# Chapter 3

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

## 3.1 Introduction

*Pectobacterium carotovorum* subsp. *carotovorum* (formerly *Erwinia carotovora* subsp. *carotovora)* is a Gram negative, non-sporing, facultative anaerobic bacterium. *Pcc* is a plant pathogen that causes soft rot and stem rot diseases in several economically important crops like cucumber (*Cucumis sativus* L.), potato (*Solanum tuberosum*), carrot (*Daucus carota*), mung bean (*Vigna radiata*), tomato (*Solanum lycopersicum*), watermelon (*Citrullus lanatus*), etc., leading to extensive in field, in transit and post-harvest losses. The pathogenesis of *Pcc* is mediated by quorum sensing which is a population density-dependent mechanism for gene regulation, utilizing freely diffusible chemical signal molecules (AHL), primarily through the coordinate and prolific production of a variety of plant cell wall degrading enzymes (PCWDEs) like proteases, pectinases, hemicelluloses and cellulases. Such virulence factors (PCWDEs) operate as part of a necrotrophic mode of action (where the invading organism causes death of host tissue and colonizes dead substrate). Therefore, this group of pathogens have been termed ‘‘brute force pathogen’’ in line with their physical attack on plant cell walls (Gorshkov et al., 2018).

Plant pathogens such as *Pseudomonas syringae* actively manipulate host defences and need living host tissue as part of their infection process. Such pathogens have been known as ‘stealth’ pathogens due to their ability to influence plant defences as part of the infection process. On the other hand, *Pcc* is called as a ‘brute force’ pathogen since it causes necrotrophic damage via physical attack on plant cell wall mainly due to production of cell wall degrading enzymes that cause the necrosis of host tissue. This co-ordinated and creative production of virulence factors at high population density is regulated by a cell-cell communication mechanism known as quorum sensing (QS) averting untimely initiation of plant defences and increasing the possibility of effective infection (Zhang et al., 2004; Liu et al., 2008). The signalling molecules of QS are *N*-acyl homoserine lactones (AHLs) responsible for controlling the virulence factors production which include plant cell wall degrading enzymes (PCWDE) pectate lyases, pectinase and polygalacturonase. Quorum sensing also controls harpin and other factors secreted by Type III secretion system, small number of virulence regulators, and the antibiotic carbapenem for the full virulence in *Pcc* (Garge and Nerurkar.,2017; Jafra, et al., 2006; Joshi et al., 2020; Moleleki et al., 2017; Põllumaa et al., 2012; Singh et al., 2021; Valente et al., 2017; Vesuna & Nerurkar, 2018). One such

phytopathogenic strain of *Pcc* from eggplant (brinjal) (*Pcc*BR1) was earlier isolated and its epidemiology was thoroughly studied in our laboratory (Maisuria & Nerurkar, 2013). It was reported to cause soft rot in wide range of fruits and vegetables like *Brassica oleracea* (Cabbage), *Lpomoea batatas* (Sweet potato), *Beta vulgaris* (Sugar beet), *Trichosanthes cucumerina* (Snake gourd), *Pyrus communis* (Pear fruit), *Daucus carota* (Carrot), *Solanum melongena* (Brinjal), *Lagenaria siceraria* (Molina), *Cucumis sativus* (Cucumber), *Solanum tuberosum* (Potato). This strain belongs to class II strains of *Pcc* and produces the quorum sensing signal molecule 3-oxo-hexanoyl homoserine lactone (3-oxo-C6-AHL) that regulates its PCWDE production to cause soft rot to different host plants. A thorough biochemical characterisation of its virulence factors viz. polygalturonase and pectate lyase has been carried out (Maisuria et al., 2010, Maisuria and Nerurkar, 2012).

Disrupting the regulation of quorum sensingthat causes pathogenicity in *Pcc* could prevent the production of virulence factors which makes it a suitable target for development of new phyto protective agents (Barnard & Salmond, 2007). The rhizosphere of plants, along with AHL producing pathogens like *Pcc*, also has the presence of AHL degrading bacteria (Chan et al., 2011; Cirou et al., 2012; Faure et al., 2009; Jafra et al., 2006). The degradation of AHLs inactivates the signal and leads to the disruption of QS which is termed as quorum quenching (QQ) (Uroz et al., 2009). The first QQ bacteria were detected from soil and belong to the Gram-positive genus *Bacillus* (Dong et al., 2000). Potato plant rhizosphere also showed the presence of large quantity of AHL degrading bacteria and they mainly belong to *Agrobacterium*, *Bacillus*, *Pseudomonas*, *Delftia, Ochrobactrum* and *Rhodococcus* genera (Jafra et al., 2006; Cirou et al., 2007). AHL degrading bacteria used for attenuation of soft rot on potato host against *P. atrosepticum* (Smadja et al., 2004) or *P. carotovorum* has also been demonstrated (Molina et al., 2003; Uroz et al., 2005, 2014). A distinct characteristic of this strategy is that it attenuates the virulence by reducing the production of AHL regulated virulence factors like PCWDEs and does not kill the pathogen while on the contrary other antimicrobial approaches give rise to resistant strains (Faure & Dessaux, 2007).

This study discusses the biochemical properties of QQ Actinomycetota their nd and assays that demonstrate itsbiocontrol potential. Additionally, studies showing absence of deleterious effect of the isolates on the host plant and the ability of the QQ isolates

to control the disease pre-infection (preventive) and during infection (attenuation) are included. This part also sheds light on the biochemical properties and mechanism of the QQ enzyme of the isolate *G. nicotianae* AI5a. The broad objective of the present study is to evaluate diverse biocontrol properties of the isolated QQ Actinomycetota for the control of soft rot caused by *Pcc*BR1.

## 3.2 Methods and Materials

### 3.2.1 Bacterial strains and conditions

Soft rot causing plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum* BR1 (Laboratory isolate) was grown and maintained on LB media at 30 °C under shaking condition. Actinomycetotal isolates *Glutamicibacter nicotianae* AI5a, *Rhodococcus pyridinivorans* AI4 and *Rhodococcus erythropolis* CRD13.3C were grown on Rich Media Agar (RMA) media at 30 °C under shaking condition.

### 3.2.2 Media used

#### 3.2.2.1 Bushnell Haas Media

Bushnell Haas Media (HiMedia Labs) was used as minimal media for carbon utilization assay. The media was autoclaved for 15 min at 15 PSI.

**Table 3.1 Composition of Bushnell Haas Broth**

| Ingredients | gram/L |
|---|---|
| Magnesium sulphate | 0.200 |
| Calcium chloride | 0.020 |
| Monopotasium phosphate | 1.000 |
| Dipotassium phosphate | 1.000 |
| Ammonium nitrate | 1.000 |
| Ferric chloride | 0.050 |
| Final pH | 7.0. |

### 3.2.3 Biochemical tests

The biochemical tests were performed with the isolates *G. nicotianae* AI5a and *R. pyridinivorans* AI4. To check the ability of the isolates to fix nitrogen, they were streaked on Jenson's agar and incubated for 12 days at 30 ºC. Isolates were also streaked on Starch Casein Agar (SCA), and pyridine agar while spots of isolates were

inoculated on Pikovskayas agar plates. SCA and Pikovskayas agar plates were incubated for 24 h at 30 ºC while pyridine agar plates were incubated for 12 days at 30 ºC. For Methyl red, Voges Proskauer, indole production , citrate utilization and nitrate reduction tests, the isolates were inoculated in respective media and incubated under shaking conditions for 24 h 30 ºC (MacFaddin, 2000).

### 3.2.4 Growth curve of AHL degrading Actinomycetotal isolates

Growth curve experiment of the *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C was performed to understand the growth pattern and calculate the generation time of the isolates for experiments ahead. Overnight grown cultures of the isolates were inoculated in the 100 ml LB in 250 ml flasks, which were incubated at 30˚C under shaking conditions, aliquots were taken to calculate the CFU/ml every hour until stationary phase was attained. The experiment was performed in triplicates.

### 3.2.5 Growth inhibition assay

100 µl of overnight grown *Pcc*BR1 was added in the 7 ml 0.8% soft agar and overlaid on the Petri plates with Luria agar basal media. 8 mm wells were bored in the LA plates and 100 µl supernatant of overnight grown cultures of *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were added to the wells. 100 µl 1% SDS was used as a positive control for zone of inhibition and LB was used as a negative control. The plates were incubated at 30 ºC for 24 h and occurrence of zone of inhibition was observed. The experiment was performed in triplicates.

### 3.2.6 *In vitro* co-culture assay

Overnight grown cultures of *Pcc*BR1, *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were subcultured in 10 ml LB and grown till $10^6$ CFU/ml for *Pcc*BR1 and $10^8$ CFU/ml was attained. All three isolates were then co-cultured with *Pcc*BR1 separately in fresh 10 ml LB tubes. The tubes were incubated at 30 ºC and aliquots were taken at every 12 h starting from 0 h until 36 h. The supernatant of aliquots from *Pcc*BR1, *G. nicotianae* AI5a, *R. pyridinivorans* AI4, *R. erythropolis* CRD13.3C, *G. nicotianae* AI5a+*Pcc*BR1, *R. pyridinivorans* AI4+*Pcc*BR1 and *R. erythropolis* CRD13.3C+*Pcc*BR1 were added to the wells bored into the basal LA which was earlier overlaid with 7 ml 0.8% soft agar inoculated with 100 µl overnight

grown *Chromobacterium violaceum* CV026. The supernatants of tubes inoculated with only *Pcc*BR1 were taken as positive control and LB as negative control. The plates were prepared in triplicates and incubated at 30 ºC for 24 h. The AHL degradation was determined by reduction in purple zone around the wells with co-cultures as compared to *Pcc*BR1 controls.

### 3.2.7 Activity of Plant Cell Wall Degrading Enzymes (PCWDEs)

The Actinomycetotal isolates ($10^8$ CFU/ml) were co-cultured with *Pcc*BR1 ($10^6$ CFU/ml) individually in 10ml LB and incubated at 30˚C for 48 hours under shaking conditions. *Pcc*BR1, *G. nicotianae* AI5a, *R. pyridinivorans* AI4, *R. erythropolis* CRD13.3C inoculated individually and LB were used as controls. The aliquots were taken at every 12 h starting from 0 h to 48 h of co-culture, and activities of three major virulence factors i.e., pectin lyase (PNL), pectate lyase (PL) and polygalacturonase (PGAse) were checked.

Lyase activity (PL and PNL) was determined by measuring the unsaturated oligomers released due to cleavage of polygalacturonic acid (PGA) using thiobarbituric acid (TBA) reagent. 50 µl of culture supernatant from co-cultures and controls were added to 250 µl of 0.5% (w/v) of substrate (Pectin for PNL and polygalacturonic acid for PL) in 50 mM Tris-Cl (pH 8.0) and the reaction mixture was incubated at 40 ºC for 30 min. This was followed by incubation at 76 ºC for 10 min after adding 1 N NaOH. Then 500 µl of 1 N HCl was added with 500 µl of 0.04 M thiobarbituric acid (TBA), and finally incubated at 76 ºC for 10 min. The reaction mixture vials were centrifuged at room temperature and absorbance of final reaction product was measured at 550 nm. One-unit of PNL/PL activity was defined as the amount of enzyme required to change absorbance by 0.01 $OD_{600}$ unit per hour. All the experiments were done in triplicates with appropriate reagent, substrate and enzyme blanks (Nedjma et al., 2001; Thite et al., 2020).

