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Exploring the Biodiversity of Rare Actinobacteria for Combating AMR: Screening for Anti-Quorum Sensing Properties

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Abstract

The rise of antimicrobial resistance (AMR) poses a significant global health challenge, necessitating the search for alternative therapeutic strategies. Targeting bacterial quorum sensing (QS), a communication system that regulates virulence and biofilm formation, has emerged as a promising anti-virulence approach. Actinobacteria, renowned for their prolific production of bioactive secondary metabolites, represent a valuable resource for discovering novel anti-quorum-sensing (AQS) compounds. This study explores the biodiversity of actinobacteria from diverse ecological niches to identify strains with anti-QS properties. Using *Chromobacterium violaceum* and *Serratia marcescens* as biosensors, actinobacterial extracts were screened for their ability to inhibit QS-regulated pigment production. Selected extracts were further evaluated for swarming motility inhibition and potent strains were identified. Four rare actinobacterial strains MG1, BD21, MG3 and VL9 identified as *Kutzneria viridogrisea*, *Microbacterium barkeri*, *Amycolatopsis thermoflava* and *Yuhushiella* sp. were studied for their biofilm inhibition activity and prodigiosin inhibition in pathogenic bacteria. Our findings are the first to highlight the potential of these rare actinobacteria as a source of novel anti-QS agents, contributing to the development of alternative strategies to mitigate AMR.

Keywords

Actinobacteria, Quorum Sensing Inhibition, Quorum Quenching, Antimicrobial Resistance, Biofilm Inhibition

1. Introduction

Antibiotics have been essential in combating bacterial infections for over a century, but their widespread use and misuse has contributed to the alarming rise of antibiotic resistance [1]. The emergence of multidrug-resistant (MDR) pathogens, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and carbapenem-resistant *Enterobacteriaceae*, has severely limited treatment options [2]. Compounding this crisis is the lack of significant new antibiotic discoveries since 1987, underscoring the urgent need for alternative strategies to combat bacterial infections.

One promising approach is targeting quorum sensing (QS), a bacterial communication system that regulates virulence and pathogenicity. QS involves the production and detection of small signalling molecules, known as autoinducers, that coordinate behaviors such as swarming, biofilm formation, motility, and pigment production [3] [4]. *Pseudomonas aeruginosa* and *Proteus mirabilis* are opportunistic pathogens, especially among hospitalized and/or catheterized patients and menopausal women, the frequency of their involvement in UTI being next only to *E. coli* [5] [6]. Both these organisms exhibit the swarming phenotype which plays a crucial role in the establishment of renal infections which begin with the colonization of the lower urinary tract followed by ascending migration of these urinary pathogens.

Swarming enables *Proteus mirabilis* and *Pseudomonas aeruginosa* to successfully counter the host defense mechanisms and establish disease. Also, swarm cells of both these pathogens exhibit enhanced ability to invade epithelial cells, an important aspect in pathogenicity. With their ability to grow in biofilms, especially over catheter surfaces they become highly resistant to most antibiotics leading to chronic or persistent UTIs [7]. With an increasing difficulty in treating such recalcitrant infections, there has been a growing interest in alternative therapies.

QS inhibition has been considered a potential alternative antipathogenic therapy. It has been demonstrated to be an efficient anti-infective technique in several host-microbe systems. Unlike conventional antibiotics that exert selective pressure for resistance, quorum sensing inhibitors (QSIs) disrupt bacterial communication without killing the bacteria directly, thereby reducing virulence while minimizing resistance development [8]. Actinobacteria, a prolific source of bioactive compounds, have historically contributed to the discovery of antibiotics such as rifamycin, erythromycin, vancomycin, and gentamycin [9] [10]. While much research has focused on well-known genera like *Streptomyces*, rare actinobacteria remain underexplored despite their potential for producing novel QS inhibitors.

This study aims to bridge this gap by isolating rare actinobacteria from diverse environmental sources and screening them for anti-QS activity. Using indicator organisms such as *Chromobacterium violaceum* and *Serratia marcescens*, we assessed QS inhibition, while anti-swarming activity and anti-biofilm activity was evaluated against UTI pathogens like *Proteus mirabilis* and *Pseudomonas aeru-*

ginosa. By exploring the untapped potential of rare actinobacteria, this research seeks to identify novel QSIs that could serve as effective alternatives or adjuncts to traditional antibiotics in the fight against MDR bacterial infections.

2. Materials & Methods

2.1. Chemicals and Media

All chemicals and solvents used in the study were of analytical grade and were purchased from Merck, Germany. The culture media used for bacterial growth and isolation were obtained from Hi-media, Mumbai, India.

2.2. Microorganisms and Media

The test strains used for screening antimicrobial activity were obtained from the Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India and NCMR, NCCS, Pune. The strains used were *Bacillus subtilis* MTCC 441, *Proteus mirabilis* 425, *Pseudomonas aeruginosa* MTCC 424, *Serratia marcescens* MTCC 86, *Chromobacterium violaceum* MCC 4212 and *Candida albicans* MTCC 227.

In addition, clinical strains of *Proteus mirabilis* and *Pseudomonas aeruginosa* were obtained from Vishakha Clinical & Microbiological Lab, Nagpur. All strains were routinely maintained on nutrient agar slants at 4˚C. An overnight liquid culture of the organisms in Luria Bertani (LB) broth was used in all experiments.

Swarm Agar:

- *Proteus mirabilis*: LB broth solidified with 1.5 g% agar.
- *Pseudomonas aeruginosa*: LB broth supplemented with 0.5 g% casein hydrolysate, 0.5 g% glucose, and solidified with 0.5 g% agar.

2.3. Isolation of Actinobacteria from Environmental Samples

2.3.1. Collection of Environmental Samples

Soil samples were collected from various sites at depths of 3 - 5 cm below the surface. The samples were carefully placed in sterile polyethylene bags, sealed tightly, and stored at 4˚C until further processing. Additionally, water samples were collected from different water bodies and stored in sterile bottles until they were needed for analysis [11]. The sample collection was conducted over a period of May 2022 to December 2023.

2.3.2. Treatment of Soil Samples

Approximately 1 g of each collected soil sample was suspended in 10 ml sterile distilled water, and from this suspension, three dilutions (10^{-2} to 10^{-4}) were prepared. Each dilution was divided into aliquots, which were subjected to different treatments to isolate actinobacteria. The treatments applied included 1.5% phenol treatment, 0.3% Chloramine T treatment, Benzethonium chloride treatment, Heat treatment at 120˚C and a combination of the above treatments [12] [13].

After treatment, the aliquots were plated on various isolation media to promote the growth of actinobacteria. The media used for isolation included Starch Casein

Agar (SCA), Humic Acid Vitamin Agar (HVA), Potato Dextrose Agar (PDA), Starch M-Protein Agar (SPA) and Actinomycetes Isolation Agar (AIA) [14].

For selective isolation of actinobacteria, the media were supplemented with antibiotics such as nystatin, nalidixic acid, gentamycin, kanamycin, and others, to inhibit the growth of non-actinobacterial microorganisms and ensure the successful isolation of actinomycete genera [15].

2.4. Characterization of the Isolates

To study the macroscopic features of the isolated strains, they were streaked onto Potato Dextrose Agar (PDA) plates and incubated at 28˚C for 7 days. The resulting colonies were examined for various morphological characteristics, including colony size, consistency, shape, elevation, margins, color of aerial/substrate mycelium and pigments diffusing into the medium. These characteristics were observed both visually and under a microscope [16].

To further characterize the isolates, the colonies were picked and teased to separate the mycelium. The mycelial samples were then subjected to Gram staining. The stained slides were examined under high-power magnification, and the morphological characteristics, such as shape, size, and arrangement of cells, were recorded [17] [18]. This approach allowed for the classification of the isolates based on their microscopic and cultural characteristics.

2.5. Production and Extraction of Bioactive Compounds

The extract was obtained using liquid-liquid extraction. The isolates were cultured in Potato Dextrose Broth (PDB) as shake flask cultures for 10 days at 130 rpm and 28˚C. After the incubation period, the cultures were centrifuged at 7800 ×g for 15 min. The supernatant was mixed with ethyl acetate (1:1 v/v) and shaken at 150 rpm for 24 h to recover the crude bioactive compounds. The extracts were collected and evaporated using a vacuum concentrator. The resulting crude extracts were stored at −20˚C in a deep freezer for further analysis [19].

The use of Ethyl acetate as an extraction solvent is recommended because of its medium polarity and minimum toxicity on test strains. Many biological compounds (polar and non-polar) can be extracted and evaluated for their bioactivities [20].

2.6. Antimicrobial Activity by Agar Well Diffusion Method

The actinobacterial extracts were resuspended in dimethyl sulfoxide (DMSO) to prepare stock solutions with concentrations of 1 mg/ml, which were then used to assess antimicrobial activity using the agar well diffusion method.

