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Research Paper

Inhibition of quorum sensing-mediated biofilm formation in *Pseudomonas aeruginosa* by a locally isolated *Bacillus cereus*

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Quorum sensing has been shown to play a crucial role in *Pseudomonas aeruginosa* pathogenesis where it activates expression of myriad genes that regulate the production of important virulence factors such as biofilm formation. Antagonism of quorum sensing is an excellent target for antimicrobial therapy and represents a novel approach to combat drug resistance. In this study, *Chromobacterium violaceum* biosensor strain was employed as a fast, sensitive, reliable, and easy to use tool for rapid screening of soil samples for Quorum Sensing Inhibitors (QSI) and the optimal conditions for maximal QSI production were scrutinized. Screening of 127 soil isolates showed that 43 isolates were able to breakdown the HHL signal. Out of the 43 isolates, 38 isolates were able to inhibit the violet color of the biosensor and to form easily detectable zones of color inhibition around their growth. A confirmatory bioassay was carried out after concentrating the putative positive cell-free lysates. Three different isolates that belonged to *Bacillus cereus* group were shown to have QSI activities and their QSI activities were optimized by changing their culture conditions. Further experiments revealed that the cell-free lysates of these isolates were able to inhibit biofilm formation by *P. aeruginosa* clinical isolates.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Introduction

With chaotic use of antibiotics, there has been a substantial increase in the number of multidrug resistant (MDR) pathogens rendering antibiotics not the thrilling drugs they previously were. Therefore, there is a burning desire for development of novel strategies to combat the infections caused by MDR pathogens [1–3].

Quorum sensing (QS) is a population density-dependent regulatory system which regulates a number of crucial processes in various pathogens. QS has been shown to regulate a number of diverse phenotypes in both Gram-negative and Gram-positive bacteria [4] including biofilm formation [5], virulence gene expression [6], and bioluminescence [7]. Bacteria are capable of using small secreted signalling molecules termed autoinducers (AIs) to coordinate group-level behaviors. Those AIs are produced by bacterial cells, then released into surrounding environment, and once a critical threshold concentration is reached, they are recognized by specific receptors leading to changes in gene regulation [4, 8, 9].

QS has been receiving a mounting attention in recent years and it has been proposed that interfering with QS will inhibit microbial pathogenicity [6, 10]. The interruption of QS or Quorum Quenching (QQ) has been pursued as a novel strategy to attenuate bacterial virulence [11, 12] and may represent a promising approach that may perhaps lead to development of novel anti-infective/anti-pathogenic drugs based on interfering with bacterial communication to block QS-mediated pathogenic infection [13, 14].

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Pseudomonas aeruginosa is a highly resistant opportunistic pathogen commonly associated with nosocomial infections [15, 16]. This pathogen exhibits intrinsic resistance to many structurally unrelated antibiotics where multiple virulence factors are involved in disease development such as biofilm formation [17]. In *P. aeruginosa*, QS plays an imperative role where signal cascade is recruited to activate expression of dozens of genes when sufficient population densities are reached. This regulates the production of several key extracellular virulence factors and promotes biofilm formation [18–20].

Several methods have been developed to detect the presence of Acyl Homoserine Lactones (AHLs) including bacterial biosensor systems [21–23]. These biosensors display a palpable phenotypic change in the presence of AHLs such as light emission or pigment production [24]. *Chromobacterium violaceum* produces the water insoluble purple pigment violacein, when subjected to transposon mutagenesis, a white mutant is obtained which no longer produces the Hexanoyl Homoserine Lactone (HHL) signal [22]. This white *C. violaceum* mutant termed CV026 turns purple following exposure to exogenous AHL making it a supreme AHL biosensor [22, 25–27]. CV026 has been shown to be exceptionally sensitive to short and medium chain length AHLs, but lacks sensitivity to AHLs with longer chains [28].

The aim of this study is to control biofilm formation in virulent *P. aeruginosa* through inhibition of its quorum sensing cascade by bacteria isolated from soil with the help of CV026 as a biosensor strain and also to optimize the production of the QQ of the promising isolates through changing the environmental and physiological production conditions.

Materials and methods

Media and chemicals

Media. Nutrient broth (NB) and brain heart infusion broth (BHI) were obtained from Biolife (Italy). Nutrient agar (NA) was obtained from Biomark (India), Eosin Methylene Blue (EMB) and MacConky were obtained from Merck (Germany), while tryptic soy broth (TSB) was obtained from Oxoid (England). Basal medium (BM) consists of $(NH4)_2SO_4$ (0.5 g), $CaCl_2.2H_2O$ (1 mg), $MgSO_4.7H_2O$ (50 mg), K_2HPO_4 (1.74 g), KH_2PO_4 (1.36 g), glucose 1.5% and dest. H_2O to 100 ml whereas modified basal medium (MBM) consists of BM + 1% peptone. Lauria bertani (LB) was separately prepared by admixing 10 g L^{-1} tryptone (Difco, USA), 5 g L^{-1} yeast extract (Difco), and 5 g L^{-1} NaCl.

Chemicals. Bovine serum albumin (BSA), dithiotheritol (DTT), N-hexanoyl-L-homoserine lactone (HHL) and Folin–Ciocalteu reagent were obtained from Sigma–Aldrich (Germany). Sodium dodecyl sulfate and tris hydrochloride were obtained from Merck (Germany). All other chemicals were of analytical grade and were purchased from local sources.

Bacterial strains

Biosensors. C. violaceum 026 (CV026) was kindly provided by Professor Dr. Mohamed Mabrouk, Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University.

Pseudomonas aeruginosa clinical isolates. Eight *P. aeruginosa* clinical isolates from cancer patients, collected from the national cancer institute (NCI, Cairo, Egypt), were employed in this study.

Recovery of bacterial isolates from soil. A total of 127 bacterial soil isolates were collected from different localities in Helwan city. Samples were collected in sterile plastic bags from different areas in Helwan and stored at 4 °C till use. One gram of each soil sample was placed into a 100 ml flask containing 20 ml sterile normal saline solution and incubated at 30 °C in a shaking incubator 200 rpm for 30 min. Then, a sample from the slurry produced was serially diluted and plated on nutrient agar. The plates were then incubated for 24 h at 37 °C and morphologically different colonies were picked up, re-streaked on nutrient agar plates for isolation of pure culture. Afterwards, individual colonies were subcultured on nutrient agar slants and stored in glycerol at −80 °C. The recovered isolates were categorized according to their microscopical characters revealed by Gram stained films.

Biochemical tests for the selected isolates

Various biochemical tests were performed to characterize the three isolates that showed highest QSI activity (Table 1). Isolates were further identified using API® CHB identification strips (Biomërieux, France).

