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BIOPROSPECTING INDIAN COASTAL NICHES FOR MICROBIAL METABOLITES TO COMBAT QUORUM SENSING AND BIOFILM FORMATION

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ABSTRACT Biofilm associated infections and antimicrobial resistance are major global health concerns. This demands for discovery of novel therapeutic strategies including use of anti-virulence agents. This study explored Indian coastal niches for bacteria having the potential to produce metabolites with anti-quorum sensing and anti-biofilm activity. Coastal water samples collected from various coastal regions were enriched in TSB, ISP1 and ISP2 media. Out of 122 isolates obtained, 39 isolates exhibited potential of inhibiting violacein production by *Chromobacterium violaceum MTCC 2656* indicating anti-quorum sensing activity. Quantitative assays determined that strains G2 and M9 exhibited 69.7% and 72.3% inhibition of violacein production respectively. Ethyl acetate extracts of these isolates exhibited strong anti-quorum sensing activity and led to significant inhibition of biofilm formation and moderate eradication of biofilms formed by *Staphylococcus aureus MTCC 3160* and *Pseudomonas aeruginosa MTCC 2453.* This study highlights Indian coastal microorganisms as potential source of bioactive metabolites that can act as novel antibiofilm and anti-virulence agents.

KEYWORDS : Biofilms, Antimicrobial Resistance, Quorum Sensing, Bioactive, Anti-Virulence

INTRODUCTION

Biofilm based infections account for eighty percent of the chronic infections across the globe. Biofilms are also a niche for dissemination of antimicrobial resistance genes making treatment of such infections challenging (Assefa & Amare, 2022; Grari et al., 2025). There is a need to prospect for new antimicrobial agents especially agents which can inhibit and break biofilms.

Quorum sensing (QS) is a communication system that allows bacteria to detect their population density and coordinate activities like production of virulence factors and biofilm formation (Vasavi et al., 2014). This communication is carried out using small signaling molecules called autoinducers which increase in concentration with increase in cell density (Al-Haidari et al., 2016). These autoinducers modulate gene expression and govern social behavior of the cells in a community (Vasavi et al., 2014).

Biofilms evade antibiotics by producing extracellular polymeric substance (EPS) thereby prohibiting the access to the pathogens (Assefa & Amare, 2022). The way to combat biofilms is by using agents which disrupt adhesion of cells, signaling mechanisms that underline quorum sensing, and emulsification of extracellular matrix (Behzadnia et al., 2024). The rise of antimicrobial resistance and biofilm associated infections has triggered interest in bioprospecting marine microorganisms as sources of novel antimicrobial agents. Rather than killing the pathogen, many marine metabolites target quorum-sensing and biofilm formation which act as potential anti-virulence strategy. This may also aid in reducing resistance development in the pathogens (Chang et al., 2017).

Coastal niches host ecological and chemical diversity, micro-organisms, algae, invertebrates and their symbionts which produce secondary metabolites having antimicrobial, antibiofilm and anti-inflammatory properties. Bioprospecting is a systematic exploration of biodiversity for useful compounds. This involves sampling, screening for activity, characterization and development of high value products (Teasdale et al., 2009). Coastal microbes are known to produce secondary metabolites like antibiotics, surfactants, peptides, polyketides or polysaccharides that inhibit quorum sensing regulated pigments, virulence factors and biofilm formation (Sukmarini et al., 2024).

This study focuses on sampling, isolating coastal bacteria and screening for their secondary metabolites which have potential anti-quorum sensing activity and can inhibit the formation of biofilm.

MATERIALS AND METHODS

Sample Collection

Water samples were collected from Girgaon, Miramar, Malgund, Bhogwe, Sindhudurg, Malvan, Tarkarli, Gorai, Ganpatipule, Murud beaches in sterile 1 liter container.