PGAse activity was determined by measuring reducing sugar released due to hydrolysis of PGA using Dinitrosalicylic acid (DNSA) reagent. 50 µl of culture supernatant from co-cultures and controls were added to 250 µl of 0.5% (w/v) of PGA substrate in 50 mM Tris-Cl (pH 8.0) and the reaction mixture was incubated at 40˚C for 10 min. The reaction mixture was then boiled for 10 min after adding DNSA. This was cooled to room temperature and the volume was made up to 1.5 ml. This reaction

mixture was boiled for 10-15 min until the crystals dissolved. The absorbance of reaction product (reducing sugars) was taken at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmole of D-galacturonic acid per minute. N=3 in all the experiments with appropriate reagent, substrate and enzyme blanks (Miller, 1959; Thite et al., 2020).

### 3.2.8 *In vitro* soft rot preventive and co-culture assay

The *in vitro* co-culture pathogenicity assay was modified from (Dong et al., 2004) and performed on potato (*Solanum tuberosum* L.) tuber and cucumber (*Cucumis sativus* L.) slices. Potato tubers and cucumbers were purchased from a local market, they were thoroughly washed under running tap water, surface sterilized by immersing sequentially in 0.01% sodium hypochlorite, 70% ethanol and sterile distilled water, and then cut into uniform slices of 4–5 mm thickness. The Actinomycetotal isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4, *R. erythropolis* CRD13.3C (~ $2.5 \times 10^6$ CFU/ml) were mixed with *Pcc*BR1 (~$2.3 \times 10^6$ CFU/ml) and 5 µl of each mixture was injected at different points on each cucumber slice and added to a bored well in the potato slice. The slices were placed in sterile Petri dishes and incubated at 30 °C for 24 h. Macerated tissue weight was measured by scooping out the macerated region and weighing it, and percentage maceration was calculated based on the weight of tissue before inoculation. Controls consisted of inoculating either only pathogen culture or PBS. Three independent trials were carried out. Each trial had three replicates.

The approach of Molina et al., (2003) was followed to perform preventive assays. The biocontrol isolates were added 12 h before (preventive biocontrol) the pathogen *Pcc*BR1 was applied to potato/cucumber slices. Controls consisted of pure *Pcc*BR1 and PBS inoculated separately on the slices. Three independent trials were performed and each trial comprised of three replicate potato slices in separate petri plates.

### 3.2.9 Degradation of AHLs

The AHL degradation bioassay was performed in 96 well microtitre plates using *C. violaceum* CV026 as a biosensor strain as mentioned in Chapter 2 (Park et al., 2005; Huang et al., 2012). Biocontrol isolates *Glutamicibacter nicotianae* AI5a, *Rhodococcus pyridinivorans* AI4 and *Rhodococcus erythropolis* CRD13.3C were grown for 24 h and centrifuged at 10000 rpm for 5 min. The cell pellet was resuspended in phosphate buffered saline (PBS, pH 7.0). 80 µl of 25 µM AHLs (C4-AHL, C6-AHL,

3-oxo-C6-AHL, C8-AHL or 3-oxo-C8-AHL in separate assays) prepared in PBS was added to 20 µl of cell suspension of biocontrol isolates and the mixture was incubated at 30 °C for 2 h (Huang et al., 2012). Untreated controls of 25 µM of C4-AHL, C6-AHL, 3-oxo-C6-AHL, C8-AHL or 3-oxo-C8-AHL prepared in PBS were incubated at similar conditions. The reaction mixture was mixed with the biosensor *C. violaceum* CV026 in 96 well microtiter plates. After overnight incubation at 30 °C under static conditions, the purple colour produced by the biosensor was observed.

### 3.2.10 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 LA plates, agar was aseptically cut into 1 cm wide bars separated by 2 to 3 mm slits as shown in Fig. 3.1. 3 mm diameter wells were made with the well borer above each agar bar. Overnight grown AHL biosensor strain CV026 was streaked uniformly below each well. 5 µl C6-AHL of different concentrations (5, 10, 25, 50, 100, 200, 500 µM) was added individually to the wells. These plates were then incubated for 24 h 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-AHL and this distance is proportional to the amount of C6-AHL in the well, which was quantified. The experiment was carried out in triplicates and a standard graph of distance of the concentration of AHL vs. diffusion of AHL was plotted.

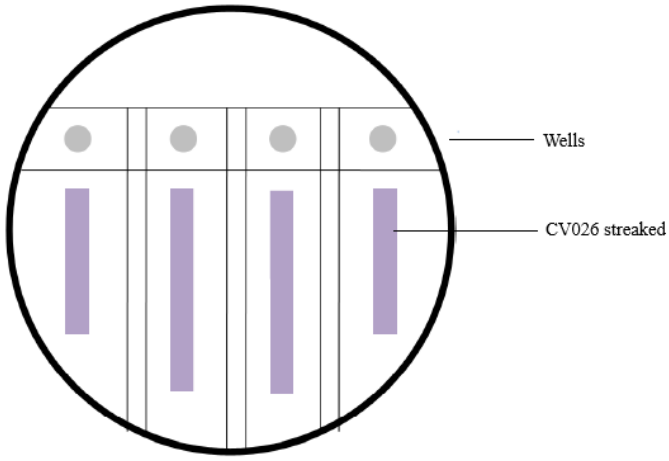


**Figure 3.1 Representative image of a plate used in bioassay for AHL degradation**

### 3.2.11 Growth on AHL as sole source of carbon

To study whether *G. nicotianae* AI5a was able to utilize AHL as a sole carbon source, a *G. nicotianae* AI5a culture was grown for 24 h, centrifuged at 7000 rpm and the pellet was washed thrice with PBS and resuspended in 100 µl of BH minimal media. 1 ml aliquots of BH minimal media containing 2.5 mM glucose or 5 mM C6-AHL was added as carbon source in the media, inoculated with 1% cell suspension of *G. nicotianae* AI5a and was grown in shaking condition at 30 °C. The OD of the culture was measured at 600 nm till 48 h at 12 h intervals. Uninoculated BH was taken as negative control while BH media supplemented with 2.5 mM glucose was taken as positive control for the growth of *G. nicotianae* AI5a. The experiment was conducted in triplicate.

### 3.2.12 AHL Restoration assay

The AHL restoration assay reported by Yates et al. (2002) was modified and used. For the assay, *G. nicotianae* AI5a was grown for 24 h, centrifuged at 10000 rpm for 5 min. The cell pellet was resuspended in phosphate buffered saline (PBS, pH 7.0). 80 µl of 25 µM C6-AHL prepared in PBS was added to 20 µl of cell suspension of *G. nicotianae* AI5a and the mixture was incubated at 30 °C for 2 h. 25 µM C6- AHL without the cell suspension of the isolates was used as an untreated control. After incubation, the reaction mix and untreated control were acidified with 25 µl of 50 mM HCl to bring the pH down to 2. The reaction mix and untreated control without acidification and after acidification were subjected to the CV026 biosensor assay.

### 3.2.13 AHL extraction after degradation and HPLC analysis

The AHL degradation assay was performed as stated above with 800 µl AHL and 200 µl of cell suspension of *G. nicotianae* AI5a. After incubation, 1 ml aliquot from the mixture was mixed with 1 ml of 0.1% acidified (HCl) ethyl acetate. The mixture was vortexed for 1 min and the phases were allowed to separate. The shaking was repeated two times before the ethyl acetate fraction was removed. This extraction process was repeated two times. The combined ethyl acetate fractions (3 ml) were evaporated to dryness in a rotary evaporator and reconstituted in 500 µl HPLC grade methanol. The same extraction protocol was used for control samples, where only C6-AHL without suspension of *G. nicotianae* AI5a was present (modified from Ravn et al., 2001)).

HPLC was carried out in Waters HPLC. The mobile phase for HPLC analysis was methanol: water (70:30). HPLC was carried out isocratically at a flow rate of 0.5 mL/min (by Waters 1500-Series HPLC pump) through a C8 5 µm (4.6 X 250 mm) reverse-phase column (SunFire®) and was detected at a wavelength of 210 nm (Waters 2489 UV/Visible detector) (Lépine & Déziel, 2011; Garge & Nerurkar, 2016).

## 3.2.14 Biochemical characterization of Quorum Quenching enzyme

### 3.2.14.1 Effect of substrate concentration

5 µl of purified and PBS washed cell suspension of overnight grown culture of *Glutamicibacter nicotianae* AI5a were added to C6-AHL (5, 10, 25, 50,100, 250, 500 µM individually) in a final volume of 25 µl and incubated at 30 °C for 2 h to determine the substrate saturation curve. C6-AHL ((5, 10, 25, 50,100,250, 500 µM individually) without pure culture were taken as controls. The remaining C6-AHL after degradation was quantified using Agar diffusion bioassay as mentioned in section 3.2.10 in Fig. 3.1 following which the enzyme activity was calculated.

### 3.2.14.2 Effect of Temperature

The effect of temperature on the enzyme activity was determined by adding 5 µl of *G. nicotianae* AI5a cell suspension to C6-AHL (25 µM) in final volume of 25 µl. Similarly, C6-AHL (25 µM) in 25 µl without culture was used as control. Both, reaction mixtures and controls, were separately incubated at 20 °C, 25 °C, 30 °C, 37 °C, 45 °C and 50 °C in 50 mM Tris-Cl buffer (pH 8.0). Degradation was checked by microtitre plate assay as mentioned section 2.2.6 in chapter 2. Quantification was done using Agar diffusion bioassay following which the enzyme activity was calculated.

### 3.2.14.3 Effect of pH

The effect of pH on enzyme activity was determined by adding 5 µl of *G. nicotianae* AI5a cell suspension to C6-AHL (25 µM) in a final volume of 25 µl. Similarly, C6-AHL (25 µM) in final volume of 25 µl without culture and only PBS were used as control. The reactions and controls were set up at different pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0) and were incubated at 30 °C. The C6-AHL remaining after degradation was checked by CV026 biosensor assay. Quantification was done using Agar diffusion bioassay following which the enzyme activity was calculated.

#### 3.2.14.4 Effect of Metal ions

The effect of different metal ions ( $Cd^{2+}$, $Mn^{2+}$, $Fe^{2+}$ , $Zn^{2+}$, $Mg^{2+}$, $Cu^{2+}$ , $Ca^{2+}$ ) on the activity of the enzyme was determined by adding 5 µl of *G. nicotianae* AI5a suspension to C6-AHL (25 µM) and 2.5 µl of 1 mM of different metal ions in a final volume of 30 µl. A reaction of *G. nicotianae* AI5a with 25 µM of C6-AHL without any metal ions was set up as control. The reaction mixtures and controls were incubated at 30 °C for 2 h. The remaining C6-AHL after degradation was checked by CV026 biosensor assay assay. Quantification was done using Agar diffusion bioassay following which the enzyme activity was calculated.

#### 3.2.14.5 Determination of $K_m$ and $V_{max}$

To determine $K_m$ and $V_{max}$ of the AHL degrading enzyme of *G. nicotianae* AI5a , 5 µl of purified culture cell suspension weas added to C6-AHL solution (5, 10, 25, 50, 100, 250, 500 µM) in a final volume of 25 µl. The reactions were incubated at 30 °C for 2 h. The remaining C6-AHL after degradation was quantified as mentioned using Agar diffusion bioassay following which the enzyme activity was calculated.

## 3.3 Results and Discussion

### 3.3.1 Biochemical characterization of the Actinomycetotal isolates

The G+C content of the DNA of *R. pyridinivorans* is 66 mol%. Phylogenetic classification shows that *R. pyridinivorans* falls within an evolutionary radiation comprising *Rhodococcus* species and is most closely related to the type strain of *Rhodococcus rhodochrous*, sharing 99% 16S rRNA gene similarity. The differences in some phenotypic characteristics and the genetic distinctiveness distinguish *R. pyridinivorans* from the *Rhodococcus* species described previously (Yoon et al., 2000). *R. pyridinivorans* is also reported to degrade phenol (Al-Defiery et al., 2018). Earlier, *Glutamicibacter nicotianae* was classified as *Arthrobacter nicotianae* but later reclassified in 2016 (Busse, 2016).As sparse information is available on biochemical characteristics of *R. pyridinivorans* and *G. nicotianae* ,basic biochemical tests as well as tests reported in literature specific for genus *Rhodococcus* and *Arthrobacter* for biochemical characterization were performed. Table 3.11 shows the results of the tests performed.

