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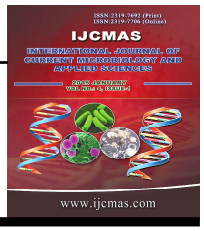

Original Research Article

Enzyme purification and kinetic characterization of AHL lactonase from *Bacillus sp*.RM1 a novel and potent quorum quencher isolated from Fenugreek root nodule rhizosphere

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Keywords

Bacillus sp., AiiA lactonase, Quorum Quenching, *Vibrio sp*.

A B S T R A C T

Rhizospheric environment is packed with complex intra species bacterial interactions. Majority of soil bacteria interact via quorum sensing (QS) and often bacteria capable of quenching this type of intracellular communication is also found in the close vicinity. Hence rhizospheric soil from fenugreek root nodule rhizosphere was chosen as a source, for isolating probable quorum quenchers. An isolate with strong ability to degrade bacterial QS signal molecules was obtained and it was phylogenetically characterized to be a novel sp. of *Bacillus.* The quorum quenching AHL Lactonase isolated from *Bacillus sp.* RM1was then purified and kinetically characterized. The V_{max} and K_m values for AHL lactonase catalyzed cleavage of C6HSL were: 52.26 mM AHL degraded/hour/mg of protein and 0.3133 mM respectively. Additionally the AiiA lactonase from *Bacillus sp.* RM1was also found to have the ability to disrupt biofilm formed by an important human pathogen *Vibrio cholerae.*

Introduction

Soil ecosystems are full of complex bacterial interaction. Both Quorum sensing and Quorum quenching are a constant feature in these mixed bacterial communities and they play an important ecological role in the soil environment. Gram negative bacteria present in soil, also communicate via AHL based QS systems as well and concentration of these signal molecules in soil have been shown to lie within the range of nano-molar to milli-molar (Dessaux et al., 2011). It is suggested that a possible diffusion zone of 4–80 mm exists for these AHL signals which allows nearby members of the same population to sense the signals and modulate gene expression accordingly (Dessaux et al., 2011). Numerous bacterial phenotypes are regulated via AHL mediated QS, like, biofilm formation and virulence genes expression (Fuqua et al., 2001). In soil environments where competition is inevitable any organism capable of quenching quorum sensing mediated gene expression of a nearby bacterial population will definitely be in a profitable situation as far as colonization of a particular niche is

concerned. Furthermore it is not surprising to find organisms that degrade AHLs,invariably in contact with large AHL-producing bacterial communities. Such a situation was first demonstrated in a biofilm isolated from a water reclamation system by Hu et al (2003). It is therefore enticing to hypothesize that origin of AHL degradative capability may have resulted from a co-evolution of degradative organisms and AHL-producing bacteria. An interesting information to support this arises from the fact AHL degradation activity is strongest in the rhizosphere of leguminous plants as compared to non-leguminous plants. This activity is almost always coupled with uninhibited presence of bacteria communicating via AHL signals, as AHL-based QS signaling required for regulation of various rhizosphere and symbiosis-related functions (Jafra et al., 2006).Thus quorum quenching (QQ) capability is widespread in soil ecosystems and effectors include both enzymes and bio-molecules. AHL lactonases are hydrolases that cleave the lactone ring of AHLs rendering them biologically inactive and they have been reported in numerous members of the firmicute clad especially *Bacillus sp.*, an organism that is found most commonly in soil ecosystem (Lee et al., 2002). It is now known that the *aiiA* gene encoding the AHL lactonase enzyme is widespread among *Bacillus sp*., especially among, strains of *Bacillus cereus* group, including *Bacillus thuringiensis*, *B. cereus*, *Bacillus anthracis* and *Bacillus mycoides* (Huma et al., 2011; Pedroza et al., 2014).

First report of an AHL lactonase from genus *Bacillus* was by Dong et al (2000). They cloned and over expressed the gene, *aiiA 240B1* encoding an AHL-inactivating enzyme (AiiA240B1), from *Bacillus sp*. strain 240B1.The enzyme was found to severely attenuate pathogenecity of an important plant pathogen *Erwinia carotovora* which elaborates its virulence genes via 3-oxo C6HSL dependent QS circuit (Dong et al., 2002). AHL lactonases have also been described in other Firmicute like genus *Arthrobacter* and in Actinobacteria (*R. erythropolis*) etc. (Park et al., 2003; Uroz et al., 2008; Huang et al., 2012; Wand et al., 2010; Mei et al., 2010). AHL lactonases generally exhibit a very broad AHL substrate range, and hence they may be used in bio-control or genetic engineering approaches to impede QS communication in different bacteria. This manuscript describes isolation of a novel species of *Bacillus* from Fenugreek (Methi) root nodule rhizosphere and investigation into its ability to quench gram negative quorum sensing by producing a potent AHL lactonase.

Materials and Methods

Microbes and culture conditions

Chromobacterium violaceum CV026, a mini tn5 mutant of wild type *C. violaceum* ATCC 31532, was used as the biosensor strain to monitor AHL degradation activity. The culture was grown in Luria bertani medium supplemented with antibiotics, 30µg ml^{-1} Kanamycin and 100µg ml^{-1} Ampicillin at $30^{o}C$ for 16-18 hours.CV026 is incapable of synthesizing AHLs of its own but responds to exogenously supplied AHL molecules by synthesizing purple pigment violacein. Quorum quenching *Bacillus sp*.RM1 was grown in Luria Bertani medium at $28^{o}C$, 185 rpm for 36 hours to obtain maximum enzyme production.

Isolation of quorum quenching bacteria from rhizospheric soil

Bacteria with quorum quenching potential were isolated from rhizospheric soil around

Methi (Fenugreek) root nodules. 1gm of rhizospheric soil was added to 10 ml of sterile saline, mixed well and it was then serially diluted to 10^6. The last three highest dilutions were plated on minimal medium. Oligotrophic conditions were maintained to prevent overgrowth on agar plates. Colonies with distinctive morphology were selected and were streaked on a fresh agar plate to get pure culture of the isolates. Selected isolated were then checked for their ability to inhibit AHL mediated gram negative QS.

