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Assessment of Quorum Quenching Activity of *Bacillus* Species Against *Pseudomonas aeruginosa* MTCC 2297

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Abstract: Bacteria communicate among themselves using N-Acyl Homoserine Lactone (AHL) and oligopeptide as signaling or quorum sensing molecules. AHL lactonase, a potent tool for biocontrol, can hydrolyze quorum sensing signal molecule into inactive products and there by blocking the quorum sensing systems. This process is known as quorum quenching. *Pseudomonas, Salmonella, E. coli, Vibrio,* the gram-negative bacteria, are highly pathogenic and lead to various dreadful diseases by their signaling molecule. Among the organisms, *P. aeruginosa*, is multi-drug tolerant, by its virulence factor secretions (protease, chitinase, pyocyanin, elastase, rhamnolipid). Keeping this in view, it is planned to determine the quorum quenching effect, the AHL lactonase activity, of *Bacillus* species such as *B. subtilis, B. cereus* and *B. licheniformis* using *Chromobacterium violaceum* MTCC 2656 as bio reporter model. The Crude Cell Extract (CCE) of the three *Bacillus* species was used to assess the quorum quenching effect against the supernatant of *P. aeruginosa.* Our screening result had shown that *B. licheniformis* has significant quorum quenching activity. Further, HPLC analysis of *P. aeruginosa* CCE had shown a significant reduction in AHL, which could be due to its breakdown by *B. licheniformis* AHL lactonase activity. From the SDS-Polyacrylamide gel electrophorestic pattern of *B. licheniformis* extract the MW of the quorum-quenching molecule was found to be 25 kDa.

Key words: Quorum Quenching · Quorum Sensing · N-Acyl Homoserine Lactone(AHL) · *Bacillus* species · *Pseudomonas aeruginosa*

INTRODUCTION

Cell-to-cell communication is widely spread in bacteria and controls a broad range of activities (*via* modulation of gene expression) that result in bacterial phenotype changes and better adjustment to environmental conditions during growth [1-8]. Many bacteria employ self-generated small diffusible signal molecules to control gene expression as a function of cell population density. In this process, termed "quorum sensing" (QS), the concentration of a signal molecule that accumulates in the extracellular environment reflects the cell number such that the perception of a threshold concentration of signal molecule determines when the population is "quorate" and ready to make a collective behavioral adaptation [9-12].

Pseudomonas aeruginosa is an important opportunistic pathogen which plays an important role in hospital intensive care units, causing a wide spectrum of nosocomial infections. The spread of this organism in health care settings is often difficult to control, due to the presence of multiple intrinsic and acquired mechanisms of resistance to a wide variety of antibiotics [13-15]. Since the majority of *P. aeruginosa* strains are resistant to most of antibacterial agents, it is considered as one of the major problems in many hospitals [16]. Infections caused by this microorganism are often severe, life threatening and difficult to treat because of the high frequency of an emergence of antibiotic resistance during therapy [17-18]. Quorum sensing within bacterial population can promote pathogenesis, symbiosis, cellular dissemination or dispersal, DNA transfer, metabolism and microbial biofilm development [19-20]. One of the important characteristics of *P. aeruginosa* is biofilm formation that helps them to survive different antibiotic therapy and antibiotic resistant strains of *P. aeruginosa* have been found exhibiting better biofilm forming ability [21-22].

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P. aeruginosa is a well-studied model bacterium whose quorum sensing system is a core mechanism to develop the biofilms formation*s*. The Quorum sensing system of *P. aeruginosa* is organized hierarchically with the Rhl I-Rhl R components. Las 1 directs the synthesis of N-S-oxo-dodeconocyl-L-homoserine lactone (OdDHL), whereas Rhl I synthesis N-butanoyl-L-homoserine Lactone (BHL) [23].

Virulence factors in *P. aeruginosa* are controlled by quorum-sensing (QS) system an intercellular communication scheme in which bacteria is able to detect the population density (via signaling molecules and receptors) and control gene expression. Virulence factors secreted via type II secretion system into the extra cellular factors such as elastase, alkaline phosphatase, exotoxin A and phospholipase C participate in invasion by destroying the protective glycocalix of the respiratory epithelium and exposing epithelial ligands to *P. aeruginosa* [24].

