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Anti-quorum Sensing Activity by Cell Free Lysates of Quorum Quenching Bacteria in *Chromobacterium violaceum* MCC 4212

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ABSTRACT: The pathogenicity of bacterial phytopathogens solely depends on cell-to-cell communication called quorum sensing (QS) which in turn regulates the genes responsible for virulence factors, phytotoxin production, biofilm formation and motility to initiate and establish the disease in hosts. Quorum quenching will be a most suitable approach to control such virulence by interfering with QS system without hampering its growth. To confirm the antiquorum sensing activity of QQ isolates, cell free lysates (10%-50%) of the isolates were tested against the growth and violacein production of *Chromobacterium violaceum* MCC 4212. Concentration dependant violacein inhibition was observed with all QQ isolates without any significant reduction in growth. The highest inhibiton percent of 97% shown by three of the isolates (HSL 31, HSL 32 and HSL 64) at 50% concentration. The results suggest the potential of QQ bacteria to interfere the QS activity of pathogens that could attenuate the pathogenicity and thereby, diseases without resistance development.

Keywords: Quorum sensing, quorum quenching, *Chromobacterium violaceum*, violacein inhibition, growth.

INTRODUCTION

The discovery of bacterial communication in 1970s called quorum sensing, an imperative mechanism widely employed by a variety of Gram positive and Gram negative bacterial species to coordinate a communal behaviour (Rutherford and Bassler 2012). As a population dependant approach, bacteria orchestrate their communication by means of producing diffusible signalling molecules called autoinducers (AI) as their language. Concentration of these AIs switches on/off the quorum sensing mechanism in turn regulates various physiological traits such as virulence factors, motility, biofilm formation etc., according to cell density and growth phase which is responsible for the bacterial survivability in the environment (Baltenneck *et al*., 2021).

Bacterial phytopathogens like *Pectobacterium* spp., *Pseudomonas syringae*, *Xanthomonas campestris*, *Dickeya* sp., (Mansfield *et al*., 2012) express their virulence factors, regulates type III secretion system, motility, biofilm formation by means of quorum sensing mechanism (Sibanda *et al.,* 2018). The importance of QS in bacterial pathogenesis has driven the research of inhibiting QS mechanism. Interfering this communication system in bacteria and control the phytopathogenicity of infectious bacterial pathogens without hampering their growth, thus avoiding the development of bacterial resistance. The process of degrading or inactivating the QS signalling molecules is called quorum quenching (QQ). QQ from different sources *viz*., animals, plants, fungi and bacteria, are capable of hitting the QS system in two ways either by quorum quenching enzymes that degrade the QS molecules or molecules that block QS (Chan *et al*., 2011).

Recently, quorum quenching from bacterial origin is given special interest in the quest for anti-virulence compounds because of their enzymatic activity that degrades the QS signals without affecting the pathogen's growth (Christiaen *et al*., 2011; Garge and Nerurkar 2016). Since virulence activity of the phytopathogens are authoritated by quorum sensing system, it became plausible for searching anti quorum sensing substances from different origin. In this report, culture extract of five quorum quenching bacterial isolates from the different environmental samples were tested for their QS inhibitory effects in a *C. violaceum* MCC 4212, a biosensor strain. Anti-quorum sensing efficiency of cell free lysate of QQ bacterial isolates are presented and discussed here.

MATERIALS AND METHODS

Bacterial cultures. Quorum quenching bacterial isolates used in this research were obtained from previous studies that isolated from different regions of Gandhi Krishi Vignyanakendra, Bengaluru, India. The

previous studies stated that a soil enrichment technique, using minimal salt media supplemented with C_6-HSL as a substrate, was performed for the isolation of QQ bacterial isolates. Enrichment cycles were repeated with increasing concentration of substrate up to 400 µM/L. Different dilutions (10^{-6}, 10^{-7}, and 10^{-8}) of the farmyard manure, vermicompost and five different rhizosphere soils enriched suspensions 100µL of each suspension were spread onto LB agar medium and incubated at 30°C for 24 h. Bioindicator bacteria *C. violaceum* wild type was cultivated onto LB agar and incubated at 28°C for 2 days. Preliminary screening studies of violacein inhibition assay and soft agar overlay assay using bioindicator *C. violaceum* was carried out.

Identification of active quorum quenching bacteria. Active QQ bacterial isolates were partially identified using Gram stain reaction, catalase, oxidase, amylase and gelatinase production.

