Summary

Chapter 2: Isolation, Screening and Identification of AHL degrading Actinomycetota

- Actinomycetota were isolated using two methods: Acetonitrile enrichment and Heat Shock method. Acetonitrile method yielded 17 bacterial isolates and Heat Shock method yielded 62 bacterial isolates. A total of 79 bacterial isolates were obtained which were screened for their AHL degrading ability which is directly related to their Quorum Quenching ability.
- The primary screening was performed using the *Chromobacterium violaceum* CV026 bioassay for AHL degradation. Out of the 79 isolates, 22 isolates were observed to be AHL degraders, rest 57 isolates were non-degraders and were discarded from further studies. Out of the 22 isolates, 7 bacterial isolates showed consistent AHL degradation. Two from the 7 isolates were obtained by the Acetonitrile enrichment method while the remaining were obtained by Head Shock method.
- The isolates were identified using 16S rRNA gene sequencing. The 16S rRNA sequencing results were analyzed using EzBioCloud database and NCBI BLAST. Two isolates two isolates AI4 and AI5a, both obtained by the Acetonitrile enrichment method were identified to be Actinomycetota. AI5a was identified as *Glutamicibacter nicotianae* and AI4 was identified to be *Rhodococcus pyridinivorans*. The two Quorum Quenching isolates *Glutamicibacter nicotianae* AI5a and *Rhodococcus pyridinivorans* AI4 were submitted at National Centre for Microbial Resource (NCMR), Pune, India. Other isolates identified were *Pseudomonas mendocina, Klebsiella pneumoniae* subsp. *ozaenae.* They were not used in the studies.
- The identity of another Actinomycetotal isolate CRD13.3C which was partially sequenced and identified as *Rhodococcus erythropolis* was confirmed using *Rhodococcus erythropolis* specific primers which amplify only *Rhodococcus erythropolis* and not any other bacteria or even any other *Rhodococcus* sp.

Chapter 3: Characterization of the selected AHL degrading Actinomycetotal isolates for attenuation of soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* BR1

- The two Actinomycetotal strains isolated and identified *G. nicotianae* AI5a and *R. pyridinivorans* AI4 were subjected various biochemical tests. The detailed results for *G. nicotianae* AI5a and *R. pyridinivorans* AI4 are mentioned in Table 3.11. The results augmented and supported the identification results of 16s RNA sequencing.
- . The generation time of the isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C was found out to be 22.2 mins, 47 mins and 58.62 mins, respectively.
- The three AHL degrading Actinomycetotal isolates did not inhibit the growth of the phytopathogen *Pectobacterium carotovorum* subsp. *carotovorum* BR1 (Fig. 3.3) which is an important attribute for any potential quorum quenching isolate.
- Upon co-culturing the quorum quenching isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C with *Pcc*BR1 *in vitro* were able to degrade the 3-oxo-C6-AHL produced by *Pcc*BR1. The QQ isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C reduced the purple zone due to violacein pigment from 49 mm in *Pcc*BR1 control to 3.9 mm, 21.6 mm and 33.4 mm, respectively.
- The Actinomycetotal isolates reduced the PCWDEs (PNL, PL, PGA) produced by *Pcc*BR1 significantly.

PCWDE Enzyme activities	***Pcc*BR1**	***G. nicotianae* AI5a**	***R. pyridinivorans* AI4**	***R. erythropolis* CRD13.3C**
PNL activity (48 h)	39.84 EU	0.25 EU	6.08 EU	5.7 EU
PL activity (24 h)	11.06 EU	0.6 EU	1.56 EU	3.1 EU
PGA activity (48 h)	18.26 EU	5.39 EU	8.53 EU	10.46 EU

- All three QQ isolates were able to degrade a wide range of AHLs viz. C4-AHL, C6-AHL, 3-oxo-C6-AHL, C8-AHL and 3-oxo-C8-AHL.

- The three QQ isolates were effective in reducing the infection of *Pcc*BR1 in preventive and attenuation *in vitro* soft rot assays on host vegetables cucumber and potato.

Assay	Vegetable host	***Pcc*BR1** (maceration)	***G. nicotianae* AI5a + *Pcc*BR1** (maceration)	***R. pyridinivorans* AI4 + *Pcc*BR1** (maceration)	***R. erythropolis* CRD13.3C + *Pcc*BR1** (maceration)
Preventive assay	**Potato**	89.7%	2%	5.5%	22.6%
	Cucumber	74%	6.1%	2.3%	15.3%
Attenuation assay	**Potato**	91.2%	2.7%	11.8%	26.5%
	Cucumber	99%	0%	3.1%	11.8%

- The effect of temperature, pH and metal ions at varying AHL concentrations for the crude putative QQ enzyme of *G. nicotianae* AI5a qualitatively and quantitatively was studied. 37 °C was the optimum temperature for enzyme activity though QQ enzyme of *G. nicotianae* AI5a was active over wide range 5-9 pH. The optimum pH of the QQ enzyme was at 8.5, though its range of activity was 7-8.5 at higher AHL concentrations. Mg^{2+} and Cu^{2+} decreased the activity of enzyme (Fig. 3.16 A and B). Zn^{2+}, Ca^{2+} and Cd^{2+} increased the activity of the enzyme. The enzyme showed K_M value of 3 µM for C6-AHL at pH 8.5 and 37°C while the maximum enzyme velocity was 0.61 µM $min^{-1.}$
- *G. nicotianae* AI5a could not use AHL as a carbon source. AHL degraded by *G. nicotianae* AI5a was reactivated (reappearance of purple colour) when treated with HCl, signifying restoration of the lactone ring. HPLC analysis showed 2 peaks instead of the usual 1 of AHL upon degradation, this and the other two results suggest that the QQ enzyme of *G. nicotianae* AI5a could be a putative lactonase.