Chromobacterium violaceum is a versatile Gram-negative â-proteobacterium [25] that produces the violet non-diffusible antibiotic pigment violacein [26, 27]. This bacterium is a common inhabitant of soil and water confined to tropical and subtropical regions. Generally, it behaves as a saprophyte, but sporadically it becomes an aggressive opportunistic animal (including human) pathogen, causing serious infections with a high mortality in immunodeficient individuals [28-30]. Since the production of violacein is quorum sensing (QS)-driven, it has become an important tool for bacterial QS signal bioassays, especially for the N-acylhomoserine lactone autoinducers (AHLs). A *C. violaceum* mini-Tn5 mutant, CV026 (dependent on exogenous AHL for violacein production), is used as an indicator organism [31, 32]. Such assays are important because AHLs that belong to the class of furanone derivatives are involved in cell-to-cell signaling in Gram-negative bacteria [33,34].

The potential for microbiological degradation of QS signals is important for several reasons. Since AHL-mediated signaling mechanisms are widespread and highly conserved in many pathogenic bacteria, they can be attractive targets for novel anti-infective therapies [35]. Quorum quenching bacteria sharing the same habitat with quorum sensing bacteria could gain a competitive advantage by degrading AHL signal molecules. Two types of AHL degrading enzymes have been documented: AHL lactonase [36] and AHL-acylase [37]. However, to date, there is no rapid and efficient method for assessing quorum-quenching activity of *Bacillus* species. To address this issue, the present study was focus to assess quorum-quenching activity of *Bacillus* species against *Pseudomonas aeruginosa* MTCC 2297.

MATERIALS AND METHODS

Microorganisms: *Bacillus subtilis* MTCC 736, *Bacillus cereus* MTCC 1305, *Bacillus lichneiformis* MTCC 429, *Pseudomonas aeruginosa* MTCC 2297 and *Chromobacterium violaceum* MTCC 2656 were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

Screening of Anti-Quorum Sensing Activity (Disc Diffusion Method): Standard disc-diffusion assay was used to detect anti-QS activity of the three *Bacillus* sp. To perform this assay, 4-10µl of culture supernatant of the *Bacillus* species was loaded into sterile discs placed into three LB plates spread with 100µl of 16 hrs culture of *C. violaceum*. 30µg of AHL was mixed prior to the plates and incubated overnight at 30°C. The QS inhibition was measured with a ring of colorless and viable cells around the disc. Measurements were made from the outer edge of the discs to the edge of the zones of anti-QS inhibition.

Preparation of Resting Cells: Three bacterial cells such as *B. subtilis, B. cereus, B. lichneiformis* were grown separately in LB media at 28°C in an orbital shaker with 220 rpm. The overnight grown cells were centrifuged at 7000 x g for 10 min. These cells were resuspended in 100ml of PBS (100mM of Tris-HCl with pH6.5) and washed twice in the same buffer. The cell pellets were finally resuspended in 10ml PBS (100mM of Tris-HCl with pH6.5), equalized to an OD of 1.0 at 600 nm and used directly as a source of resting cells for *in vitro* AHL inactivation assays.

AHL Inactivation Assay: Aliquots of 3-oxo-C6-HSL (10µg/µl) in absolute ethanol were dispensed into sterile tubes and the solvent evaporated to dryness under sterile conditions. Resting cells were used to rehydrate the 3-oxo-C6-HSL to a final concentration of 0.1µg/µl. The three mixtures were separately incubated at 37ºC for 4.5 hrs with gentle shaking in a hybridization oven. Heat denatured suspension (10µl) was inoculated onto three LB agar seeded with the bioreporter of *C. violaceum* separately and incubated at 28ºC. Disappearance of 3-oxo-C6-HS from the mixture was assessed at t + 0 hrs, t + 1.5 hrs, t + 4.5 hrs using bioreporter. Degradation of 3-oxo-C6-HSL is evident by loss of purple pigmentation shown by *C. violaceum* and the results were digitally recorded.

Supernatant for Virulence Factor Assay: *Pseudomonas aeruginosa* (MTCC 2297) cultures was grown either for 16 to 20 hrs to $O.D_{600}$ of 2.0 (for the protease, chitinase, elastase, rhamnolipid, pyocyanin assays) treated with 1ml

Bacillus sp. supernatant. Cells were harvested by centrifugation and the supernatant were filter sterilized. The supernatant were either used immediately or stored at-20°C.