Screening of the recovered bacterial soil isolates for QSI activity

Primary screening using the synthetic signal molecule (HHL). This was done as described elsewhere [29]. Briefly, isolates and CV026 were cultured in LB broth at 28 °C. Fifty-microliter aliquots of 10 μM HHL were mixed with equal volumes of tested isolates culture in microtitre plate. The plate was then incubated at 28 °C for 4 h and subsequently was exposed to UV germicidal lamp for 90 min at a 10 cm distance. Afterwards, 50 μl of CV026 culture ($OD_{600\,nm}$ of 0.3–0.4) was added to each well and

Table 1. Biochemical characterization of the selected isolates.

Test name	Isolate 31	Isolate 39	Isolate 52
Growth on nutrient agar	Opaque white colonies with irregular margins		
Growth on MacConky agar	–	–	–
Growth on EMB agar	–	–	–
Production of catalase	+	+	+
Growth in thioglycolate broth	Facultative anaerobe		
Growth in 2% NaCl	+	+	+
Growth in 5% NaCl	+	+	+
Growth in 7% NaCl	+	+	+
Growth in 10% NaCl	–	–	–
Growth in 15% NaCl	–	–	–
Starch hydrolysis	+	+	+
Gelatin liquefaction	+	+	+
Decomposition of casein	+	+	+

the plates were incubated overnight at 28 °C. Positive and negative control wells were included by adding 50 μl phosphate buffered saline (PBS) instead of the HHL signal or by adding 50 μl plain LB broth instead of the bacterial culture suspension.

Secondary screening of the bacterial soil isolates with HHL-degrading activity. Secondary screening was done, for isolates that have been shown to degrade HHL in the primary screening, using a previously described protocol [30]. Briefly, 300 μl of an overnight culture of CV026 ($OD_{600\,nm}$ of 0.3–0.4) were diluted with 10 ml PBS and then 1 ml of this diluted suspension was added to sterile Petri dish containing 50 μl of 100 μM HHL. Subsequently, 15 ml warm LB agar (50 °C) was poured and mixed well before being left for solidification. Later, fresh cultures of the test isolates were streaked on the agar surface. Those plates were incubated at 28 °C for 24 h and QQ activity of the test isolates was indicated by zones of colorless growth of the biosensor surrounding the test isolate compared to purple colored growth in the whole plate.

Preparation of crude cell lysate of the selected isolates

Isolates that have been confirmed to have QSI activity were grown overnight on LB agar then suspended in PBS ($OD_{600\,nm}$ of 0.1–0.2) and 100 μl of these suspensions were added to 5 ml aliquots of LB broth and incubated for 24 h at 28 °C with shaking at 160 rpm. Cells were centrifuged at 6000 rpm for 10 min, washed twice with cell washing buffer (25 mM Tris-HCl, pH 7.5), and resuspended in 2 ml cracking buffer (25 mM Tris-HCl, pH 7.5 + 1 mM DTT). The cells were intermittently sonicated and centrifuged at 6000 rpm for 20 min, supernatants were collected and sterilized through 0.2 μm filter, and total protein were estimated by Lowry method using BSA as a standard [31]. The protein concentrations of test samples were adjusted to 1.5 mg ml^{-1} (unless otherwise mentioned) for different experiments involving use of crude cell lysates.

Assay of the isolates that proved QSI activity using serial concentrations of HHL signal

Crude cell lysates of the selected isolates were adjusted to 1.5 mg ml^{-1} total protein and used in this assay. The assay method is similar to the primary screening method, but the test samples in that assay were the cell lysates instead of the bacterial cultures and various concentrations (0, 5, 10, 20, 30, and 40 μM) of the signal were used. The cell lysates that showed violet color inhibition at the highest signal concentration were chosen and subjected to the same assay again with increasing signal concentration up to 100 μM. PBS replacing tested lysate was used as a negative control.

Quantification of HHL degradation

This has been carried out as previously described [14, 32] with some modifications as equal volumes of the filter-sterilized cell-free lysates and HHL (200 μM) were incubated at 28 °C and 50 μl of this mixture was added to 5 ml aliquots of LB broth inoculated with 100 μl of CV026 ($OD_{600\,nm}$ of 0.3–0.4) and incubated at 28 °C, 160 rpm for 24 h. Then, 2 ml were centrifuged at 10,000 rpm for 10 min, cells were then washed twice with cell washing buffer and resuspended in 1 ml DMSO, and incubated in water bath at 60 °C for 15 min, then centrifuged at 10,000 rpm for 10 min. The supernatant was used to measure violacein pigment at 580 nm and inhibition was calculated as a percentage of control containing PBS instead of the test lysate. All experiments were done in triplicates and cell dry weight was measured in parallel with violacein inhibition.

Determination of cell dry weight

In order to make sure that the inhibition of violacein production is reflecting quorum quenching effect of the test lysate and not due to growth inhibiting effect on CV026, cell dry weight was measured in all experiments and was compared to control.

The samples taken for dry weight measurement were centrifuged at 10,000 rpm for 10 min. Cells were washed twice with cell washing buffer then the Eppendorfs were dried at 40 °C for 18 h. The cell dry weight was calculated by subtracting the weight of empty Eppendorfs from the final weight of Eppendorfs containing dried pellets. Cell dry weight was measured in triplicates and represented

as percentage of control containing PBS instead of the test lysate.

Studying the effect of reaction time on HHL degradation

Filter-sterilized cell lysates were treated as previously mentioned but samples were withdrawn from the reaction mixtures at 1, 2, 3, and 4 h reaction times and were used for determination of QSI effect and cell dry weight as previously mentioned in quantification of HHL degradation and determination of cell dry weight.

Studying the environmental factors influencing the QQ productivity of the selected isolates

This was done by changing the environmental growth conditions of the test isolates once per time. After growing the test isolate at the specified conditions, cells were collected and treated as previously mentioned under preparation of crude cell lysate of the selected isolates. The cell lysates were then filter-sterilized and used for determination of QSI effect and cell dry weight as previously mentioned in quantification of HHL degradation and determination of cell dry weight.

Effect of different types of growth media

Six different media were tested including LB, NB, BHI, TSB, BM, or MBM. Briefly, selected isolates were grown overnight on LB agar then suspended in PBS to an $OD_{600\,nm}$ of 0.1–0.2. Hunfred microliters of these suspensions were used to inoculate 5 ml aliquots of each tested media and incubated for 24 h at 28 °C with shaking at 160 rpm. For each isolate, the medium that showed highest QQ activity was selected and used in subsequent experiments.