Preparation of cell free lysate. The bacterial isolates were grown in the Luria Bertani broth containing (per liter) 15·0 g tryptone, 0·5% yeast extract, 0·5% NaCl, incubated at 30 °C with shaking (150 rpm) for 24 h followed by harvesting by centrifugation at 12,000 rpm for 10 min. The cell pellets were collected and resuspended in 10 ml of phosphate buffer saline (pH 7.2) and ground to get cell-free lysate and centrifuged; supernatant was filtered through 0.22 µm filter and the filtrate was collected as "cell-free lysate" which was stored at −20 °C until use. All assays were performed with phosphate buffer saline as assay control.

Assay for the inhibition of violacein production in *Chromobacterium violaceum* MCC 4214. *C. violaceum* MCC 4214 was cultured overnight in 5 mL of Luria-Bertani (LB) broth at 30°C with or without the addition of varying concentrations (10%, 20%, 30%, 40% and 50 %) of cell free lysates of QQ isolates. The growth was measured spectrophotometrically (Thermo scientific, Biomate 3s, China) at 600 nm. One milliliter (mL) aliquot of the culture was centrifuged at 13000 rpm for 10 min. The culture supernatant was discarded, and the pellet (precipitated violacein) was re-solubilized in DMSO (1 mL) followed by centrifugation at 13 000 rpm for 10 min to precipitate the cells. Violacein content in the solution was quantified using a spectrophotometer at a wavelength of 585 nm. Inhibition of the purple pigment-violacein, produced by wild-type strain *C. violaceum* is indicative of anti-quorum sensing activity. The percentage of violacein inhibition was calculated using the following formula:

$$\frac{OD_{585}\text{control} - OD_{585}\text{sample}}{OD_{585}\text{control}} \times 100$$

Statistical analysis. The results are presented as mean±SD of three independent experiments. Statistical differences were determined by one way ANOVA using Graph pad Prism software. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Diverse physiological functions including virulence and pathogenicity in most of the Gram-negative bacteria are regulated by quorum sensing mechanism, quenching of cell-cell communication in bacteria could be a promising strategy to attenuate the expression of virulence genes, and thus can thwart the pathogenic infections (Abraham *et al.*, 2011) causing the transition of pathogenic bacteria in to nonpathogenic (Zhang and Dong, 2004). Because of extended emergence and spread of multidrug-resistant bacteria, antivirulent strategy to combat bacterial pathogenicity has received increased attention in recent years. In the present study, from enrichment culture technique using C_6-HSL as a substrate, 39 distinct colonies that varied in size, pigmentation, texture, elevation, and margin surface originating from different samples from eight different locations of Gandhi Krishi vignyanakendra, Bengaluru, were picked up, purified. The pure cultures were screened for anti-quorum sensing activity using *Chromobacterium violaceum* MCC4212 as a bioindicator strain, by quantitative (violacein inhibition) and qualitative (soft agar overlay assay) methods. Five isolates from the study showed antiquorum sensing activity without antibacterial activity. Those positive isolates were extracted and continued to the next step.

Partial identification of potential isolates. All fiveisolates were Gram negative and showed positive for catalase activity with three of these isolates were oxidase positive while all the isolates were negative for both amylase and gelatinase assay.

Effect of cell-free lysate on *Chromobacterium violaceum* MCC 4212. The most important facet in the development of quorum quenching strategy is targeting the virulence of bacteria rather than killing them. Population density of bioindicator strain grown in LB broth incubated at 30°C for 24 h showed no significant difference ($p \leq 0.05$) when measured spectrophotometrically at 600 nm in all tested concentrations. The results are expressed as OD_{600} and compared with treatments without cell free lysates (Fig. 1a). These results substantiate the anti-quorum sensing activity of the cell free lysates of bacterial isolates rather than any bacterial growth inhibition (Abudoleh and Mahasneh 2017).

The bacterial cell free lysates (CFLs) showed anti-quorum sensing activity with the reference bioindicator strain. Different concentrations of bacterial extract were used to quantify the inhibition of violacein. A purple-coloured pigment, violacein is produced by the bioindicator *C. violaceum* and this activity is tightly regulated by QS controlled genes. The interaction of cell free lysates with the bioindicator results in degradation of AHL signals and thwart the violacein production in *C. violaceum* (Aliyu *et al.*, 2020). This was indicated by loss in purple pigmentation (Fig. 2) quantified spectrophotometrically and determined as anti-QS activity. The QSI activity exhibited by each isolate was diverse in their performance at the concentrations tested in the range of 10% – 50% (Zhu *et al*., 2011) given in the Fig. 1.

