pectin agar under the same incubation conditions. The presence of clear zones around the colonies confirmed the presence of cellulase or pectinase activity [24]. Furthermore, the protease activity of endophytes was examined using skim milk agar plates, which showed clear zones of hydrolysis around the colonies. For crude extract preparation, bacterial (MM01–04, MM07, MM08, MM10, and MM11) and fungal (MM05, MM06, MM09, and MM12) isolates were incubated in nutrient broth and potato dextrose media (200 mL in a 500 mL flask) for 48 h at 37 °C and 120 h at 28 °C with agitation rate of 150 rpm in a rotatory shaker, respectively. After the desired incubation, bacterial cells were harvested by centrifugation at 4000 × g (20 min), and fungal cells were harvested by filtration (Whatman paper). The resulting

supernatants were mixed with ethyl acetate (1:1, *v/v*) and kept overnight at 4 °C [25]. Furthermore, the solvent phase containing bioactive compounds was extracted using a separating funnel, followed by drying using a rotary evaporator at 40 °C (90 rpm) to produce a crude extract. The amount of crude extract was measured by weighing. For further studies, the extract was dissolved in methanol and stored at 4 °C. The MM07 extract was characterized using confirmatory tests for alkaloids, flavonoids, tannins, and terpenoids, including Wagner's, Shinoda's, ferric chloride, and Salkowski's tests, as described in the standard procedures [26,27]. Furthermore, the MM07 extract's total phenolic content was determined using the Folin–Ciocalteu procedure [27].

2.4. Antibacterial Activity of Endophytic Microbial Extract

The antimicrobial potential of the endophytic microbial crude extract (10 µg/mL) was assessed against *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*, with 10 µL of gentamicin (1 mg/mL) and methanol (10%) as the positive and negative controls, respectively. Briefly, 100 µL of overnight cultures (OD₆₀₀, 0.40) were spread on NA plates, followed by the addition of crude extract at the central spot. These plates were incubated for 24 h at 37 °C, and the zone of inhibition (ZOI) was analyzed in mm [28]. Furthermore, the minimum inhibitory concentration (MIC) of endophytic extracts against *C. violaceum* and *P. aeruginosa* was determined by growth inhibition assay at concentrations ranging from 1.25 to 30.0 µg/mL at 8 h of incubation.

2.5. Molecular Characterizations of Efficient Endophyte

To molecularly characterize the endophytic isolate MM07, which exhibits efficient antimicrobial activity, genomic DNA was extracted from the pure culture, and its quality was verified on a 1.0% agarose gel. A ~1500 bp fragment of the 16S rRNA gene was amplified via polymerase chain reaction (PCR) using the universal bacterial primers as forward primer (27F, 5'-AGAGTTTGATCATGGCTCAG-3') and reverse primer (1492R, 5'-TACGGCTACCTTGTACGACTT-3') as described previously [29]. The resulting PCR amplicon was purified and subjected to cycle sequencing using the BDT v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) on an ABI 3730xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). A consensus sequence was generated and analyzed using BLAST⁺ (2.17.0) at the National Center for Biotechnology Information (NCBI) for identification [29]. The phylogenetic analysis was evaluated using ClustalW (2.1), and MEGA 11 for sequence alignment and neighbor-joining phylogenetic tree construction, respectively [30].

2.6. Antibiofilm Activity of Endophytic Extract

The antibiofilm activity of the crude extract metabolites was analyzed using the tissue culture plate procedure. 10 µL of 0.4 (OD₆₀₀) culture of overnight-grown *P. aeruginosa* was dispensed into wells of a flat-bottom tissue culture plate (96-well), and a concentration of 10 µg/mL of endophyte extracts was added, maintaining a final volume of 200 µL per well with TSB (Tryptic soy broth). The plate was incubated for 24 h at 37 °C. Thereafter, the wells were washed (three times) with PBS (pH 7.4) to detach the non-adherent planktonic bacteria or media residues. The surface-attached cells were stained with crystal violet (0.1%, *v/v*) for 30 min at 37 °C. The excess stain was eliminated through rigorous washing with PBS and air-drying. Biofilm quantification was assessed at 570 nm after solubilizing bound crystal violet with methanol. Biofilm inhibition was calculated based on the amount of biofilm grown in the absence of endophytic extract (100%) and in media sterility control (0%) as follows (Equation (1)):

$$\text{Biofilm inhibition (\%)} = (\text{OD}_c - \text{OD}_t) / \text{OD}_c \times 100 \quad (1)$$

OD_c and OD_t represented the absorbance of the untreated and treated cultures, respectively.

For EPS (Extracellular polymeric substances) inhibition analysis, 25 µL of *P. aeruginosa* (OD₆₀₀, 0.4) was incubated overnight in 8 mL of LB broth containing endophytes extracts (10–30 µg/mL) or furanone/methanol (controls) in 6-well plates for incubation for 24 h at 37 °C [31]. The planktonic cells were eliminated, and the biofilms were cleaned with PBS, scraped, and resuspended in TE buffer, followed by 500 µL of phenol (5%, *w/v*) and 1 mL of concentrated H₂SO₄. The resulting mixture was incubated for 1 h in the dark, and absorbance was monitored at 490 nm to estimate the total carbohydrates using glucose as a standard. Similarly, the total protein content of EPS was analyzed using the Lowry method, with Bovine serum albumin as a standard, as described previously [27]. The EPS inhibition was calculated based on the total carbohydrates and protein content as follows (Equation (2)):

$$\text{EPS inhibition (\%)} = (X_c - X_t) / X_c \times 100 \quad (2)$$

whereas X_c and X_t represent the total carbohydrates and protein contents (EPS) of the control and treated samples, respectively.

