Chapter 2

Isolation, Screening and Identification of AHL degrading Actinomycetota

2.1 Introduction

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. Additionally, the call for pesticide free food by the consumer and growing rates of pesticides has led for a hunt for alternatives for these items. Chemical solutions are also unsuccessful and unavailable for certain diseases. Biological control or Biocontrol is thus being contemplated as a substitute method of cutting down the role of chemicals in agriculture (Azaiez et al., 2018). The use of microorganisms that antagonize plant pathogens (biological control) is risk-free when it results in enhancement of resident antagonists against the pathogen. Moreover, the combination of such biological control agents (BCAs) with reduced levels of pesticides (integrated control) promotes a degree of disease suppression similar to that achieved with full pesticides treatment (Abd-El-Khair et al., 2021).

Actinomycetota is one of the largest phyla within domain *Bacteria* and comprises of phenotypically diverse organisms, with widespread distribution in nature but largely found in soil. While some Actinomycetota are known pathogens of plants and animals many are reported to produce enzymes that are used for inhibition of quorum sensing pathogens (Polkade et al., 2016).

Rhodococcus has been the most studied Actinomycetota and is known to have a number of AHL-inactivating lactonases. *R. erythropolis* has been frequently used as a biocontrol agent to prevent soft-rot in plants. All three AHL degrading enzymes i.e., AHL lactonase, acylase and oxidoreductase have been reported in *R. erythropolis* (Uroz et al., 2005, 2008; Ryu et al., 2020). *Streptomyces* sp. which are abundantly found in soil are also known to have an AHL degrading enzyme, an acylase and can be a useful biocontrol agent against phytopathogens (Park et al., 2005). *Arthrobacter* sp. have been found to reduce the virulence of soft rot causing *Pcc* by suppressing the AHL and pectate lyase. *Arthrobacter* sp. showed AHL degrading activity due to the presence of AHL Lactonase QQ enzyme (Park et al., 2003)

Another Actinomycetota *Glutamicibacter* sp. have been found in a variety of environments like soil, sewage, animals and plants. *Glutamicibacter* sp. shares almost 99% sequence similarity with the *Arthrobacter* sp. *Arthrobacter* sp. have been reported to produce quorum quenching enzyme AhlD lactonase (Park et al., 2003; Busse, 2016). Hence, these Actinomycetota and their enzymatic quorum quenching abilities could also be studied further as an alternative approach for the biocontrol of microbial

infections. Taking these facets into consideration the present study involves isolation, screening and identification of AHL degrading Actinomycetota.

2.2 Methods and Materials

2.2.1 Isolation Strategies

To isolate AHL degrading Actinomycetota two isolation strategies were applied:

1) Acetonitrile enrichment
2) Heat shock method

These strategies were adopted based on reports indicating role of Actinomycetota in nitrile hydrolysis (Langdahl et al., 1996) and that Actinomycetota can withstand high temperature up to 55 ºC, the temperature at which most of the bacteria cannot survive (Balagurunathan et al., 2020).

2.2.2 Collection of samples for isolation of Actinomycetota

Soil samples were collected from farmland, garden, soil containing organic solvents etc. and different plant samples were also collected to isolate plant associated Actinomycetota. Rhizospheric soil from tobacco, corn, Ivy gourd, pigeon pea farms were collected. Further, soil containing organic solvents was collected from Kavi Kamboi, Gujarat and garden soil from The M.S. University of Baroda campus was collected. Plants used for isolation of Actinomycetota included roots, stem and leaves of wild growing grass, *Calotropis* plant and *Datura* were collected. Soil containing organic solvents and soil from petrol pumps were processed using acetonitrile enrichment whereas farmland soil and plant samples were processed using heat shock treatment. Soil samples were collected using sterile spatula and collected in sterile centrifuge tubes.

2.2.3 Media used

Rich Media Agar (RMA)

Table 2.1 RMA Composition

Constituents	g/L
Glucose	10
Nutrient broth	8
Yeast extract	0.5
Agar	20

M3 Agar

Five separate stock solutions were prepared and mixed after autoclaving to avoid precipitation. The 5 stock solutions are mentioned below in Table 2.2.

Table 2.2 M3 Agar media composition

	Constituents	**g/100 ml**
Solution A	KH_2PO_4	0.233
	Na_2HPO_4	0.336
Solution B	NaCl	0.145
	KNO_3	0.05
Solution C	$CaCO_3$	0.01
Solution D	Na-propionate	0.10
Solution E	Agar	10

Defined Media (DE)

Defined media (DE) was used for acetonitrile enrichment and was prepared as separate stock solutions of trace elements, vitamins etc. Media was prepared as mentioned in the table below and separately 90 ml distilled water was added to make up volume to 500 ml. 0.5 ml of 100 mg/ml cyclohexamide was added to 500 ml of media after autoclaving to prevent fungal growth (Fig. 2.1 and Table 2.3) (Borisova, 2011).