Isolation of Bacteria from Coastal Water Samples

Each water sample was passed through 0.4µ membrane filter. The filter was cut into three pieces under aseptic conditions and inoculated in Tryptic Soy broth (TSB) (17 g/L pancreatic digest of casein, 3 g/L papaic digest of soybean meal, 5 g/L sodium chloride, 2.5 g/L dextrose, and 2.5 g/L dipotassium phosphate, pH 7.3), International Streptomyces Project 1 broth (ISP1) (5g/L tryptone, 3g/L yeast extract, pH 7.0), International Streptomyces Project 2 broth (ISP2) (4g/L yeast extract, 10g/L malt extract, 4g/L dextrose, pH 7.3) for isolation of halotolerant bacteria (Azmin et al., 2024) (Juboi, H. et al., 2019). The inoculated media were incubated at 27^{0}C for 24 hours. Post incubation the bacteria were isolated on respective agar plates and incubated at 27^{0}C for 24 hours. Colonies were selected and reisolated to obtain pure bacterial colonies. Gram nature of all isolates was determined by Gram staining technique.

Anti-quorum Sensing Spot Assays

Molten tryptic soy/ISP1/ISP2 agar butts (20mL) were bulk seeded with culture suspension of 24 hours old Chromobacterium violaceum MTCC 2656 having optical density of 0.2 at 600 nm having cell density of approximately 1*10^8 cells/mL and poured into sterile Petri dishes. Post solidification, loopful of culture suspension of test isolates having an optical density of 0.1 at 600nm were spotted on these plates. The plates were then incubated at 27^{0}C for 24 hours. Inhibition of violacein pigment production by *Chromobacterium violaceum MTCC 2656* around the growth spots were considered as positive for anti-quorum sensing potential of the test isolates (Kachhadia et al., 2022). Triplicates of the assay were performed.

Quantitative Anti-quorum Sensing Assay

The positive strains from the spot assay where subjected to the quantitative estimation. In microfuge tubes, 800 µL of sterile tryptic soy /ISP1/ISP2 broth was added. To these 100 µL of culture suspension of 24 hours old *Chromobacterium violaceum MTCC 2656* having optical density 0.1 at 600 nm was added. 100 µL culture suspension of 24 hours old test isolates having optical density 0.1 at 600 nm was added to respective medium. Tubes containing 200 µL of sterile tryptic soy broth were maintained as sterility control. While tubes with 900 µL of sterile tryptic soy broth and 100 µL culture suspension of 24 hours old *Chromobacterium violaceum MTCC 2656* having optical density 0.1 at 600 nm were maintained as assay control for active quorum-sensing. The tubes were incubated at 27^{0}C for 24 hours. The tubes were then centrifuged at 10000rpm for 10 minutes at 4^{0}C. The supernatant was discarded and the pellet was dissolved in 1mL of DMSO. The tubes were centrifuged at 10000rpm for 10 minutes at 4^{0}C and the supernatant was transferred to 96 well plate and the absorbance was measured using ELISA plate reader at 585 nm (Chenia, 2013; Luís & Domingues, 2025). The anti-quorum sensing activity was calculated as follows:

% Inhibition of violacein production= (Absorbance of control$_{585nm}$-Absorbance of test$_{585nm}$)/ Absorbance of control$_{585nm}$

This assay was performed in triplicates for assessing repeatability of the anti-quorum sensing potential. The data was statistically analysed by one-way ANOVA and the p value was determined.

Extraction of the Secondary Metabolite:

1mL culture suspension of the active isolate having optical density 0.1 at 600nm was inoculated in 500mL Tryptic soy broth. The medium was incubated at 27^{0}C for 24 hours. Post incubation, the medium was centrifuged at 10000rpm for 30minutes at 4^{0}C. The supernatant was collected in a fresh flask and equal volume of ethyl acetate was added. The flask was then kept on shaker at 150rpm for 3 hours. The mixture was then poured into a separating funnel and was let to stand for 3 hours. The ethyl acetate fraction was then collected in a beaker and was evaporated at 60^{0}C (Abdel-Nasser et al., 2023) . The powder obtained post evaporation was then dissolved in 5mL of sterile distilled water and stored at 4^{0}C until further use.