After performing various biochemical tests, it was observed that *R. pyridinivorans* AI4 and *G. nicotianae* AI5a are sugar non-fermenting though they can utilize glucose, lactose, maltose, sucrose. Both organisms cannot produce indole (indole negative), cannot produce high amounts of mixed acids (methyl red negative) and unable to produce acetoin (VP negative). Being aerobic organisms, both the isolates showed effervescence in presence of $H_2O_2$ (catalase positive). *G. nicotianae* AI5a is able to utilize citrate as carbon source (Citrate positive). These results are similar to the ones reported by (Busse, 2016) during the reclassification. *R. pyridinivorans* AI4 is able to reduce nitrate. Many *Rhodococcus sp.* have been reported to reduce nitrate under oxic conditions for example, *Rhodococcus rhodochrous* (Shah, 2014). Blasco et al., (2001) reported that *Rhodococcus* can grow in presence of high nitrate contents due to their nitrate reductase activity. Starch Casein Agar, a nutrient rich media used for isolation of Actinomycetota supported pigment formation in case of both the isolates. Both organisms can utilize pyridine as sole carbon source but even after 12 days of incubation neither isolate as able to produce pigment on it. Both the isolates were unable to solubilize phosphate however both were able to grow on nitrogen free Jenson's Agar media indicating that they possess nitrogen fixation ability. Many *Rhodococcus* have been reported to fix nitrogen, for example Thamhesl et al., (2015) reported ability of *Rhodococcus erythropolis* to fix atmospheric nitrogen. Free living *Arthrobacter* are also known to fix atmospheric nitrogen (Sellstedt et al., 2013).

**Table 3.2 Result of Biochemical Tests for that *R. pyridinivorans* AI4 and *G. nicotianae* AI5a**

| **Tests** | | ***R. pyridinivorans* AI4** | ***G. nicotianae* AI5a** |
|---|---|---|---|
| Sugar fermentation | Glucose | Growth, no bubble formation | Growth, no bubble formation |
| | Lactose | Growth, no bubble formation | Growth, no bubble formation |
| | Maltose | Growth, no bubble formation | Minimal growth, no bubble formation |
| | Sucrose | Growth, no bubble formation | Minimal growth, no bubble formation |
| Indole test | | No violet ring, negative | No violet ring, negative |
| Methyl red test | | No acid production, negative | No acid production, negative |

| | | |
|---|---|---|
| Voges- Proskauer test | No red complex, negative | No red complex, negative |
| Citrate utilisation test | No blue colouration, negative | Blue colouration, positive |
| Nitrate reduction test | Red complex formation, positive | No red complex formation, negative |
| Catalase test | Effervescence, positive | Effervescence, positive |
| Starch Casein Agar (3 days incubation) | Small pink coloured colonies | Yellow coloured colonies |
| Pikovskayas Agar for phosphate solubilization (3 days incubation) | No zone of solubilization, negative | No zone of solubilization, negative |
| Pyridine Agar (12 days incubation) | Small colourless isolated colonies | Small colourless isolated colonies |
| Jenson's Agar for nitrogen fixation (12 days incubation) | Small colourless colonies | Small colourless colonies |

### 3.3.2 Growth curve of Actinomycetotal isolates

*G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were the AHL degrading Actinomycetotal isolates used for biocontrol of *Pcc*BR1. Overnight grown isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were sub-cultured in 3 sets of 100 ml LB and after every 1 h absorbance was measured and dilutions of aliquots at each time point were plated on LA Petri plates. and CFU/ml was calculated at each time point. The increase in cell number ($\log_{10}$ CFU/ml) with respect to time (hours) was calculated based on absorbance at 600 nm data and the growth curve was plotted as depicted in Fig. 3.2. The generation time as calculated from the growth curve was 22.2 min for *G. nicotianae* AI5a, 47 min for *R. pyridinivorans* AI4 and 58.62 min for *R. erythropolis* CRD13.3C.

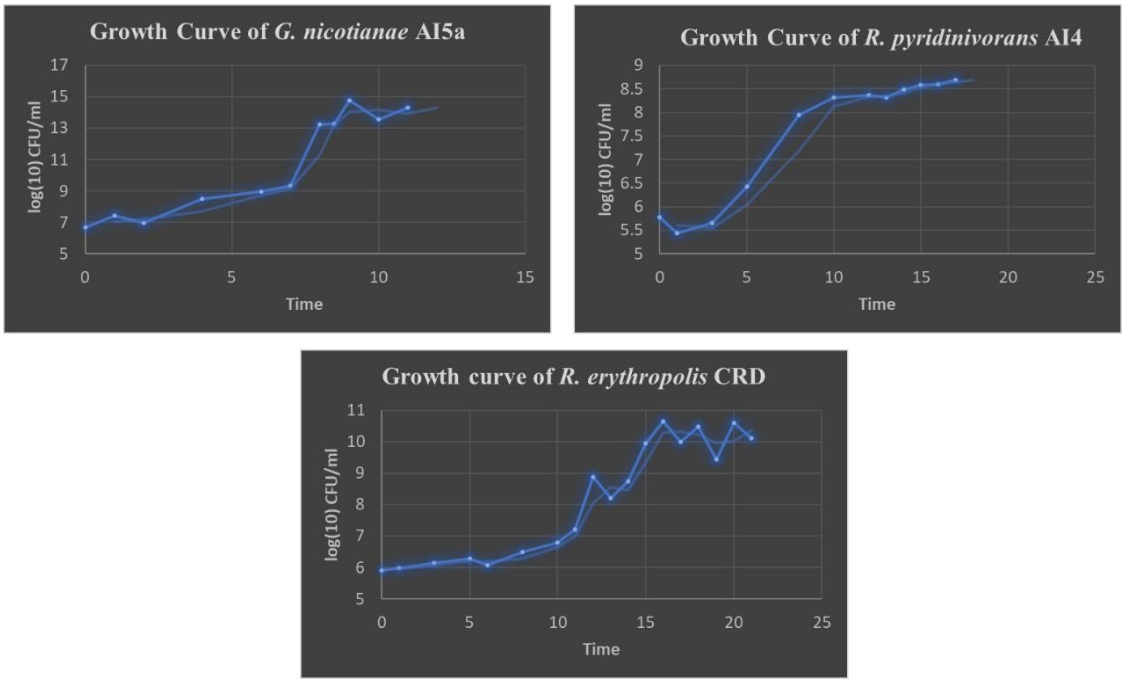


**Figure 3.2 Growth curves of the three Actinomycetotal isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C.**

### 3.3.3 Growth inhibition assay

Quorum quenching (QQ) biocontrol strategy only interferes with the QS signaling molecule that is 3-oxo-C6-AHL in case of *Pcc*BR1, and does not interfere with the growth of pathogen. An approach like this is desirable since it does not directly affect the survival of bacteria, consequently providing low selection pressure, yielding a lower resistance development in the pathogen (Bzdrenga et al., 2017). Hence, isolates with such properties are useful for quorum quenching approach. Supernatants of the isolates ($10^8$ CFU/ml) showed no inhibitory effect against *Pcc*BR1 while 1% SDS showed zone of inhibition as it lyses the bacterial cell (Fig. 3.3). *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were selected for quorum quenching biocontrol studies. Further on, the three isolates can be termed as Quorum Quenching isolates.


**Figure 3.3 *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C do not inhibit the growth of *Pcc*BR1 while the SDS shows growth inhibition.**

### 3.3.4 *In vitro* co-culture bioassay

The ExpI/ExpR (virulence and production of PCWDEs) and CarI/CarR quorum sensing circuits of *Pectobacterium carotovorum* subsp. *carotovorum* are regulated by the AHL 3-oxo-C6-AHL only. *Pcc*BR1 synthesizes 3-oxo-C6-AHL which acts as an autoinducer for regulating its QS circuit that governs virulence as mentioned earlier (Pirhonen et al., 1993; McGowan et al., 2005).The three strains *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C under study when tested against the AHL produced by *Pcc*BR1 during coculture were able to degrade it in *in vitro* co-culture assay (Fig. 3.4). The violacein zone size after 36 hours for *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C was reduced to 3.9 mm, 21.6 mm and 33.4 mm, respectively when compared to that the violacein zone size of average 49 mm produced by *Pcc*BR1 after 36 h of incubation. *G. nicotianae* AI5a

showed the most significant decrease in violacein zone size after 36 h as compared to the two *Rhodococcus* isolates (Vesuna & Nerurkar, 2020).

Thus, the co-culture bioassays performed to check the degradation of 3-oxo-C6-AHL produced by *Pcc*BR1 demonstrated that the three Actinomycetotal isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were able to degrade 3-oxo-C6-AHL without affecting the growth of the pathogen (Fig. 3.4).

### 3.3.5 Actinomycetotal isolates reduce Plant Cell Wall Degrading Enzymes produced by *Pcc*BR1

Among the PCWDEs (PNL, PL and PGA) studied quantitatively, the PNL activity of *Pcc*BR1 was found out to be 37.67 enzyme units (EU) and 39.84 EU at 24 and 48 h, respectively, which was highest amongst the three PCWDEs. The PNL activity was significantly reduced by all three quorum quenching biocontrol isolates. At 24 h when co-cultivated with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C separately, *Pcc*BR1 showed reduced PNL activity of 1.61, 29.29 and 8.54 EU, respectively. At 48 h when co-cultivated with *G. nicotianae* AI5a, *Pcc*BR1 showed a maximum reduction at 0.25 EU while when co-cultivated with *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C, *Pcc*BR1 showed reduced activity of 6.08 EU and 5.7 EU, respectively (Fig. 3.5a).

*Pcc*BR1 produced less PGA and PL when compared to PNL. PGA activity by *Pcc*BR1 at 24 h was 15.71 EU which was reduced to 10.31, 10.80 and 8.67 EU by co-culture with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 or *R. erythropolis* CRD13.3C, respectively. At 48 h, the PGA activity by *Pcc*BR1 was 18.26 EU which reduced to 5.39, 8.53 and 10.46 EU by co-culture with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 or *R. erythropolis* CRD13.3C, respectively (Fig. 3.5b). PL activity by *Pcc*BR1 at 24 h was 11.06 EU which reduced to 0.6, 1.56 and 3.1 EU by co-culture with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 or *R. erythropolis* CRD13.3C, respectively (Fig. 3.5c).

The *Pectobacterium carotovorum* subsp. *carotovorum* quorum sensing circuit regulates production of plant cell wall degrading enzymes (PCWDEs) like pectin lyase, polygalacturonase and pectate lyase, which affect the plant and the vegetable tissue, leading to blackleg and soft rot (Andresen et al., 2010). Thus, degradation of 3-oxo-C6 AHL by *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C has direct effect on virulence factor production by *Pcc*BR1. Among the

three PCWDEs considered here, pectin lyase is produced in the highest amount by *Pcc*BR1, followed by polygalacturonase and pectate lyase. This may show that pectin lyase produced by *Pcc*BR1 plays a major role in plant cell wall degradation in comparison to polygalacturonase and pectate lyase. Comparing the three biocontrol isolates, *G. nicotianae* AI5a reduced the enzyme activity of pectin lyase drastically at 24 h. The Actinomycetotal isolate *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were able to reduce the production of PCWDEs pectin lyase, pectate lyase and polygalacturonase (Fig. 3.5). On the whole, activity of *G. nicotianae* AI5a in reduction of the PCWDEs was best among the three isolates as the PCWDEs rapidly dropped to insignificant amounts.