2.7. Anti-QS Activity of Endophytic Extract

The anti-QS activity of the extract was performed through violacein production by *C. violaceum*. *C. violaceum* (0.4, OD₆₀₀) was grown in LB broth containing endophyte extracts (10–30 µg/mL) or furanone/methanol (controls) at 30 °C for incubation of 18 h [27]. After the desired incubation, 1.0 mL of culture was withdrawn and centrifuged (10,000 rpm for 10 min) to recover the insoluble violacein pigment as a pellet. Thereafter, the pellets were suspended in dimethyl sulfoxide, and the inhibition of violacein production was calculated as follows (Equation (3)):

$$\text{Violacein inhibition (\%)} = (OD_c - OD_t) / OD_c \times 100 \quad (3)$$

2.8. Influence of Endophytic Extract on Virulence Factors and Protease Production of *P. aeruginosa*

Initially, the swimming motility assay was performed [31]. In brief, 10 mL of swim agar (LB medium containing agar (0.3%, *w/v*)) supplemented with various concentrations of endophytic extracts was dispensed into Petri dishes and allowed to solidify. Thereafter, fully grown *P. aeruginosa* (5 µL) was then point-inoculated onto the center of each plate and incubated for 24 h at 37 °C. Swimming motility was quantified by measuring the radial expansion of bacterial migration relative to the untreated control. The inhibitory influence of the endophytic extract on pyocyanin production by *P. aeruginosa* was evaluated using the assay described earlier [27]. In brief, extracts at different concentrations were incubated with *P. aeruginosa* for 24 h at 37 °C with agitation at 200 rpm. After the desired incubation, full-grown cells were recovered through centrifugation (4000 rpm for 20 min), and the supernatant was used after the addition of chloroform (3.5 mL) and vigorous mixing. The organic (chloroform) phase was separated, and 1.5 mL (0.2 M HCl) was added to initiate phase separation. Approximately 200 µL of the pink aqueous layer was used for estimation at 595 nm. The pyocyanin inhibition was determined as follows (Equation (4)):

$$\text{Pyocyanin inhibition (\%)} = (OD_c - OD_t) / OD_c \times 100 \quad (4)$$

The azocasein proteolytic assay was evaluated to measure the effects of the endophytic extract on *P. aeruginosa* protease secretion. Briefly, 100 µL of overnight-grown *P. aeruginosa* was cultivated in TSB (10 mL) containing extract (10–30 µg/mL) and incubated at 37 °C for 48 h. Thereafter, cells were recovered through centrifugation, and the supernatant

(150 µL) was supplemented with 1 mL of 0.3% (*w/v*) azocasein (0.05 M Tris-HCl, pH 7.5) and incubated for 15 min at 37 °C. After the desired incubation, the reaction was stopped by adding 0.5 mL of trichloroacetic acid (TCA; 10% *v/v*). Absorbance was measured spectrophotometrically at 440 nm.

Alginate production by *P. aeruginosa* was quantified using the method described earlier [32]. For alginate production, an overnight-grown *P. aeruginosa* culture was diluted (OD₆₀₀, 0.05) and re-cultivated in LB broth with/without MM07 extract for 24 h at 37 °C under shaking conditions (180 rpm). Following incubation, the bacterial cells were collected by centrifugation (4000 × *g* for 20 min), and the resulting supernatant was assessed for extracellular alginate content using a colorimetric uronic acid-based assay. In brief, the supernatant was mixed with a borate-sulfuric acid reagent and incubated in a boiling water bath to hydrolyze the polysaccharide into uronic acid residues. After cooling, the carbazole reagent was added, and the resulting mixture was incubated to develop a pink-purple chromophore for absorbance measurement at 530 nm [32]. The alginate concentration was determined using a standard of sodium alginate. The alginate production inhibition was calculated as follows (Equation (5)):

$$\text{Alginate production inhibition (\%)} = (A_c - A_t) / A_c \times 100 \quad (5)$$

whereas A_c and A_t represent the alginate concentrations in the control and treated cultures, respectively.

2.9. Gene Expression Analysis

The quantitative real-time polymerase chain reaction (qRT-PCR) was used to evaluate the expression levels of QS regulatory genes (*lasI*, *lasR*, *rhlI*, and *rhlR*) in *P. aeruginosa* PAO1 after treatment with the MM07 extract (30 µg/mL), as described previously [27]. In brief, *P. aeruginosa* PAO1 was cultured in LB medium with/without MM07 extract for 24 h of incubation at 37 °C. For total cellular RNA extraction, the grown cells were collected by centrifugation (4000 rpm for 20 min at 16 °C), followed by resuspension of the cell pellets in TRIzol reagent as per the supplier's guidelines. Furthermore, single-stranded cDNA was synthesized using the RevertAid First Strand kit, Thermo Scientific, MA, USA. cDNA template (10 µL) was amplified using desired primers and SYBR Green master mix (Thermo Scientific, MA, USA) by RT-PCR LightCycler 480 (Roche Diagnostics, Risch-Rotkreuz, Switzerland) (Table S1). The program was run under cycling conditions: pre-denaturation (95 °C, 5 min), denaturation (95 °C, 15 s), annealing (at the desired temperature, 0.5 min), and extension (72 °C, 0.5 min) for 40 cycles with a final extension (72 °C, 5 min). Finally, the samples were evaluated in triplicate and normalized using *rpsL* expression via the $2^{-\Delta\Delta C_t}$ method [27,33].

2.10. Microscopy, Instrumental and Statistical Measurements

The inhibitory effect of the endophytic extract on biofilm formation was assessed by microscopy. Briefly, *P. aeruginosa* was inoculated into TSB containing different concentrations of MM07 extract and cover slips, and incubated for 48 h at 37 °C. Thereafter, the cover slips were recovered and washed with PBS (two times) to remove non-adherent cells and air-dried. Furthermore, the cover slip was incubated in a crystal violet (0.4%, *w/v*) solution for 10 min, followed by excess stain removal via washing through PBS (three times). After air-drying, the cover slip was visualized under a light microscope (Olympus CX21i, Tokyo, Japan) at 100× magnification to assess biofilm inhibition. The endophytic extract MM07 Fourier transform infrared (FTIR) spectroscopy analysis was performed in the range of 4000–400 cm^{−1} using a SpectrumTM 3 FTIR spectrometer from PerkinElmer, Springfield, IL, USA [34]. The gas chromatography-mass spectroscopy (GC-MS) analysis

of the endophytic extract was performed using a GC–MS 2400™, PerkinElmer, Springfield, IL, USA. All experiments were evaluated in triplicate and replicated twice. Each data values were presented as mean $\pm$ standard deviation. The difference between groups was assessed using one-way analysis of variance followed by Dunnett's post hoc test (ANOVA, $\alpha = 0.05$; GraphPad Prism 5, USA).

