EVALUATION OF ANTI-QUORUM SENSING ACTIVITY OF N-HEXADECANOIC ACID PRODUCED BY PSEUDOMONAS STUTZERI SJ4 – A MARINE EPIBIOTIC BACTERIUM

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ABSTRACT
Quorum sensing (QS) mechanism is cell communication that plays vital role in the development of infection by many pathogenic microorganisms. It controls multiple virulence factors such as pigmentation, biofilm formation, swarming motility, resistance towards antibiotics, extracellular polysaccharide production (EPS) and expression of several collective traits. The disruption of quorum sensing mechanism can be a solution to the emerging problem of multi-drug resistance among pathogenic bacteria. The effector molecule for Quorum sensing inhibition may be enzymatic or non-enzymatic in nature termed as Quorum Quenching (QQ) or Quorum sensing inhibitory (QSIs)/anti-QS compound, respectively. We used marine epibiotic bacteria as a source to obtain novel bacterial strain as QSI producer. One of the potent isolate, SJ4, was identified as Pseudomonas stutzeri SJ4, it is a short rod, gram negative bacterium. The ethyl acetate extract from P. stutzeri SJ4, showed highest QSI activity against monitor strain Chromobacterium violaceum (MTCC 5526). The extracted compound was tested against P. aeruginosa PAO1 at minimum inhibitory concentration (MIC) and sub-MIC to study the effect on virulence factors. The significant inhibition of pycocyanin pigment, EPS production, rhamnolipid production and reduced swimming and swarming motility was observed. In addition, biofilm formation was notably inhibited which was confirmed by staining and spectrometric method. Based on this observation, QS inhibition by extract which contain QSI remarkably reduced the virulence of pathogen hence, can be use as therapeutic agents. The Thin Layer Chomatography (TLC) and Gas chromatography-Mass Spectrometry (GC-MS) identified major compound as n-Hexadecanoic acid. However, further research is required on purification of compound and its potential applications for the treatment of infections.

Keywords: Quorum sensing, Quorum sensing inhibition, marine bacteria, Pseudomonas stutzeri, Pseudomonas aeruginosa

INTRODUCTION
The discovery of antibiotics was a boon but its excessive use and decreased efficiency turned it into a bane. The pathogens Which are become completely or partially resistant to one or more antibiotics/antimicrobial compounds are referred to as Multi-Drug Resistant (MDR) microbes. This global issue drew attention of scientific community to search for a novel approach which can combat pathogenic infections without triggering selection pressure which leads to development of drug resistance (World Health Organization, 2017; Reina et al., 2019). Many pathogenic bacteria, have mechanisms which control the expression of virulence genes such as pigmentation, antibiotic resistance, extracellular proteases and biofilm formation termed as Quorum sensing (QS) (Whitehead et al., 2001; Quinones et al., 2005; Jayaraman and Wood, 2008; Garg et al., 2014; Papenfort et al., 2016; Whiteley et al., 2017). It is a cell population dependent process where cells communicate, coordinate and express genes leading to the production of signalling molecules called as autoinducers in the surrounding environment (Fuqua et al., 1994; Fuqua et al., 2001). The concentration of signalling molecules inducing characteristic phenotypes is directly proportional to the cell density. Gram positive bacteria produce oligopeptides and Gram negative bacteria produce N-Acyl Homoserine Lactones (AIHLs) as QS signal molecules (Ng WL et al., 2009; Parker et al., 2009). QS regulation plays vital role in establishment of infection within the host by several pathogen such as P. aeruginosa, Staphylococcus aureus, Escherichia coli, Vibrio cholerae and Vibrio harveyi, therefore the disruption or silencing of virulence gene expression can be a novel strategy to combat MDR. (Ng WL et al., 2009; Kalia et al., 2011; Nathra et al., 2011; Lasrre et al., 2013; Grandclement et al., 2016; Defoirdt et al., 2018; Kalia et al., 2019; Zhao et al., 2019).

P. aeruginosa PAO1 is an infectious strain associated with the urinary tract infections (UTIs) and hospital acquired infections. It is a Gram-negative, motile organism and becoming resistant towards conventional antibiotics. QS inhibitory approach can be an effective way to control MDR clinical strain P. aeruginosa PAO1. The four QS systems of P. aeruginosa PAO1 are divided broadly into two groups, (i) AHL-based signalling system namely Las/LasR and Rhl/RhlR system and (ii) non-AHL or quinolone based such as Pseudomonas quinolone signal (PQS) and integrated QS signal (IQS) (Rutherford and Bassler 2012; Pérez-Pérez et al., 2017; Li et al., 2018; Hemmati et al., 2020). LasR and rhlR code for a receptor protein that binds to the signal molecule and initiate transcription of QS genes. The LasR system uses 3-oxo-C12-HSL as a cognate signal molecule, and RhlR utilizes C4-HSL. These two systems work in a hierarchical manner, as the las-encoded system affects the expression of the rhl-encoded system (Hentzer and Givskov 2003; Mostafa et al., 2020). The PQS system is monitored by 2-alkyl-quinoilones while IQS is controlled by 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Lasarre & Federle 2013; Li et al., 2018).