Chapter 4: *In planta* greenhouse and storage biocontrol studies with Quorum Quenching biocontrol isolate *Glutamicibacter nicotianae* AI5a against *Pectobacterium carotovorum* subsp. *carotovorum*

- Fast growing Cucumber *in planta* infection model was developed for phytopathogen *Pcc*. The effect of QQ isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 or *R. erythropolis* CRD13.3C on the pathogenicity of *Pcc*BR1 was examined. The growth parameters of the plants treated with the QQ isolates was much higher than that of the plants infected with only *Pcc*BR1

(Fig. 4.7 and 4.8). The root lengths and shoot lengths of plants bacterized with a mixture of *Pcc*BR1 and *G. nicotianae* AI5a, *R. pyridinivorans AI4 or R. erythropolis* CRD13.3C were 5.5, 4.2 and 3.5- fold and 5.4, 4.6 and 4.4- fold higher respectively, than the plants bacterized with only *Pcc*BR1.

- The efficiency of QQ isolate *G. nicotianae* AI5a against the virulent *Pcc*BR1 was observed in its natural host Potato. Three different modes of infection were administered: I) Soil inoculation II) Stem inoculation and III) Leaf and lateral stem inoculation.

Mode of inoculation	**Growth Parameters**	***Pcc*BR1**	***G. nicotianae* AI5a + *Pcc*BR1**
Soil Inoculation	**Shoot length**	17.8 cm	108.2 cm
	Number of leaves	16	115
	% of diseased leaves	99 %	8 %
	Wet Weight	9.3 g	48.5 g
	Dry weight	2.2 g	15.14 g
Stem inoculation	**Shoot length**	102.8 cm	126.6 cm
	% of diseased leaves	50.9 %	11.52 %
Leaf and lateral stem inoculation	**Shoot length**	67.4 cm	127.8 cm
	% of diseased leaves	100 %	13.36 %

- *Pcc*BR1 was tagged with GFP using pHC60 GFP plasmid containing *E. coli* S17- 1 λpir strain by biparental mating method. 16S rRNA gene specific to *G. nicotianae* AI5a was transformed into *E. coli* DH5α using pTZ57R/T cloning vector. 16S rRNA gene specific to *Pcc*BR1 pHC60 was transformed into *E. coli* DH5α using pTZ57R/T cloning vector pJET cloning vector. The plasmids obtained from the two clones were used to generate standard graphs for qPCR for studies ahead.
- The biocontrol ability of *G. nicotianae* AI5a was tested against soft rot causing *Pcc*BR1 on different vegetables and fruit under storage conditions. *G. nicotianae* AI5a reduced the soft rot symptoms and maceration caused by *Pcc*BR1 on various hosts.

Vegetable host	**Number of days of Storage**	***Pcc*BR1** (maceration)	***G. nicotianae* AI5a + *Pcc*BR1** (maceration)
Potato	10	59.98%	6.51%
Tomato	3	43.23%	9.1%
Capsicum	3	50.83%	6.17%
Brinjal	7	60.1%	14.6%

- The two bacteria were quantified using qPCR under storage conditions in potato host. The amount of *Pcc*BR1 did not decrease upon treatment with *G. nicotianae* AI5a but the pathogenicity of *Pcc*BR1 was definitely reduced.
- Further, ability of *G. nicotianae* AI5a to reduce the pathogenicity and virulence of *Pcc*BR1 pHC60 was checked with mung bean *in planta* model in sterile conditions. The growth parameters were observed and the bacteria were quantified using qPCR. It was observed that the treatment with *G. nicotianae* AI5a did not reduce the counts of *Pcc*BR1 pHC60 in the plants but the growth parameters improved.

Mung Bean growth parameters	***Pcc*BR1**	***G. nicotianae* AI5a + *Pcc*BR1**
Shoot length	1.96 cm	15.18 cm
Root length	0.7 cm	3.46 cm
Copy number of *Pcc*BR1 pHC60 specific 16S rRNA gene	8.57 X 10^5 copies/µl	7.6 X 10^5 copies/µl
Copy number of *Gn*AI5a specific 16S rRNA gene	2.16 X 10^8 copies/µl	1.93 X 10^8 copies/µl

- Fluorescence microscopy results suggested that the pathogen *Pcc*BR1 did not enter inside of the root with the phloem and most of the xylem were bacteria free.