3. Results

3.1. Isolation of Endophytes from Therapeutic Plants

A total of 12 microbial isolates were obtained from leaves of *C. asiatica*, *H. cordata*, and *P. foetida*. The morphological characteristics of these isolates, such as form (Circular, irregular, or rhizoid), margin (Flat, raised, or umbonate), and elevation (Curled, entire, filiform, or undulate), are presented in Table S2. Based on the cultivation media, the strains were classified as bacteria (MM01–04, MM07, MM08, MM10, and MM11) and fungi (MM05, MM06, MM09, and MM12). The bacterial and fungal endophytes across *C. asiatica*, *H. cordata*, and *P. foetida* plants exhibit distinct distributions, reflecting their roles in plant growth, properties, and metabolic activities.

3.2. Biochemical Characterization of Endophytes, Extract Yield, and Antimicrobial Activity Assessment

Initially, the confirmatory examination of the activities of cellulase, pectinase, and protease in endophytes was evaluated (Table S3). The isolates MM02, MM04, MM06, MM07, MM09, MM10, and MM12 exhibited cellulase activity. MM01, MM04, MM05, MM09, and MM10 showed pectinase activity, suggesting their role in the breakdown of the plant cell wall. Similarly, isolates MM03 and MM06–MM10 displayed protease activity, as evidenced by a clear zone of hydrolysis (Figure S1). The endophytic isolates resulted in a high crude extract yield of 0.22–0.43 and 0.36–0.51 g for bacterial and fungal cultures, respectively (Table S4). Furthermore, the antimicrobial properties of these microbial isolates were examined against *B. subtilis*, *E. coli*, *P. aeruginosa*, and *S. aureus* using an agar-based well-diffusion assay (Table 1). The microbial isolates MM02, MM05, MM07, and MM12 showed efficient antimicrobial activity, with ZOI values of up to 8.3 mm for *B. cereus*, 12.8 mm for *B. subtilis*, 8.1 mm for *E. coli*, 8.9 mm for *K. pneumoniae*, 18.8 mm for *P. aeruginosa*, and 16.9 mm for *S. aureus*. This finding demonstrated that *B. subtilis*, *P. aeruginosa*, and *S. aureus* were highly susceptible to these endophytes, as indicated by large zones of inhibition. Similarly, MIC values of isolated endophytes against *C. violaceum* and *P. aeruginosa* ranged from 2.5 to 30.0 $\mu\text{g/mL}$ (Table S4). Among these isolated strains, MM07 exhibited MICs of 2.5 and 5.0 $\mu\text{g/mL}$ for *C. violaceum* and *P. aeruginosa*, respectively.

3.3. Antibiofilm Activity and Molecular Characterization

The endophytic isolates exhibited antibiofilm activity, with a maximum of 64.3%, compared with 89.5% for furanone at 10 $\mu\text{g/mL}$ (Figure 1A). Based on the high antimicrobial properties of the MM07 endophyte, the isolate was identified by 16S rRNA gene sequencing. The partial gene sequence of MM07 was deposited in NCBI GenBank under accession number PX498110. Based on BLAST results for similarity and phylogenetic analysis, MM07 was identified as the *Bacillus xiamenensis* strain MM07 (Figure S2). MM07 exhibited a dose-dependent inhibition of *P. aeruginosa* biofilm formation at concentrations ranging from 5 to 30 $\mu\text{g/mL}$ (Figure 1B). The maximum inhibition of 83.5% was noted at 30 $\mu\text{g/mL}$, compared to the control at 89.5%. Furthermore, MM07 extract showed a dose-dependent decline in EPS formation by *P. aeruginosa* (Figure 1C). The maximum inhibition of 60.3% was observed at 30 $\mu\text{g/mL}$, compared with 65.8% for the furanone.

Table 1. Antimicrobial activity of the endophytic isolates extract.

Isolates	Zone of Inhibition (mm) ^a					
	<i>B. cereus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
MM01	7.1	6.2	7.1	8.9	10.1	9.8
MM02	7.8	7.1	7.6	12.5	16.7	14.3
MM03	6.7	6.5	7.0	9.1	9.7	8.6
MM04	6.2	6.8	6.5	7.3	8.2	7.9
MM05	6.1	6.7	7.2	8.3	8.0	7.5
MM06	6.3	6.4	6.7	6.9	7.1	6.8
MM07	8.3	12.8	8.1	8.9	18.8	16.9
MM08	6.7	7.0	6.3	7.4	9.6	9.1
MM09	6.2	6.6	6.9	6.7	8.5	8.6
MM10	6.8	6.5	7.2	6.1	7.9	9.7
MM11	7.3	7.0	7.5	9.2	9.0	8.5
MM12	6.3	6.8	7.0	8.8	7.9	8.2
Gentamicin	16.8	14.9	17.7	17.9	20.6	18.2

^a The ZOI was not observed in the methanol (negative) control.

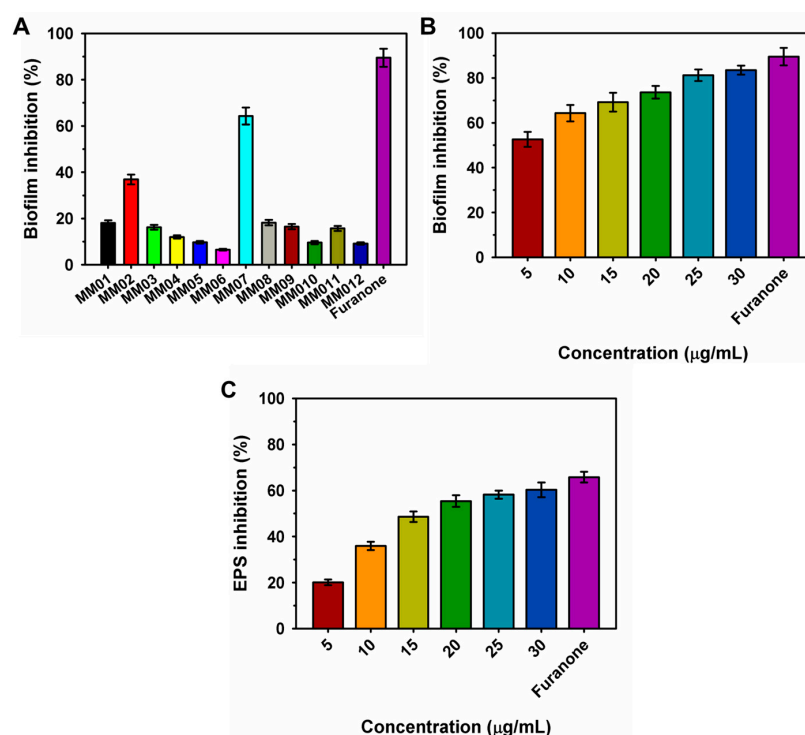


Figure 1. Biofilm inhibition potential of endophytic microbial extract (A), influence of MM07 extract concentration on biofilm inhibition (B), and EPS inhibition (C). Significant differences ($p \leq 0.05$) were observed using one-way ANOVA statistical analysis.

