Ind. J. Pharm. Edu. Res., 2027; 10.5530/ijper.20273790.
https://www.ijper.org

Original Article

Screening of Rare Actinobacteria for Antiquorum Sensing Properties against *Proteus mirabilis* and *Serratia marcescens*

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ABSTRACT

Objectives: Multidrug-Resistant (MDR) bacteria are a global health threat, driving interest in alternative, non-antibiotic therapies. Quorum-Sensing Inhibition (QSI) disrupts bacterial communication, reducing virulence and antibiotic resistance. The current study is focused on isolating new actinobacteria from various biodiverse regions of Maharashtra, India, and their screening for antimicrobial activity against *Proteus mirabilis*, the most prevalent causative agent of urinary tract infections, and anti-quorum-sensing activity against *Serratia marcescens*, primarily known for hospital-acquired infections. **Materials and Methods:** Actinobacteria are prolific producers of bioactive compounds and are emerging as promising sources of QSI agents. The focus was on isolating new actinobacteria from various biodiverse regions of Maharashtra, India. **Results:** Successful isolation of 66 actinobacteria was done. Of these, 25 isolates showed potent inhibition against *Bacillus subtilis*, 11 isolates showed activity against *Proteus mirabilis*, and 24 isolates exhibited activity against *Serratia marcescens*. The organic extracts of 18 isolates demonstrated significant inhibition of swarming behaviour compared to the control. The study reveals a few rare genera of actinobacteria, like *Amycolotopsis tucumanensis, Amycolotopsis orientalis, Microbacterium barkeri, Yuhushiella*, and *Nocardia*. **Conclusion:** Our findings highlight that the Bhushi Dam, Venna Lake, and Melghat forest of Maharashtra are underexplored and unique ecological habitats harboring previously uncharacterized actinomycetes with significant potential for novel antibiotic discovery.

Keywords: 16S rRNA, Actinobacteria, Autoinducers, Multi-drug resistance, Quorum sensing, Quorum-sensing inhibitors.

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Received: 21-07-2026;
Revised: 18-08-2026;
Accepted: 09-10-2026.

INTRODUCTION

Actinobacteria are Gram-positive bacteria characterised by a filamentous structure and a high Guanine-Cytosine (G+C) content in their DNA.[1] 70% of known antibiotics have evolved from the *Streptomyces* spp.[2] Many screening programs have been conducted, discovering many new bioactive molecules utilised in various clinical applications, from infection control to cancer treatment.[3] While new metabolites are being discovered, already-known species are also being rediscovered. In recent years, many researchers have shifted their focus to rare Actinobacteria or non-*Streptomyces*, considering their potential to synthesize innovative and effective antimicrobial substances.[4] Rare Actinobacteria have become promising resources for the discovery of not only new antimicrobial compounds but also other bioactive metabolites with diverse properties.[5] Multidrug-Resistant Bacteria (MDR) evolving due to antibiotic overuse are a major healthcare concern nowadays, and hence, there is a need for non-antibiotic therapies for bacterial infections.[6] Bacterial cells communicate with each other through a mechanism also known as Quorum Sensing (QS). Autoinducers (AIs) are the tiny diffusible signaling molecules that help with Quorum sensing among the bacteria. An anti-QS compound activity involves obstructing the QS process of pathogenic microorganisms. Comparatively, a conventional antimicrobial agent controls through its ability to either restrain cell growth (microbiostatic effect) or induce cell death (microbiocidal effect).[7]

Proteus mirabilis poses a major healthcare challenge as it is an important urinary tract pathogen, due to its biofilm-forming ability, multi-drug-resistant infections, necessitating alternative therapies.[8] *Serratia marcescens* is a recognized opportunistic, Gram-negative bacterium associated with healthcare-acquired respiratory and urinary tract infections, and it belongs to the *Enterobacteriaceae* family. Intensive care units often experience epidemics of colonization and infection caused by *Serratia marcescens*.[9] The expression of virulence factors in *S. marcescens* and *P. mirabilis* is a density-dependent behaviour and is regulated by Quorum Sensing (QS).[10] Quorum sensing thus contributes

DOI: 10.5530/ijper.20273790

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to antibiotic production and exoenzyme secretion, swarming behaviour, surface adhesion, and formation of biofilm.[11] Hence, all these Quorum Sensing-related virulence and pathogenesis in *Serratia marcescens* and *Proteus mirabilis* can be controlled by disrupting QS.

The major focus of this study was aimed at exploring rare actinobacteria. Soil samples were collected from Melghat Forest, which is a tropical dry deciduous forest in Vidarbha, and Vandangad Fort, located in the Sahyadri hills, which is rich in biodiversity, and from Bhushi Dam, a Biodiversity Heritage Site in Lonavala. Alluvial samples were from Guhagar Beach, which has a diverse coastal ecosystem, and Venna Lake (Satara, medicinal plant-rich forests). No reports are available for the collection of rare actinobacteria (non-*Streptomyces*) from these sites, and we aim to isolate rare and unexplored actinobacteria from these sites.

Our study establishes a foundational understanding of the rich biodiversity of actinobacteria isolated from unexplored ecological niches and underscores their significant potential as a source for the discovery of novel antibiotics. This research focuses on identifying novel quorum-sensing inhibitors from rare actinobacteria as promising alternatives or adjuncts for combating multidrug-resistant bacterial infections.

MATERIALS AND METHODS

Test Organism

Antimicrobial activity was performed on *Proteus mirabilis, Bacillus subtilis*, and *Serratia marcescens* bacterial strains. These were procured from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India, and were: *Bacillus subtilis* MTCC 441, *Proteus mirabilis* MTCC 425, and *Serratia marcescens* MTCC 86. The organisms were preserved at 4°C on nutrient agar slants, with overnight-grown LB broth cultures used throughout the experiments. The study involved only microbial strains and no vertebrate animals or humans.

Reagents and Culture Media

Analytical-grade chemicals and solvents were obtained from Merck (Germany), and microbial culture media, as well as antibiotics, were obtained from Hi-Media (Mumbai, India).

Sample Collections

Soil/alluvial sample collection was done from Dec 2023 to April 24, usually from a 5-10 cm depth, transferred into sterile, airtight plastic bottles/jars, and stored in the refrigerator at 4°C until further processing.[12]

Sample Preparation

After air drying the samples, they were manually triturated using a mortar and pestle and passed through a sieve to remove clumps and stones before use.[13]

Pretreatment

A 1 g free-flowing sample was exposed to different preliminary treatments, like air drying, application of dry heat at 120°C, Benzethonium Chloride (0.01%), 1.5% phenol, and Chloramine T (0.03%) treatment.[14]

For air-dry treatment, the samples were allowed to stand at room temperature until all moisture was removed. Soil samples were heat-treated by keeping them in Petri dishes and exposing them to 120°C for 1 hr inside a hot-air oven. The sample slurry was treated with a 1.5% phenol solution for phenol treatment, followed by incubation for 60 min at room temperature. To the sample slurry, Benzethonium Chloride (0.01%) was weighed and added, along with 1 hr of incubation at room temperature. The same was repeated for Chloramine T (0.03%) treatment.[15]

Isolation of actinobacteria and different isolation media

One gram of soil sample from each pre-treatment was added to 10 mL sterile distilled water, while for marine samples, sterile seawater was utilized. The mixture was vortexed for 2 min. Dilutions up to 10^{-5} were generated using sterile distilled water as well as sterile seawater for both samples. 100 µL from each dilution was added to various isolation media and poured into sterile petri plates. For isolation, selective media, like AIA (Actinobacteria Isolation Agar), SCA (Starch Casein agar), PDA (Potato Dextrose agar), HV Agar (Humic Acid Vitamin agar), and ISP-7 Agar were prepared, each fortified with Cycloheximide (100 µg/mL) to inhibit fungal growth. And Nalidixic acid (50 µg/mL), the antibacterial agent.[16] Distilled water was used for media preparation of soil samples, and seawater was used for marine samples.[17] Incubation of the plates was carried out at 28°C for a period of 15 to 40 days. Isolates displaying morphological traits analogous to actinobacteria were identified. Potato Dextrose Agar plates were used for single-colony streaking, and plates were maintained at 28°C incubation for typical actinobacterial colony formation.

Colony Characterization

Colony characterization of the isolates was carried out based on colour, texture, extracellular pigment production, and aerial and substrate mycelium colour. Microscopic observations were performed along with Gram's staining. The mycelium and spores were observed using 100X magnification with a compound microscope.

Cultivation of Actinobacterial Isolates for Bioactive Compound Collection

PDB (Potato Dextrose Broth) 100 mL was prepared in a 250 mL flask, and a pure culture of each isolate was inoculated. These flasks were kept at 28°C in a rotary shaking incubator for 8 to 10 days. After an 8-day incubation period, the cells and culture

filtrates were collected separately. Recovery of Crude bioactive compounds was done using ethyl acetate (1:1, w/v) for solvent extraction. The extracts were concentrated under vacuum, and the resulting crude material was preserved at -20°C for future activity.

Antimicrobial assay

For assessing the antimicrobial activity of the extracts, LB agar was used, employing the agar well diffusion method. 100 µL of 1 mg/mL stock was used for the tests.[18] After 24 hr of incubation at 37°C for *Bacillus subtilis* and *Proteus mirabilis*, the diameter of the zone of inhibition was determined.