### 3.3.6 *In vitro* soft rot reduction on host vegetables by Actinomycetotal isolates

It is imperative that the effect of the quorum quenching isolates is observed performing *in vitro* studies before working with the quorum quenching Actinomycetotal strains *in planta*. Among the wide host range of *Pcc*BR1, potato and cucumber slices were used to perform this *in vitro* soft rot assay (Maisuria & Nerurkar, 2013). Noticeable symptoms like tissue maceration, foul smell, oozing liquid etc. are observed for soft rot disease on host tissue following infection (Crépin et al., 2011). Preventive assay comprises application of the biocontrol isolates before the infection so as to stop or reduce the infection to a minimum. While the attenuation assay applies the biocontrol isolates simultaneously with the pathogen and then its ability to avoid/reduce infection is observed.

For preventive biocontrol on cucumber, the *Pcc*BR1 infected slices showed 74% maceration but when treated with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C for 12 h prior to infection with *Pcc*BR1, the maceration percentages were reduced to 6.1%, 2.3% and 15.3%, respectively (Fig. 3.6a, c). For soft rot attenuation on cucumber, the *Pcc*BR1 infected slices showed 99% maceration after 24 h, but when simultaneously treated with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C, the maceration percentages were reduced to 0%, 3.1% and 11.8%, respectively (Fig. 3.6a, d).

For preventive biocontrol on potato, the *Pcc*BR1 infected slices showed 89.7% maceration but when treated with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R.*

*erythropolis* CRD13.3C for 12 h prior to infection with *Pcc*BR1, the maceration percentages were reduced to 2%, 5.5% and 22.6%, respectively (Fig. 3.6b, e). For attenuation of soft rot on potato, the *Pcc*BR1 infected slices showed 91.2% maceration but when simultaneously treated with *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C, the maceration percentages were reduced to 2.7%, 11.8% and 26.5%, respectively (Fig. 3.6b, f). The slices were also inoculated with the biocontrol isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C alone, as control to confirm that they do not cause any infection to the host tissue themselves (Fig. 3.6a, b).

The ability of strains *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C to prevent and attenuate soft rot caused by *Pcc*BR1 in *in vitro* assays with two hosts potato and cucumber in terms of the symptoms produced clearly determined their biocontrol ability (Fig. 3.6). Zamani et al., (2013) had previously identified *Bacillus cereus* having biocontrol properties against *Pcc*. They reported a decrease of 66% in the macerated tissue area in potato upon inoculating the *Bacillus cereus* U92 along with the pathogen. Our results showed higher reduction by 88.4%, 79.3% and 64.6% for *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C, respectively.

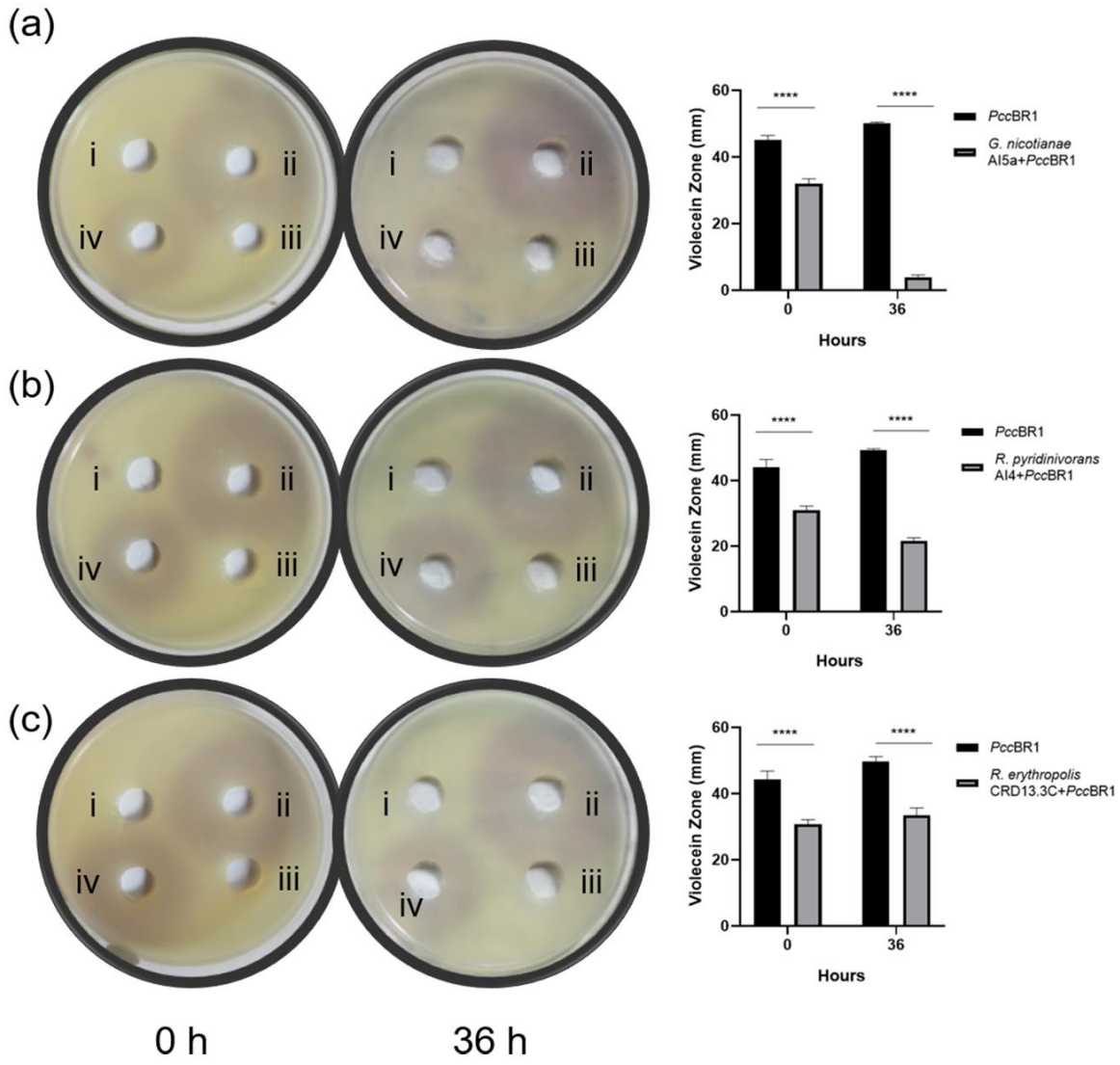


**Figure 3.4 *In vitro* co-culture bioassay for degradation of 3-oxo-C6 AHL by *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C.**

The plates show images of violacein production and the graphs show the corresponding quantitative results in LB media. Three rows are (**a)** *G. nicotianae* AI5a. (**b)** *R. pyridinivorans* AI4. (**c)** *R. erythropolis* CRD13.3C. Each panel is **(i)** LB broth control **(ii)** *Pcc*BR1 culture broth **(iii)** Actinomycetotal culture broth **(iv)** *Pcc*BR1 + Actinomycetotal co-culture broth. **** indicated $P < 0.0001$ as per Holm-Sidak's multiple comparisons test using Two-way ANOVA (n = 3).

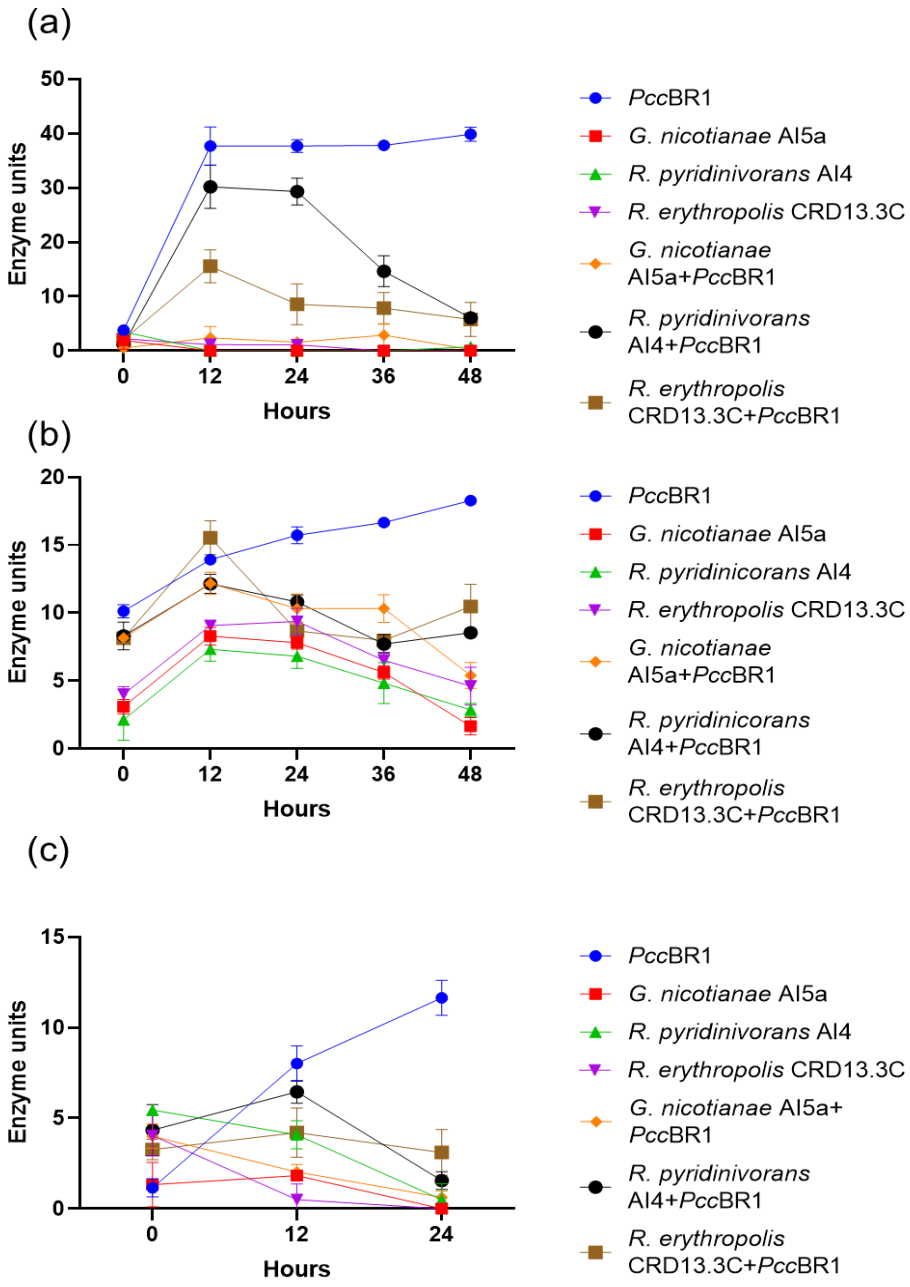


**Figure 3.5 Activity levels of plant cell wall degrading enzymes in pure culture and in co-culture broths. (a) pectin lyase; (b) polygalacturonase; (c) pectate lyase**

