# 4.1 Introduction

Many bacteria communicate with each other and respond collectively to a changing environment. In this cell to cell communication mechanism known as quorum sensing (QS) bacteria release, detect and respond to accumulation of small signal molecules in a cell density dependent manner (Camilli and Bassler 2006). Quorum sensing regulates expression and synchronization of a set of target genes in bacteria with functional coordination among bacterial communities (Dong and Zhang 2005; Miller and Bassler 2001). Many human and plant pathogenic Gram negative bacteria belonging to the genera *Agrobacterium, Brucella, Burkholderia, Erwinia, Enterobacter, Pseudomonas, Ralstonia, Serratia, Vibrio* and *Yersinia* regulate their virulence factors production for pathogenesis through the signalling molecules known as *N*- acyl homoserine lactones (AHLs) produced by them (Whitehead et al. 2001; Von Bodman et al. 2003). With the increase in population density of bacteria more AHL accumulates in the environment and achieves its critical threshold concentration, AHL binds and activates its cognate transcriptional regulator to trigger the expression of the target genes (Fuqua et al. 2001). One of the plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) relies majorly upon 3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) mediated quorum sensing to sense its population density and in turn resulting in the production of its virulence factors. These are majorly secretory plant cell wall degrading enzymes (pectate lyases, pectinase and cellulase) that macerate plant tissues and contribute to the soft rot phenotype (Barnard and Salmond 2007; Liu et al. 2008). As quorum sensing regulates key virulence factors of pathogen for infection to the host plant, the strategy of disrupting quorum sensing holds a potential to be used as biocontrol approach against quorum sensing pathogens.

Interference with quorum sensing is termed as quorum quenching (QQ) and can be caused in many different ways. The production of signal molecules, the signal molecule itself and/or sensing of the signal molecule by the cognate regulatory protein could be targets for quorum quenching. The mechanisms involve use of quorum sensing inhibitors or quorum quenching enzymes which could be either of abiotic or biotic origins (Uroz et al. 2009; Hirakawa et al. 2013). Majority of quorum quenching enzymes of bacterial origin can be categorized into two distinct groups: AHL lactonases and AHL acylases. AHL lactonases catalyse hydrolytic ring opening of the lactone to form an *N*-acyl homoserine product. This kind of hydrolysis may also occur spontaneously at alkaline pH and can be reversed at acidic pH (Yates et al. 2002).The first reported QQ enzyme was AHL lactonase type (AiiA) form a Gram-positive

*Bacillus* sp. 240B1 (Dong et al. 2000). AHL lactonase producing bacteria were found from phylum Firmicutes such as *B. thuringiensis*, *B. cereus*, *B. anthracis*, and *B. mycoides*, *B. subtilis*, *B. amyloliquefaciens*, *Geobacillus* sp. (Dong et al. 2002; Lee et al. 2002; Pan et al. 2008; Yin et al. 2010; Augustine et al. 2010; Seo et al. 2011); Actinobacteria such as *Arthrobacter sp*., *Microbacterium testaceum*, *Rhodococcus erythropolis* (Park et al. 2003; Wang et al. 2010; Uroz et al. 2008); Proteobacteria, such as *Agrobacterium*, *Ochrobactrum* sp., *Klebsiella pneumoniae* (Carlier et al. 2003; Mei et al. 2010; Park et al. 2003) and Bacteroidetes such as *Chryseobacterium* sp., *Muricauda olearia* (Wang et al. 2012; Tang et al. 2014). These AHL lactonases belong to either metallo- β-lactamase, phosphotriesterase (PTE) or α/β hydrolase-fold family of proteins. The second group of quorum quenching enzyme is AHL acylases which catalyse hydrolytic cleavage of the amide bond to form homoserine lactone and free fatty acid. AHL acylases belong to the Ntn-hydrolase superfamily (Amara et al. 2011). AHL acylases are identified from Proteobacteria, such as *Variovorax paradoxus*is, *Ralstonia* sp., *P. aeruginosa*, *Comamonas* sp. (Leadbetter and Greenberg 2000; Lin et al. 2003; Huang et al. 2003; Uroz et al. 2007) and Actinobacteria such as *Streptomyces* sp. (Park et al. 2005).

Heterologous expression of quorum quenching enzyme either in pathogenic bacteria or in the host plant has been shown to attenuate virulence. Over expression of *aiiA* (AHL lactonase of *Bacillus* sp.) in *E. carotovora* resulted in reduced AHL accumulation and significantly decreased the secretion of pectolytic enzymes which contribute majorly in causing infection (Dong et al. 2000). On the other hand, genetically modified potato and tobacco plants where *aiiA* was inserted into their genome resulted in enzymatic degradation of 3-oxo-C6-HSL in the plant environment which reduced the maceration caused by *E. carotovora* (Dong et al. 2001). Application of native bacteria with quorum quenching enzyme has been shown to prevent maceration of plant tissue by the quorum sensing pathogens (Uroz et al. 2003; Faure and Dessaux 2007). This strategy gave the opportunity for direct application of AHL degrading bacteria as biological control agents of plant bacterial diseases.

The next objective of the present study was to identify the mechanism of AHL degradation exhibited by *Lysinibacillus* sp. Gs50 involved in attenuation of quorum sensing regulated pathogenesis of *Pcc*BR1 (discussed in section 3.4). Accordingly, in this chapter the results of the studies comprising identification, purification and characterization of the AHL-lactonase (AdeH) from the soil isolate *Lysinibacillus* sp. Gs50 have been discussed.

# 4.2 Materials and method

## 4.2.1 Bacterial strains and culture conditions

*Lysinibacillus* sp. Gs50 was grown in Luria-Bertani (LB) at 30° C. *Pcc*BR1 was grown in LB medium at 30°C. *C. violaceum* CV026 used as biosensor strain for detecting exogenous AHLs (AHLs having acyl chains of C4 to C8 in length) was grown in LB with 30 µg/ml of Kanamycin. *Escherichia coli* strains DH5α and BL21 (DE3) were grown in LB at 37° C and 100 µg/ml of Ampicillin was added when necessary. *N*-butanoyl-L-homoserine lactone (C4-HSL), *N*-hexanoyl-DL-homoserine lactone (C6-HSL), 3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL) and 3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8HSL) were purchased form Sigma-Aldrich. Strains and plasmids used in this study are given in Table 8.

**Table 1 Bacterial strains and plasmids used for cloning and expression**

| Strain/plasmid | Characteristics | Source |
|---|---|---|
| *E. coli* DH5α | $F^-$ 80dlacZ M15 (lacZYA-argF) U169 recA1 endA1hsdR17($rk^-$, $mk^+$) phoAsupE44 -thi-1 gyrA96 relA1. Highest transformation efficiency, general cloning ,blue-white selection | Laboratory stock |
| *E. coli* BL21(DE3) | $F^-$ ompT hsdS($rB^-$ $mB^-$) gal dcm λ(DE3) λ(DE3): lacI, lacUV5-T7 gene 1, ind1, sam7, nin5 ). Inducible for high-level protein expression | Laboratory stock |
| pTZ57R/T | TA cloning vector, $Amp^r$ | Thermo Scientific |
| pTZ57R/T/*adeH* | Contains ORF of gene *adeH*, $Amp^r$ | This study |
| pET22b(+) | Expression vector, $Amp^r$ | Novagen |
| pET22b(+)/*adeH* | Upon IPTG induction expresses AdeH, $Amp^r$ | This study |

## 4.2.2 Degradation of different AHLs

The AHL degradation bioassay was performed according to Park et al. (2006). Briefly, overnight grown *Lysinibacillus* sp. Gs50 culture was centrifuged at 7000 rpm for 5 min and pellet was resuspended in phosphate buffer saline (PBS) pH 7.4. 20 µl of *Lysinibacillus* sp. Gs50 cell suspension was added to 80 µl of 100µM AHL (C4-HSL, C6-HSL, 3-oxo-C6-HSL,

C8-HSL or 3-oxo-C8HSL in separate assays). This reaction mixture was incubated at 30°C for 2 hours under static conditions. Untreated controls of 100µM of C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL and 3-oxo-C8HSL prepared in PBS were incubated at similar conditions. The amount of AHL remaining after exposure to *Lysinibacillus* sp. Gs50 was quantified by adding 30 µl of supernatant of the reaction mixture to suspensions of the biosensor *C. violaceum* CV026 (0.002 OD) in a 96 well microtiter plate. This was incubated overnight under static conditions at 30°C and the amount of purple pigment was quantified in terms of the ratio $A_{585nm}$ /$A_{660nm}$. Percentage degradation of AHL during treatment with *Lysinibacillus* sp. Gs50 was calculated with reference to the $A_{585nm}$ /$A_{660nm}$ of untreated AHL controls.

The AHL degrading activity of appropriately induced *E.coli* BL21(DE3) pET22b(+)/*adeH* culture was assayed by 50 µl of cell suspension with 50 µl of 25µM of C6-HSL. Similar reactions were set up with *E.coli* BL21(DE3), *E.coli* BL21(DE3) pET22b(+) without IPTG induction, *E.coli* BL21(DE3) pET22b(+) with IPTG induction and *E.coli* BL21(DE3) pET22b(+)/*adeH* without IPTG induction as controls. 25µM of C6-HSL was taken as no treatment control. Degradation of different chain length AHLs by appropriately induced *E.coli* BL21(DE3) pET22b(+)/*adeH* was carried out in a 100 µl reaction mixture that contained 50 µl of cell suspension and 50 µl of 100µM of C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL and 3-oxo-C8HSL. Untreated controls consisted of 100µM of C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL and 3-oxo-C8HSL. The activity of purified AdeH was assayed by mixing 100µg of AdeH and 30µM of C6-HSL in total volume of 150 µl. Untreated control consisted of 30µM of C6-HSL without purified AdeH. Biosensor assay was performed as above and appearance of purple pigment was observed. All bioassays for AHL degradation were conducted in triplicates.

### 4.2.3 Localisation of AHL degrading enzyme in *Lysinibacillus* sp. Gs50

To investigate whether AHL degrading enzyme is intracellular or extracellular, *Lysinibacillus* sp. Gs50 was grown overnight and culture supernatant was collected after centrifugation at 7000 rpm for 5 min. The culture pellet was resuspended in PBS. The resuspended cells were lysed using Sonics Vibracell TM sonicator (30% amplitude for 3 minutes at alternating intervals of 9 seconds) and supernatant and cell content were collected after centrifugation at 10,000 rpm for 10 min at 4°C. 20 µl of three fractions designated culture supernatant, supernatant after sonication and pellet after sonication were assayed individually for AHL

degradation by mixing with 80 µl of 25µM C6-HSL and incubating at 30°C for 2 hours under static conditions followed by the biosensor assay described above. Reaction mixture containing whole cell of *Lysinibacillus* sp. Gs50 with 25µM of C6-HSL was taken as degradation positive control and 25µM C6-HSL was no treatment control. The assay was conducted in triplicates.

### 4.2.4 AHL restoration assay

An AHL restoration assay was performed as per Yates et al. (2002). For the assay, degradation reaction of overnight grown *Lysinibacillus* sp. Gs50 cells and 25µM of C6-HSL was carried out for 2 hours at 30°C. Untreated control of 25µM of C6-HSL was taken. After incubation, an aliquot of supernatant from both, reaction mixture and untreated control, was acidified with 25 µl of 50mM HCl to bring the pH to 2. Further, the reaction mixture and untreated control without acidification and after acidification were subjected to the biosensor assay. The assay was conducted in triplicates.

