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Marine Microorganisms as Potential Source of Quorum-Sensing Inhibitory Compounds

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Abstract

Antibiotics are extensively utilized globally, and the rate of antibiotic resistance is increasing more rapidly than ever. Since the introduction of antibiotics, the overuse of antibiotics caused selection pressure, and almost all pathogenic bacteria have developed resistance against commonly used antibiotics, which is particularly known as antimicrobial resistance (AMR). It has proven challenging to combat and resolve the issue of microbial resistance. A growing number of microorganisms have developed different resistance mechanisms and turned into "superbugs". The identification of quorum-sensing systems has given researchers a new hope for understanding drug resistance processes. The quorum-sensing system controls a number of cellular functions, including the expression of pathogenic genes, the synthesis of toxins, and the synthesis of extracellular polysaccharides. To targeting QS systems marine bacterial samples were collected by different coastal sites of Gujarat, like Mandvi, Dwarka and Diu. A total of 52 marine isolates were obtained, out of which 16 were associated with various marine macro-organisms like sponges and algae, whereas 36 were free-living. The study has done by screening those marine isolates shown potential to inhibiting the quorum sensing molecule by co culture study against Chromobacter violaceium, followed by growth inhibition assay. The G14 and G82 isolate shows highest degradation of N-acyl homoserine lactone by AHL study. The QSI compounds were extracted using ethyl acetate extraction method. Biofilm inhibition assay with Pseudomonas aeruginosa shows potent inhibitory effect of extract. These results prove that marine microorganism have potential to inhibits quorum sensing and virulence factors regulated by Quorum Sensing phenomenon.

Keywords: *Antimicrobial resistance; Biofilm inhibition; Pseudomonas aeruginosa infection; Quorum sensing; Quorum sensing inhibition; Violacein inhibition*

1. Introduction

The pathogenicity of bacteria depends on its capacity to sense and react to its surroundings as well as to adapt to a new environment. Numerous mechanism that bacteria have evolved to sense a wide range of environmental conditions, including pH, osmolarity, food availability and population density. Bacteria sense changes in their surroundings and adjust their production of several components. Quorum sensing is process of cell-to-cell communication which allows bacteria to perceive the density of the surrounding bacterial population and to coordinately respond to this signal by regulating various genes. QS Was first discovered in marine luminescent bacterium Vibrio fischeri (Neaslon and Hastings 1979) in which it regulates the luminescent activity of luciferase in the light organ of its symbiotic partner Hawaii squid (Euprymna scolopes, Waters & Bassler, 2005). Bacteria uses three types of signaling communication mechanisms. Gram negative bacteria use LuxI – LuxR signaling system which typically produced acylated homoserine lactone

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(LaSarre & Federle, 2013) (Papenfort & Bassler, 2016) whereas Gram positive bacteria use small peptides or oligopeptides. (Bhatt, 2018) The autoinducer-2 signal molecule has been detected in both Gram positive and Gram negative bacteria, therefore thought to be a universal signaling molecule allowing bacteria to sense other bacterial population. (Haque et al., 2019). Quorum sensing is the regulation of gene expression and plays effective role in virulence, biofilm formation, pigment production and gene transcription. Some important human pathogens show QS dependent virulence includes Pseudomonas aeruginosa, Vibrio harveyi, Vibrio cholera, Staphylococcus aureus, Staphylococcus pneumonia, and Escherichia coli (LaSarre & Federle, 2013). P. aeruginosa responsible for nosocomial infections, (Reynolds & Kollef, 2021) Vibrio harveyi causes diseases in vertebrates and invertebrates includes vasculitis, gastro- enteritis and eye lesions. (Zhang et al., 2020) Some plant pathogens includes Agrobacterium tumefactions causing crown gall and Erwinia carotovora responsible for soft rots are the most popular plant pathogens showing QS dependent virulence. (Gohlke & Deeken, 2014) (Andersson et al., 2000). Antibiotics are widely used to treat these pathogens. Many microorganisms have ability to resist against it and it has proven challenging to combat and resolve the issue of microbial resistance due to the presence of numerous antibiotic resistance mechanisms. (Zhao et al., 2020) Therefore, development of alternative therapeutic approach is in demand [1-5]. Quorum quenching pathways and quorum sensing inhibitor mechanism may lead to reduce the virulence. In order to control bacterial pathogenesis, quorum quenching can be employed as an antivirulence strategy. (Tang & Zhang, 2014). Marine ecosystem consists of vast diversity and marine microbial species have been unexplored. Novel compounds with QS inhibitory (QSI) properties have been found in abundance in the marine environments, which is well known for its biodiversity. It has been discovered that certain of these substances are made by marine bacteria, either as defense mechanism against rival microbes QS systems or as a component of their own quorum sensing mechanism. (Zhao et al., 2020), (Borges & Simões, 2019) Two novel QSI depsipeptides were identified from marine Photobacterium are Solonamide A and B which shows inhibition against Staphylococcus aureus arg dependent QS pathway. It was a first report of arg QS inhibitory pathway from marine bacteria. Another such study finds the isolation of three active metabolites from Oceanobacillus sp. The active metabolites are identified as 2-Methyl-N-(2-phenylethyl)-butyramide, 3-Methyl-N-(2-phenylethyl)-butyramide and benzyl benzoate. The three metabolites are first reported to inhibit QS activities against C. violaceium and P. aeruginosa (Chen et al., 2019). In this article, we demonstrate potential of marine microorganism producing QS inhibitory molecule. Finding of these molecules may be the future resources in new drug discovery. It carries less chance of getting resistance as QSI molecules do not kill the microorganism or inhibits the growth, rather it only suppresses the virulence factors produce by the pathogen.