2.2.4 Isolation and enrichment protocol

2.2.4.1 Heat Shock Method

Soil suspension was prepared by taking 0.5 g of soil samples in 5 ml of autoclaved distilled water. Plant samples were washed with autoclaved distilled water and crushed using mortar and pestle. The crushed samples were added to 10 ml sterile distilled water. The soil suspension and crushed plant samples were then processed in same manner by mixing them thoroughly using vortex following which heat shock treatment was given. Heat shock was provided by incubating samples at 55 ºC for 6 min (Borisova, 2011). Samples were then serially diluted and spread on M3 Agar plates. Plates were incubated at 30 ºC for 5 to 7 days and different colony types based on colony characteristics were selected and plated on RMA plates and incubated at 30 ºC for 2-3 days.

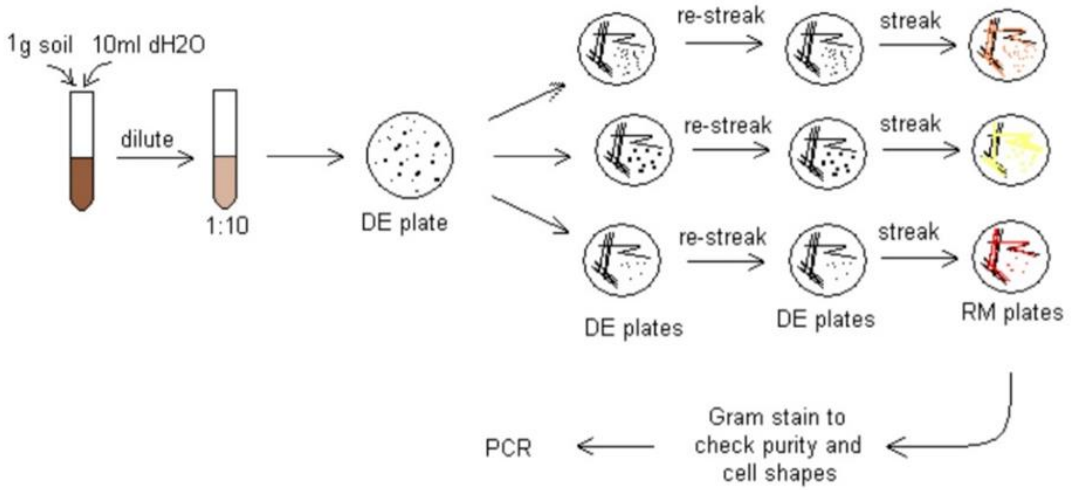

Figure 2.1 Acetonitrile enrichment using solid defined basal media (DE)

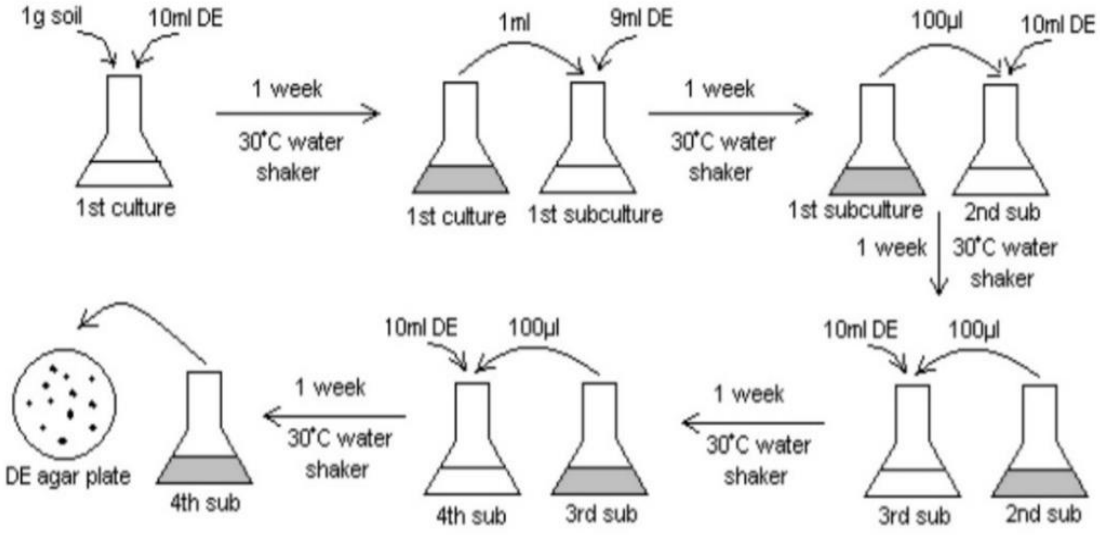

Figure 2.2 Acetonitrile enrichment using liquid defined basal media (DE)