### 4.2.5 Carbon source utilisation

To investigate whether *Lysinibacillus* sp. Gs50 was able to utilise AHL as sole carbon source, overnight grown *Lysinibacillus* sp. Gs50 culture was centrifuged at 7000 rpm and the pellet was washed three times with PBS and resuspended in 100 µl of M9 minimal medium. This stock was used to inoculate (1% dilution) 1 ml aliquots of M9 minimal medium and supplemented with 2.5mM sucrose and 5mM C6-HSL as carbon sources in the media (Park et al. 2003). *Lysinibacillus* sp. Gs50 was allowed to grow in shaking condition at 30°C. The optical density of the culture was monitored using Tecan InfinitePro 2000 microtiter-plate reader at 600nm till 50 hours. Uninoculated M9 was taken as negative control while M9 supplemented with 2.5mM was taken as positive control for the growth of *Lysinibacillus* sp. Gs50. The experiment was conducted in triplicates.

### 4.2.6 Genomic DNA extraction

Genomic DNA from the overnight grown cells of *Lysinibacillus* sp.Gs50 was extracted using the same protocol as mentioned in section 2.2.5.

### 4.2.7 Primer design and PCR amplification of putative *adeH* gene

Primers for the gene coding for AHL degrading enzyme were designed on the basis of gene bsph_3377 in *Lysinibacillus sphaericus* C3-41 chromosome (NCBI Reference Sequence: NC_010382.1) shown *in-silico* to code for a hypothetical protein which might possess AHL lactonase activity (Kalia et al. 2011). The sequence of the forward primer was 5': CG**GGATCC**GATGCGAATGGATAAGAATTA: 3' and reverse primer was 5': C**GAGCTC**GATTCATAATATCCTTCTGTCGATTT: 3' (Sigma- Aldrich). The sequences in bold show the restriction sites for the restriction enzymes *BamHI* in the forward primer and *SacI* in the reverse primer. The gene was amplified using *pfu* polymerase using genomic DNA of *Lysinibacillus* sp. Gs50 as template. The PCR was carried out in TaKaRA (U.S.A) thermal cycler for 35 cycles having initial denaturation at 94°C for 5 min followed by each cycle having denaturation at 94°C for 50 sec, annealing at 47°C for 1 min, elongation at 72°C for 2 min and a final elongation at 72°C for 10 mins. The PCR product obtained was incubated with *Taq* polymerase in presence of dNTP's at 72°C for 15 mins to include 3'-A overhangs.

### 4.2.8 Plasmid isolation

Plasmid isolation was carried out from *E. coli* strains using alkaline lysis method (Sambrook et al. 2001).

### 4.2.9 Restriction digestion of plasmid

For restriction digestion, 0.5-1.0 µg of DNA sample and 0.2-0.4 U of each individual restriction enzyme was used with respective buffer (1X or 2 X concentrations) in a final reaction volume of 20 µl. The digestion reaction mixture kept at 37°C for 4-5 hours. The DNA digestion pattern was visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels.

### 4.2.10 Gel elution and purification of DNA by freeze thaw method

After running digestion product in low melting agarose, the desired DNA band was cut by sterile scalpel. The gel slice obtained was chopped in small pieces and molten at 65°C for 10 min. To this 2 volumes of TE (10:1) was added, then vortexed vigorously for 5 min and kept at -20°C for 2 hours. This was thawed after 2 hours and centrifuged at 10,000 rpm and the

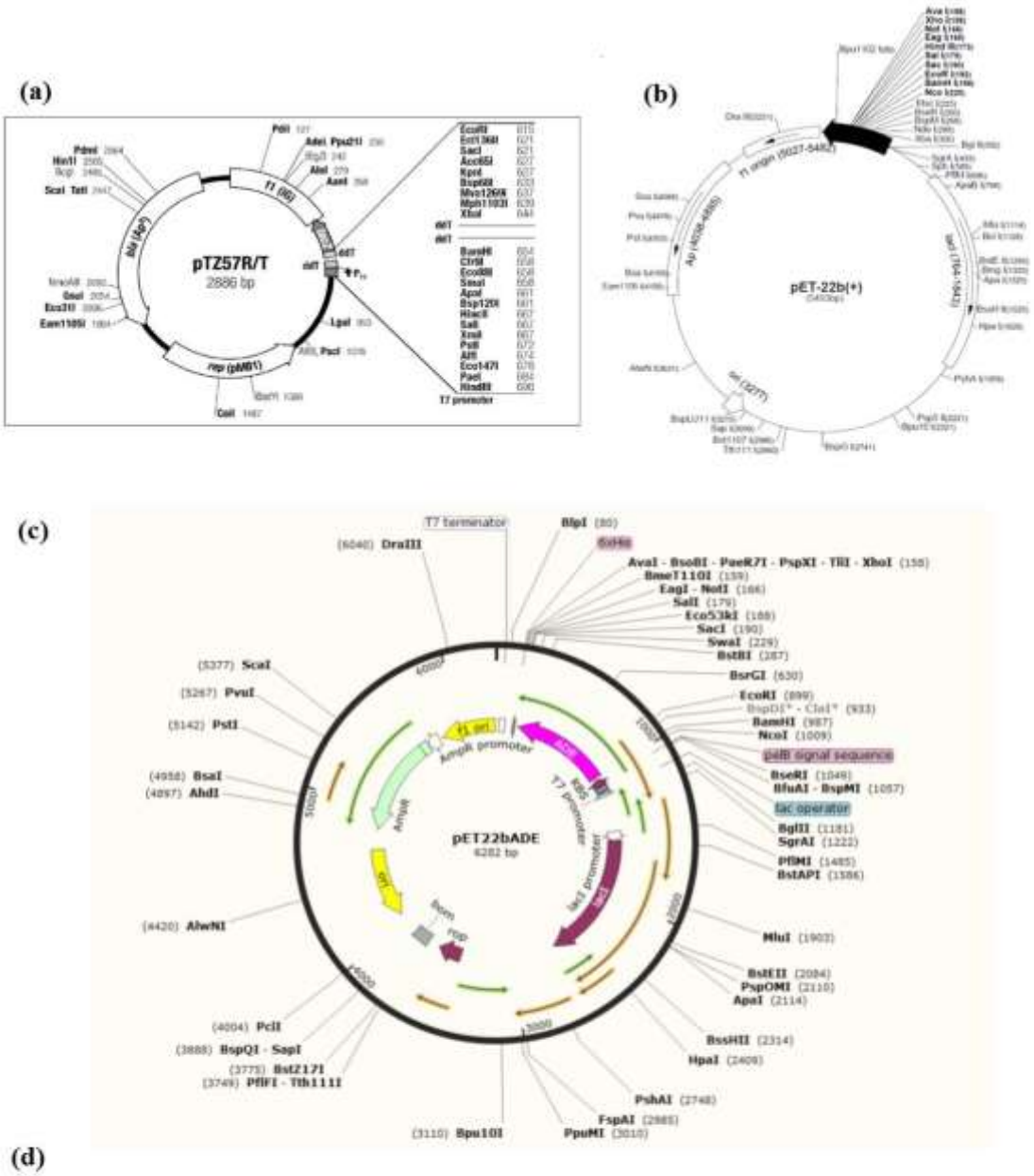
supernatant was collected. This process was repeated thrice and the supernatant from each cycle was pooled. The DNA from the supernatant was precipitated by adding 1/10th volume of 3M sodium acetate and double volume ethanol by keeping it overnight at -20°C. After centrifugation for 10 min at 12,000 rpm the precipitate was collected and washed with 70% ethanol and thereafter dissolved in miliQ water for further use.

## 4.2.11 Cloning and heterologous expression of *adeH*

The PCR product having 3'-A overhangs as obtained in 4.2.7 was ligated using T4 DNA ligase into pTZ57R/T cloning vector. Map of pTZ57R/T cloning vector is given in Figure 19 (a). The ligation mixture was prepared by following the instructor's manual and after incubating at 22°C for 1 hour was transformed into *E.coli* DH5α cells using TransformAid Bacterial Transformation Kit from Thermo-Scientific. The transformants were obtained on LA plates containing Amp (100 µg/ml) and X- gal (40 µl per plate) after incubation at 37°C for 16-18 hours and the resultant recombinant plasmid pTZ57R/T/*adeH* from the transformant was digested with *BamHI* and *SacI* and the purified insert was ligated into *BamHI/SacI* digested pET22b(+) (Novagen) (Figure 19 (b)) to give pET22b(+)/*adeH* which was then transformed into *E.coli* BL21(DE3) cells. The transformants were selected on LA plates containing Amp (100 µg/ml). Map of the recombinant pET22b(+)/*adeH* is given in Figure 19 (c). The transformed *E.coli* BL21(DE3) pET22b(+)/*adeH* culture grown till 0.4 $OD_{600}$ was subjected to 0.5mM of IPTG (isopropyl-D-thiogalactopyranoside) and incubated at 30°C shaking condition for 6 hours for induction of AdeH. The expression of AdeH was detected on 10% SDS-PAGE.

## 4.2.12 SDS PAGE and staining

SDS PAGE was performed according to standard laboratory protocol. Protein bands in the gel were visualised by either silver staining or Commassie staining method. For silver staining the gel was kept in fixing solution (50:10:40 methanol: Glacial acetic acid: Distilled water) for half an hour and then washed twice with 5% methanol. After this, the gel was washed with distilled water for 5 min twice and subsequently immersed in 0.02% sodium thiosulphate for 3 min. The gel was washed with distilled water and kept in 0.2% $AgNO_3$




ATGAAATACTTGCTGACCGAACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCAGGCGATGCCCAT
GGATATCGGAATTAATTCGGATCCGATGCGAATGGATAAGAATTATATGATTGCGATGCACAATCCCGCAAC
CATCGATCATCCAAACCAGCCAAATGAATTTGTTGAATTCCCTGTTTATACAGTATTGATTGATCATCCAGAA
GGCAAAATTCTATTTGATACTGCATGCAATCCAAACTCGATGGGTCCAGAAGGGCGATGGGGTGAATTTACG
CAAAAAGCATTTCCTATTAATATGCCAGAAGAATGCTATTTACATCATCGTCTAGAAGAATTAAATGTTCGTCC
AGAGGATATCAAATATGTTGTTGCTTCCCATTTGCATCTCGACCATGCAGGGTGCTTAGAACTCTTTACAAAT
GCCACAATTATTGTACAGGAAGATGAGTTTAATGGCACATTACAAACATATGCTAGAAACGTGAAAGACGGT
GCCTATGTTTGGGGTGACATTGATATGTGGATTAAAAACAATCTACAATGGCGCTTAATCAAACGTGGCGAG
GATCAGGTAAAACTAGCTGATGGGATACAAGTATTAAATTTTGGTAGTGGTCATGCTTGGGGAATGCTAGGT
TTGCATATTCAAATGCCTGACACAGGTGGTATTATTTTAGCCTCAGATGCCATTTATACGGCAGAAAGCTTTG
GTCCTCCTGTCAAGCCACCAGGCATTATCTACGATTCTGTAGGTTATAATACAACAGTGGAACGGATTCGAA
GACTAGCAAATGAAACAAATTCACAGGTATGGTTTGGCCATGATCCTATTCAATTTAAATCATTTAGAAAATCG
ACAGAAGGATATTATGAATCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCA

**Figure 1 Strategy for cloning of *adeH***

**(a)** Restriction map of pTZ57R/T cloning vector **(b)** Restriction map of pET-22b(+) expression vector **(c)** restriction map of recombinant pET22b(+)/adeH **(d)** Gene sequence of *adeH*

solution for 30 min. Next the developing solution (2 ml of 0.02 % $Na_2S_2O_3$ + 2.19 % $Na_2CO_3$ + 125 µl formaldehyde) was added till the bands appeared. The staining was stopped by adding 1.4% EDTA solution. For Commassie staining the Commassie stain (0.25g commassie G-250 in 90ml methanol: water (1:1) and 10ml glacial acetic acid) was added on the gel and kept for 3 hours. Destaining solution (50:10:40 methanol: glacial acetic acid: water) was added on to the gel every 20 min till the bands were clearly seen and the background of gel was clear. The molecular weight of the expressed protein was determined using higher range protein molecular weight marker (Merck, India).