Pigment Inhibition Assay

Pigment inhibition potential of the extracts against *Serratia marcescens* was evaluated by the method of agar well diffusion. Autoclaved 2% LB agar medium was poured into Petri plates by adding an overnight-grown culture of *Serratia marcescens and* was set aside for solidification. Using a sterile cork borer, wells were prepared for Solvent control, Cell extract, and Broth extract for each isolate. 100 µL of 1 mg/mL stock was dispensed to each well, and plates were subsequently incubated for 24 hr at 28°C. A positive QSI response was indicated by the loss of pigment production in the indicator strain adjacent to the test organisms. No pigmentation inhibition suggests negative results.[19]

Inhibition of Swarming in *P. mirabilis*

The impact of anti-QS compounds derived from actinobacterial isolates on the swarming motility of *Proteus mirabilis* was assessed. From the previous results, extracts were added to the swarm agar plates at appropriate concentrations. A positive control (no additives) and solvent control (Ethyl acetate) plate was included. An overnight-grown culture of *Proteus mirabilis* (2 µL) was inoculated onto the swarm agar plates at the centre, followed by incubation at 37°C for 20 hr. The reduction in swarm zone diameter was determined relative to the control, and the percentage decrease was calculated. Each assay was performed three times, and the mean values were reported for the extracts.

Molecular Identification/16S rRNA for Potent Isolates Identification

Selected isolates were identified based on 16S rRNA sequencing. Amplification of the 16S rRNA gene of selected actinobacterial isolates was carried out with Actino-specific (Forward Primer -5'-CCTAACACATGCAAGTCGA-3' and reverse primer - 5'-CGTATTACCGCGGCTGCTGG-5'. Each 25 µL PCR reaction consisted of 0.2 mM of dNTPs, 1 mM primers, 12.5 ng genomic DNA, 0.5% (v/v) DMSO, and 0.6 U of Ex-Taq Hot Start polymerase in 1× Ex-Taq buffer.[20] The amplification protocol consisted of an initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation (94°C, 30 sec), annealing (53°C, 30 sec), and extension (72°C, 1 min), and a final extension at 72°C for 5 min. The expected amplicon size was approximately 1500 bp. PCR products were analysed on 1% agarose gel, excised, purified using a gel extraction kit, and subsequently sequenced at Barcode/Eurofins (Bangalore, India).[21]

Bacterial Growth Curve Assay

The bacterial growth curve assay was executed to monitor bacterial growth in the presence of extracts. An overnight-grown *P. mirabilis* (0.1%) culture was added to a sterile broth flask of 100 mL LB broth. Cultures were sampled every hour and transferred to a shaker incubator. O.D. at 600 nm was read at 2, 4, 6, 8, and 24 hr. The obtained readings were compared to the control.[22]

MIC of extracts

To determine the MIC of the extracts, the broth dilution technique was employed.[23] Extract concentrations from 1 to 200 µg/mL were supplemented in the medium, and *Proteus mirabilis* culture was exposed to these concentrations and incubated overnight. Cultures were incubated at 37°C for 24 hr. MIC was determined as the lowest concentration of the compound that suppressed*Proteus mirabilis* growth at 600 nm. Assays were carried out in triplicate, and mean MIC values were calculated for each extract.

Statistical Analysis

The Data obtained was statistically analysed. All experiments were conducted in triplicate, and the data are presented as mean±Standard Deviation (SD). *p-value* ≤0.05 was considered significant unless otherwise mentioned.

RESULTS

The soil samples were given different pre-treatments, and a very good number of actinobacteria were isolated. The types of samples, their respective locations, and associated physical features are outlined (Table S1), while Figure S1 presents the impact of various pre-treatments and media on actinobacterial isolation.

Pre-treatment Methods and Culture Media Formulations for the Successful Actinobacterial Isolations

The number of actinobacteria obtained from five different locations-Bhushi Dam, Melghat Forest, Vandangad Fort, Venna Lake, and Guhagar Beach-showed significant variation (Figure S2a). Bhushi Dam yielded the highest number of isolates (27). Melghat Forest (12), Vandangad Fort (10), and Venna Lake (9), followed by Guhagar Beach, recorded the lowest number of isolates (8). The impact of various pretreatment methods was significant, as different isolates were isolated from the various pretreatments. Among the given pretreatment conditions, the Benzethonium Chloride (BC) pretreatment yielded the highest number (18 isolates), followed by air drying (13 isolates) and

heat treatment (12 isolates). Chloramine T treatment resulted in a moderate yield (11 isolates), while 1.5% phenol (4 isolates) and the combined heat + 1.5% phenol treatment (2 isolates) produced markedly fewer isolates (Figure S2b). The combined heat + BC treatment also led to reduced isolate recovery (6 isolates). PDA (Potato Dextrose Agar), AIA (Actinomycete Isolation Agar), ISP 7 (International *Streptomyces* Project Medium 7), Humic Acid Vitamin Agar (HVA), and Starch Casein Agar (SCA) were used for the isolation of actinobacteria. Among all media, PDA (40 isolates) was the most effective medium, supporting the highest microbial diversity, followed by AIA (7 isolates), ISP 7 (10 isolates), HVA (4 isolates), and SCA (5 isolates) (Figure S2c).

Colony characterization, growth patterns, and microscopy

Various types of colonies, such as robust ones that adhered to the medium and exhibiting whitish aerial mycelium exhibiting a chalky and powdery texture, were observed. A few colonies showed powdery aerial mycelium. In contrast, a small number of pigmented colonies with dark brown, yellow, white, and pink aerial mycelium were also isolated. Dark brown colonies produced pigment that diffused into the medium. Additionally, some colonies were off-white to yellow, with smooth surfaces. These colonies were smaller than the chalky white ones but more tightly embedded in the agar medium.

Microscopic examination of the isolates revealed cross-linked filamentous structures and clusters of spores. Isolate SNN 11 from Bhushi Dam showed spindle-shaped cells under microscopic observation. Isolate SNN 19 and SNN 7 showed slow growth and, upon Gram staining, exhibited long spore chains and filamentous morphology.

Table S2 summarizes the colony characteristics and microscopic observations of the chosen isolates.

Growth pattern and Characteristics of isolates

All isolates exhibited pellet morphology, a distinctive cell growth pattern of actinobacteria in broth culture. Soil samples collected from the Bhushi Dam site yielded a diverse range of actinomycete isolates, many of which varied in colours, including dark brown, yellow, bright yellow, and violet. Isolates obtained from the Melghat forest predominantly produced yellow pigment and formed soft, large, white, puffy pellets. Notably, isolates such as Ca and Isolate 6 produced dark-coloured pigments in broth cultures, while Isolate R1 produced a pink pigment. Further, isolates MG2, Q5, and V1 yielded dark orange pigments, while VL 9 exhibited a dark yellow pigment. These pigments were recovered following overnight extraction with ethyl acetate. Figures S2-S5 summarise the pigment production profiles of pure cultures, including broth cultures, and both broth and cell extracts.

Antimicrobial Activity

Antimicrobial activity of all the isolates was evaluated using the agar well diffusion method. *Bacillus subtilis and Proteus mirabilis, and* for pigment inhibition, *Serratia marcescens,* a red pigment-producing organism, was used.

Antibacterial activity against *Bacillus subtilis*

Isolates SNN 1, SNN 2, SNN 4, SNN 8, SNN 9, SNN 13, SNN 14, SNN 15, SNN 16, SNN 18, SNN 21, SNN 23, MG2, Q5, VL9, Ca, Ci, AD3, and Daisy were found to have antimicrobial activity against *Bacillus subtilis* (Figures S6a and S6b).

Antimicrobial activity against *Proteus mirabilis*

Isolates SNN 2, SNN 6, SNN 13, SNN 14, SNN 16, SNN 21, VL9, VL3, Ca, and Daisy were found to have activity against *Proteus mirabilis* (Figures S7a and S7b).

Pigment Inhibition in *S. marcescens*

Isolate SNN 6, SNN 21, SNN 2, SNN 10, MG 2, SNN 13, SNN 20, and SNN 15 exhibited anti-QS activity against *Serratia marcescens* (Figures S8a and S8b), where a zone of pigment inhibition was observed in the agar well diffusion method.

Anti-Swarming activity of the isolates against *Proteus mirabilis*

Extracts from thirty-five potent actinobacterial isolates were tested for anti-swarming activity against*Proteus mirabilis.* Out of these, significant swarming inhibition was seen in extracts of 18 isolates, compared to the control (Figure S9). The percentage inhibition values were quantified and are presented in Figure 1. The inhibition levels varied markedly among the isolates, from 3.4% to 70.0%. The isolate VL 9 demonstrated the strongest inhibitory effect, significantly suppressing swarming by 70.0% (Figure). Other notable isolates included Ca (37.0%), R1 (27.8%), and SNN 21 (21.2%), all of which exhibited moderate swarming inhibition. Several isolates, such as SNN 16 (3.4%), SNN 1 (5.0%), and SNN 15 (7.8%), showed minimal or weak inhibition of swarming motility (Figure 2). These finding reveals the potential role of actinobacterial extracts in impairing motility-associated properties, presumably through inhibition of QS-mediated signalling pathways. VL 9 possesses significant anti-swarming activity and may serve as a promising candidate for further characterisation of its bioactive metabolites or mechanisms of action in inhibiting bacterial motility.

The extracts of isolate VL 9, Ca, SNN 21, Iso 6, Daisy, SNN 10, and SNN 18 have likely interfered with flagellar function, quorum sensing, which are essential for sustained swarming. The reduction rings imply the extract does not completely kill the bacteria, but inhibits motility progression, making it a promising candidate for anti-virulence or biofilm-prevention strategies.

Bacterial Growth Assay

To confirm non-toxicity, the growth of*Proteus mirabilis* was pursued for 48 hr in the presence of the extracts (5 µg/mL). The setup involved a solvent control and a positive control, with results showing no considerable inhibition of bacterial growth rate (Figure 3).

MIC of Potent Extracts

The MIC for all extracts against *Proteus mirabilis* was found to be 200 µg/mL.

