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ASSESSMENT OF QUORUM QUENCHING ACTIVITY OF *SERRATIA* SPP. ISOLATED FROM PLANT RHIZOSPHERE AGAINST QUORUM SENSING CONTROLLED BIOFILM-FORMING PATHOGENS

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ABSTRACT

Quorum quenching is the process of blocking quorum sensing activity that can be used as an effective way to control quorum sensing dependent virulence in pathogens. Quorum quenching mechanisms prevent the development of antibiotic resistance in biofilm-forming microorganisms. Rhizospheric bacteria shows great diversity along with complex intra species interactions. Within this diversified environment many bacterial species with quorum sensing and quorum quenching ability are found in close vicinity. In this study rhizospheric soil of different plants was used to screen potential quorum quenchers. The rhizospheric bacterial isolates were tested for their ability to degrade/inactivate N-hexanoyl-l-homoserine lactone (C6HSL) molecules. Pigment inhibition bioassays were performed using *Chromobacterium violaceum CV026* and *C. violaceum 2656* to screen bacterial isolates possessing quorum quenching activity. The biofilm formation inhibition by six screened isolates was studied against different human pathogens such as *Vibrio parahaemolyticus* MTCC451, *V. cholerae* MTCC3906 *and Pseudomonas aeruginosa* PA01. Results revealed that biofilms were efficiently inhibited. Significant biofilm inhibition was revealed by two isolates and identified as *S. marcescens subsp. sakuensis* KRED, and *S. nematodiphila* using 16S rRNA gene sequencing technique. The partial purification of C6HSL inactivating quorum quenching molecule resulted in maximum pigment inhibition of *C. violaceum* 2656 at 80% saturation of ammonium sulphate. These findings revealed that *S. marcescens subsp. sakuensis* KRED and *S. nematodiphila* are found to be potential candidates to control biofilms in bacterial human pathogens.

Keywords: Quorum Quenching, Quorum Sensing, biofilm inhibition, rhizospheric bacteria, N-hexanoyl-l-homoserine lactone degradation, *Serratia* sp.

INTRODUCTION

Recently, multidrug resistance in several pathogens has been increased due to misuse of antibiotics. Cytotoxic approaches are widely employed to control several diseases which result in the emergence of drug-resistant organisms **(Kalia *et al.*, 2011)**. Conventionally used antibiotics can interfere with the cellular housekeeping functions like DNA, RNA and protein synthesis. These antibiotics may play a crucial role in the development of multidrug-resistant microbial pathogens **(Hentzer & Givskov, 2003)**.

Therefore, non-cytotoxic approaches are alternative to cytotoxic approaches like hindrances in bacterial communication that can prevent antibiotic resistance in pathogens. Recently, antibiofilm agents like quorum sensing (QS) inhibitors have become one of the most promising approaches to control human pathogens. Thus, antimicrobial agents employed in QS inhibition process are considered as a novel category of antimicrobial agents **(Y.-H. Dong *et al.*, 2007; Hentzer & Givskov, 2003)**. Such antimicrobial agents target the virulence mechanisms of several biofilm-forming pathogens. Thus, targeting virulence instead of killing pathogens is a very promising approach to control diseases without creating life-death pressure on pathogens **(Waters & Bassler, 2005)**. Quorum quenchers act as anti-virulent agents that can reduce the emergence of antibiotic resistance in pathogens **(Y.-H. Dong & Zhang, 2005)**.

QS is a well-known cell-to-cell communication system that monitors bacterial population density by secreting and detecting signaling molecules. Once the bacterial population reaches the required density then the expression of a different set of genes takes place and all bacterial cells in quorum respond simultaneously **(Waters & Bassler, 2005)**. QS is observed in both Gram-positive and Gram-negative bacteria **(Whitehead *et al.*, 2001)**. Several types of signaling molecules employed in QS are identified as acyl homoserine lactones (AHL) in Gram-negative bacteria, auto inducing peptides (AIPs) in Gram- positive bacteria. However, Auto-inducer-2 molecules are synthesized by both Gram-positive and Gram-negative bacteria **(Bassler, 2002)**.