For antimicrobial testing, 15 ml of Mueller-Hinton agar was poured into sterile Petri dishes, followed by inoculation with the test bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. For antifungal activity testing, Potato Dextrose Agar (PDA) was used, with *Candida albicans* as the test organism.

Wells were created in the agar, and 50 µl of each extract was introduced into the wells. The plates were incubated at 37˚C for 24 hours for bacterial growth and at 28˚C for 48 hours for yeast growth. The diameter of the zones of inhibition surrounding the wells was measured to determine the antimicrobial activity.

Each experiment was repeated three times to ensure reproducibility, and the mean zone of inhibition was calculated. Control wells containing 50 µl DMSO were included in each experiment to rule out any inhibitory effect of the solvent on the test organisms.

2.7. Pigment Quenching Assay

The quorum sensing inhibition (QSI) activity of the bioactive extracts was evaluated by studying pigment quenching using the agar well diffusion method with *Serratia marcescens* and *Chromobacterium violaceum* as the indicator organisms.

To perform the assay, agar plates were prepared by inoculating the respective indicator strains onto Luria Bertani (LB) agar. Wells were created on the agar plates, and 50 µl of the bioactive extracts were added to the wells.

After incubation at 28˚C for 24 - 48 hours, the presence or absence of pigmentation around the well was observed. A positive QSI result was indicated by the lack of pigmentation in the vicinity of the well, suggesting that the bioactive extract was able to inhibit the quorum sensing mechanism of the indicator strain. In contrast, a negative result was indicated by no inhibition of pigmentation, and the indicator organism showed its characteristic pigmentation [11] [21] [22]. The zones of pigment inhibition surrounding the wells were measured and recorded to assess the extent of anti-QS activity. p-nitrophenyl glycerol (PNPG) was used as a standard anti-quorum sensing compound [23] [24].

2.8. Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) of extracts was determined using the broth microdilution method [25]. *Proteus mirabilis* and *Pseudomonas aeruginosa* cultures were exposed to extracts supplemented in the medium to a final concentration of 1 to 200 µg/ml and incubated overnight. After 24 h of incubation at 37 ˚C, MIC was calculated as the lowest concentration of compound that inhibited visible growth of *Proteus mirabilis* and *Pseudomonas aeruginosa* as well as read at 600 nm. Each experiment was repeated in triplicates, and the average MIC value was calculated for each extract.

2.9. Anti-Swarming Assay

To evaluate the effect of potent anti-QS compounds from actinobacteria on the swarming motility of *Proteus mirabilis* and *Pseudomonas aeruginosa*, swarm agar plates were prepared for each bacterial species. Extracts were incorporated into the agar plates at appropriate concentrations, based on previous screening results. Two control plates were included for each bacterial strain, one containing no additives and termed positive control and the other containing DMSO and termed

solvent control. A 2 µl aliquot of overnight culture of each bacterial strain was centrally inoculated onto the swarm agar plate. All the plates were incubated at 37˚C for 20 h. Thereafter, the diameter of the swarm zone was measured and compared to the control and percent reduction was calculated [8] [26]. Each experiment was conducted in triplicate, and the average percent reduction was calculated for each extract.

2.10. Molecular Identification of Potent Actinobacteria

The 16S rRNA gene of potent actinobacteria was amplified with Actino specific forward Primer—5'-CCTAACACATGCAAGTCGA-3' and Actino Specific Reverse primer—5'-CGTATTACCGCGGCTGCTGG-5'. The reaction mixture contained 0.2 mM of each dNTP, 0.5 % (v/v) DMSO, 1 mM of degenerate primers, 12.5 ng of genomic DNA and 0.6 units of Ex-Taq Hot Start Version in a final volume of 25 µl of 1 × Ex-Taq PCR buffer. PCR reaction was started with denaturation at 94˚C for 5 min followed by a total of 25 cycles that consisted of denaturation at 94˚C for 30 sec, annealing at 53˚C for 30 sec and extension at 72˚C for 1 min. The reaction was terminated with a final extension at 72˚C for 5 min [27]. The size of PCR products was around 1500 bp in length.

The amplified DNA fragment was separated on 1% agarose gel, eluted from the gel and purified using gel extraction kit. The purified PCR product was sent for sequencing to Barcode BioSciences Pvt. Ltd. or Eurofins India, Bangalore.

2.11. Bacterial Growth Assay

To study the effect of rare actinobacterial extracts on growth of Proteus mirabilis, growth was monitored turbidometrically for 48 hours in LB broth in the presence of extracts at a concentration of 5 µg/ml in liquid culture at 37˚C. A solvent control was also set up and both were compared to a positive control [8] [14] [28].

2.12. Biofilm Inhibition Assay

The biofilm formation of *Proteus mirabilis* and *Pseudomonas aeruginosa* in the presence of rare actinobacterial extracts (2.5 - 20 µg/ml) was evaluated using the microtiter plate assay. Treated and untreated cultures of *Proteus mirabilis* and *Pseudomonas aeruginosa* in LB broth were incubated overnight at 37 ˚C. Free cells were removed and biofilms were washed three times with sterile PBS and fixed with 99% (v/v) methanol. The adhered cells were stained with 0.2% (w/v) crystal violet. The additional crystal violet was removed by washing with distilled water. Finally, crystal violet was solubilized using ethanol for 20 min and the absorbance was measured at OD_{590} nm [29] [30].

2.13. Prodigiosin Assay (for *S. marcescens*)

Overnight culture of *S. marcescens* was used for development of assay. 1% of broth culture was inoculated in sterile nutrient broth tubes containing different concentrations of extracts (5 - 25 µg/ml) and incubated overnight. After incuba-

tion the cells were pelleted by centrifugation at 10,000 rpm for 10 minutes and resuspended in acidified ethanol solution (96 ml of ethanol containing 4% of 1 M HCl) for extraction of prodigiosin. The extracted prodigiosin was determined by reading absorbance at 534 nm and percent inhibition of prodigiosin production was calculated [31].

Simultaneously, Relative Prodigiosin production was determined by determining the ratio of the absorbance of the extracted prodigiosin solution at 534 nm to the turbidity of the culture suspension as optical density at 600 nm. The effects of inhibitors were evaluated using the relative prodigiosin production (A534/optical density at 600 nm), for which the control value was 100% [32] [33].

2.14. Statistical Analysis

All the experiments were performed in triplicates and the results were expressed in the form of mean ± standard deviation (SD). Student's t-test was performed using SYSTAT Software (Systat Software, Inc., Chicago, IL, USA). P value ≤ 0.05 was considered significant unless otherwise mentioned.

3. Results

3.1. Isolation of Actinobacteria from Environmental Samples

A total of 192 putative actinobacterial isolates were successfully obtained by employing different soil treatment methods, selective media, and cultivation conditions. Among the various sampling sites, the highest number of isolates was obtained from the Melghat forest (37 isolates), followed by Bhushi Dam (30 isolates) and Palolem Beach (30 isolates). A summary of the sampling sites and their respective locations is provided in Table 1.

Table 1. Location of soil samples collection sites.

Sampling Site	Location	Type of environment	No. of isolates
Bhushi Dam	18˚43'21.86"N 73˚23'47.26"E	Dam	30
Melghat	21˚26'45"N 77˚11'50"E	Forest	37
Malgund	17˚9'48"N 73˚16'7"E	Marine	27
Guhagar	17˚29'21"N 73˚11'34"E	Marine	10
Palolem Beach	15˚01'35"N 74˚01'60"E	Marine	20
Karla	18˚78'36"N 73˚46'99"E	Mountain soil	8
Chikhaldhara	21˚24'30"N 77˚19'42"E	Forest	15
Katol	21˚27'33"N 78˚58'60"E	Farm	14
Vena Lake	17˚56'3"N 73˚39'54E	Lake sediment	9
Pratapgarh Fort	25.8971˚N 81.9409˚E	Mountain	22

Among the different soil treatment methods, 1.5% phenol treatment of various soil suspensions resulted in the highest number of colonies, yielding a total of 73 colonies. These were primarily obtained on Potato Dextrose Agar (PDA) supplemented with gentamycin, followed by Starch Casein Agar (SCA) with the same antibiotic. Heat treatment at 120˚C produced 47 colonies, with 34 colonies growing on PDA and 13 on AIA supplemented with nalidixic acid. Air-dried soil samples yielded 21 colonies, 15 of which were on PDA with gentamycin, and 6 colonies were obtained on AIA supplemented with streptomycin. Benzethonium chloride treatment resulted in the isolation of 43 colonies. The effect of pretreatment on isolation of rare actinobacteria is depicted in Figure 1.