Preliminary screening of its quorum quenching activity

The isolated organisms were screened for their quorum quenching potential by the method established by McLean et al (2004) with slight modifications. This method employs the use of *Chromobacterium violaceum* CV026 as a biosensor strain. CV026 is a mini Tn5 mutant of the wild type *C. violaceum* which is unable to produce violacein because it is defective in AHL production. Violacein production in CV026 is induced by externally supplied synthetic AHLs with a medium chain length of C6-C8 or a 3-oxo-acyl side chain. In this screening method, the isolates were streaked on a Luria Bertani (LB) Agar plate and then incubated for 36 hours at 30^{o}C. The plate is then overlaid with 10 ml LB soft agar (0.8% agar) containing 50µl of CV026 culture and 2.5 µM cognate signal molecule-C6HSL. After 24 hours of incubation at 30^{o}C, isolates with quorum sensing inhibitory activity show zone of inhibition of violacein synthesis around the streak region. After this qualitative detection of QQ potential of the isolates a more quantitative assay was performed to provide further confirmation.

16rRNA based identification of positive isolate

Genomic DNA from the positive culture was isolated according to the modified method reported by Sambrook et al. (1987). The extracted genomic DNA was quantified and the 16S rRNA gene was amplified using universal primers 8F and 1525R (Stackebrandt et al., 1991) in a PCR reaction mixture containing 2.5U of AmpliTaq Gold (Invitrogen) and PCR conditions used were as follows: an initial denaturation at 94°C for 5min, with 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min and extension at 72°C for 1 min with additional extension at 72°C for 10 min and finally held at 20°C. The amplicons were then subjected to PCR clean up and sequenced using internal primers. Chromas Pro version 1.34 (Technelysium Pty Ltd., Tewantin, Queensland, Australia) was used for assembling the reads into a contig. The sequence was subjected to database matching in NCBI BlastN (http://www.ncbi.nlm.nih.gov). Similar sequences were subjected to multiple sequence alignment using CLUSTAL W. Phylogenetic tree was computed in MEGA 4 using the neighbor joining method (Tamura et al., 2007).

Whole cell AHL degradation bioassay to understand the mode of AHL degradation

The positive culture showing potent QQ capability was then subjected to whole cell AHL inactivation assay to understand whether an AHL lactonase or an AHL acylase was responsible for its degradative activity. *C. violaceum* CV026 was used as a biosensor for this assay. Thoroughly washed 20 mg cells of *Bacillus sp*. were suspended in 500 µl of 0.1 M phosphate buffer pH 7.0 and 0.5 mM of C6HSL was added to the reaction mixture. At 0 hour and after 2 hours of incubation at 40^{o}C, 50 µl of reaction mixture was taken, after spinning down the cells and added to wells dug in a LB agar plate overlaid with 10ml of LB soft agar

containing 75µl of overnight culture of *Chromobacterium violaceum* CV026.The plates were incubated at 30^{o}C overnight and checked for zones of violacein synthesis around the wells. The diameter of the zone of violacein synthesized by the biosensor strain CV026 is proportional to the amount of AHL remaining in the reaction mixture after incubation period. Appropriate controls were maintained for each concentration of AHL used and it was observed that there was no spontaneous degradation of AHL molecule at the said pH of reaction mixture.

To understand whether this enzymatic degradation of QS signal molecules is due to an AHL lactonase or AHL acylase a simple test was performed. The reaction tubes containing appropriate amount of AHL and 20 mg washed cells were incubated at 40^{o}C for two hours or more to allow complete degradation of AHLs. After complete degradation of provided AHL the reaction mixture was acidified to pH 2.0 .This was done to promote re-lactonization of AHL ring structure if it has been acted upon by a lactonase. The tubes were incubated at room temperature for two hours. Presence of re-lactonized, biologically active, AHLs, can be detected by the ability of CV026 to perceive them and in-turn synthesize violacein. Inability of degraded AHL to regain their activity even after acidification indicates presence of AHL acylase activity, which is an irreversible reaction.

Growth of Bacillus sp. RM1 in minimal medium containing AHL as sole source of carbon

KG Chan et al (2009) first introduced the KG Medium. It is a chemically defined medium supplemented with AHL as a sole source of carbon. Ability of an organism to grow in this basal medium depends on its capacity to degrade AHL molecules and use it for its growth and metabolism (Chan et al., 2010). 10ml of KG medium with 500µM of three different AHL molecules namely C6 HSL,C7 HSL and 3-OXO-C6 HSL were used to check for growth of the test organism. The medium was incubated for 48 hours at 28^{o}C and 180 rpm to determine growth. Two Control tubes were used, KG medium with AHLs only (no test culture) and KG medium without AHL (hence devoid of any carbon source) but inoculated with test organism were used.

Purification of QQ enzyme (AHL lactonase) from Bacillus sp RM1

Crude enzyme preparation

Maximum enzyme production was obtained after growing the culture in Luria Bertani broth at 28^{o}C for 36 hours and 180 rpm. After incubation period cells were harvested by centrifuging 50ml of 36 hours old culture broth at 8000rpm for 5 minutes. Spent medium was discarded and the cell pellet (approx. 1gm) was then suspended in 0.1M phosphate buffer pH 7.0 (ratio of cell pellet to buffer was 1:3,i.e. 1gram cells suspended in 3ml buffer).The cell suspension was then subjected to sonication for 10 minutes at 70 amplitude, with 10 seconds ON and 10 seconds OFF pulse cycle. Sonicated cell suspension was then centrifuged at 10000 rpm for 30 minutes at 4^{o}C, to remove cell debris and obtain clear cell lysate. Cell lysate was subsequently subjected to ammonium sulfate fractionation to obtain crude enzyme fraction. Initially the cell lysate was saturated with 30% of ammonium sulfate (w/v) for 4 hours. The precipitate thus obtained was collected by centrifugation and the supernatant was further saturated with 80% of ammonium sulfate (w/v) for 6 hours. The protein precipitate obtained with 80% saturation was also collected and the supernatant was discarded after checking for absence of AHL

degradative activity. All the samples, i.e. sonicated cell debris, sonicated supernatant before ammonium sulfate saturation, 30% saturation fraction and 80% saturation fraction were checked for their ability to degrade AHLs using CV026 based bioassay. The 80% saturation precipitate was found to contain the crude AHL lactonase. Crude protein preparation was then dialyzed overnight in 100X volume of 10mM phosphate buffer pH 7.0 and purified further using Q-Sepharose column chromatography.