Compounds that can inhibit or interfere with bacterial cell to cell communication systems and biofilm formation are known as quorum quenchers (QQ). They specifically target the cell signaling molecules which affect the bacterial virulence without growth inhibition and thus, reducing the chances of drug resistance. Recently, a variety of QQ biomolecules have been identified from a wide range of sources that control pathogenic infections without altering them to antibiotic-resistant pathogen **(Dong *et al.*, 2001)**. Thus, QQ is an alternative and effective approach reported earlier **(Reimmann et al., 2002)**. In QQ strategy, AHL signal molecules are degraded which results into inhibition of the QS process. AHL degrading enzymes are AHL lactonase and AHL acylase which are studied extensively. These enzymes are encoded by different genes in many species which has been studied previously **(Y.-H. Dong & Zhang, 2005)**. Both Gram-positive and Gram-negative bacteria isolated from soil, rhizosphere and phyllosphere environments are reported for their AHL degrading activity **(Ma *et al.*, 2013)**. Similarly, several AHL lactonase producing bacteria isolated from agricultural lands of Indonesia are identified as genus *Bacillus* and *Serratia marcescens* **(Fitriyah *et al.*, 2015)**. AHL degrading bacteria isolated from the rhizosphere of potato plants are identified as the genus *Bacillus*, *Arthrobacter*, and *Pseudomonas*. These bacteria are reported for their AHL degrading activity of plant pathogen *Pectobacterium atrosepticum* and thus it could reduce the pathogenicity in potato tubers **(Esmaeil Mahmoudi, 2011)**.

Thus, isolation and characterization of quorum quenching molecules from bacteria can be beneficial to develop the most promising strategy to exploit them as potential bio-control agents. The main aim of this study is to isolate bacterial species from the rhizosphere having quenching activity against biofilm- forming pathogens. In the present study *Serratia* species possessing the potential ability to inactivate AHL signaling molecule were isolated and their ability to interfere with biofilm formation in pathogens were studied.

MATERIALS AND METHODS

Cultivation and maintenance of bacterial strains

In this study, two bacterial strains *Chromobacterium violaceum* 2656 and *C. violaceum* CV026 were used as biosensor strains to monitor Acyl Homserine Lactone N-hexanoyl-L –Homoserine lactone (C6-HSL) inactivation activity**(Rajesh & Ravishankar Rai, 2014)**. *C. violaceum* CV026 is a mini tn5 mutant of wild-type *C. violaceum*. *C. violaceum* 2656 was obtained from MTCC, IMTECH, Chandigarh, India, and *C. violaceum* CV026 is obtained from NCMR-NCCS, Pune, Maharashtra. *C. violaceum* 2656 was cultivated and maintained in nutrient broth tubes and incubated at 30ºC. Similarly, *C. violaceum* CV026 was also cultivated and maintained at 30ºC in nutrient broth tubes supplemented with 25µg ml^{-1} Kanamycin.

Isolation of bacteria from plant root surfaces

All plant root samples from 36 different plants were collected from different areas of Pune, Maharashtra, India. The roots were kept in a sterile container at 4ºC. The roots were dispensed in 10ml of sterile distilled water separately and shaken vigorously to dissolve amount of soil adhered on the root surfaces. From each sample, a loopful of sample was streaked on the sterile nutrient agar plates (media nutrients were 5 times diluted). All plates were incubated at 30ºC for 24h. Based on morphological features, selected colonies were sub-cultured and purified on the sterile nutrient agar plates. All purified cultures were maintained on sterile nutrient agar slants at 4ºC. Further, these cultures were screened to test the ability to inhibit AHL mediated quorum sensing **(Alinejad *et al*., 2020)**.

Primary Screening of Quorum Quenching activity

All bacterial isolates were screened to check their ability for the production of quorum quenching molecules by using a previous method with some modifications **(Mclean *et al*., 2004)**. In this method, *C. violaceum* CVO26 was used as a biosensor strain which is a mutant for violacein pigment production. In this organism, induction of violacein pigment production was achieved by supplying synthetic AHLs with a chain length of C4-C8 or a 3-oxo-acyl side-chain cognate signal molecules in media. For screening, all isolates were streaked on the sterile nutrient agar plates and incubated at 30ºC for 48h. Further, the surface of the plates was overlaid with 10ml of nutrient soft agar (0.8% agar) supplemented with cognate signal molecule 12.5 µM C6-HSL and 100 µl cell suspension of *C. violaceum* CVO26. All plates were incubated at 30ºC for 24 h and quorum sensing inhibitory activity was detected in terms of zone of violacein pigment production inhibition. Appropriate controls were kept throughout the experimentation. Further, quorum quenching potential of isolates was tested by using quantitative assays **(Mclean *et al*., 2004)**.