Agar Well Assay for Anti-quorum Sensing Activity

Tryptic soy agar butts (20mL) were bulk seeded with 1mL of culture suspension of 24 hours old *Chromobacterium violaceum MTCC 2656* having optical density of 0.2 at 600 nm having cell density of approximately 1*10^8 cells/mL and poured into sterile Petri dishes. Post solidification, wells were bored using a sterile cork borer. 50 µL of the test extracts were added to the wells, 50 µL of 1% acetic acid was used as positive control for anti-quorum sensing activity while 50 µL of sterile distilled water was used as negative control. The plates were incubated at 27^{0}C for 24 hours (Dereli et al., 2023; Vasavi et al., 2014). The assay was performed in triplicates.

Assay for Inhibition of Biofilm Formation

180 µL of trypticase soy broth was added to each well of a 96 well plate excluding the edges to avoid edge effect in biofilm formation. 10 µL of the test extract was added to the wells. 10 µL of culture suspension of 24 hours old biofilm forming cultures *Pseudomonas aeruginosa MTCC 2453* or *Staphylococcus aureus MTCC 3160* having optical density 0.1 at 600 nm was added. Wells containing 200 µL of trypticase soy broth was maintained as sterility control. While wells containing 190 µL of trypticase soy broth and 10 µL of the biofilm forming culture suspension was maintained as biofilm formation control. The plates were incubated at 37^{0}C for 24 hours. Post incubation, the spent broth was aspirated and the wells were washed with 200 µL of sterile saline. The wells were dried for an hour and 200 µL of 0.1% crystal violet solution was added to the wells and the plate was incubated at room temperature for 30 minutes. The stain was aspirated and the wells were washed with sterile distilled water. Post this 200 µL of 33% acetic acid was added to the wells and the plates were incubated at room temperature for 20 minutes. Absorbance was measured at 599 nm (Cruz et al., 2018; Gupta, 2015; Zai et al., 2021). The assay was performed in five replicates. Percentage inhibition of biofilm formation was calculated as follows:

% inhibition = (Absorbance of biofilm formation control $_{599nm}$ – Absorbance of test $_{599nm}$)/ O.D. of biofilm formation control $_{599nm}$

The data was statistically analysed by one-way ANOVA and the p value was determined.

Assay for Eradication of Biofilm

180 µL of trypticase soy broth was added to each well of a 96 well plate excluding the edges to avoid edge effect in biofilm formation. 20 µL of culture suspension of 24 hours old biofilm forming cultures *Pseudomonas aeruginosa MTCC 2453* or *Staphylococcus aureus MTCC 3160* having optical density 0.1 at 600 nm was added. Wells containing 200 µL of trypticase soy broth was maintained as sterility control. The plates were incubated at 37^{0}C for 24 hours (Rajagopalan & Lokur, 2026). Post incubation, the spent broth was aspirated and the wells were washed with 200 µL of sterile saline. To the test wells 200 µL of the test extracts were added. To some of the wells 200 µL of sterile saline was added. These wells act as untreated controls. The plates were incubated at 37^{0}C for 24 hours. Post incubation, the solution was aspirated from the wells and were washed using sterile distilled water. The wells were dried for an hour and 200 µL of 0.1% crystal violet solution was added to the wells and the plate was incubated at room temperature for 30 minutes. The stain was aspirated and the wells were washed with sterile distilled water. Post this 200 µL of 33% acetic acid was added to the wells and the plates were incubated at room temperature for 20 minutes. Absorbance was measured at 599 nm (Thieme et al., 2019; Zai et al., 2021). The assay was performed in five replicates. Percentage eradication of biofilm formation was calculated as follows:

% eradication = (Absorbance of untreated control $_{599nm}$ – Absorbance of test $_{599nm}$)/ Absorbance of untreated control $_{599nm}$

The data was statistically analysed by one-way ANOVA and the p value was determined.

RESULTS

Isolation of Bacteria from Coastal Water Samples

A total of 122 bacterial isolates were obtained from enrichment of the coastal water samples in TSB, ISP1 and ISP2 broth. TSB supported the growth of highest number of isolates both Gram positive and Gram negative while ISP1 and ISP2 enriched Gram positive organisms.

Table 1: Details of Isolates Obtained from Enrichment of Coastal Water Samples

Medium	Total Isolates	Gram Positive	Gram Negative	Gram nature variable
TSB	58	43	11	4
ISP1	32	30	2	-
ISP2	32	32	-	-

Anti-quorum Sensing Spot Assays

Out of 122 strains, 39 strains exhibited inhibition of violacein production around the test colonies. Since violacein synthesis is regulated by quorum sensing, the reduction of the purple pigment around test colonies indicates interference with quorum sensing signaling. Around 32% of the isolates exhibited potential anti-quorum sensing activity.