## 4.2.13 Soft rot attenuation by *E.coli* BL21 (DE3) pET22b(+)/*adeH*

The virulence assay was performed on potato tuber slices as described in section 3.2.6. *Pcc*BR1 was cultivated to achieve ~2.8 $\times 10^6$ CFU/ml in LB broth at 30°C. Overnight grown culture of *E.coli* BL21(DE3) pET22b(+)/*adeH* sub-cultured in 100 ml LB at 37°C. As the OD of the culture reached 0.4, 0.5 mM IPTG was added and kept at 30°C shaking condition for 6 hours for induction. 50 µl of induced *E.coli* BL21(DE3) pET22b(+)/*adeH* was mixed with 50 µl *Pcc*BR1. 5 µl of this mixture was inoculated on each potato slice by injecting at three points using sterile disposable syringe in aseptic condition. The inoculated slices were placed in Petri dishes and further incubated at 30 °C for 24 hours. The controls were consisted of *Pcc*BR1 culture alone, IPTG induced *E.coli* BL21(DE3) pET22b(+) (vector control) + *Pcc*BR1 co-culture and mock inoculation with PBS. Three independent trials were carried out. Each trial had three replicates.

## 4.2.14 Purification of AHL degrading enzyme (AdeH)

The $His_6$-AdeH was purified to homogeneity using Ni-NTA affinity column chromatography, for which appropriately induced 100 ml of *E.coli* BL21(DE3) pET22b (+)/*adeH* was taken and the cells were centrifuged at 7000 rpm for 10 mins, resuspended in 10 ml lysis buffer 1 (50mM Tris-Cl buffer pH 8.0, 1% sarcosine, 0.3% TEA, 0.5% triton X 100, 20mM imidazole and 300mM NaCl) and incubated on ice for 2 hours. Cells were sonicated (Sonics VibracellTM) at 30% amplitude for 10 minutes at alternating intervals of 9 seconds and were then centrifuged at 7,000 rpm for 10 minutes at 4°C. The supernatant was loaded onto the column having Ni-NTA beads charged with lysis buffer 2 (50mM Tris-Cl buffer pH 8.0 and 300mM NaCl) and then kept on rocker for 2 hours. After washing the column with 50 ml wash buffer (50mM Tris-Cl buffer pH 8.0, 20mM imidazole and 300mM NaCl) it was eluted

with the elution buffer (50mM Tris-Cl buffer pH 8.0, 300mM imidazole and 300mM NaCl). The fractions collected every 15 min were pooled and precipitated with acetone. The purified enzyme was resuspended in 50 mMTris-Cl buffer (pH 8.0) after evaporating acetone and purity of the enzyme was assessed on 10% SDS-PAGE.

### 4.2.15 Protein estimation

Bradford method of protein estimation with BSA (bovine serum albumin) as standard was used. 2ml system contained 1ml of the Bradford reagent and different concentration of BSA. The system was assayed by taking OD at 595nm. A standard graph was plotted. After constructing the standard graph the recombinant AdeH protein was estimated.

### 4.2.16 Mass spectrometry analysis of the products of AHL degradation by AdeH

To determine the chemical structure of enzymatic reaction products of AdeH, the substrate C6-HSL was subjected to degradation by purified AdeH and the resulting enzymatic reaction product was analysed by electrospray ionization (ESI)-mass spectrometry (MS). The reaction mixture contained purified AdeH (~5µg) mixed with C6-HSL (100µM) in 500 µl of 50mM Tris-Cl buffer pH 8.0 (Dong et al. 2001; Wang et al. 2004). A control with only C6-HSL (100µM) in 500 µl of 50mM Tris-Cl buffer pH 8.0 was included. After 2 hours of incubation at 35°C, both the reaction mixture and control were extracted three times with 0.1% acidified ethyl acetate and the combined organic phase was evaporated in a rotary evaporator. The samples were dissolved in 100 µl methanol, ionized by positive-ion electrospray and subjected to ESI-MS analysis in HCT Ultra PTM Discovery System (ETD II- Bruker Daltonics) with 1100 series HPLC (Agilent) at Indian Institute of Science, Bangalore, India.

### 4.2.17 Agar diffusion bioassay for quantification of AHL

The Agar diffusion bioassay protocol from Zhang et al. (2007) was modified and used for quantification for AHL. In sterile Luria agar (LA) plates, agar was aseptically cut into 1cm wide bars separated by 2 to 3 mm slits. 3mm diameter wells were made with the well borer in each agar bar. Overnight grown AHL biosensor strain *C. violaceum* CV026 was streaked uniformly below each well. 6 µl C6-HSL of different concentrations (60, 120, 180, 240, 300, 420, 600 picomols) was added individually to the wells. These plates were then incubated at static condition for 48 hours at 30°C. After the incubation the bioassay plates were examined

for the development of purple coloured streak and length of the purple streak so developed was measured. *C. violaceum* CV026 growth turns purple appearing as a streak up to the point of diffusion of C6-HSL and this distance is proportional to the amount of C6-HSL in the well, which is quantified. The experiment was carried out in triplicates.

## 4.2.18 Biochemical characterization of AdeH

### 4.2.18.1 Effect of substrate concentration

To determine the substrate saturation curve, purified AdeH (2µg) was added to C6-HSL (60, 120, 180, 240, 300, 600 picomol) in 50mM Tris-Cl buffer (pH 8.0) in a final volume of 6µl and incubated at 30°C for 2 hours. C6-HSL (60, 120, 180, 240, 300, 600 picomol) in 50mM Tris-Cl buffer (pH 8.0) without purified AdeH were taken as controls. The reactions were stopped by adding 2% SDS after incubation. The remaining C6-HSL after degradation was quantified using Agar diffusion bioassay as mentioned in section 4.2.17 following which the enzyme activity was calculated.

### 4.2.18.2 Temperature range and temperature optimum

The effect of temperature on purified AdeH activity was determined by adding 2µg of AdeH to C6-HSL (240 picomol) in a final volume of 6 µl. Similarly C6-HSL (240 picomol) in a final volume of 6 µl without AdeH was kept as control. Both, reaction mixtures and controls, were incubated at 5°C, 15°C, 20°C, 25°C, 30°C, 37°C, 45°C or 50°C in 50mM Tris-Cl buffer (pH 8.0). The reaction was stopped by adding 2% SDS after incubation. The remaining C6-HSL after degradation was quantified using Agar diffusion bioassay as mentioned in section 4.2.17 following which the enzyme activity was calculated.

### 4.2.18.3 pH range and pH optimum

The effect of pH on purified AdeH activity was determined by adding 2µg of AdeH to C6-HSL (240 picomol) in a final volume of 6 µl. C6-HSL (240 picomol) in a final volume of 6 µl without purified AdeH was kept as control. The reactions and controls were set up at different pH (3.6, 4.0, 4.6, 5.0, 5.6, 6.0, 6.6, 7.0, 7.6, 8.0, 8.6, 9.0) and were incubated at 35°C. The reaction was stopped by adding 2% SDS. The remaining C6-HSL after degradation was quantified using Agar diffusion bioassay as mentioned in section 4.2.17 following which the enzyme activity was calculated.

#### 4.2.18.4 Effect of metal ions

The effect of different metal ions ( $Cd^{2+}$, $Mn^{2+}$, $Fe^{2+}$ , $Zn^{2+}$, $Mg^{2+}$, $Cu^{2+}$ , $Ca^{2+}$ ) and EDTA, on the activity of purified AdeH was determined by adding 2µg of AdeH to C6-HSL (240 picomol) and 1mM of different metal ions in a final volume of 6 µl. A reaction of purified AdeH with 240 picomol of C6-HSL without any cations or EDTA was set up as control. The reaction mixtures and controls were incubated at 35°C in 50mM Tris-Cl buffer (pH8.0). The reaction was stopped by adding 2% SDS. The remaining C6-HSL after degradation was quantified using Agar diffusion bioassay as mentioned in section 4.2.17 following which the enzyme activity was calculated.

#### 4.2.18.5 Determination of $K_M$ and $V_{max}$

To determine $K_M$ and $V_{max}$ of AdeH, 2µg of purified AdeH was added to C6-HSL solution (60, 120, 180, 240, 300, 600 picomoles) in 50mM Tris-Cl buffer (pH 8.0) in a final volume of 6 µl. The reactions were incubated at 35 °C for 2 hours, stopped by adding 2% SDS. The remaining C6-HSL after degradation was quantified as mentioned using Agar diffusion bioassay in section 4.2.17 following which the enzyme activity was calculated.

#### 4.2.18.6 Thermal-stability

Thermal stability of AdeH was determined by pre-incubating the purified enzyme at different temperatures (10°C, 20°C, 30°C, 40°C and 50°C) for 2 hours. To this pre-incubated enzyme 240 picomoles of C6-HSL was added and incubated at 35°C for 2 hours. The reaction was stopped by adding 2% SDS. The remaining C6-HSL after degradation was quantified as mentioned in section 4.2.17 following which the enzyme activity was calculated.

### 4.2.19 Nucleotide sequence accession number

The sequences of the *adeH* gene of *Lysinibacillus* sp. Gs50 have been deposited in GenBank with accession number KU219945. The isolate *Lysinibacillus* sp. Gs50 has been deposited in the Microbial Culture Collection, NCCS, Pune (India) with the accession no of MCC3181.

# 4.3 Results

## 4.3.1 Characterization of AHL degrading *Lysinibacillus* sp. Gs50

### 4.3.1.1 Localisation of AHL degrading enzyme

In order to find out if the AHL degrading enzyme of *Lysinibacillus* sp. Gs50 was intracellular or extracellular, the activity was observed in different cellular fractions, Culture supernatant, Supernatant after sonication and Pellet after sonication. *C. violaceum* CV026 failed to develop purple colour in the wells containing reaction mixture of pellet after sonication. This indicated that the AHL degrading activity was associated with the pellet after sonication fraction (Figure 22 B (1,2,3)). While *C. violaceum* CV026 developed purple colour due to the presence of intact C6-HSL in case of culture supernatant and the supernatant after sonication fractions indicating absence of AHL degrading activity (Figure 20). Based on this, it can be inferred that the AHL degrading enzyme in *Lysinibacillus* sp. Gs50 was not extracellular but was intracellular. Though intracellular the enzyme seemed to be associated with pellet and not the cytosolic fraction (supernatant after sonication).

