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Chromobacterium violaceum Based Screening of Quorum Quenching Bacteria for the Biocontrol of Quorum Sensing Phytopathogens

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Abstract

Bacterial phytopathogens attack host by a series of mechanisms like virulence factor synthesis which is a consequence of quorum sensing (QS) mechanism as a group behavior in unison. Acyl homoserine lactones (AHLs) are major signaling auto inducer molecules of most of the QS systems. Hindering the AHLs to reach threshold concentration by quenching the signal can upset the QS. Soil bacterial community possesses quorum quenching (QQ) population which devitalize the quorum system by interfering with AHL signals using an array of QQ enzymes. Eight samples were collected from different experimental sites of University of Agricultural Sciences, GKVK, Bengaluru. A total of thirty-nine AHL degrading (QQ) bacterial isolates were obtained by *in vitro* enrichment culture technique in which N-hexaonyl-L-homoserine lactone (C_6-HSL) served as a sole carbon source. Preliminary screening studies were conducted using bioindicator *Chromobacterium violaceum* MCC 4212 where the inoculum level and incubation period were standardized as 0.1 per cent and 24-36 h, respectively. In violacein quantification assay, per cent (%) inhibition of violacein exhibited by the bacterial isolates varied from 53.58 to 80.89 per cent followed by soft agar overlay assay wherein 31 isolates showed violacein inhibion and eight isolates exhibited both growth and pigment inhibition. Fifteen efficient isolates were screened out of 39 for further QQ assays against the soft rot pathogen *Pectobacterium carotovorum* subsp. *carotovorum*.

Keywords : Acyl homoserine lactones, Bioindicator strain, Quorum quenching, Quorum sensing, Violacein

In the current farming trend, major food crops suffer from a lack of genetic diversity allowing pathogens and pests to rapidly spread throughout fields and devastate crops, causing yield losses up to 32 per cent worldwide (Rooney *et al*., 2020). Gram negative phytopathogens *viz., Pectobacterium carotovorum, Pseudomonas syringae, Ralstonia solanaearum* tops the list in causing serious crop damage leading to severe yield loss (Mansfield *et al.*, 2012). These bacterial pathogens recruit their pathogenicity as a population dependent behavior called quorum sensing, wherein diffusible signaling molecules, majorly N- acyl homoserine lactones (autoinducers), play an important role as a language for communication. Concentration of these molecules regulates various pathogenicity traits *viz.*, biofilm formation, production of plant cell wall degrading enzymes (Chandrashekar and Prasannakumar, 2021), phytotoxin and also regulation of secretion systems (Baltenneck *et al*., 2021). Hence, quorum sensing has turned out as a notable target for plant disease control recently.

The activity of disturbing the quorum sensing by disruption of signaling molecules is termed 'quorum quenching' (Fan *et al.,* 2020). Such anti-quorum

sensing systems are found to be conserved in many prokaryotic and eukaryotic systems. QQ bacteria accounts for about 10 per cent of the total culturable bacteria recovered from several soils and rhizospheres (Dessaux *et al*., 2011). Attenuating virulence by quorum quenching is preeminent rather than bactericidal and bacteriostatic drugs since it is less likely to incite the evolution of resistance in bacteria. Quorum quenching, in addition to plant disease biocontrol, extend its application in the field of bioremediation by controlling biofouling in bioreactors, in aquaculture and so on (Malik *et al*., 2021).

Chromobacterium violaceum, a Gram negative proteobacterium, is known to be a well-established biosensor organism (Poli *et al*., 2018). It produces violacein, a purple pigment as an outcome of quorum sensing mechanism and can be used as a tool for measuring the impact of various quenching substances on quorum sensing (Kothari *et al*., 2017). It is also reported that *C. violaceum* can be employed as an indicator organism capable of detecting a range of AHLs to validate if quorum sensing inhibition is due to AHL interference (McLean *et al*., 2004).

Based on the above observations experiments were conducted to isolate quorum quenching bacteria by enrichment culture technique using C_6 HSL as substrate, followed by the screening for efficient QQ bacterial isolates using the bioindicator strain *Chromobacterium violaceum* MCC 4212.

Material and Methods

Collection of Soil Samples

A total of eight samples were collected from the experimental sites of University of Agricultural Sciences, GKVK, Bengaluru and represented with GIS coordinates (Table 1). Different experimental sites were targeted for sampling such as farmyard manure (FYM), vermicompost and rhizosphere soil. The samples were collected at a depth of 10 - 15 cm from the surface, transferred into the polypropylene bags and transported immediately to the laboratory. Sampling was carried out in the year 2020. The samples were stored at 4°C for further use.