Table 2.3 DE Media composition

Ingredients		Amt. needed	Working stock solution	100x concentrated solution	10x concentrated solution	Amt. mixed	Amt. per 500ml
Main Ingredients (500ml)	NaCl	10g	10g/100ml	---------	---------	100ml	100ml
	$MgCl_2 \cdot 6H_2O$	1.5g	---------	---------	30g/100ml	5ml	5ml
	KCl	0.25g	0.25g/100ml	---------	---------	100ml	100ml
	$MgSO_4 \cdot 7H_2O$	0.15g	---------	30g/100ml	---------	0.5ml	0.5ml
	$CaCl_2 \cdot 2H_2O$	0.075g	---------	15g/100ml	---------	0.5ml	0.5ml
	K_2HPO_4	0.5g	0.5g/100ml	---------	---------	100ml	100ml
	KH_2PO_4	0.375g	0.375g/100ml	---------	---------	100ml	100ml
Trace Element Solution (TES-3) (100ml)[a]	$FeCl_2 \cdot 4H_2O$	200mg	----------	---------	100mg/10ml EtOH	-------[a]	100µl
	$CoCl_2 \cdot 6H_2O$	25mg	---------	---------	----------	25mg	0.5ml
	$MnCl_2 \cdot 4H_2O$	10mg	---------	---------	----------	10mg	
	$ZnCl_2$	7mg	---------	---------	----------	7mg	
	H_3BO_3	0.6mg	---------	60mg/100ml	----------	1ml	
	$Na_2MoO_4 \cdot 2H_2O$	4mg	---------	---------	----------	4mg	
	$NiCl_2 \cdot 6H_2O$	7mg	---------	---------	----------	7mg	
	$CuCl_2 \cdot 2H_2O$	0.2mg	---------	20mg/100ml	----------	1ml	
	$AlCl_3 \cdot 6H_2O$	6mg	---------	---------	----------	6mg	
	$NaWO_4 \cdot 2H_2O$	0.6mg	---------	60mg/100ml	----------	1ml	
Vitamin Mixture (VM) (100ml)[a]	Pyridoxamine dihydrochloride	10mg	---------	---------	----------	10mg	0.5ml
	Calcium D (+) – pentothenate	5mg	---------	---------	----------	5mg	
	Nicotinic acid	10mg	---------	---------	----------	10mg	
	DL-α-lipoic acid	1mg	---------	---------	10mg/100ml	10ml	
	D-(+)-biotin	1mg	---------	---------	10mg/100ml	10ml	
	4-aminobenzoic acid	4mg	---------	---------	----------	4mg	
	Folic acid	3mg	3mg/100ml	---------	----------	-------[a]	0.5ml
B_{12} Solution (100ml)[a]	cyanocobalamin	5mg	5mg/100ml	---------	----------	5mg	0.5ml
Thiamine HCl solution (100ml)[a]	NaH_2PO_4	160mg	---------	---------	----------	160mg	0.5ml
	$Na_2HPO_4 \cdot 7H_2O$	0.1mg	---------	10mg/100ml	----------	1ml	
	Thiamine HCl	10mg	---------	---------	----------	10mg	
C and N source[b]	Acetonitrile	2.5ml	---------	---------	----------	2.5ml	2.5ml
H_2O	----------	---------	---------	---------	----------	--------	90ml

a: Added separately to the main ingredients of 500ml DE medium. b: 2.5ml added to 500ml of DE medium.

2.2.4.2 Acetonitrile Enrichment Method

1 g soil sample was mixed in 10 ml of sterile DE media and incubated at 30 ºC at shaking conditions for 7 days. After 7 days, 1 ml aliquot was taken from this mixture and added to 9 ml of fresh DE media and incubated for 7 days at 30 ºC. Subsequently 2 more rounds of enrichment were carried in same way and after last round, 0.1 ml of aliquot was spread on DE agar plates and incubated at 30 ºC for 7-10 days. Different types of colonies were selected and streaked on DE agar plates and incubated at 30 ºC for 7

days. Isolated colonies from these plates were selected and streaked on RMA plates and incubated at 30 ºC for 2-3 days (Fig. 2.2).