2. Materials and Method

2.1 Sample Collection

Marine water and/with marine macroorganisms such as marine algae with sediment samples were collected from various undisturbed costal sites of Gujarat which include Diu, Devbhoomi-Dwarka and Mandvi. These samples were collected at depth of 0.5 to 1 m. All the samples were collected in sterile plastic container and processed within 24 h to avoid spoilage of macroorganism. Samples were stored at 4°C until use.

2.2 Isolation of Marine Bacteria

Samples pretreatment and isolation procedures were carried out according to (Singh et al., 2020). To isolate free-living marine bacterial each marine water sample were diluted up to 10-3 dilution in Luria Bertani Broth (LB; Himedia, India) and uniformly spreaded over Luria Bertani Agar plate (LB; Himedia, India) containing 2% NaCL, followed by incubation at 37°C for 1 to 7 days. To isolate macroorganisms associated marine bacteria, all macroorganism were surface-sterilized with 70%

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ethanol and washed twice with sterile seawater then macerated individually in sterile seawater and the homogenate was diluted up to 10-3 dilution in LB Broth [6]. From each dilution tube 100 µL of sample was uniformly spread on LB Agar plate containing 2% NaCL, followed by incubation at 37°C for 1 to 7 days. For pure culture, all morphologically diverse colonies were transfer into fresh LB plate.

2.3 Screening of Isolates for Quorum Sensing Inhibition

Co-culture study was done with Chromobacter violaceium of marine isolates: To check weather marine isolates, have capacity to produce quorum quenching compounds or have potential to inhibit the purple pigment violacein produced by Chromobacter violaceium (CV). Violacein is QS mediated pigment produced by the strains of Chromobacterium. All the marine isolates were inoculated with Chromobacter violaceium in 96 well plate. The inoculation was done in LB broth medium. Over LB broth Chromobacter violaceium culture was added and overnight grown marine respective cultures were inoculated. The plates were incubated at 28□C for 24 hrs. under shaking. After 24 hrs. of incubation observation taken as positive if purple pigment violacein was inhibited by marine respective cultures. Absence of purple pigment shows the ability of marine isolates to produce quorum quenching compounds. CV with LB taken as negative control and CV with marine respective cultures taken as positive control. To observe the more accurate result, spread the cultures on LB agar plate.

Growth inhibition assay of marine respective culture gives positive result: To check the capacity of marine isolates to inhibit CV, LB agar plates were prepared. Centrifuge out the supernatant from isolates. Pour 100□l of supernatant in well and incubate for 24hrs. The zone surrounding the well shows positive result.

2.4 Extraction of Quorum Quenching Compounds from Marine Isolates

Selected marine isolates were overnight grown in LB broth. Overnight grown cultures were centrifuged. For extraction of supernatant Ethyl Acetate was used as solvent. On hot plate magnetic stirrer supernatant was overnight mixed with equal volume of Ethyl Acetate. Ethyl acetate is then evaporated using rotary vacuum evaporator at 60ºC to dryness. The compound was then mixed with 5ml of methanol and used for bioassay with CV. To determine the potential of crude, extract the following assay was done (a) Well diffusion method- LB agar plates were prepared, LB agar plates were previously overlaid with overnight grown CV. 100µl of crude extract was poured in well and incubate at 28ºC for 24 hrs. The zone surrounding the well shows the Quorum Sensing inhibitory activity of the compound.