Effect of incubation time, incubation temperature, and agitation rate

Isolates were grown as previously mentioned under effect of different types of growth media (using the appropriate medium for each isolate). To examine the effect of incubation time, isolates were grown for 6, 18, 24, 30, and 48 h at 28 °C with shaking at 160 rpm. For studying the effect of incubation temperature, isolates were grown for 24 h at (28, 33, or 37 °C) with shaking at 160 rpm. To investigate the optimal agitation rate, isolates were grown with or without shaking at 160 and 250 rpm.

Studying the physiological factors affecting the QQ productivity of the selected isolates

For each isolate, the environmental growth conditions that provoked highest QQ activity were chosen and used with the subsequent tested physiological conditions. This was carried out by changing the medium composition once per time. After growing the test isolate at the specified conditions cells were collected and treated as previously mentioned under preparation of crude cell lysate of the selected isolates. The cell lysates were then filter-sterilized and used for determination of QSI effect and cell dry weight as previously mentioned in quantification of HHL degradation and determination of cell dry weight.

Effect of changing/addition of carbohydrate source and concentration

For isolates coded 31 and 52, the carbohydrate source (glucose) in the selected growth medium (MBM) was replaced by fructose, lactose, sucrose, or starch at the same concentration (1.5%), while for isolate 39, the tested carbohydrate (glucose, fructose, lactose, sucrose, or starch) was added to the selected medium (LB) to a final concentration of 0.5%. To examine the optimal carbohydrate concentration, the selected carbohydrate was tested at 0.5, 1, 1.5, and 2% for isolates 31 and 52, while in case of isolate 39, the concentrations tested were 0.1, 0.5, 1, and 2%. Isolates were grown as previously described using the specified incubation condition for each isolate. Lysates were tested for their QS inhibitory activity as indicated before. In all cases filter-sterilized sugars were added to the medium after autoclaving.

Effect of changing/addition of different minerals and changing mineral concentration

For isolates coded 31 and 52, the minerals in the selected growth medium (MBM) was replaced by calcium, ferrous, manganous, or magnesium at concentration (100 μM), while for isolate 39, the tested mineral was added to the selected growth medium (LB) at the same concentration. For investigating the effect of changing mineral concentration, the selected mineral was tested at 50, 100, and 200 μM. After growing the isolates at the specified conditions, the cell lysates prepared were adjusted to total protein concentration of 0.75 mg ml^{-1} instead of 1.5 mg ml^{-1} before being tested for QS inhibitory activity as previously mentioned.

Effect of QQ activity of cell lysates on biofilm formation by *P. aeruginosa* clinical isolates

Cell-free lysates were prepared as previously mentioned and adjusted to total protein concentration of 1.5 mg ml^{-1} and used as test lysates in the static microtitre plate assay [14, 33]. Briefly, *P. aeruginosa* clinical isolates were grown in TSB at 37 °C for 18 h and 160 rpm. The cultures were centrifuged at 6000 rpm for 10 min and cells were

washed twice with PBS and resuspended in M9 medium to an $OD_{600\,nm}$ of 0.1–0.2. Afterwards, 150 μl of this culture and 50 μl of the test lysate (PBS for control) were dispensed into 96-well plate. The plate was incubated at 37 °C for 24 h without agitation. Planktonic cells were removed to another plate, read at 600 nm while biofilms that have been formed were detected by staining with 0.2% crystal violet, incubated for 15 min at room temperature, and then washed thoroughly with d. H_2O. Ethanol 95% was used to remove crystal violet from produced biofilms and absorbance was measured at 590 nm.

Results

Recovery of bacterial isolates from soil

A total of 127 bacterial isolates were recovered from various soil samples on nutrient agar, these isolates were categorized based on their Gram reaction and their morphology under the microscope into: 53.5% Gram-positive bacilli, 29.9% Gram-negative bacilli, 8.7% Gram-positive cocci, and 7.9% Gram-negative coccobacilli.

Screening of the recovered bacterial soil isolates for QSI activity

Primary screening using HHL. Out of 127 isolates, only 43 were shown to have the ability to breakdown the added synthetic signal molecule HHL as indicated by loss of the purple color in the negative control (Supporting Information Fig. S1). Thirty two of these isolates (79%) were Gram-positive bacilli while the remaining nine isolates (20.9%) were Gram-negative bacilli.

Secondary screening of the bacterial soil isolates with HHL-degrading activity. The isolates that were shown to have HHL-degrading activity in the primary screening method were screened again using the method of Ref. [30] against CV026. Results obtained demonstrated that 38 isolates out of the selected 43 isolates were able to inhibit the violet color of the growing biosensor and to form easily detectable zones of color inhibition around their growth.

Assay of the isolates that showed QSI activity using serial concentrations of HHL signal

Cell lysates of seven isolates were found to inhibit the violet color production up to HHL concentration of 40 μM. These lysates were tested again after raising HHL concentration to 100 μM. Only three lysates were able to continue color inhibition till the highest concentration. These isolates were coded 31, 39, and 52.

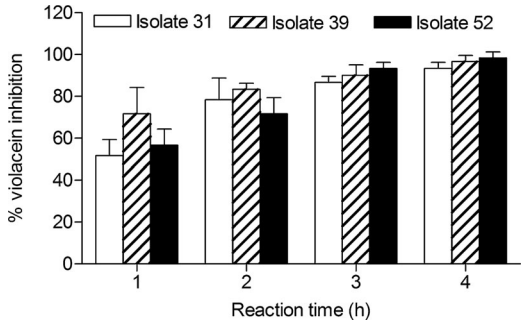

Figure 1. Effect of reaction time on HHL degradation by selected isolates. Cell lysates were incubated with HHL (200 μM) at 28 °C and 50 μl of this mixture was added to 5 ml aliquots of LB broth inoculated with 100 μl of CV026 ($OD_{600\,nm}$ of 0.3–0.4) and incubated at 28 °C, 160 rpm for 24 h. Then, 2 ml were centrifuged, washed, resuspended in 1 ml DMSO and incubated in water bath at 60 °C for 15 min then centrifuged. Supernatant was used to measure violacein pigment at 580 nm and samples were withdrawn from reaction at 1, 2, 3, and 4 h and were used for determination of QSI. $n = 3$ experiments; means ± standard deviations are shown.

Biochemical characterization of the selected isolates

The three isolates that showed highest QSI activity, namely 31, 39, and 52 were then subjected to biochemical tests. Microscopical examination revealed the presence of Gram-positive spore forming rods. The results of various biochemical tests performed to identify the isolates are listed in Table 1.

The three isolates were further identified using API® CHB identification system and were found to belong to *Bacillus cereus* group.