Quantitative estimation of violacein pigment inhibition

Studies on inhibition of violacein production in *C. violaceum* strains by all positive isolates were performed as described by **Khan *et al*. (2009)** with slight modifications. All screened isolates were grown for 48 h in test tubes (10ml) containing 5ml of nutrient broth. Further, the broth was centrifuged at 10000g for 10 minutes. Cell-free supernatant (CFS) were collected and stored at 4◦C. The obtained cell pellets were washed thrice with phosphate buffer saline and suspended in 1ml of phosphate buffer saline. Further, cells were disrupted by the bead beating method by using Fastprep-24^{TM} bead meter 5G. In this method, cell samples were processed for two cycles of 30 seconds. The processed samples were centrifuged at 10000g for 10 minutes and supernatants were collected. Further, previously obtained CFS and supernatants obtained after cell lysis were tested for their QQ activity using pigment inhibition assay which was performed in triplicates for each sample separately. In this assay, 50 µl of CFS and cell free lysate were inoculated separately in 2 ml of 1/100th dilution of freshly grown biosensor culture of *C. violaceum 2656* in nutrient broth. Control sets were kept for intracellular and extracellular activity. All these cultures were incubated at 30º C with shaking at 120 rpm till complete pigmentation is achieved in the blank. Initially, 2ml of treated and untreated cultures were lysed by adding an equal volume of 10% SDS and mixing for 5 s with vortex, and incubating at room temperature for 5 minutes. Further, 4 ml of water-saturated butanol (50ml n-butanol mixed with 10ml distilled water) was added to cell lysate, vortexing for 5 seconds was carried out. Further cell lysates were centrifuged at 13000g for 5 minutes. The upper (butanol) phase containing the violacein was collected and the absorbance was read at 585nm. Reduction in violacein pigment production in presence of CFS was measured in terms of percentage inhibition as [(OD of control)-(OD of treated)/OD of control]* 100.

Whole-cell AHL inactivation assay:

The positive isolates obtained from plant root rhizosphere were confirmed for QS inhibitory activity by whole-cell AHL inactivation assay accompanied by AHL biosensor strain *C. violaceum* CV026 **(Rehman & Leiknes, 2018)**. In this method, all isolates were cultivated in sterile 10 ml tubes containing 2ml nutrient broth and incubated at 30ºC and 120 rpm. After 24h of incubation broth was centrifuged at 10000g for 10 min. to get a bacterial cell pellet. Cell pellet was washed with sterile phosphate-buffered saline by centrifuging at 10000 g for 5 min. The cell pellets were suspended in phosphate-buffered saline and C6-HSL was added in order to maintain final concentration of 12.5 µM. This mixture was incubated at 30◦C for 24h. The pH of this mixture was maintained in order to avoid degradation of C6-HSL because of alkaline condition **(Yates *et al*., 2002).** In this experiment, C6-HSL was mixed with the cell-free phosphate buffered saline and considered as a negative control.

At 0 h and 24 h of incubation at 30º C, the bacterial cultures were centrifuged at 10000g for 10 minutes to pellet the cells. Residual C6-HSL in the CFS was detected by the pigment inhibition assay method. In this method, nutrient agar plates were covered with a 100-fold dilution of 24 h grown *C. violaceum* CV026 culture mixed with 7ml soft nutrient agar (0.8%). After the soft agar biosensor layer was solidified, 6-mm wells were prepared in the medium by using a surface-sterilized cork borer. In each well, about 50 µl CFS was added separately and incubated at 30ºC for 24 h. A similar procedure was followed for negative control as well. After incubation, the presence of a violet colored zone around the well-specified the absence of QS inhibitory activity. The diameter of the zone of violacein synthesized by the biosensor strain CVO26 is proportional to the amount of AHL remaining in the reaction mixture after the incubation period **(Mukherji & Prabhune, 2015)**.