Quorum sensing inhibition by quorum quenching bacteria is because of the enzymes that capable of degrading the QS signaling molecules. Cell free lysates inhibited violacein with minimal percentage of ≥70 at their lower concentration (10%), irrespective of the QQ bacterial isolates. The violacein production reduced concomitantly with increase in concentration of CFLs.

Dose dependant inhibition of QS mediated violacein production was reported by Akdamar *et al.* (2016). The highest percentage recorded was 97% by the CFL of isolates HSL 31, HSL 32 and HSL 64 at its maximum concentration (50%). Anti-quorum sensing activity by CFL of isolate HSL 64 increased significantly with each increasing concentration. The results of Rajesh and Rai (2014) supports the study where quantification of violacein inhibition using cell-free lysate of endophytic bacteria, *Bacillus firmus* PT18 and *Enterobacter asburiae* PT39 exhibited inhibition of about 80% violacein production in bioindicator strain.

Comparison results yielded no significant variation among the quorum quenching activity of CFL of five isolates except for their inhibition at varying concentrations. The results are given in the Fig. 2. QS inhibitions at high concentration (≥ 40 %) showed ≥ 90% effects with no statistically significant difference ($p \leq 0.05$) between the CFLs of QQ bacterial isolates except for the isolate HSL 61. Least inhibition of 78.49% was recorded at the maximum concentration of HSL 61. The results are in agreement with the findings of Venkatramanan *et al.* (2020) who reported that ethyl acetate extract of *Passiflora edulis* inhibited violace in production up to 88%. Thus, cell free lysates of QQ bacteria have potent antiquorum sensing activity without antibacterial activity against the *C. violaceum.* Without any negative impact on the growth of pathogens, quorum quenching bacteria are able to inhibit quorum sensing system in pathogens as well as reduce the virulence factors towards the hosts (Defoirdt *et al.*, 2004).

The results from bioindicator assay using cell free lysate further confirms the anti-quorum sensing activity of the quorum quenching isolates which may be enzymatic and inhibition is not due to halting the growth. The actual role of quorum quenching bacteria in inactivating AHL and their activity in attenuating the virulence of bacterial phytopathogens is under experimentation and yet to be confirmed.