3.4. Endophytic Extract Characterization

Endophytes produce diverse bioactive compounds that play a vital role in plant metabolism and function. Therefore, the qualitative analysis of major biomolecules present within the crude extract of MM07, like alkaloids, flavonoids, tannins, and terpenoids, was examined by Wagner's, Shinoda's, ferric chloride, and Salkowski's tests via cream or orange precipitation, light yellow, blue-black, and reddish-brown color at interface formation, respectively (Table S5). Here, the total phenolic content of the extract was 295 mg/g. FTIR has been widely used to identify the prominent functional groups of biomolecules in the endophytic extract. Therefore, in FTIR measurement, the strong peaks at 3400–3200, 2964, 1716–1664, 1516–1450, 1410–1223, 1126, 1045, and 822–490 cm^{-1} were detected, corresponding to hydroxyl (-OH), -CH/-CH₂ stretching, -C=O (amid I)/quinone

compounds, C-N stretching and N-H bending (amide II), C-N stretching, N-H bending, and C-C stretching (amide III), -C-O-C- of polysaccharides, -C-O stretching, and -CH (out of plane in aromatic band), respectively (Figure 2A). In GC-MS analysis, the prominent peaks were observed at retention times of 3.89, 10.76, 11.01, 12.44, 12.54, 21.79, and 28.86 min, respectively, corresponding to 3,3-dimethyl-4-methylamino-butan-2-one, 6-amino-2-methyl-1-iodo-2-methylundecane, heptadecane, undecane, hexadecanoic acid, and cyclotrisiloxane (Table 2 and Figure 2B).

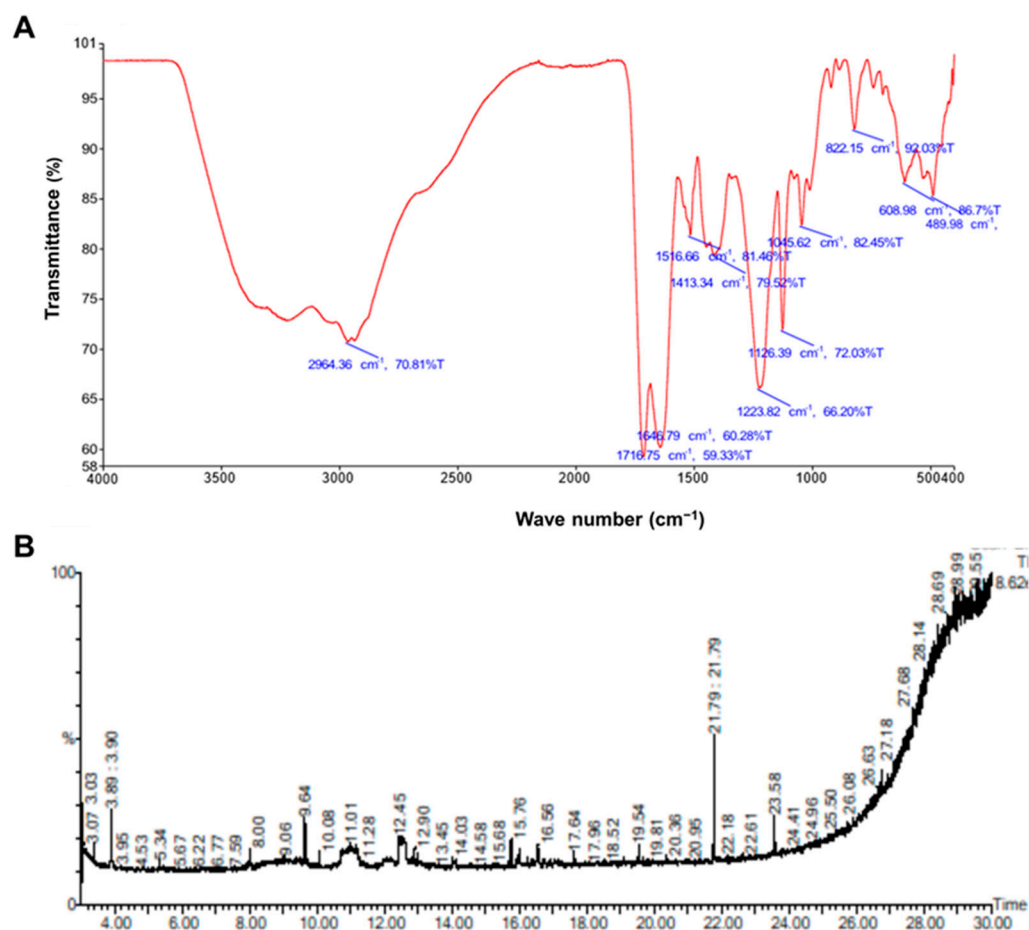


Figure 2. Endophytes MM07 extract characterization: FTIR spectra (A), and GC-MS spectra (B).

Table 2. GC-MS analysis of the MM07 endophytic isolate extract.

S. No.	Retention Time (min)	Name of Compound or Group	Molecular Formula	Molecular Weight (g/mol)	Content (%)	Match Score (R-Value) ^a
1	3.89	3,3-Dimethyl-4-methylamino-butan-2-one	C ₇ H ₁₅ NO	129	1.9	804
2	10.76	6-amino-2-methyl-1-iodo-2-methylundecane	C ₈ H ₁₉ NO	115	3.6	807
3	11.01	Heptadecane	C ₁₂ H ₂₅ I	296	2.3	813
4	12.45	Undecane	C ₁₇ H ₃₆	240	2.9	835
5	12.54	Hexadecanoic acid	C ₁₁ H ₂₄	156	4.2	817
6	21.79	Cyclotrisiloxane	C ₁₆ H ₃₂ O ₂	256	3.5	891
7	28.86		C ₆ H ₁₈ O ₃ Si ₃	222	2.1	882

^a The confidence level is considered a good match for the R-values > 800 [35].