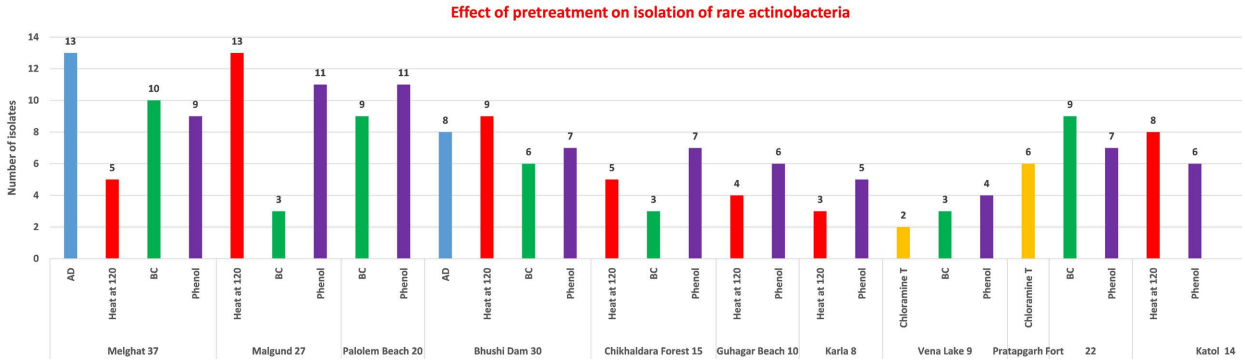

Figure 1. Effect of pretreatment on isolation of rare actinobacteria from soil samples.

3.2. Characterization of Actinobacterial Isolates

A total of 192 actinomycete strains were identified based on distinct differences in colony characteristics, including color, colony size, filamentous structure, mycelial growth, and pigment production. Among the 192 isolates, 125 (65.1%) exhibited distinctive pigment production on the reverse side of the colonies, while 80 (41.6%) produced soluble pigments.

The isolates were categorized based on their colony colors, and four main color classes were observed, with some intergradation between classes. The predominant colors were white (85 isolates, 44.3%), brown (42 isolates, 21.8%), yellow (29 isolates, 15.2%), orange (22 isolates, 11.5%), and gray (14 isolates, 7.2%). Figure 2 showcases the colony characteristics of a few actinobacterial isolates.

3.3. Antimicrobial Activity by Agar Well Diffusion Method

The antimicrobial activity of rare actinobacterial extracts was assessed using the agar well diffusion method against a variety of test organisms: *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. Additionally, the resistance profiles of these test organisms to commonly used antibiotics were evaluated using the disc diffusion method.

Among the test organisms, *Bacillus subtilis* was found to be sensitive to all antibiotics tested. In contrast, *Proteus mirabilis* exhibited resistance to tetracycline and vancomycin, and a clinical isolate of *Proteus mirabilis* demonstrated re-

sistance to multiple antibiotics, including tobramycin, trimethoprim, penicillin, tetracycline, and vancomycin. *Staphylococcus aureus* showed resistance to tetracycline, vancomycin, and trimethoprim, while *Pseudomonas aeruginosa* was resistant to tetracycline and vancomycin.

Following the antibiotic resistance screening, the organic extracts of all actinobacterial isolates were tested for antimicrobial activity against the aforementioned pathogens. Among the isolates, 60% exhibited activity against *Bacillus subtilis*, while 10% were active against *Staphylococcus aureus*, 12% against *Proteus mirabilis*, and only 10% against *Pseudomonas aeruginosa*. Additionally, 30% of the actinobacterial extracts showed antifungal activity against *Candida albicans* (Figure 3).

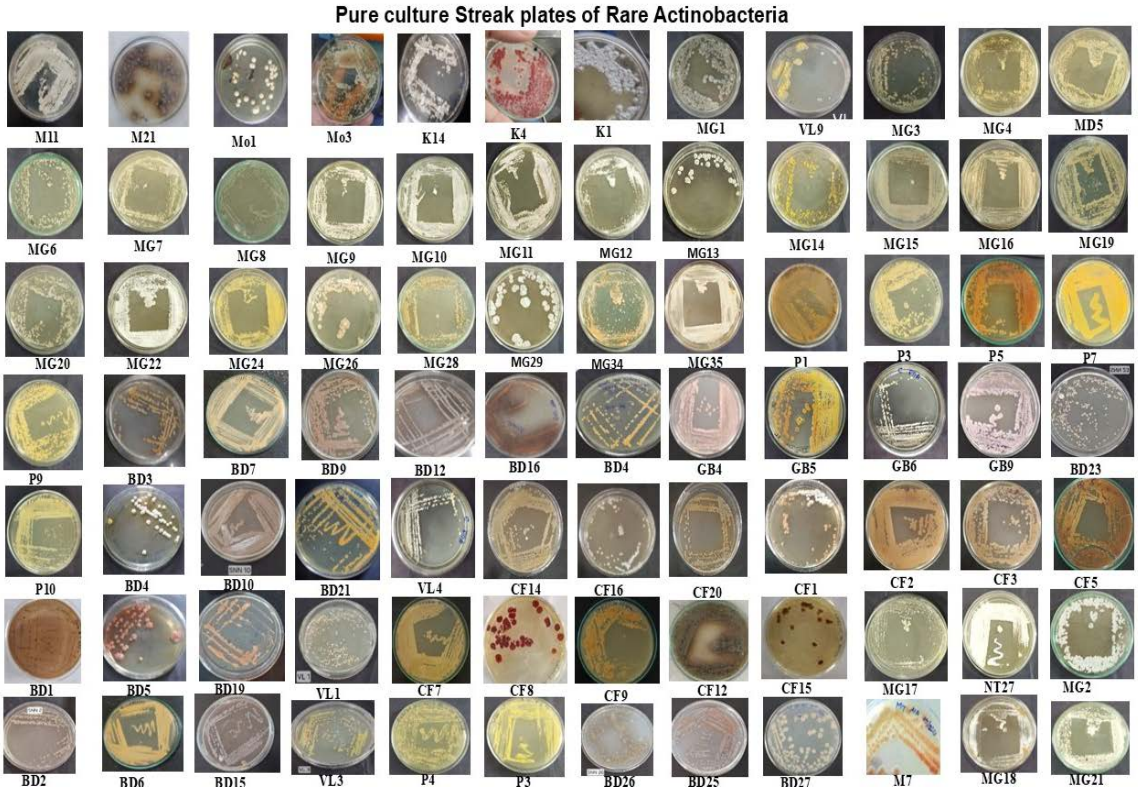

Figure 2. Colony characteristics of pure cultures of actinobacterial isolates.

3.4. Pigment Quenching Assay

Further investigation was conducted to assess the anti-quorum sensing (QS) activity of the actinobacterial extracts by testing them for pigment inhibition against *Serratia marcescens* and *Chromobacterium violaceum*. A significant proportion of the isolates demonstrated anti-QS activity, with 66% of the actinobacterial extracts inhibiting pigment production in *Serratia marcescens*. Additionally, 53% of the isolates exhibited anti-QS activity against *Chromobacterium violaceum*.

These findings suggest that a substantial number of the actinobacterial extracts possess potential anti-QS properties, indicating their capacity to interfere with

bacterial communication and virulence.

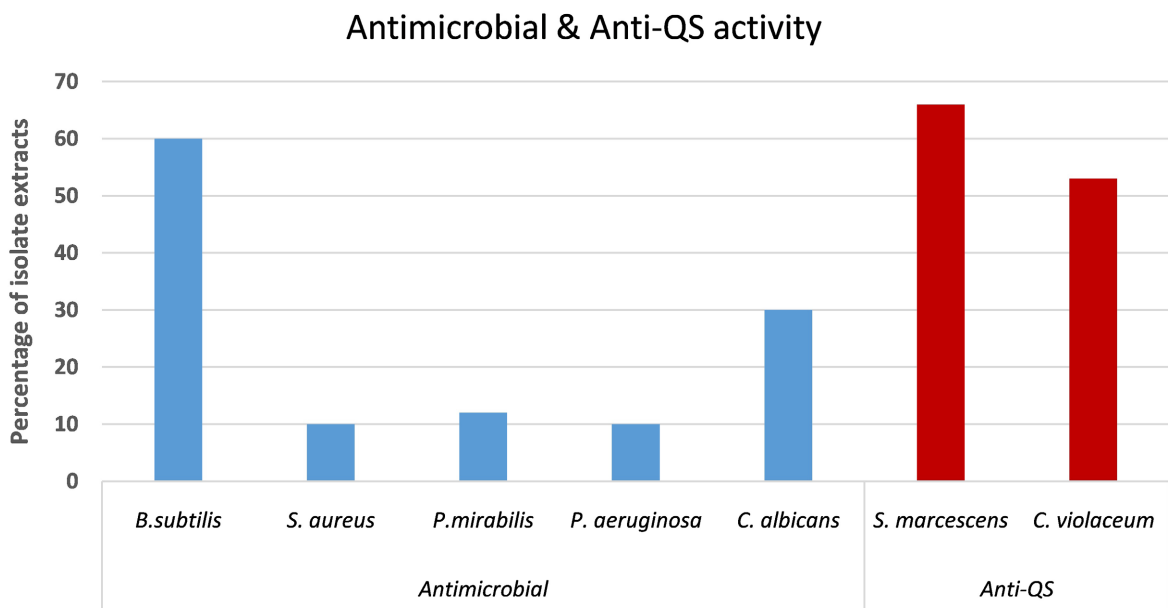

Figure 3. Antimicrobial and anti-quorum sensing activity of actinobacterial extracts.