Molecular Identification of Potent Isolates by 16S rRNA

The twelve selected potent isolates were identified through 16S rRNA gene sequencing. The resulting sequences were deposited in GenBank, and their corresponding accession numbers are presented in Table 1.

DISCUSSION

In this study, the successful isolation of rare actinobacterial strains was achieved through a strategic combination of physical and chemical pretreatments, focusing on non-Streptomycete genera. These findings are consistent with previous research showing that pretreatments such as phenol exposure, air-drying, dry heat application, and the use of selective antibiotics effectively aid in isolating rare actinobacteria.

Significant differences in sampling sites and the corresponding number of isolates indicate the presence of diverse ecological environments. Habitat-specific environmental conditions play a crucial role in shaping microbial diversity, underscoring the importance of exploring different ecological niches for effective microbial prospecting. Microbial isolation from soil is highly influenced by the choice of pretreatment. Heat treatment at 120°C, with or without Benzethonium chloride, along with air-drying and chemical pretreatments like Benzethonium chloride and Chloramine T, effectively suppresses common bacteria and enriches rare actinobacteria. This approach successfully targeted spore-forming genera, including *Streptomyces, Nocardia, Amycolatopsis, Microbacterium*, and *Yuhushiella*. PDA proved the most effective medium, yielding 40 isolates, followed by ISP 7 media with 10 isolates, supporting genera such as *Streptomyces, Amycolatopsis, Microbacterium*, and *Nocardia*. AIA (Actinomyces Isolation Agar) medium and SCA (Starch Casein Agar) selectively supported *Streptomyces* species through tailored nutrient compositions. ISP 7 facilitated the isolation of rare taxa like *Yuhushiella* and *Amycolatopsis orientalis* by accommodating metabolically versatile strains.[24,25]

For the screening of QSI, we used *Serratia marcescens* as an indicator organism in our assay instead of *Chromobacterium violaceum,* a routinely used organism. In *S. marcescens,* prodigiosin production, a red coloured pigment, is regulated through AHL-mediated quorum-sensing systems, such as SmaI/SmaR and SpnI/SpnR,[26] which control biosynthesis and related virulence

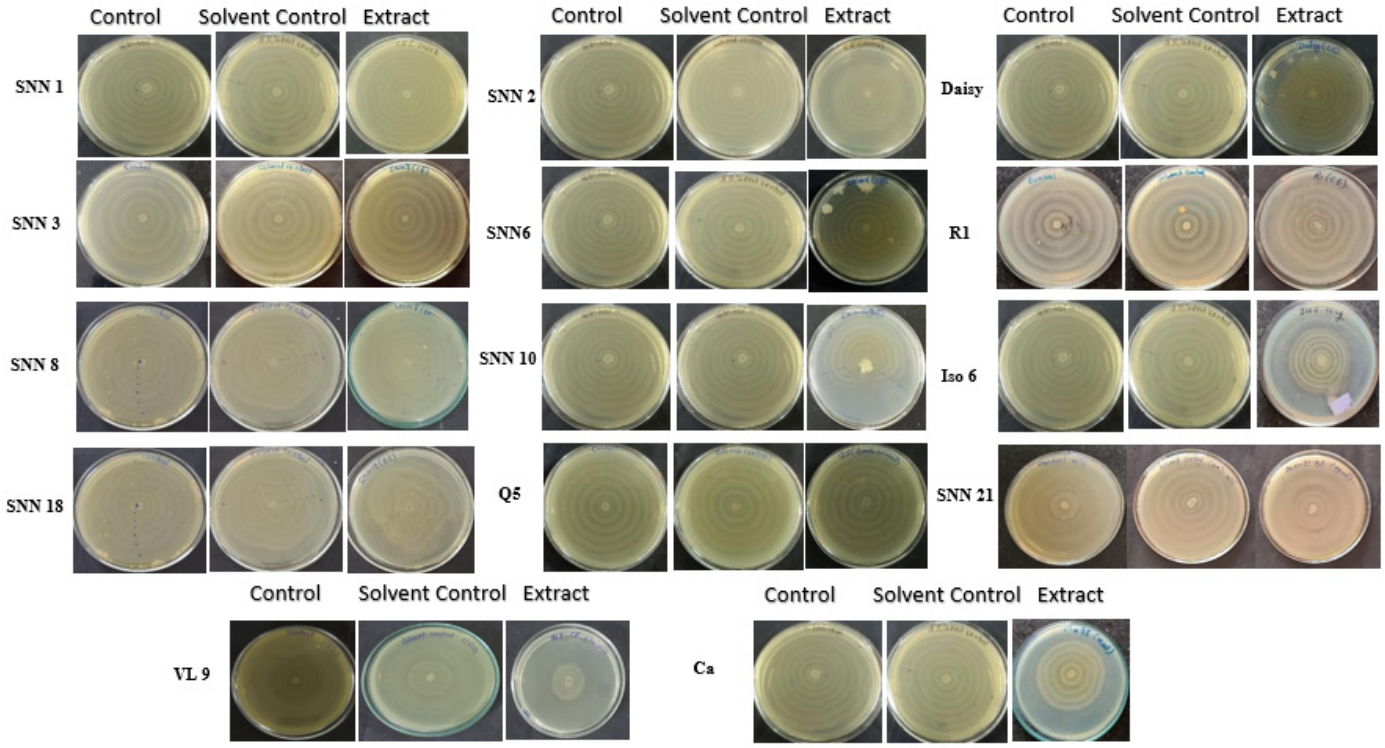


Figure 1: Antiswarming Assay of the Extracts against *Proteus mirabilis.* (Isolate VL9 and Ca Showing reduction in the swarm front).

factors, and extracts disturb this quorum-sensing phenomenon by the production of a zone of pigment inhibition around the agar wells, disrupting the QS system of the *S. marcescens*. The isolates Ca, SNN 1, SNN 2, SNN 6, SNN 13, SNN 21, and MG 2 have shown notable pigment inhibition, and overall, 24 extracts (36%) were effective in pigment inhibition. Rare actinobacterial extracts, including those from *Microbacterium, Amycolatopsis*, and *Yuhushiella*, have been shown to inhibit prodigiosin production in *Serratia marcescens* in a dose-dependent manner. This suggests that rare actinobacteria isolated in this study have the potential to produce compounds that disrupt QS-regulated pigment biosynthesis and virulence traits, offering potential novel anti-virulence agents against antimicrobial resistance.

According to Echeverrigaray et al, there is an alteration in the *P. mirabilis* swarm pattern in the presence of pure terpenes.[27] The same is observed in the present study, where the extract of the isolate VL 9 has reduced the number of rings, and synchronisation of rings occurred. Reduction in the swarming motility ($p<0.05$) in *P. mirabilis* was observed by several extracts, particularly VL 9 (*Yuhushiella*), SNN 6 (*Amycolatopsis thermoflava*), and SNN 21

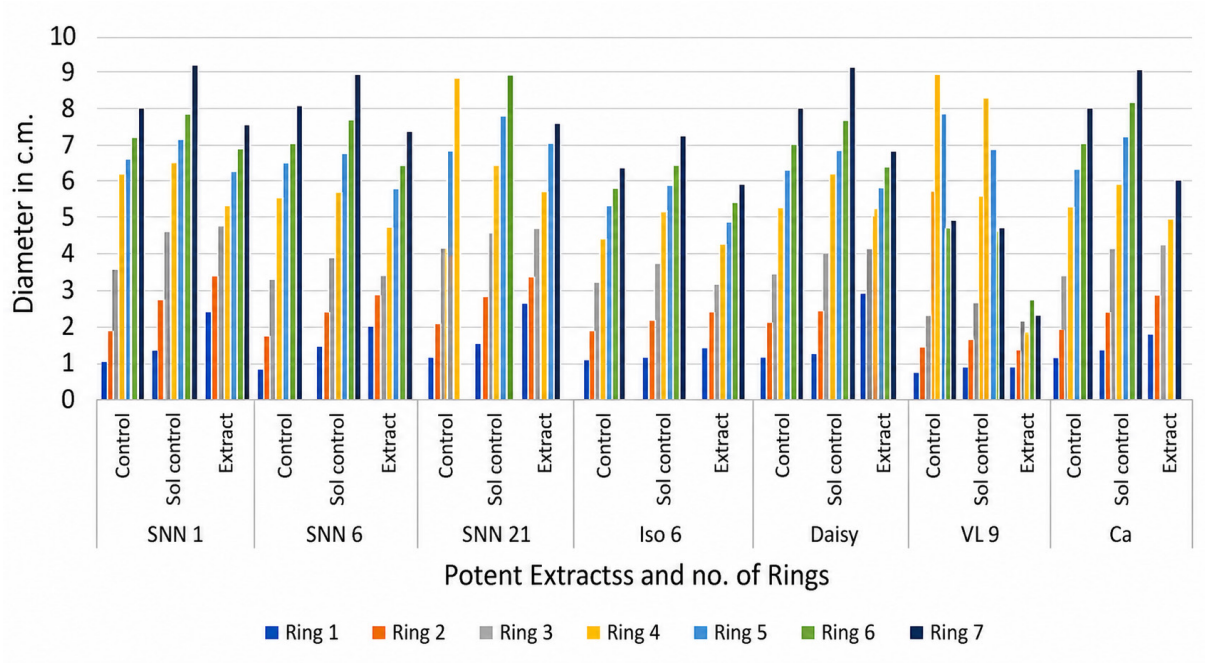


Figure 2: Comparison of the Number of Rings of the Swarm front of the potent isolate and Diameter comparison among the swarm front of each ring (Data expressed as mean ± SD ($n = 3$); $p \leq 0.01$).