Isolation of AHL Degrading Quorum Quenching Bacteria

The QQ bacteria were isolated by enrichment culture technique as given by Ye *et al*. (2019). In the present study N-Hexanoyl-L-homoserine lactone (C_6-HSL; e' 96% (HPLC)) (Product No. 56395, Sigma Aldrich, USA) was used as a substrate for isolation of QQ bacteria. The stock solution of concentration one mM L^{-1} was prepared using methanol and stored at -20°C for further use. Working solution was made by diluting the stock solutions with culture medium and filtering them through a 0.2 µM nylon membrane syringe filter. The working solution of concentration 20 µM L^{-1} was prepared by diluting 0.4 mL of stock solution in 1.96 mL of minimal salt medium (MSM) (Fan *et al*., 2020).

Suspension was prepared by mixing samples (50 mg) with 2 mL of the MSM without a carbon source and the mixture was vigorously vortexed. The suspension was then centrifuged using centrifuge model 5420 (Eppendorf India Pvt Ltd.) at 3000 rpm for 5 mins, the supernatant was inoculated (5% v/v) with 0.2 mL of the MSM containing 20 µM of C_6-HSL and the mixture was incubated at 30 °C for three days. After three days, the suspension was transferred to fresh MSM containing C_6-HSL (100 µM L^{-1}) at 5 per cent inoculum and cultivated under the same conditions. This step was repeated until the C_6-HSL supplementation was increased in three enrichment cycles (100, 200 and 400 µM L^{-1}). The final suspension was serially diluted (10^{-1} to 10^{-8}) and 0.1 mL of 10^{-6}, 10^{-7} and 10^{-8} dilutions was spread on Luria Bertani (LB) agar plates in triplicates, incubated for 24h at 30°C. The bacterial colonies that showed distinct morphology were picked for isolation. After several passages on LB agar, pure cultures were obtained and maintained on LB agar slants for further studies.

Standardization of inoculum level of *Chromobacterium violaceum*

The primary screening for AHL degrading bacteria was carried out by co-inoculation of isolated bacterial cultures with a quorum sensing induced chromogenic

bioindicator strain. Accordingly, the standard strain *Chromobacterium violaceum* MCC 4212 was chosen as the bioindicator. The standard culture of *C. violaceum* MCC 4212 was procured as freeze-dried ampoule from National Centre for Culture Supply (NCCS), National Centre for Microbial Resources (NCMR), Pune, Mumbai, India. The culture was revived by the standard protocol as given in NCMR guidelines using LB broth. The fresh culture was stored in agar slant and glycerol stock at 4°C and -20°C, respectively, for further use.

The screening of bacterial isolates for QQ involved *Chromobacterium violaceum,* hence, standardization of inoculum level and respective violacein production was required. For standardization, the culture of overnight grown *C. violaceum* was adjusted to the OD_{600} = 0.1 using phosphate buffer (pH 7.2). A set of conical flasks containing 10 mL of LB broth were inoculated with 0.1, 0.2, 0.5, 1 and 2 per cent of *C. violaceum*, respectively and medium without inoculum served as control. All the flasks were incubated at 30°C with proper shaking (150 rpm). The samples were drawn at different time intervals (0, 3, 6, 9, 12, 24 and 36 h respectively) for the analysis of growth and violacein production (Kusari *et al*., 2014) that were assessed using UV visible spectrophotometer (Thermo scientific, Biomate 3s, China) at 600 and 585 nm respectively. One mL of *C. violaceum* culture collected from each flask were centrifuged using Eppendorf® Minispin® personal microcentrifuge (Eppendorf India Pvt. Ltd.) at 13000 rpm for 10 min for the analysis of violacein production. One mL of dimethyl sulfoxide (DMSO) was added to the cell pellet and vortexed thoroughly to extract the violacein in to the solvent. The cell debris was removed by centrifugation (13000 rpm, 10 min). The supernatant was then quantified spectrophotometrically at OD_{585}. Each setup was prepared in triplicates.