Many natural and synthetic compounds are known to function as quorum sensing inhibitors (QSI). QSI are non-enzymatic compounds, which can be cyclic or linear peptides, amides, AHL analogs. Further, inactivation of QS signal molecules by enzymes (Acylases, AHL Lactonases, and AHL Reductase) are termed as Quorum Quenching molecules (QQ) (Vadakkan et al., 2018). QSI or QQs can be obtained naturally from plants such as Myrmecodia pendors (Saturi et al., 2021), Custardia suitea (Eksi et al., 2020), Passiflora edulis (Fruit) (Venkatramanan et al., 2020); fungi Daldinia eschscholtzii (Mishra et al., 2020); host associated bacteria Esigubacterium indicum (Singh et al., 2019), Desemzia incerta and Bacillus sp., (Singh et al., 2020); and corals Sarcotragus spinulosus (Saurav et al., 2019).

The marine environment has plethora of biological components which are not studied yet, therefore there are chances of getting novel bacterial species with potential anti-QS application (Borges et al., 2019). Some of the reports of QSI producing marine bacteria are Bacillus cereus from marine sediments of Rhode Island,USA (Teasdale et al., 2009), Bacillus amyloquilocuciens from mangrove rhizophore rhizophore, Bay of Bengal, India (Gowrishankar et al., 2014, Gowrishankar et al., 2019) and Marinobacter sp. from Arabian coastal region, Oman (Abed et al., 2013).Gujarat has vast coastal region and its microbial diversity is yet to be explored. Hence, the present study is focused on the exploration of marine epibiotic bacteria, isolated from the surface of macro-
organism like algae, sponges, and seaweeds, for QSI activity. Isolates were screened for the anti-QS activity using *C. violaceum* (MTCC 5526) as a monitor strain. Total 20 isolates showed anti-QS activity, isolate showing highest anti-QS activity was selected for further studies on clinical strain of *Pseudomonas aeruginosa* PAO1.

**MATERIALS AND METHODS**

**Sample Collection**

The marine seaweeds, algae and sponges were collected in sterile sample bottles from coastal site of Okha, Gujarat, India. (22°28’38.7”N 69°04’51.7”E). The samples were kept in the sea water at 4°C until use.

**Isolation of marine epibiotic bacteria**

A 1 gram sample (marine seaweeds, algae and sponges) was washed thrice with sterile sea water. The washings were collected (Total 30 mL) and treated as samples. All the samples were diluted up to 10⁶ times; 100 µL was spread on Zobell Marine Agar (ZMA, Himedia, Mumbai, India) and incubated at 37 °C for 5-7 days. Morphologically distinct colonies were transferred to the ZMA plates to obtain pure colonies (Singh et al., 2020).

**Screening of epibiotic bacteria for anti-QS activity**

Isolates were screened for anti-QS activity by soft agar overlay method using *C. violaceum* (MTCC 2656) as a monitor strain (McLean et al., 2004). The secondary screening was done by quantification of violaercin pigment. The bacterial isolates (1×10⁶ CFU/mL cells) and *C. violaceum* (1×10⁶ CFU/mL cells) were inoculated in Luria broth (LB) and incubated at 37 °C for 24 h under shaking condition. Next day, 1 mL sample was withdrawn and centrifuged at 10,000 rpm for 15 min, pellet was dissolved in DMSO and centrifuged again at 10,000 rpm for 15 min. Absorbance of the supernatant was measured at 585 nm. Cell density was also measured at 600 nm to check the growth of isolates (Choo et al., 2006; Musthafa et al., 2011).

**Extraction of Anti-QS compounds**

The select marine isolates were grown in ZMB at 37 °C for 24 h under shaking condition. The cultures were centrifuged and the supernatant was extracted with equal volume of organic solvents of varying polarity like hexane, chloroform, ethyl acetate, n-butanol, isopropanol and methanol. The extract was concentrated by evaporation and the residue was dissolved in 500 µL of respective solvent. The compound was used for *C. violaceum* based bioassay by well diffusion method (Nithya and Pandian SK., 2009; Nithya et al., 2010).

