Chapter 2
Isolation of special strains of *Bacillus* spp. possessing AHL degrading activity and their identification

2.1 Introduction

Biological control of plant pathogens is an alternative way of reducing the use of chemicals in agriculture (Compant et al. 2005). Microorganisms that can grow in rhizosphere are ideal as biocontrol agents, because the rhizosphere affords primary defense for roots against attack by pathogens. Pathogens encounter antagonism from rhizosphere microorganisms before and during primary infection and also in the course of secondary spread on the root (Weller 1988). An ideal biocontrol agent should have a good degree of persistence and aggressiveness but be non-pathogenic to the host. The *Bacillus* sp. comprises a great number of strains that act as biological control agents, the antagonistic properties of which are based on the activation of multiple mechanisms. Members of the *Bacillus* genus are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for phytopathogen growth. *Bacillus* sp. is involved in the control of plant diseases through a variety of mechanisms of action, such as competitive root colonization, antibiosis, lytic enzyme production, detoxification and degradation of virulence factors and induction of systemic resistance (Tomashow and Weller 1996). Their spore-forming ability is an attribute that makes these bacteria suitable candidates for developing biopesticide products that are efficient from a technological point of view as *Bacillus* spores have a high level of resistance to the dryness necessary for formulation into stable products.

Moreover, *Bacillus* sp. are also a pool for identifying potential isolates which produce quorum quenching enzymes. A soil bacterial isolate of Gram-positive *Bacillus* species produced a quorum quenching enzyme encoded by the *aiiA* gene (Dong et al. 2000), and characterized as an AHL-lactonase was the first to be identified (Dong et al. 2001). Later, a range of other bacterial isolates and strains that produce AHL degradation enzymes were identified from soil, plant and biofilm samples as well as from laboratory bacterial culture collections. The data suggest that ~ 43% of the quorum quenching bacteria are reported from *Bacillus* genera (Uroz et al. 2009; Dong and Zhang 2005, Czajkowski and Jafra 2009). Antibiotic production has been the major mechanism of microbial antagonisms commonly exploited in the biocontrol of bacterial and fungal diseases. Thus finding that quorum sensing could be a widely conserved mechanism in the regulation of virulence suggests that quorum quenching mechanisms might have promising potentials in biocontrol (Dong and Zhang 2005). Hence, these quorum quenching enzymes could also be explored as a new version of antagonism for the biocontrol of microbial infections. Taking these aspects into consideration

present study involves isolation and screening of *Bacillus* sp. for AHL degradation phenotype and identification of the selected isolates.

2.2 Materials and methods

2.2.1 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). 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 and Venturi 2007). Biosensor strain *C. violaceum* CV026 was grown overnight and maintained in LB at 30°C with Kanamycin (30µg/ml).

2.2.2 Isolation of sporulating bacteria from root

Root samples of carrot (*Daucus carota)*, beetroot (*Beta vulgaris L*), maize (*Zea mays*), fenugreek (*Trigonella foenumgraecum*), potato (*Solanum tuberosum*), pigeon pea (*Cajanus cajan*), radish (*Raphanus sativus*), pearl millet (*Pennisetum glaucum*), and eggplant (*Solanum melongena*) were either obtained from local market or from the fields. Roots were rinsed with distilled water for several times to remove the adhering soil. The terminal portion of roots (approximately 2.5 inch long) was dipped in Ringer's salt solution and sonicated for 2 min for 1-5 cycles in sonic bath. Ringer salt solution (500 ml) contained 1.25 g NaCl, 0.05 g KCl, 0.06 g $CaCl_2.2H_2O$ and 0.25 g $NaHCO_3$. Root samples were given heat treatment at 80°C for 20 min after sonication and were dried in the laminar flow hood. The dried roots were placed on the surface of isolation medium (CM semi solid medium), after overnight incubation at 30°C different isolated colonies growing surrounding the root were picked up. The method was adapted form Fall et al. (2004)

The composition of isolation medium is as follows:

Casein mannitol medium (CM) composition: (g/L)