3.5. Anti-Quorum-Sensing Activity

The ability of the MM07 anti-QS potential was assessed using violacein inhibition, a key QS-regulated pigment in *C. violaceum*. The treatment with the endophytic supernatant resulted in a concentration-dependent decline in violacein production (Figure 3A). At

30 µg/mL, a high inhibition of 73.2% in violacein production was observed, compared to 90.2% using furanone.

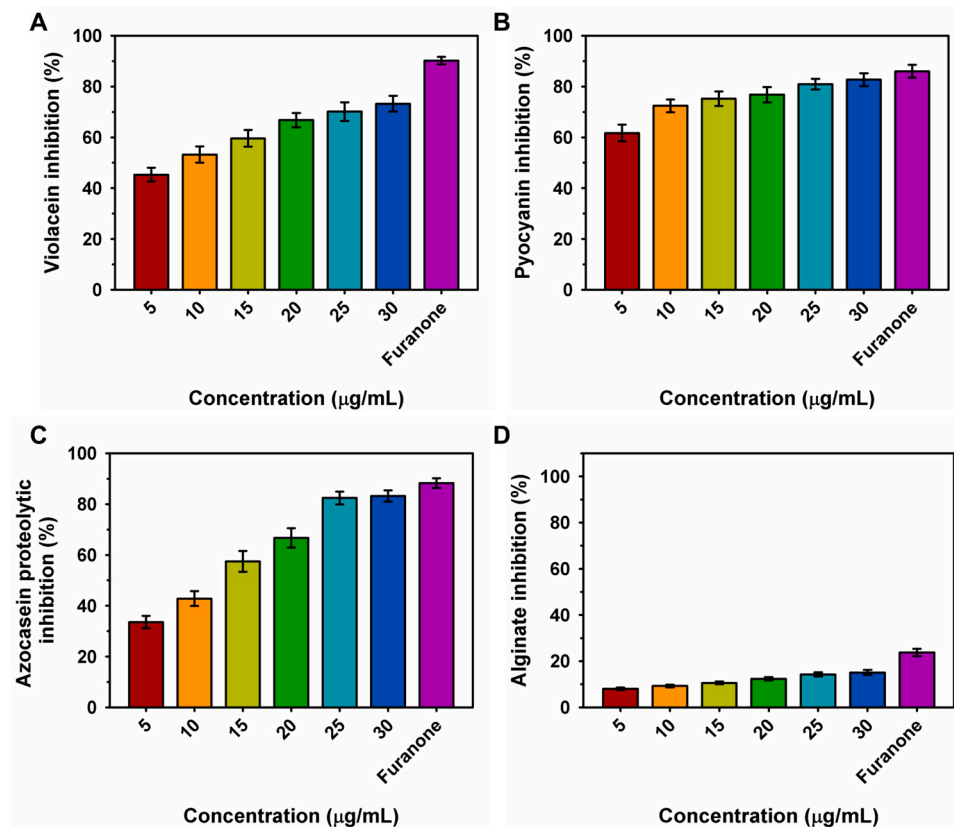


Figure 3. Anti-QS, virulence factor, and protease production inhibition: Violacein production (A), pyocyanin production (B), protease production (C), and alginate production (D). Significant differences ($p \leq 0.05$) were observed using one-way ANOVA statistical analysis.

3.6. Effect on Virulence Factors and Protease Production of *P. aeruginosa*

MM07 extract significantly altered virulence factor expression in *P. aeruginosa*, as assessed by pyocyanin production. An 82.7% inhibition of pyocyanin production was observed at 30 µg/mL, closely matching that of the positive control furanone (86.0%) (Figure 3B). To further evaluate the impact of the endophyte on QS-regulated virulence traits, total proteolytic activity was quantified using the azocasein assay. MM07 exhibited a significant, dose-dependent inhibitory effect on extracellular protease production in *P. aeruginosa*. At a concentration of 30 µg/mL, protease activity was inhibited by 83.2% compared to the untreated control (Figure 3C). Furthermore, the biochemical profiling showed that the MM07 supernatant inhibited alginate production by 15.1% at 30 µg/mL, as compared to 23.8% in the control (Figure 3D). Motility assays demonstrated that the MM07 supernatant restricted the swimming ability of *P. aeruginosa*. The untreated control displayed a swimming diameter of 33.0 mm (100%), whereas treated cultures showed 63.2% inhibition of motility at 30 µg/mL (Figures 4A and S3). The growth curve of *P. aeruginosa* in the presence of 30 µg/mL of extract is presented in Figure S4. In *P. aeruginosa* PAO1, QS is controlled by regulatory genes (*lasI*, *lasR*, *rhlI*, and *rhlR*) that modulate biofilm formation and virulence factor synthesis. Hence, these gene expression analyses can validate QS-mediated inhibition of *P. aeruginosa* PAO1 by the endophytic extract (30 µg/mL). The qRT-PCR analysis revealed downregulation of 40.3%, 37.8%, 32.3%, and 37.1% for *lasI*, *lasR*, *rhlI*, and *rhlR*, respectively, compared to the control (Figure 4B).

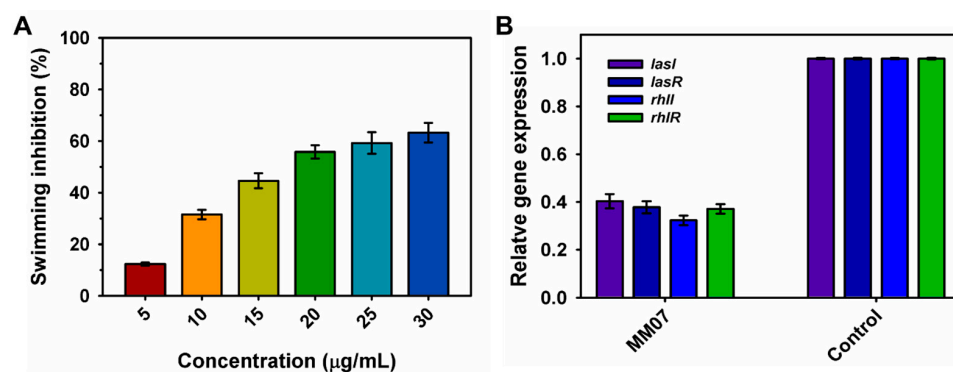


Figure 4. Motility and gene regulation analysis by treatment of *P. aeruginosa* with MM07 extract: Swimming inhibition (A), and qRT-PCR-based gene expression (B). Significant differences ($p \leq 0.05$) were observed using one-way ANOVA statistical analysis.