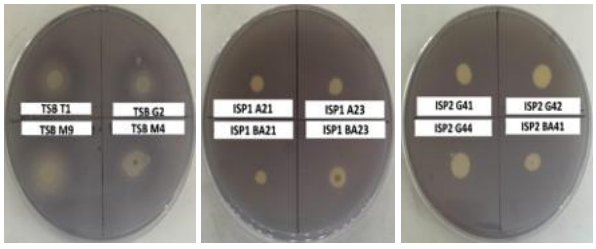


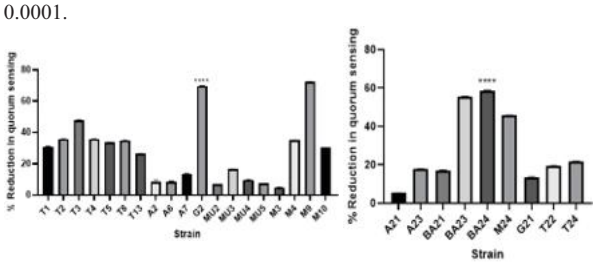
Figure 1: Spot assay for Screening Anti-quorum Sensing Potential of Isolates Against C. violaceum MTCC 2656

Table 2: Details of Strains Indicating Inhibition of Violacein Production in Spot Assay

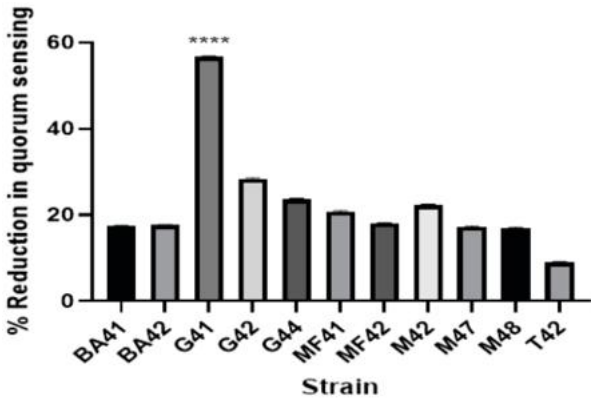
Medium	Number of strains exhibiting inhibition of violacein production	Strain codes exhibiting inhibition of violacein production
TSB	19	T1, T2, T3, T4, T5, T8, T13, A2, A6, A7, G2, MU2, MU3, MU4, MU5, M3, M4, M9, M10
ISP1	09	A21, A23, BA21, BA23, BA24, M24, G21, T22, T24
ISP2	11	BA41, BA42, G41, G42, G44, MF41, MF42, M42, M47, M48, T42

Quantitative Anti-quorum Sensing Assay

TSB strains G2 and M9 exhibited 69.7% and 72.3% inhibition of violacein production respectively, while ISP1 strains BA 23 and BA24 exhibited 55.4% and 58.6% inhibition of violacein production respectively, and ISP2 strain G41 exhibited 56.6% inhibition of violacein production. These findings indicate that strains M9 and G2 have strong quorum quenching activity. This also indicates that these organisms produce diffusible metabolites that inhibit quorum sensing signaling in *C. violaceum MTCC 2656.* The data obtained was analysed using one-way ANOVA and the p value was found to be < 0.0001.



(a) **(b)**



(C)

Figure 2: Quantification of Anti-quorum Sensing Activity Against *C. violaceum MTCC 2656:* Quorum Sensing Inhibition Potential of a) TSB Strains b) ISP1 Strains c) ISP2 Strains

Extraction of the Secondary Metabolite

Post evaporation of ethyl acetate, 100mg and 96mg of the metabolite was obtained from 500 mL of production system of G2 and M9 strain respectively. These metabolites were dissolved in sterile distilled water and used for further assays.