Casein digest	10 g
D-mannitol	10 g
Agar	10 g

Metals solution 500X stock

$CaCl_2 \cdot 2H_2O$	1.5 g
$MgSO_4 \cdot 7\ H_2O$	50 g
$MnSO_4 \cdot H_2O$	0.5 g
$CoCl_2 \cdot 6H_2O$	100 mg
$Na_2MoO_4 \cdot 2H_2O$	100 mg
$CuSO_4 \cdot 5H_2O$	50 mg
$ZnSO_4 \cdot 7H_2O$	50 mg
H_3BO_3	20mg

This isolation medium was sterilized and cooled to 50°C. A working stock of metal solution was obtained by diluting 500X to 100X and for each 100 ml, 50 mg $FeSO_4 \cdot 7H_2O$ and 50mg ascorbic acid were added, and the solution was filter sterilized and was used to prepare plates. CM agar plates were usually allowed to dry for 4–6 hour before use.

2.2.3 Isolation of sporulating bacteria from rhizospheric soil

The rhizospheric soil samples were collected from garden from the different areas of The Maharaja Sayajirao University of Baroda (Gujarat, India) campus and were taken into distilled water, soil particles were allowed to settle down (Wahyudi et al. 2011). Heat treatment was given to supernatant as above and bacteria were isolated on LA medium.

2.2.4 Bioassay for AHL degradation

N- Hexanoyl DL- homoserine lactone (C6-HSL) degrading activity of the isolates was detected using the biosensor *C. violaceum* CV026, which produces the purple pigment violacein in response to externally added C6-HSL (Blosser and Gray 2000). Bacterial isolates to be tested were grown overnight and centrifuged at 7000 rpm for 5 mins. Cell pellet was resuspended in phosphate buffered saline (PBS pH 7.4). 80 µl of 25µM C6-HSL (Sigma Aldrich) prepared in PBS was added to 20 µl of pelleted cell suspension and the mixture was incubated at 30°C for 2 hours. 25µM C6-HSL without the cell suspension of the isolates was

used as a control. For the estimation of remaining C6-HSL the reaction mixture was added to the biosensor *C. violaceum* CV026 in 96 well microtiter plates. *C. violaceum* CV026 was grown overnight with Kanamycin (30µg/ml) and was diluted to 0.002 OD by adding sterile LB before adding to the well of microtiter plates. After overnight incubation at 30°C under static conditions, O.D. at 585nm and 660nm was taken. Visible purple pigmentation in wells indicated presence of AHL. The amount of C6-HSL left in the wells was quantified using Violacein Units which has the formula as follows:

$$\text{Violacein units (V.U.)} = (A_{585\ nm} / A_{660\ nm}) \times 100$$

2.2.5 Genomic DNA extraction

Genomic DNA extraction of isolates was done by CTAB method (Sambrook and Russell, 2001). Overnight grown bacterial cultures (1.5 ml) were resuspended in 300 µl of TES (25mM Tris-Cl pH 8.0, 25mM EDTA pH 8.0, 300mM sucrose) and 10 µl of lysozyme (40mg/ml) was added to it and were kept for incubation at 37°C for an hour. Samples were treated with 4 µl of proteinase K (20mg/ml) and 20 µl of 10% SDS (sodium dodecyl sulphate) and were kept for incubation at 55°C for an hour. Samples were mixed thoroughly after adding 67 µl of 5M NaCl and to this 44 µl of (10% CTAB/0.7M NaCl) was added and again kept for incubation at 65°C for 10 min. Genomic DNA was extracted using phenol, chloroform and iso-amyl alcohol treatment and precipitated using isopropanol. The genomic DNA was washed with 70% ethanol and the pellet was dissolved in 20 µl of distilled water. RNAase treatment was given at 65°C for 10 min and presence of DNA was verified by running the samples in 0.8% agarose gel electrophoresis in 1X TAE buffer and visualized by staining with ethidium bromide (0.5µg/ml) and photographed using transmitted UV light at 295 nm.