3.5. Anti-Swarming Assay

The anti-swarming activity of thirty-five potent actinobacterial extracts was evaluated against *Proteus mirabilis* (clinical isolate) and *Pseudomonas aeruginosa*. Out of these, twenty-six extracts demonstrated significant inhibition of swarming behaviour compared to the control. The potency of the extracts varied, with a reduction in the diameter of the swarm zone ranging from 10% to 70% in *Proteus mirabilis* and 10% to 60% in *Pseudomonas aeruginosa*. Notably, extracts from BD2, BD3, BD10, BD16, BD21, BD24, MG1, MG13, MG22, MG23, MG30, P5, CF20, K6, and VL9 showed substantial inhibition (**Figure 4**). The anti-swarming activity of a few actinobacterial extracts against *Proteus mirabilis* is shown in **Figure 5** and against *Pseudomonas aeruginosa* is shown in **Figure 6**.

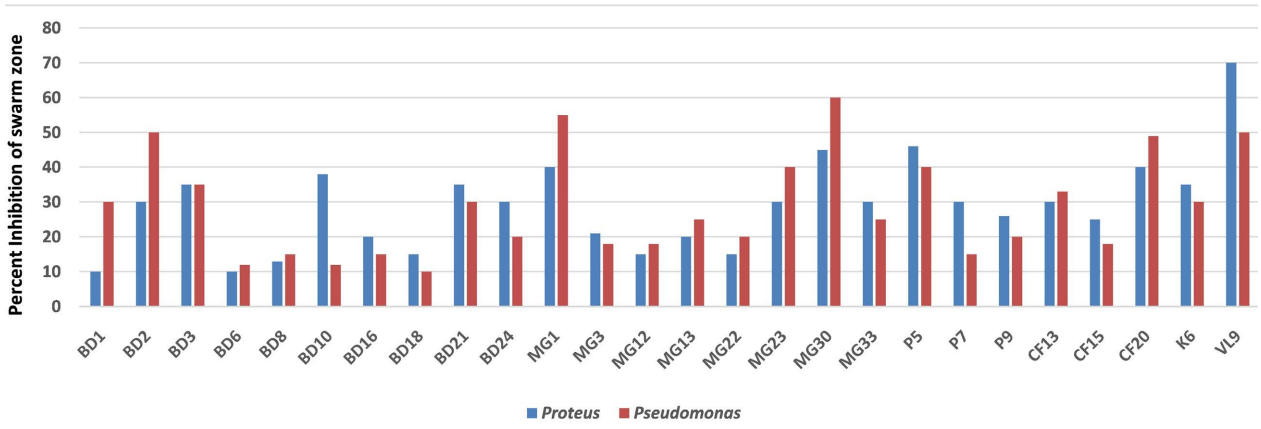

Figure 4. Anti-swarming activity of actinobacterial extracts.

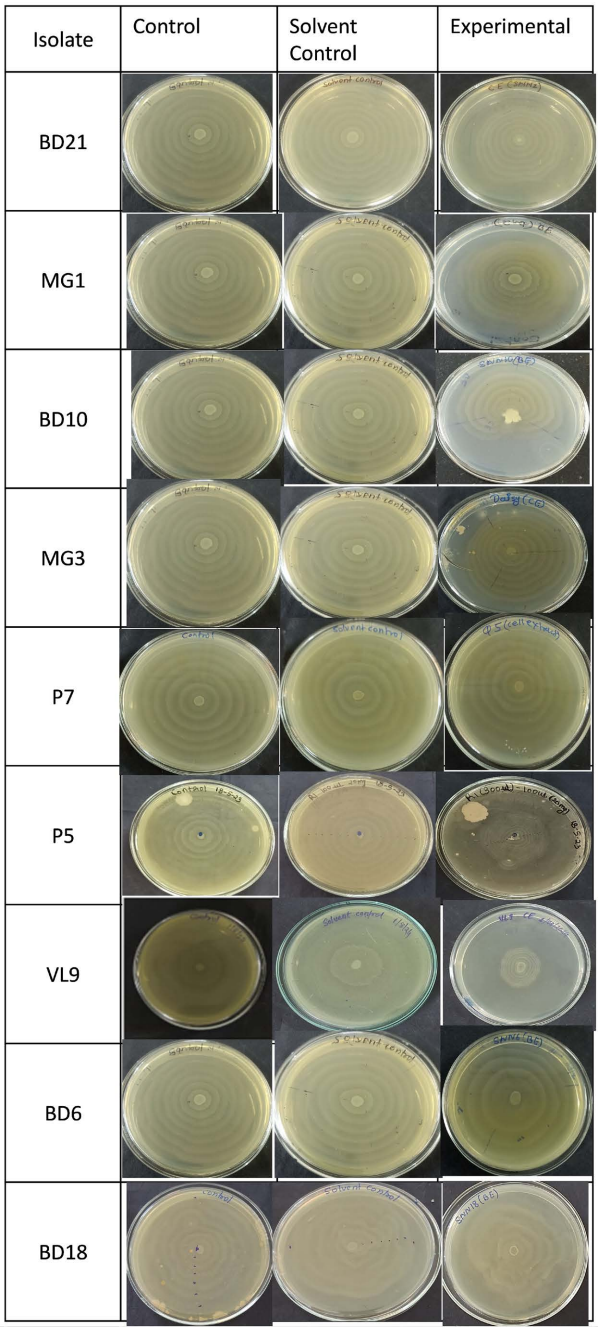

Figure 5. Anti swarming activity of actinobacterial extracts against *Proteus mirabilis*.

Of particular interest, VL9 exhibited the highest inhibition (70%) against *Proteus mirabilis*, while MG30 and MG1 showed 60% and 55% inhibition, respectively, against *Pseudomonas aeruginosa*. These results highlight the potential of these actinobacterial extracts in modulating bacterial motility, likely by interfering with quorum sensing (QS)-mediated behaviours. Furthermore, the extracts inhibited swarming in a dose-dependent manner, as observed with MG1 and VL9, reinforcing their potential as effective modulators of bacterial motility.

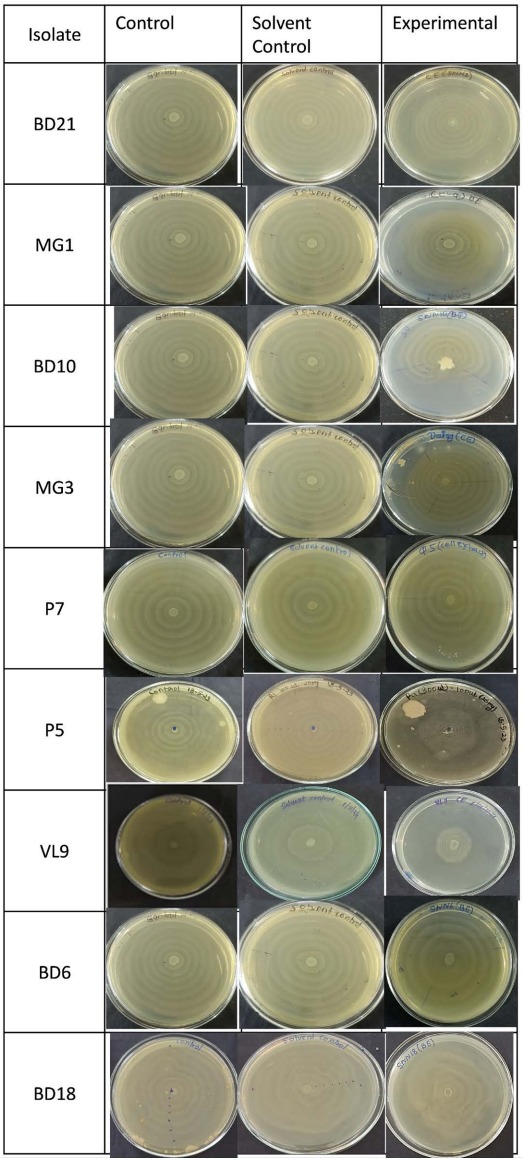

Figure 6. Anti-swarming activity of actinobacterial extracts against *Pseudomonas aeruginosa*.