Studying the effect of reaction time on HHL degradation as indicated by violacein pigment inhibition

Figure 1 shows that increasing the contact time of the test lysate with the synthetic signal HHL enhances the percentage of inhibition of pigment production, while the percentage of cell dry weight remains constant around 100% of the test control (in all the following optimization steps, not represented), which indicates increasing percentage of degraded HHL with time and emphasize that the color inhibition is due to quorum quenching and not growth inhibition.

One hour incubation time was chosen for the quantification of QSI activity in all optimization steps as in all cases it refers to reasonable inhibition percentage whereby any change can be detected.

Studying the environmental factors affecting the QQ activity of the selected isolates

Effect of different types of growth media. As demonstrated in Fig. 2, six types of growth media were tested

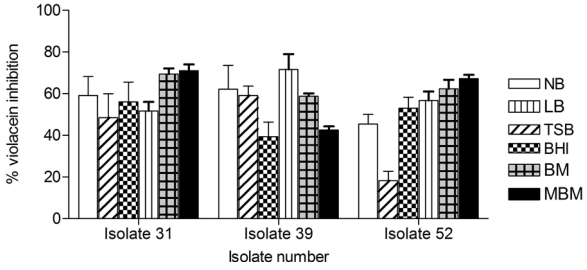

Figure 2. Effect of different types of growth media on QSI activity of selected isolates. Selected isolates were grown overnight on LB agar then suspended in PBS to an $OD_{600\,nm}$ of 0.1–0.2. Hundred microliters of these suspensions were used to inoculate 5 ml aliquots of each tested media and incubated for 24 h at 28 °C with shaking at 160rpm. $n = 3$ experiments; means ± standard deviations are shown.

including NB, TSB, BHI, LB, BM, and MBM. Results obtained demonstrated that MBM was the optimal growth medium for cultivation of isolates 31 and 52 for highest QS inhibitory activity, while for isolate 39 LB medium was selected for further optimization steps to donate highest QQ activity.

Effect of incubation time. Lysates were prepared at five incubation time intervals 6, 18, 24, 30, and 48 h. For all of the tested isolates, 24 h was selected as the incubation time that showed the highest QSI activity as demonstrated in Fig. 3. At 6 h incubation, both isolates 31 and 52 failed to give detectable growth under the specified growth conditions so the color inhibition was considered to be zero at this time interval.

Incubation temperature influences violacein pigment production. As shown in Fig. 4, three incubation temperatures (28, 33, and 37 °C) were tested. Results obtained revealed that 33 °C was selected as the incubation temperature for cultivation of isolate 31 in further

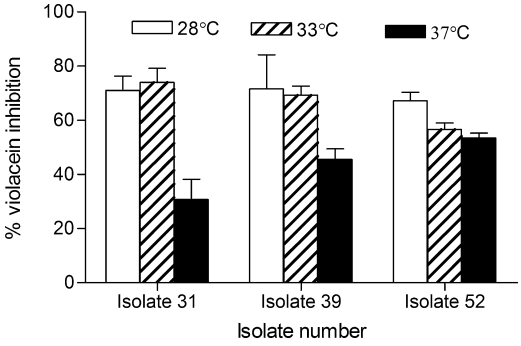

Figure 4. Effect of incubation temperature on QSI activity of selected isolates. Selected isolates were grown overnight on LB agar and suspended in PBS to an $OD_{600\,nm}$ of 0.1–0.2. Hundred microliters of these suspensions were used to inoculate 5 ml aliquots of the appropriate medium for each isolate and incubated for 24 h at (28, 33, or 37 °C) with shaking at 160 rpm. $n = 3$ experiments; means ± standard deviations are shown.

optimization experiments, while isolates 39 and 52 were grown at 28 °C instead.

Effect of agitation rate. The QSI activity of cell-free lysates of the test isolates grown in static condition and in agitation rate of 160 and 250 rpm was tested. As demonstrated in Fig. 5, results obtained showed that 160 rpm was the optimal agitation rate for cultivation of the three isolates in further optimization steps as it referred to the highest QQ activity.

Studying the physiological factors affecting the QQ productivity of the selected isolates

Effect of changing/addition of carbohydrate source. For isolates 31 and 52, glucose, fructose, lactose, sucrose, or starch were used as carbohydrate source (at 1.5%). As shown in Fig. 6, glucose was carbohydrate source that

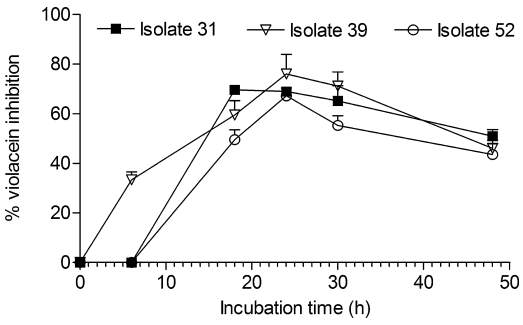

Figure 3. Effect of incubation time on QSI activity of selected isolates. Selected isolates were grown overnight (using the appropriate medium for each isolate) and suspended in PBS to an $OD_{600\,nm}$ of 0.1–0.2. Hundred microliters of these suspensions were used to inoculate 5 ml aliquots of each tested media and incubated for 6, 18, 24, 30, or 48 h at 28 °C with shaking at 160 rpm. $n = 3$ experiments; means ± standard deviations are shown.

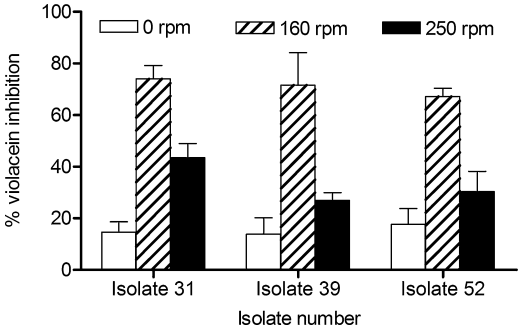

Figure 5. Effect of agitation rate on QSI activity of selected isolates. Selected isolates were grown overnight on LB agar and suspended in PBS to an $OD_{600\,nm}$ 0.1–0.2. Hundred microliters of these suspensions were used to inoculate 5 ml aliquots of the appropriate medium for each isolate and incubated for 24 h with or without shaking at 160 and 250 rpm. $n = 3$ experiments; means ± standard deviations are shown.