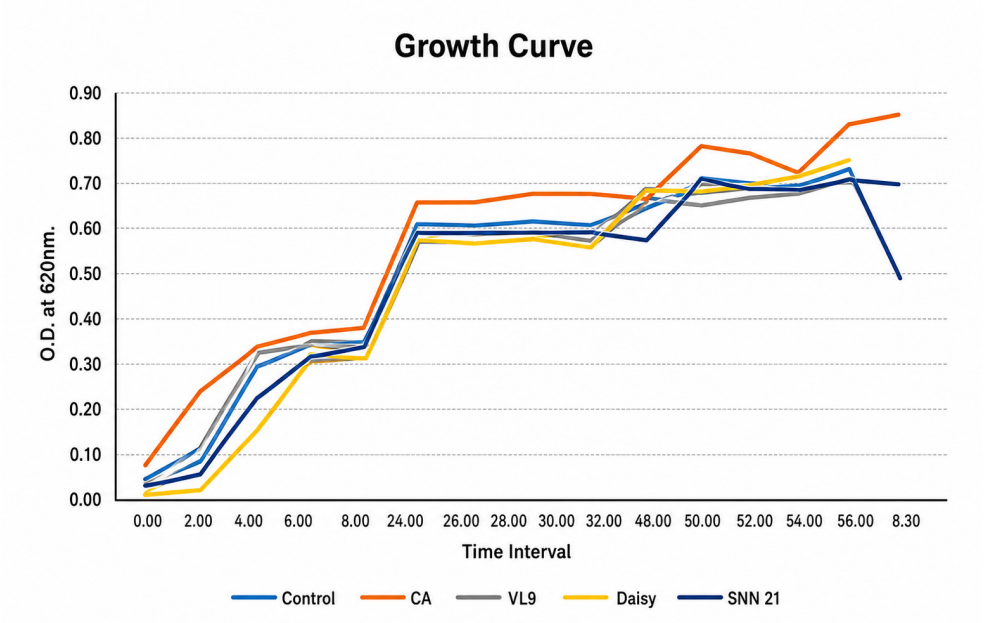


Figure 3: Evaluation of extracts in relation to the growth of*Proteus mirabilis.*

Table 1: Identification of Potent Isolates through 16s-RNA Sequencing.

Isolate	GenBank accession No.	No. of nucleotides sequenced	Query cover	Similarity	Closest cultivated species (GenBank accession no.)
SNN 1	KQ948892	1385	100	99.63	*Streptomyces bungoensis* DSM 41781(T)
SNN 2	AB184537	1509	98	99.33	*Streptomyces abikoensis* NBRC 13860(T)
SNN 6	KI421511	1511	100	99.79	Amycolatopsis thermoflava N1165(T)
SNN 13	BDBN01000167	1439	100	99.93	*Nocardia* nova *NBRC 15556(T)*
SNN 16	PV290304	1048	90	95.78	*Amycolatopsis* sp. *HW47 KF194351.1*
SNN 21	PV262557	630	100	100	*Microbacterium barkeri* NR026164.1
SNN 24	PV258785	1030	99	97.46	*Amycolatopsis tucumanensis* strain ABO NR_115883.1
Ca	AB184537	1509	100	99.51	*Streptomyces abikoensis* NBRC 13860(T)
Daisy	NR114917.1	1003	100	99.01	*Streptomyces erumpens DSM* 40941
Q5	DQ026631	1435	100	99.58	*Streptomyces ardesiacus* NRRL B-1773(T)
VL 9	PV262564	980	100	100	Yuhushiella spp.
Isolate 6	PV258786	1063	99	97.34	*Amycolatopsis orientalis* strain KSI 153

(*Microbacterium barkeri*). Since swarming is key to infection and UTI colonisation, these extracts may hold potential in controlling ascending UTIs caused by *P. mirabilis*. The differentiated swarm cells

of *P. mirabilis* are highly invasive and are responsible for the colonisation of cells and ascending migration of bacteria against the flow of urine and mucous in UTI.[28] The UTI pathogens are unlikely to develop resistance to the anti-QS compounds because they pose no selective pressure on bacteria. Thus, a compound in the extracts that inhibits swarming would presumably be effective in controlling ascending UTI by *P. mirabilis*. Notably, isolate VL9 reduced *P. mirabilis* swarming by 70%, indicating strong quorum-quenching potential. This study successfully isolated rare actinobacterial genera, including *Amycolatopsis tucumanensis, Microbacterium barkeri, Yuhushiella*, and *Nocardia*, many showing antimicrobial activity against *P. mirabilis* and *S. marcescens*. The rare genera investigated in this study, such as *Yuhushiella* (VL 9) and *Amycolatopsis thermoflava* (SNN 6), 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. The compounds from these rare actinobacteria can be further identified and utilised and formulated or coated on the instruments used in the ICU units, so that prevention of biofilm can be done.

CONCLUSION

So far, there are no published studies on isolating bioactive actinobacteria from Bhushi Dam or Venna Lake in Maharashtra's Sahyadri range, with few studies in the Vidarbha region. This research provides the first comprehensive overview of actinobacterial diversity from these areas. Our results establish a foundation for the use of innovative isolation and culture methods to obtain rare actinobacterial taxa that may have therapeutic applications.

Rare Actinobacterial genera *Amycolatopsis thermoflava, Amycolatopsis tucumanensis, Amycolatopsis orientalis, Microbacterium barkeri, Yuhushiella*, and *Nocardia* were obtained from the recent study. This indicates that sites such as Bhushi Dam, Melghat Forest, and VandanGad Fort were promising hotspots for future bioprospecting using expanded isolation strategies.

Several rare isolates, notably VL 9 (*Yuhushiella*), SNN 21 (*Microbacterium barkeri*), SNN 6 (*Amycolatopsis thermoflava*), and SNN 13 (*Nocardia nova*), exhibited strong anti-quorum-sensing activity against *P. mirabilis* and *S. marcescens*. These findings emphasise the potential of rare actinobacteria from these sites for discovering novel antimicrobial compounds. Advanced studies, such as fermentation under submerged

culture conditions, followed by fractionation, purification, and molecular elucidation, could be done to assess the therapeutic and commercial prospects of these antibiotics.

ACKNOWLEDGEMENT

The authors would like to thank Hislop College, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, for providing laboratory facilities.

ABBREVIATIONS

AIA: Actinomycetes Isolation Agar; **AIs:** Autoinducers; **BC:** Benzethonium Chloride; **DMSO:** Dimethyl sulfoxide; **DNA:** Deoxyribonucleic Acid; **DMSO:** Dimethyl sulfoxide; **G+C:** Guanine-cytosine; **HVA:** Humic Acid Vitamin Agar; **ISP 7:** International Streptomyces Project Medium 7; **LB:** Luria-Bertani; **MDR:** Multidrug-resistant; **MIC:** Minimum Inhibitory; **SD:** Standard deviation; **PCR:** Polymerase Chain Reaction; **PDA:** Potato Dextrose Agar; **RNA:** Ribonucleic Acid; **QSI:** Quorum-sensing inhibition; **QS:** Quorum sensing; **UTI:** Urinary tract infections.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

This research received no specific grant from any funding agency.

AUTHORS CONTRIBUTION

All authors contributed to the conception and design of the study. **Nisha D. Margode:** Performed material preparation, microbiological work, data collection and analysis. **Dr. Sunita Bundale and Dr. Varaprasad Kolla:** Evaluated the work and results. The first draft of the manuscript was written by Nisha D. Margode, and it was assessed by Varaprasad Kolla and Sunita Bundale and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

SUMMARY

Emerging multidrug-resistant microorganisms are causing a serious health challenge worldwide, as many infections are becoming harder to treat despite the wide availability of broad-spectrum antibiotics. Actinobacteria, the producer of natural bioactive compounds, are gaining significant attention due to their exceptional capacity to synthesise diverse secondary metabolites with strong pharmacological properties. Actinomycetes, widely distributed in nature, are key producers of diverse antibiotic classes and remain intensively studied across various habitats.

The locations we selected for our study were never reported for the isolation of actinobacteria. In our study, we successfully isolated 66 pure cultures of actinobacteria. A variety of actinobacteria were obtained from forest soil, from biodiverse sites like Bhushi dam and Venna lake. The study led to the isolation of rare genera of actinobacteria (non-*Streptomyces*). These rare isolates exhibited strong antimicrobial activity against selected pathogenic microorganisms. Isolate VL9 has shown strong swarming inhibition of pathogenic *Proteus mirabilis*, both in swarm front reduction, along with a reduction in the number of swarm rings, followed by isolates Ca and R1. Rare actinobacteria have shown remarkable pigment inhibition activity against *Serratia marcescens*, thus proving the quorum quenching capacity of these rare actinobacteria.

Future commercialization of these actinobacterial antibiotics necessitates systematic downstream investigations, including optimized fermentation protocols, compound purification, and comprehensive structural characterization through advanced analytical methods. This will validate the therapeutic potential and economic feasibility of these natural metabolites against emerging drug-resistant pathogens.

Hence, our study builds a groundwork and proves the biodiversity of actinobacteria isolated from unexplored sites selected in our study and their potential for novel antibiotic discovery.