3.7. Microscopy Measurements

The untreated sample, serving as a control, exhibited a dense, thick biofilm matrix of *P. aeruginosa* under the microscopic analysis (Figure 5). Samples treated with MM07 extract showed a dose-dependent decline in matrix surface coverage at concentrations ranging from 5.0 to 30 µg/mL, resulting in a scattered, disintegrated biofilm architecture. In contrast, nearly complete disintegration of the biofilm matrix was observed using furanone. This finding suggests that the MM07 extract-associated biomolecules effectively prevent surface colonization by *P. aeruginosa* by disrupting the EPS matrix.

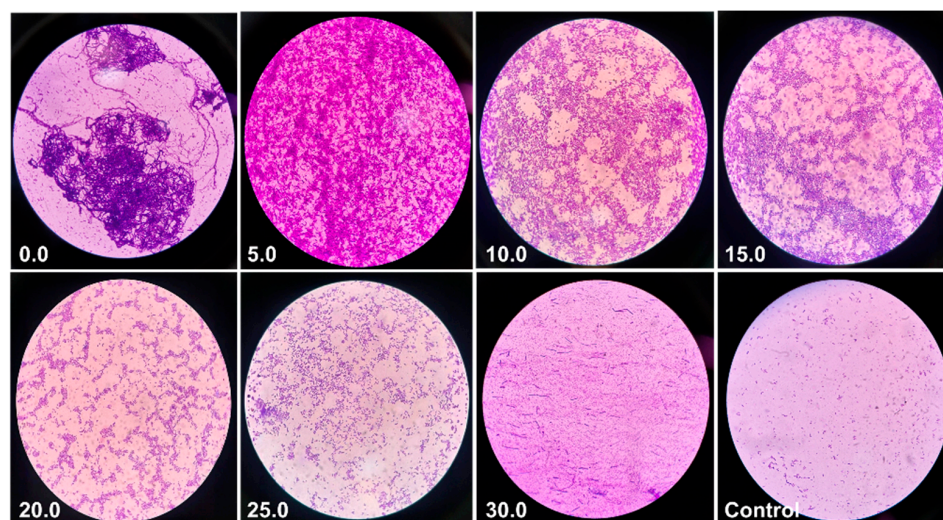


Figure 5. Microscopic images of biofilm formation at different concentrations of MM07 extract (µg/mL) with a control (furanone) using a light microscope (100×, magnification).

4. Discussion

Plant-associated endophytes, such as bacteria and fungi, produce diverse bioactive molecules, including steroids, terpenes, flavonoids, and peptides with broad-spectrum efficacy towards pathogens [36]. Therefore, the development of sustainable bioactive therapeutics, rather than antibiotics, has attracted considerable attention due to their broad secondary metabolite diversity with potent antimicrobial properties. In the present study, a medicinal plant-associated endophytic culture with potential to produce therapeutic biomolecules to inhibit biofilm formation and QS properties of *P. aeruginosa* PAO1 was investigated. During screening of endophytes from *C. asiatica*, *H. cordata*, and *P. foetida*, a total of 12 isolates (MM01–MM12) were obtained, including bacteria (MM01–04, MM07, MM08, MM10, and MM11) and fungi (MM05, MM06, MM09, and MM12)-based on their

morphological characteristics, including form, margin, and elevation. These endophytic isolates exhibited distinct patterns of confirmatory biocatalytic activity, as determined by NA plate methods, including cellulase, pectinase, and protease. Among these endophytic isolates, MM07 produced an extract with high antimicrobial activity against *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*, with a ZOI of up to 18.8 mm, comparable to the control ZOI of up to 20.6 mm with gentamicin. A high ZOI of 12.8, 16.9, and 18.8 mm towards *B. subtilis*, *S. aureus*, and *P. aeruginosa* for the MM07-based extract demonstrated its superior efficacy over other isolated endophytes. Previous studies have shown that endophytic cultures isolated from these medicinal plants exhibit diverse populations with broad-spectrum antimicrobial activity against diverse pathogens [8,16,37,38]. An endophytic bacterium, *Bacillus* sp. VITLTMJ4, isolated from a Citrus plant, inhibited *P. aeruginosa* with a lower ZOI of 15.6 mm [39]. Similarly, *Xylocarpus granatum*-based endophytic *P. aeruginosa* XGDE7 crude extract showed a lower ZOI of up to 12.3 towards *B. subtilis*, *E. coli*, *E. faecalis*, and *S. aureus* [40]. In contrast, *Alternanthera philoxeroides*-based endophytic *K. pneumoniae* did not exhibit antimicrobial activity against *B. cereus*, *Streptococcus pneumoniae*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* [30]. These findings suggest that the diversity of plants as endophyte hosts or origin sources (such as leaves, roots, and stems), along with the broad alterations in the composition and content of therapeutic bioactive compounds in the extracts produced by these plants, can alter antimicrobial activity against various pathogenic organisms [38].

P. foetida-based endophytic isolates exhibited MIC in the range of 2.5–30.0 µg/mL against *C. violaceum* and *P. aeruginosa*. Among isolated endophytes, MM07 exhibited the lowest MIC of 2.5 µg/mL for *P. aeruginosa*. Previously, various *Bacillus* spp. such as *B. mesophilus*, *B. xiamenensis*, *B. halotolerans*, and *B. subtilis* isolated from Red Sea mangrove sediments showed significantly higher MIC values up to 5.00, 7.81, 5.62, and >5.0 mg/mL towards *E. coli*, *S. aureus*, *P. aeruginosa*, and *K. pneumoniae* as bacterial pathogens, respectively [41]. In contrast, *B. xiamenensis* PM14 isolated from the sugarcane rhizosphere exhibited antimicrobial activity against various pathogenic fungi with an inhibition coefficient up to 77.6 for *Colletotrichum falcatum*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Macrophomina phaseolina*, *Pythium splendens*, and *Rhizoctonia solani* [42]. Similarly, these endophytic isolates exhibited distinct antibiofilm activity, reaching up to 64.3% against *P. aeruginosa* at 10 µg/mL. Based on the 16S rDNA analysis, the efficient endophytic isolate MM07 was identified as *B. xiamenensis* strain MM07. The MM07 extract-based biomolecules demonstrated dose-dependent inhibition of *P. aeruginosa* biofilm formation, with up to 83.5% inhibition at 30 µg/mL. *Bacillus amyloliquefaciens* NWR-14 endophyte isolated from *Piper chaba* exhibited only 68.2% of biofilm inhibition of *K. pneumoniae* at a much higher bacterial extract concentration of 6.0 mg/mL [43]. In contrast, endophytic *P. aeruginosa* XGDE7 crude bioactive resulted in biofilm inhibition in the range of 40.0–80.1% of *B. subtilis*, *E. coli*, *E. faecalis*, and *S. aureus* at concentrations of 62.5, 62.5, 31.2, and 125 µg/mL, corresponding to 2× of their MIC values, respectively [40]. In contrast, sponge-based endophytic *Bacillus mobilis* SP2-AB7 was reported to inhibit only 8.3% of *P. aeruginosa* ATCC 27853 at a significantly higher concentration of 10 mg/mL [44]. 89.5%. During biofilm formation, the synthesis of EPS as adhesive molecules is vital. MM07 extract inhibited EPS formation by up to 60.3%, comparable to the furanone as a control. The mechanisms and roles of EPS in biofilm formation have been widely demonstrated against plant and human pathogens. The infections by these pathogens can be readily countered by bacterial endophytes [36]. These findings suggest that the MM07 extract, enriched in bioactive compounds, may inhibit *P. aeruginosa* through a synergistic effect.