2.2.6 PCR amplification of 16S rRNA gene of isolates

Full length 16S rRNA genes were amplified using the 16S ribosomal DNA universal bacterial primer set 27F (5'-AGAGTTTGATCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGCA3'). 50 µl PCR reactions were performed in an Eppendorf thermocycler and contained 200µM deoxynucleoside triphosphate mix, 0.2µM of each primer, 1.5mM $MgCl_2$, PCR buffer, 1.5 U Taq DNA polymerase and 1 µl genomic DNA template. 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 sec, annealing at 58°C for 30sec and

extension at 72°C for 1 min. A final extension at 72°C for 10 min was then performed. The amplification of PCR products was verified by 1.5% (w/v) agarose gel electrophoresis in 1X TAE buffer and visualized by staining with ethidium bromide (0.5µg/ml) and photographed using transmitted UV light at 295 nm.

2.2.7 Amplified ribosomal DNA restriction analysis (ARDRA)

The 16S rRNA gene amplicons were used for ARDRA. Following three restriction enzymes (RE) were used: *RsaI, AluI* and *HhaI*. Each 15µl digestion reaction consisted of 1X Tango Buffer, 7U of RE, and 8µl of 16S amplicon. Reaction was incubated overnight at 37°C. RE patterns were visualized in 2.5% (w/v) agarose gel electrophoresis in 1X TAE buffer by staining with ethidium bromide (0.5µg/ml) and photographed using transmitted UV light at 295 nm. The RE patterns were then analyzed using Alphaquant and NTSys softwares to construct a phylogenetic tree.

2.3 Results

2.3.1 Isolation of sporulating bacteria

Total 97 isolates were obtained by two different isolation methods. Isolation of bacteria from different plant roots (Figure 7) yielded 38 isolates and 59 isolates were obtained from rhizospheric soil. Vegetative cells of bacteria were eliminated through heat treatment and consequently sporulating bacteria were enriched. At preliminary level, Gram staining of all isolates confirmed them to be Gram positive sporulating rod shaped bacteria.

2.3.2 Screening of isolates for C6-HSL degrading ability

Total of 97 isolates were screened using C6-HSL degradation bioassay for selecting the isolates with AHL degrading ability. C6-HSL degradation bioassay using *C. violaceum* CV026 as the biosensor allowed categorising the isolates into three classes based on violacein pigment production and Violascein unit (V.U.). The observed V.U. was inversely proportional to the C6-HSL degrading ability of the isolate. Out of 97 isolates, 20 isolates had