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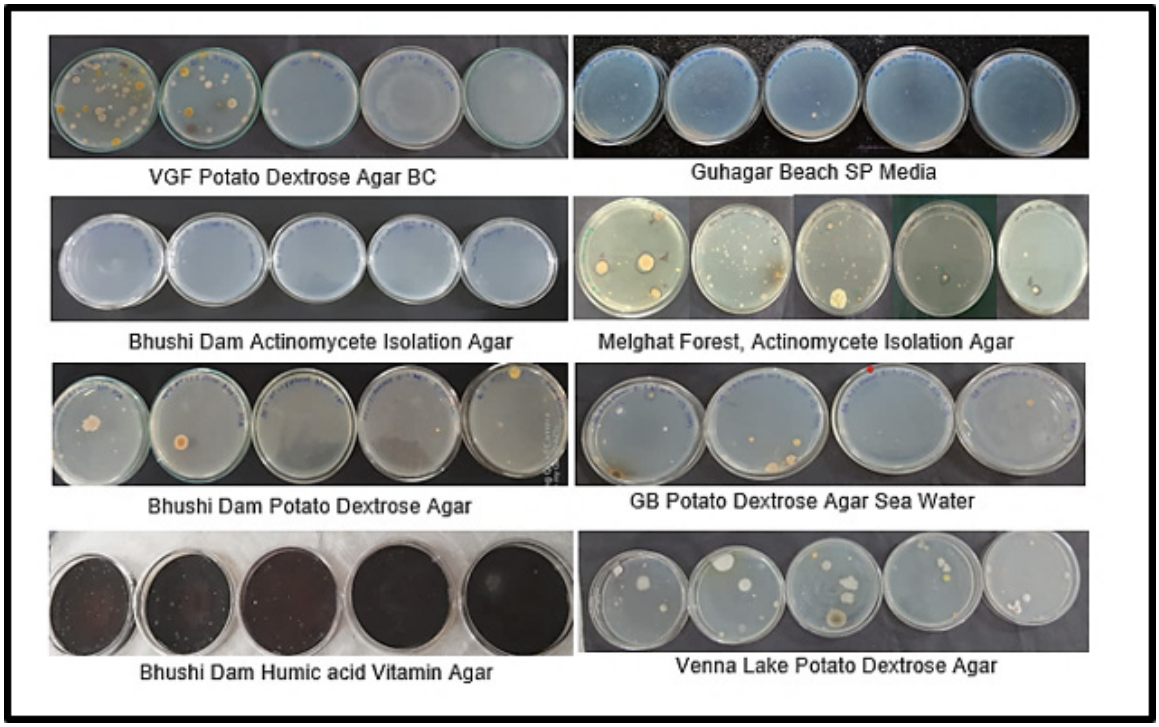


Figure S1: Pretreatment Plates with Location and Media.

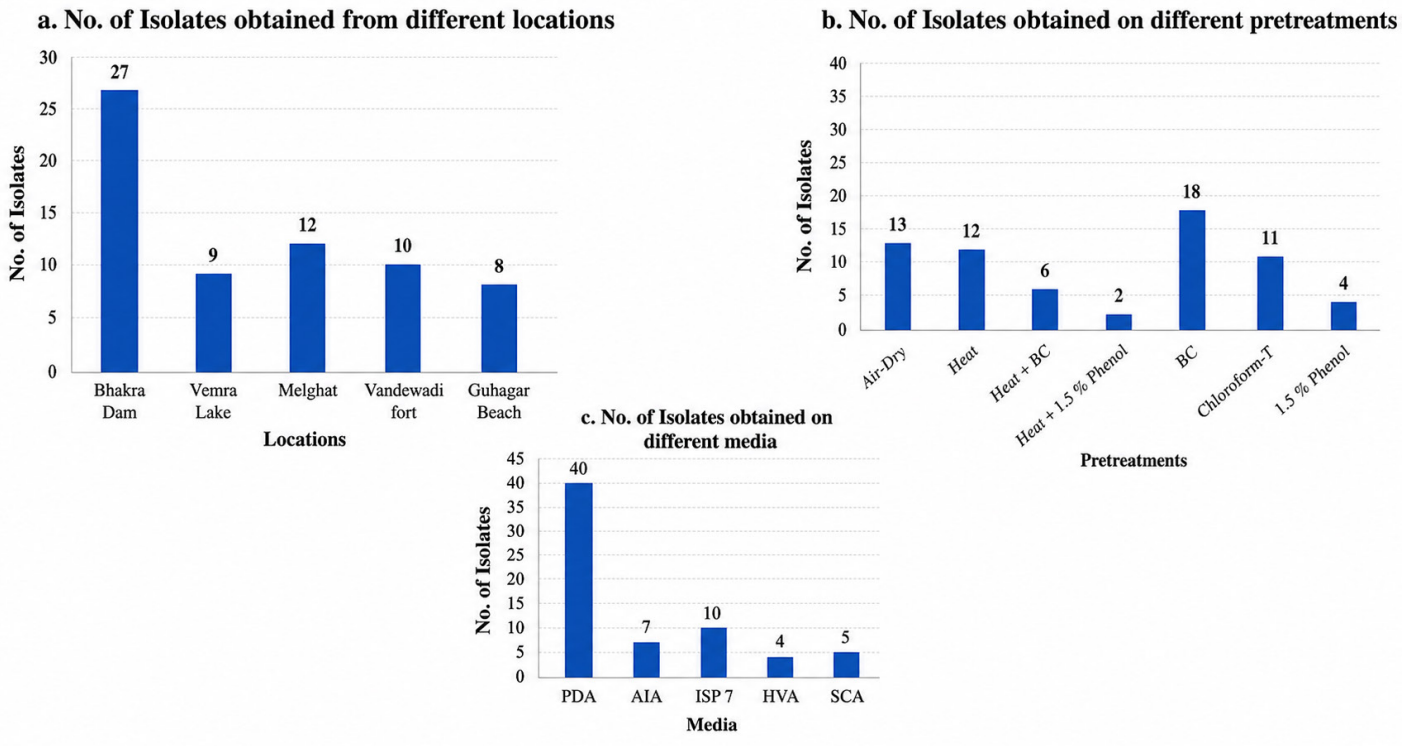


Figure S2: a) Number of Isolates obtained from different locations b) Number of Isolates obtained from the given pretreatments c) Number of Isolates obtained from the different media.

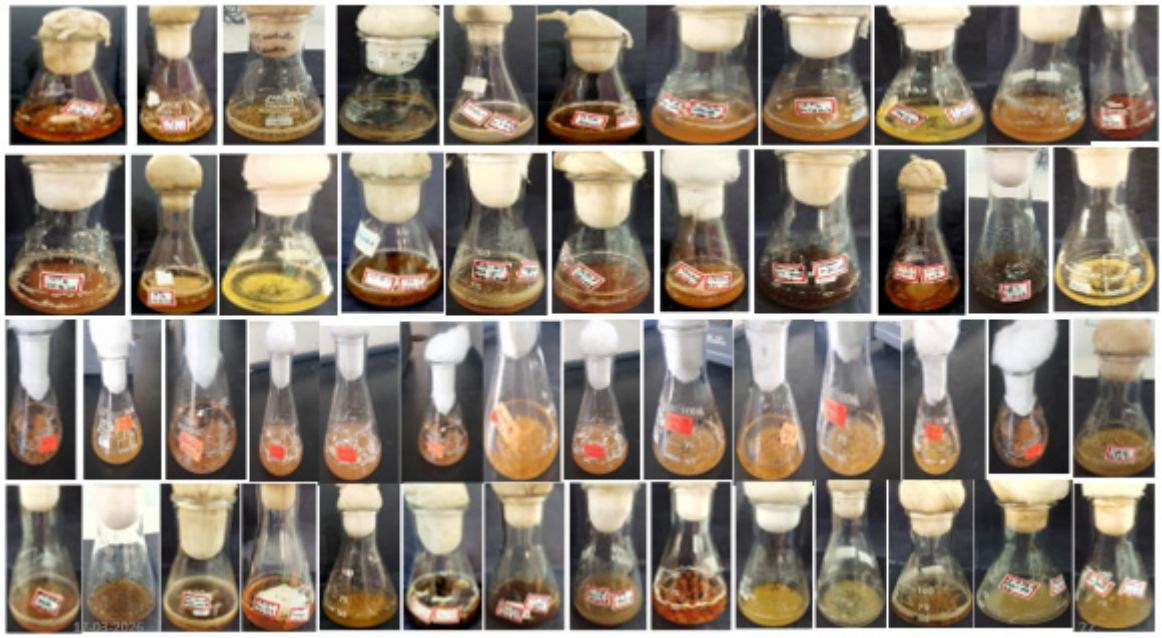


Figure S3: Production of bioactive compounds in broth culture.

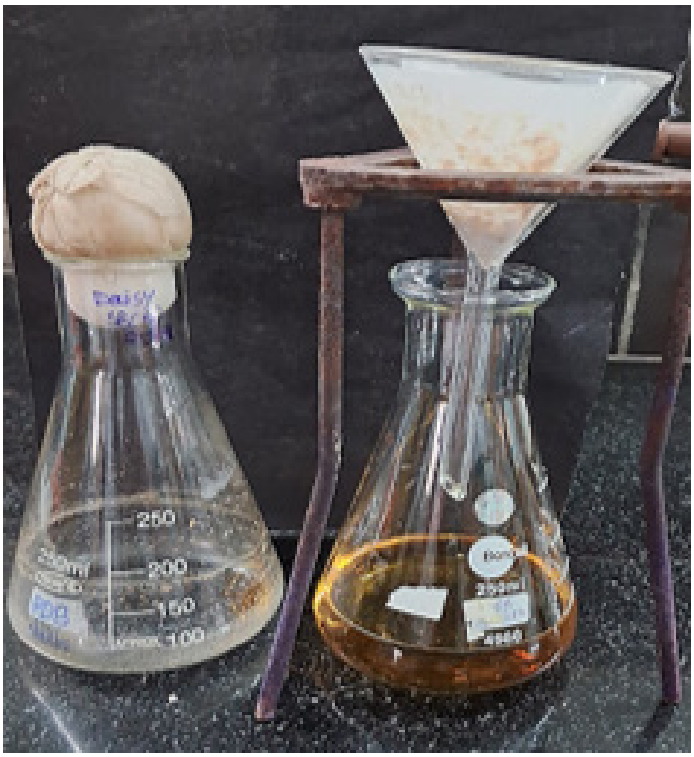


Figure S4: Extraction of Bioactive Compounds.