The confirmatory results of the tests, including Wagner's, Shinoda's, ferric chloride, and Salkowski's, validated the presence of biomolecules within the extract of MM07, such

as alkaloids, flavonoids, tannins, and terpenoids, with a high total phenolic content of 295 mg/g. Previously, the endophytic extract of *B. subtilis*, derived from *Fagonia indica*, exhibited a lower phenolic content of 243 mg/g [45]. FTIR analysis has been widely used to identify the prominent functional groups of biomolecules in the endophytic extract [39]. Here, FTIR analysis suggested the presence of strong peaks for hydroxyl (-OH), -CH/-CH₂ stretching, -C=O (carbonyl), C-N stretching and N-H bending, C-N stretching, N-H bending, and C-C stretching, -C-O-C- of polysaccharides, and -C-O stretching. These peaks indicate the presence of diverse bioactive compounds with functional groups in the MM07 extract. The GC-MS analysis confirmed that the bioactive compounds in the extract include aminoketones, alcohol, and hydrocarbons. These bioactives have been reported to inhibit the growth of microbial pathogens and exhibit antibiofilm properties [45–47]. The inhibition of pathogens, including MRSA, *K. pneumoniae*, and *E. coli*, has been successfully reported by aminoketones and alcohols, such as 3,3-dimethyl-4-methylaminobutan-2-one [48,49]. In mechanism, aminoketone downregulated the expression of *icaC* and *icaD*, which are critical for intercellular adhesion and biofilm formation [49]. Similarly, 6-amino-2-methyl-based compounds inhibit microbial growth by disrupting membranes [50]. In another study, the inhibitory potential of 1-iodo-2-methylundecane towards both Gram-positive and Gram-negative bacteria, including multidrug-resistant *P. aeruginosa*, has been well demonstrated [51,52]. Previously, endophytic extracts containing the branched alkane heptadecane were shown to exhibit anti-biofilm and anti-QS activity against *E. coli* and *Proteus mirabilis* [53]. Undecane derivatives are known to inhibit pathogens via anti-biofilm or anti-QS activity [54]. Similarly, hexadecanoic acid exhibited antibiofilm activity in MDR strains through its membrane-permeabilizing effect [55,56]. Previously, the presence of siloxane compounds in the *Combretum erythrophyllum* seed-based endophytic *Methylobacterium radiololerans* MAMP4754 extract exhibiting potent antimicrobial activity against *B. subtilis* ATCC 9659, *B. cereus* ATCC1076, *E. coli* ATCC1053, *K. oxytoca* ATCC13182, and *M. smegmatis* ATCC21293 was demonstrated, which supports the antibacterial properties of MM07 containing cyclotrisiloxane [57]. Overall, these findings validated that MM07 is enriched in biomolecules with potential antimicrobial, antibiofilm, and anti-QS activities.

The anti-QS potential of MM07, measured by inhibition of *C. violaceum*-based violacein production, showed dose-dependent inhibition, reaching 73.2% at 30 µg/mL. Similarly, a diverse zone of violacein inhibition (0.0–9.0 mm) of *C. violaceum* ATCC 12472 by various endophytic *B. cereus* SP1-AB4, *B. mobilis* SP2-AB7, *B. pumilus* SP2-W6, *Bacillus thuringiensis* SP-AB2, and *Bacillus wiedmannii* SP5-AB7 at a much higher concentration of 1.0 mg/mL on mannitol and 5294 media as compared to the control (Furanone) with a ZOI of 11.0 mm [44]. In mechanism, anti-violacein activity of endophytic *Bacillus* was demonstrated via the production of N-acyl homoserine lactone lactonase enzymes [58]. In another study, the endophytic *Bacillus toyonensis*, based on *Arthrocaulon* sp., exhibited anti-QS activity against *Pseudomonas syringae* pv. tomato DC3000 due to its degrading potential towards a broad range of N-acyl-homoserine lactones [59].

MM07 inhibited pyocyanin production by *P. aeruginosa* in a dose-dependent manner, with a maximum inhibition of 82.7% at 30 µg/mL. Previously, *Thymus vulgaris*-based endophytic *B. cereus* AL1 exhibited much lower pyocyanin inhibition of 15.0% at a higher extract concentration of 1.0 mg/mL towards *P. aeruginosa* PAO1 [6]. Similarly, various sponge-associated endophytic *Bacillus* spp., including *B. cereus* SP1-AB4, *B. mobilis* SP2-AB7, *B. pumilus* SP2-W6, *B. thuringiensis* SP-AB2, and *B. wiedmannii* SP5-AB7, were reported to inhibit *P. aeruginosa* pyocyanin production only by up to 37.6% at a much higher extract concentration of 1000 µg/mL [44]. The MM07-based extract reduced the swimming ability of *P. aeruginosa* by 63.2% at 30 µg/mL, as assessed by the mortality assay. Endophytic *B. cereus* AL1 displayed significantly lower *P. aeruginosa* PAO1 swarming motility inhibition