Figure 1 Representative culture of *Bacillus* sp. isolated from root sample

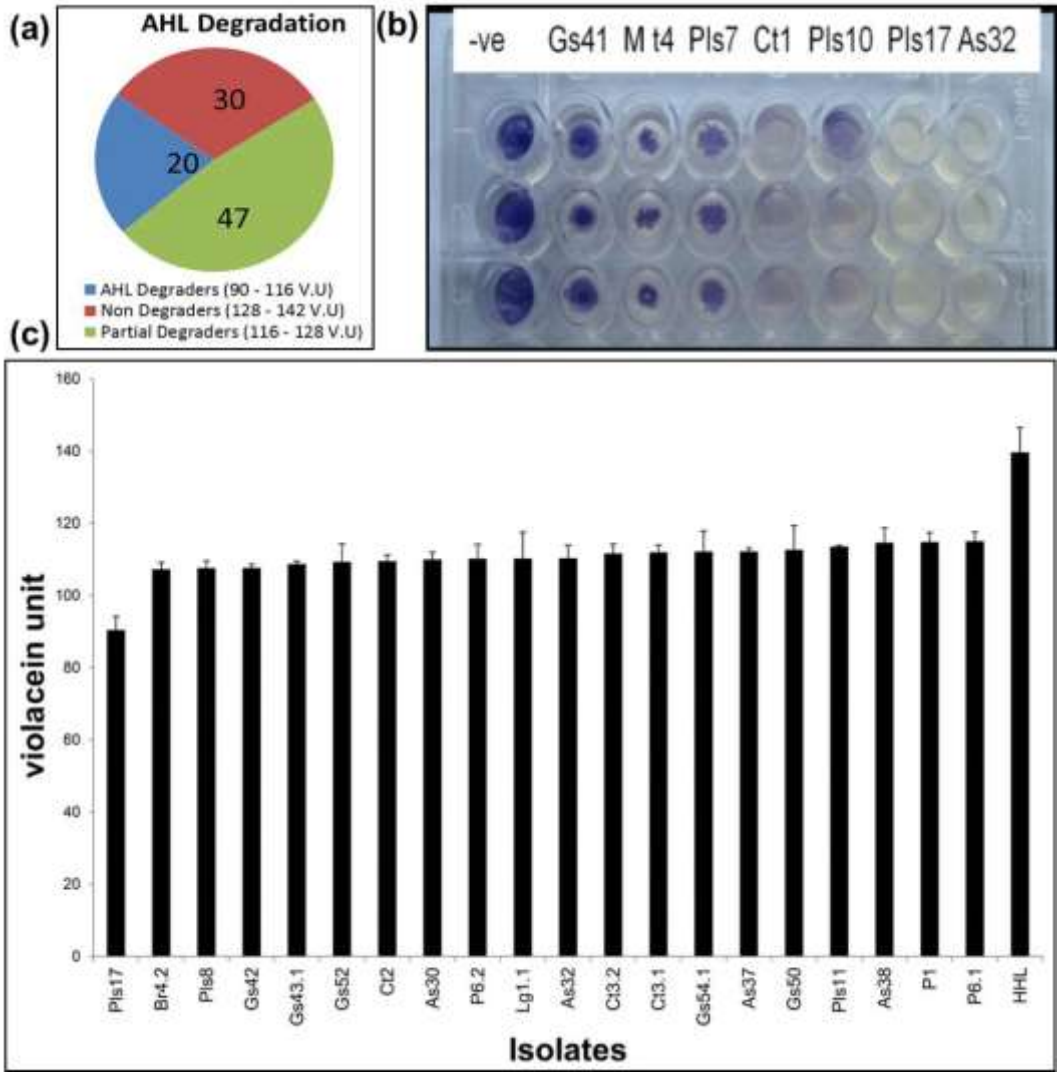

Figure 2 Screening of isolates for C6-HSL degrading ability

(a) Grouping of AHL degrading isolates **(b)** Microtiter plate representing AHL degrader (As32, Pls17), partial degrader (Pls10, Ct1) and non-degrader (Pls7, Mt4, Gs41) and no degradation control (-ve) **(c)** Comparison of violacein units of selected twenty AHL degrading isolates. Error bars indicate standard deviation of three independent measurements.

V.U. of 90-117, 47 isolates had V.U. of 116-128 and 30 isolates had V.U of 128-142 and were categorised as C6-HSL degraders, partial degraders and non-degraders respectively (Figure 8 (a) (b)). However, it was observed that there was only marginal difference in the V.U. (C6-HSL degrading ability) of the C6-HSL degradation positive isolates (Figure 8 (c)).

2.3.3 Genotypic characterization of selected isolates

In order to obtain non redundant isolates among the selected 20 C6-HSL degraders, they were genotypically characterised using an amplified rDNA restriction analysis (ARDRA) to cluster the isolates on the basis of phylogenetic similarity. 16S rRNA gene of all 20 isolates was amplified (Figure 9(a)) and the amplicons when digested with restriction enzymes *RsaI*, *AluI* and *HhaI* (Figure 9 (b), (c) and (d)) gave three different restriction digestion patterns which divided the isolates in three groups. Group 1 included P1, Gs52, As37, As38, Gs50, Ct2, Gs54.1 and Pls11. Group 2 included P6.1, P6.2, Ct3.1, Ct3.2, As32, Lg1.1, Pls17, Br4.2, Pls8, Gs42, Gs43.1 and Group 3 contained only As30 (Figure 10). Six isolates (Pls8, Pls17, As30, Gs42, Gs50 and Gs52) based on Violacein Unit, colony morphology and ARDRA patterns were chosen for further studies. 16S rRNA gene sequences (sequenced at Xcelris, Ahemdabad, India) for the selected six isolates were compared with 16S rRNA gene sequences available in Nucleotide database using NCBI blast tool and Ribosomal Database project (http://rdp.cme.msu.edu/). Five isolates belonged to *Bacillus* genus while one isolate Gs50 belonged to *Lysinibacillus* genus. Table 5 gives the source of isolation, identification of the isolates and their Genbank accession numbers.