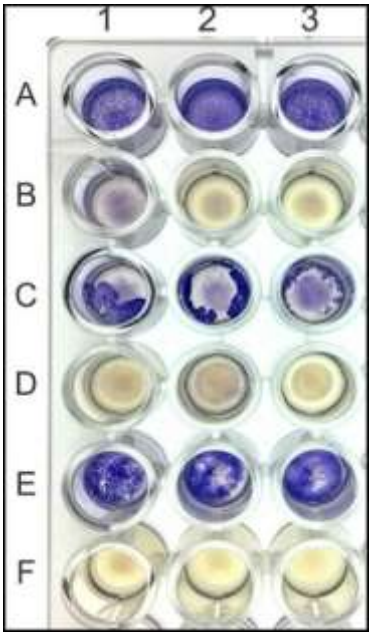



**Figure 2 AHL degrading enzyme in *Lysinibacillus* sp. Gs50 cells**

25µM C6-HSL was added to culture supernatant (A 1, 2, 3), sonicated pellet (B 1, 2, 3), supernatant after sonication (C 1, 2, 3) and whole cell of *Lysinibacillus* sp. Gs50 (D 1, 2, 3). *C. violaceum* CV026 (E 1, 2, 3) + 25µM C6-HSL is no treatment control and only *C. violaceum* CV026 (F 1, 2, 3) is biosensor control

### 4.3.1.2 Growth of *Lysinibacillus* sp. Gs50 on AHL as sole carbon source

If the AHL is cleaved by amidohydrolysis, the resulting products can serve as carbon source for the growth of the bacteria (Figure 21 (a)). However, *Lysinibacillus* sp. Gs50 could not utilise C6-HSL provided in minimal medium as sole carbon source as it demonstrated no growth on C6-HSL whereas it showed significant growth when sucrose was provided as sole carbon source (Figure 21 (c)).

### 4.3.1.3 Restoration of AHL degraded by *Lysinibacillus* sp. Gs50

If the cleavage of AHL is due to enzymatic/non-enzymatic lactone ring hydrolysis, it can be restored to intact AHL at acidic pH (Figure 21 (a)). As observed, C6-HSL degraded by *Lysinibacillus* sp. Gs50 showed reappearance of purple colour upon acidification (Figure 21 (b) B (1,2,3)). This suggested that *Lysinibacillus* sp. Gs50 possibly cleaved the lactone ring of AHL which was restored to intact at low pH. Summing up the above results, it was concluded that AHL degradation activity of *Lysinibacillus* sp. Gs50 may be due to intracellular AHL lactonase.

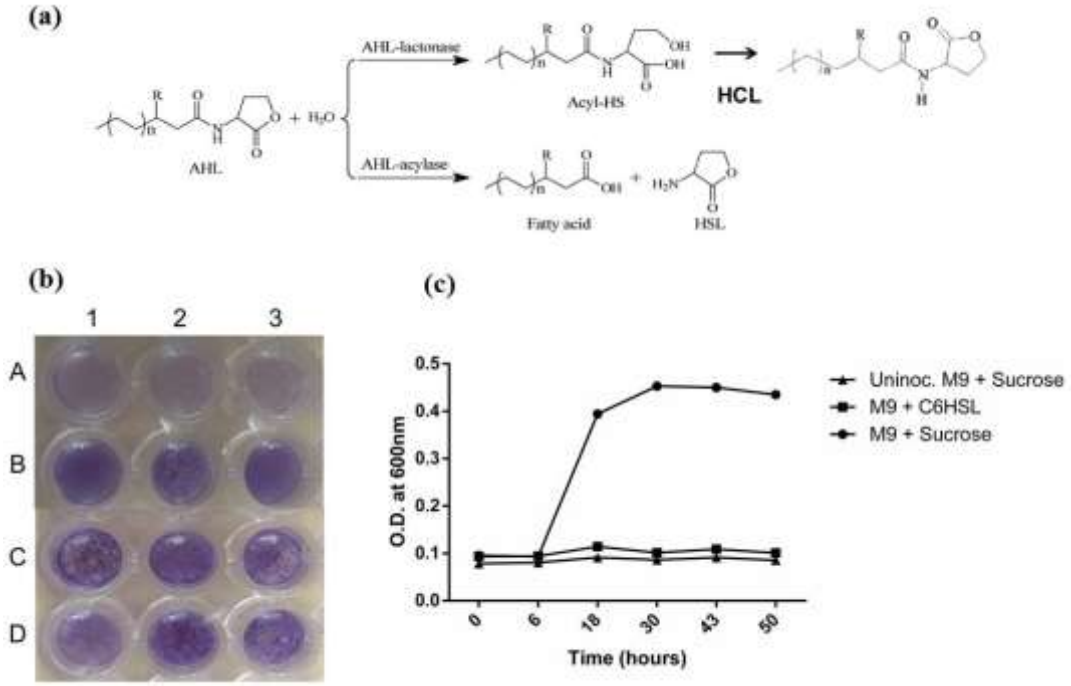



**Figure 3 Characterisation of AHL degrading enzyme of *Lysinibacillus* sp. Gs50**

(a) Two different catalytic mechanisms of AHL degradation (b) Recovery of *Lysinibacillus* sp. Gs50 degraded C6-HSL by acidification. Reaction mixture of *Lysinibacillus* sp. Gs50 + 25µM C6-HSL (A 1, 2, 3) and acidified reaction mixture of *Lysinibacillus* sp. Gs50 + 25µM C6-HSL with 25µl of 50mM HCl (B 1, 2, 3). *C. violaceum* CV026 + 25µM C6-HSL (C 1, 2, 3) and acidified *C. violaceum* CV026 + 25µM C6-HSL (D 1, 2, 3) are controls. (c) Growth of *Lysinibacillus* sp. Gs50 in minimal medium containing C6-HSL as sole carbon source measured at $OD_{600}$

## 4.3.2 Cloning of the gene responsible for AHL degradation in *Lysinibacillus* sp. Gs50

Kalia et al. (2011) mined sequenced whole genome database and revealed a list of organisms possessing conserved domains of AHL lactonases. Accordingly, *Lysinibacillus sphaericus* C3-41 genome showed the conserved domain of AHL lactonase. We identified a sequence of hypothetical protein encoded by bsph_3377 in *Lysinibacillus sphaericus* C3-41 (NCBI Reference Sequence: NC_010382.1) genome showing presence of the conserved domain of AHL lactonase. As the isolate Gs50 also belonged to *Lysinibacillus* genus, primers were designed to target the gene sequence of hypothetical protein encoded by bsph_3377 and used for amplification of the AHL lactonase gene from the genomic DNA of *Lysinibacillus* sp. Gs50.

### 4.3.2.1 PCR amplification of putative gene coding for AHL degrading enzyme

*Pfu* polymerase PCR amplification was carried out using forward primer 5': CG**GGATCC**GATGCGAATGGATAAGAATTA: 3' and reverse primer 5': C**GAGCTC**GATTCATAATATCCTTCTGTCGATTT: 3'. PCR amplicon of size 792 bp was obtained (Figure 22 (a)). This amplicon was further incubated with *Taq* polymerase and dNTPs to include 3'-A overhangs in order to clone it into TA cloning vector.

### 4.3.2.2 Cloning into pTZ57R/T vector in *E.coli* DH5α

PCR product containing overhangs was ligated with pTZ57R/T vector and transformed into *E.coli* DH5α cells. Positive clones (white) were selected and plasmid was isolated. Clone was confirmed by digesting the plasmid individually by *BamHI* and *XbaI* restriction enzymes. There was a restriction site of *BamHI* at 5' of *adeH* and the other was in the pTZ57R/T (Figure 19 (a)) therefore upon digestion with *BamHI* a fragment of 808 bp was released as expected (Figure 22 (b) lane 1). Digestion with *XbaI* resulted in linearization of the recombinant pTZ57R/T/*adeH* showing a band of 3.6 kb (Figure 22 (b) lane 3). Undigested cloned plasmid and pTZ57R/T were run as controls.

### 4.3.2.3 Sub-cloning into pET22b(+) vector in *E.coli* BL21(DE3)

Recombinant pTZ57R/T vector was sequentially digested using *BamHI* and *SacI* .The digested insert was purified further and ligated with *BamHI/SacI* double digested pET22b(+) vector. Recombinant pET22b(+) was then transformed into *E.coli* DH5α cells. Transformants containing recombinant pET22b(+) were selected from LA-Ampicillin plates and plasmid

was isolated. In the recombinant pET22b(+)/*adeH* (Figure 19 (c)) there was a single *BamHI* restriction site therefore digestion with *BamHI* resulted in linearization of recombinant pET22b(+)/*adeH* showing a band of 6.2 kb (Figure 22 (c) lane 1) as expected . Clone was also confirmed by *NcoI* and *SacI* digestion which showed an insert release of 827 bp (Figure 22 (c) lane 3) where *NcoI* restriction site was contributed by pET22b(+) (Figure 19 (c)) and *SacI* was present at the 3' end of *adeH* (Figure 19 (c)). The clone carrying the gene was confirmed by sequencing and was found to be putative AHL lactonase gene.

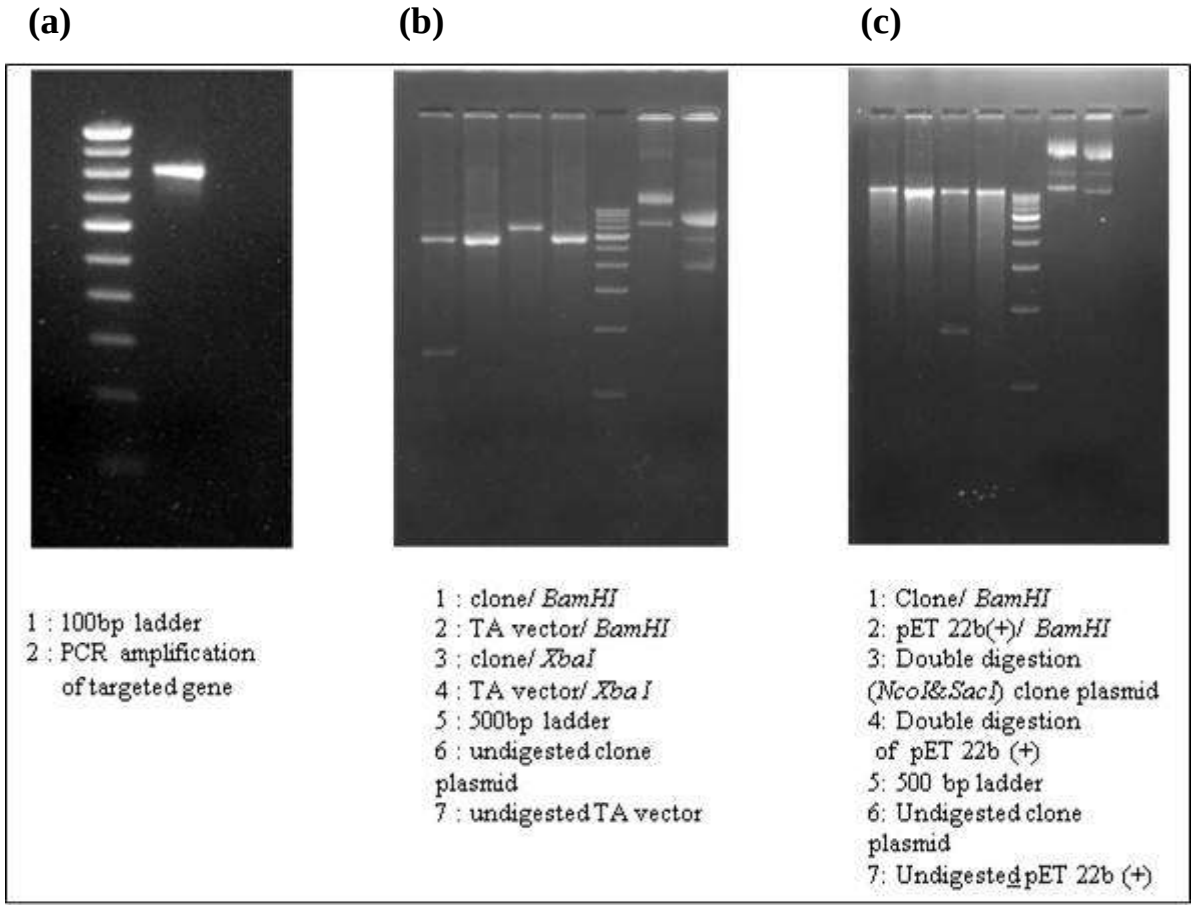



**Figure 4 Cloning of gene responsible for AHL degradation in *Lysinibacillus* sp. Gs50**

**(a)** PCR amplification of putative gene coding for AHL degrading enzyme **(b)** Cloning of AHL degrading gene into pTZ57R/T vector in *E.coli* DH5α **(c)** Sub-cloning of AHL degrading gene into pET22b(+) vector