QQ activity of extracellular extracts on biofilm-forming bacteria

Extracellular extracts of positive isolates have been tested against biofilm formation of *V. parahaemolyticus* MTCC451, *V. cholerae* MTCC 3906 *and P. aeruginosa* PA01 by static microtiter plate assay previously reported by Rajesh *et al* with some modifications **(Rajesh & Ravishankar Rai, 2014)**. The *P. aeruginosa PA01* and *V. parahaemolyticus* MTCC451were grown in 10ml of test tubes containing 3ml of sterile nutrient broth. For the growth of *V. cholerae* MTCC 3906, the final concentration of 3% NaCl was adjusted in 3ml sterile nutrient broth. The bacterial suspension was added to M9 minimal medium supplemented with 0.4% (w/v) glucose to obtain roughly $1.5X10^7$ cfu/ml which was monitored by optical density. Further 180 µl of above- mentioned biofilm-forming cultures were inoculated along with 50 µl of extracellular extract of positive isolates were dispensed in a 96-well microtiter plate. The plates were incubated at 37ºC for 24 h without agitation. Further, planktonic cells were removed and transferred to separate microtiter plates to measure absorbance at 600 nm. Biofilm formation was measured by a crystal violet assay method. In this method, 125µl of crystal violet (0.1% w/v in water) was added into microtiter wells containing biofilms and incubated for 10 minutes at room temperature. Then wells were washed thoroughly with sterile distilled water. Further, crystal violet was extracted in 95% ethanol and absorbance was measured at 590 nm to quantitate biofilm formation **(Haney *et al*., 2018)**.

Partial purification of QQ molecules by ammonium sulfate precipitation

Ammonium sulfate precipitation of BG5 and K1 was carried out to concentrate the QQ molecule by using a previously reported method with some modifications **(Mukherji & Prabhune, 2015)**. The crude enzyme was obtained by growing BG5 and K1 cultures in nutrient broth at 30ºC and 120 rpm for 24 h. Further, the broth was centrifuged at 13000g for 10 minutes at 4ºC to obtain CFS. Ammonium sulfate powder was added slowly at 4◦C to 100ml CFS using a magnetic stirrer to concentrate QQ enzyme in crude broth. The 100ml CFS was treated with ammonium sulfate powder separately to obtain 40, 50, 60, 70, 80, and 90 percent saturation of ammonium sulfate. The precipitates were collected for each percent saturation after overnight incubation at 4◦C with ammonium sulfate powder (w/v) followed by centrifugation at 10000g for 10 minutes. The precipitates obtained for each percent saturation of ammonium sulfate were dissolved in a phosphate buffer (pH 7).The fractions were dialyzed overnight in 100X volume of 10 mM phosphate buffer (pH, 7.0) by using a 3.5 kDa dialysis membrane (Spectrum™ Labs Spectra), and dialysates were concentrated in powder form by using freeze dryer (ScanVac, Labogene). The dialyzed fraction of each percent saturation was transferred to 2ml centrifuge tubes and frozen overnight at -80◦C. Further fractions were vacuum dried in a freeze dryer to obtain the powdered form of precipitates. The concentrated freeze dried protein fractions were further dissolved in 100 µl of Mili Q water. The violacein pigment inhibition assay was performed using *C. violaceum* 2656 to detect QQ activity in partially purified fractions **(Mukherji & Prabhune, 2015)**. Reduction in violacein pigment production in presence of CFS was measured in terms of percentage inhibition as described earlier in quantitative estimation of violacein pigment inhibition.