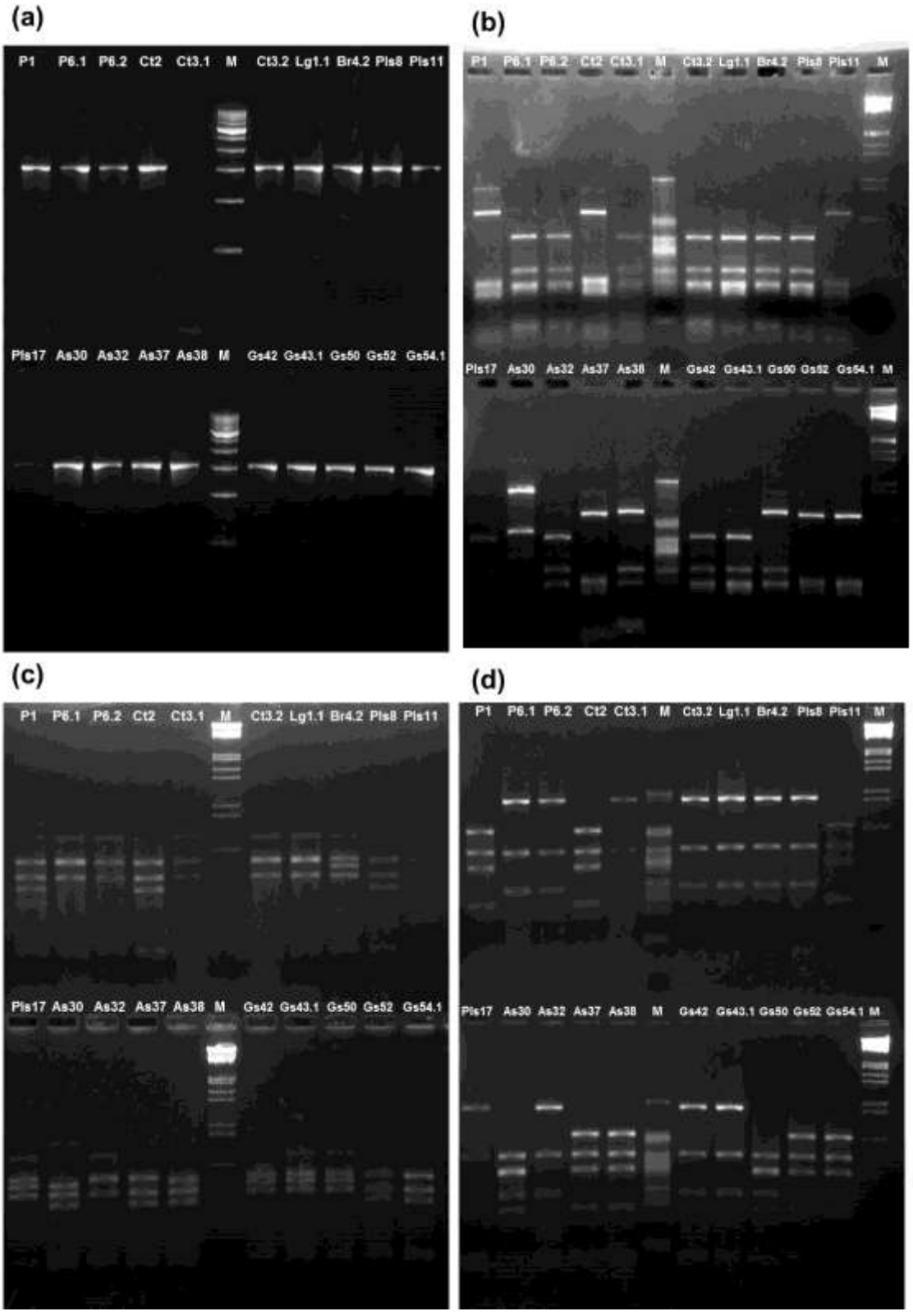

Figure 3 Genotypic characterization of selected isolates

(a) 16S rDNA fragment amplicons of twenty selected isolates. ARDRA patterns of the twenty isolated with **(b)** *Alu*I **(c)** *RsaI* and **(d)** *HhaI*. M represents molecular weight marker