Screening of Quorum Quenching Bacteria

Violacein Inhibition Assay

A total of thirty-nine isolates were tested for their QQ ability quantitatively using violacein inhibition assay. Overnight grown *C. violaceum* and bacterial isolates were adjusted to OD_{600} = 0.1, using phosphate buffer. Each bacterial isolate was co-inculated with *Chromobacterium violaceum* both at 0.1 per cent. The bioindicator strain *C. violaceum* alone at 0.1 per cent without bacterial isolate served as negative control. All samples were incubated at 30°C on a rotary shaker (150 rpm) for 24-36h. Violacein unit was estimated spectrophoto metrically (OD_{585}) using the method as explained earlier. Inhibition of violacein production was calculated based on the following formula :

$$\text{Violacein inhibition percentage (\%)} = \frac{OD_{585}\ \text{control} - OD_{585}\ \text{sample}}{OD_{585}\ \text{control}} \times 100$$

Soft Agar Overlay Method

The QQ ability of the isolates was qualitatively assessed using soft agar overlay assay as given by McLean *et al.* (2004). Three µL of test organisms were spot inoculated onto the center of the full-strength LB agar plate (2%) and grown overnight at 30 °C. Following overnight growth, the organisms were then overlaid with 5 mL LB soft agar (full strength LB broth containing 0.7% agar) at 45 °C, seeded with 0.1 per cent of overnight cultured *C. violaceum* (OD_{600} = 0.1) in 10 mL of LB broth and mixed thoroughly. The seeded media was poured over the surface of solidified LB plate containing overnight bacterial growth, to form the overlay and incubated at 30 °C for 24 - 36 h. The QQ activity was assessed by measuring the diameter of turbid halos created due to inhibition of violacein pigment.

Statistical Analysis

Statistical significance of variance for data collected was determined using ANOVA. The inhibition of violacein production and zone of pigment inhibition were analyzed by one-way ANOVA. Analysis was performed using Graphpad Prism 8.0.1 software.

Results and Discussion

Isolation of QQ Bacteria from different Samples

Occurrence of QQ and QS bacteria in soil microbial diversity entertains the balance in the natural

Sample	Site	Crop	GIS co-ordinates	Bacterial isolates
FYM	Agroforestry, GKVK	-	13.08670°N 77.57432°E	HSL 11 HSL 12 HSL 13 HSL 14
Vermicompost		-		HSL 21 HSL 22 HSL 23 HSL 24
Rhizosphere soil	CPH research plot, GKVK	Tomato (*Solanum lycopersicum* L.)	13.08290°N 77.57211°E	HSL 31 HSL 32 HSL 33 HSL 34 HSL 35 HSL 36 HSL 37 HSL 38
Rhizosphere soil	Horticultural field, GKVK	Dolichos bean (*Lablab purpureus*)	13.08104°N 77.56773°E	HSL 41 HSL 42 HSL 43 HSL 44 HSL 45
Rhizosphere soil		Cabbage (*Brassica oleracea*)	13.08113°N 77.56799°E	HSL 51 HSL52 HSL 53 HSL 54 HSL 55
Rhizosphere soil	Krishimela site, GKVK	Chilli (*Capsicum annuum* L.)	13.08429°N 77.57308°E	HSL 61 HSL 62 HSL 63 HSL 64
Rhizosphere soil	Agronomy field unit, E block, GKVK	Radish (*Raphanus sativus*)	13.07796°N 77.56986°E	HSL 71 HSL 72 HSL 73 HSL 74
Rhizosphere soil		Ragi (*Eleusine coracana*)	13.07833°N 77.57089°E	HSL 81 HSL 82 HSL 83 HSL 84 HSL 85

ecosystem (Zapata *et al.,* 2017). QS systems use short chain AHLs as cue for most of the bacterial phytopathogenicity (Morohoshi *et al*., 2019). These AHLs act not only as language but also as a competitive advantage as it results in formation of tetrameric compound which will be toxic for most of the co-existing bacterial population. Such competitive advantage of QS can be countered by a variety of bacteria in the environment due to their heterogeneity. Enzymatic degradation of those