2.5 Biofilm Inhibition Assay

Biofilm inhibition assay was done as per (Díaz et al., 2020) with minor modification. P. aeruginosa culture was overnight grown in LB medium. The culture was diluted to 1:100 for biofilm assay. The assay was done in 96 well plate in triplicates for quantitative analysis. For determination of biofilm inhibition P. aeruginosa was inoculated with supernatant of marine respective cultures. After 48 hrs. of incubation at 37°C the cells were drained out by inverting the plate and shaking out the liquid. The plate was washed in distilled water 2-3 times. Biofilm staining was done by 0.1% Crystal Violet stain. The stain was washed after 10-15 mins and biofilm get fixed by methanol [7-9]. The absorbance of solubilized dye was measured by micro titer reader.

2.6 Identification of Positive Isolates by Various Biochemical Tests

Biochemical tests were done according to (Holding & Collee, 1971) with minor modifications.

Gram's staining: Gram's staining was done to determine the nature of isolate whether it is Gram positive or Gram negative. Gram's staining was done by four steps: 1) Applying 2-3 drops of Crystal Violet (Primary stain) over thin smear. 2) Add 2-3 drops of Gram's Iodine (Mordant stain). 3) Decolorizing the stain with ethanol or alcohol. 4) Add safranin (Counter stain).

Catalase test: Catalase test is done to check the presence of catalase enzyme in bacteria. The

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principal states that if catalase is present, bacteria neutralize hydrogen peroxide (H2O2) and bubbles will produce. The test is carried out using slide method, which involves combining the bacterial colony with few drops of H2O2 over slide.

Oxidase Test: The test is done to determine the ability of bacteria to produce cytochrome oxidase enzyme. Principal based on transfer of electrons. The test is done by using oxidase disc. Loop full of bacterial culture was spreaded over the disc. The appearance of purple color is indication of positive test.

IMViC Test:

- **Indole Test:** The principal states that presence of tryptophanase enzyme in bacteria will covert tryptophan amino acid to indole gas. Positive test cultures were overnight grown in Tryptophan Broth (Tryptophan broth; Himedia India). The presence of indole gas is checked by using Kovac's reagent or Ehrlich's reagent. Presence of red color ring considered as positive test.
- **Methyl Red:** To check the ability of bacteria to utilize glucose. Positive isolates were overnight grown in Methyl Red-Voges Proskauer Broth (MR-VP Broth; Himedia, India). Methyl red was added to broth culture and color change from yellow to red is observed.
- **Voges-Proskauer test:** The test is done to determine the ability of bacteria to produces acetyl methyl carbinol from glucose fermentation. Positive isolates were overnight grown in MR-VP broth. α-naphthol and 40% KOH were used as reagents. After adding reagent, the color change from yellow to pink red indicates the positive result.
- **Citrate Utilization Test:** Positive isolates were overnight grown in citrate agar medium. The test is done to determine the ability of bacteria to utilize citrate as a sole energy source. The color change from green to blue indicates the positive result.

Starch Hydrolysis: The test is done to determine the ability of positive isolates to hydrolyze starch. Isolates were overnight grown on Starch Agar medium. After incubation iodine solution was added to starch agar plate the clear zone observed around the line of growth indicates positive test.

MacConkey Broth: The test is done for the isolation of gram-negative bacteria
and differentiation of lactose fermenting and non-fermenting. Positive isolates were overnight grown in MacConkey Broth medium (MacConkey; Himedia, India) and the change of color od medium from red to pink indicates the bacteria is lactose fermenting.

Triple Sugar Iron Agar (TSI): Principal of TSI based on the carbohydrate fermentation and hydrogen sulfide production by bacteria. Positive isolates were overnight grown in TSI agar (TSI; Himedia, India) containing glucose, lactose or sucrose. The results observed by five different observations which are (shown in Table 1)

Table 1 Observations of Triple Sugar Iron Agar Test

Expected result	Color change	Indication
Alkaline or acid reaction	Orange to red slant and yellow butt	Dextrose fermenter
Acid /acid reaction	Orange to yellow slant and yellow butt	Dextrose, lactose and sucrose fermenter
Alkaline/ alkaline reaction	Orange to red slant and red butt	Absence of Carbohydrate fermentation
Blacking of medium	Orange to blank slant	H_2S production
Bubbles formation	-	Gas production (CO2 and H_2)

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3. Results and Discussion

3.1 Results

Isolation and screening of marine isolates: A total of 53 different isolates recovered from marine samples with different marine sample and macroorganism.