**Morphological characterization of bacterial isolate**

Isolate SJ4 showing highest QSI inhibition, was gram stained and its morphologies were examined at 100X magnification using Magnus MLM light microscope.

**Biochemical characterization of bacterial isolate**

Biochemical characterization of selected bacteria was done, which included tests such as catalase, oxidase and sugar utilization (lactose, glucose, sucrose, mannitol, rhamnose and arabinose).

**Molecular identification of bacterial isolate**

Genotypic characterization of SJ4 was by sequencing its 16S rDNA gene. The extracted genomic DNA was shipped to Eurofins Scientific India Pvt Ltd. (Banglore, India) and served as a template for PCR amplification of the nearly complete 16S rDNA gene using the universal oligonucleotide primer pair 27F (5’-AGA GGT TGA TCC TGG CTC AG-3’) and 1492R (5’-TAC GGT TAC CTT GGT ACG ACT T-3’), as recommended by Lane (Lane, 1991).

**Testing QSI activity of extract**

QSI activity of the extract was determined using well diffusion method. LB agar plates were inoculated with 100 µL of *C. violaceum* 50 µL of extract was added into the wells. The plates were then incubated at 37 °C for 24 h. Extract showing zone of pigment inhibition in *C. violaceum* were shortlisted and then tested against *P. aeruginosa* PAO1.

**Determination of Minimum Inhibition Concentration (MIC) of QSI extract**

The MIC was determined by broth dilution method using *P. aeruginosa* PA01 as a test strain. The extract was serially diluted to make different concentrations from 1 mg/mL to 0.098 mg/mL. Test strain was inoculated in LB broth and incubated at 37 °C for 18 h. The control was prepared without extract. The tube showing no turbidity was considered as MIC. Consequently, sub-MICs were selected for further studies (CLSI, 2012).

**Inhibition of virulence factors in *P. aeruginosa***

**Pyocyanin inhibition**

Pyocyanin inhibition was assayed by growing *P. aeruginosa* overnight with QSI extract (MIC and sub-MIC) and without extract at 37 °C for 24 h. After incubation, 5.0 mL culture supernatant was extracted with 2.5mL of chloroform and was centrifuged at 3000 rpm for 2 min for separation of chloroform layer. 500µL of 0.2N HCL was added to the separated layer and vortexed vigorously which produced a red colored thick ring. The ring was carefully removed, the absorbance was measured at 520 nm and inhibition percentage was calculated (Ganesh and Rai, 2016).

**Inhibition of Exopolysaccharide (EPS)**

EPS was quantified from supernatant of *P. aeruginosa*. EPS was precipitated by three volumes of iso-propyl-alcohol (100%) and incubated for 24 h at room temperature. The precipitated EPS was pelleted by centrifugation (10000 rpm, 15 min) and dissolved in distilled H₂O. EPS was quantified using the phenol-sulphuric acid method, wherein 1 mL of 5% cold phenol and 5 mL of conc. H₂SO₄ were mixed with 1 mL of EPS suspension, which was measured spectrophotometrically at 490 nm (Packiavathy et al., 2014).

**Rhamnolipid inhibition**

A 1 mL cell free supernatant was extracted with twice the volume of ethyl acetate and dried. The dried extract was suspended in 900 µL of orcinol solution (0.19% orcinol dissolved in 53% (v/v) sulfuric acid). The mixture was incubated for 30 min at 80 °C and quantified at 421 nm spectrophotometrically (Packiavathy et al., 2014).

**Biofilm inhibition**

The test strain was mixed with QSI extract (MIC and sub-MIC) and incubated for 24 h at 37 °C; a control was prepared without QSI extract. After incubation, the wells were washed with sterile phosphate-buffered saline (PBS) to remove unadhered cells. The biofilm was stained with 1% crystal violet for 5 min and again washed with sterile PBS to remove excess stain. The crystal violet stained biofilm was dissolved with 33% acetic acid and was quantified by absorbance at 595 nm (Luo et al., 2016).

**Motility inhibition**

Swimming and swarming was studied as described by Rashmi et al. (2018). Growth media used for swimming assay comprised nutrient broth along with extract or without extract. Actively growing culture was point inoculated and plates were incubated at 37 °C for 24-48 h. In Swarming, test strain was inoculated in nutrient agar with or without QSI extract. Actively growing culture was point inoculated and plates were incubated at 37 °C for 24-48 h.