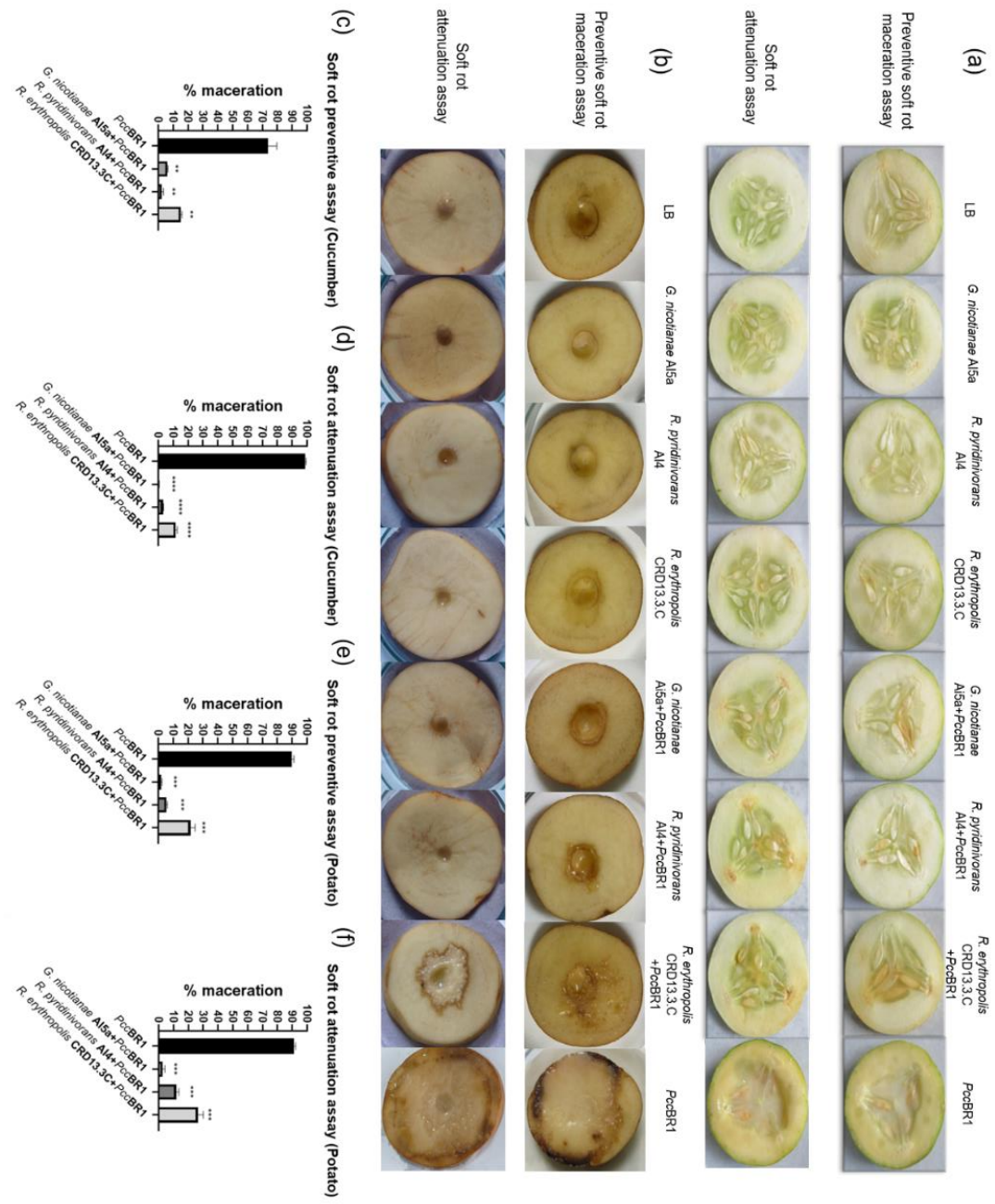


**Figure 3.6 *In vitro* soft rot reduction on potato and cucumber hosts by Actinomycetotal isolates.**

Images show **(a)** assays for preventive and attenuation biocontrol of soft rot on cucumber; and **(b)** assays for preventive and attenuation biocontrol of soft rot on potato. Corresponding quantitative data (percent maceration) for **(c)** assay for preventive biocontrol on cucumber; **(d)** assay for soft rot attenuation on cucumber; **(e)** assay for preventive biocontrol on potato; **(f)** assay for soft rot attenuation on potato.

**** indicates $P < 0.0001$, *** indicates $P < 0.001$, ** indicates $P < 0.01$, per one-way ANOVA with Holm-Sidak's multiple comparisons test (n = 3)

### 3.3.7 Degradation ability of biocontrol isolates on different AHLs

Biosensor *C. violaceum* CV026 produces violet pigmentation upon induction by short acyl chain AHLs. The biocontrol isolates *Glutamicibacter nicotianae* AI5a, *Rhodococcus pyridinivorans* AI4 and *Rhodococcus erythropolis* CRD13.3C were subjected to separate CV026 bioassays utilizing five different AHLs- C4-AHL, C6-AHL, 3-oxo-C6-AHL, C8-AHL and 3-oxo-C8- AHL as substrates. It was observed that the three isolates, *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C were able to degrade all the five short acyl chain AHLs (Fig. 3.7).

In *C. violaceum* CV026, violacein is inducible by all the AHLs possessing N-acyl side chains from C4 to C8 in length. However,AHL compounds with N-acyl side chains from C10 to C14 are unable to induce violacein production (McClean et al., 1997). There is a dual purpose in performing this experiment: i) It gives an idea about the range of AHLs degraded by the quorum quenching Actinomycetotal isolates ii) Synthetic C6-AHL is used in most of the experiments which is the most commonly used AHL in laboratory conditions, while the phytopathogen *Pcc*BR1, belongs to Class II strain of Pectobacteria which generates 3-oxo-C6-AHL; this experiment justifies the use of C6-AHL in most of the experiments at the same time giving proof that it also degrades 3-oxo-C6- AHL produced by *Pcc*BR1 (Garge & Nerurkar, 2016) (Fig. 3.7). Our isolates were able to degrade different types of AHL in addition to 3-oxo-C6-AHL, which is the AHL produced by our target phytopathogen *Pcc*BR1.

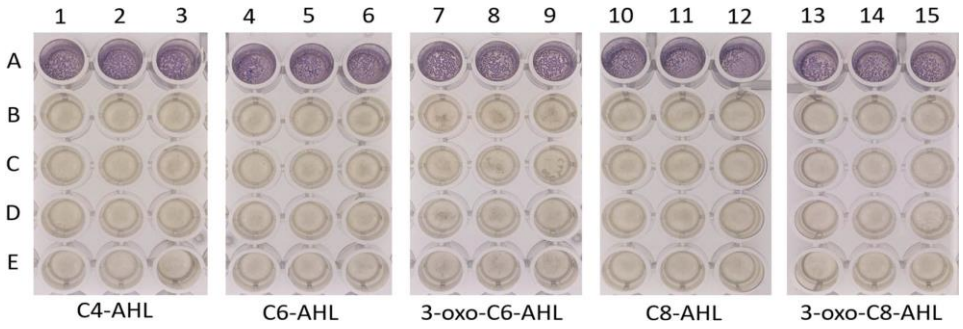


**Figure 3.7 CV026 assay for isolates *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C with five different AHLs.**

*C. violaceum* CV026 + 25 µM AHL Control (row A), *C. violaceum* CV026 + whole cell of *G. nicotianae* AI5a + 25 µM AHL (row B), *C. violaceum* CV026 + whole cell of *R. pyridinivorans* AI4 + 25 µM AHL (row C), *C. violaceum* CV026 + whole cell of R. erythropolis CRD13.3C + 25 µM AHL (row D), F- *C. violaceum* CV026 + PBS (row E). Biological replicates used. (n = 3)

### 3.3.8 Quantification of C6-AHL using Agar Diffusion Method

For biochemical characterization of the QQ enzyme of *G. nicotianae* AI5a, agar diffusion bioassay of Zhang et al., (2007) was used to quantify the residual C6-AHL in the degradation reaction. Different amount of C6-AHL (5, 10, 25, 50, 100, 200, 500 µM) was loaded into the wells made in Luria agar plates and dthe distance travelled by *C. violaceum* CV026 were detected as purple streaks of varying lengths as shown in Fig. 3.8a. The distance of C6-AHL diffusion was measured and the plot of amount of C6-AHL versus the distance travelled was obtained. By doing regression analysis, $R^2$ value of 0.924 and equation for the trend line as y = 206x-200 were obtained (Fig. 3.8b). Using this standard graph, the residual C6-AHL concentration was calculated in the further AHL degrading enzyme activity experiments.

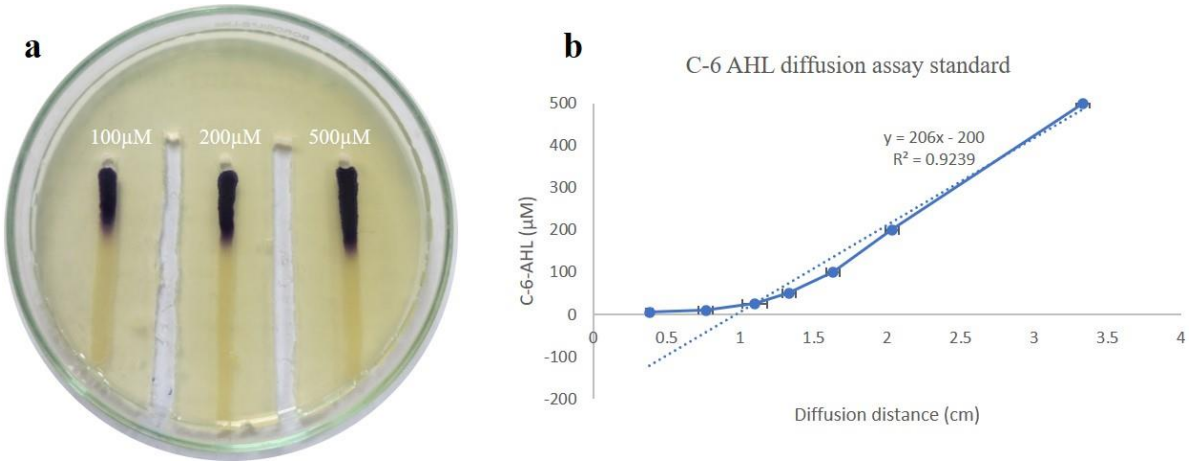


**Figure 3.8 (a) Plate representing different amount of C6-AHL in the wells and development of purple streak by CV026 due to diffusion of C6-AHL (b) Plot of amount of C6-AHL versus the distance travelled. Values are represented as mean ±SD for three replicates.**

### 3.3.9 Qualitative microtitre plate assay for biochemical characterization of AHL degradation by Actinomycetotal isolates

#### 3.3.9.1 Qualitative analysis of C6-AHL degradation

**Effect of temperature on C6-AHL degradation by QQ isolates**

The influence of temperature on QQ ability of isolates *G. nicotianae* AI5a, *R. pyridinovorans* AI4 and *R. erythropolis* CRD13.3C was checked. AHL was degraded by all the three isolates at a temperature range of 20-45 °C. *G. nicotianae* AI5a and *R. erythropolis* CRD13.3C are unable to perform degradation at a high temperature of 50

°C. AHL+PBS was used as positive control for all the temperatures and PBS as negative control of the assay.