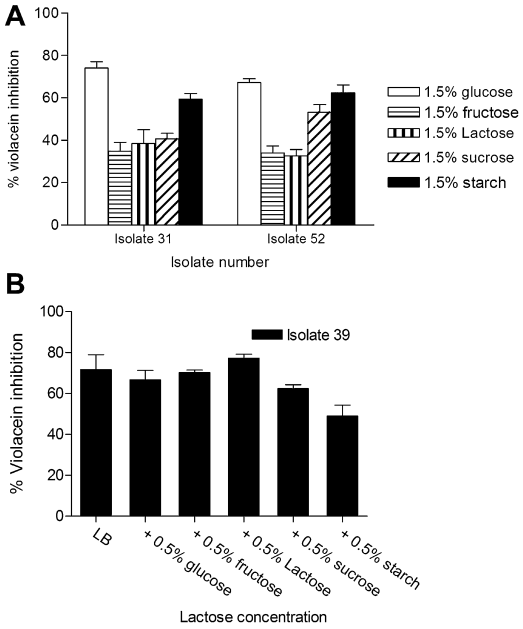

Figure 6. Effect of changing/addition of carbohydrate source on QSI activity of selected isolates (A: isolates 31 and 52; B: isolate 39). Selected isolates were grown overnight and suspended in PBS to an $OD_{600\,nm}$ of 0.1–0.2. Hundred microliters of these suspensions were used to inoculate 5 ml aliquots of the appropriate medium using the specified incubation condition for each isolate. $n = 3$ experiments; means ± standard deviations are shown.

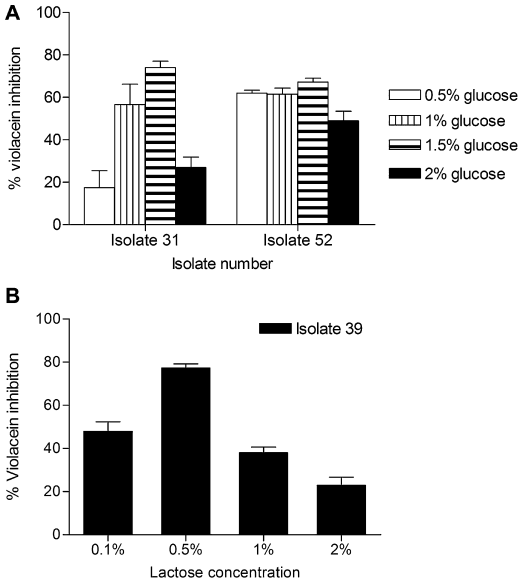

Figure 7. Effect of changing carbohydrate concentration on QSI of selected isolates (A: isolates 31 and 52; B: isolate 39). Selected isolates were grown at the specified incubation conditions and their lysates were tested for QS inhibitory activity. The selected carbohydrate was tested at 0.5, 1, 1.5, and 2% for isolates 31 and 52, while in case of isolate 39, the concentrations tested were 0.1, 0.5, 1, and 2%. $n = 3$ experiments; means ± standard deviations are shown.

gave rise to the maximal QQ activity and, therefore, it has been selected to be used for isolates 31 and 52 in the following steps. In case of isolate 39, glucose, fructose, lactose, sucrose, or starch were added to LB broth (at 0.5%) and were tested as carbohydrate sources. Results obtained showed that lactose was the carbohydrate source that referred to the highest inhibition of violacein pigment production for isolate 39. Hence lactose was selected as the carbohydrate source for cultivation of this isolate in further optimization steps.

Effect of changing carbohydrate concentration. For isolates 31 and 52, glucose was selected as the carbohydrate source and was tested at concentrations of 0.5, 1, 1.5, and 2%. This has showed that 1.5% was the optimal glucose concentration that showed highest QQ activity and was used in subsequent steps.

On the other hand, for isolate 39, the lactose concentrations tested were 0.1, 0.5, 1, and 2%. The highest percentage inhibition (77.3%) was obtained with 0.5% lactose and hence, 0.5% was selected for subsequent steps. In all cases, percent cell dry weight was constant around 100% of the test control (Fig. 7).

Effect of changing/addition of different minerals. For isolates coded 31 and 52, the minerals in the selected growth medium (MBM) was replaced by magnesium, manganous, calcium, or ferrous at concentration of 100 μM. Results obtained, as demonstrated in Fig. 8, showed that the maximal percentage of violacein inhibition (90.5%) by isolate 31 was obtained when ferrous was used. Likewise, the maximal percentages of violacein inhibition (91.6%) by isolate 52 and (98.9%) by isolate 39 were achieved when manganous was used. Consequently, ferrous was chosen to be added to the growth medium for cultivation of isolate 31 while

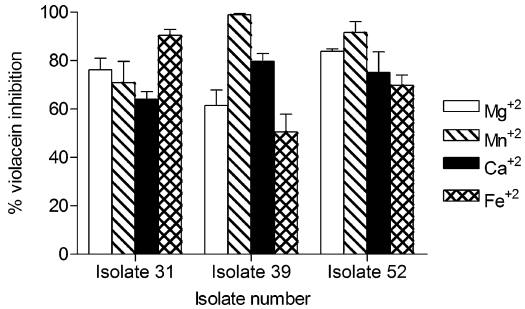

Figure 8. Effect of changing/addition of different minerals on QSI activity of selected isolates. Selected isolates were grown at the specified incubation conditions For isolates coded 31 and 52, the minerals in the selected growth medium (MBM) was replaced by calcium, ferrous, manganous, or magnesium at concentration (100 μM), while for isolate 39, the tested mineral was added to the selected growth medium (LB) at the same concentration. $n = 3$ experiments; means ± standard deviations are shown.

manganous was selected to be added to the growth medium for cultivation of isolates 39 and 52 in the subsequent optimization steps as they referred to highest QQ activity.

Effect of changing mineral concentration. The selected mineral was tested at concentrations of 50, 100, and 200 μM. After growing, the isolates at the specified conditions, the prepared cell lysates were adjusted to total protein concentration 0.75 instead of 1.5 mg ml^{-1} before testing them for QSI activity as indicated before.

For isolates 39 and 52, manganous ion was tested at the same concentrations. This showed that the maximal percentage of inhibition of violacein pigment production (62.9%) by isolate 39 was obtained with 200 μM. Similarly, the maximal percentage of inhibition of violacein pigment production (50.7%) by isolate 52 was achieved with 100 μM (Fig. 9A). On the other hand, ferrous was the selected metal ion for isolate 31 and was tested at concentrations of 50, 100, and 200 μM. This has showed that maximal percentage of inhibition (65.1%) was achieved with 100 μM (Fig. 9B). Thus, 100 μM ferrous ion was selected for cultivation of isolate 31 while manganous at concentrations 200 and 100 μM was used for isolates 39 and 52, respectively.