competent signaling molecules *i.e.,* quorum quenching by bacteria has evolved as one such competing strategies. In the present study enrichment of minimal salt medium supplemented with C_6-HSL as a sole carbon source was used to boost the growth of AHL degrading bacteria and three enrichment cycles were carried out. After enrichment, the cell suspensions of different dilutions were spread on the LB agar plates. The dilutions of 10^{-8} and 10^{-7} yielded colonies that were too less to count, hence, colonies from 10^{-6} dilution were also taken for isolation. A total of thirty-nine isolates showing distinct colony morphology, were obtained from FYM, vermicompost and six different rhizosphere soils collected from experimental sites of UAS, GKVK, Bengaluru (Table 1). AHL signal molecules can be utilized by QQ bacteria *via* synthesis of AHL degrading/ modifying enzymes and the resulting products can be exploited as energy and nitrogen source (Chong *et al*., 2012). The culturable QQ bacterial population was reported to be 10 per cent in the soil and rhizospheres (Dessaux *et al*., 2011). Christaen *et al*. (2011) isolated 59 isolates that were able to use AHL signal as a carbon and nitrogen source, from sixteen different environmental sample. The highest number of isolates (8) were obtained from rhizosphere of Tomato. Amin *et al*. (2016) isolated 45 bacterial isolates from rhizosphere soil of tomato and rice in Malaysia and screened six isolates with QQ activity, all belonged to *Bacillus* spp. A study conducted by Saranya *et al*. (2021) revealed that 25 per cent of the bacterial isolates possessed QQ ability among 96 rhizobacteria isolates. This study is in consistence with previous studies regarding the existence of QQ activity in bacteria from the soil (Li-Xing *et al*., 2012; Magdalena *et al*., 2021 and Lohith kumar and Krishna Naik, 2021).

Standardisation of Inoculum Level and Violacein Production by *Chromobacterium violaceum*.

Bioindicator strain based screening of QQ bacterial isolates is the most prevalent approach for studying their efficiency in QS inhibition. Gram-negative biosensor strain, C*hromobacterium violaceum* is known to produce violacein, a purple pigment, as a result of quorum sensing utilizing the CviI/CviR synthase-receptor signaling (McClean *et al.,* 1997). Loss of this pigment is an index of quorum quenching behavior. Therefore, standardization of *C. violaceum* was done for inoculum size and their respective violacein production. The growth of *C. violaceum* and its violacein production at different intervals where inoculation level varied from 0.1 to 2 per cent was estimated spectrophotometrically at 600 nm and 585 nm, respectively. The results are presented in the Fig. 1.

In all the inoculum levels, *C. violaceum* showed lag phase of growth till 6 h, later it entered log phase which lasted upto 24 h of incubation. After 24 h, it entered early stationary phase. With regards to violacein production, which is a population dependent QS attribute, it was at basal level till mid log phase, later there was an inflation at all the

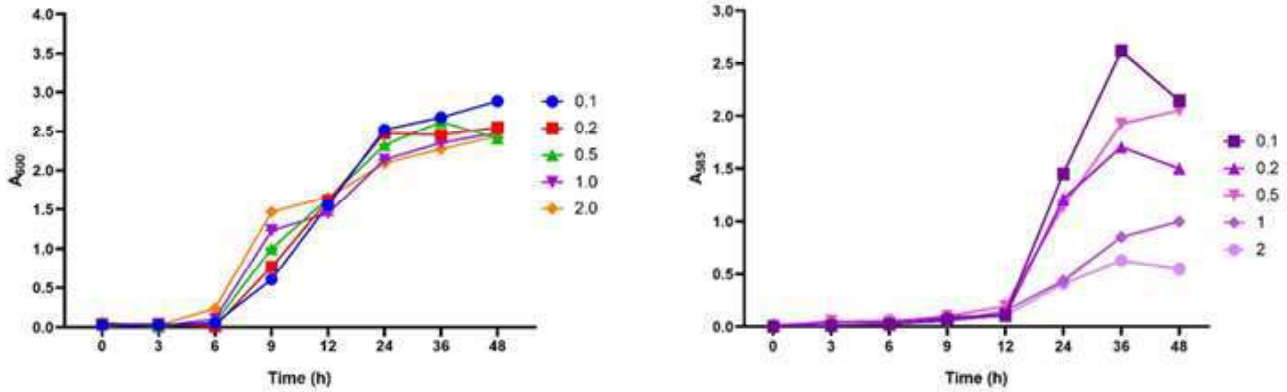

Fig. 1 : Growth and production of violacein by *Chromobacterium violaceum* at different inoculum levels. (a) at OD_{600} and (b) at OD_{585}