Column chromatography using Q Sepharose for enzyme purification

A 9cm high 2 cm wide column of Q-Sepharose was packed and was equilibrated with 0.1M phosphate buffer 7.0.Washed and equilibrated column was then loaded with 3-4 ml of dialyzed crude protein sample containing enzyme of interest was slowly allowed to pass through the column once. Flow through was collected and the column was washed with 3 times its volume of equilibration buffer to remove unbound proteins. Protein fractions were eluted (4 ml each fraction) using elution buffer of 0.1M phosphate buffer pH 7.0 with 300mM NaCl. Fractions were then checked for AHL lactonase activity with the help of CV026 based bioassay as described previously using: 200µl of each fraction and 0.25mM of C6HSL and two hours incubation at 40^{o}C. Protein estimation of fractions was done using Bradford assay and the fraction showing lactonase activity were subsequently pooled and dialyzed with 100X buffer to remove excess of salts and concentrated using Amicon Centricons with 3kDa MWC.

Native PAGE gel electrophoresis

Fractions showing enzyme activity were also run on NATIVE PAGE to confirm the molecular weight of the protein of interest when compared with a molecular weight standard.

Optimization of reaction parameters to obtain maximum AHL degradation

To determine optimum conditions for AHL degradation by concentrated AHL lactonase obtained from *Bacillus sp*. RM1 a standard plot of zone diameter of violacein synthesis v/s AHL concentration was plotted. Buffers in the pH range of 6-8.5 were used to determine the optimum pH for AHL cleavage by the enzyme. Reaction incubation temperatures of 20, 30, 40, 50, and 60^{o}C were tested to determine the optimum temperature which favored maximum AHL degradation by *Bacillus sp*.RM1 AHL lactonase. Incubation time of one hour, Substrate (C6HSL) at a concentration of 0.5mM and 36µg of AHL lactonase was used for the optimization studies. Appropriate controls for pH and temperature were used to eliminate false positive reaction obtained due to spontaneous degradation of AHLs.

Kinetic characterization of enzyme

Kinetic parameters for the enzyme reaction were determined under previously optimized conditions of pH and temperature. Range of substrate (C6HSL) concentration used was from 0.05 mM to 0.75 mM. Cleavage of C6HSL by 18µg of *Bacillus sp*. RM1 AHL lactonase was monitored and the reaction was kinetically characterized. V*max*, K*m* values were calculated from Michaelis Menten plot.

***Vibrio cholerae* based anti-biofilm assay**

A majority of virulence genes expressed by human pathogen *Vibrio cholerae* is regulated by quorum sensing pathways

including its ability to form biofilms so any enzyme that can degrade quorum sensing signal molecules may have the potential to inhibit biofilm formed by *Vibrio sp*. Partially Purified AHL lactonase from *Bacillus sp*. RM1 was checked for its ability to inhibit V.*cholerae* biofilm using method described by Augustine et al (2010) with slight modifications. A range of protein concentrations was used for this assay namely, 10, 20,50, 75 and 100µg. Briefly 10µl of overnight culture of *V.cholerae* was added to 2ml of culture media with or without presence of appropriate concentration of *Bacillus sp*. RM1 AHL lactonase, in a 6 well plate containing a sterilized cover slip in each well. The plate was then incubated at 37^{o}C for 16-18 hours .After incubation period all the spent media from the wells along with planktonic cells was discarded and the biofilm formed on the coverslip surface was gently washed twice with deionized water. The biofilm left on the coverslip was then allowed to air dry. Subsequently it was stained with 0.1% crystal violet for 10 mins. After staining, excess of crystal violet was discarded and the biofilm was washed with deionized water twice. After air drying the stained coverslips were visualized under light microscope at 40X magnification. Control coverslips (Without activity of AHL lactonase) were compared with that of test coverslips to determine the extent of biofilm inhibition by *Bacillus sp.* RM1 AHL Lactonase.

Results and Discussion

Isolation of quorum quenching bacteria from rhizospheric soil and preliminary characterization of their QQ potential

QS is known to control virulence of a variety of plant pathogens. QQ bacteria and enzyme have the potential to be used as biocontrol agents which can protect plants from its pathogens (Molina et al., 2003). Rhizospheric soil has significant density of both beneficial and harmful bacteria which are constantly competing with each other to occupy specific niche. Rhizosphere environment exhibits presence of both AHL-dependent QS and AHL-degrading activities, since both beneficial rhizosphere bacteria and pathogens use AHLs as QS molecules. This fact prompted us to look for AHL degrading bacteria in novel rhizospheric environments and in this present manuscript isolation of probable QQ bacteria from Fenugreek root nodule rhizosphere has been described. Total of 28 isolates were selected based on their colony morphology from minimal medium plates. Colony characteristics and gram nature were noted for all the 28 isolates. Initial screening of the AHL degradation activity of the isolates was performed using the centre streak method put forward by McLean et al (2004). AHL degradation activity of selected isolates can be seen in Figure 1. A number of isolates showed promising quorum quenching activity especially ones seen in Panel A, C and D. However one of the most potent isolate (seen in panel F) was selected and studied further to understand the basis of this QQ activity.