Agar Well Assay for Anti-quorum Sensing Activity

The extracted metabolites exhibited clear zone of pigment inhibition in the agar well diffusion assay. These zones indicate that metabolites are extracted and are functional. Also, it confirms that the activity can be attributed to extracellular metabolites rather than competitive growth effect.

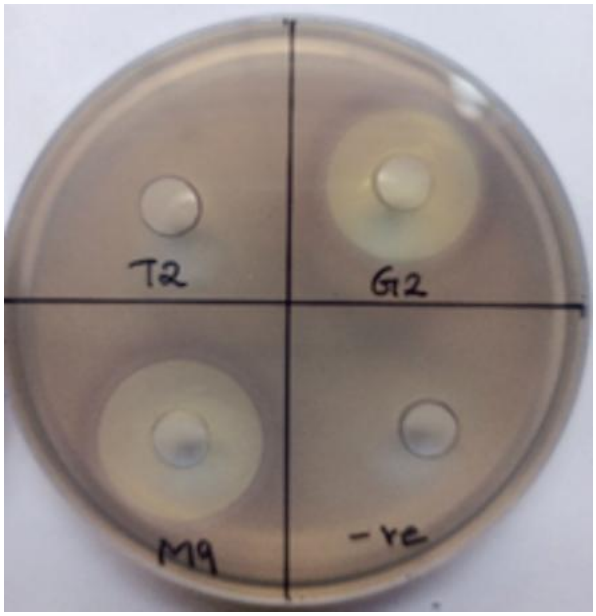


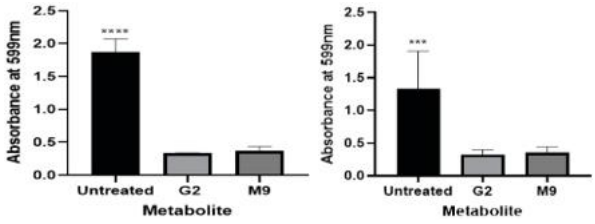
Figure 3: Inhibition of Violacein Production by Metabolites G2 and M9

Table 3: Average Zone Diameter of Inhibition of Violacein Production by Metabolites G2 and M9

Metabolite obtained from strain	Average Zone diameter of inhibition (mm)
G2	24
M9	22.5

Assay for Inhibition of Biofilm Formation

Metabolites extracted from G2 exhibited 82.16% and 75.32% inhibition while those from M9 exhibited 80.31% and 73.41% against *Staphylococcus aureus MTCC 3160* and *Pseudomonas aeruginosa MTCC 2453.* These results indicate potent anti-biofilm activity against both Gram positive and Gram negative bacteria used in this study. The data obtained was analysed by one-way ANOVA and the p value was found to be <0.0001 and 0.0007 for *Staphylococcus aureus MTCC 3160* and *Pseudomonas aeruginosa MTCC 2453* respectively.



(a) (b)

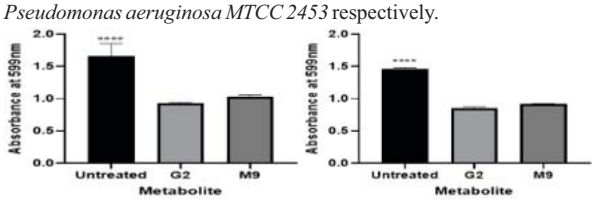
Figure 4: Inhibition of Biofilm Formation by Metabolites G2 and M9 Against a) *Staphylococcus Aureus MTCC 3160* b) *Pseudomonas Aeruginosa MTCC 2453*

Table 4: Biofilm Inhibition Potential of Metabolites G2 and M9 Against *Staphylococcus Aureus MTCC 3160* and *Pseudomonas Aeruginosa MTCC 2453*

Metabolite obtained from strain	% Inhibition of *S. aureus MTCC 3160* Biofilm formation	% Inhibition of *P. aeruginosa MTCC 2453* Biofilm formation
G2	82.16	75.32
M9	80.31	73.41

Assay for Eradication of Biofilm

The metabolites exhibited partial eradication of preformed biofilms. Metabolites extracted from G2 exhibited 82.16% and 75.32% while those from M9 exhibited 80.31% and 73.41% eradication potential against *Staphylococcus aureus MTCC 3160* and *Pseudomonas aeruginosa MTCC 2453* biofilms. Mature biofilms are highly resistant to antibiotics and disinfectants. The ability of the extracts to partially eradicate preformed biofilms indicates that the metabolites may disrupt extracellular polymeric substances, alter the stability of biofilm or emulsify the matrix thereby weakening the adherence of cells. The data obtained was analysed by one-way ANOVA and the p value was found to be <0.0001 for both *Staphylococcus aureus MTCC 3160* and *Pseudomonas aeruginosa MTCC 2453* respectively.