2.2.5 Bacterial strains and culture conditions

All bacterial cultures including isolates were grown in Luria Bertani (LB) at 30 °C under shaking condition and were maintained on Luria Bertani Agar (LA).

i) Biosensor strain *Chromobacterium violaceum* CV026 [violacein and AHL-negative double miniTn5 mutant, in which one transposon is inserted into the *cviI* (AHL synthase gene) and the other is inserted into a putative violacein repressor locus] was used to detect AHL. Exposure of this strain to exogenous AHLs (AHLs having acyl chains of C4 to C8 in length), that are able to interact with CviR, results in rapid production of a visually clear purple pigmentation (McClean et al., 1997; Steindler et al., 2007). Biosensor strain *C. violaceum* CV026 was grown overnight and maintained in LB at 30°C with Kanamycin (30 µg/ml).

ii) *Pectobacterium carotovorum* subsp. *carotovorum* BR1 was used as the phytopathogenic strain in all the studies. *Pcc*BR1 was isolated in our laboratory from brinjal showing soft rot (Maisuria & Nerurkar, 2013). *Pcc*BR1 is quorum sensing isolate produces 3-oxo-hexanoyl homoserine lactone which regulates production of different virulence factors like pectin lyase, pectate lyase, polygalacturonase etc. that cause soft rot in various plant hosts.

iii) *Lysinibacillus sp*. GS50 is a laboratory isolate which produces AHL degrading lactonase enzyme and have been shown to possess the ability to attenuate soft rot was used as positive control during AHL degradation assays (Garge & Nerurkar, 2016).

iv) AHL degrading bacteria *Rhodococcus erythropolis* CRD13.3C isolated in another laboratory and being known AHL degrader was included in the studies.

2.2.6 CV026 biosensor based AHL degradation assay

CV026 bioassay was used to check the AHL degradation activity of isolates, the bioassay was based on the method used by Blosser-Middleton & Gray, 2001 for detection of exogenous AHL. 1.5 ml of overnight grown isolate was centrifuged at 12000 rpm for 5 min to obtain cell pellet. The cell pellet was washed with PBS (pH 7.0) and resuspended in 500 µl PBS. Reaction mixture was prepared by adding 20 µl of resuspended pellet to 80 µl of 25 µM C6-AHL and it was incubated at 30 ºC for 2

h. To check amount of C6-AHL remaining in reaction system biosensor strain *Chromobacterium violaceum* CV026 was used. 100 µl of 0.002 OD *C. violaceum* CV026 was added to each well of the microtitre plate and 30 µl of reaction mixture was added to the wells. Plates were then incubated at 30 ºC for 24 h and presence of purple colour due to violacein production was observed which indicated presence of undegraded AHL in the reaction system. Absence of purple colour represented the degradation of C6-AHL by the isolate.

2.2.7 Genomic DNA isolation

Genomic DNA isolation of the isolates was carried out using CTAB method as described by Russell and Sambrook, (2001). with modifications. 3 ml of overnight grown culture samples were centrifuged at 10000 rpm for 5 min and the pellet was washed using 5 M NaCl. Washed pellet was resuspended in 500 µl TES buffer to which 20 µl of lysozyme (40 mg/ml) was added. This mixture was incubated at 37 ºC for 1 h. 40 µl of 10% SDS and 8 µl of Proteinase K was added and the samples were incubated at 55ºC for 1 h. Then 67 µl 5 M NaCl was added to the samples and after gentle mixing 88 µl of CTAB/NaCl solution was added and the samples were incubated at 65 ºC for 10 min. Equal amount of phenol: chloroform: isoamyl alcohol (25:24:1) was added to remove protein impurities and centrifuged at 12500 rpm for 20 min. Supernatant was taken and to it equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12500 rpm for 20 min. Supernatant was collected and to that $1/6^{th}$ volume of 6 M Sodium acetate was added and 70% ethanol was added twice the volume of the supernatant. The genomic DNA preparation was then incubated at -20 ºC overnight for precipitation. After incubation the preparations were centrifuged and DNA pellets obtained were washed with 70% ethanol. Samples were centrifuged at 10000 rpm for 5 min, ethanol supernatant was discarded and DNA pellets were dried and dissolved in 20 µl sterile water and treated with 1 µl RNase for 10 min at 65 ºC. DNA samples were run on 0.8% agarose gel in 1X TAE buffer to check presence of DNA and visualized using AlphaImager™ Gel imaging system from Alpha Innotech.

2.2.8 PCR amplification of 16S rRNA gene of isolates and identification

Full length 16S rRNA genes were amplified using the 16S ribosomal RNA gene universal bacterial primers

27F (5'-AGAGTTTGATCCTGGCTCAG-3'),

1541R (5'-AAGGAGGTGATCCAGCCGCA-3'),

1107R (5'-RGCTCG TTGCGGGACTTAACC-3') and

926F (5′-AAA CTY AAA KGA ATT GAC GG-3′).

50 µl PCR reactions that contained 200 µM deoxynucleoside triphosphate mix, 0.2 µM of each primer, 1.5 mM MgCl2, PCR buffer, 1.5 U Taq DNA polymerase and 1 µl genomic DNA template were performed in Applied Biosystems thermocycler. PCR conditions used were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. A final extension at 72 °C for 10 min was then performed. The sequencing results obtained were analyzed using EzBioCloud and NCBI BLAST for identification of the isolates from their respective sequences (Yoon et al., 2017).