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inoculum levels. The threshold population density for the quorum concentration was estimated as OD_{600}> 1.25 and it was observed that the bacterial culture could produce pigment. Results of growth studies of *C. violaceum* were similar to the observations of Kanekar and Devasya (2022). With increase in inoculum size, the violacein escalation fold size was reduced and it was also found to be less than 0.1 per cent inoculum size. This increase in decreasing trend with step up in inoculum level may be due to competence within population for nutrient and also due to the fact that higher concentration of AHL counters the quorum sensing system by negative feedback mechanism, since AHL synthesis is a costly step. Further a detailed study needs to be carried out to elucidate the reason behind this reduced level of violacein production with increase in bacterial inoculum. *Chromo bacterium violaceum* at 0.1 per cent (OD_{600}=0.1) showed steady growth with distinct growth phases over a period of time and increased violacein production with growth. Violacein production was found to be higher during 24-36 h of growth. Accordingly, inoculum size of 0.1 per cent and incubation period of 24-36 h for *C. violaceum* were used for further screening studies.

Screening of Quorum Quenching Bacteria using *Chromobacterium violaceum* MCC 4212

The QQ efficacy of the test isolates were determined by quantification of violacein extracted using dimethyl sulfoxide (DMSO). The violacein unit was measured spectrophotometrically and inhibition percentage of violacein production by the 39 bacterial isolates were calculated. Test isolates inhibited violacein pigment when co-inoculated with the biosensor organism.

Chromobacterium violaceum is known to produce hydrogen cyanide and violacein that pose toxicity towards interfering prokaryotes and eukaryotes. Bacteria with quorum quenching activity drastically reduce such toxicity by unleashing enzymes (Mion *et al*., 2021). Violacein synthesis in *C. violaceum* was tested in presence and absence of test isolates and was found to be reduced in coculture conditions. The results of violacein inhibition rendered by the bacterial isolates is presented in Fig. 2. The percentage inhibition of violacein pigment by the quorum quenching isolates ranged between 53.57 and 80.87 per cent. Significantly higher percentage of violacein inhibiton was exhibited by the isolate HSL 52 (80.87±1.07mm) followed by HSL 61 (80.24±0.64 mm). The lowest was recorded by the isolate HSL 84 (53.57±1.4 mm). The results are in agreement with the findings of Rajesh and Rai (2014) and Venkatramanan *et al.* (2020) who reported that the endophytic bacteria of *Pterocarpus santalinus* and ethyl acetate extract of *Passiflora edulis* inhibited violacein production up to 80 and 88 per cent, respectively.