3.6. Identification of Potent Isolates

Nineteen potent isolates were identified on the basis of 16S rRNA gene sequencing. The sequences have been submitted in Genbank and accession numbers obtained as shown in Table 2.

3.7. Minimum Inhibitory Concentration (MIC)

MG1, BD21, MG3, and VL9, identified as rare actinobacterial species, were selected for further studies based on their demonstrated antibacterial activity. Among these, MG1 exhibited notable activity against both *Proteus mirabilis* and *Pseudomonas aeruginosa*, prompting the determination of their Minimum Inhibitory Concentration (MIC) to further explore their anti-infective properties. The MIC

values for all four extracts were found to be 200 µg/ml for *Proteus mirabilis* and 400 µg/ml for *Pseudomonas aeruginosa*. These MIC values were used as a reference for selecting sub-MIC concentrations to evaluate the extracts' potential to modulate biofilm formation and quorum sensing in UTI pathogens.

Table 2. Identification & bioactivities of Actinobacteria.

Isolate	Closest Cultivated species	GenBank Accession Number	No. of nucleotides sequenced	Query cover	Similarity	Anti-swarming activity (Percent reduction of swarm zone)		Pigment Inhibition (Zone of pigment inhibition)	
						Proteus mirabilis	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Chromobacterium violaceum*
BD24	*Amycolatopsis tucumanensis* strain ABO	PV258785	1030	99	97.46	++	+	++	++
MG1	*Kutzneria viridogrisea* strain MCRL 0044	PV262340	1113	98	95.32	++	+++	+++	++++
BD 2	*Streptomyces abikoensis* NBRC 13860(T)	AB184537	1509	98	99.33	++	+++	++	++
MG3	*Amycolatopsis thermoflava* strain N1165	PV257661	1157	96	96.95	++	++	+++	++
K 6	*Amycolatopsis orientalis* strain KSI 153	PV258786	1063	99	97.34	++	++	++	++
BD21	*Microbacterium barkeri*	PV262557	630	100	100	++	++	++	++
BD16	*Amycolatopsis* sp. HW47	PV290304	1048	90	95.78	+	+	++	++
MG13	*Nocardia yamanashiensis* strain W8187	PV249100	1042	99	99.71	+	+	++	++
MG18	*Streptomyces erumpens strain* DSM 40941	NR_114917.1	1003	100	99.01	+	+	++	++
MG22	*Streptomyces sclerotialus strain* W25	PV252810	818	99	99.90	+	+	++	+++
MG23	*Streptomyces cellulosae strain* MJM7075	PV252811	919	99	99.89	++	++	++	++
BD10	*Streptomyces bungoensis DSM* 41781(T)	KQ948892	1385	98	99.63	++	+	++	++
P5	*Streptomyces salinarius* SS06011(T)	LC430995	1273	100	99.29	++	++	++	++

Continued

MG33	*Streptomyces tuirus* NBRC 15617(T)	AB184690	1208	100	*99.83*	++	+	+++	++
MG30	*Amycolatopsis japonica* MG417-CF17(T)	CP008953	1182	100	*99.45*	++	+++	++	++
BD6	*Amycolatopsis thermoflava* N1165(T)	KI421511	1511	99	*99.79*	+	+	++	++
VL9	*Yuhushiella* sp.	PV262564	980	100	*100*	+++	+++	++	++
BD13	*Nocardia nova* NBRC 15556(T)	BDBN01000167	1439	100	99.93	++	+	++	+
P7	*Streptomyces ardesiacus* NRRL B-1773(T)	DQ026631	1435	100	99.58	++	+	++	++

Anti-swarming activity: + (0 - 1 cm); ++ (1 - 2 cm); +++ (2 - 3 cm); ++++ (3 and above); Pigment inhibition activity: + (10% - 25%); ++ (25% - 50%); +++ (50% - 75%).

3.8. Effect of Rare Actinobacterial Extracts on the Growth of *Proteus mirabilis*

MG1, BD21, MG3, and VL9, identified as rare actinobacterial species, were selected for further studies based on their observed activity. The swarming motility assay demonstrated that the rare actinobacterial extracts did not exhibit microbiocidal effects on *Proteus mirabilis*. The extracts did not kill or inhibit bacterial growth under the experimental conditions, despite effectively inhibiting quorum sensing-related behaviours such as swarming motility.

To confirm their non-toxic nature, the growth of *Proteus mirabilis* was monitored over a 48-hour period in the presence of the extracts (5 µg/ml), comparing it to a solvent control and a positive control. The results showed that the extracts had no significant inhibitory effect on the bacterial growth rate. *Proteus mirabilis* reached similar cell densities after 48 hours in the presence or absence of either the solvent or the extracts, at concentrations that significantly inhibited swarming behaviour (**Figure 7**). Therefore, the inhibitory effects observed in QS-related behaviours are not due to toxicity or growth inhibition. Instead, the extracts appear to specifically target QS pathways involved in regulating motility and other virulence factors, without affecting overall bacterial growth.

3.9. Biofilm Inhibition Assay

MG1, BD21, MG3 and VL9 actinobacterial extracts demonstrated biofilm inhibition activity against both *Proteus mirabilis* and *Pseudomonas aeruginosa*.

For *Proteus mirabilis*, biofilm inhibition increased with higher extract concentrations, except for the MG3 extract, where the inhibition remained consistent between 43% - 45% at concentrations ranging from 10 µg/ml to 20 µg/ml. At 10 µg/ml (1/20 MIC), substantial inhibition was observed, with MG1, BD21, MG3, and VL9 showing inhibition rates of 32.2%, 27.5%, 43.6%, and 33.4%, respectively (**Figure 8**).

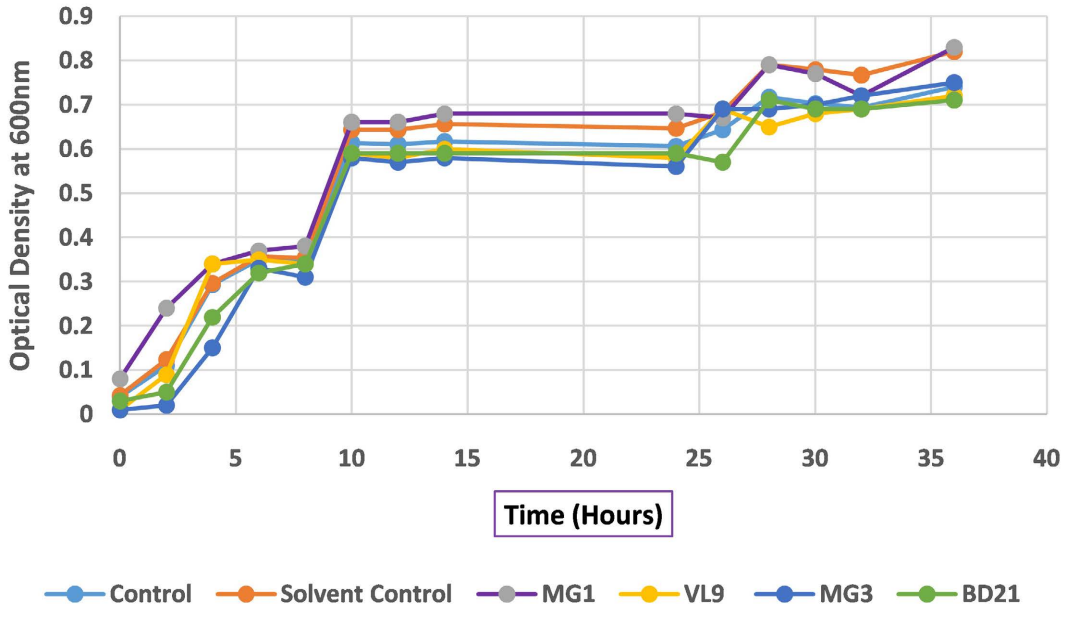

Figure 7. Effect of rare actinobacterial extracts on the growth of *Proteus mirabilis*.

