Conclusion

Quorum quenching strategies are emerging biocontrol strategies because they do not aim to kill bacteria or limit bacterial growth but affect the expression of a specific function exerting a limited selective pressure for microbial survival than biocide treatment. The objective of work undertaken was to isolate plant associated *Bacillus* sp. either form root surface or from soil and screen them for their AHL degrading ability to ebe further used as biocontrol agents. Total 97 isolates were screened for AHL degrading activity and post-screening 20 isolates were found out to be positive for the phenotype. This screening provided three *Bacillus* species namely, *B. aerius* Pls17, *Lysinibacillus* sp. Gs50 and *B. firmus* As30 which are not yet reported for their AHL degradation ability along with two known *Bacillus* species namely *B. thuringiensis* and *B. subtilis*. Quorum quenching though is potentially effective method of biocontrol, the AHL degrader should have certain additional but desirable characteristics in order to be used in a sustainable biocontrol strategy against phytopathogens. In the present study the selected isolates, in addition to their AHL degrading capability, were evaluated for relevant biocontrol attributes. *B. aerius* Pls17 was eliminated from further studies since it produced potato tissue maceration, a detrimental effect on host. Thus, all bacterial species with AHL degrading ability are not potential biocontrol agents and it is necessary to negate any detrimental effect on the host before there use as biocontrol agents. Four AHL degrading isolates (*B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52) could degrade signalling molecule of *Pcc*BR1 and did not affect pathogen growth. These isolates also decreased the production of PG and PNL *in vitro* which suggested the possible involvement of quorum quenching in decreasing the virulence enzyme production indicating their biocontrol potential against this phytopathogen. *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp. Gs50 and *B. thuringiensis* Gs52 could survive on potato, carrot and cucumber and in virulence bioassays showed significant attenuation of soft rot symptoms in all of these hosts, implying that these isolates have potential as broad-range biocontrol agents. Further, the ability to control symptoms pre and post infection is a valuable trait in a biocontrol agent, and these four isolates demonstrated both successful preventive and curative biocontrol of *Pcc* soft rot. In addition to the biocontrol experiments on vegetable slices, biocontrol potential of the isolates was explored for their ability to prevent systemic infection of *Pcc*BR1 through seeds and roots. All four isolates prevented the spoilage of beans at the germination stage and in *in planta* experiments while colonising and persisting on the roots of mung bean. This further strengthens the possibility of using AHL degrading bacteria for the

attenuation of quorum sensing mediated soft rot at pre and post-germination stage of plant growth.

Though all four isolates *B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp.Gs50 and *B. thuringiensis* Gs52 showed QQ based biocontrol potential, *Lysinibacillus* sp. Gs50 demonstrated maximum AHL degradation and biocontrol ability. Moreover, *Lysinibacillus* genus is not yet reported for QQ based biocontrol potential, therefore identification of the mechanism of AHL degradation in *Lysinibacillus* sp. Gs50 was of interest. 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. 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. 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. The AHL degrading enzyme was found located intracellularly. 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 and could decrease maceration symptoms appreciably when co-inoculated with *Pcc*BR1on potato slices. Further confirmation of AdeH as a lactonase was afforded by Mass spectrometry analysis of the AdeH enzymatic reaction products. The mass spectrum of AdeH reaction product gave a definite peak corresponding to acylhomoserine at 217.9 m/z in ESI-MS analysis clearly established that AdeH catalysed lactone ring hydrolysis. Moreover, *in silico* analysis revealed the presence of zinc-binding motif "HXHXDH" in the amino acid sequence of AdeH, which is conserved among AHL lactonases. 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. Thus, the enzyme was found active in the physical conditions prevalent in the soil environment.

Studies on effects of environmental factors on the quorum quenching ability of *Lysinibacillus* sp. Gs50 cells further demonstrated that it is suitable for varied environmental conditions found in nature. *Lysinibacillus* sp. Gs50 was able to degrade varying chain length AHLs suggesting its broad specificity. It was also observed that *Lysinibacillus* sp. Gs50 can perform quorum quenching in a wide range of pH, temperature and substrate concentration hence has potential to be used as biocontrol agent against quorum sensing plant pathogens.

