

# Summary

In order to obtain AHL degrading *Bacillus* isolates sporulating bacteria were isolated from different plant roots and rhizospheric soil samples. Total 97 isolates were obtained by two different isolation methods.

All 97 isolates were screened for their C6-HSL degrading ability using AHL degradation *Chromobacterium violaceum* bioassay. The isolates were categorised into three classes based on violacein pigment production and represented as Violacein unit (V.U.). Out of 97 isolates, 20 isolates were categorised as C6-HSL degraders showing V.U. of 90-117, 47 isolates as partial degraders showing V.U. of 116-128 and 30 isolates as non-degraders showing V.U. of 128-142. It was observed that there was marginal difference in the V.U. (C6-HSL degrading ability) of the C6-HSL degradation positive isolates showing V.U. above 117.

In order to obtain non redundant isolates among the selected 20 C6-HSL degraders, ARDRA was performed using restriction enzymes *RsaI*, *AluI* and *HhaI*. This produced three different restriction digestion patterns depending on which a dendrogram was obtained which sorted the isolates in three groups. Group 1 included isolates P1, Gs52, As37, As38, Gs50, Ct2, Gs54.1 and Pls11. Group 2 included isolates P6.1, P6.2, Ct3.1, Ct3.2, As32, Lg1.1, Pls17, Br4.2, Pls8, Gs42, Gs43.1 and Group 3 contained only As30. Six isolates (Pls8, Pls17, As30, Gs42, Gs50 and Gs52) screened on the basis of Violacein Unit, colony morphology and ARDRA patterns were chosen for further studies. The selected isolates were identified as *Bacillus subtilis* Pls8, *Bacillus aerius* Pls17, *Bacillus firmus* As30, *Bacillus subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *Bacillus thuringiensis* Gs52. The genbank accession numbers of them are KR709146, KR709147, KR709148, KR709151, KR79144 and KR709145 respectively.

The work was initiated with the objective to evaluate quorum quenching based biocontrol potential of the selected isolates. As this approach targets the pathogen by attenuating its virulence without affecting its growth, the selected isolates were tested for their ability to inhibit the growth of *PccBR1*. *B. subtilis* Pls8 though useful inhibited the growth of the pathogen therefore it was excluded from further studies. *B. aerius* Pls17 was also eliminated from further studies as it showed maceration of the potato slice, a deleterious effect on host.

The selected four isolates *Bacillus firmus* As30, *Bacillus subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *Bacillus thuringiensis* Gs52 in co-culture studies with the quorum sensing phytopathogen *Pectobacterium carotovorum* subsp. *carotovorum* *PccBR1*, was found to

degrade the 3-oxo-C6HSL produced by *PccBR1*. The activities of the major virulence enzymes such as PG and PNL were also reduced appreciably. It was further verified that the decrease in 3-oxo-C6HSL and concomitant reduction in PG and PNL activities was not due to reduction of the growth of *PccBR1* as the growth of *PccBR1* was unaffected when grown in co-culture with *Bacillus* isolates.

In *in vitro* biocontrol assays on different hosts like potato, carrot and cucumber of *PccBR1*, the simultaneous cultivation of AHL degrading isolates (*Bacillus firmus* As30, *Bacillus subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *Bacillus thuringiensis* Gs52) with *PccBR1* resulted in attenuation of soft rot on the slices of potato, carrot and cucumber. The maceration area and tissue maceration (%) were significantly reduced in case of the slices which were co-inoculated with *Bacillus* isolates and *PccBR1* compared to the slices which were inoculated with *PccBR1* alone. For all three hosts (potato, carrot and cucumber) *Lysinibacillus* sp. Gs50 showed significant attenuation of soft rot of *PccBR1* exhibiting its biocontrol potential on broad host range.

In curative biocontrol studies, when the AHL degrading isolates *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 were individually applied 12 hours after *PccBR1* substantial decrease in disease symptoms was observed as compared to treatment with *PccBR1* alone. *Lysinibacillus* sp. Gs50 particularly showed maximum decrease in the maceration in curative biocontrol assay. In *in vitro* preventive biocontrol assay, when the four AHL degrading isolates *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 were individually applied 12 hours before the pathogen *PccBR1* soft rot was almost completely prevented.

All four isolate *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 showed  $\sim 10^6$  CFU/ml of adhesion on germinated mung bean sprouts and  $\sim 10^8$  CFU/ml root colonisation. This demonstrated the root colonisation ability and persistence of *Bacillus* isolates on the roots of mung bean. In *planta* assay for biocontrol of spoilage of bean sprouts caused by *PccBR1* on susceptible variety of mung bean (*Vigna radiata*) showed that *PccBR1* treated sprouts resulted in retarded growth after seven days whereas the sprouts treated with AHL degrading isolates even in presence of *PccBR1* resulted in healthy plants. In this assay as well, AHL degrading isolate *Lysinibacillus* sp. Gs50 showed better ability of stopping spoilage of bean sprouts caused by *PccBR1* as compared to the other three isolates.