Total Proteolytic Assay and Pyocyanin Assay: To the 100µl of each culture supernatant of *P. aeruginosa* treated with 1ml of *Bacillus* supernatant containing AHL degrading molecules, 900µl of 0.5% of azocasien prepared in 50mM Tris buffer containing 2mM $CaCl_2$ was added. After this 100µl of 15% TCA were added to stop the reaction and centrifuged at 8000 rpm in 4°C for 10 min. The absorbance of the supernatant from both control and treatment were measured at 520nm.

The 3ml of chloroform was mixed with 5ml of filter-sterilized supernatant of *P. aeruginosa* treated with 1ml of three *Bacillus species* supernatant separately containing AHL degrading molecules. Chloroform layer was transferred to fresh tube and mixed with 1ml of 0.2M HCl. After centrifugation top layer (0.2M HCl) was removed and measured at 520nm. Chitinase Assay and Elastase Assay:

The filter-sterilized supernatant of *P. aeruginosa* treated with 1ml of three *Bacillus* sp. supernatant separately containing AHL degrading molecules, were mixed in the ratio of 2:1 with 0.1M of sodium citrate buffer at pH 4.8 and 0.5mg/ml of chitin azure. The supernatant of chitin azure mixtures were incubated at 37°C with shaking (200 rpm) for 1 week. These samples were then centrifuged at 15000 x g for 10 mins and the absorbance at 570 nm was determined. The samples were compared to blanks incubated with medium only.

To 200µl of each culture supernatant of P. *aeruginosa*, 1ml of three *Bacilli* sp. supernatant were treated separately containing AHL degrading molecules, add 1ml of 0.5% elastin congored solution (in 10 mM PBS). The mixer was incubated at 37°C for 3 hours in water bath. Samples were vortexed, centrifuged at 1200xg for 10 min at 10°C and measure at 570nm.

Rhamnolipid Assay: *Pseudomonas aeruginosa* (MTCC 2297) was grown in Mineral Salt medium with little modification and the pH of the medium was adjusted to 7.0 ± 0.2. The collected supernatant was then acidified using medium containing 0.7g KH_2PO_4, 0.9g Na_2HPO_4, 2g $NaNO_3$ and 0.4g $MgSO_4.7H_2O$, 0.1g $CaCl_2.2H_2O$. 2ml of trace element (2g $FeSO_4.7H_2O$, 1.5g $MnSO_4.H_2O$, 0.6g (NH_4) $6Mo_7O_{24}.4H_2O$). After incubation, the cultures were purified by separating the cells 12M HCl to pH 2.0 and the precipitated rhamnolipids were collected by centrifugation. It was extracted thrice with chloroform-ethanol (2:1) mixture, which was then evaporated. The oily residues were treated with 1ml supernatant of each *Bacillus* sp. separately. The reduction was quantified by UV spectroscopy for identifying the variation in reduction among three *Bacillus* sp.

SDS-PAGE Separation of Proteins: The protein sample from the supernatant of *B. lichneiformis* was electrophoresed on 6% SDS-PAGE. The sample was prepared by mixing the protein sample with equal volume of sample loading buffer containing SDS and 2-mercaptoethanol. To denature the protein, sample mixture was heated at 40^0C for 2min. After polymerization 20µl of sample and dye mixture (marker in 1st well, test samples in other wells) were loaded into each well. Then the lower and upper electrophoresis reservoirs were filled with electrode buffer. The voltage was applied until the tracking dye reaches the bottom of the plate. To detect the protein bands in electrophoresis gels, the slabs was removed and stained.

AHL Inactivation Assay Using HPLC: The HPLC analysis was performed using SHIMADZU LC 10 AS HPLC pumps and a varian prostar 210 autosampler were used (Shimadzu, Japan). The HPLC column Security Guard kit and Security Guard cartridges (4 mm x 2 mm) were purchased from Phenomenex (Torrance, CA, USA). The HPLC column used was CLC-ODS C18, 50 mm x 2 mm, 5µm particle size were used. 30µl of AHL standard mixtures, blanks or samples were injected with the conditions of mobile phase methanol-water (70:30 v/v) with 0.1% formic acid via microliter pickup in the column, at a flow rate of 0.25 ml/min. The elution method utilized for HPLC separation of AHLs included an isocrastic profile of methanol-water (70:30 v/v) for 5 min, followed by a linear gradient from 35-95% methanol in water over 10 min were used for flushing the column for the following run.