of 25.0% at 1.0 mg/mL with maximum biofilm inhibition of 38.0% [6]. In contrast, the maximum swimming and swarming motility of *P. aeruginosa* ATCC 27853, up to 51.9%, was inhibited by various sponge-based *Bacillus* spp. endophytes at a similar concentration [44]. MM07 showed dose-dependent inhibition of extracellular protease activity in *P. aeruginosa*, with a maximum decline of 83.2% at 30 µg/mL, as validated by a skim milk agar assay. Endophytic *B. cereus* SP1-AB4, *B. mobilis* SP2-AB7, *B. pumilus* SP2-W6, *B. thuringiensis* SP-AB2, and *B. wiedmannii* SP5-AB7 were reported for inhibiting maximum up to 59.9% protease production by *P. aeruginosa* ATCC 27853, based on azocasein assay at a higher extract concentration of 1000 µg/mL [44]. Alginate production by *P. aeruginosa* PAO1, which is involved in biofilm formation, has been demonstrated [60]. Here, alginate production was inhibited up to 15.1% by MM07 extracts. A comparative alginate synthesis inhibition of 22.3% by 10 µM of commercially produced DZ2002 as a selective S-adenosylhomocysteine hydrolase inhibitor [60]. QS regulation in *P. aeruginosa* PAO1 is associated with genes such as *lasI*, *lasR*, *rhlI*, and *rhlR*. Therefore, the expression levels of these genes can be used effectively to validate the anti-QS properties of endophyte-derived biomolecules [33]. The decline in the expression of these genes, up to 40.3% in qRT-PCR analysis using MM07-based biomolecules, confirms the potential inhibition of *P. aeruginosa* PAO1. In contrast, the biofilm inhibition of *P. aeruginosa* PAO1 by DZ2002 was unrelated to the expression of *lasI*, *lasR*, *rhlI*, and *rhlR* [60]. Instead of these gene regulations, DZ2002 inhibits biofilm formation via downregulating extracellular DNA production, alginate synthesis, and swarming motility. In the mechanism, *Bacillus* spp., including *Brevibacillus borstelensis*, *Bacillus megaterium*, and *B. amyloliquefaciens*, have demonstrated anti-QS activity by interfering with QS signaling or by producing lactonase enzymes. These endophytes produce a plethora of bioactive molecules, including QS-quenching enzymes such as lactonase and acylase, which can effectively degrade QS signals from pathogenic microbes and disrupt intercellular communication [61]. Overall, various factors, such as metabolic dependency, strain-specific effects, environmental conditions, and extract concentrations, may contribute to QS suppression leading to growth inhibition. Previous studies have shown that bacterial endophytic extracts are eco-friendly antimicrobial agents with low cytotoxicity toward normal mammalian cells [36,62]. Therefore, the unique properties and high selectivity of *B. xiamenensis* MM07 can be proven beneficial for developing novel therapeutic drugs with reduced side effects.

5. Conclusions

Medicinal plant endophytes, such as bacteria and fungi, produce a broad range of bioactive compounds, making them a source of novel, alternative, sustainable therapeutic agents to synthetic antibiotics. This study reported that a novel *P. foetida*-based bacterial endophyte, identified as *B. xiamenensis* MM07, showed efficient antimicrobial activity against various pathogenic bacteria. MM07 extract exhibited dose-dependent inhibition of biofilm-forming potential and QS activity in *P. aeruginosa*. The biochemical and compositional analyses, along with microscopy and analysis of QS regulatory gene downregulation, validated the presence of various biomolecules with antimicrobial, antibiofilm, and anti-QS potential. These findings suggest that selective screening of unique endophytes and their hosts could be beneficial for achieving desirable therapeutic applications, such as discovering new antimicrobial agents to combat the rising tide of drug-resistant pathogens.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microbiolres17040065/s1>, Figure S1: Plate-based clear zone of hydrolysis assay for cellulose (A), pectinase (B), or protease (A) of the few selected bacterial isolates MM02, MM03, MM07, MM09, and MM10 (represented as numbers on corresponding plates); Figure S2: Plate culture of MM07 (A), and phylogenetic analysis of *Bacillus xiamenensis* strain MM07 (Accession number PX498110)

and similarity details based on 16S rRNA gene sequences (B); Figure S3: Influence of MM07 extract on swimming ability of *P. aeruginosa*: Control (A), and 30 µg/mL of extract (B); Figure S4: Growth curve of *P. aeruginosa* in the presence of 30 µg/mL of extract; Table S1: The details of the PCR primers used in RT-PCR analysis of QS-associated gene expression; Table S2: Morphological properties and characteristics of endophytic microbial isolates; Table S3: Biocatalytic activity of endophytic microbial isolates; Table S4: Yields of endophytic microbial isolate extracts and their minimum inhibitory concentrations (MICs); Table S5: Qualitative assessment of biomolecules presents in the MM07 crude extract.

Author Contributions: Conceptualization, M.M. and S.K.S.P.; methodology, M.J.N.; software, M.M.; validation, M.J.N. and S.R.C.; formal analysis, S.R.C. and S.K.; investigation, M.J.N., S.R.C. and P.; resources, M.M.; data curation, S.R.C. and P.; writing—original draft preparation, M.J.N. and S.K.S.P.; writing—review and editing, M.M. and S.K.S.P.; visualization, S.K.S.P.; supervision, M.M. and S.K.S.P.; project administration, M.M.; funding acquisition, M.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work is supported by Anusandhan National Research Foundation (ANRF) and JNU—PAIR Network for sustainable Future [FILE NO. ANRF/PAIR/2025/000029/PAIR].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Acknowledgments: The authors thank the Department of Molecular Biology and Biotechnology (MBBT), Department of Science and Technology (DST) PURSE, and Department of Biotechnology (DBT). Government of India for their support. Also, the author would like to thank the Hemvati Nandan Bahuguna Garhwal University (A Central University), Srinagar, for providing support. The authors would also like to thank the “Central Sophisticated Instrumentation Facility” of Tezpur University for GC–MS and FTIR analysis. The plants are housed in the Tezpur University Herbarium, Department of Economic Studies, Tezpur University, Assam, India.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
EPS	Extracellular polymeric substances
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas-Chromatography Mass Spectroscopy
LB	Luria–Bertani
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NCBI	National Center for Biotechnology Information
OD	Optical Density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
QS	Quorum sensing
TE	Tris EDTA
TSB	Tryptic Soy Broth
ZOI	Zone of inhibition

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