### 4.3.3 Heterologous expression of the gene for AHL degradation from *Lysinibacillus* sp. Gs50

The expected size 792 bp fragment was amplified and cloned in pTZ57R/T, subcloned in pET22b(+) followed by transformation in *E.coli* BL21(DE3) (designated *E.coli* Bl21(DE3) pET22b(+)/*adeH*) for its expression. Upon IPTG induction of *E.coli* Bl21(DE3) pET22b(+)/*adeH*, a distinct band of ~29 kDa protein was observed on SDS PAGE gel

(Figure 23 (a) Lane 5). Other controls viz. *E.coli* BL21(DE3), *E.coli* BL21(DE3) pET22b(+) without IPTG induction, *E.coli* BL21(DE3) pET22b(+) with IPTG induction and *E.coli* BL21(DE3) pET22b(+)/*adeH* without IPTG induction did not show the protein band of interest (Figure 23 (a) Lane 1-4).

C6-HSL degrading activity shown by *E.coli* Bl21(DE3) pET22b(+)/*adeH* (Figure 23 (b) F (1,2,3)) was comparable to the activity of wild type *Lysinibacillus* sp. Gs50 (Figure 23 (b) A (1,2,3)) indicating that the cloned gene had imparted AHL degradation phenotype and the enzyme was therefore designated *adeH* (AHL degrading hydrolase).

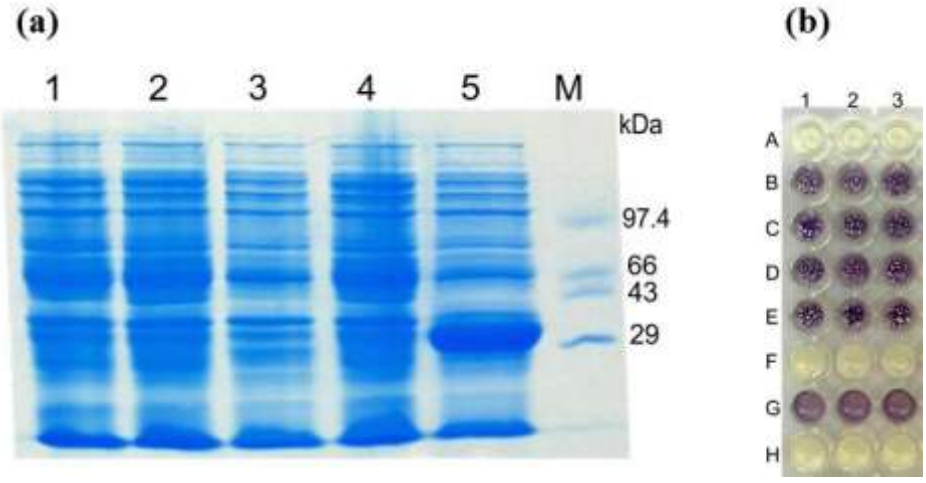



**Figure 5 Expression of recombinant *adeH* in *E.coli* BL21(DE3)**

**(a)** SDS PAGE of *E.coli* BL21(DE3) pET22b(+) expressing AdeH. Lane 1: *E.coli* BL21(DE3) lysate, Lane 2: Lysate of *E.coli* BL21(DE3) pET22b(+) without IPTG induction, Lane 3: Lysate of *E.coli* BL21(DE3) pET22b(+) with IPTG induction, Lane 4: Lysate of *E.coli* BL21(DE3) pET22b(+)/*adeH* without IPTG induction, Lane 5: Lysate of *E.coli* BL21(DE3) pET22b(+)/*adeH* with IPTG induction, M : Protein molecular weight marker. **(b)** AHL degradation assay of *E.coli* BL21(DE3) pET22b(+)/*adeH*. 25µM C6-HSL was added to *Lysinibacillus* sp. Gs50 (A 1, 2, 3), *E.coli* BL21(DE3) (B 1, 2, 3), *E.coli* BL21(DE3) pET22b(+) without IPTG induction (C 1, 2, 3), *E.coli* BL21(DE3) pET22b(+) with IPTG induction (D 1, 2, 3), *E.coli* BL21(DE3) pET22b(+)/*adeH* without IPTG induction (E 1, 2, 3), *E.coli* BL21 (DE3) pET22b(+)/*adeH* with IPTG induction (F 1, 2, 3). *C. violaceum* CV026 (G 1, 2, 3) + 25µM C6-HSL is no treatment control and only *C. violaceum* CV026 (H 1, 2, 3) is biosensor control.

AHL degradation bioassay performed with different AHLs as substrates suggested that *Lysinibacillus* sp. Gs50 and *E.coli* BL21(DE3) pET22b(+)/*adeH* both have the ability to inactivate different AHLs as *C. violaceum* CV026 failed to develop purple pigment in the microtiter plate wells which contained reaction mixture of *Lysinibacillus* sp. Gs50 or *E.coli* BL21(DE3) pET22b(+)/*adeH* and different AHLs (Figure 24).

Further, *E.coli* Bl21 (DE3) pET22b(+)/*adeH* when co-inoculated with *Pcc*BR1 on the potato slice resulted in the virulence attenuation of *Pcc*BR1 as it decreased the soft rot symptoms

caused by *Pcc*BR1 (Figure 25). These results confirmed that the cloned gene from *Lysinibacillus* sp. Gs50 was the one that encoded the AHL degrading enzyme.

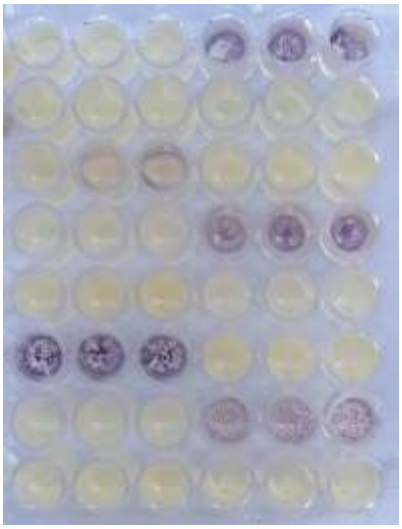


**Figure 6 Degradation of different chain length AHLs by *Lysinibacillus* sp. Gs50 and *E.coli* BL21(DE3) pET22b(+)/*adeH***

A (1, 2, 3) *Lysinibacillus* sp. Gs50+C4-HSL, B (1, 2, 3) *E.coli* BL21(DE3)pET22b(+)/*adeH* +C4-HSL, C (1, 2, 3) C4-HSL+ *C. violaceum* CV026, D (1, 2, 3) *Lysinibacillus* sp. Gs50+C6-HSL, E (1, 2, 3) *E.coli* BL21(DE3)pET22b(+)/*adeH* +C6-HSL, F (1, 2, 3) C6-HSL+ *C. violaceum* CV026, G (1, 2, 3) *Lysinibacillus* sp. Gs50+3-oxo-C6HSL, H (1, 2, 3) *E.coli* BL21(DE3)pET22b(+)/*adeH*+3-oxo-C6HSL, A (4, 5, 6) 3-oxo-C6HSL+ *C. violaceum* CV026, B (4, 5, 6) *Lysinibacillus* sp. Gs50+C8-HSL, C (4, 5, 6) *E.coli* BL21(DE3)pET22b(+)/*adeH*+C8-HSL, D (4, 5, 6) C8-HSL+ *C. violaceum* CV026, E (4, 5, 6) *Lysinibacillus* sp. Gs50+ 3-oxo-C8HSL, F (4, 5, 6) *E.coli* BL21(DE3)pET22b(+)/*adeH*+ 3-oxo-C8HSL, G (4, 5, 6) 3-oxo-C8HSL+ *C. violaceum* CV026, H (4, 5, 6) only *C. violaceum* CV026.

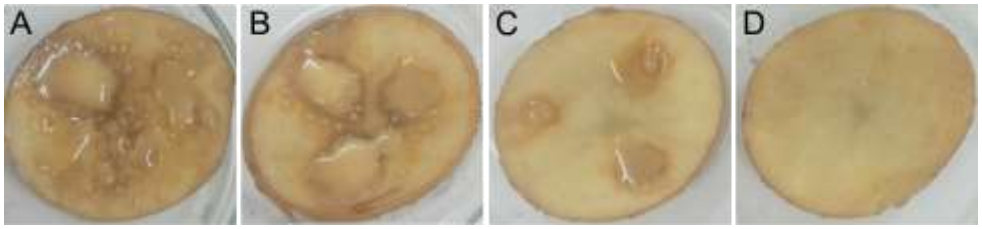



**Figure 7 Soft rot attenuation assay of *E.coli* BL21 (DE3) pET22b(+)/*adeH* caused by *Pcc*BR1**

Potato slices were inoculated with A : *Pcc*BR1, B : IPTG induced *E.coli* BL21(DE3) pET22b(+) and *Pcc*BR1, C : IPTG induced *E.coli* BL21 (DE3) pET22b(+)/*adeH* and *Pcc*BR1, D : PBS control.

## 4.3.4 AdeH is an AHL lactonase

Purification of AdeH from *E.coli* BL21(DE3) pET22b(+)/*adeH* was carried using Ni affinity chromatography as the AdeH expressed in pET22b(+) had a 6-His tag at its C terminal. The purified AdeH detected on 10% SDS PAGE was visualised as a single band of ~29 kDa molecular weight (Figure 26 (a) lane 4-6). Further the acetone precipitated purified AdeH

completely degraded the C6-HSL which confirmed that the purified AdeH was the enzyme responsible for AHL degradation (Figure 26 (b)).