Identification of bacterial isolates using 16S rRNA Gene sequencing

A single colony of culture BG5 and K1was inoculated separately in nutrient broth and incubated at 30ºC overnight. Culture suspensions (~ 4ml) were centrifuged and cells pelleted at 5000g for 10 min. The cell pellet was washed with 500 µl of 1X PBS (phosphate-buffered saline, pH 7.4). Genomic DNA was isolated from the cell pellet using FavorPrep Soil DNA Isolation Mini Kit according to the manufacturer's instructions. Amplification of the 1.5 kb 16S rDNA was carried out with forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') using genomic DNA as the template. The PCR cycle consisted of a first denaturation step at 94°C for 5 min, followed by 30 denaturation cycles at 94°C for 60s, and further annealing was done at 56 °C for 60 s. Then extension was at 72°C for 60s and the final extension was at 72°C for 20 min. A Purified 1.5 kb fragment was used as a template for the cycle sequencing reactions with the forward and reverse primers: 27F and 1492R. Sequencing of the 1.5 kb fragment was performed on an ABI-3700 automated sequencer. The sequences obtained were compared with the reference sequences available in the EzBioCloud Database, to determine the closest known relative species.

RESULTS

Isolation, screening and identification of bacterial isolates for quorum quenching activity

Total 132 bacterial isolates were obtained based on their colony morphological differences on five times diluted nutrient agar plates. These isolates were sub-cultured and maintained on a nutrient agar medium. These isolates were further screened for their QQ ability. Nine bacterial cultures were detected positive for QQ activity. Two potential isolates BG5 and K1 were further selected based on their inhibition zone of violacein pigment as shown in fig. 1.

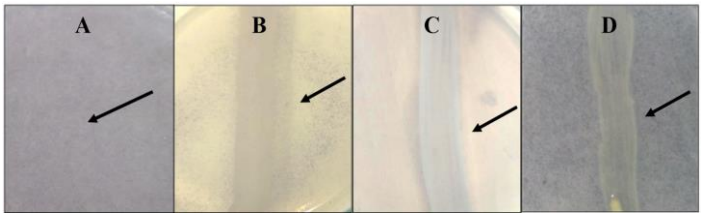

Figure 1 Representative images of Quorum quenching (QQ) activity of bacterial isolates: (A) *Chromobacterium violaceum CV026* without bacterial isolate (B) *Chromobacterium violaceum CV026* with bacterial isolate BG5 and (C) *Chromobacterium violaceum CV026* with bacterial isolate K1 (D) *Chromobacterium violaceum CV026* with bacterial isolate M5.

Bacterial isolates B5 and K1 were identified by 16srRNA gene sequencing. The obtained sequences were compared with the reference sequences available in the EzBioCloud Database. It was seen that these bacterial isolates were close to genera *Serratia*. The sequence data of these isolates were submitted to the GenBank database and accession numbers were obtained. Isolates BG5 and K1 are identified as *S. marcescens subsp. sakuensis* KRED (GenBank accession ID MT705010) and *S. nematodiphila* DSM21420 (GenBank accession ID MT754555), respectively.

Quantitative estimation of violacein pigment inhibition

The QQ activity of positive isolates was studied by using a pigment inhibition assay with biosensor strain *C. violaceum 2656*. Extracellular and intracellular extracts of positive isolates were assessed for their QQ activity by a pigment inhibition assay. The highest pigment inhibition (>50%) was observed with extracellular extracts of BG5 and K1 isolates indicating them as potential QQ molecule producers as shown in Fig.2.

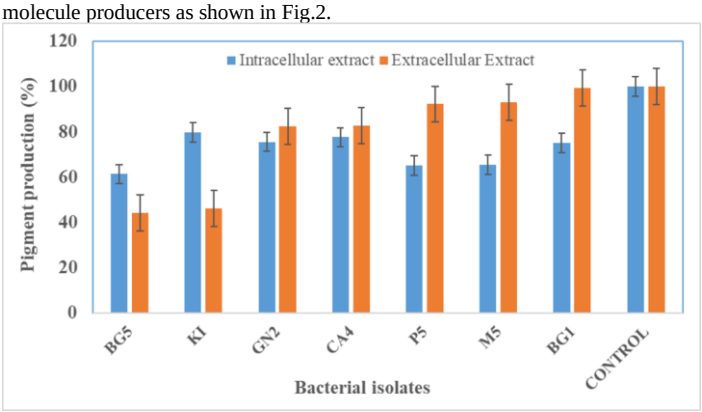

Figure 2 QQ activity of intracellular and extracellular extracts of bacterial isolates against *Chromobacterium violaceum 2656* as determined by pigment inhibition assay.