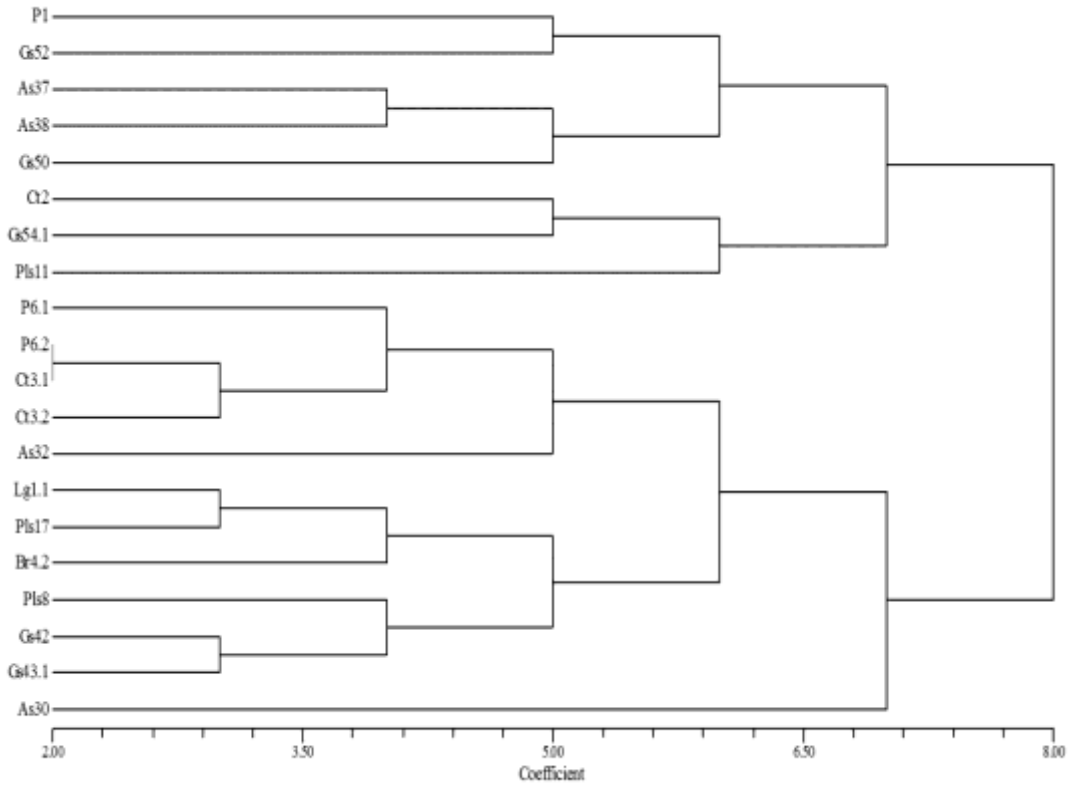

Figure 4 Dendrogram based on ARDRA analysis showing three major clusters of the twenty AHL degrading isolates

Table 1 Identification of six selected isolates based on 16S rDNA sequencing

Source	Isolate	NCBI match	Similarity	Accession no.
Soil	Pls8	*Bacillus subtilis*	99%	KR709146
Soil	Pls17	*Bacillus aerius*	99%	KR709147
Soil	As30	*Bacillus firmus*	98%	KR709148
Soil	Gs42	*Bacillus subtilis*	99%	KR709151
Soil	Gs50	*Lysinibacillus sp.*	99%	KR709144
Soil	Gs52	*Bacillus thuringiensis*	99%	KR709145