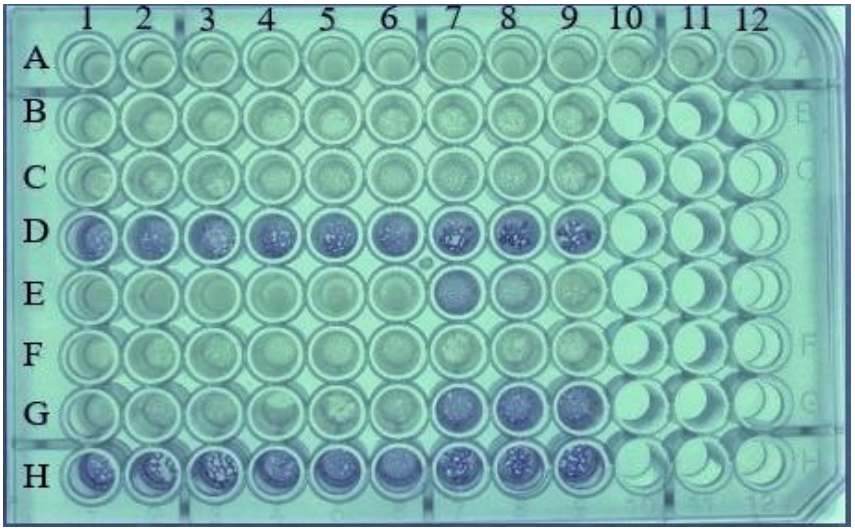


**Figure 3.9 Influence of varying incubation temperature on C6-AHL degradation by *G. nicotianae* AI5a, *R. pyridinivorans* AI4 and *R.* erythropolis CRD13.3C.**

In Figure 3.9 the samples were added to the wells as follows:

A 1,2,3- AI5a 20ºC, B 1-3- AI4 20ºC, C 1-3- CRD13.3C 20ºC, D 1-3- AHL 20ºC, E 1-3- AI5a 25ºC, F 1-3- AI4 25ºC, G 1-3- CRD13.3C 25ºC, H 1-3- AHL 25ºC, A 4-6- AI5a 30ºC , B 4-6- AI4 30ºC, C 4-6- CRD13.3C 30ºC , D 4-6- AHL 30ºC, E 4-6- AI5a 37ºC, F 4-6- AI4 37ºC, G 4-6- CRD13.3C 37ºC, H 4-6- AHL 37ºC, A 7-9- AI5a 45ºC, B 7-9- AI4 45ºC, C 7-9- CRD13.3C 45ºC, D 7-9- AHL 45ºC, E 7-9- AI5a 50ºC, F 7-9- AI4 50ºC, G 7-9- CRD13.3C 50ºC, H 7-9- AHL 50ºC, A 10-12-PBS control (purple colour indicates the controls and non-degraded samples)

**Effect of pH on C6-AHL degradation by QQ isolates**

*G. nicotianae* AI5a, exhibited AHL degradation activity over a wide pH range from pH 5-9 as it could degrade AHL in acidic as well as basic conditions. *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C could not degrade AHL in wide range of pH. This result indicates that the *G. nicotianae* AI5a can perform QQ in soil environment where there is a fluctuation in the pH. *G. nicotianae* AI5a was better compared to the other two Actinomycetotal isolates in the studies carried out until now. Therefore, further studies were focused on *G. nicotianae* AI5a.

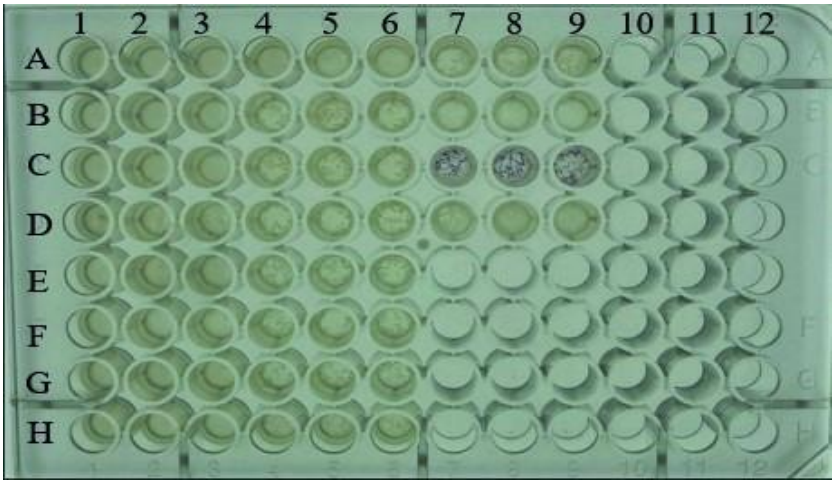


**Figure 3.10 C6-AHL degradation at different pH by *R. pyridinivorans* AI4 and *R. erythropolis* CRD13.3C**

In Figure 3.10, the samples were added to the wells as follows:

**A 1-3**- AI4 pH 5, **B 1-3**- AI4 pH 5.5, **C 1-3**- AI4 pH 6, **D 1-3**- AI4 pH 6.5, **E 1-3**- AI4 pH 7, **F 1-3**- AI4 pH 7.5, **G 1-3**- AI4 pH 8, **H 1-3**- AI4 pH 8.5, **A 4-6**- AI4 pH 9, **B 4-6**- CRD13.3C pH 5, **C 4-6**- CRD13.3C pH 5.5, **D 4-6**- CRD13.3C pH 6, **E 4-6**- CRD13.3C pH6.5, **F 4-6**- CRD13.3C pH 7, **G 4-6**- CRD13.3C pH 7.5, **H 4-6**- CRD13.3C pH 8, **A 7-9**- CRD13.3C pH 8.5, **B 7-9**- CRD13.3C pH 9, **C 7-9**- AHL control, **D 7-9**- PBS control

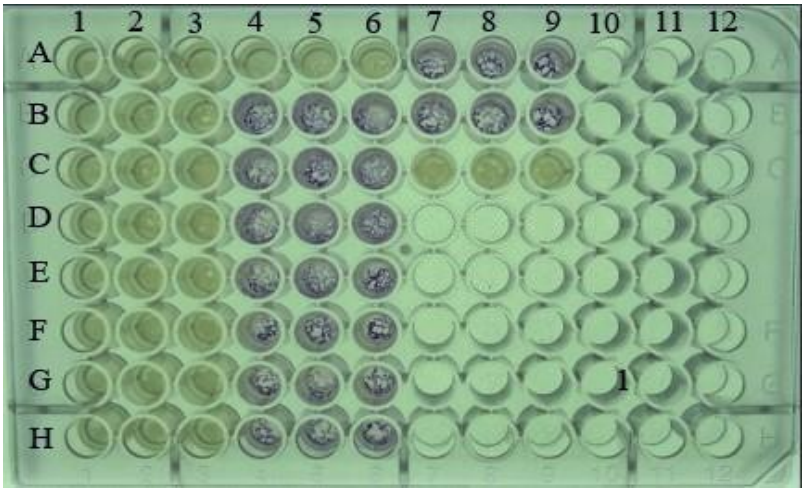


**Figure 3.11 C6-AHL degradation at different pH by *G. nicotianae* AI5a**

In Figure 3.11, the samples were added to the wells as follows:

**A 1-3**- AI5a pH 5, **B 1-3**- AI5a pH 5.5, **C 1-3**- AI5a pH 6, **D 1-3**- AI5a pH 6.5, **E 1-3**- AI5a pH 7, **F 1-3**- AI5a pH 7.5, **G 1-3**- AI5a pH 8, **H 1-3**- AI5a pH 8.5, **A 4-6**- AI5a pH 9, **B 4-6**- AHL pH 5 , **C 4-6**- AHL pH 5.5, **D 4-6**- AHL pH 6, **E 4-6**- AHL pH6.5, **F 4-6**- AHL pH 7, **G 4-6**- AHL pH 7.5, **H 4-6**- AHL pH 8, **A 7-9**- AHL pH 8.5, **B 7-9**- AHL pH 9, **C 7-9**- PBS control (purple colour indicates non-degradation of the AHL controls at different pH)

## 3.3.10 Quantitative bioassay for biochemical characterization of the quorum quenching enzyme of *G. nicotianae* AI5a

The effect of physical and chemical parameters such as temperature, pH and metal ions that may affect the enzyme activity was investigated. Enzyme activity was defined as the hydrolyzed µmoles of C6-AHL per minute.

### 3.3.10.1 Effect of temperature on AHL degrading enzyme activity of *G. nicotianae* AI5a

The AHL degrading enzyme of *G. nicotianae* AI5a could exhibit C6-AHL degrading activity in the temperature range of 20 °C to 37 °C for 25 µM AHL concentration giving maximum activity of 0.20 µM $min^{-1}$ (Fig. 3.12 and 3.13 (A)). For a higher concentration of 50 µM, a bell-shaped curve was obtained in the activity of the enzyme where maximum enzyme activity was seen at 37 °C. Thus, the optimum temperature for the enzyme activity was 37°C where the maximum enzyme activity of 0.22 µM $min^{-1}$ was achieved (Figure 3.12 (A) and (B)). Thereafter the enzyme activity declined in both substrate concentrations at temperature 50 °C possibly due of enzyme inactivation (Fig. 3.12 and 3.13). In case of 50 µM substrate the degradation was absent at 25 °C as well as 50 °C.

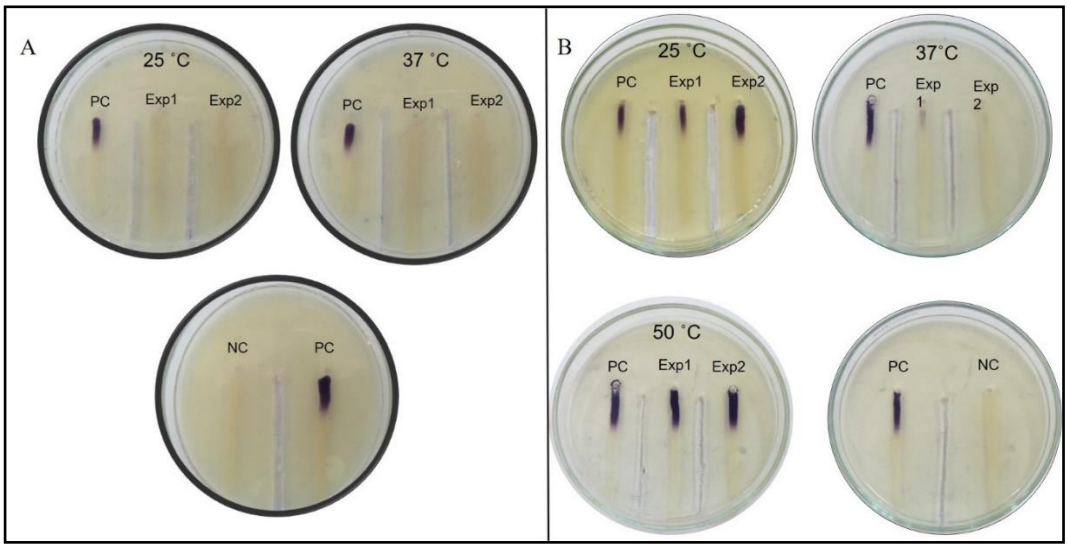


**Figure 3.12 Effect of temperature on AHL degrading enzyme of *G. nicotianae* AI5a at A) 25µM B) 50µM C6-AHL concentrations.**

Plates represent AHL degrading enzyme activity at different temperatures where PC is the positive control, NC is negative control. Exp1 and Exp2 represents 2 of the 3 replicates of experimental set. Values are represented as mean ±SD for three replicates.