Aliquots of AHLs in ethyl acetate were dispensed into sterile tubes and the solvent evaporated to dryness under a stream of sterile nitrogen. These tubes were filled with 1ml of resting *Bacillus licheniformis* cell suspensions separately obtained as indicated above, with rehydrating the AHLs and providing a final AHL concentration of 100µM. For AHL inactivation assays *in vitro* using (CCE) Crude Cell Extract, the reaction mixture contained 0•5mgml^{-1} of bacterial protein and also 100µM of AHLs in a final volume of 500µl. Assays were incubated at 25°C or 37°C for up to 360min and stopped at regular intervals by addition of 3 volumes (1•5ml) ethyl acetate. For CCE assays, ethyl acetate containing the residual AHL was removed and evaporated to dryness.

The solution was reconstituted in methanol (100µl) and residual AHL concentrations determined using the AHL biosensors and HPLC. Control experiments involving un-inoculated medium or extraction buffer incubated with AHLs and cells or extracts incubated without AHLs were always performed.

RESULT AND DISCUSSION

Screening of Anti-quorum Sensing Activity: (Disc Diffusion Method): Inhibitory effect of bacterial supernatant such as *B. subtilis, B. cereus* and *B. licheniformis* were observed with the indicator strain, *C. violaceum*. The inhibition was observed as opaque zones. We further confirmed that bacterial supernatant had the ability to control the QS system and with the three bacterial extracts *B. licheniformis* showed highest inhibitory effect ranges from (7mm-11mm) at various concentration of (4µl-10µl) in Table 1. Our result demonstrated that three bacterial extracts showed varying levels of AHL mediated violacein pigment inhibition.

The present research focused on the use of bacterial species for the anti-quorum-sensing activity due to the lack of investigation on the current scenario. *C. violaceum* bioassay was performed in the presence of natural AHL extracted from *C. violaceum* CV31532. The five plants extract namely *Hemidesmus indicus* (root), *Holarrhena antidysenterica* (bark), *Psoralea corylifolia* (seed), *Punica granatum* (pericarp) and *Mangifera indica* (leaf) demonstrated varying level of AHL mediated violacein pigment inhibition in the reporter strains [38]. Findings of the present investigation indicated the potential of various *Bacillus* sp. as a source of anti-QS compounds and highlight the importance of evaluating the nature and activity of the enzyme lactonases. Among three *Bacillus* species *B. lichneiformis* showed inhibition of 7mm even at 4µl concentration.

AHL Inactivation Assay: Inactivation of 3-oxo-C6-HSL was confirmed with the loss of purple pigmentation using the resting cells of *B. licheniformis* at different time intervals as (0, 4, 6, 8 hours) at different concentrations of (4µl, 6µl, 8µl, 10µl) by using *C. violaceum* as bio-reporter model. The lawn produced in our results ranges from (7mm-15mm) in Table 2. Our results suggested that *B. licheniformis* showed high AHL inhibition of (11mm) even at (4µl) concentration at 8hrs when compared to other two bacterial extracts. AHL molecules of 3-oxo-C6-HSL are known to undergo lactonolysis in basic medium and at high temperatures [39]. Disappearance of 3-oxo-C6-HSL was observed with the quorum quenching activities of bacterial extracts on CV lawn and nearly all 3-oxo-C6-HSL was degraded after incubation of 1.5hrs [40], but our extracts degraded after 2hrs. This is because of difference in growth condition. From this report our result showed best activity in degrading AHL.

Table 1: Screening of anti-quorum sensing activity Disc diffusion method

	Concentration in (µl) Zone formation in mm			
Bacterial extracts	4	6	8	10
B. subtilis	-	7	8	8
B. cereus	-	7	8	8
B. licheniformis	7	9	11	11

Table 2: AHL-inactivation assay

Concentration of AHL molecules	4µl	6µl	8µl	10µl
Time (hours)	Zone formation in mm			
0	-	-	-	-
2	7	7	8	8
4	8	8	9	9
6	9	10	11	11
8	11	13	15	15

Total Proteolytic Assay and Pyocyanin Assay: The Total proteolytic activities in the supernatant of *P. aeruginosa* treated bacterial supernatant were quantified by measuring the reduction of azocasein at 520nm. The concentration of inhibitory effect of bacterial extracts on extra cellular protease was tested against *P. aeruginosa* supernatant in LB broth. Among the bacterial extracts *B. subtilis* showed highest proteolytic activity up to 33% when compared to other two bacterial extracts treated with *P. aeruginosa* supernatant, where as *B. cereus* showed 22% and *B. lichneiformis* showed 17% in Figure 1. The secretion of total proteolytic activity was 6% or less of that secreted by the control cells in the presence of antibiotic Azithromycin (AZM), Cefprozil (CPR) [41]. Our results predicted that it reduced up to 33% less than that of control cells when treated with bacterial extracts.