(a) (b)

Figure 5: Eradication of a) *Staphylococcus Aureus MTCC 3160* and b) *Pseudomonas Aeruginosa MTCC 2453* Biofilms by Metabolites G2 and M9

Table 5: Biofilm Eradication Potential of Metabolites G2 and M9 Against *Staphylococcus aureus MTCC 3160* and *Pseudomonas Aeruginosa MTCC 2453* Biofilms

Metabolite obtained from strain	% eradication of *S. aureus MTCC 3160* Biofilm	% eradication of *P. aeruginosa MTCC 2453* Biofilm
G2	44.00%	41.67%
M9	33.94%	37.28%

DISCUSSION

Biofilm associated infections are a major healthcare challenge across the globe. Their persistence, antibiotic resistance and ability to evade host immune response makes it challenging to treat. In this study, Indian coastal niches were explored as potential reservoirs of bacteria having the ability to produce metabolites that can interfere with quorum sensing and biofilm formation (Sukmarini et al., 2024).

Coastal niches are competitive environments due to fluctuating salinity, nutrient availability, temperature, pollutants and microbial diversity. These stress factors promote the evolution of micro-organisms which can produce unique secondary metabolites for survival (Ojha & Kachhadiya, 2024). Among 122 isolates screened, 39 isolates inhibited violacein pigment production in *C. violaceum MTCC 2656.* Out of which strains M9 and G2 exhibited strong quorum quenching activity with 72.3% and 69.7% reduction in violacein production respectively. This indicates that these two strains may be producing metabolites which are extracellular, diffusible and have the ability to interfere with synthesis, transport or receptor binding of signaling molecules (Borges & Simões, 2019).

The extracellular metabolites extracted using ethyl acetate exhibited

inhibition zones of 24mm and 22.5mm for G2 and M9 respectively in the agar well diffusion assay. Ethyl acetate extraction is commonly used for recovering semi-polar secondary metabolites like phenolics, cyclic peptides, lactones or polyketides etc (Borges & Simões, 2019). Further purification and structural characterization of these metabolites using chromatographic and spectroscopic methods is necessary to understand the chemical nature and identity of these metabolites (Abdel-Nasser et al., 2023; Vijay et al., 2021).

It was also found that G2 and M9 metabolites exhibited significant inhibition of biofilm formation of *Staphylococcus aureus MTCC 3160* and *Pseudomonas aeruginosa MTCC 2453.* Slightly lower inhibition observed in case of *Pseudomonas aeruginosa MTCC 2453* may be due to its complex quorum sensing network and its robust extracellular polymeric matrix (Thi et al., 2020; Zhang et al., 2025). They also exhibited biofilm eradication activity against preformed biofilms. Though the potential to eradicate biofilms is low, it still offers an avenue to use these agents in combination with other antibiofilm agents to achieve better removal of preformed biofilms.

Molecular identification of the isolates, purification and characterization of active compounds, toxicity assessment and mechanism studies will provide insights into their therapeutic applicability.

CONCLUSION

This study proves that Indian coastal niches are valuable reservoirs of microorganisms which have the capability to produce anti-quorum sensing and anti-biofilm metabolites. 32% of the isolates exhibited anti-quorum sensing activity against C. violaceum MTCC 2656. Metabolites extracted from isolates G2 and M9 exhibited strong activity, reducing violacein production and potent inhibition and eradication of biofilms formed by *Staphylococcus aureus MTCC 3160* and *Pseudomonas aeruginosa MTCC 2453.* The study indicates the potential of coastal microbial metabolites as anti-virulence agents to combat biofilm associated infections. Further characterization of these metabolites may aid in development of unique therapeutic strategies.

Acknowledgement

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