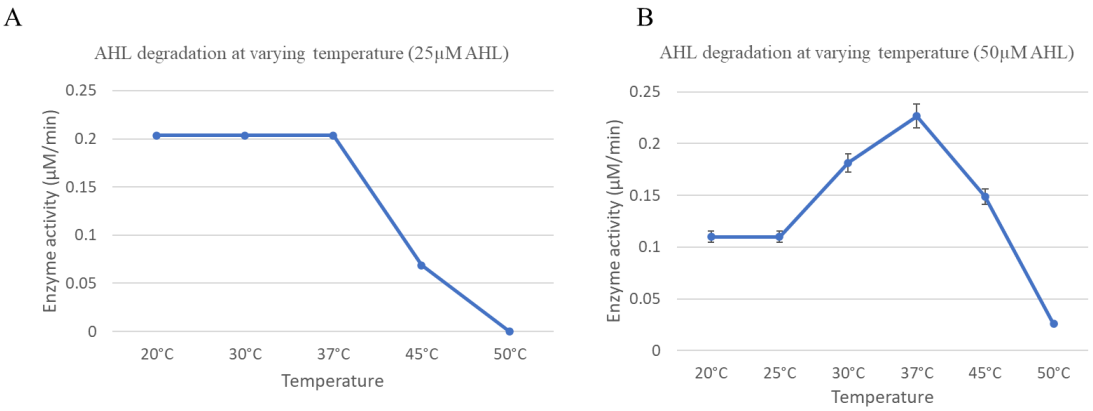


**Figure 3.13 *G. nicotianae* AI5a AHL degrading Enzyme activity v/s temperature curve at (A) 25 µM and (B) 50 µM AHL concentration**

### 3.3.10.2 Effect of pH on AHL degrading enzyme activity of *G. nicotianae* AI5a

The AHL degrading enzyme of *G. nicotianae* AI5a could exhibit C6-AHL degrading activity in the pH range from 5 to 9 at 25 µM AHL concentration. The enzyme activity at all the pH was seen to be 0.20 µM $min^{-1}$ as seen in Fig. 3.14 and 3.15. This might be due to the saturation of the substrate during the reaction. Optimum pH could be obtained by decreasing the time of reaction and then checking the degradation. When the concentration of the substrate was increased to 50 µM, the enzyme showed maximum activity of 0.25 µM $min^{-1}$ at pH 8.5 (Fig. 3.14 and 3.15). The enzyme activity declined slightly as the pH was raised to 9 at higher AHL concentration. The potential interference of non-enzymatic pH-dependent lactone hydrolysis was precluded by analysis of the controls in which same amount of C6-AHL was incubated in the reaction system of different pH without the *G. nicotianae* AI5a culture.

### 3.3.10.3 Effect of metal ions on AHL degrading enzyme activity

Among the various metal ions tested, $Mg^{2+}$ and $Cu^{2+}$ decreased the activity of enzyme as seen in Fig. 3.16 A and B . On the other hand, $Zn^{2+}$, $Ca^{2+}$ and $Cd^{2+}$ increased the activity of the enzyme as compared to untreated. Maximum enzyme activity was seen in the presence of $Ca^{2+}$ cations. Whereas, $Cu^{2+}$ completely inhibited the enzyme activity suggesting it interferes and deactivates the enzyme. We can conclude that $Ca^{2+}$ aids the AHL degradation by *G. nicotianae* AI5a the most, followed by $Zn^{2+}$, $Ca^{2+}$ and $Cd^{2+}$ ions.

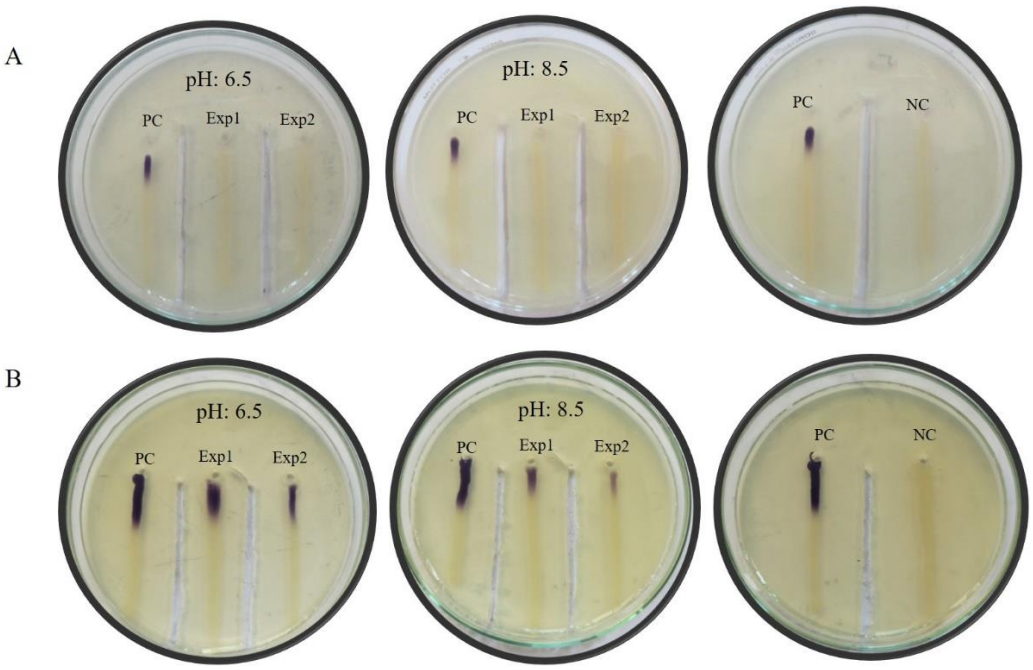


**Figure 3.14 Effect of pH on AHL degrading enzyme activity of *G. nicotianae* AI5a at A) 25 µM B) 50 µM**

Representative plates representing degradation at pH 6.5 and 8.5 where PC is the positive control, NC is negative control for experiment and Exp1, Exp2 represent 2 of the 3 replicates of experimental set. Values are represented as mean ±SD for three replicates.

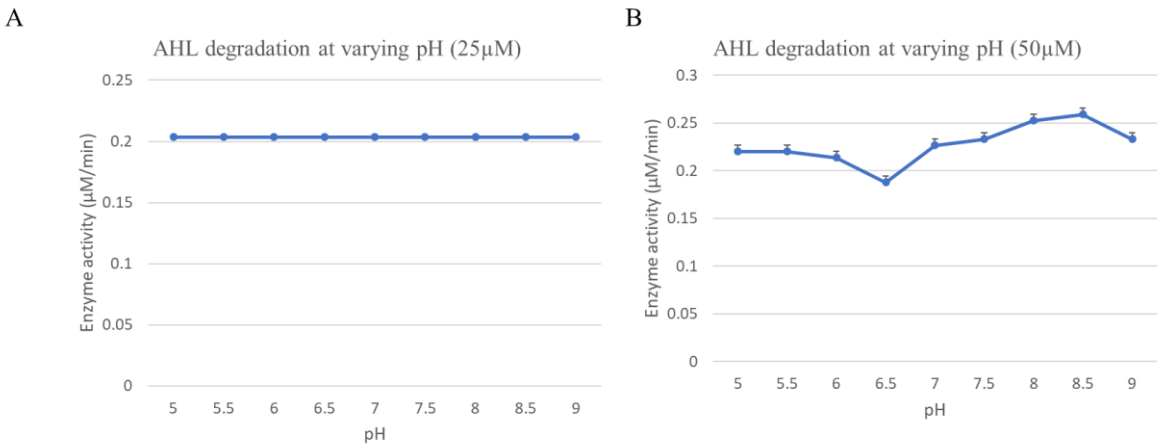


**Figure 3.15 Plot representing pH v/s AHL degrading enzyme activity at 25 µM and 50 µM AHL concentration**

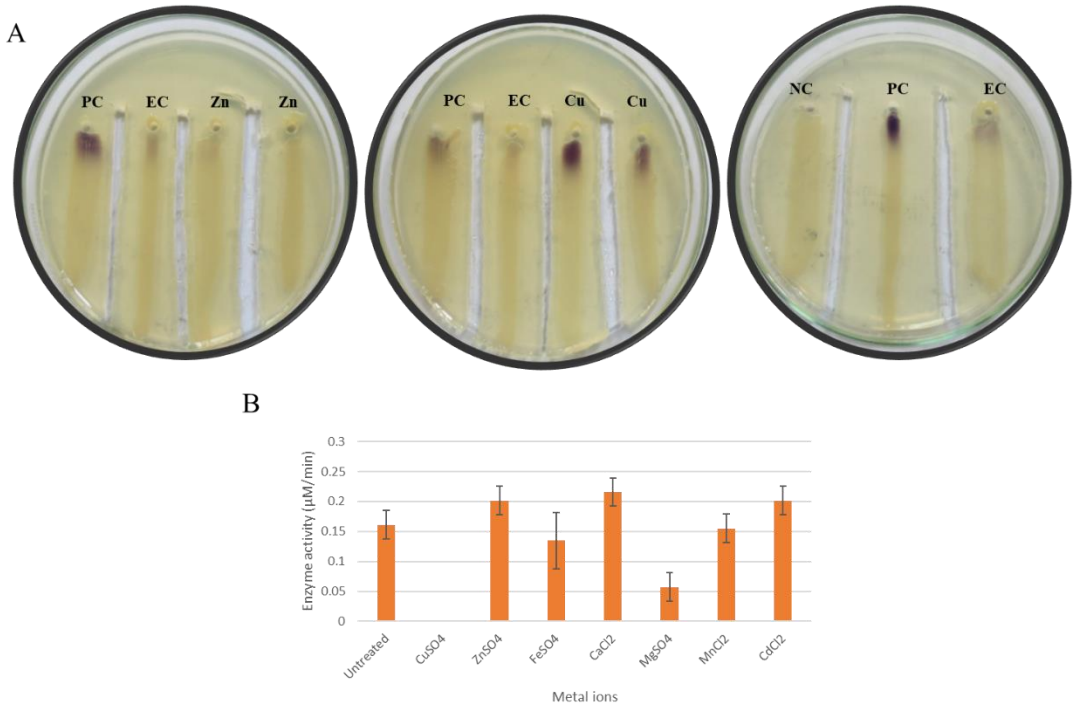


**Figure 3.16 (A) Representative plates showing AHL degrading enzyme activity of *G. nicotianae* AI5a in presence of Zn2+, Cu2+ .PC is the positive control, EC experiment control and streak under Zn shows the degraded AHL and streak under Cu shows the undegraded AHL (B) Plot representing enzyme activity as a function of different metal ions supplemented.**

Values are represented as mean ±SD for three replicates.

### 3.3.10.4 Determination of $V_{max}$ and $K_m$ by Michaelis-Menton curve

AHL degradation kinetics of the QQ enzyme was determined by plotting velocity as a function of substrate concentration (Fig. 3.17). The $K_M$ was calculated by fitting the data to the Michaelis-Menten equation. 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 $min^{-1.}$

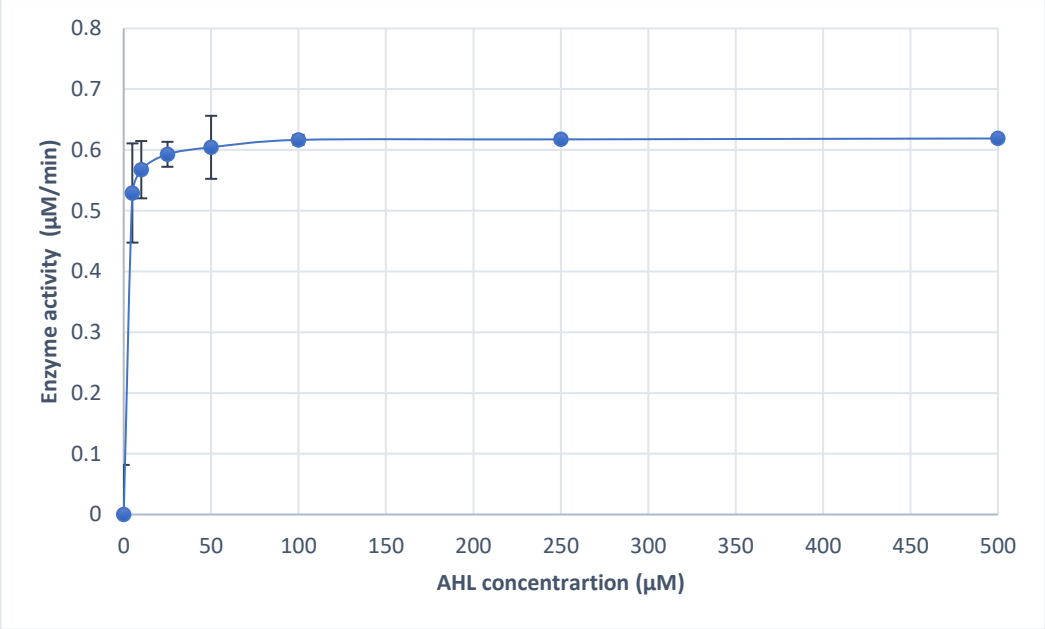


**Figure 3.17 Substrate saturation curve of C6-AHL degrading enzyme from *G. nicotianae* AI5a**

A comparative summary of the results obtained in the study and available literature is shown below in Table. 3.12. The results obtained show more or less similarity with cited literature for the isolate *G. nicotianae* AI5a's AHL degrading enzyme and other reported lactonases. Further study was carried out to prove that the AHL degrading enzyme present in *G. nicotianae* AI5a is an AHL lactonase.