2.4 Discussion

Faure and Dessaux (2007) have highlighted that quorum sensing is an emerging biocontrol target for phytopathogens because of two main reasons (1) Bacteria of the genera *Agrobacterium, Brucella, Burkholderia, Erwinia, Enterobacter, Pseudomonas, Ralstonia,*

Serratia, Vibrio and *Yersinia* utilize the quorum sensing mechanism for regulation of the virulence factors synthesis (2) Quorum quenching strategies do not aim to kill bacteria or limit bacterial growth but affect the expression of a specific function exerting a limited selective pressure for microbial survival than biocide treatment. This is a valuable trait for the development of sustainable biocontrol or therapeutic procedures in the present context of rising antibiotic resistance. After knowing this fact researcher started isolating and screening bacterial strains which were capable of AHL degradation. Till date, bacterial genera such as *Bacillus, Pseudomonas, Rhodococcus* and *Ralstonia* and other organism including *Acidobacteria, Agrobacterium tumefaciens, Arthrobacter* sp., *Klebsiella pneumoniae, Photorhabdus luminescens, Rhizobium leguminosarum, Variovorax paradoxus, Streptomyces* sp., *Nostoc* sp., *Shewanella* sp., *Comamonas*, sp., and *Anabaena* sp. are reported to exhibit AHL degrading ability (Uroz et al. 2009; Dong and Zhang 2005; Czajkowski and Jafra 2009). Moreover, in *Bacillus* genera majority of its species like *B. thuringiensis, B. cereus, B. anthracis, B. mycoides, B. subtilis, B. amyloliquefaciens, Geobacillus* sp. are known to produce quorum quenching enzyme (Dong et al. 2002; Lee et al. 2002; Pan et al. 2008; Yin et al. 2010; Augusine et al. 2010; Seo et al. 2010). Major attention to be given to the fact that in spite of so many microorganisms reported with quorum quenching ability very few of them have been explored for their biocontrol potential against quorum sensing plant pathogens. Research carried out in this area mainly involves heterologous expression of genes encoding for quorum quenching enzymes in pathogen cell or in plant tissue (Dong et al. 2001; 2002) or employing natural quorum quenching bacteria as biocontrol agents (Steidle et al. 2001; Morello et al. 2004; D'Angelo-Picard et al. 2004, 2005). The latter biocontrol strategy is more acceptable and doesn't involve any ethical issue of usage of genetically modified plant or bacteria to be used for biocontrol. Therefore, it is preferred to isolate and screen for the bacteria with quorum quenching ability and use them for the biocontrol of quorum sensing phytopathogens.

Hence, the objective of work undertaken was to isolate plant associated *Bacillus* sp. either from root surface or from soil and screen them for their AHL degrading ability. Total 97 isolates were screened for AHL degrading activity, post screening 20 isolates were found out to be positive for the phenotype in the present conditions of screening. As could be observed the screening on the basis of violacein unit did not allow much differentiation in the AHL degrading abilities of the twenty isolates ARDRA was carried out. For this reason and also in order to obtain non redundant isolates the 20 isolates were subjected to ARDRA and eight

isolates were selected for further studies. It is noted that this study resulted in identifying three *Bacillus* sp. namely, *B. aerius* Pls17, *Lysinibacillus* sp. Gs50 and *B. firmus* As30 which are not yet reported for their AHL degradation ability along with known *Bacillus* like *B. thuringiensis* and *B. subtilis*. Special attention to be given to *Lysinibacillus* sp. Gs50 as organisms in this genus were previously regarded as members of the genus *Bacillus*, but the taxonomic status of these microorganisms was changed to the genus *Lysinibacillus* in 2007 and it is commonly found in soil (Ahmed et al. 2007). *Lysinibacillus* is a Gram-positive, rod-shaped, and round-spore forming genus in the family *Bacillaceae*. This is the first report of demonstrating the AHL degrading potential of *Lysinibacillus* genus. Long term implication of the study is the use selected efficient AHL degraders as biocontrol agents against quorum sensing phytopathogen therefor exhaustive evaluation of their biocontrol attributes and potential is mandatory.