Pyocyanin is a secondary metabolite and has antimicrobial activity against several species of bacteria and fungi. We investigated that the effect of our bacterial supernatant on the ability to reduce the virulent pigment indicated that the pyocyanin pigment was reduced up to 25% in *B. lichneiformis* which showed high reduction activity when compared to other two bacterial supernatant such as *B. subtilis* 10% and *B. cereus* 15% against *P. aeruginosa*. The effects of pyocyanin are of

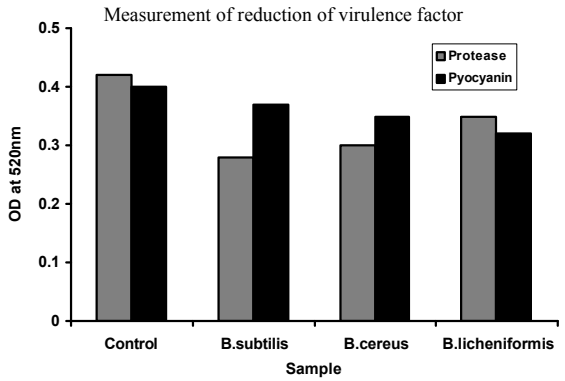

Fig. 1: Total Proteolytic assay and Pyocyanin assay

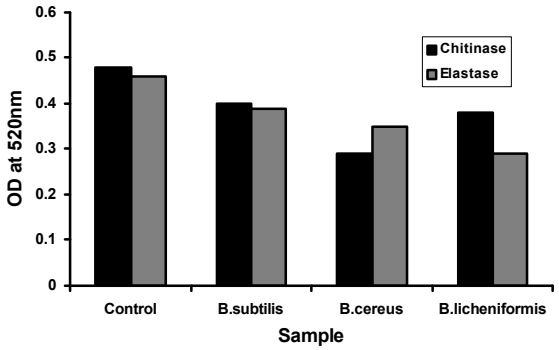

Fig. 2: Chitinase and Elastase assay

importance not only through virulence determination, but also for direct physiological affects on host tissues. Therefore pyocyanin degradation is critical because it also acts antimicrobially towards other organisms by oxidative means [42]. So, this may also be the reason for low reduction of pyocyanin among the bacterial species in case of our results.

Chitinase Assay and Elastase Assay: The chitinase activities in the supernatant of *P. aeruginosa* treated bacterial supernatant were quantified by measuring the degradation of chitin azure. Bacterial supernatant reduced the chitinolytic activity almost to the level of 40%. The optimum reduction of chitinolytic activity was observed in the treatment with three bacterial supernatant. *B. cereus* showed highest activity in controlling chitinase up to 40%, whereas other two bacterial extracts such as *B. subtilis* showed up to 17% and *B. lichneiformis* showed up to 20% in Figure 2. The chitinase activities of *P. aeruginosa* had been reduced by the presence of antibiotics such as AZM, CPR and Cefotaxime (CFT), AZM and CPR reduced the chitinolytic activity of *P. aeruginosa* almost to the level of that for the mutant strains. CFT did not decrease chitinase activity as much as AZM and CPR did, although it still had an effect [43].

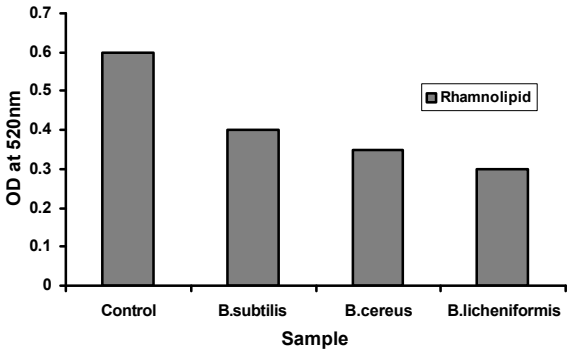

Fig. 3: Rhamnolipid assay

So in case of bacterial sp. each possesses variation in its genetic nature, that's why these three bacterial sp. showed variation in reducing chitinase.