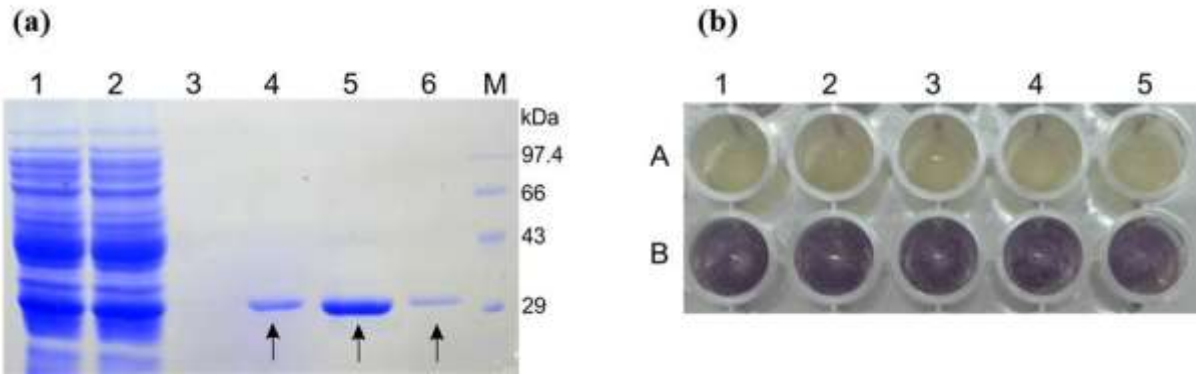



**Figure 8 Purificcation of active AdeH**

**(a)** SDS PAGE analysis of purified His6 tagged AdeH. Lane 1: Unbound Fraction, Lane 2: First wash fraction, Lane 3: Last wash fraction, Lane 4: First elution fraction (AdeH), Lane 5: Second elution fraction (AdeH), Lane 6: Third elution fraction (AdeH), M: Protein molecular weight marker **(b)** AHL degradation assay of purified AdeH. Reaction mixture of 30µM C6-HSL + 100µg of purified and acetone precipitated AdeH was added into *C. violaceum* CV026 (A 1, 2, 3, 4, 5) and untreated control of 30µM C6-HSL was added into *C. violaceum* CV026 (B 1, 2, 3, 4, 5)

To elucidate the mechanism of AdeH catalysis, C6-HSL was treated with purified AdeH and the resulting degradation products were analysed. The ESI-MS profile of C6-HSL (substrate for enzymatic reaction) showed peaks of Na- conjugate and K- conjugates of C6-HSL having an m/z of 221.9 (M + Na) and 239.9 (M + K) respectively (Figure 27 (a)). Subtraction of the mass of conjugates from their m/z values yielded the m/z of 199.9 which corresponds to that of C6-HSL. The enzymatic product of AdeH reaction with C6-HSL as substrate gave a peak with m/z of 217.9 showing a mass increase of 18 as compared to the m/z of intact C6-HSL (199.9). The m/z 217.9 represented the *N*- hexanoyl homoserine (C6HS) which is obtained by the addition of a water molecule to the ester bond of *N*- hexanoyl homoserine lactone (C6-HSL) as a result of the hydrolytic cleavage of lactone ring (Figure 27 (b)). These results confirmed that AdeH hydrolyses the lactone ring of C6-HSL and hence proved to be an AHL lactonase.

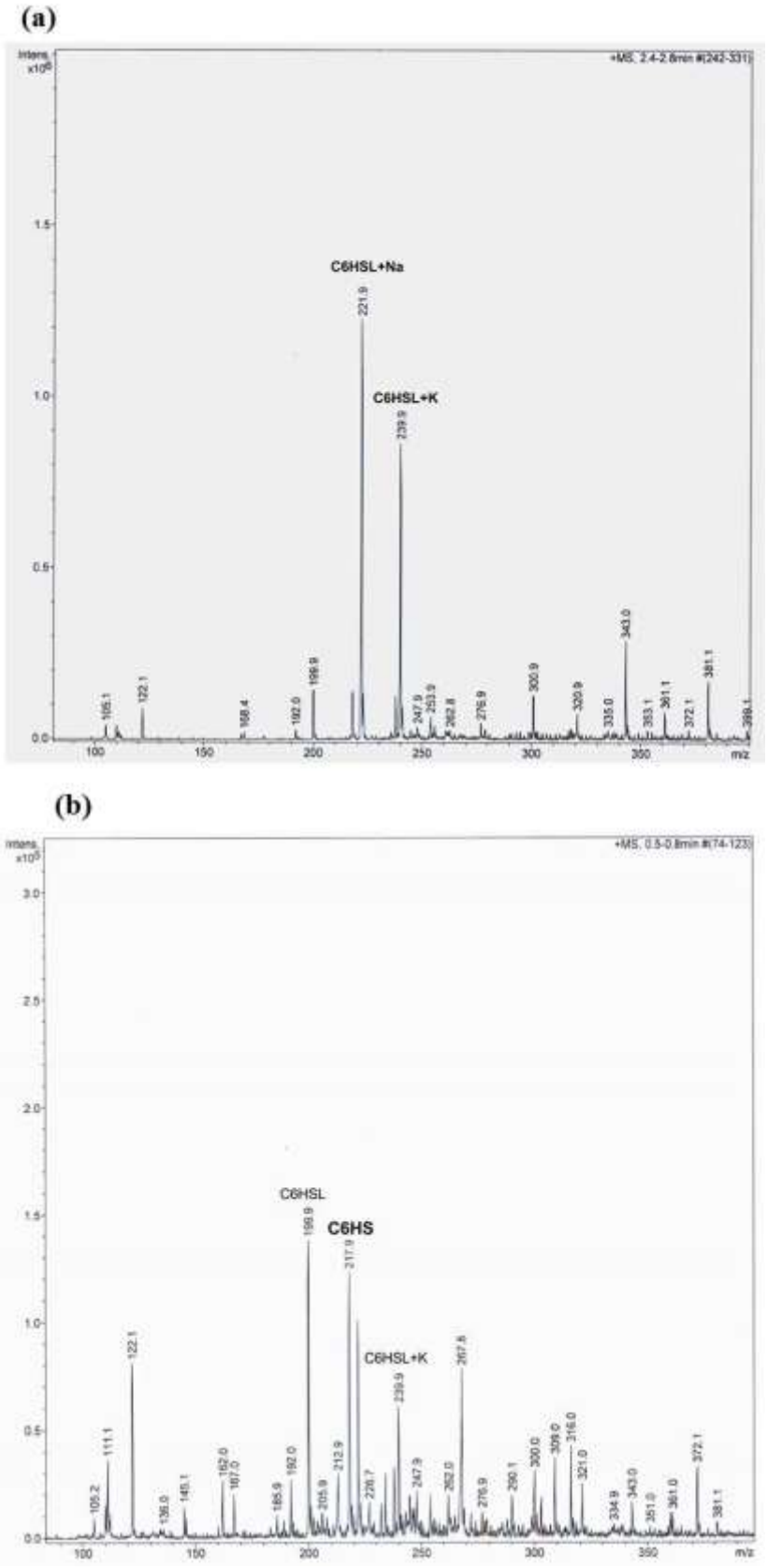



**Figure 9 AdeH is an AHL lactonase**

**(a)** ESI-MS analysis of C6-HSL **(b)** ESI-MS analysis of C6-HSL degraded by AdeH showing a peak of C6HS

The deduced 264 amino acid sequence of AdeH was used to perform local BLASTP search in NCBI database which revealed 99% identity with MBL fold metallo-hydrolase from strains *Lysinibacillus* sp. F5 (accession number WP 058844592.1), *Lysinibacillus sphaericus* (accession number WP 054550471.1) and *Lysinibcillus fusiformis* (accession number WP 004231440.1) which suggested that AdeH belongs to the metallo- β-lactamase superfamily of protein. Many AHL lactonases belong to this superfamily of proteins. The amino acid sequence of AdeH was compared with those of the known AHL lactonases of metallo- β-lactamase superfamily through multiple sequence alignment. The zinc-binding motif "HXHXDH" which is conserved among them was also found in the amino acid sequence of AdeH (Figure 28).

To determine the relationship between AdeH and the known representative AHL lactonases from various bacteria, the amino acid sequence of AdeH was subjected to the phylogenetic analysis using the neighbor-joining method in Mega7 software. The representative amino acid sequences taken were AiiA from *Bacillus* sp. 240B1, AttM and AiiB from *A. tumefaciens* C58, AhlD from *Arthrobacter* sp. IBN110, AhlK from *Klebsiella pneumoniae*, AidC from *Chryseobacterium* sp. StRB126 and MomL from *Muricauda olearia* belonging to metallo- β-lactamase superfamily; QsdA from *R. erythropolis* W2 which belong to phosphotriesterase (PTE) family; AiiM from *Microbacterium testaceum* StLB037 and AidH from *Ochrobactrum* sp. T63 belonging to the α/β hydrolase-fold family. Although AdeH belongs to the metallo- β-lactamase superfamily it was distant from the other AHL lactonase clusters representatives of metallo- β-lactamase superfamily (Figure 29).

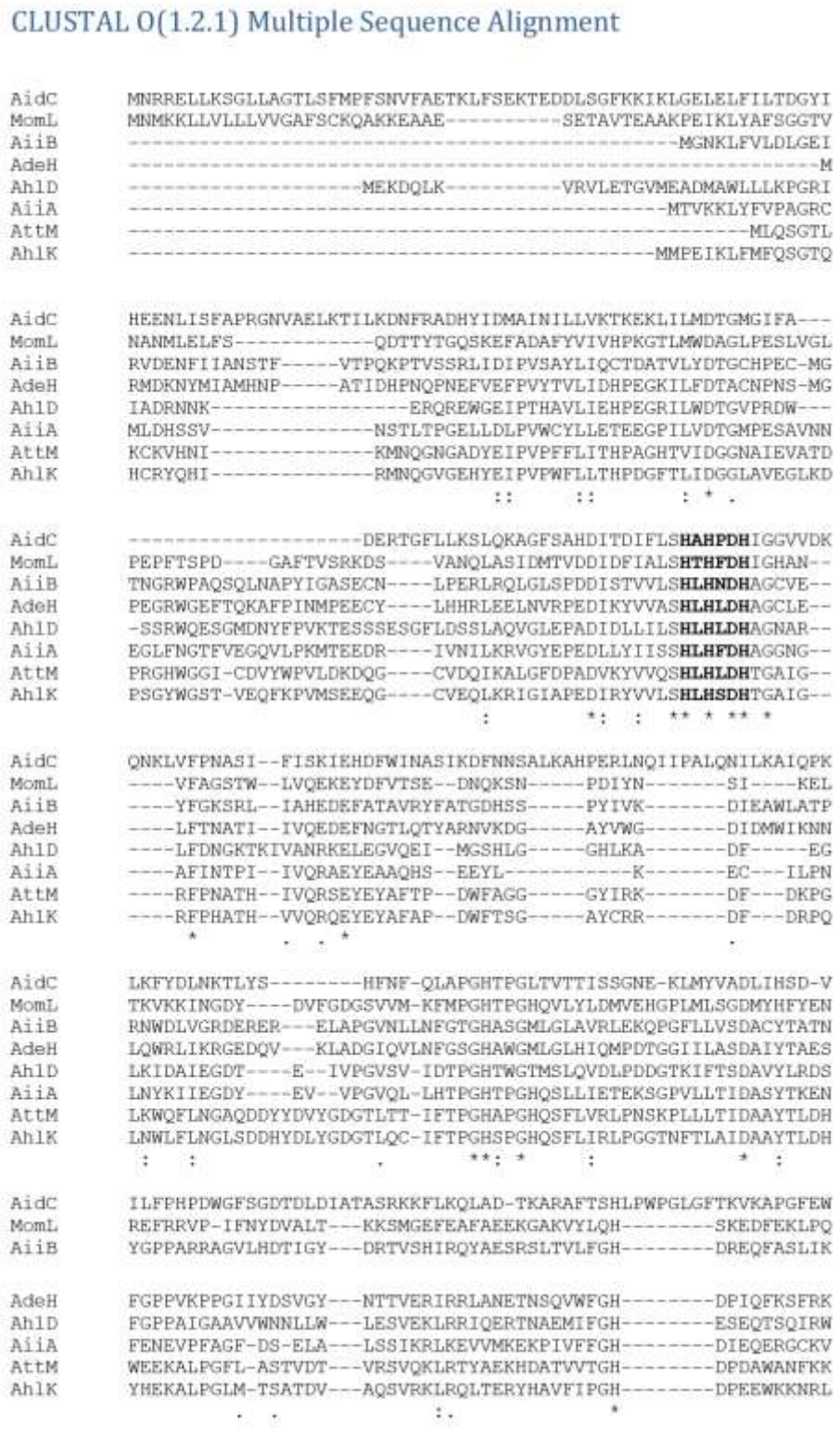



**Figure 10 Multiple sequence alignment of AdeH and other representative AHL lactonases**

Sequence alignment was performed by Clustal Omega online software. Representative AHL lactonases are AidC (Accession: BAM28988), MomL (Accession: AIY30473), AiiB (Accession: NP_396590), AhlD (Accession: AAP57766.1), AiiA (Accession: AAF62398.1), AttM (Accession: AAD43990.1) and AhlK (Accession: AAO47340.1). The shared "HXHXDH" motifs highlighted in bold. Asterisk indicates positions which have a single, fully conserved residue. Colon indicates conservation between groups of strongly similar properties. Period indicates conservation between groups of weakly similar properties.