**Identification of active compound(s) in E/A extract of *P. stutzeri* SJ4**

Thin layer chromatography (TLC) was performed to partially purify active compound (QSI) from Ethyl acetate crude extract of *P. stutzeri* SJ4 using chloroform: methanol (10:1) as solvent system (Basaran et al., 2020). The eluted bands were checked for the anti-QS activity. The band showing highest activity was analysed by Gas chromatography-Mass Spectrometry (GC-MS) (Perkin Elmer, Autosystem XL GC with Turbomass). The chromatogram was matched with NIST library database in order to predict the compound.

**Anti-QS activity of n-Hexadecanoic acid**

QSI inhibitory activity of different concentrations of commercially available n-hexadecanoic acid (HDA) (Himedia, Mumbai, India) was compared with the crude extract from *P. stutzeri* SJ4 against *C. violaceum* MTCC 2656. The concentration showing highest decolorization zone was used for further studies.

**RESULTS AND DISCUSSION**

**Epiobiotic bacterium *P. stutzeri* SJ4 as a QSI producer**

Total 60 bacteria having distinct morphology were isolated from the surfaces of 8 samples of seaweed, algae and sponges. All isolates were screened for QSI activity using *C. violaceum* MTCC 2656 as a monitor strain. 20 isolates showed QSI activity by inhibiting the violacein pigment production. SJ4 showing highest activity was selected for further study (figure 1).
The colony of SJ4 was small, reddish brown, dry, and irregular in shape. It was gram negative, short rod, arranged singly (figure 2 a & b). SJ4 was catalase negative, oxidase positive, fermented lactose, glucose, sucrose, mannitol, rhamnose and arabinose sugars. Apart from morphological and biochemical characterisation, molecular identification was carried out by 16S rDNA sequencing. SJ4 was identified as P. stutzeri SJ4. The nucleotide sequences were submitted to Genebank (Accession No. MW547117) and analysed using NCBI Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was done using MegaX (Downloaded from https://www.megasoftware.net/) software.

The phylogenetic tree was constructed by Neighbor-Joining method with the bootstrap value of 1000 (figure 2c).

Figure 1 Primary screening by soft agar overlay method using C. violaceum as a monitor strain (a) Control (b) SJ4.

Figure 2 Identification and phylogenetic analysis of P. stutzeri SJ4 (a) The colony morphology (b) Gram’s Reaction (c) Phylogenetic tree using Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA X.

Extraction of QSI Substances

The supernatant was extracted with different organic solvents such as hexane, chloroform, ethyl acetate, n-butanol, isopropanol and methanol. The extracts were checked for the QSI activity against C. violaceum by well diffusion method. The zone of pigment inhibition was highest in ethyl acetate extract compared to other solvents (Table 1). Teasdale et al., (2009), also found highest anti-QS activity of Halobacillus salinaris C42 in the ethyl acetate extract. On the other hand, Abudoleh et al., (2017), reported more anti-QS activity from three isolates in butanol, chloroform and hexane extracts. Hence, further virulence assays of P. aeruginosa were performed using ethyl acetate (EA) extract.

In a previous study, Venkatramanam et al., (2020) reported 2 mg.mL⁻¹ and 1mg.mL⁻¹ as MIC and sub-MIC respectively, 75.8% inhibition of violacein pigment in C. violaceum was observed, Busetti et al (2015) have reported significant decrease in pyocyanin production (60%) at 1 mg.mL⁻¹ concentration from Pseudalteromonas sp.

EPS is a fundamental component of the biofilm formation and it is crucial to maintain integrity of biofilm. EPS production was reduced by 77% at MIC while 19.23% at sub-MIC (figure 3). Mishra et al., (2018) studied effect of extract of Alternaria alternata on EPS production of P. aeruginosa PAO1; 66.6% reduction was observed.

Rhamnolipid is one of the important aspects in the formation of biofilm. In the present study, QSI reduced rhamnolipid production up to 45% at MIC; while 29% reduction was observed at sub-MIC (figure 3). Rajkumari et al., (2018) observed 16.51% reduction in rhamnolipids of P. aeruginosa when cinnamic acid was used as QSI.

Table 1 QSI activity of compound extracted with various solvents

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Solvent</th>
<th>Diameter of Zone of Pigment Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexane</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Ethyl acetate</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>n-Butanol</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Isopropanol</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>Methanol</td>
<td>2</td>
</tr>
</tbody>
</table>

Pyocyanin is a green, fluorescent compound produced by P. aeruginosa which plays a vital role in the infection. To investigate, the effect on pyocyanin and other parameters, the MIC of QSI extract was tested and it was found to be 6.25 mg.mL⁻¹ and the sub-MIC was 3.125 mg.mL⁻¹. These two concentration were used for the study. QSI extract inhibited pyocyanin production up to 79% and 34% at 6.25 and 3.125 mg.mL⁻¹ concentration respectively (figure 3).