Finally, *Lysinibacillus* sp. Gs50 was selected for elucidation of the AHL degradation mechanism the next objective of the study as it showed better quorum quenching based biocontrol potential compared to other isolates. Also *Lysinibacillus* sp. Gs50 was of special interest as organisms in this genus were previously regarded as members of the genus *Bacillus*, but the taxonomic status of these microorganisms was changed to genus *Lysinibacillus* so this is the first report of AHL degrading *Lysinibacillus* strain. The isolate has been deposited in the Microbial Culture Collection with the accession no of MCC3181. The AHL degrading enzyme in *Lysinibacillus* sp. Gs50 was found to be located intracellularly. Moreover, *Lysinibacillus* sp. Gs50 could not utilise C6-HSL provided in minimal medium as sole carbon source as it demonstrated no growth on C6-HSL. At the same time C6-HSL degraded by *Lysinibacillus* sp. Gs50 showed reappearance of purple colour upon acidification. This suggested that *Lysinibacillus* sp. Gs50 possibly produce AHL lactonase type of enzyme which cleaved the lactone ring of AHL that was restored to intact AHL at low pH.

In order to identify the gene responsible for AHL degradation, primers were designed from information obtained from available literature and used for amplification of the AHL lactonase gene from the genomic DNA of *Lysinibacillus* sp. Gs50. The expected size 792 bp amplified fragment was 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. Also C6-HSL degrading activity shown by *E.coli* BL21(DE3) pET22b(+)/adeH was comparable to the activity of wild type *Lysinibacillus* sp. Gs50 indicating that the cloned gene had imparted AHL degradation phenotype and the enzyme was therefore designated adeH (AHL degrading hydrolase). Moreover, *E.coli* BL21(DE3) pET22b(+)/adeH was also able to degrade AHLs of different chain length. *E.coli* BL21(DE3) pET22b(+)/adeH when co-inoculated with *PccBR1* on the potato slice resulted in the virulence attenuation of *PccBR1*. These results confirmed that the cloned gene from *Lysinibacillus* sp. Gs50 encoded an AHL degrading enzyme. Further the AdeH was purified using Ni-affinity chromatography as the protein contained His tag at its C terminal.

To confirm the lactone hydrolysis mechanism of AdeH catalysis, C6-HSL was treated with purified AdeH and the resultant degradation products were analysed by ESI-MS. 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. This result confirmed that AdeH hydrolyses the lactone ring of C6-HSL and hence was an AHL lactonase. The amino acid sequence of AdeH was compared with the known AHL lactonases of metallo- $\beta$ -lactamase superfamily through multiple sequence alignment and the zinc-binding motif "HXHXDH" which is conserved among them was also found in the amino acid sequence of AdeH. This further reiterated the identity of AdeH as a lactonase.

For biochemical characterisation of AdeH, the reported agar diffusion bioassay optimised with modification was used to quantify the residual C6-HSL after AdeH reaction. 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 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<sup>-1</sup> was achieved. 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<sup>-1</sup> activity. The activity declined slightly as the pH was raised to 8.6 and at pH 9 AdeH activity was completely abolished. Various metal ions Ca<sup>2+</sup>, Mn<sup>2+</sup>, Cu<sup>2+</sup> and Cd<sup>2+</sup> completely inhibited AdeH at 1mM concentration and AdeH activity was abolished in the presence of these cations. On the other hand, Mg<sup>2+</sup>, Fe<sup>2+</sup> and Zn<sup>2+</sup> partially inhibited AdeH activity and decreased it to varying extent at 1mM concentration. Chelating agent EDTA at 1mM concentration completely abolished the AdeH activity suggesting that AdeH could be a metallo-enzyme. Purified AdeH exhibited excellent thermal stability at 30°C as it maintained maximum activity of 1.4 picomol min<sup>-1</sup>. 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. Finally, AdeH showed  $K_M$  value of 3.089  $\mu$ M for C6-HSL at pH 8.0 and 35°C while the specific activity was 0.8 picomol min<sup>-1</sup>  $\mu$ g<sup>-1</sup>.