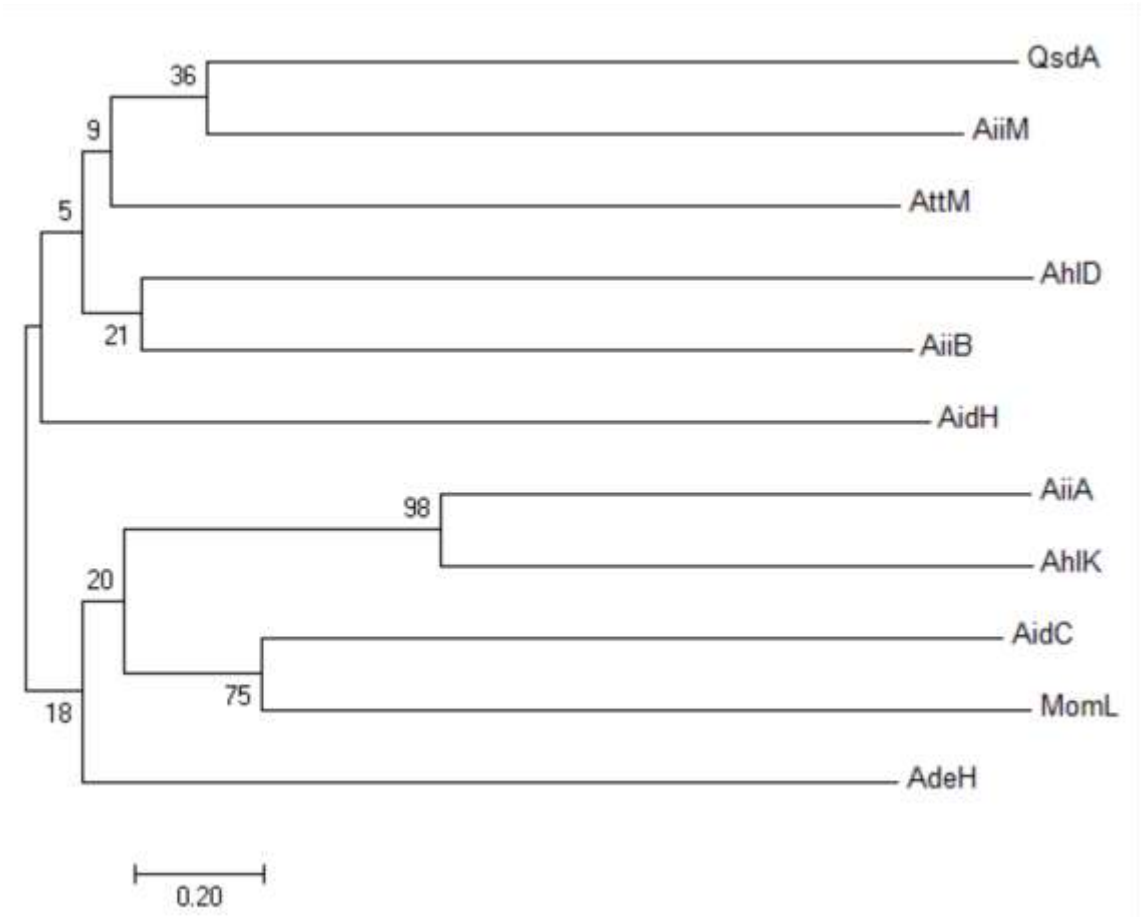



**Figure 11 Neighbor-joining tree of AHL lactonases belonging to the metallo- β-lactamase, phosphotriesterase and α/β hydrolase-fold family based on amino acid sequences**

The dendrogram was constructed by neighbor joining method with the ClustalW program in the MEGA 7 software package (1,000 bootstrap replicates). Scale bar, 0.2 substitutions per amino acid position.

## 4.3.5 Quantification of C6-HSL using agar diffusion bioassay

For biochemical characterisation of AdeH, agar diffusion bioassay of Zhang et al. (2007) was used to quantify the residual C6-HSL in the AdeH reaction. Different amount of C6-HSL (60, 120, 180, 240, 300, 420, 600 picomols) loaded into the wells made in agar plates distance travelled detected by *C.violaceum* CV026 showed purple streaks of varying lengths as shown in Figure 30 (a). The distance of C6-HSL diffusion was measured and the plot of log of amount of C6-HSL versus the distance travelled was obtained. By doing regression analysis, $R^2$ value of 0.85 and equation for the trend line as Y = 0.7739*X + 0.6628 were obtained (Figure 30 (b)). Using this standard graph the residual C6-HSL concentration was calculated in the further AdeH activity experiments.

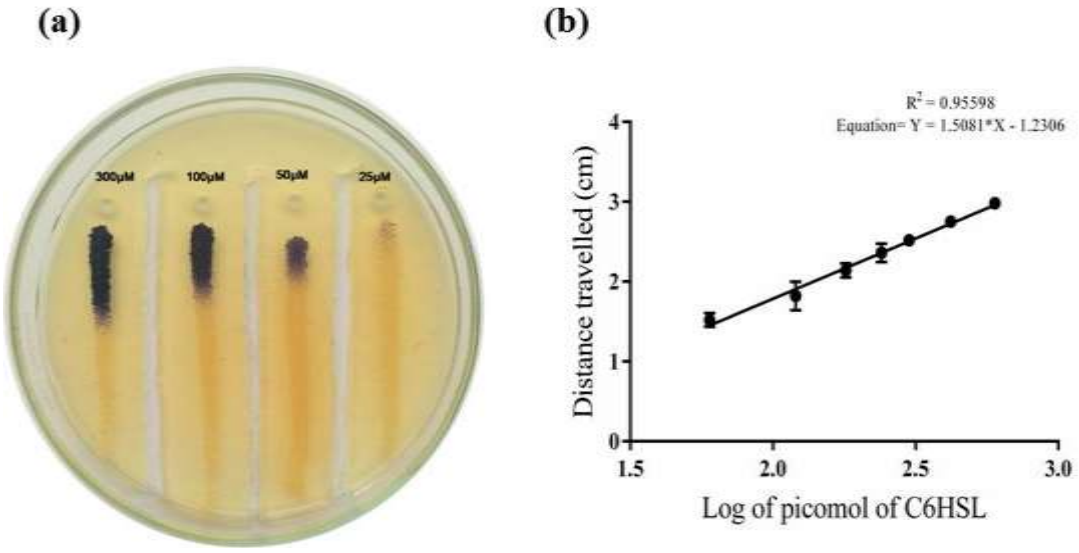



**Figure 12 Quantification of C6-HSL using Agar diffusion bioassay**

**(a)** Plate representing different amount of C6-HSL in the wells and development of purple streak by *C.violaceum* CV026 due to diffusion of C6-HSL **(b)** Plot of log of amount of C6-HSL versus the distance travelled. Values are represented as mean ±SD for three replicates.

## 4.3.6 Biochemical characterization of AdeH

The effect of physical and chemical parameters such as pH, temperature, EDTA and metal ions that may affect the enzyme activity of purified AdeH was investigated. AdeH enzyme activity was defined as the hydrolysed picomols of C6-HSL per minute.

### 4.3.6.1 Effect of temperature on AdeH activity

AdeH could exhibit C6-HSL degrading activity in the temperature range of 20°C to 37°C. The optimum temperature for AdeH activity was 35°C where the maximum activity of 1.5 picomol $min^{-1}$ was achieved. Thereafter the activity declined at temperature 40°C possibly because of enzyme inactivation (Figure 31 (a)).

### 4.3.6.2 Effect of pH on AdeH activity

AdeH could exhibit C6-HSL degrading activity in the pH range from 5 to 8.6 and the activity was enhanced with increase in pH from 5 to 8. The AdeH activity reached maximum at pH 8 giving 1.8 picomol $min^{-1}$ activity (Figure 31 (b)). The activity declined slightly as the pH was raised to 8.6 and at pH 9 AdeH activity was completely abolished. The potential interference of non-enzymatic pH-dependent lactone hydrolysis was precluded by analysis of the controls in which same amount of C6-HSL was incubated in the reaction buffers of different pH

without the purified AdeH. AdeH appeared unstable at pH 9, hence it failed to degrade C6-HSL. Moreover, very little activity was detected when pH was adjusted below 5.

#### 4.3.6.3 Effect of metal ions on AdeH activity

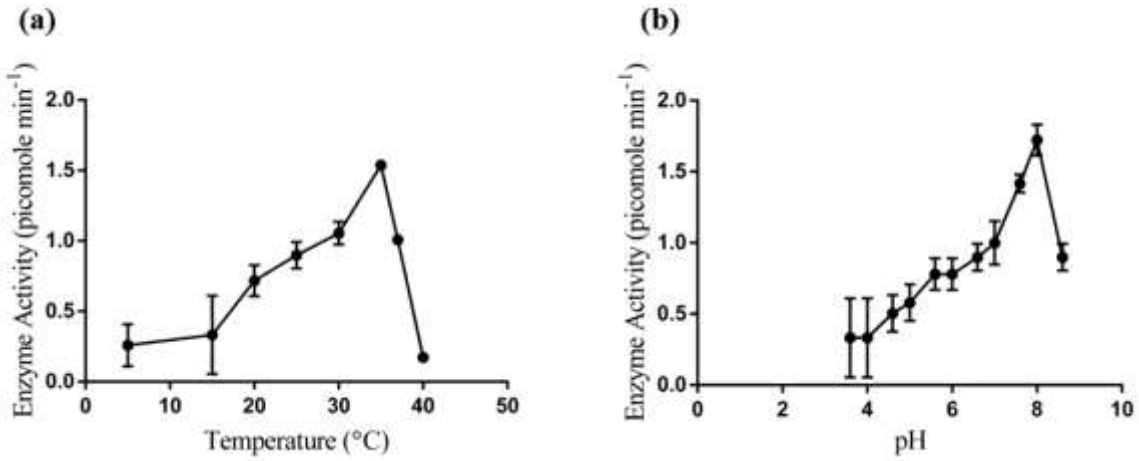
Various metal ions $Ca^{2+}$, $Mn^{2+}$, $Cu^{2+}$ and $Cd^{2+}$ completely inhibited AdeH at 1mM concentration and AdeH activity was abolished in the presence of these cations. On the other hand, $Mg^{2+}$, $Fe^{2+}$ and $Zn^{2+}$ partially inhibited AdeH activity and decreased it to different extents at 1mM concentration compared to AdeH activity without any metal ion which was found out be maximum of 1.5 picomol $min^{-1}$ (Figure 31 (c)). Chelating agent EDTA at 1mM concentration completely abolished the AdeH activity (Figure 31 (c)).