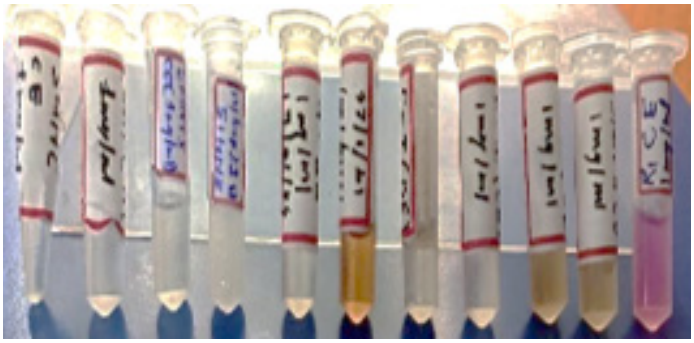
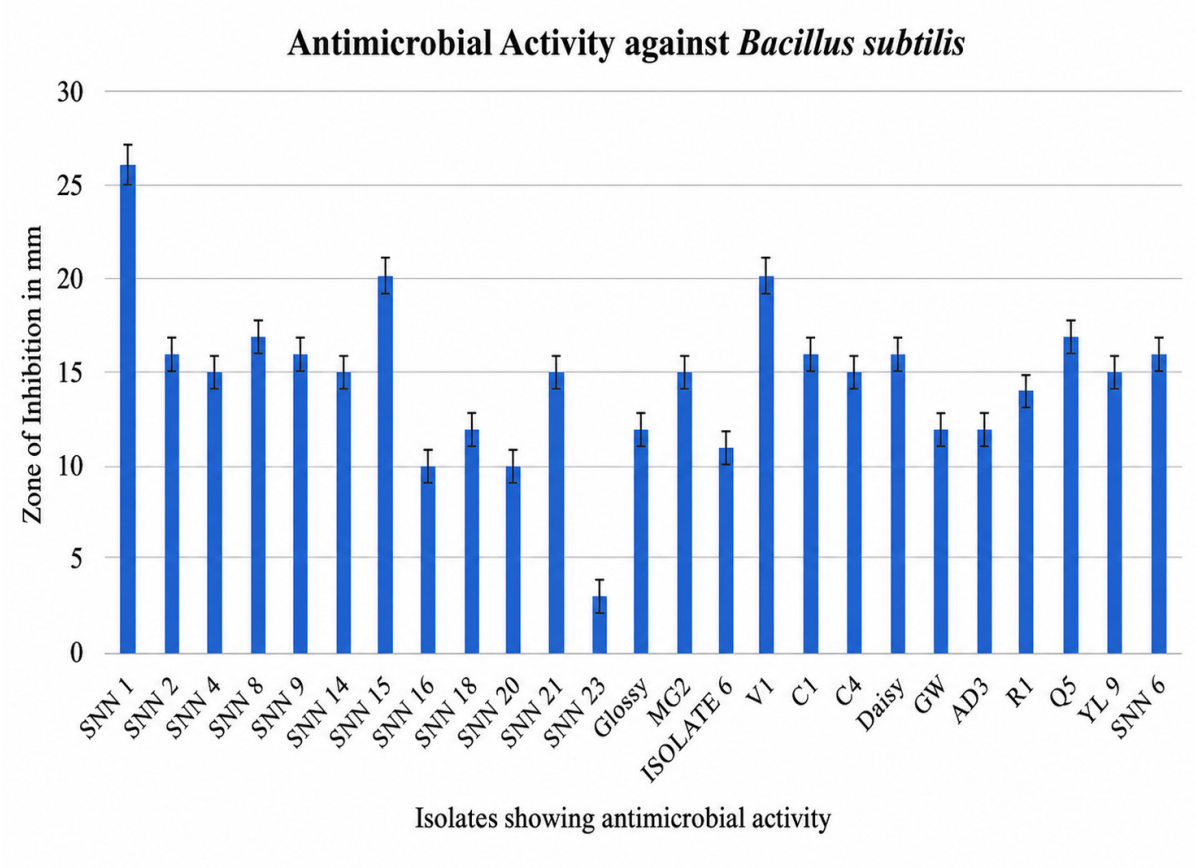
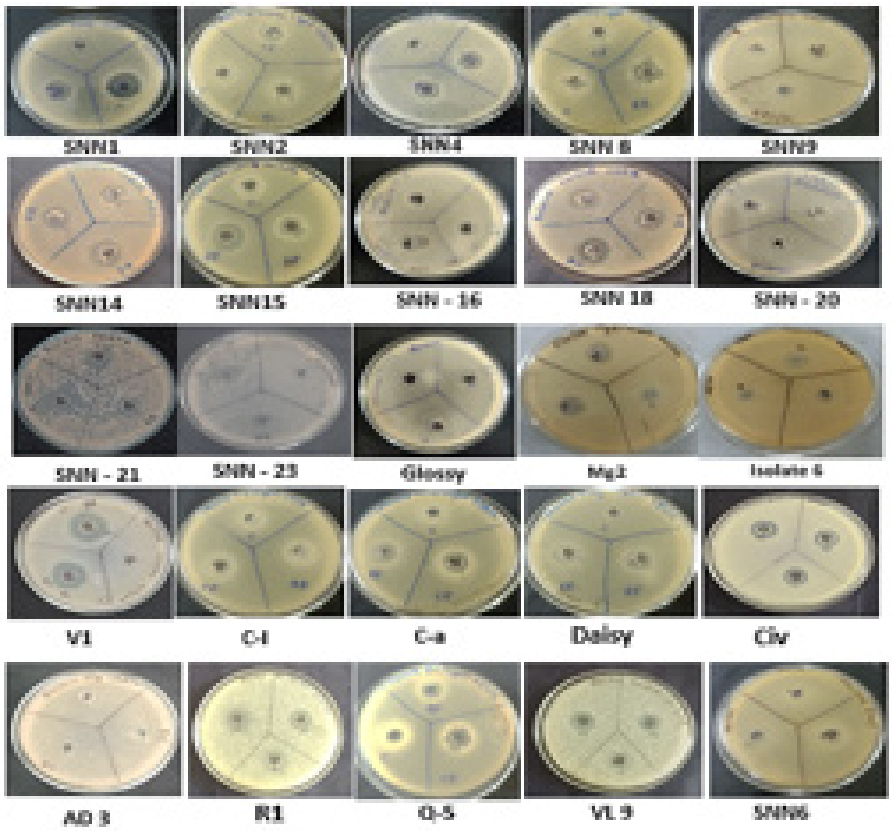


Figure S5: Collection of Extracts.

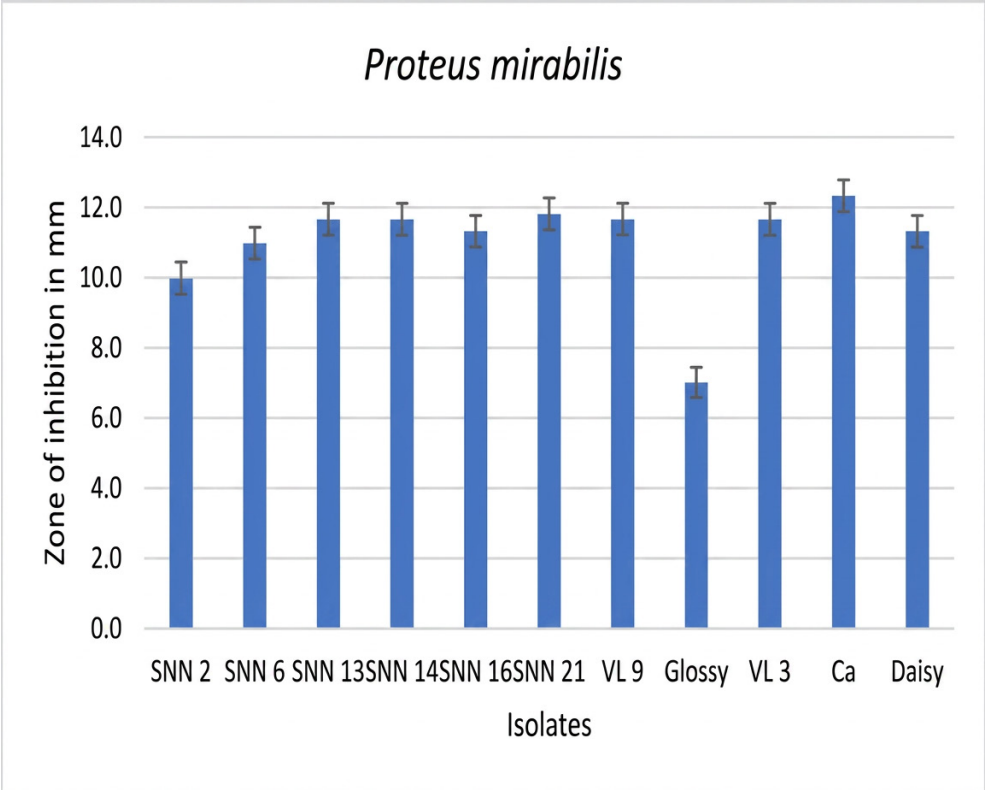


a)