Studies further were conducted to understand the effect on the external environment on the *Lysinibacillus* sp. Gs50 to explore its suitability as biocontrol agent. To understand the

influence of commonly encountered soil factors on the quorum quenching activity of *Lysinibacillus* sp. Gs50, effect of various physiological parameters was studied. *Lysinibacillus* sp. Gs50 showed the ability to inactivate different AHLs like C4-HSL, C6-HSL, 3OC6-HSL, C8HSL and 3OC8HSL. *Lysinibacillus* sp. Gs50 showed C6-HSL degradation between the temperatures 20°C to 37°C wherein the maximum activity was achieved at 37°C. The activity was completely lost at temperatures below 20°C and above 37°C. *Lysinibacillus* sp. Gs50 showed C6-HSL degradation between pH 3.6 to pH 8.0. Unlike temperature range, *Lysinibacillus* Gs50 was able to perform quorum quenching at all the pH in acidic as well as in basic conditions and temperatures prevalent in tropical climate and on concentrations of C6-HSL prevalent in the soil environment.

Therefore, apart from influence of environmental factors the juxtaposition of the pathogen and QQ strain would also be crucial for the biocontrol by QQ approach. To demonstrate the efficacy of quorum quenching based virulence attenuation *in situ*, it was necessary to understand the spatial distribution of the quorum quenching strain and the quorum sensing pathogen. For this purpose an infection model of mung bean and *Pseudomonas aeruginosa* was developed. *P. aeruginosa* PAO1 showed  $1.92 \pm 1.64 \times 10^8$  CFU/ml of adhesion on mung bean sprout and  $1.6 \pm 0.6 \times 10^6$  CFU/mg of root colonization 10 days post inoculation. Whereas the quorum sensing mutant *P. aeruginosa* PAO1 (*lasI rhII*) showed  $1.80 \pm 1.65 \times 10^6$  CFU/ml of adhesion on mung bean sprout and  $3.5 \pm 1.4 \times 10^5$  CFU/mg of root colonization 10 days post inoculation. *P. aeruginosa* PAO1 caused mortality of mung bean sprouts and the remaining sprouts which survived showed retarded growth. On the other hand, the QS mutant *P. aeruginosa* PAO1 (*lasI rhII*) did not show any mortality of mung bean sprouts. Sprouts treated with the QS mutant *P. aeruginosa* PAO1 (*lasI rhII*) developed into healthy plants after 10 days. However, the values for *P. aeruginosa* PAO1 (*lasI rhII*) treated plant growth parameters (root length, shoot length and no. of lateral root branches) were intermediate, between the PBS treated plants and *P. aeruginosa* PAO1 wild type treated plants implying that QS is not the only regulatory mechanism but partially involved in regulation of one of the major *P. aeruginosa* virulence determinants in mung bean plant infections. The role of quorum sensing in the pathogenesis of *P. aeruginosa* PAO1 on mung bean was further verified by imaging GFP labelled *P. aeruginosa* PAO1-*gfp*, an AHL producer strain and the QS mutant *P. aeruginosa* PAO1 *lasI rhII* was labeled with pMHLAS::*rfp*, an AHL sensor strain on mung bean roots. The AHL producing strain activated the sensor strain as it showed red fluorescence after encountering AHL produced by AHL producing strain. It was also

observed in microscopy that AHL producing strain and sensor strain were co-localized on the root of mung bean.

After verifying the role of QS in *P. aeruginosa* PAO1 the studies on QQ of *P. aeruginosa* PAO1 by *Lysinibacillus* sp. Gs50 were undertaken. As *P. aeruginosa* produces two different signalling molecules C4-HSL and 3-oxo-C12HSL to regulate some of its virulence factor production, *in vitro* AHL degradation assay with synthetic C4-HSL and 3-oxo-C12HSL by *Lysinibacillus* sp. Gs50 was carried out. *Lysinibacillus* sp. Gs50 showed better efficiency in degradation of 3-oxo-C12HSL, while it degraded comparatively less amount of C4-HSL. Next, *in planta* experiments were set up to understand the interactions between QS (pathogen) and QQ bacterial populations on mung bean roots when they were co-inoculated. Here also *P. aeruginosa* PAO1 wild type strain caused mortality of the mung bean sprouts. While the co-inoculated plants with *Lysinibacillus* sp. Gs50 and *P. aeruginosa* PAO1 resulted in no mortality. Also effect of QQ by *Lysinibacillus* sp. Gs50 was visible on the plants as the plants were healthy compared to plants infected with *P. aeruginosa* PAO1. Root length, shoot length and number of lateral roots of plants was significantly higher in plants co-cultured with *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 compared to only *P. aeruginosa* PAO1 treated ones. The CSLM image of root co-inoculated with *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 showed that root was massively covered with two different types of bacterial population. Both these populations were co-localized throughout the root surface and their spatial distribution was homogeneous. *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50 did not show any niche specificity in our studies and were not found in separate microcolonies of their own rather there were bacterial cell clusters having both the type of cells in close vicinity of each other. This observation confirmed that usefulness of a quorum quenching *Lysinibacillus* sp. Gs50 on plant roots for attenuating the infections caused by quorum sensing plant pathogens.