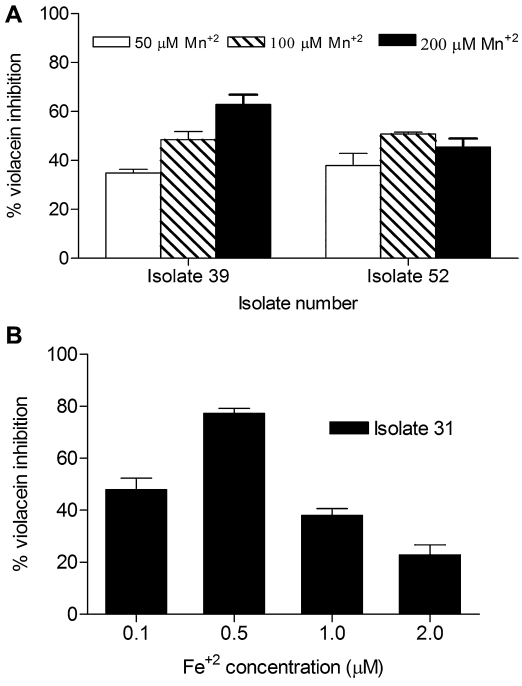

Figure 9. Effect of changing mineral concentration on QSI activity of selected isolates (A: isolates 39 and 52; B: isolate 31). The selected mineral was tested at 50, 100, and 200 μM. After growing the isolates at the specified conditions, the cell lysates prepared were adjusted to total protein concentration of 0.75 instead of 1.5 mg ml^{-2} before being tested for QS inhibitory activity. $n = 3$ experiments; means ± standard deviations are shown.

Effect of quorum quenching activity of the cell lysates on biofilm formation by P. aeruginosa clinical isolates. The optimized growth conditions which gave rise to the highest QQ activity for each isolate were found to be MBM with 1.5% glucose and 100 μM ferrous sulfate incubated at 33 °C and 160 rpm for 24 h for isolate 31, LB with 0.5% lactose and 200 μM manganous sulfate incubated at 28 °C and 160 rpm for 24 h for isolate 39 and MBM with 1.5% glucose and 100 μM manganous sulfate incubated at 28 °C and 160 rpm for 24 h for isolate 52. The effect of cell-free lysates of isolates grown at these conditions on the biofilm and planktonic growth of eight *P. aeruginosa* clinical isolates was measured as a percentage of control containing PBS instead of the test lysate. In almost all cases, the test lysates showed higher OD values of planktonic cell compared to the corresponding control (Supporting Information Fig. S2); however, in many cases, the test isolates showed detectable biofilm inhibition despite of the higher growth compared to the control.

As shown in Fig. 10, isolates 31, 39, and 52 were able to inhibit biofilm formation by various *P. aeruginosa* clinical isolates by various degrees.

Discussion

QS is a mechanism whereby bacteria can elicit a biological response to an external stimulus. It includes expression of an array of genes that endow bacteria with the ability to work in unison to preclude any kind of afflictions during microbe–microbe or host–microbe

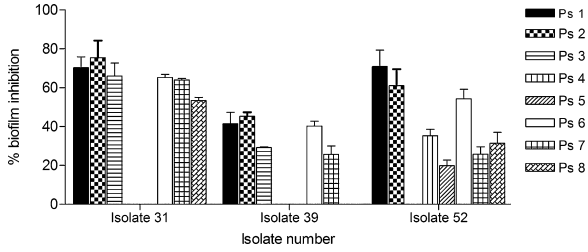

Figure 10. Effect of QQ activity of cell lysates on biofilm formation by *P. aeruginosa* clinical isolates after correction of growth effect. *P. aeruginosa* clinical isolates were grown in TSB at 37 °C for 18 h and 160 rpm. Cells were centrifuged, washed with PBS, and resuspended in M9 medium to an $OD_{600\,nm}$ of 0.1–0.2. One hundred and fifty microliters of this culture were added to 50 μl of test lysate or PBS in 96-well plate and incubated at 37 °C for 24 h without agitation. Planktonic cells were removed to another plate, read at 600 nm while biofilms were detected by staining with 0.2% crystal violet, incubated for 15 min at room temperature, and then washed thoroughly with d. H_2O. Ethanol 95% was used to remove crystal violet from produced biofilms and absorbance was measured at 590 nm. $n = 3$ experiments; means ± standard deviations are shown.

interactions [34]. With the help of QS, bacteria regulate plethora of activities and diverse cellular functions [11]. AHLs-dependent QS is essential for virulence in many pathogens and interfering with its signaling cascade could prevent virulence of pathogenic bacteria [35]. Currently, this approach is gaining more attention to control infection caused by MDR bacteria since QQ may have the potential to circumvent the challenge of the development of MDR in bacteria [33].

P. aeruginosa has an incredible metabolic versatility which is mainly attributed to its genomic diversity. One of the important characteristics of *P. aeruginosa* is the ability to form biofilms that endow it with the ability to resist different antibiotics therapy. Antibiotic resistant strains of *P. aeruginosa* have been shown to exhibit better biofilm forming ability [36, 37].

In this study, we initially screened 127 soil isolates for their ability to degrade HHL and 43 isolates have shown the ability to completely breakdown the HHL which is in line with previously published work reporting the wide distribution of QSI-producing bacteria in soil environments [30, 38].

Further screening showed that 38 isolates out of the selected 43 isolates (88%) were able to inhibit the violet color of the growing biosensor with the prevalence of Gram-positive bacilli (79%). Having screened isolates for HHL degrading ability, we set out to examine the cell-free lysates of isolates for their QQ activity and this showed that only seven lysates were found to inhibit violet color production up to HHL concentration of 40 μM while only three lysates continue color inhibition after raising HHL concentration to 100 μM. These isolates coded 31, 39, and 52 and were further identified as different strains of *Bacillus cereus* which is in concordance with previously published data reporting the wide distribution of homoserine lactonases among this class of microorganisms [39, 40].

Afterwards, we thought to study factors that may have the potential to influence QQ activity of the tested isolates. Several parameters have been adjusted including contact time between lysate and HHL, type of media, incubation time, incubation temperature, agitation rate, carbohydrate source, and minerals. Results obtained showed that increasing the contact time of the test lysate with the synthetic signal HHL has led to increase the percentage of inhibition of pigment production, while the percentage of cell dry weight remained constant around 100% of the test control. This indicates increasing percentage of degraded HHL with time while emphasizing that the color inhibition is due to quorum quenching not growth inhibition and this is in line with previously published data [38]. One hour incubation time was chosen in the quantification of QSI activity in all the optimization steps as in all cases it led to maximal percentage of inhibition whereby any change can be detected.