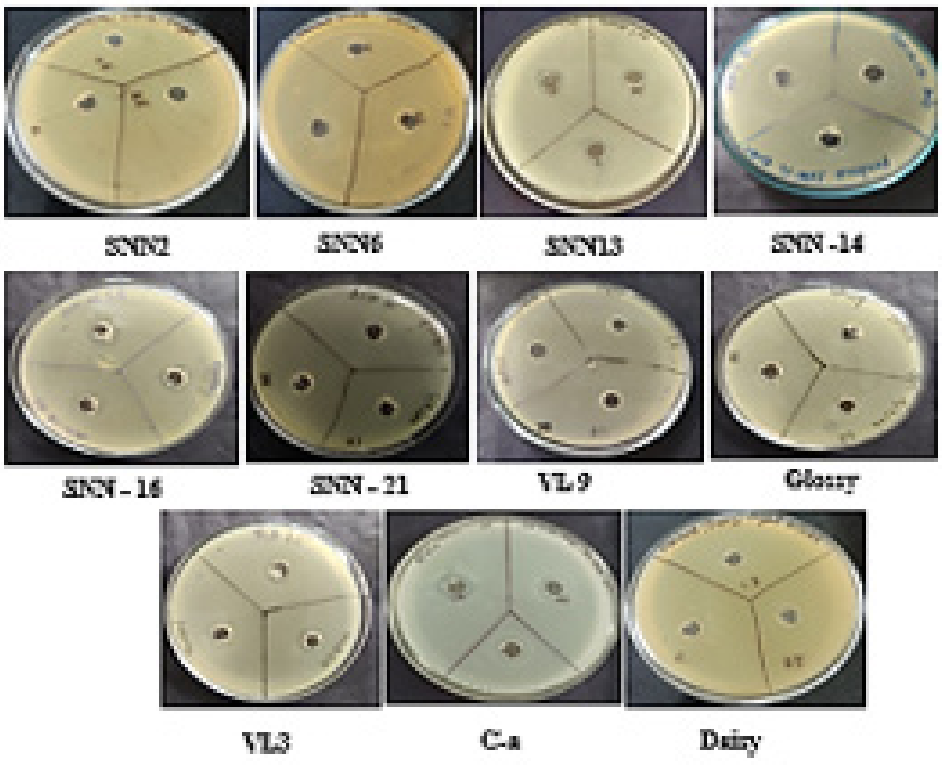


b)

Figure S6: a) and b) Extract having antimicrobial activity against *Bacillus subtilis*.

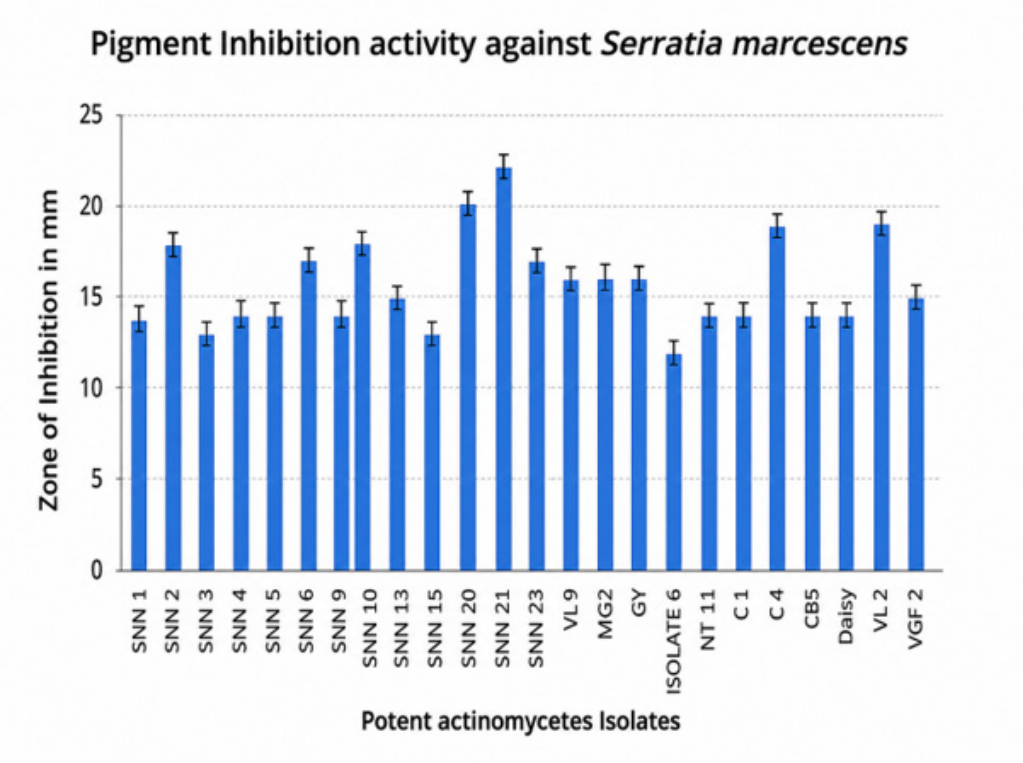


a)

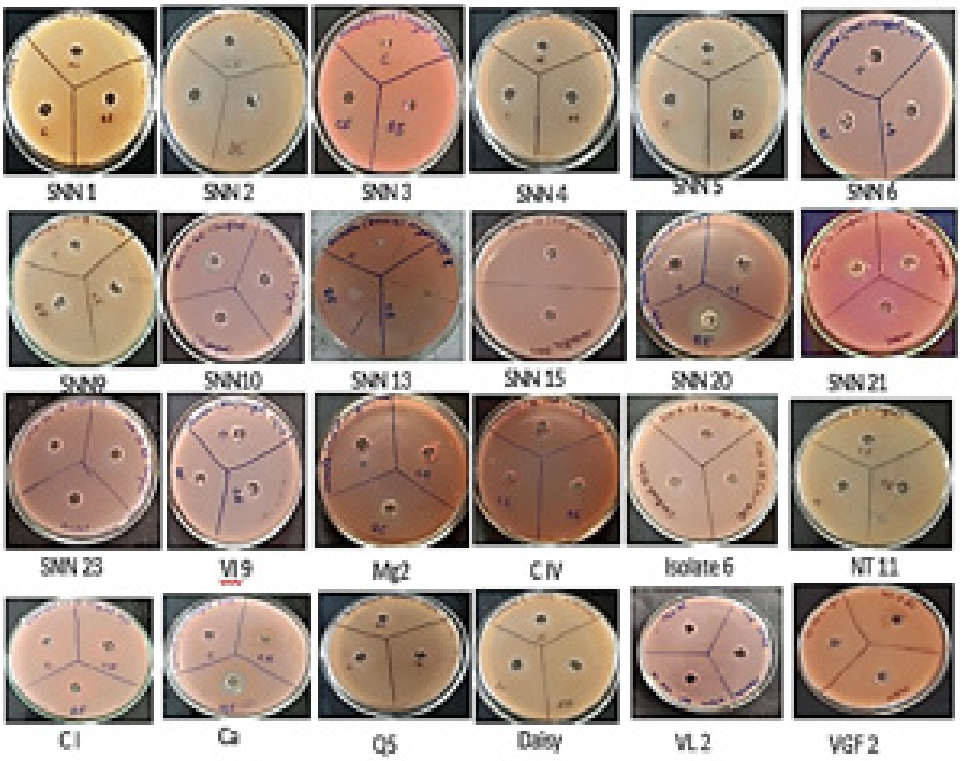


b)

Figure S7: a) and b) Anti-microbial activity of extracts against Proteus mirabilis.



a)



b)

Figure S8: a) and b) Pigment Inhibition activity of Extracts against *Serratia marcescens*.

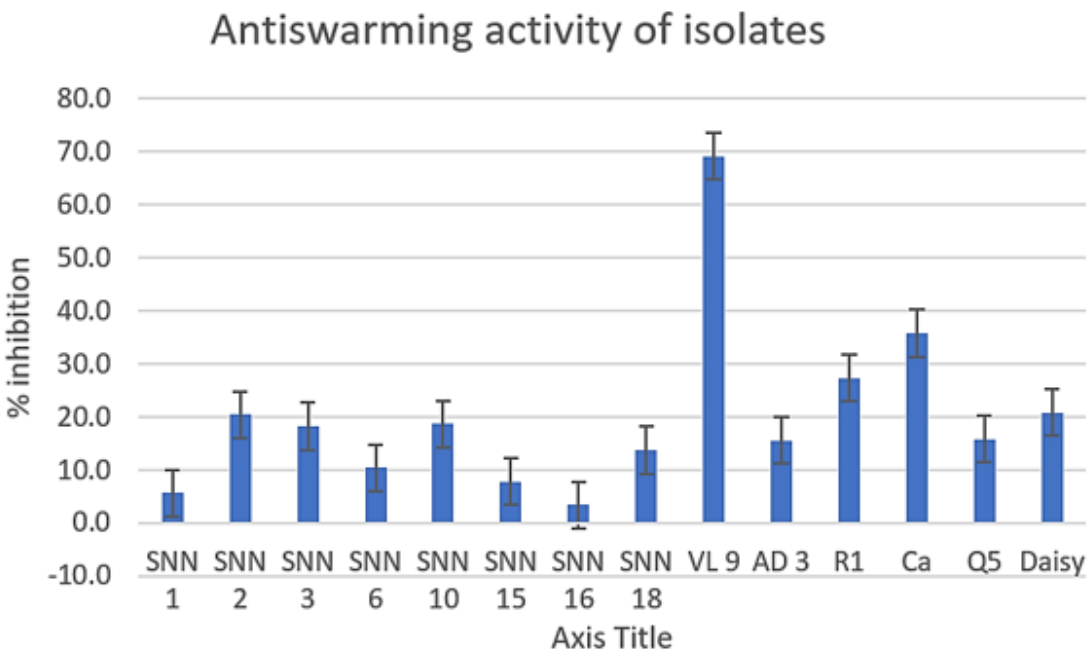
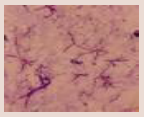
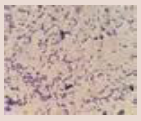
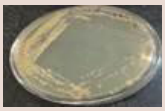
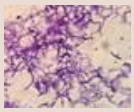
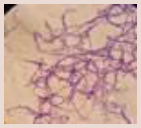
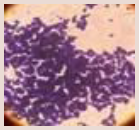
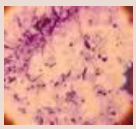


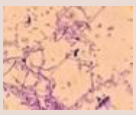
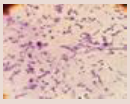
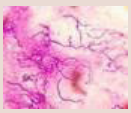
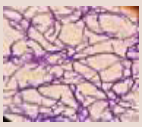
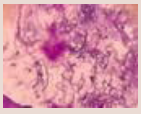
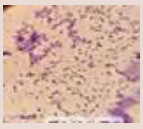
Figure S9: Anti-swarming activity of isolates.