Chapter 5: Virulence attenuation of *Pcc*BR1 by quorum quenching phytochemical compounds

- Twelve phytochemical compounds were checked for their ability to inhibit the virulence of phytopathogen *Pcc*BR1 at their sub-lethal concentrations against *Pcc*BR1. Out of those Eugenol (400 µM), Carvacrol (400 µM) and Salicylic acid (210 µg/ml) inhibited AHL as no purple pigment (violacein)

was detected when tested in the bioassay with the biosensor strain *Chromobacterium violaceum* CV026.

- Eugenol (400 µM), Carvacrol (400 µM) and Salicylic acid (210 µg/ml) were effective in reducing the infection of *Pcc*BR1 in preventive and attenuation *in vitro* soft rot assays on host vegetables cucumber and potato.

Assay	Vegetable host	***Pcc*BR1** (maceration)	**Eugenol (400 µM) + *Pcc*BR1** (maceration)	**Carvacrol (400 µM) + *Pcc*BR1** (maceration)	**Salicylic acid (210 µg/ml) + *Pcc*BR1** (maceration)
Soft rot Attenuation assay	**Potato**	77.73%	10%	0%	15%
	Cucumber	99%	0%	1.3%	3.31%

- The effect of the three phytochemical compounds on the virulent traits of *Pcc*BR1 such as motility, PCWDEs and biofilm formation was checked. Eugenol (400 µM), Carvacrol (400 µM) and Salicylic acid (210 µg/ml) were able to reduce the virulent traits of *Pcc*BR1 significantly as depicted below.

PCWDEs	***Pcc*BR1**	**Eugenol (400 µM) + *Pcc*BR1**	**Carvacrol (400 µM) + *Pcc*BR1**	**Salicylic acid (210 µg/ml) + *Pcc*BR1**
PNL activity (48 h)	41.3 EU	0.81 EU	1.62 EU	2.11 EU
PL activity (48 h)	20.1 EU	1.36 EU	1.31 EU	2.7 EU
PGA activity (48 h)	19.73 EU	1.32 EU	1.33 EU	1.9 EU

Virulence traits	***Pcc*BR1**	**Eugenol (400 µM) + *Pcc*BR1**	**Carvacrol (400 µM) + *Pcc*BR1**	**Salicylic acid (210 µg/ml) + *Pcc*BR1**
Motility	18 mm	12.5 mm`	10.5 mm	11 mm
Biofilm formation (Percent reduction)	-	31.8 % reduction compared to *Pcc*BR1	24.2 % reduction compared to *Pcc*BR1	37.5 % reduction compared to *Pcc*BR1

- The three phytochemicals Eugenol (400 µM), Carvacrol (400 µM) and Salicylic acid (210 µg/ml) reduced the soft rot symptoms and maceration caused

by *Pcc*BR1 on potato under storage conditions as shown below. qPCR was used to quantify the amount of *Pcc*BR1 in the host with and without treatment with the phytochemicals. The copy number of *Pcc*BR1 did not decrease upon treatment with the phytochemicals as shown below.

Potato	***Pcc*BR1**	**Eugenol (400 µM) + *Pcc*BR1**	**Carvacrol (400 µM) + *Pcc*BR1**	**Salicylic acid (210 µg/ml) + *Pcc*BR1**
Maceration	59.1%	2.2%	3.35%	5.1%
Copy number of *Pcc*BR1 pHC60 specific 16S rRNA gene	1.77 X 10^6 copies/µl	1.64 X 10^6, copies/µl	1.66 X 10^5 copies/µl	1.6 X 10^5 copies/µl

- The three phytochemicals Eugenol (400 µM), Carvacrol (400 µM) and Salicylic acid (210 µg/ml) reduced the pathogenicity and virulence of *Pcc*BR1 pHC60 in mung bean *in planta* model under sterile conditions. It was observed that the treatment with *G. nicotianae* AI5a did not reduce the copy number of *Pcc*BR1 pHC60 in the plants but the growth parameters improved as shown below.

Mung Bean	***Pcc*BR1**	**Eugenol (400 µM) + *Pcc*BR1**	**Carvacrol (400 µM) + *Pcc*BR1**	**Salicylic acid (210 µg/ml) + *Pcc*BR1**
Shoot length	1.90 cm	15.05 cm	14.53 cm	14.41 cm
Root length	0.71 cm	3.53 cm	3.55 cm	3.28 cm
Copy number of *Pcc*BR1 pHC60 specific 16S rRNA gene	8 X 10^5 copies/µl	5.5 X 10^5 copies/µl	3.1 X 10^5 copies/µl	2.8 X 10^5 copies/µl

- Actinomycetota *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were good Quorum Quenching bacteria that reduced the pathogenicity phytopathogen *Pcc*BR1. *G. nicotianae* AI5a was explored further for its mechanism of QQ and the AHL degrading enzyme was found to be a putative lactonase.
- Eugenol (400 µM), Carvacrol (400 µM) and Salicylic acid (210 µg/ml) were phytochemicals which were able to reduce the QS based virulence of *Pcc*BR1 without killing the bacteria.