2.3 Results and Discussion

2.3.1 Isolation of Actinomycetota from soil and plant samples

Specific isolation strategies were adopted to isolate Actinomycetota as they are slow growing and many a times outgrown by other types of fast-growing bacteria (Balagurunathan et al., 2020; Yandigeri et al., 2012). Previously isolation strategy using Actinomycetes Isolation Agar (AIA) and Starch Casein Agar (SCA) was followed where Actinomycetota were not obtained as they were outgrown by many *Bacillus* sp. Acetonitrile enrichment technique for selectively enriching and isolating Actinomycetota was applied, more specifically *Rhodococcus sp.* as shown by Langdahl et al., 1996 and also adopted by Borisova, 2011. Heat shock method was also used as described by Borisova, 2011 for isolation of Actinomycetota by reducing number of other heat intolerant bacteria. Addition of cyclohexamide in the media helped minimizing fungal contamination as the incubation period was long. Using Acetonitrile enrichment, 17 isolates were obtained whereas by heat shock method 62 isolates were obtained (Table 2.4 and 2.5). It was observed that during isolation when the isolates were shifted from minimal defined media like M3 or DEA to a rich media

like RMA, isolates were able to produce EPS and pigmentation (Fig. 2.3). This can be attributed to nutrient rich composition of RMA. Combining both the strategies 79 isolates were obtained in total which were then screened for their AHL degrading ability.

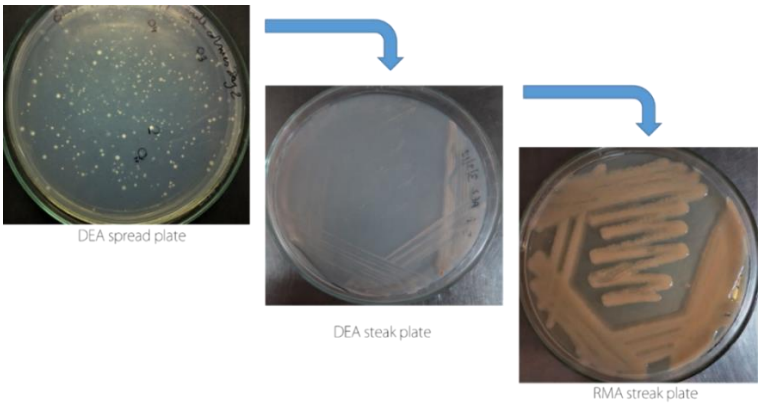

Figure 2.3 Representative example of isolation and enrichment technique for Actinomycetota

Table 2.4 Bacterial isolates obtained using Acetonitrile enrichment method

Sample used	Isolates
Soil containing organic solvents	AA1, AA2, AA3
Soil sample, Kavi Kamboi	AI1, AI2, AI3, AI4, AI5a, AI5b, AI6
Underbridge soil, The M.S. University of Baroda	AE1, AE2, AE3
Garden soil, The M.S. University of Baroda	AC1, AC2
Pigeon pea rhizosphere soil	AC3, AG1

Table 2.5 Bacterial isolates obtained using heat shock method

Sample used	Isolates
Underbridge soil, The M.S. University of Baroda	VMS1, VMS2, VMS3, VMS4
Petrol Pump soil, Sayajigunj	HP1. HP2. HP3. HP4
Garden Soil, The M.S. University of Baroda	MGS1. MGS2
Corn Farm soil, Anand Agriculture University	SC1, SC2, SC3, SC4, SC5, SC6
Tobacco Farm soil, Anand Agriculture University	SC7, CPT1, CPT2, CPT3, CPT4, CPT5, CPT6, CPT7, CPT8
Ivy gourd farm soil, Anand Agriculture University	CPZ1, CPZ2, CPZ3, CPZ4, CPZ5, CPZ6, CPZ7
Calotropis leaves	CL1, CL2, CL3, CL4
Garden grass, The M.S. University of Baroda	GRL1, GRL2, GRL3, GRL4, GRL5, GRL6, GRL7, GRL8

Tobacco Farm soil 2, Anand Agriculture University	CPR1, CPR2, CPR3, CPR4, CPR5, CPR6, CPR7, CPR8, CPR9,CPR10
Datura fruit and stem	DFS1, DFS2
Datura leaves	DL1, DL2, DL3, DL4, DL5

2.3.2 Primary Screening for isolates- CV026 bioassay

Primary screening of the isolates was based on the ability of isolates to degrade AHL. CV026 bioassay for AHL degradation was used with *C. violaceum* CV026 as biosensor strain. The isolate is able to degrade AHL in the reaction mixture and no purple colour is observed in the microtitre plate if the isolate produces an AHL degrading enzyme (Garge & Nerurkar, 2016) (Fig. 2.4). The isolates that showed no purple colouration upon incubation were AHL degrading isolates. Out of 79 isolates screened for AHL degradation 22 isolates were observed to be AHL degraders. a) PBS and b) *Lysinibacillus sp*. GS50, an AHL degrading lab isolate (Garge & Nerurkar, 2016) were used as positive control which gave no colour whereas PBS+AHL system was used as negative control gave purple colour. The assay was carried out in biological triplicates (n=3). (Fig. 2.4)

Isolates that showed purple colour were AHL non-degraders and were excluded from further studies. The isolates showing mild purple colouration were discarded. The 22 samples which showed no colour repeatedly were taken for further studies.