For the quorum sensing strategy to be effective, the juxtaposition of the pathogen and QQ strain is crucial. Quorum sensing signalling molecules diffuse out to the extracellular environment for communication with other bacteria whereas the AHL lactonase produced by *Lysinibacillus* sp. Gs50 is intracellular. Therefore the efficacy of quorum quenching based biocontrol by *Lysinibacillus* sp. Gs50 would rely upon the spatial structure of the quorum sensing pathogen and quorum quenching *Lysinibacillus* sp. Gs50 on the roots of the host plant. To address this and to demonstrate that *Lysinibacillus* sp. Gs50 has a broad range of quorum quenching, an infection model of another well studied quorum sensing pathogen *Pseudomonas aeruginosa* and mung bean was developed. The role of *las* and *rhl* quorum sensing systems in *P. aeruginosa* PAO1 pathogenesis was confirmed as there was no mortality caused by *P. aeruginosa* PAO1 (*lasI rhlI*) and the health of the plants was significantly scored higher than *P. aeruginosa* PAO1 wild type infected plants. This suggested the importance of *las* and *rhl* QS systems in the expression of virulence factors (protease, elastase, alkaline phosphatase) of *P. aeruginosa* PAO1 which are responsible for degradation of the plant tissues and cause the death/infection of the plant. Further, the active QS of wildtype *P. aeruginosa* PAO1 was confirmed by confocal images of roots which were co-inoculated with of GFP tagged *P. aeruginosa* PAO1 wild type (an AHL producer strain) and RFP tagged QS mutant *P. aeruginosa* PAO1 (*lasI rhlI*) pMHLAS-*rfp* (an AHL sensor strain) where the latter prominently showed the induction of red fluorescence suggesting that the 3-oxo-C12HSL produced by *P. aeruginosa* PAO1 wild type was able to diffuse and was perceived by sensor strain leading to RFP expression. Further the quorum quenching based virulence attenuation by *Lysinibacillus* sp. Gs50 was demonstrated by the good health of the plants which were co-inoculated with *P. aeruginosa* PAO1 and *Lysinibacillus* sp. Gs50. The co-localization of *Lysinibacillus* sp. Gs50 with *P. aeruginosa* PAO1 on mung bean roots imaged by CSLM has suggested that there are interactions taking place between QQ and QS populations in natural environments like root surface. The co-localization of these two populations makes the availability of the AHLs (substrate) to the AHL lactonase (enzyme)

easier for AHL degradation (interference in QS or QQ) as shown in the mung bean model. The fact that the plants that were co-inoculated with QS and QQ bacteria showed better plant growth strengthens the role of QQ in attenuating the pathogenesis.

Finally to conclude, it is clearly demonstrated that while some isolates (*B. firmus* As30, *B. subtilis* Gs42, *Lysinibacillus* sp.Gs50 and *B. thuringiensis* Gs52) along with strong AHL degradation ability have many preferred biocontrol attributes which can make them effective biocontrol agents while others do not. Notably, AHL degradation is necessary criterion but is not sufficient in developing quorum quenching based biocontrol agent. Evaluation of biocontrol properties is a prerequisite before the isolate is put to use in the field. Further, Plant-associated microorganisms can be beneficial, deleterious or neutral to the plant and some beneficial bacteria (*Rhizobium* and *Pseudomonas*) depend on quorum sensing for their beneficial traits. As *Lysinibacillus* sp. Gs50 has demonstrated its broad specificity for different AHLs produced by different pathogens like *Pcc* and *P. aeruginosa* PAO1, quorum quenching biocontrol strategy using *Lysinibacillus* sp. Gs50 may suffer from the limitation of affecting the non-target bacteria in addition to the pathogen (target) like other antimicrobial approach. This approach may result in the interference of quorum sensing of beneficial bacteria as well but it can be argued that the impact of non-target effects would depend on the factors like specificity of the AHL degrading enzyme and proximity of AHL degrading bacteria and pathogen/beneficial bacteria. Therefore the knowledge about the spatial distribution of the QS and QQ populations involved in more complex multispecies natural environments like rhizosphere will help in establishing whether QQ has future biocontrol potential. The use of QQ biocontrol agents should also be carefully evaluated as it may also be noted that the debate on pathogen evolving resistance against quorum sensing disruption cannot be ignored. Further studies comprising field applications of biocontrol agent like *Lysinibacillus* sp. Gs50 against *Pcc* and other quorum sensing pathogens will prove the usefulness of quorum quenching based biocontrol approach.