### 3.3.11 AHL utilization and restoration assay

After degradation of AHL if the cleavage is in the lactone ring as in AHL lactonase enzyme then the AHL degradation assay shows no colour in the *C. violaceum* CV026 bioassay.The lactone ring of AHL can be restored in acidic conditions resulting in purple coloration in *C. violaceum* CV026 bioassay. In the AHL restoration assay, AHL degraded by *G. nicotianae* AI5a was reactivated (reappearance of purple colour) when treated with HCl, signifying restoration of the lactone ring (Fig. 3.18). On the other hand *G. nicotianae* AI5a could not grow in the Bushnell Haas minimal media supplemented with AHL as the sole carbon source, but showed good growth when supplemented with glucose (Fig. 3.19a and b). Degraded AHL products such as acyl homoserine and lactone have been reported to work as a source of carbon or/and nitrogen for AHL lactonase (AhlD) producing *Arthrobacter* sp. strains. *V. paradoxus*

has been reported to utilize the fatty acid released from AHL as carbon source. AHL lactonases hydrolyse the lactone ring of AHL, producing N-acyl homoserine which can be restored to N-acyl homoserine lactone at acidic pH (Yates et al., 2002; Czajkowski & Jafra, 2009). The product of C6-AHL degradation by *G. nicotianae* AI5a was restored upon acidification by HCl hence the enzyme must be AHL lactonase.

### 3.3.12 AHL degradation and HPLC analysis

HPLC analysis was used to detect the number of products of AHL degraded by *G. nicotianae* AI5a,. The chromatogram shows the C6-AHL control peak of 0.1 absorbance units (AU) with a 20 min retention time (Fig. 33a). In C6-AHL degraded by *G. nicotianae* AI5a, a reduction in the C6-AHL peak area (to 0.06 AU) was observed, and another peak was observed at a retention time of 15 min which was absent in the control (Fig. 3.20b).

HPLC analysis of AHL treated with *G. nicotianae* AI5a yielded one new peak along with the peak of C6-AHL suggesting that the AHL had been degraded in the system producing a single product (Huang et al., 2012; Park et al., 2003).Degradation of AHL by lactonase and acylase yield one and two product(s) respectively in HPLC.

Lack of growth of *G. nicotianae AI5a* on AHL as sole carbon source, AHL restoration assay and HPLC analysis, suggests that *G. nicotianae* AI5a could be inactivating the AHL by cleaving it to give a new product leading to quorum quenching and could be a putative lactonase (Vesuna & Nerurkar, 2020) (Fig. 3.19 and 3.20).

**Table 3.3 Comparison of biochemical characteristics of *G. nicotianae* AI5a lactonase with reported lactonases**

| | ***G. nicotianae* AI5a putative lactonase** | **Other reported lactonases** |
|---|---|---|
| | | |
| **Temperature range** | 20-37 ℃ | 6 ℃-37 ℃<br>(Wang et al., 2004)<br>20 ℃ -37 ℃<br>(Garge & Nerurkar, 2016) |
| **Optimum temperature** | 37 ℃ | 37 ℃<br>(Park et al., 2003)<br>35 ℃<br>(Garge & Nerurkar, 2016) |
| **pH range** | 5-9 | 5-8.6<br>(Garge & Nerurkar, 2016) |
| **Optimum pH** | 8.5 | 8<br>(Wang et al., 2004) |
| **Metal ions enhancing degradation** | $Zn^{2+}$, $Ca^{2+}$, $Cd^{2+}$ | - |
| **Metal ions inhibiting degradation** | $Cu^{2+}$, $Mg^{2+}$ | $Cr^{2+}$, $Fe^{2+}$, $Pb^{2+}$, $Cu^{2+}$<br>(Wang et al., 2004)<br>$Ca^{2+}$, $Mn^{2+}$, $Cu^{2+}$, $Cd^{2+}$<br>(Garge & Nerurkar, 2016) |
| **Substrate specificity** | C6 AHL, 3-oxo C6 AHL (needs to be checked with more AHLs) | C4 AHL, C6 AHL, C8 AHL, 3-oxo C6 AHL, 3-oxo C8 AHL<br>(Wang et al., 2004)<br>C6 AHL, 3-oxo C6 AHL C8 AHL, 3-oxo C8 AHL<br>(Garge & Nerurkar, 2016) |
| **$V_{max}$ and $K_m$** | $V_{max}$: 0.61µM $min^{-1}$<br>$K_m$: 3 µM | $K_m$: 0.0038 mM<br>(Wang et al., 2004) |

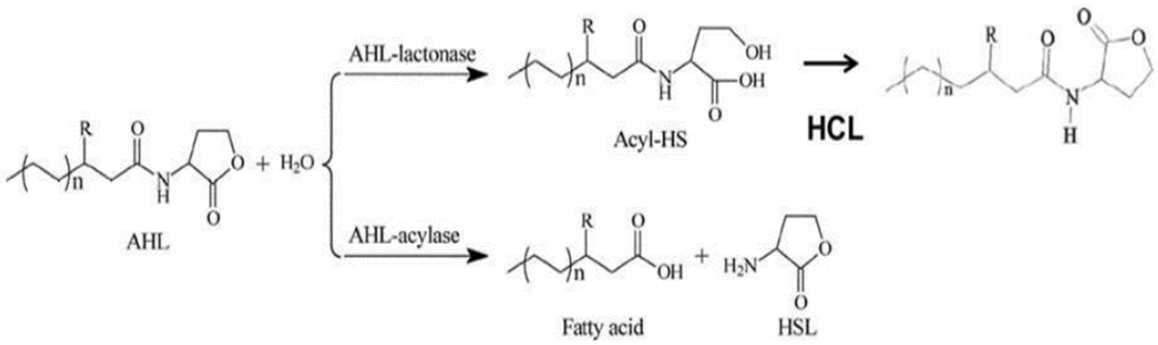


**Figure 3.18 AHL restoration process via acidification by HCL**

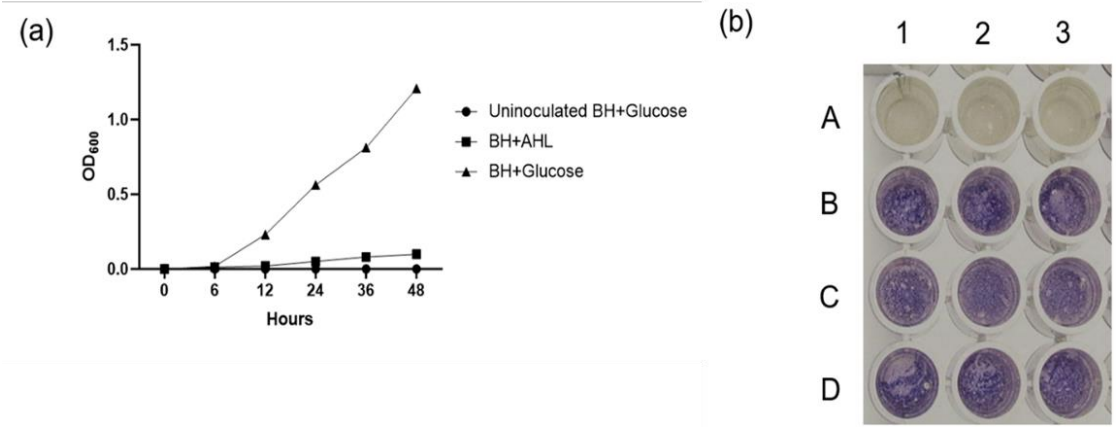


**Figure 3.19 (a) Growth of *G. nicotianae* AI5a in BH minimal media containing either C6-AHL or glucose as the sole carbon source (n =3). (b) Representative image of the AHL restoration assay.**

The reaction mixture of *G. nicotianae* AI5a + 25 μM C6-AHL abolishes violacein production (row A); acidifying the reaction mixture of *G. nicotianae* AI5a + 25 μM C6- AHL with 25 μl of 50 mM HCl restores violacein production (row B); *C. violaceum* CV026 + 25 μM C6-AHL (row C) and acidified *C. violaceum* CV026 + 25 μM C6-AHL (row D) are controls. Within rows, columns 1, 2, and 3 are biological replicates.

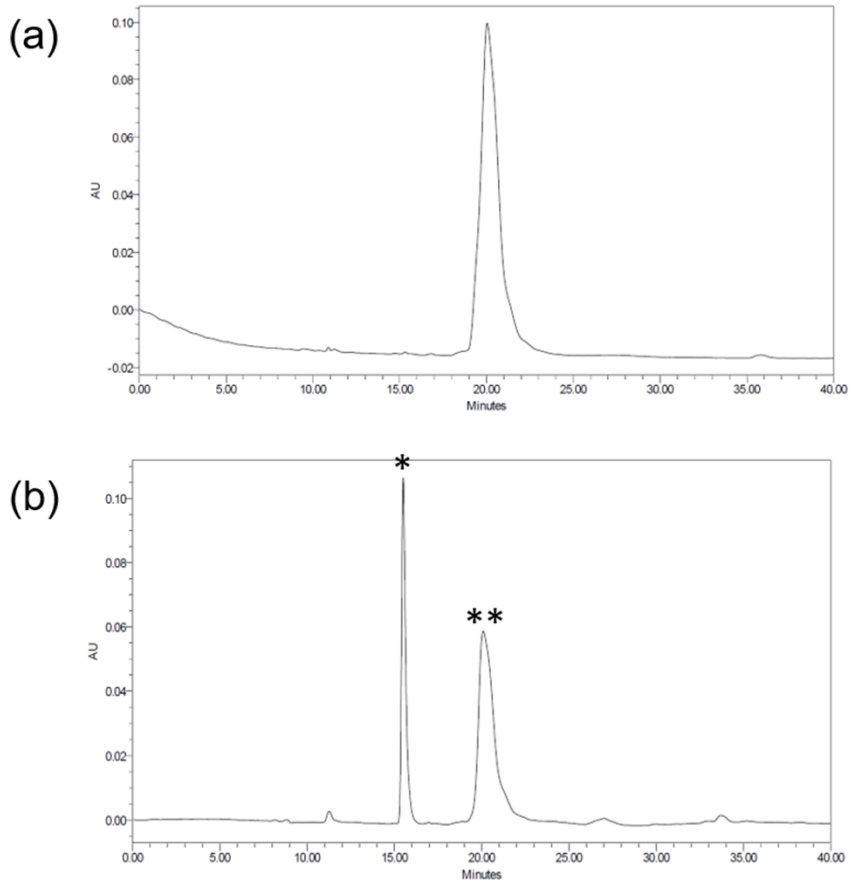


**Figure 3.20 HPLC analysis of the product of AHL degradation by *G. nicotianae* AI5a (a) C6-AHL peak (control) (b) Peaks of degradation product (*)of C6-AHL by *G. nicotianae* AI5a and of residual C6-AHL (**)**