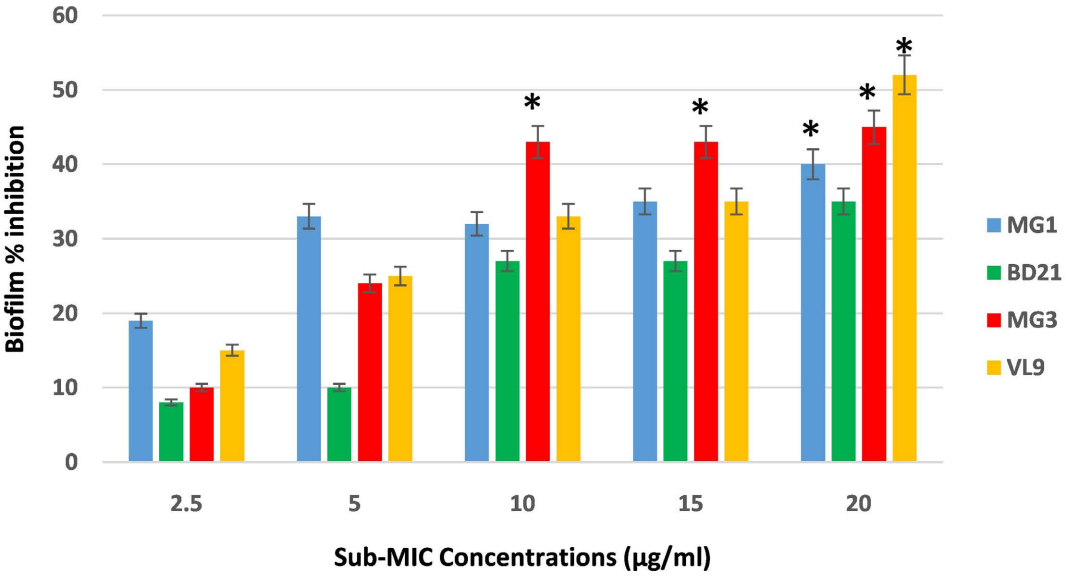

Figure 8. Biofilm percent inhibition against *Proteus mirabilis*. Data expressed as mean ± SD (n = 3); *p ≤ 0.03.

In the case of *Pseudomonas aeruginosa*, biofilm inhibition was more pronounced across all concentrations. At 2.5 µg/ml (1/80 MIC), biofilm inhibition ranged from 20% to 40%, while at 20 µg/ml (1/10 MIC), the inhibition increased to 55% to 65%. At 10 µg/ml, the highest inhibition was observed with BD21 (51.2%), followed by VL9 (40.7%), MG3 (38.5%), and MG1 (36.3%) (Figure 9). ANOVA on ranks was carried out against pH control group and the actinobacterial extracts were found to have a significant effect on biofilm inhibition at a p value of 0.03 for *Proteus mirabilis*, and p value of 0.01 for *Pseudomonas aeruginosa*.

Interestingly, at both the lowest and highest concentrations, all extracts showed more biofilm inhibition against *Pseudomonas aeruginosa* compared to *Proteus mirabilis*. Both *Proteus mirabilis* and *Pseudomonas aeruginosa* are known to form biofilms through quorum sensing-controlled mechanisms, which play a crucial role in their ability to adhere to bladder cells and indwelling catheters. The statistically significant reduction in biofilm formation by both *Proteus mirabilis* ($p < 0.03$) and *Pseudomonas aeruginosa* ($p < 0.01$) in the presence of rare actinobacterial extracts further supports the presence of anti-QS compounds within these extracts.

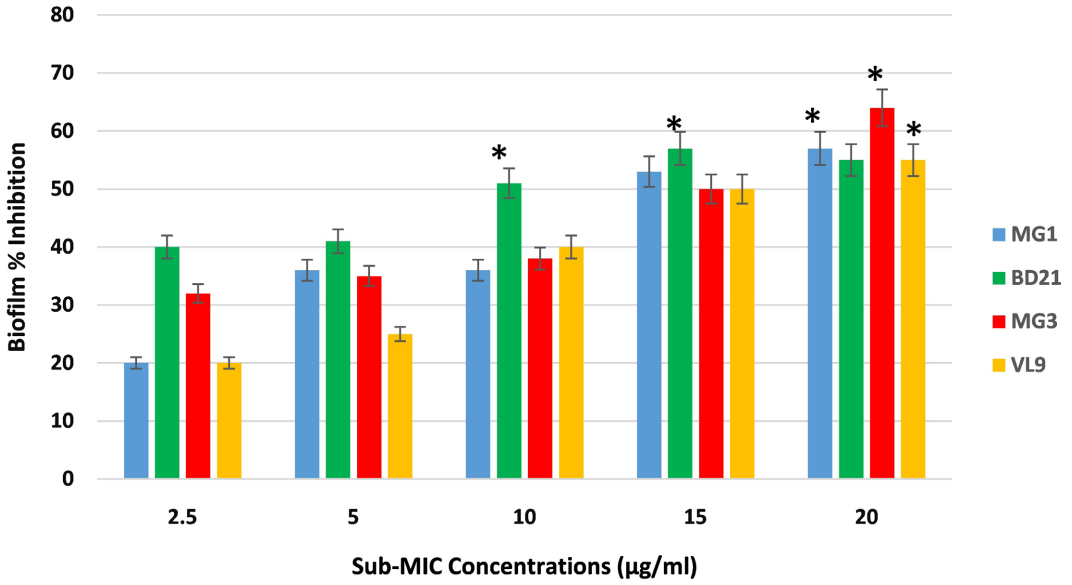

Figure 9. Biofilm percent inhibition against *Pseudomonas aeruginosa*. Data expressed as mean ± SD (n = 3); *$p \leq 0.01$.

3.10. Prodigiosin Assay

The production of prodigiosin, a red pigment synthesized by *Serratia marcescens*, is regulated by quorum sensing. In this study, the effect of rare actinobacterial extracts on prodigiosin production was assessed. A concentration-dependent reduction in prodigiosin pigment production was observed as the concentration of the actinobacterial extracts increased. However, at higher concentrations, certain extracts, specifically BD21 and VL9, showed a reversal of inhibition, with an increase in prodigiosin production. This effect was consistent with the pigment inhibition assay conducted using the agar well diffusion method, where higher concentrations of the extracts led to enhanced pigmentation.

Maximum inhibition of prodigiosin production was observed at 10 µg/ml for all extracts, with the following inhibition percentages: MG1 (62.5%), BD21 (46.7%), MG3 (76.7%), and VL9 (35.6%) (**Figure 10**). There was a significant inhibition as compared to control for MG1 and MG3 ($p < 0.001$) and for BD21 and VL9 ($p < 0.05$).

To further assess the impact of the extracts, the relative prodigiosin production per cell was calculated at various concentrations. At 10 μg/ml, a significant reduction in prodigiosin production per cell was observed compared to the control group. For MG3, the relative prodigiosin production per cell decreased from 64.3 in the control group to 17.5 at 10 μg/ml of extract ($p < 0.0001$). Similarly, in the presence of VL9 extract at 20 μg/ml (1/10 MIC), the prodigiosin production decreased to 15.6 relative units per cell, compared to 43.5 for the control ($p < 0.0001$). For BD21, the lowest prodigiosin production (8.82 relative units per cell) was observed at 15 μg/ml, compared to 25.07 in the control group ($p < 0.005$). Finally, for MG1, the prodigiosin production dropped to 7.89 relative units per cell at 10 μg/mL, compared to 17.78 in the control ($p < 0.0001$) (Figure 11).

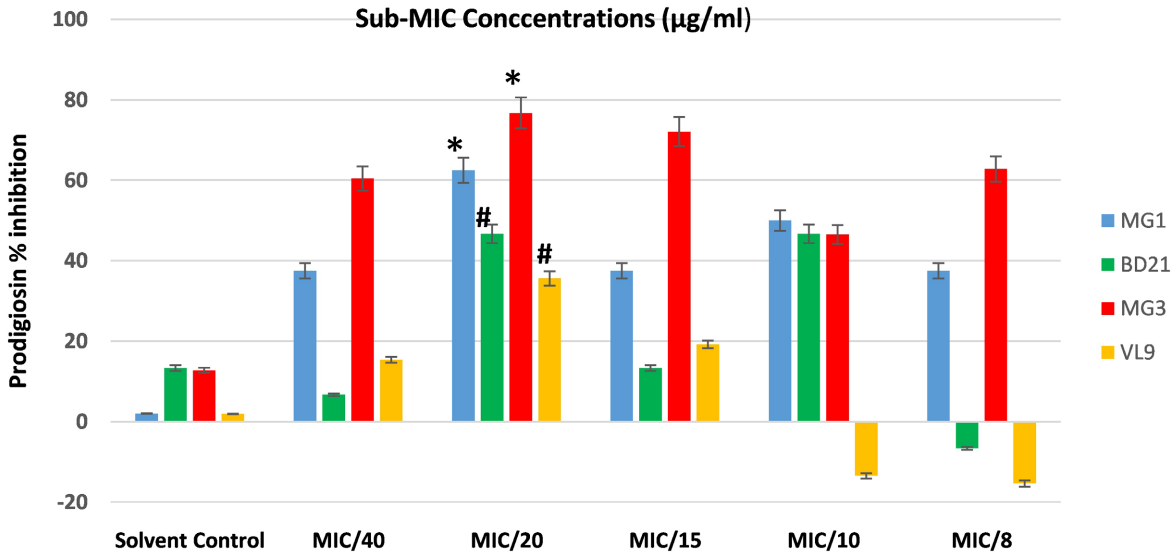

Figure 10. Prodigiosin percent inhibition against *Serratia marcescens*. Data expressed as mean ± SD (n = 3). *$p < 0.001$; #$p < 0.05$ vs Solvent control.