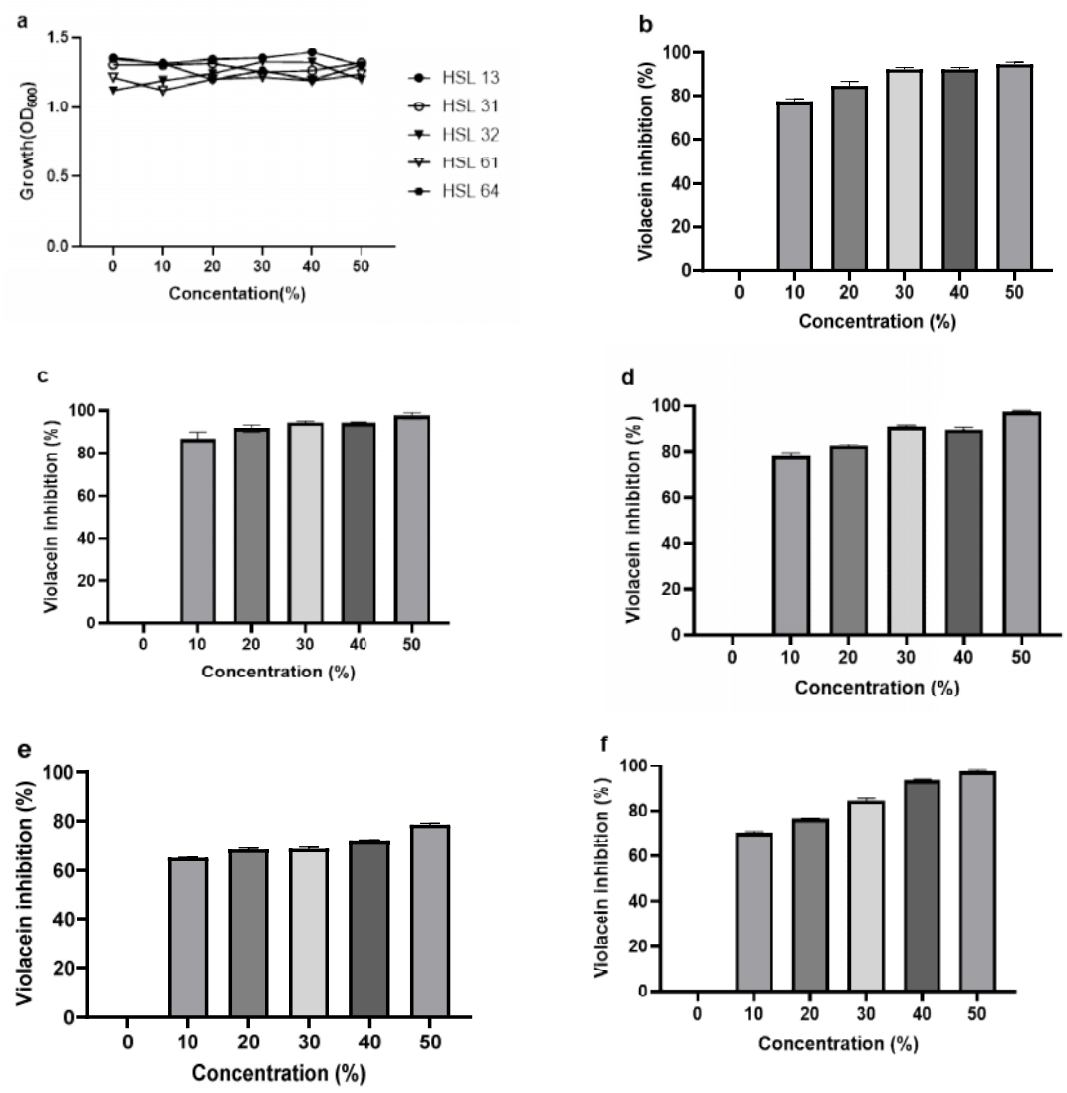

Fig. 1 a. Effect of cell free lysates on growth of *C. violaceum* MCC 4212.Quantitative analysis of the concentration-dependent inhibitory effects of cell free lysate of QQ bacterial isolates on violacein production by *C. violaceum* MCC 4212. b. HSL 13; c. HSL 31 d. HSL 32; e. HSL 61 and f. HSL 64.

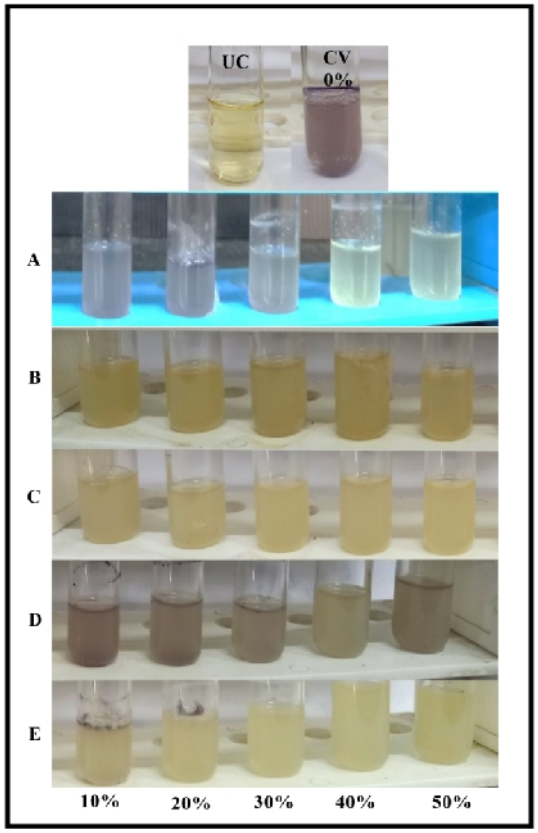

Fig. 2. Violacein production inhibition by cell free lysates with loss of purple pigmentation on *C. violaceum*(MCC 4212). A. HSL 13; B. HSL 31 C. HSL 32; D. HSL 61 and D. HSL 64. UC- Uninoculated control, CV – *C. violaceum* alone without CFL.

CONCLUSION

All the isolates have the quorum quenching property and capable of inhibiting the quorum sensing mediated virulence without affecting the growth. Therefore, this will be further experimented for their biocontrol efficiency against bacterial phytopathogens and this will reduce the risk of resistance development in pathogens.

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Conflict of Interest. None.

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