Phylogenetic identification of the positive isolate based on 16S rDNA sequencing

Genomic DNA of the positive isolate was extracted and its 16S rDNA was amplified using a set of universal primers. The amplicon thus obtained was sent for sequencing and FASTA format of the sequence of 1490 base pairs is appended below:

CATAACGCCGAATGGGATTAAAGAG
CTTGGCTCTTATGAAGTTAGCGGCGG
ACGGGTGAGTAACACGTGGGTAACCT

GCCCATAAAACTGGGATAACTCCGGG
AAACCGGGGCTAATACCGGATAACAT
TTTGAACCGCATGGTTCGAAATTGAA
AGGCGGCTTCGGCTGTCACTTATGGA
TGGACCCGCGTCGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCAA
CGATGCGTACCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCGCAATGGACAA
AAGTCTGACGGAGCAACGCCGCGTG
AGTGATGAAGGCTTTCGGGTCGTAAA
ACTCTGTTGTTAGGGAAGAACAAGTG
CTAGTTGAATAAGCTGGCACCTTGAC
GGTACCTAACCAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTATCCGGAA
TTATTGGGCGTAAAGCGCGCGCAGGT
GGTTTCTTAAGTCTGATGTGAAAGCC
CACGGCTCAACCGTGGAGGGTCATTG
GAAACTGGGAGACTTGAGTGCAGAA
GAGGAAAGTGGAATTCCATGTGTAGC
GGTGAAATGCGTAGAGATATGGAGG
AACACCAGTGGCGAAGGCGACTTTCT
GGTCTGTAACTGACACTGAGGCGCGA
AAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTAGAGGGTTT
CCGCCCTTTAGTGCTGAAGTTAACGC
ATTAAGCACTCCGCCTGGGGAGTACG
GCCGCAAGGCTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACA
TCCTCTGAAAACCCTAGAGATAGGGC
TTCTCCTTCGGGAGCAGAGTGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGATCTTAGTT
GCCATCATTAAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCA
TGCCCCTTATGACCTGGGCTACACAC
GTGCTACAATGGACGGTACAAAGAG
CTGCAAGACCGCGAGGTGGAGCTAAT
CTCATAAAACCGTTCTCAGTTCGGAT
TGTAGGCTGCAACTCGCCTACATGAA
GCTGGAATCGCTAGTAATCGCGGATC
AGCATGCCGCGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACAC
CACGAGAGTTTGTACACCCGAAGTCG
GTGGGGTAACCTTTTTGGAGCCAGCC
GCCTAAGTGGGACAGATGATTGGGGT
GAAGTCGTAACAGGTAGCCGTATCGG
AAGGTGCGGCTGGGATCCACCTCCTC
AG

The sequence was deposited in the GenBank database under the accession number KM281156. The sequence was then subjected to BLAST analysis in NCBI BlastN (http://www.ncbi.nlm.nih.gov). An un-rooted phylogenetic tree was then constructed using MEGA 4 and the isolate was found to belonging to genus *Bacillus* showing close relatedness to *Bacillus cereus* group (Fig. 2). As mentioned before AiiA homologs are widespread among *Bacillus sp*. (Huma et a., 2011) and AHL lactonase from *Bacillus thuringiensis* has already been crystallized (Liu et al., 2005). Consequently AHL lactonase activity demonstrated by our isolate, *Bacillus sp*. RM1, obtained from rhizospheric soil sample is not unanticipated.

Whole cell AHL degradation assay to confirm AHL lactonase activity of the isolate

In order to understand the mode of AHL degradation by any bacterium it is extremely important to be acquainted with the mechanism of enzyme reaction catalyzed by an AHL lactonases and an AHL acylases. Scheme 1 presents enzymatic degradation of AHLs by a lactonase and an acylase and it is easy to see that both the enzymes differ markedly in their mechanism of AHL inactivation. However it is very interesting to note that AHL cleavage by a lactonase is a reversible reaction however that mediated

via an acylase is not. Inactivation of AHLs by lactonases can be overcome by simply acidifying the reaction mixture for a specific period of time and allowing the AHL molecule to re-lactonize. This forms the basis of a simple test that allowed differentiation between lactonase or acylase activity of any given bacterial isolate. 20mg washed cells of *Bacillus sp*. RM1 was found to degrade up to 0.5mM of AHL within an hour of incubation.

The reaction period was increased for a little longer to allow complete degradation of substrate and the reaction mixture was then allowed to undergo acidification to pH 2.0 for period of 2 hours at room temperature to allow re-lactonization of degraded AHL. Recovery of biological activity of AHL as evidenced by the ability of biosensor strain to synthesize violacein was proof of AHL lactonase activity of *Bacillus sp*. RM1 (Fig. 3)

Growth of Bacillus sp. RM1 in minimal medium with AHL as sole source of energy

To further confirm the ability of the isolate under study to synthesize a QQ enzyme it was allowed to grow in KG minimal medium containing AHL as a sole source of carbon for a period of 48 hours. This novel chemically defined minimal medium, named KG medium, supplemented with AHLs was used for the first time with the intention of isolating AHL-degrading bacteria from sewage sample by KG Chan et al (2009). They used media containing 3-oxo-C6-HSL at a final concentration of 500µg/ml. In our study three different AHL molecules at a final concentration of 500µM were used as substrates, namely C6 HSL, C7 HSL and 3-OXO-C6 HSL, and *Bacillus sp*. RM1 showed good growth with all three substrates (Fig. 4). No growth was obtained in the control tubes. This confirms the ability of *Bacillus sp*. RM1 to synthesize an AHL lactonase with broad substrate specificity which is in accordance with previous reports.

Purification, optimization of reaction parameters and kinetic characterization of AHL lactonase from Bacillus sp. RM1

Intracellular AHL lactonase produced by *Bacillus sp*. RM1 was isolated by sonicating the cells and precipitating out the crude enzyme from the clear cell lysate using ammonium sulfate saturation. Q-Sepharose based Ion exchange chromatography was then utilized to get partially purified enzyme fraction. AHL lactonase activity in the collected fractions was checked using CV026 based bioassay and fractions showing decrease in violacein synthesis were considered as positive fractions containing enzyme of interest (Fig.5).