Screening marine isolates of the QS inhibition activity: All marine isolates were screened against the bioreporter strain Chromobacterium violaceum using co-culture technique with necessary modification as describe by (Chu et al., 2011). Out of 53 free living isolates obtained from marine samples, 14 showed inhibition of pigment (shown in Table 2), None of positive screened isolated were found to inhibit the growth of Chromobacterium violaceum. Result (shown in Figure 1, Table 3)

Table 2 Anti-Quorum Sensing Potential of Marine Microorganism

Sr. no.	Sample Site	Isolate	Pigment Inhibition in Co-culture Screening
1	Mandvi	MY1	+
2		MY5	++
3		MY10	ND/+
4		MY15	+
5	Devbhoomi-Dwarka	G14	+++
6		G82	+++
7		G35	+
8		G65	+
9	DIU	DG20	+
10		DG10	+
11		DG56	ND/+
12		DG12	+
13		DG13	++
14		DG8	+

Table 3 Pigment Inhibition by Co-cultivation of Marine isolates against C

Figure	Marine Isolates	Result/ Observation	Interpretation
	G14	Inhibition of violacein pigment	Isolates producing QSI compounds
	G82		
	E5		
	G8	No inhibition of violacein pigment	Isolates not producing QSI compounds
	G13		
	G81		
	Positive control	No violacein production	Positive control
	Negative control	CV culture	Negative control

Pigment inhibition by co-cultivation of marine isolates against C. violaceum, the figure represents violacein inhibition. C. violaceum alone taken as positive control and Luria broth without any co-culturing taken as negative control.

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Growth inhibition assay: Isolates showed positive result in growth inhibition assay. The assay was done against C. violaceium. G14 and G82 marine isolate inhibits the purple pigment violacein produced by C.V (shown in Figure 1).

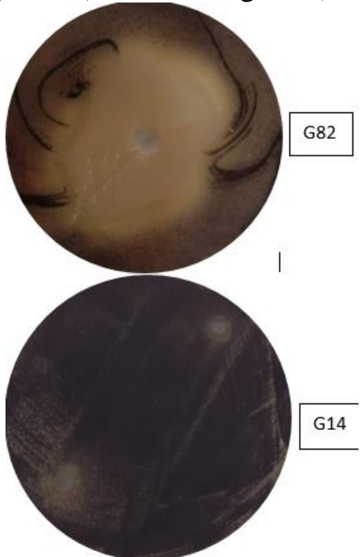

Figure 1 G82 and G14 isolate inhibited the growth of violacein pigment produced by C. violaceium.

Extraction of quorum quenching compounds from marine isolates: Pigment inhibition in CV upon treating with crude extract derived from cell free supernatant of specific isolates G14 and G82 shows quorum quenching activity. The quorum quenching capacity of G14 and G82 isolates was indicated by zone of pigment inhibition surrounding the well. (shown in Figure 2)

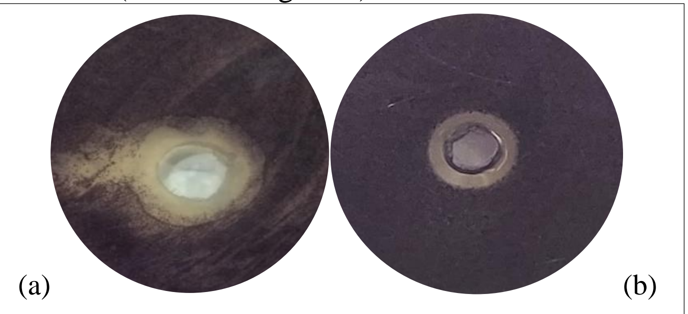

Figure 2 Pigment Inhibition by cell free supernatant (a) G82 isolates shows the quorum quenching ability against C. violaceium, (b) G14 isolate shows quorum quenching ability against C. violaceium

Biofilm inhibition assay: P. aeruginosa was cultivated with supernatant of marine isolates for 48 hrs. After 48 hrs. biofilm inhibition was observed in the wells having P. aeruginosa culture with marine isolate supernatant. Isolate G14, G82 and E5 shows the biofilm inhibition of P. aeruginosa (shown in Figure 3). Luria broth without any inoculation was taken as positive control and P. aeruginosa alone was taken as negative control.