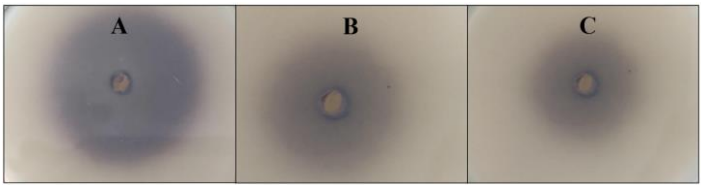

Figure 3 Whole-cell AHL degradation assay by using bacterial isolates K1 and BG5. Arrow indicates violet zone of violacein pigment inhibition. A) Control indicating violet zone of violacein pigment in absence of QQ molecule, B) Reduction in zone of violacein pigment in presence of cell-free supernatant of K1 B) Reduction in zone of violacein pigment in presence of cell-free supernatant of BG5

Whole-cell AHL degradation assay

To understand the pigment inhibition mechanism, whole-cell AHL degradation assay was carried out.
Enzymatic degradation of the AHL molecule may be responsible for the reduction in the diameter of the zone of violacein pigment produced by the biosensor strain CV026. Maximum reduction in the zone of pigment synthesis was detected in isolates K1 and BG5, which indicates the degradation of C_6HSL molecule.

QQ activity of extracellular extracts of K1 and BG5 on biofilm-forming organisms

The cell-free supernatants were tested for QQ activity on biofilm formation by *V. cholerae* MTCC 3906, *V. parahaemolyticus* MTCC 451, and *P. aeruginosa* PA01 by a static microtiter plate method. The results showed that maximum inhibition of biofilm formation was observed in all three biofilm-forming bacteria after treatment of CFS of BG1 and K1 bacterial isolates. The quantification of biofilms and planktonic cells revealed that biofilm inhibition has taken place however, planktonic cells were not inhibited. Thus, these results suggested that biofilm formation in the quorum sensing dependent pathogens can be controlled by K1 and BG5.

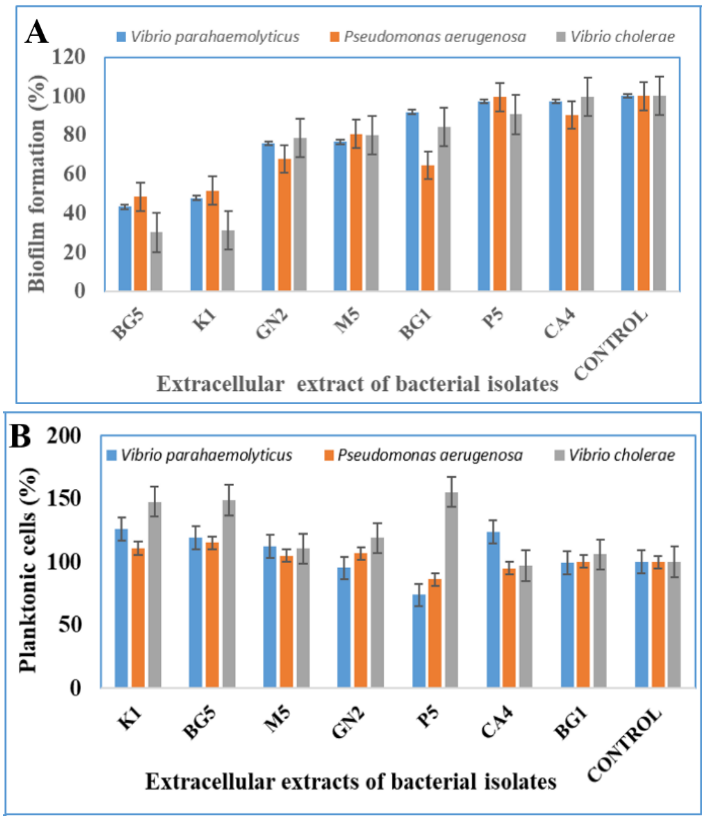

Figure 4 Effect of QQ activity of extracellular extracts on planktonic cell growth and biofilm-formation of *P. aeruginosa PA01, V. parahaemolyticus* MTCC 451, and *V. cholerae* MTCC 3906.