2.3.3 16S rRNA gene sequencing and identification of isolates

Out of the 22 AHL degrading isolates, 7 isolates showed consistent AHL degradation activity, the other 15 over time lost their AHL degradation ability. Genomic DNA isolated from these 7 isolates was used as template for 16S rRNA gene amplification using PCR. Primers used for amplification were 27F and 1541R which gave amplicon of around 1500 base pair length in 0.8% Agarose gel (Fig. 2.5). These 7 isolates were outsourced for 16S rRNA gene sequencing. Out of the 7 isolates, 2 isolates AI4 and AI5a were obtained by acetonitrile enrichment while 5 isolates AC2, DFS1, DFS2, CL4, DL3 were isolated using heat shock treatment. Partial sequencing of these 7 isolates was carried out using 27F primer and sequence analysis was done using NCBI BLAST and bacterial 16S EzBioCloud database (Yoon et al., 2017).

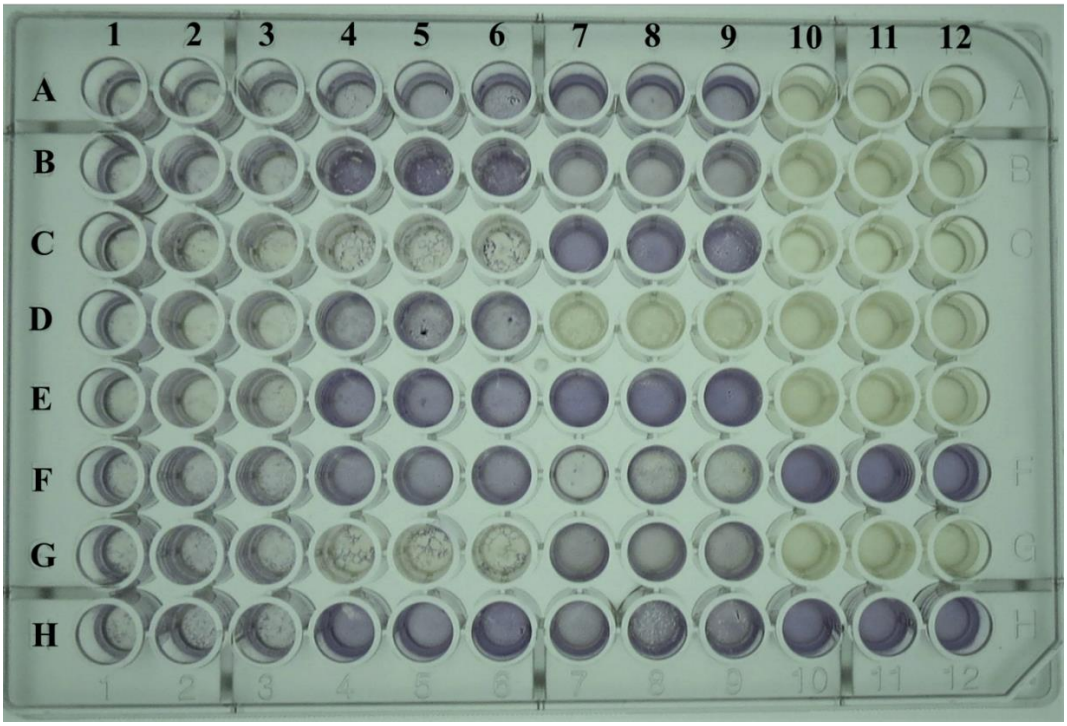

Figure 2.4 Representative Image of Microtiter plate of AHL degradation bioassay.

E 10-11-12 PBS positive control and G 10-11-12 *Lysinibacillus* sp. GS50 Positive controls, F 10-11-12 and H 10-11-12 Negative controls.

Based on NCBI BLAST and EzBioCloud database, two isolates AI4 and AI5a were identified to be Actinomycetota while the rest were identified as *Pseudomonas mendocina* (AC2) and *Klebsiella pneumoniae* subsp. *ozaenae* (DFS1, DFS2, CL4, DL3). Isolate AI4 was identified to be *Rhodococcus pyridinivorans* showing 100% similarity with 56% completeness of the sequence while isolate AI5a was identified as *Glutamicibacter nicotianae* showing 99.79% similarity with 66.5% completeness of sequence based on the partial 16S rRNA gene sequencing on EzBioCloud database (Yoon et al., 2017).