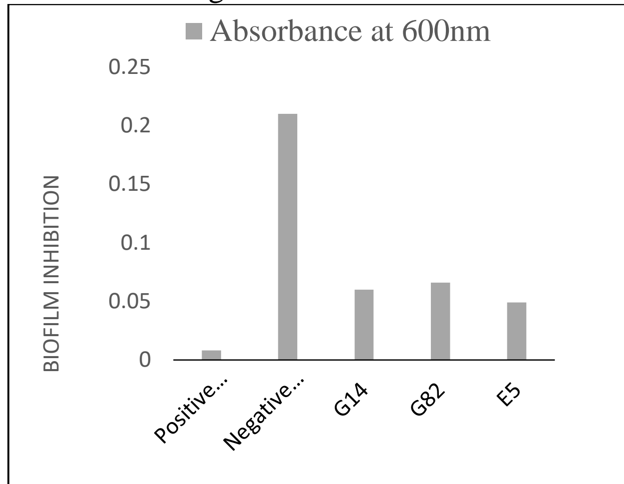

Figure 3 Marine isolates (G14, G82, E5) shows biofilm inhibition capacity against P. aeruginosa

Identification of positive isolates from various biochemical tests: Positive isolates were determined by different biochemical tests. The biochemical characteristics of both G14 and G82 isolate are shown in table 4.

Table 4 Determined biochemical characteristics of G14 and G82 isolates.

Tests	Observation	Result
Gram's staining	Rod shaped; pink color bacteria observed	Gram negative
Catalase test	No bubble formation	Catalase Negative
Oxidase test	Purple color appeared immediately	Oxidase positive
Indole test	Red ring observed	Indole positive

Methyl red test	Red color observed	Positive
Voges Proskauer test	No color change	Negative
Starch hydrolysis	No zone observed	Negative
MacConkey test	No color change	Negative
TSI test	Red color slant and yellow butt observed	Dextrose fermenter

3.2 Discussion

The above study was carried out to determine the potential of marine isolates having quorum quenching or quorum sensing inhibitory ability. QS is the mechanism that allows bacteria to show virulence, sporulation, gene transcription, production of metabolites and so on. Quorum quenching compounds derived from marine microorganisms can interfere the QS mechanisms. QSI can help decrease the continuous emergence of pathogenic microorganisms that are resistant to antibiotics [13]. Microorganisms derived from marine environments have capacity to produce quorum quenching compounds such as secondary metabolites, peptide-based quorum quenching compounds and so forth (Borges & Simões, 2019; Papenfort & Bassler, 2016, Saurav et al., 2017). Inhibition of bacterial signalling by QSI can be done by three strategies: a) degrading the signalling molecule, b) inhibiting the biosynthesis of signalling molecule, c) inhibiting the signal detection by receptor (González & Keshavan, 2006). For screening out most valuable isolates agar plate screening methods were widely used but the plate methods are time consuming and sometimes nonreproducible. Therefore, this study shows the co-cultivation method for screening out the most potent marine isolate. All the marine isolates were screened out by co-cultivation method against C. violaceium. The method is fast and easy and can screen out the isolates have capacity to produce QSIs. To isolate particularly the quorum quenching compounds extraction method is used. In extraction method ethyl acetate was used as a solvent to extract quorum quenching compounds. To target on multidrug resistance mechanisms biofilm inhibition assay of P. aeruginosa was carried out. The marine isolates showed very promising role in inhibiting the biofilms [10-12]. Biofilms are generally controlled by antibiotics, biocides and ion coatings traditionally. Recent research said that bacteria can easily resist to these antibiotics and can easily increase virulence factor. Cell free supernatant obtained from marine isolates inhibits the biofilm and shows an alternative therapeutic approach.
Therefore, marine microorganisms play a promising role in QS inhibition. An impartial method of screening as applied in the current study enables the simultaneous checking of every potential QSI for its activity. Assessing the quorum quenching capability of marine bacteria with different mechanisms was shown to be significantly aided by co-cultivating method. Marine-derived quorum-quenching antagonists can combat pathogens without growth disruption, potentially reducing resistance. Further studies on G14 and G82 isolates are ongoing [14-18].

Conclusion

For any organism to survive in a group, communication is one of the most important processes. Thus, the fundamental process found in all organisms is quorum sensing [19]. By modifying gene expression, QS enables bacteria to react and adapt to changes in cell population density. In this investigation, we found two marine bacterial isolates G14 and G82 which shows anti QS properties and inhibition of pigment violacein produced by Chromobacter violaceium without inhibiting growth. Extraction of Quorum sensing inhibitory compounds were successfully achieved. This would greatly contribute to trat the infections of C. violaceium and multi-drug resistance. Research on the mechanism of QS inhibition is necessary. It has been proposed that QSIs will be the next focus for therapeutic agent development aimed at combating multidrug-resistant pathogens [20]. QSIs may prove beneficial for healing society in the future.

e ISSN: 2584-2854
Volume: 02
Issue: 04 April 2024
Page No: 939-947

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