Partial purification of QQ molecules by ammonium sulfate precipitation

Quorum quenching molecules secreted by *S. marcescens* subsp. *sakuensis* KRED and *S. nematodiphila* are precipitated from CFS using ammonium sulfate saturation. AHL lactonase activity in the collected dialysate fractions was detected using *C. violaceum 2656* based pigment inhibition bioassay and the fractions showing the decrease in violacein synthesis were considered as positive dialysate fractions containing quorum quenching biomolecule. The total violacein pigment inhibition was detected at 80% saturation of ammonium sulfate dialysates of both *Serratia* species (figure 5).

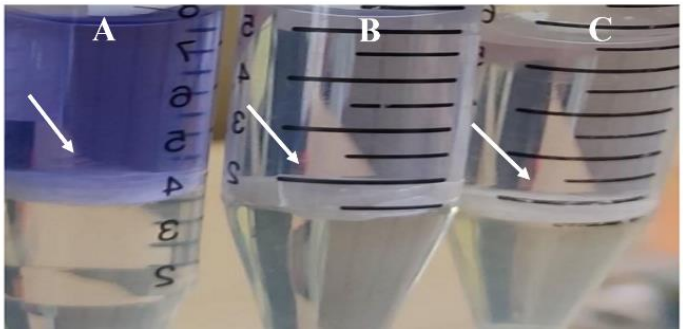

Figure 5 Ammonium sulphate precipitation of extracellular extracts of BG5 and K1 showing complete inhibition of violacein pigment synthesis. Arrow indicates butanol extracted violet coloured violacein pigment A) Negative Control – production of violacein pigment in absence of dialysate, B) *S. marcescens* subsp. *sakuensis* KRED 80% saturation of ammonium sulfate dialysate treatment showing 100% inhibition of violacein pigment, C) *S. nematodiphila* 80% saturation of ammonium sulfate dialysate treatment showing 100% inhibition of violacein pigment

Bacterial identification based on 16S rRNA Gene sequencing:

Two potent isolates B5 and K1, with quorum quenching ability, were identified by 16S rRNA gene sequencing. The sequences were compared with the reference sequences available in the EzBioCloud Database, to determine the closest known relative species. These two AHL degrading strains were closely related to genus *Serratia*. The sequence data of isolates were submitted to the NCBI GenBank database and accession numbers were obtained. Isolates BG5 and K1 were identified as *S. marcescens subsp. sakuensis* KRED (GenBank accession ID MT705010), and *S. nematodiphila* DSM21420 (GenBank accession ID MT754555), respectively.

DISCUSSION

Inhibition or inactivation of QS is the most potential novel approach in order to control antibiotic resistant, biofilm formation and infection in bacterial pathogens **(Dong and Zhang, 2005, Vesuna and Nerurkar, 2020)**. From various microbial communities quorum quenching molecules have been detected and studied to use them to control QS system in biofilm-forming bacterial pathogens **(Saurav *et al*., 2016; Tan *et al*., 2015)**. Diverse species of rhizospheric bacteria assists in development of plant health due to their beneficial activities. The QQ activity has also been previously reported in several bacterial isolates from rhizosphere soil **(Alinejad *et al., 2020)***. Extensive reports are available on QQ activity of rhizospheric *Bacillus* species against QS signaling systems of animal and plant associated bacterial pathogens **(Augustine *et al*., 2010; Y. H. Dong *et al*., 2001; Rosier *et al*., 2021)**.Therefore investigation of quorum quenching molecules that interfere with the QS mechanism of biofilm-forming pathogens, ultimately results in control of virulence in pathogens. In current study, total 132 bacterial isolates were obtained from several agricultural and wild plant root rhizosphere. Amongst these isolates, two isolates from Chickpea (*Cicer arietinum*) and Coriander (*Coriandrum sativum*) plants were identified as *S. marcescens* subsp. *sakuensis* KRED and *S. nematodiphila* DSM21420 respectively, showed significant QQ activity.