4. Discussion

The rising global threat of antibiotic resistance has necessitated the exploration of alternative therapeutic strategies, among which, quorum sensing inhibition (QSI) has emerged as a promising approach to disrupt bacterial communication and combat the emergence of multidrug-resistant strains [34] [35]. As the search for new antimicrobial agents intensifies, the discovery of quorum sensing inhibitors (QSIs) from underexplored microbial sources, such as rare actinobacteria, represents a significant and exciting development in this field [36].

Actinobacteria are known to produce an extensive range of bioactive compounds, including antibiotics and enzymes, but much of this diversity remains untapped [37] [38]. Rare actinobacteria, in particular, are not as frequently studied as their more common counterparts, such as Streptomyces, and they represent an underexplored reservoir of bioactive agents [19]. In this context, the discovery of anti-QS compounds from these organisms is of high relevance.

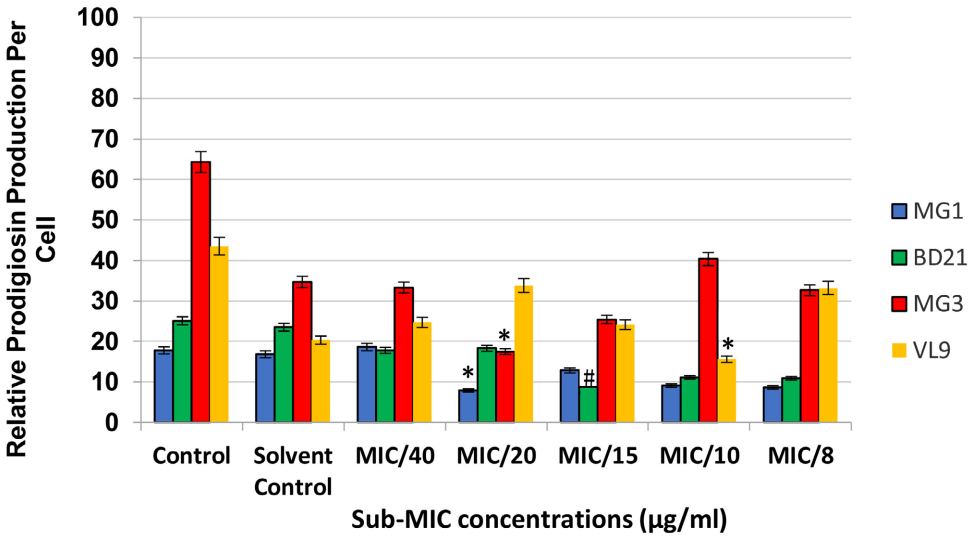

Figure 11. Relative prodigiosin production by *Serratia marcescens* in presence of rare actinobacterial extracts. Data expressed as mean ± SD (n = 3). $^{\#}p < 0.005$; $^{*}p < 0.0001$ vs Solvent control.

4.1. Isolation & Characterization of Actinobacteria from Environmental Samples

In the present study, the successful isolation of the rare actinobacterial strains was achieved through a combination of physical and chemical pretreatment methods, tailored to select for non-Streptomycete genera. Our study corroborates previous research, which has shown that pre-treatments such as phenol, air-drying, dry heat, and the use of selective antibiotics can be effective in isolating rare actinobacteria [11] [12] [39]. The phenol treatment method, for example, yielded approximately 38% of the total isolates, with *Amycolatopsis*, *Kutzneria*, and *Yuhushiella* being among the most notable genera [40].

In addition, the use of different culture media maximizes the chances of isolating a wide array of actinobacterial strains, as different media can provide varied nutritional conditions that support the growth of diverse microbial species [41]. For marine samples, specific media like Starch M-Protein agar was used to mimic the natural saline environment, further enhancing the isolation of marine actinobacteria [9] [42]. Of the 57 colonies obtained from marine samples, 20 each were obtained on PDA and SCA and 17 on Starch M-Protein agar plates. The use of seawater-based media led to the successful isolation of isolate P5, identified as *Streptomyces salinarius* from Palolem Beach.

4.2. Antimicrobial Activity by Agar Well Diffusion Method

The bioactivity of actinobacteria is a critical factor in their potential to serve as sources of novel therapeutic compounds, particularly in the search for anti-quorum sensing (QS) agents. In this study, we observed that nearly 36% of the actinobacterial isolates displayed broad-spectrum bioactivity, showing both antibacterial and antifungal properties, with majority of them exhibiting activity primarily against Gram-positive bacteria (60%). This finding is consistent with pre-

vious reports highlighting the wide-ranging bioactive potential of actinomycetes [43] [44]. The relatively low antibacterial activity against *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Staphylococcus aureus* may be attributed to the antibiotic resistance mechanisms that these bacteria have developed over time.

4.3. Pigment Quenching Assay

We further focused on the pigment inhibition activity of rare actinobacteria and found that they exhibited significant anti-QS activity, as evidenced by their ability to inhibit pigmentation in indicator organisms, *Chromobacterium violaceum* (53%) and *Serratia marcescens* (66%). Both of these indicator organisms are known to produce pigments—violacein in *C. violaceum* and prodigiosin in *S. marcescens*—controlled by AHL-mediated QS systems.

Our results are quite promising in comparison to previous studies, which reported much lower rates of positive results for anti-QS activity in actinobacteria. For example, anti-QS activity of crude extracts from actinobacterial strains against *C. violaceum* was demonstrated by several researchers [22] [45] [46]. Further, in a study 56 actinobacterial strains were screened for pigment inhibition activity against *C. violaceum* and only 5 (approximately 9%) were found to be positive [47]. In contrast, the higher percentage of positive isolates in our study (53%) may be attributed to the diversity of actinobacterial strains isolated.

4.4. Anti-Swarming Assay

Swarming motility is a critical virulence factor for *Proteus mirabilis* and *Pseudomonas aeruginosa* causing urinary tract infections (UTIs). This form of collective bacterial movement on solid surfaces plays a crucial role in the pathogenesis of catheter-associated UTIs, as it contributes to biofilm formation and host tissue invasion [48].

In this study, several actinobacterial extracts demonstrated significant anti-swarming activity against *Proteus mirabilis* and *Pseudomonas aeruginosa*. In our study, MG1 exhibited the highest swarming inhibition, with 40% inhibition against *Proteus mirabilis* and 55% inhibition against *Pseudomonas aeruginosa*. This was followed by BD21 and MG3, which inhibited swarming by 35% in *Proteus mirabilis* and 30% - 35% in *Pseudomonas aeruginosa*. Interestingly, the most potent anti-swarming activity was observed in VL9, which showed significant inhibition against both UTI pathogens. To our knowledge, this is the first report of anti-swarming activity against these UTI pathogens by these rare actinobacterial genera.

In addition to the inhibition of swarming motility, we observed significant changes in the swarm patterns of both *Proteus mirabilis* and *Pseudomonas aeruginosa* in the presence of the actinobacterial extracts. In the case of *Proteus mirabilis*, there was a marked increase in the width of the individual swarm rings, coupled with a decrease in the number of rings. This pattern alteration is similar to that reported previously [49]. For *Pseudomonas aeruginosa*, a noticeable altera-

tion in the radiating tendrils was observed. The tendrils, which were thin and closely spaced in the control plates, became broader and more widely spaced in the presence of higher concentrations of the extracts.

The anti-swarming activity observed in this study is noteworthy when compared to the results of other studies on bacterial motility. Zhang *et al.* (2023) reported swarming inhibition by *Streptomyces* sp. at a concentration of 5 mg/mL, while Li *et al.* (2024) observed that *Nocardiopsis metallicus* inhibited the surface colonization of *C. violaceum* CV026 during swarming, with inhibition rates of 33.7% at 100 µg/mL and 37.8% at 150 µg/ml. In contrast, the actinobacterial extracts in this study demonstrated anti-swarming activity at much lower concentrations—specifically, at a final concentration of 5 µg/mL, which is approximately 1/40 of the minimum inhibitory concentration (MIC). This suggests that the rare actinobacteria identified in this study may produce highly potent compounds capable of inhibiting swarming at much lower concentrations and may help in preventing or treating catheter-associated UTIs.

4.5. Identification of Potent Isolates

The identification of the actinobacterial isolates was carried out through molecular methods, and despite the use of pretreatment methods of samples and selective media supplemented with antibiotics to isolate rare actinobacteria, only 60% of the identified strains were found to belong to rare genera. The most common genus identified was *Amycolatopsis*, a known producer of bioactive secondary metabolites [50].