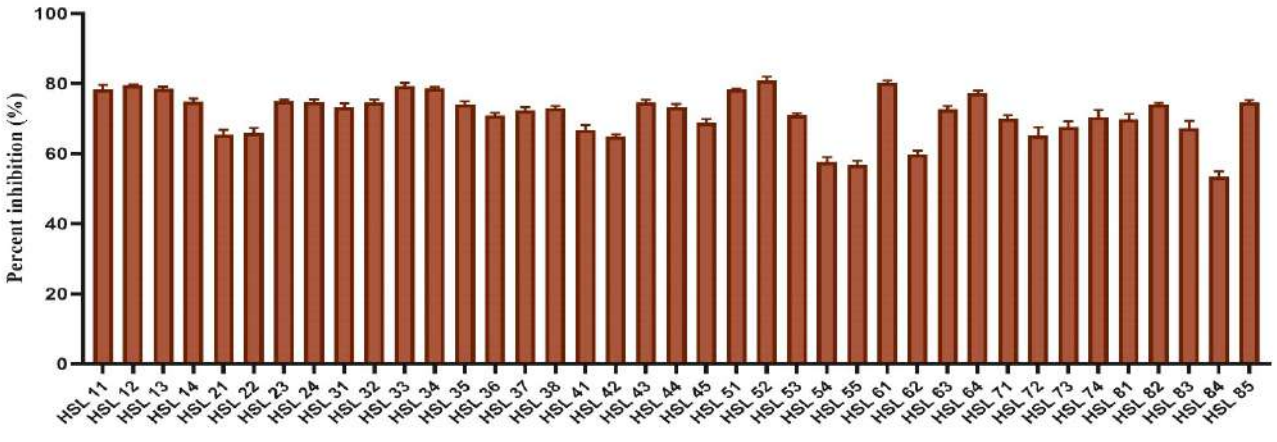

Fig. 2 : Inhibition of violacein production (%) by QQ bacterial isolates

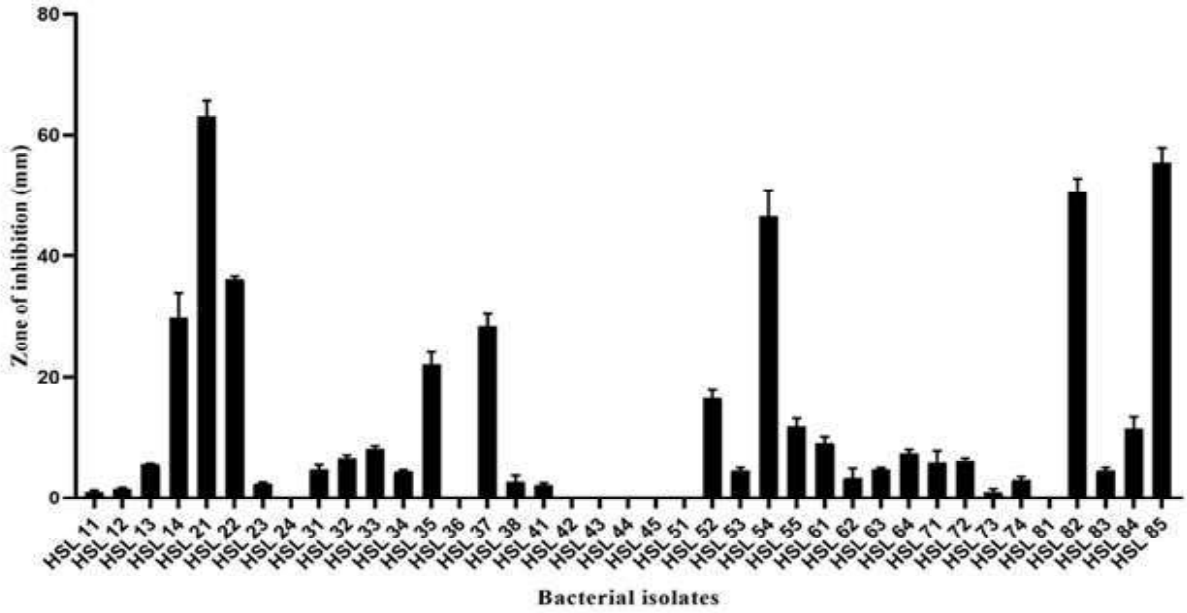

Fig. 3: Zone of pigment inhibition exhibited by bacterial isolates in soft agar overlay technique

The bacterial isolates were further assayed qualitatively by soft agar overlay technique. This was done to confirm whether the violacein inhibition is due to attenuation of quorum sensing and not due to growth inhibition. QQ mechanism mainly aims for antivirulence strategy rather than antibacterial activity. *Chromobacteriun violaceum* as a model for screening of QQ isolates was reported previously on screening of QQ bacteria from antlion (Christianto, 2011) and bacteria from soil (Chong *et al*., 2012). Out of thirty-nine bacterial isolates, thirty-one bacterial isolates showed only pigment inhibition and eight isolates (HSL 24, HSL 36, HSL 42, HSL 43, HSL 44, HSL 45, HSL 71 and HSL 81) have shown clear halo and turbid halo depicting both growth and pigment inhibtion (Plate 1). Thus, inhibition of violacein by these bacterial isolates (around 65% - 75%) was not merely due to quorum sensing inhibition but by killing of the bioindicator, exemplifying the fact given by Abudoleh and Mahasneh (2017). The ability of bacterial isolates to inhibit violacein production was indicated by varying zone of pigment inhibition. The zone of pigment inhibition of bacterial isolates is depicted in Fig. 3. Khoiri *et al*. (2016) could show that efficient strains showed inhibition zone diameter equal or more than 4mm, with the highest quorum quenching activity recorded by the isolates GG3 (10.17 mm) and B37 (10 mm). In the present preliminary screening studies, 15 efficient bacterial isolates performed consistently in both quantitative and qualitative assay with better pigment inhibition, and hence were chosen for further biocontrol studies.

Plate 1 : Soft agar overlay method : a. Control; b. Pigment inhibition only and c. Growth and pigment inhibition

Due to bacterial resistance development there is need for safe and efficient alternative in biocontrol and quorum quenching is arising as a pinch-hit in the field of bacteria pathogen biocontrol. In this study, thirty-nine soil bacteria were isolated from different environment samples by enrichment with AHL as sole carbon source. Primary screening studies using the bioindicator strain, *C. violaceum* yielded fifteen efficient QQ bacterial isolates. Our further studies will

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focus on the biocontrol potential of these QQ isolates against *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) and their mechanism of action in attenuating the virulence of pathogens.

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