Several Gram-negative bacterial pathogens from genus *Vibrio* and *Pseudomonas* control their virulence by QS mechanism, producing AHLs as major signaling molecules. Therefore many infectious pathogenic bacteria could potentially be controlled by inhibiting their QS systems. Studies on QS control may provide a means of treating many common and damaging chronic infections without the use of killing agents like antibiotics which distinctly select for resistant organisms by causing life-death pressure**(Hentzer & Givskov, 2003; Raafat *et al*., 2019; Saurav *et al*., 2016)**. Inactivation of QS signaling molecules result in the lowering of pathogenicity which may reduce the dose of the antibiotic and may further prevent the emergence of multiple drug resistant organisms **(Dong *et al.* 2000)**. In current study, it has been studied that *Serratia* species isolated from the rhizosphere soil showed its ability to inactivate C6HSL cognate signaling molecule and inhibited biofilm formation in Gram-negative pathogens. Various AHL signaling molecules are reported in *P. aeruginosa*, *V. cholerae* and *V. parahaemolyticus* and are responsible for biofilm-associated infections and drug resistance **(Vallet *et al*., 2004)**. In present study, the CFS of *Serratia* isolates exhibited interference in biofilm formation of *V. cholerae* MTCC 3906, *P. aeruginosa* PAO1, and *V. parahaemolyticus* MTCC 451 without inhibiting planktonic cell growth. The study result showed that QQ molecules are present in CFS of *Serratia* isolates. Our results are supported by previous reports of QQ activity by *Bacillus* species against biofilm forming pathogens such as *V. Parahaemolyticus*, *Proteus* species and *Pseudomonas aeruginosa* **(Augustine *et al*., 2010; Raafat *et al*., 2019; Vinoj *et al*., 2014)**. Previously, Rehman and Leiknes, also showed that *Erythrobacter* and *Labrenzia* bacterial species isolated from red sea sediments had potential to inhibit biofilm formation by *Pseudomonas aeruginosa* PAO1**(Rehman & Leiknes, 2018).**

It was also noted that the concentration of quorum quenching molecule is a key factor in controlling biofilm formation. These findings are supported by the report of Malesevic *et al*., which showed that biofilm forming ability of *P. aeruginosa* MMA83 was significantly reduced by the QS inhibitory molecule extracted from *Delftia tsuruhatensis* 11304 in dose dependent manner, without affecting overall bacterial cell growth**(Malešević *et al*., 2019).** The quorum quenching molecules were concentrated and partially purified by ammonium sulphate precipitation technique. AHL inactivating activity in the collected fractions was detected using *C. violaceum* 2656 based on violacein pigment inhibition assay. The observations revealed that dialysate obtained from 80% saturation of ammonium sulphate exhibits highest quorum quenching activity. It has been already reported in previous studies that maximum QQ activity by lactonase enzyme produced by various *Bacillus* species against AHL signaling molecule was obtained between 70- 80% ammonium sulphate saturation **(Eka *et al*., 2016; Mukherji & Prabhune, 2015)**. In another study, bacterial wilt pathogen *Ralstonia solanacearum* associated QS molecule 3-hydroxy palmitic acid methyl ester (3OH-PAME) was inactivated by 3OH-PAME degrading enzyme obtained in the CFS of endophytic bacteria was concentrated using ammonium sulphate at 80% saturation**(Achari & Ramesh, 2015)**. Thus, QQ molecule needed to be further purified and characterized to study structure and mechanism of action of inactivation of signaling molecule for its application in combined antibiotic therapies to control virulence in QS regulated pathogens.

CONCLUSION

The QS mechanism in Gram-negative bacteria regulate important biological functions, including virulence and biofilm formation that can be targeted by QQ molecules produced by rhizospheric bacterial species. This study revealed the capability of rhizospheric *Serratia* isolates, to degrade AHL signaling molecules in Gram-negative bacteria indicating the presence of QQ molecules. It clearly suggests that *Serratia* isolates can be used as potential sources of quorum quenching molecules. In current study we report that *Serratia* species isolated from plant root rhizosphere exhibits QQ activity which inhibited biofilm formation in pathogenic bacterial strains i.e. *P. aeruginosa* PAO1, *V. cholerae* MTCC 3906, and *V. parahaemolyticus* MTCC 451. Therefore, present study has the medical relevance and QQ molecules from these bacterial strains needs to be studied further to explore mechanism of action. Discovery of the more and more QQ molecules in diverse bacterial systems will provide a promising tools to control pathogen menace.

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