Elastase activities in supernatant of *P. aeruginosa* were quantified using UV spectrometer at 570nm. Bacterial extracts treated culture supernatant showed reduced elastase activity. Elastase is an important enzyme that allows the bacteria to invade and colonize host tissues. We investigated that, the optimum ability to reduce the activities of elastase was observed high in *B. lichneiformis* up to 37% when compared to other two bacterial extracts such as *B. cereus* up to 28% and *B. lichneiformis* up to 15% respectively. The reductions of elastase activities in the supernatant of *P. aeruginosa* were quantified using antibiotics such as AZM, CPR and CFT. AZM treatment reduced the elastase activity of *P. aeruginosa* almost to the level of that for the mutant strains. CPR and CFT also reduced the elastase activity [44]. So, our results predicted that *B. lichneiformis* showed higher reduction. This is because antibiotic may be of both narrow and broad spectrum that kills the microbes or reduce its virulence power with its highly sensitive nature.

Rhamnolipid Assay: Rhamnolipid synthesis is turned on in early stationary phase of *P. aeruginosa* cultures. The precipitated rhamnolipid, oil like residues were treated with three bacterial supernatant separately. The concentration of rhamnolipid was reduced up to 50% in the presence of bacterial supernatant such as *B. lichneiformis* whereas the concentration of *B. cereus* reduced upto 41% and *B. subtilis* up to 35%. Therefore our results revealed that *B. lichneiformis* supernatant showed more activity than other two extracts in Figure 3. The concentration of rhamnolipid in the supernatant exceeds 50µg/ml, which is sufficient to lyses the erythrocytes within 20min. This was tested against blood

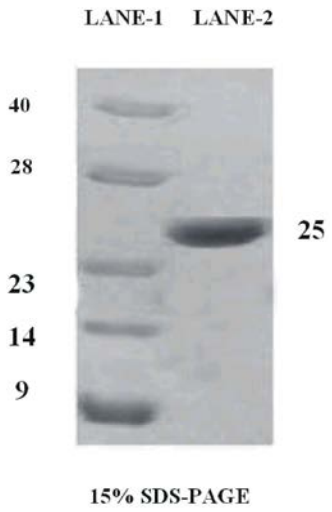

Fig. 4: LANE 1-Marker protein
LANE 2-Molecular wt of AHL molecule of *B.lichneiformis* (25kDa)

for its lysis by rhamnolipid. Presence of hemolysis indicates the presence of rhamnolipid. Antibiotics as AZM, CFT and CPR were treated to reduce the rhamnolipid content. AZM reduced the rhamnolipid contents of supernatant by 80%, whereas CPR and CFT reduced upto 55% to 65% [45]. Our results suggested that *B. lichneiformis* showed highest reduction as much as antibiotics.

SDS-PAGE: The molecular weight of AHL lactonase of *B. lichneiformis* was found to be ~25kDa by performing the SDS-PAGE. The molecular weight of AHL lactonase enzyme isolated from *Bacillus* species was found to be ~28 kDa [46], but our result shows that the molecular weight of AHL lactonase enzyme of *B. lichneiformis* was slightly lesser. The known molecular weight of certain protein was used in the gel as marker protein for separating the protein of *B. lichneiformis* is showed in Figure 4.

AHL Inactivation Assay Using HPLC: AHL degradation activity was found in the crude cell extracts of *B. licheniformis*, when compared to other two extracts of *B. cereus* and *B. subtilis* respectively. Lactone-acylase degrade C8-HSL, C10-HSL and C12-HSL and showed the highest degrading activity against C10-HSL but do not degrade C6-HSL after 110min [47]. But, our result shows that the crude cell extracts of *B. lichneiformis*, which contains AHL-lactonase enzyme, degrade upto 20%. The utility of AHL degrading enzymes in combating pathogenic bacteria shows that strategy to attenuate AHL-mediated virulence. Despite their promise, the structure or catalytic mechanism of AHL lactonases is still poorly understood. Understanding the detailed structure of AHL lactonase will help us design more effective catalysts for disrupting the QS pathways of pathogenic organisms.

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