Fractions containing enzyme of interest were also run on 10% Native PAGE along with a molecular weight standard, Carbonic Anhydrase 29 kDa (SIGMA, Molecular weight markers) , to confirm the molecular weight of the AHL lactonase obtained (Fig. 6). The protein showed band at the expected size of ~28 kDa, which is similar to previous reports (Lee et al., 2008;Huma et al., 2011;Pedroza et al., 2014;Dong et al., 2000) (Only a single molecular weight marker was used because all AiiA lactonase have be known to have molecular weights between 28-29 kDa).

Column fractions showing AHL lactonase activity were then pooled together and protein content of pooled fractions was found to be approximately 0.6mg/ml. The pooled sample was then dialyzed and concentrated using Amicon's centrifugal protein concentrator. Partially purified and

concentrated AHL lactonase had a protein content of 1.8mg/ml. This was used further for optimization and kinetics studies.

Optimum parameters to get maximum AHL degradation by *Bacillus sp*. RM1 AHL lactonase were then determined. Of all the pH and temperature values tested pH 7.0 and temperature of 50^{o}C (Fig. 7) were found to give maximum AHL degradation with 1 hour incubation period. Kinetic parameters of the enzyme reaction catalyzed by 18µg of AHL lactonase against C6HSL (the AHL substrate) were then estimated under these previously optimized conditions. The V_{max}, K_m and K_{cat}/K_m values for the enzyme reaction are 52.26 mM AHL degraded/hour/mg of protein ,0.31 mM and 2.6 X 10^3 $M^{-1}sec^{-1}$ (Fig. 7 and Table 1) respectively.

Kinetic characterization of some other AHL lactonases can be found elsewhere (Table 1) (Momb et al., 2008; Wang et al., 2004). However it is important to note that method used to study enyzyme kinetics (i.e. bioassay or chemical assay) and source of AHL lactonase (organism from which the enzyme was isolated and whether the enzyme is a wild type or mutant one) makes a significant difference to the values of kinetic parameters, so direct comparision with previous reoprts is easier said than done.

Inhibition of Vibrio cholerae biofilm formation by Bacillus sp. RM1 AHL lactonase

Vibrio cholerae is a well known human enteric pathogen which causes a severe patho-physiological condition that can prove fatal in many cases. Biofilm formation is an important part of pathogenesis of *V.cholerae* and it is a quorum sensing mediated phenotype. Partially purified AiiA Lactonase from the isolate under study could successfully inhibit *V.cholerae* biofilm at a final concentration of 75µg (Fig.8). AHL lactonase at concentrations of 50µg or less did not show significant inhibition of *V.cholerae* biofilm .Also inactivating the enzyme by heat treatment abolished its anti-biofilm potential (Fig.8).

Inhibition of *Vibrio* sp. biofilm by AiiA class of AHL lactonases has also been described previously by Augustine et al (2010) however here cell extract of recombinant *E.coli* expressing AiiA lactonase was used instead of purified enzyme and also concentration of AHL lactonase mediating this effect was not mentioned. Vinoj *et al.* have also used AiiA lactonase from *Bacillus licheniformis* and shown its ability to disrupt *Vibrio parahaemolyticus* biofilm, which is an important fish intestinal pathogen (Vinoj et al., 2014).

The present report talks about isolation and identification of a quorum quenching bacteria from Fenugreek root nodule rhizosphere. The isolated bacteria was found to be belonging to genus *Bacillus* and was named as *Bacillus sp.* RM1.It was demonstrated to be a potent producer of an AHL degrading enzyme, AHL lactonase. AHL lactonase from *Bacillus sp. RM1* was partially purified and kinetically characterized. The enzyme was also found to mediate significant disruption of *Vibrio cholerae* biofilm which highlights the possible use of this enzyme as an anti-biofilm agent against important gram negative pathogens.

Table.1 Comparison of kinetic characteristics of some selected wild type and mutant AHL lactonase

	AHL Substrate used	Origin of protein	Km	Kcat/Km	Assay method	Reference
1.	C6HSL	*Bacillus thuringiensis* aiiA gene (WT)	5.6	1.6 X 104	Spectro-photometric or chemical assay	Momb et al. 2008
2.	C8HSL		0.55	1.1 X 105		
3.	C6HSL	D108N mutant of wild type	1.6	8.8 X 102		
4.	C6HSL	*Bacillus sp.* aiiA gene (WT)	3.83	9.31 X 103	HPLC based method	Wang et al. 2004
5.	C8HSL		2.61	10.5 X 103		
6.	C6HSL	*Bacillus sp.*RM1	0.31	2.6 X 103	Bioassay based method	THIS study

Fig.1 AHL degradation activity of selected isolates as determined by centre streak method selected isolates were streaked on basal LA agar plate and after 36 hours plates were overlayed with soft agar containing CV026 + AHL.A colorless zone of AHL degradation was seen around the central streak region of the isolates under study