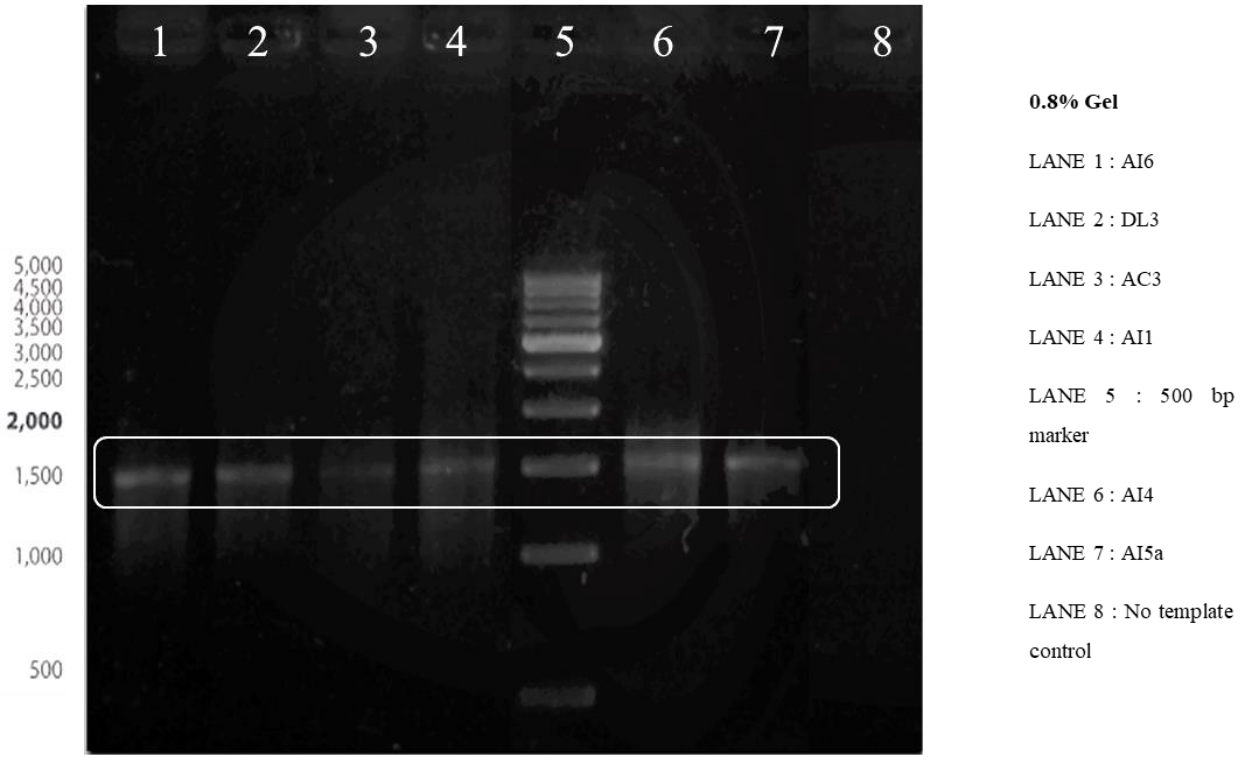

Figure 2. 5 PCR amplification of 16S rRNA gene using 27F and 1541R primers.

Table 2.6 Identification of selected AHL degrading bacterial isolates based on partial 16S rRNA gene sequencing

Isolate	Identified as	Sequence length used for Identification	Hit strain	% Identity
AI4	*Rhodococcus pyridinivorans*	1405 bp	DSM 44555(T)	100
AI5a	*Glutamicibacter nicotianae*	1513 bp	DSM 20123(T)	99.79
AC2	*Pseudomonas mendocina*	940 bp	NBRC 14162(T)	99.15
DFS1	*Klebsiella pneumoniae* subsp. *ozaenae*	815 bp	ATCC 11296(T)	99.75
DFS2	*Klebsiella pneumoniae* subsp. *ozaenae*	917 bp	ATCC 11296(T)	99.56
CL4	*Klebsiella pneumoniae* subsp. *ozaenae*	886 bp	ATCC 11296(T)	99.66
DL3	*Klebsiella pneumoniae* subsp. *ozaenae*	854 bp	ATCC 11296(T)	99.53