4.6. Biofilm Inhibition Assay

Biofilm formation plays a very important role in the pathogenesis of UTI pathogens. It has been amply demonstrated that in the biofilm mode of growth, bacteria can cause chronic infections and the cells growing in such biofilms are resistant to phagocytes, antibodies and antibiotics [51]. These properties are responsible for the characteristic failure of the host defense system and antimicrobial chemotherapy to clear biofilm pathogens, especially in device related infections. Hence, compounds that inhibit biofilm formation will presumably be able to control or expedite clearance of such infections.

In this study, we evaluated the potential of rare actinobacterial extracts to inhibit biofilm formation by *Proteus mirabilis* and *Pseudomonas aeruginosa*. The minimum inhibitory concentrations (MICs) for the actinobacterial extracts against *Proteus mirabilis* and *Pseudomonas aeruginosa* were determined to be 200 µg/ml and 400 µg/ml, respectively. At sub-MIC concentrations, the extracts of *Kutzneria viridogrisea* (MG1), *Microbacterium barkeri* (BD21), *Amycolatopsis thermoflava* (MG3), and *Yuhushiella* sp (VL9) showed significant biofilm inhibition. Specifically, at 1/10 MIC for *Proteus mirabilis,* biofilm inhibition ranged from 35% to 52%. The highest inhibition (52%) was observed with *Yuhushiella* sp. (VL9). For *Pseudomonas aeruginosa*, at 1/20 MIC, biofilm inhibition ranged from 55% to

64%, with *Amycolatopsis thermoflava* (MG3) showing the greatest inhibition (64%).

The biofilm inhibition observed in this study compares favorably with previous research using actinobacteria extracts. This study represents the first report of rare actinobacterial genera exhibiting anti-biofilm activity against these UTI pathogens at such sub MIC concentrations. Biofilm inhibition of *Pseudomonas aeruginosa* by *Streptomyces rameus* crude extracts at MIC values ranging from 64 - 512 μg/mL have been reported. The biofilm inhibition achieved was 83.39%, 54.33%, and 15.56% at 1/2, 1/4, and 1/8 MIC, respectively [14]. In contrast, our study demonstrated comparable or even more potent biofilm inhibition at significantly lower concentrations—at 1/20 MIC for *Pseudomonas aeruginosa*, extracts showed 55% to 64% inhibition.

Li *et al.* (2024) evaluated the anti-biofilm activity of *Nocardiopsis metallicus* extracts against *C. violaceum* CV026, reporting biofilm reductions of 36.5% and 65.9% at concentrations of 100 μg/mL and 150 μg/mL, respectively. In a study by Mulya and Waturangi (2021), eight actinobacterial extracts were evaluated for their antibiofilm activity against *Bacillus cereus* and *Shewanella putrefaciens*, with the most potent extract showing an 89.60% reduction in Bacillus biofilm and a 93.06% reduction in Shewanella biofilm at a concentration of 20 mg/ml. The rare actinobacterial extracts in this study, however, achieved similar levels of inhibition at sub-MIC concentrations (1/10 and 1/20 MIC), which suggests that these rare actinobacteria genera might be more efficient at lower concentrations compared to other strains.

4.7. Prodigiosin Assay

The inhibition of prodigiosin production by *Serratia marcescens,* a well-established model for quorum sensing (QS) studies, offers a promising approach for exploring novel bioactive compounds with potential therapeutic applications [52]. In this study, we evaluated the effects of rare actinobacterial extracts on prodigiosin production in *Serratia marcescens*.

The results of the prodigiosin inhibition assay reveal that rare actinobacterial extracts can inhibit prodigiosin production in *Serratia marcescens* in a dose-dependent manner. Specifically, at lower concentrations, the extracts of *Kutzneria viridogrisea* (MG1), *Microbacterium barkeri* (BD21), *Amycolatopsis thermoflava* (MG3), and *Yuhushiella* sp. (VL9) showed varying degrees of inhibition. Notably, at a concentration of 0.005 mg/mL, the inhibition of prodigiosin production was found to be 37.5%, 6.67%, 60%, and 15.3% for MG1, BD21, MG3, and VL9, respectively. Interestingly, higher concentrations of the BD21 and VL9 extracts were associated with an increase in prodigiosin production, suggesting a potential dose-dependent response. It is possible that at higher concentrations, these extracts induce a stress response in *S. marcescens* that leads to increased prodigiosin production as a defense mechanism. Prodigiosin has been associated with various physiological roles, such as antioxidative activity, cytotoxicity against competing

microbes, and modulation of quorum sensing. The upregulation of prodigiosin under stress conditions may serve as a defensive strategy, allowing the bacterium to mitigate oxidative damage, enhance survival in a competitive environment, or disrupt the colonization of antagonistic species. Further investigation into these molecular mechanisms could clarify the role of actinobacterial compounds in modulating stress-induced secondary metabolite pathways in *S. marcescens*.

Zhang *et al.* (2024) examined the effects of crude extracts on the purple pigment production in *C. violaceum* CV026, another common QS model. Their study found that at low concentrations (0.005 mg/mL), crude extracts could reduce pigment production by 27%. In comparison, our study showed higher inhibition at the same concentrations for some actinobacterial extracts, particularly *Amycolatopsis thermoflava* (MG3), which achieved 37.5% inhibition of prodigiosin production.

Previous studies have explored pigment inhibition in *Serratia marcescens* by synthetic analogs of N-acylhomoserine lactone and marine-derived compounds providing valuable insights into how certain compounds can modulate prodigiosin biosynthesis [31] [32]. Few studies on pigment inhibition in *C. violaceum* by Streptomyces sp. have been reported suggesting that actinobacteria possess compounds capable of disrupting QS and pigment biosynthesis [53] [54]. While the inhibition of prodigiosin production by actinobacteria has not been extensively studied, our results suggest that rare actinobacteria may be a promising source of novel QS inhibitors.

The growing challenge of antimicrobial resistance (AMR) has prompted the search for novel therapeutic strategies, particularly those that do not directly kill bacteria but rather modulate their virulence mechanisms. Quorum sensing inhibition (QSI) has emerged as a promising alternative approach, and recent studies have highlighted the potential of actinobacteria as rich sources of natural products with anti-QS activity. This study further emphasizes the value of exploring rare actinobacterial genera for their ability to interfere with bacterial communication pathways, potentially offering new avenues to combat multidrug-resistant pathogens.

The role of actinobacteria, particularly Streptomyces species, as prolific producers of natural products that exhibit quorum sensing inhibition (QSI) activity have been reported [55] [56]. In a recent review, the contribution of Streptomyces and other rare actinobacterial genera—such as *Microbacterium*, *Nocardiopsis*, *Kribella*, *Brevibacterium*, and *Arthrobacter*—in producing AHL-degrading enzymes and other compounds that modulate microbial virulence has been summarized [36]. However, the rare genera investigated in this study, such as *Yuhushiella* and *Kutzneria*, have not been previously reported for their QSI activity, adding a new dimension to the growing body of research on actinobacteria and their potential as sources of anti-virulence compounds.

In the present study, we observed that rare actinobacterial extracts from *Yuhushiella* sp. (VL9), *Kutzneria viridogrisea* (MG1), *Microbacterium barkeri* (BD21), and *Amycolatopsis thermoflava* (MG3) not only inhibited pigment production in *Serratia marcescens* and *Chromobacterium violaceum* but also exhibited anti-

swarming and anti-biofilm activity against *Proteus mirabilis* and *Pseudomonas aeruginosa* at sub-MIC concentrations. These findings highlight the fact that rare actinobacterial extracts may have various anti-QS compounds which interfere with various QS regulated attributes of pathogens and can be potentially tapped in the fight against antimicrobial resistance. The UTI pathogens are unlikely to develop resistance to the anti-QS compounds because they pose no selective pressure on bacteria [57] [58].

Such anti-QS compounds present in rare actinobacterial extracts can attenuate the virulence of these pathogens without challenging their growth, thereby preventing the emergence of resistant strains and facilitating the elimination of pathogens by the host's immune system. These compounds could be used as coating agents in medical devices like catheters to prevent emergence of AMR strains of *Proteus mirabilis* and *Pseudomonas aeruginosa* causing UTI.

5. Conclusion

The development of anti-QS agents derived from rare actinobacteria presents a promising strategy to combat the global threat of antimicrobial resistance. By interfering with the bacterial communication system, QSIs can mitigate virulence factors such as swarming and biofilm formation, without directly killing the bacteria. This approach could serve as an adjunct to traditional antibiotics, reducing the likelihood of resistance development and offering a sustainable alternative to conventional therapies.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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