#### 4.3.6.4 Thermal stability of AdeH

Purified AdeH exhibited excellent thermal stability at 30°C as it maintained maximum activity of 1.4 picomol $min^{-1}$. There was a reduction of 50% activity when subjected to 20°C for 2 hours. AdeH was not stable at low and high temperatures as it did not demonstrate any activity at 10°C and 40°C and above (Figure 31 (d)).

#### 4.3.6.5 AHL degradation kinetics of AdeH

AHL degradation kinetics of AdeH was determined by plotting velocity versus substrate concentration (Figure 31 (e)). The $K_M$ was calculated by fitting the data to the Michaelis-Menten equation. AdeH showed $K_M$ value of 3.089 μM for C6-HSL at pH 8.0 and 35°C while the specific activity was 0.8 picomol $min^{-1}\mu g^{-1}$.



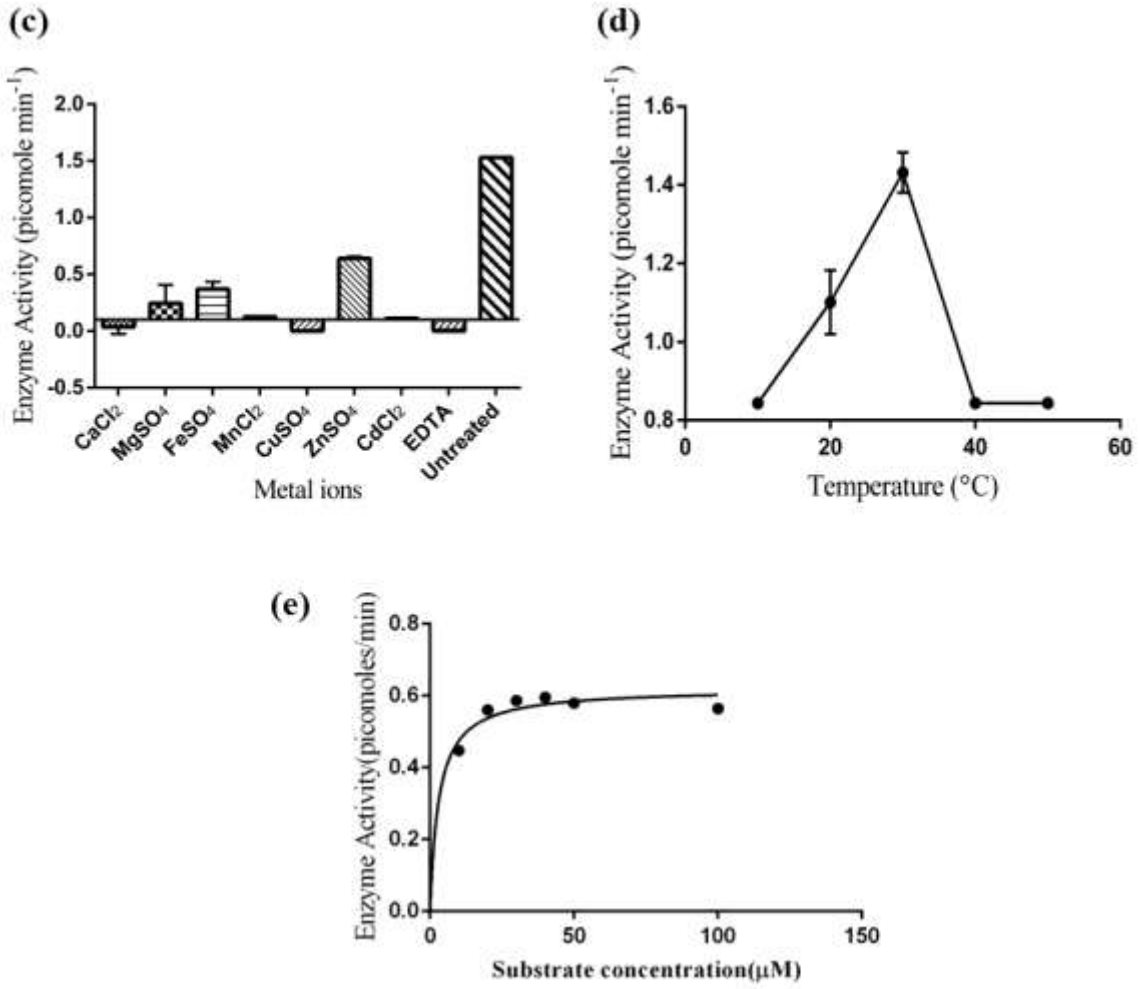



**Figure 13 Biochemical characterization of AdeH**

**(a)** Effect of Temperature on AdeH activity **(b)** Effect of pH on AdeH activity **(c)** Effect of cations and EDTA on AdeH activity **(d)** Thermal stability of AdeH **(e)** Michaelis-Menten constants of purified AdeH on C6-HSL at 35°C and pH 8.0. Values represent the mean of three replications. Bars indicate standard deviation of the mean.

## 4.4 Discussion

Soil isolate Gs50 with notable AHL degrading activity was identified as *Lysinibacillus* sp. on the basis of partial 16S rRNA gene sequence. This is the first instance of AHL degrading ability from genus *Lysinibacillus.* Since *Lysinibacillus* sp. Gs50 demonstrated appreciable biocontrol attributes as discussed in chapter 3, the characterisation and subsequent identification of its AHL degrading enzyme it is undertaken here. Major QQ enzymatic mechanisms operate either through lactone hydrolysis carried out by AHL lactonases or amidohydrolysis carried out by AHL acylases. AHL lactonases hydrolyse the lactone ring of AHL, yielding the corresponding *N*-acyl homoserine which can be restored to *N*-acyl

homoserine lactone at acidic pH (Yates et al. 2002). In accordance with this, the product of C6-HSL degradation after treatment with *Lysinibacillus* sp. Gs50 could be restored at pH 2 after addition of HCl. This suggested the possibility of *Lysinibacillus* sp. Gs50 producing AHL lactonase type of enzyme. *Lysinibacillus* sp. Gs50 was able to degrade varying chain length AHLs suggesting its broad specificity and the AHL degrading enzyme was found located intracellularly. Interestingly, the activity was not detected in the cytosol but was associated with the pellet after sonication. Majority of the AHL lactonases are either cytoplasmic or intracellular (Czajkowski and Jafra 2009) and display a wide spectrum for the substrate by cleaving different carbon acyl side chain length AHLs (Dong and Zhang 2005; Wang et al. 2004; Liu et al. 2007; Kim et al. 2005). AHL degradation products have been shown to serve as a source of nitrogen or/and carbon. *V. paradoxus* and AHL lactonase (AhlD) producing *Arthrobacter* sp. strains utilize the fatty acid released from AHL as an energy source (Park et al. 2003; Leadbetter and Greenberg 2000). *Lysinibacillus* sp. Gs50 could not utilize C6-HSL as carbon source and was unable to grow on it.

Interest of the study further was to identify the AHL degrading mechanism of *Lysinibacillus* sp. Gs50. The gene encoding for AHL lactonase (*adeH*) in *Lysinibacillus* sp. Gs50 was identified by cloning and its heterologous expression in *E.coli* BL21 (DE3). *E.coli* Bl21(DE3) pET22b(+)/*adeH* was able to degrade varying chain length AHLs like wild type *Lysinibacillus* sp. Gs50 showing its broad specificity. Moreover, it could decrease maceration symptoms appreciably when co-inoculated with *Pcc*BR1on potato slices. These observations demonstrated that the gene which was cloned in pET22b(+) imparted an AHL degradation phenotype to *E.coli* Bl21 (DE3) upon IPTG induction and caused soft rot attenuation on potato slices. Further confirmation of AdeH as a lactonase was afforded by Mass spectrometry analysis of the AdeH enzymatic reaction products. AHL lactonases hydrolyse the lactone ring by the addition of water molecule into AHLs to produce acylhomoserine (Seo et al. 2011; Mei et al. 2010; Dong et al. 2001; Liu et al. 2007; Gao et al. 2012; Liu et al. 2005). Two peaks of Na and K substrate conjugates were visible in mass spectrum of C6-HSL (substrate for AdeH). While the mass spectrum of AdeH reaction product gave a definite peak corresponding to acylhomoserine at 217.9 m/z in ESI-MS analysis clearly confirmed that AdeH catalysed lactone ring hydrolysis. Along with the product peak there were peaks of the substrate (C6-HSL) at m/z 199.9 and of substrate conjugate (M + K) at m/z 239.9 (Figure 30 (b)). These peaks might be the result of either incomplete reaction by AdeH or the possible recircularization of the hydrolysed product due to the presence of $H^+$ during

its run in positive mode of ionization. While MS analysis established that AdeH was AHL lactonase, *in silico* analysis revealed the presence of zinc-binding motif "HXHXDH" in the amino acid sequence of AdeH, which is conserved among AHL lactonases. Moreover, identity of AdeH to the known AHL lactonases from the metallo- β-lactamase superfamily were AiiA (30% identity) from *Bacillus* sp. 240B1, AttM (31% identity) and AiiB (40% identity) from *A. tumefaciens* C58, AhlD (30% identity) from *Arthrobacter* sp. IBN110, AhlK (31%) from *Klebsiella pneumoniae*, AidC (19%) from *Chryseobacterium* sp. StRB126 and MomL (24%) from *Muricauda olearia*. AdeH showed 26% identity to QsdA from *R. erythropolis* W2 which belong to phosphotriesterase (PTE) family. AdeH showed 30% identity to AiiM from *Microbacterium testaceum* StLB037 and 25% identity to AidH from *Ochrobactrum* sp. T63, the AHL lactonases belonging to the α/β hydrolase-fold family. QQ bacteria belonging to Firmicutes phylum such as *B. thuringiensis*, *B. cereus*, *B. anthracis*, and *B. mycoides*, *B. subtilis*, *B. amyloliquefaciens*, *Geobacillus* sp. (Dong et al. 2002; Lee et al. 2002; Pan et al. 2008; Yin et al. 2010; Augustine et al. 2010; Seo et al. 2011) are reported to produce AiiA type of AHL lactonases. Surprisingly AHL lactonase (AdeH) of *Lysinibacillus* sp. Gs50 displayed only 30% identity with AiiA. Based on above mentioned identities and the phylogenetic relationship of AdeH with other AHL lactonase of metallo- β-lactamase, phosphotriesterase and α/β hydrolase-fold family, AdeH can be classified as distinctly different kind of AHL lactonase from metallo- β-lactamase family. Biochemical characterization of AdeH revealed its optimum temperature and range at 35°C and 10°C to 40°C respectively. The pH optimum and range was 8.0 and 3.6 to 8.6 respectively. Metal ions $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and EDTA had a negative effect on the enzyme activity at 1mM concentration . The $K_M$ for C6-HSL was 3.089 μM while the specific activity was 0.8 picomol of C6-HSL degraded per minute per μg of AdeH. Further, having identified and characterised the enzyme responsible for quorum quenching activity of soil isolate *Lysinibacillus* sp. Gs50, the next and final aim of the study was to evaluate the efficacy of the quorum quenching mechanism as an approach to attenuate virulence of pathogen.