Next, six types of growth media were tested including NB, TSB, BHI, LB, BM, and MBM and results obtained demonstrated that MBM was optimal for cultivation of isolates 31 and 52 while LB was ideal for cultivation of isolate 39 leading to the highest QSI activity. These results indicate that organisms preferred lower nutrient availability for more QQ activity which may explain the need of QSI activity to mimic nature as many organisms use QQ as a mechanism for fighting for limited resources [41, 42].

Here, we showed that incubation time influences QQ activity of the isolates and 24 h was the optimal incubation time which corresponded to the highest QQ activity. Likewise, we showed that 33 °C was optimal incubation temperature for isolate 31 in contrast to 28 °C for isolates 39 and 52. Studying the impact of agitation rate on QQ production revealed that 160 rpm was ideal for highest QSI activity while on the other hand, incubation of the tested isolates in static conditions resulted in cell-free lysates that showed minimal QQ activity.

Subsequently, the effect of changing of carbohydrate source and concentration in the media has been addressed. The maximal QSI activity was achieved when glucose was used. With regard to carbohydrate concentration, 1.5% glucose was chosen as optimal glucose concentration for isolates 31 and 52, while 0.5% lactose was optimal for isolate 39. Likewise, the optimal QQ activity was obtained when ferrous was used for isolate 31 and manganous for isolates 39 and 52. Previous studies have reported that some metal are crucial for the activity of QQ enzymes from different microbial sources [43–45].

Biofilm formation is increasingly being recognized as an important virulence factor in *P. aeruginosa* and many previous studies have shown that some QQ enzymes and chemicals act as potent biofilm inhibitors. Rajesh and Ravishankar have shown that the cell-free lysates extracted from the endophytic bacteria *Bacillus firmus* PT18 and *Enterobacter asburiae* PT39 had a significant inhibitory effect on biofilms formed by *P. aeruginosa* PAO1 [14]. These endophytic bacteria were shown to have lactonase activity.

Accordingly, we tested the effect of cell-free lysates of the three selected isolates on the planktonic cells and biofilm formed by eight *P. aeruginosa* clinical isolates. The effect of the cell-free lysates on the planktonic cells was surprising as in almost all cases, the test lysate showed

higher growth than the corresponding control which may be due to the effect of the test lysate itself as an added nutrition. This would in turn make the result of the biofilm inhibition unreal; therefore, a calculation correction was made to rectify the effect of different growth on the test and control. Interestingly, the test lysates showed reproducible biofilm inhibition against the *P. aeruginosa* clinical isolates that reached 75.45% for isolate 31 against the clinical isolate *Ps*2.

To conclude, screening of 127 soil samples for HHL quenching activity showed that 43 isolates have been able to breakdown the HHL signal. Out of the selected 43 isolates, 38 isolates were able to inhibit the violet color of the growing biosensor. Three different isolates belong to *Bacillus cereus* group were confirmed to have QSI activity and their QSI activity was optimized by changing their culture conditions. Further experiments revealed that the cell-free lysates of these isolates were able to inhibit biofilm formation of *P. aeruginosa* clinical isolates. Altogether, the data presented in the current study support the notion that antagonism of quorum sensing is a promising approach that could pave the way toward a novel anti-virulence strategy for the treatment of bacterial infection as well as combating drug resistance.

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Conflicts of interest

The authors declare no conflict of interest.

References

[1] Shepherd, N.E., Harrison, R.S., Fairlie, D.P., 2012. Targeting quorum sensing and competence stimulation for antimicrobial chemotherapy. Curr. Drug Targets, **13**, 1348–1359.

[2] Varga, Z.G., Armada, A., Cerca, P., Amaral, L., et al., 2012. Inhibition of quorum sensing and efflux pump system by trifluoromethyl ketone proton pump inhibitors. In Vivo, **26**, 277–285.

[3] Heras, B., Scanlon, M.J., Martin, J.L., 2014. Targeting virulence not viability in the search for future antibacterials. Br. J. Clin. Pharmacol., **79**, 208–215.

[4] Li, Z., Nair, S.K., 2012. Quorum sensing: how bacteria can coordinate activity and synchronize their response to external signals? Protein Sci., **21**, 1403–1417.

[5] de Kievit, T.R., Iglewski, B.H., 2000. Bacterial quorum sensing in pathogenic relationships. Infect. Immun., **68**, 4839–4849.

[6] Antunes, L.C., Ferreira, R.B., Buckner, M.M., Finlay, B.B., 2010. Quorum sensing in bacterial virulence. Microbiology, **156**, 2271–2282.

[7] Li, Y.H., Tian, X., 2012. Quorum sensing and bacterial social interactions in biofilms. Sensors (Basel), **12**, 2519–2538.

[8] Sifri, C.D., 2008. Quorum sensing: bacteria talk sense. Clin. Infect. Dis., **47**, 1070–1076.

[9] Miyashiro, T., Oehlert, D., Ray, V.A., Visick, K.L., et al., 2014. The putative oligosaccharide translocase sypk connects biofilm formation with quorum signaling in *Vibrio fischeri*. Microbiologyopen, **3**, 836–848.

[10] Vasavi, H.S., Arun, A.B., Rekha, P.D., 2014. Anti-quorum sensing activity of flavonoid-rich fraction from Centella asiatica L. against *Pseudomonas aeruginosa* PAO1. J. Microbiol. Immunol. Infect., **14**, 1–8.

[11] Asad, S., Opal, S.M., 2008. Bench-to-bedside review: quorum sensing and the role of cell-to-cell communication during invasive bacterial infection. Crit. Care, **12**, 236.

[12] Bhardwaj, A.K., Mohanty, P., 2012. Bacterial efflux pumps involved in multidrug resistance and their inhibitors: rejuvenating the antimicrobial chemotherapy. Recent Pat. Anti-Infect. Drug Discovery, **7**, 73–89.

[13] Chen, F., Gao, Y., Chen, X., Yu, Z., et al., 2013. Quorum quenching enzymes and their application in degrading signal molecules to block quorum sensing-dependent infection. Int. J. Mol. Sci., **14**, 17477–17500.

[14] Rajesh, P.S., Ravishankar Rai V., 2014. Quorum quenching activity in cell-free lysate of endophytic bacteria isolated from Pterocarpus santalinus Linn., and its effect on quorum sensing regulated biofilm in *Pseudomonas aeruginosa* PAO1. Microbiol. Res., **169**, 561–569.

[15] Lambert, M.-L., Suetens, C., Savey, A., Palomar, M., et al., 2011. Clinical outcomes of health-care-associated infections and antimicrobial resistance in patients admitted to european intensive-care units: a cohort study. Lancet Infect. Dis., **11**, 30–38.