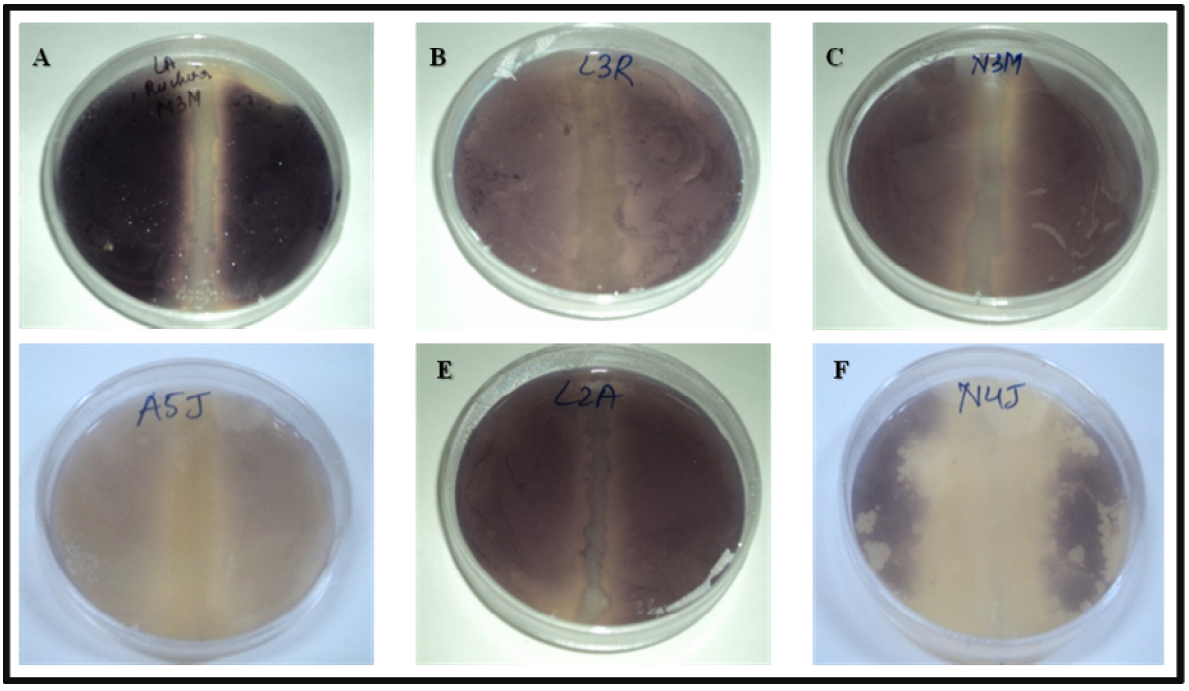


Fig.2 An unrooted phylogenetic tree describes the evolutionary relatedness of isolate *Bacillus sp*. RM1 (N4J).The isolate was seen to be closely related to the *Bacillus cereus* group but found to be evolutionary distinct

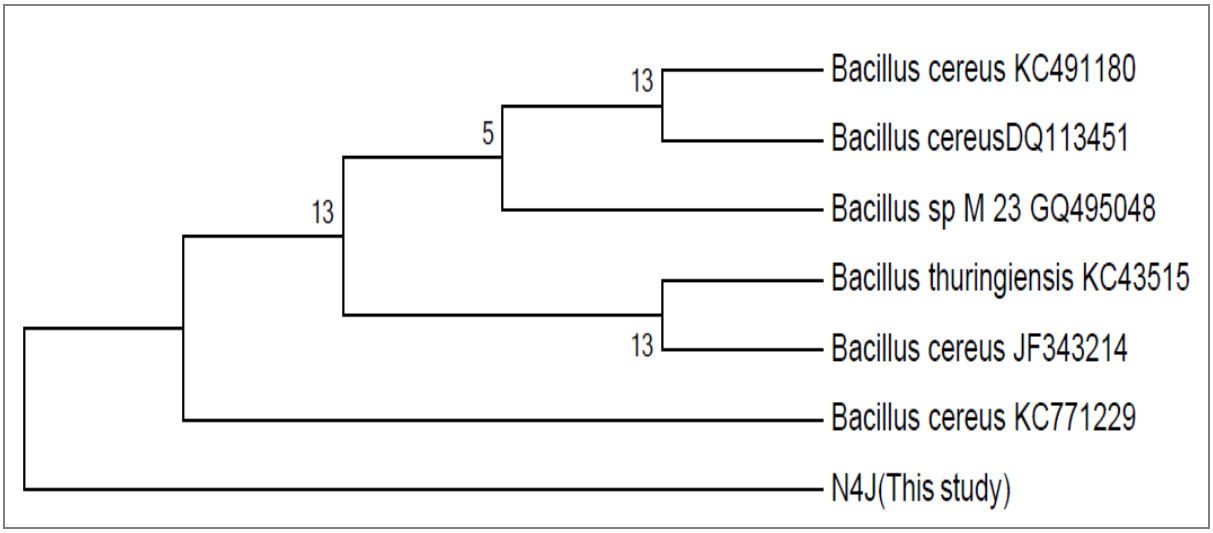


Fig. 3 AHL lactonase activity of *Bacillus sp.* RM1 confirmed by CV026 based bioassay. Thoroughly washed whole cells of Bacillus spp. RM1 were suspended in phosphate buffer containing appropriate amount of C6HSL.The reaction mixture was then incubated at 40oC and aliquots withdrawn every hour shows gradual degradation of added AHL. Re-lactonization and return of biological activity of degraded AHLs upon acidification can be seen after 6 hours which is indicative of AHL lactonase activity.

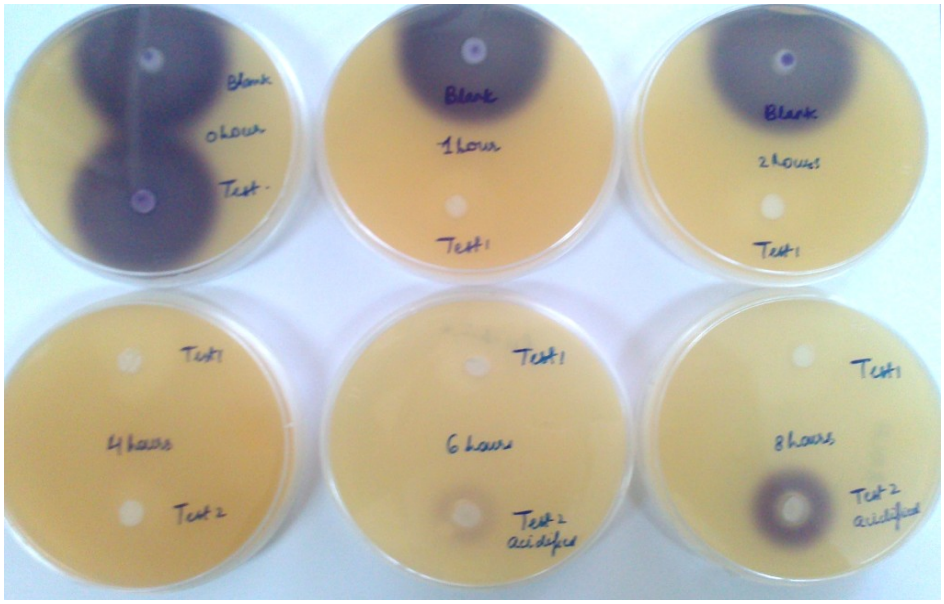


Fig. 4 Growth of *Bacillus sp. RM1* in KG medium, with AHLs as sole source of carbon: Three different AHLs, namely C7HSL, 3oxo-C6 HSL and C6 HSL were added respectively to basal KG medium as sole source of energy and nitrogen and the isolate showed good growth in these tubes when compared to control media which was devoid of any AHL.