2.3.4 Complete 16S rRNA sequencing of isolates AI5a and AI4

Complete sequencing of the 16S rRNA gene of isolates AI5a and AI4 was done using the 4 Universal 16S rRNA primers mentioned earlier. Using EzBioCloud (Yoon et al., 2017) analysis, isolate AI5a showed 100% similarity to *Glutamicibacter nicotianae*

with 100% completeness of sequence and isolate AI4 showed 100% similarity to *Rhodococcus pyridinivorans* with 97% completeness of sequence in EzBioCloud. *Glutamicibacter nicotianae* (previously, *Arthrobacter nicotianae*) is one of the reclassified *Arthrobacter* species (Busse, 2016; Busse & Wieser, 2018). The two AHL degrading isolates *Glutamicibacter nicotianae* AI5a and *Rhodococcus pyridinivorans* AI4 were submitted at National Centre for Microbial Resource (NCMR), Pune, India. The 16S rRNA sequences were also submitted on the NCBI website. Their MCC and NCBI accession numbers are as follows- *Glutamicibacter nicotianae* AI5a (NCMR Accession no. MCC 4086, NCBI Accession no. MH707177) and *Rhodococcus pyridinivorans* AI4 (NCMR Accession no. MCC 4021, NCBI Accession no. MH707179).

2.3.5 Confirmation of identity of partially sequenced CRD13.3C isolate using *Rhodococcus erythropolis* specific 16S rRNA gene primers

Isolate CRD13.3C has been partially sequenced and identified as *Rhodococcus erythropolis*. To further confirm the identity of isolate CRD13.3C we used *Rhodococcus erythropolis* specific 16S rRNA primers

ReF1- 5' CGTCTAATACCGGATATGACCTCCTATC 3' and

ReR2- 5' GCAAGCTAGCAGTTGAGCTGCTGGT 3' (Bell et al., 1999).

Rhodococcus pyridinivorans AI4 was used as negative control where no amplicon was observed in spite of it being genus *Rhodococcus*. This proves that the primers ReF1 and ReR2 were specific to 16S rRNA gene of *Rhodococcus erythropolis* only and does not amplify any other *Rhodococcus* species. Amplicon size obtained after amplification using ReF1 and ReR2 primers was expected around 450 base pairs. Accordingly, around 450 base pair band was observed in case of isolate CRD13.3C samples whereas in case of *R. pyridinivorans* AI4 no amplification was observed (Fig. 2.6). The isolate CRD13.3C was thus confirmed to be *Rhodococcus erythropolis*.

Rhodococcus pyridinivorans is a bacterium which has been reported to reduce and degrade pyridine. Its use has been reported in biodegradation and bioremediation (Sun et al., 2011; Obi et al., 2020). *Rhodococcus pyridinivorans* is a non-pathogenic bacterium and has also been reported in biosynthesis of extracellular zinc oxide particles which is used in *in vitro* drug delivery (Kriszt et al., 2012; Kundu et al., 2014).

Its bioaugmentation is used for remediation of Di (2- ethylhexyl) phthalate contaminated soil (Zhao et al., 2018). Though many other *Rhodococcus* species have been reported to be quorum quenching isolates there have been no reports of *Rhodococcus pyridinivorans* being a quorum quenching isolate. Our study is the first to do so.

Glutamicibacter nicotianae is a recently classified bacteria, it was earlier classified as *Arthrobacter nicotianae* as mentioned earlier. *Arthrobacter* sp. has been reported to contain an AHL degrading enzyme AhlD (Park et al., 2003) but since being separated from *Arthrobacter* sp., *Glutamicibacter* sp. has not been reported as a quorum quenching bacteria. As it was reclassified in 2018, the reports for *Glutamicibacter nicotianae* are not much but it has been reported to be heavy metal tolerant and its use has been observed in remediation of distillery effluent contaminated soil (Duraisamy et al., 2020). It has been also reported to work alongside *Acinetobacter tandoii* to resist cadmium pressure to stop the biodegradation of Dipropyl Pthalate (Wang et al., 2021). These three isolates *Glutamicibacter nicotianae* AI5a and *Rhodococcus pyridinivorans* AI4 and *Rhodococcus erythropolis* CRD13.3C are used as quorum quenching biocontrol isolates against pathogenicity of phytopathogen *Pectobacterium carotovorum* subsp. *carotovorum* BR1 in further studies.

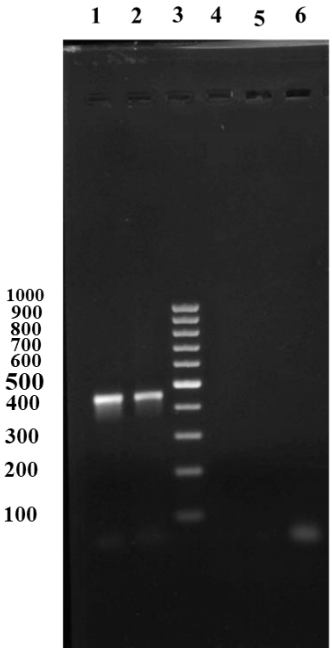

Figure 2.6 Confirmation of *Rhodococcus erythropolis* CRD13.3C using *Rhodococcus erythropolis* specific primers. *R. pyridinivorans* AI4 was used as negative control which shows no amplification (Lanes 4 and 5).