[16] Zafer, M.M., Al-Agamy, M.H., El-Mahallawy, H.A., Amin, M.A., et al., 2014. Antimicrobial resistance pattern and their beta-lactamase encoding genes among *Pseudomonas aeruginosa* strains isolated from cancer patients. BioMed Res. Int., **2014**, 8, 1–8.

[17] Lyczak, J.B., Cannon, C.L., Pier, G.B., 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect., **2**, 1051–1060.

[18] Fuqua, C., Parsek, M.R., Greenberg, E.P., 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu. Rev. Genet., **35**, 439–468.

[19] Wagner, V.E., Bushnell, D., Passador, L., Brooks, A.I., et al., 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol., **185**, 2080–2095.

[20] Wang, M., Schaefer, A.L., Dandekar, A.A., Greenberg, E.P., 2015. Quorum sensing and policing of *Pseudomonas aeruginosa* social cheaters. Proc. Natl. Acad. Sci. USA, **112**, 2187–2191.

[21] Shaw, P.D., Ping, G., Daly, S.L., Cha, C., et al., 1997. Detecting and characterizing n-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. USA, **94**, 6036–6041.

[22] McClean, K.H., Winson, M.K., Fish, L., Taylor, A., et al., 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology, **143**, 3703–3711.

[23] Winson, M.K., Swift, S., Fish, L., Throup, J.P., et al., 1998. Construction and analysis of luxCDABE-based plasmid sensors for investigating N-acyl homoserine lactone-mediated quorum sensing. FEMS Microbiol. Lett., **163**, 185–192.

[24] Chhabra, S.R., Philipp, B., Eberl, L., Givskov, M., et al., 2005. Extracellular communication in bacteria. Top. Curr. Chem., **240**, 279–315.

[25] Latifi, A., Winson, M.K., Foglino, M., Bycroft, B.W., et al., 1995. Multiple homologues of luxR and luxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Mol. Microbiol., **17**, 333–343.

[26] Swift, S., Karlyshev, A.V., Fish, L. Durant, E.L., et al., 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the luxRI homologues AhyRI and AsaRI and their N-acylhomoserine lactone cognate signal molecules. J. Bacteriol., **179**, 5271–5281.

[27] Milton, D.L., Hardman, A., Camara, M., Chhabra, S.R., et al., 1997. Quorum sensing in *Vibrio anguillarum*: characterization of the vanI/vanR locus and identification of the autoinducer *N*-(3-oxodecanoyl)-L-homoserine lactone. J. Bacteriol., **179**, 3004–3012.

[28] Cha, C., Gao, P., Chen, Y., Shaw, C.P.D., et al., 1998. Production of acyl-homoserine lactone quorumsensing signals by gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact., **11**, 1119–1129.

[29] Jafra, S., van-der-Wolf, J.M., 2004. Fast screening method for detection of acyl-HSL-degrading soil isolates. J. Microbiol. Methods, **57**, 415–420.

[30] Weng, L.-X., Zhang, Y.-Q., Meng, H., Yang, Y.-X., et al., 2012. Screening and isolating quorum sensing inhibitor from bacteria. Afr. J. Microbiol. Res., **6**, 927–936.

[31] Lowry, H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., **193**, 265–275.

[32] Zhu, H., He, C.C., Chu, Q.H., 2011. Inhibition of quorum sensing in *Chromobacterium violaceum* by pigments extracted from *Auricularia auricular*. Lett. Appl. Microbiol., **52**, 269–274.

[33] Cady, N.C., McKean, K.A., Behnke, J., Kubec, R., et al., 2012. Inhibition of biofilm formation, quorum sensing and infection in *Pseudomonas aeruginosa* by natural products-inspired organosulfur compounds. PloS ONE, **7**, e38492.

[34] Dong, Y.H., Wang, L.Y., Zhang, L.H., 2007. Quorum-quenching microbial infections: mechanisms and implications. Philos. Trans. R. Soc., B, **362**, 1201–1211.

[35] O'Loughlin, C.T., Miller, L.C., Siryaporn, A., Drescher, K., et al., 2013. A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. Proc. Natl. Acad. Sci. USA, **110**, 17981–17986.

[36] Chakraborty, D., Basu, S., Chatterjee, P., Dey, S.K., et al., 2011. Concurrent determination of collagenase and biofilm formation activities in metallo-beta-lactamase producing *Pseudomonas aeruginosa*. Int. J. Microbiol. Res., **2**, 208–212.

[37] Mani, A., Hameed, S.S., Ramalingam, S., Narayanan, M., 2012. Assessment of quorum quenching activity of bacillus species against *Pseudomonas aeruginosa* MTCC 2297. Global J. Pharmacol., **6**, 118–125.

[38] Sakr, M.M., Aboulwafa, M.M., Aboshanab, K.M.A., Hassouna, N.A.-H., 2014. Screening and preliminary characterization of quenching activities of soil bacillus isolates against acyl homoserine lactones of clinically isolated *Pseudomonas aeruginosa*. Malays. J. Microbiol., **10**, 80–91.

[39] Chan, K.-G., Wong, C.-S., Yin, W.-F., Sam, C.-K., et al., 2010. Rapid degradation of N-3-oxo-acylhomoserine lactones by a *Bacillus cereus* isolate from Malaysian rainforest soil. Antonie van Leeuwenhoek, **98**, 299–305.

[40] Chong, T.-M., Koh, C.-L., Sam, C.-K., Choo, Y.-M., et al., 2012. Characterization of quorum sensing and quorum quenching soil bacteria isolated from Malaysian tropical montane forest. Sensors (Basel), **12**, 4846–4859.

[41] Czajkowski, R., Jafra, S., 2009. Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules. Acta Biochim. Pol., **56**, 1–16.

[42] Hong, K.-W., Koh, C.-L., Sam, C.-K., Yin, W.-F., et al., 2012. Quorum quenching revisited from signal decays to signalling confusion. Sensors (Basel), **12**, 4661–4696.

[43] Elias, M., Dupuy, J., Merone, L., Mandrich, L., et al., 2008. Structural basis for natural lactonase and promiscuous phosphotriesterase activities. J. Mol. Biol., **379**, 1017–1028.

[44] Chow, J.Y., Wu, L., Yew, W.S., 2009. Directed evolution of a quorum-quenching lactonase from *Mycobacterium avium* subsp. Paratuberculosis k-10 in the amidohydrolase superfamily. Biochemistry, **48**, 4344–4353.

[45] Mei, G.-Y., Yan, X.-X., Turak, A., Luo, Z.-Q., et al., 2010. AidH, an alpha/beta-hydrolase fold family member from an Ochrobactrum sp. Strain, is a novel N-acylhomoserine lactonase. Appl. Environ. Microbiol., **76**, 4933–4942.