Figure 3 Inhibition of virulence factors of P. aeruginosa PAO1 by EA extract of P. stutzeri SJ4 at MIC (6.25 mg.mL⁻¹) and sub-MIC (3.125 mg.mL⁻¹)
Effect of EA extract of *P. stutzeri* SJ4 on Biofilm formation of *P. aeruginosa*

Biofilm formation is most important factor responsible for the virulence of any pathogenic bacteria. It majorly contributes in antibiotic resistance and development of severe infection. Our study showed 69% reduction in biofilm formation in comparison with control. The staining with crystal violet showed marked difference in the color intensity in the presence and absence of EA extract (figure 4). In a study by Christiaen et al. (2014), Diaphorobacter sp. and Delftia sp., had reduced *P. aeruginosa* PAO1 biofilm formation by 20–30% whereas marine derived strain Rhizobium sp. NA01, reduced biofilm formation by 72.3% in *P. aeruginosa* PAO1 (Chang et al., 2017).

Figure 4 Biofilm inhibition of *P. aeruginosa* PAO1 after staining with crystal violet (a) control (without extract) (b) at 6.25 mg.mL^{-1} (MIC) (c) at 3.125 mg.mL^{-1} (Sub-MIC).

Motility helps in attachment to host cell surface and responsible for the movement. *P. aeruginosa* is a highly motile strain so swimming and swarming patterns of mobility were studied in the absence and presence of QSI. A significant decrease in the colony diameter was noticed in the presence of QSI compound (figure 5). According to earlier studies, aspirin inhibited the swimming motility of *P. aeruginosa* by 34% (El-Mowafy et al., 2014).

Figure 5 Inhibition of Motility in *P. aeruginosa* PAO1 (a) Swimming (b) Swarming (1a & 1b) control, (2a&2b) extract concentration 6.25 mg.mL^{-1} (3a &3b) extract concentration 3.125 mg.mL^{-1}.

Characterization of anti-QS compound of EA extract of *P. stutzeri* SJ4

A total of 4 prominent bands were observed on TLC plate under the UV light. QS inhibition was observed in only two eluted bands, one band with highest anti-QS activity was selected for the GC-MS analysis. The chromatogram of partially purified extract showed 15 peaks with different retention time (RT) (figure 6); 3 peaks were selected for MS based on the percentage of area. According to NIST library, major compound was *n*-hexadecanoic acid (18.47%) at 23.307 RT followed by Undecanoic acid (12.18%) at 21.451 RT and Hexadecane (7.82) at 25.903 RT (table 2). Basaran et al. (2020), obtained 7 bands after TLC separation of EA extract of Natrinema versiforme. Venkatramanam et al., (2020) also reported *n*-hexadecanoic acid as the main QSI compound after GC-MS analysis of *Passiflora edulis* extract.

Table 2 List of compounds at 23.307 RT obtained after searching in NIST library

Comparative study of crude and standard compound

Different concentrations (1 mg.mL^{-1}, 0.5 mg.mL^{-1} and 0.25 mg.mL^{-1} of HDA was tried, figure 7a represents that crude extract showed 12 mm zone of inhibition and HDA showed 9 mm zone. Further, quantification was done using 1 mg.mL^{-1} HDA where as the concentration of crude extract was 6 mg.mL^{-1}. As shown in the figure 7b, the percentage production of violacein pigment was reduced to 71.06% and 90.32% with HDA and crude extract respectively. A significant difference in the activity of the two compounds was observed, however the concentrations were also different. Since the purity of the standard and test compound is different their potency can not be compared. Chemmugil and co-workers have also reported the anti-QS activity of HDA against *Staphylococcus aureus* using qualitative method. They observed 12 mm zone of inhibition which is similar to our results (Chemmugil et al., 2019).
CONCLUSION
A total of 60 marine epibiotic bacteria were screened for the anti-QS activity. _Pseudomonas stutzeri_ SL4 significantly inhibited the virulence factors which are associated with the quorum sensing of clinical strain of _Pseudomonas aeruginosa_ PAO1. The active anti-QS compound present in the crude extract is tentatively identified as n-hexadecanoic acid. Further investigations regarding the mechanism of inhibition and genetic level expression is required.

Conflict of interest: The authors declare that there is no potential conflict of interest.

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