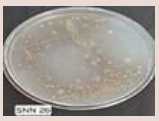
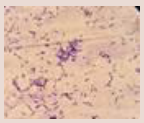
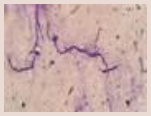
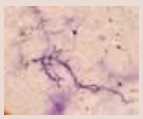
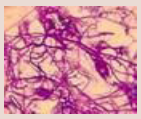
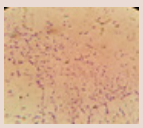
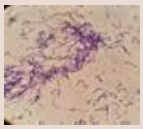
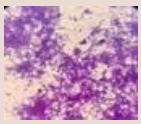
Table S1: Types of soil, location, and texture.

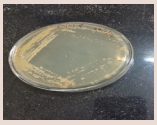
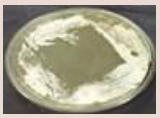
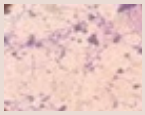
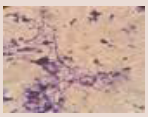
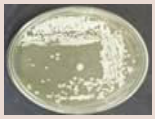
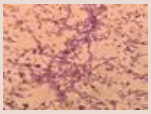
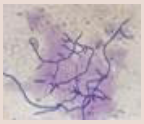
Sample No.	Type of Sample	Site	Latitude and Longitude.	Region	Texture	Soil
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2	Soil	Venna Lake	17.934°N 73.665°E	Mahabaleshwar, Maharashtra	Humous, coarse	
3	Soil	Melghat soil	210 45' N 770 31' E	Amravati, Maharashtra	Humous course	
4	Soil	Guhagar Beach	17.4890°N, 73.1847° E	Ratnagiri, Maharashtra	Coarse	
5	Soil	Vandangad Fort	17°50'20.7"N, 74°02'05.3" E	Satara, Maharashtra	Humus	

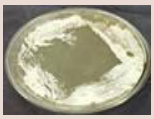
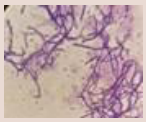
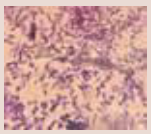
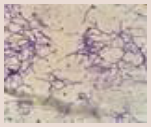
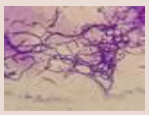
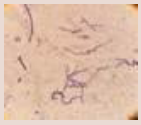
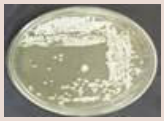
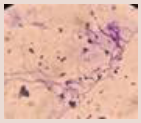
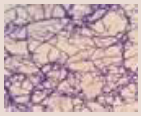
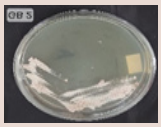
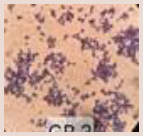
Table S2: Colony Characterization of Isolated Actinobacteria.

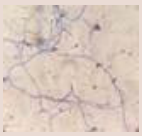
Sl. No.	Isolates	Colony characteristics		Pigment	Pretreatment	Pure culture	Microscopy
		Aerial	Substrate				
1.	SNN 1	Dark brown	Dark brown	Diffused	Heat at 120°C + BC		
2.	SNN 2	Creamish	Creamish	Non diffused	Heat at 120°C		
3.	SNN 3	Creamish	Yellow	Non diffused	Air Dry		
4.	SNN 4	White	Yellow	Non diffused	Heat at 120°C + BC		
5.	SNN 5	Pink	Pink	Diffused	Heat at 120°C + BC		
6.	SNN 6	Yellow	yellow	Non diffused	Air Dry		
7.	SNN 7	Yellow	Light yellow	Non diffused	Heat at 120°C		
8.	SNN 8	Yellow	yellow	Non diffused	Air Dry		
9.	SNN 9	Dark brown	Dark brown	Diffused	Air Dry		
10.	SNN 10	Dark blackish brown	Dark brown	Diffused	Heat at 120°C		
11.	SNN 11	Lemon yellow	Lemon yellow	Non diffused	Heat at 120°C + 1.5 % Phenol		
12.	SNN 12	Dark Brown	Dark brown	Diffused	Heat at 120°C		

13.	SNN 13	Whitish	Yellow white	Non diffused	Heat at 120°C + BC		
14.	SNN 14	White	Brown	Diffused	Air Dry		
15.	SNN 15	Brown	Dark brown	Diffused	Heat at 120°C + BC		
16.	SNN 16	Light Brown	Dark Brown	Diffused	Benzethonium Chloride		
17.	SNN 17	White	Yellow	Non-diffused	Benzethonium Chloride		
18.	SNN 18	Creamish	Creamish	Non-diffused	Heat at 120°C		
19.	SNN 19	Orange	Orange	Non-diffused	Heat at 120°C + BC		
20.	SNN 20	White	Yellow	Non diffused	Heat at 120°C		
21.	SNN 21	White	Yellow	Non diffused	Heat at 120°C		
22.	SNN 22	White	Brownish-yellow	Non diffused	Air Dry		
23.	SNN 23	White	White	Non diffused	Benzethonium Chloride		
24.	SNN 24	White	Yellow	Non diffused	Air Dry		
25.	SNN 25	Reddish brown	Brown	Diffused	Benzethonium Chloride		

26.	SNN 26	Whitish yellow	Brownish	Diffused	Benzethonium Chloride		
27.	SNN 27	White	White	Non diffused	Benzethonium Chloride		
28.	VL1	Whitish Yellow	White	Non diffused	Chloramine T		
29.	VL2	White	White	Non diffused	Benzethonium Chloride		
30.	VL3	Yellow	Yellow	Non diffused	Chloramine T		
31.	VL4	White	White	Non diffused	Chloramine T		
32.	VL5	Yellow	White	Non diffused	Benzethonium Chloride		
33.	VL6	Bright White	White	Non diffused	Chloramine T		
34.	VL7	Brownish white	Brown	Non diffused	Benzethonium Chloride		
35.	VL 8	Gray	White	Non diffused	1.5 % Phenol		
36.	VL 9	Yellow	Yellow	Non diffused	Benzethonium Chloride		
37.	MG 1	Brown	Dark brown	Diffused	Air Dry		
38.	MG 2	Yellow	Yellow	Non diffused	Chloramine T		

39.	MG 3	Gray	Gray	Non diffused	Air Dry		
40.	MG 4	Pale Yellow	Yellow	Non diffused	Air Dry		
41.	NT 11	White	White	Diffused	Air Dry		
42.	Ci	Dark Brown	Black	Diffused	Benzethonium Chloride		
43.	Civ	Whitish brown	Whitish yellow	Diffused	Benzethonium Chloride		
44.	Q5	Brown	Brown	Diffused	Benzethonium Chloride		
45.	Daisy	Pinkish white	White	Non diffused	Chloramine T		
46.	Ca	Dark Brown	Dark brown	Diffused	Benzethonium Chloride		
47.	Isolate 6	White	White	Non diffused	Chloramine T		
48.	R1	Pale Yellow	Yellow	Non diffused	Benzethonium Chloride		
49.	VGF 1	White	White	Non diffused	Heat at 120°C + 1.5 % Phenol		
50.	VGF 2	Gray	Gray	Diffused	Heat at 120°C + 1.5 % Phenol		

51.	VGF 3	Powdery white	White-Yellow	Non diffused	Benzethonium Chloride		
52.	VGF 4	White	White	Non diffused	Benzethonium Chloride		
53.	VGF 5	Yellow	Yellow	Non diffused	Benzethonium Chloride		
54.	VGF 6	White	White	Non diffused	Benzethonium Chloride		
55.	VGF 7	Brown	Yellow	diffused	Benzethonium Chloride		
56.	VGF 8	Yellowish white	Pale yellow	Non diffused	Benzethonium Chloride		
57.	VGF 9	White	White	Non diffused	Benzethonium Chloride		
58.	VGF 10	Milky White	Milky white	Non diffused	Benzethonium Chloride		
59.	GB 1	Gray	Brown	Non diffused	1.5 % Phenol		
60.	GB 2	Powdery white	White	Non diffused	Benzethonium Chloride		
61.	GB 3	Yellow	Yellow	Non diffused	1.5 % Phenol		
62.	GB 4	Yellow	Yellow	Non diffused	Benzethonium Chloride		

63.	GB 5	White	White	Non diffused	Benzethonium Chloride		
64.	GB 6	Orange	White	Non diffused	Benzethonium Chloride		
65.	GB 7	Brilliant yellow	White	Non diffused	Benzethonium Chloride		
66.	GB 8	Gray	Gray	Non diffused	Benzethonium Chloride		