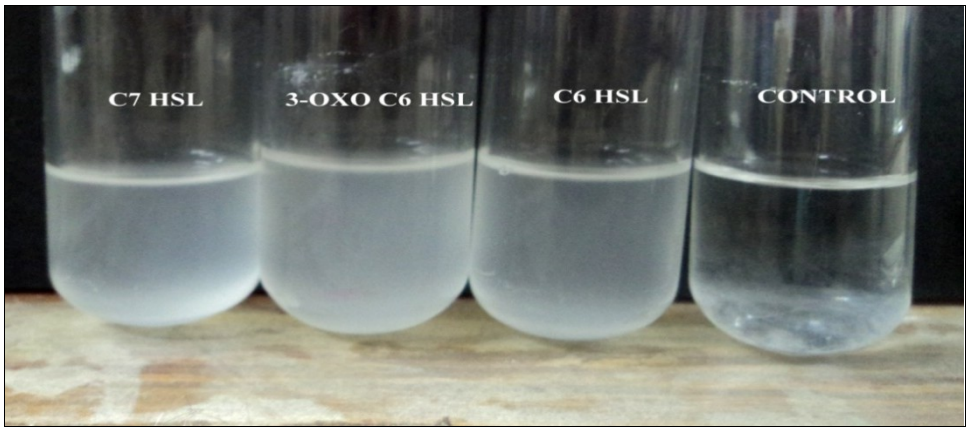


Fig. 5 Bioassay based identification of column fractions containing enzyme of interest (AHL lactonase).*Chromobacterium violaceum* CV026 based bioassay was performed using column fraction showing appreciable protein content and suspected to contain *Bacillus sp. RM1* AHL lactonase. Most potent fractions were able to completely degrade 0.5mM C6HSL within 1 hour incubation at 40^{o}C.

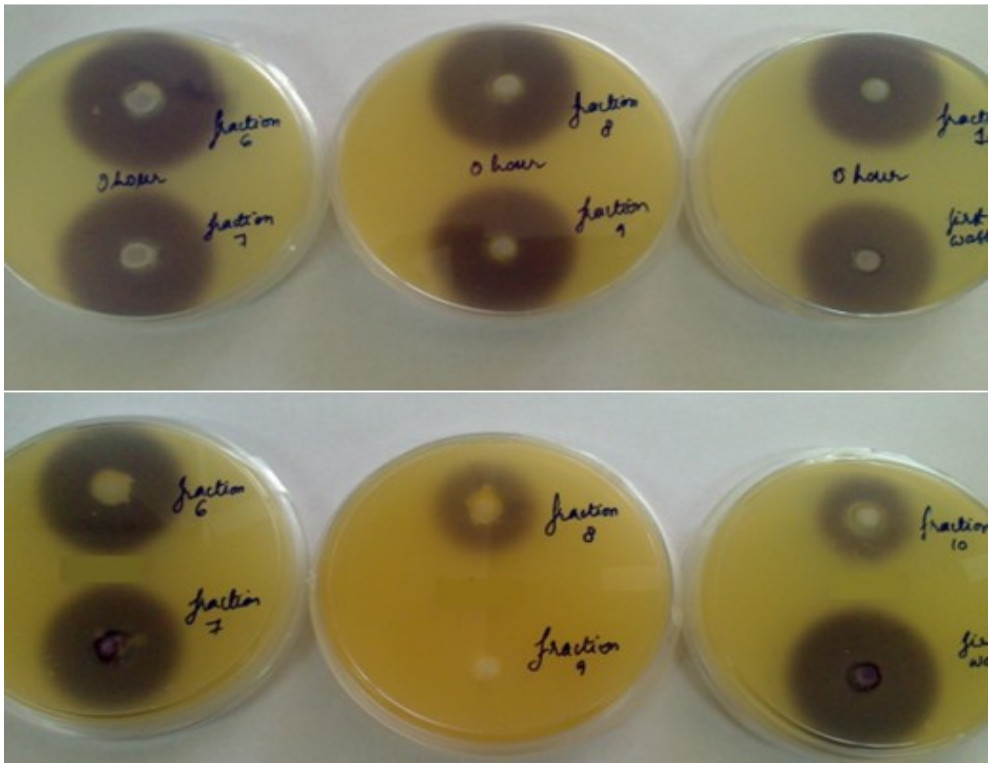


Fig.6 Native poly-acrylamide gel electrophoresis showing protein of interest, From left to right- Molecular weight marker: Carbonic Anhydrase 29 kDa (Sigma-Aldrich, India), Column Fraction 7, 8, 9 and 10 containing *Bacillus sp. RM1* AHL lactonase, as determined by CV026 based bioassay.

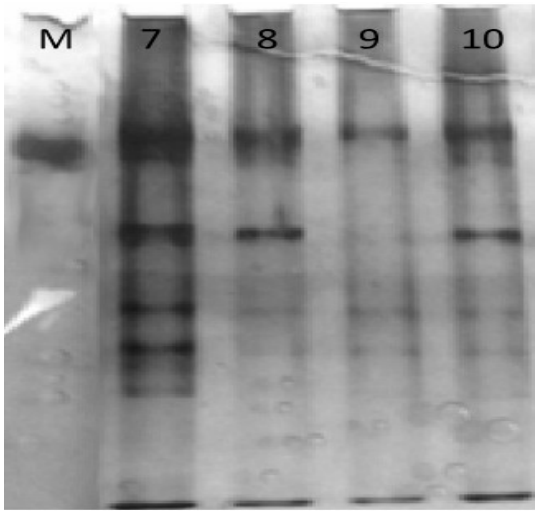


Fig.7 From top left: (A) Standard plot showing linear correlation between amount of violacein synthezized and mM of substrate(C6HSL) required; (B) and (C) Optimum temperature and optimum pH of the enzyme catalyzed reaction;(D) Kinetics of the C6HSL clevage by *Bacillus sp.* RM1 AHL lactonase as determined by Michaelis Menten plot

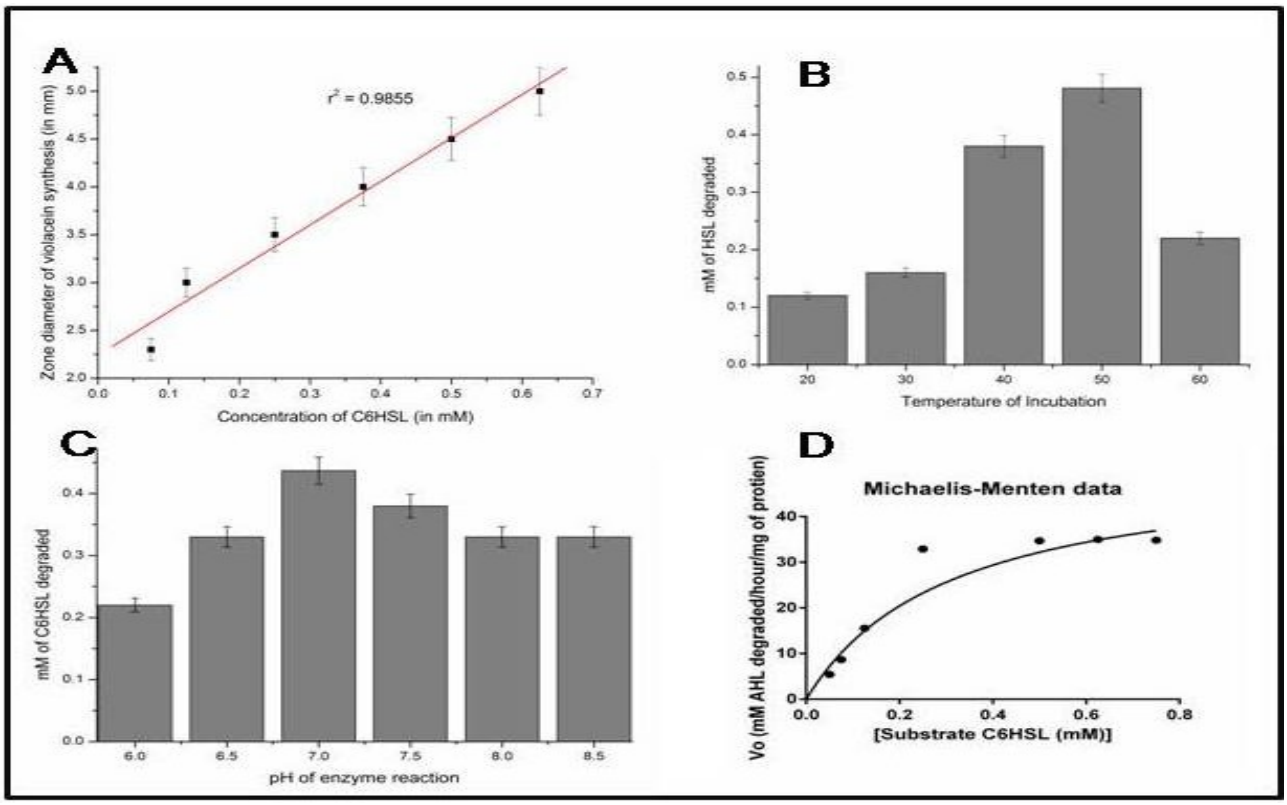
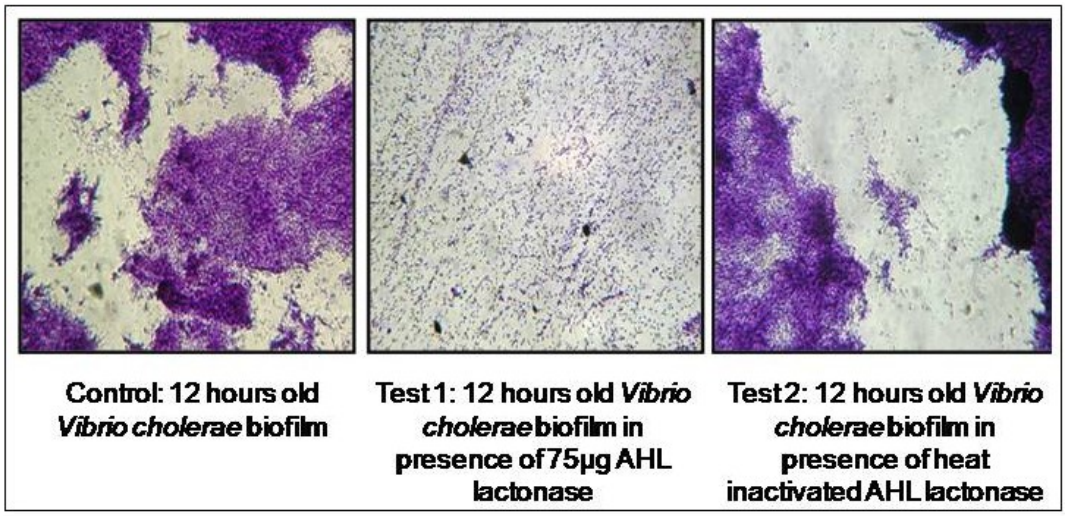
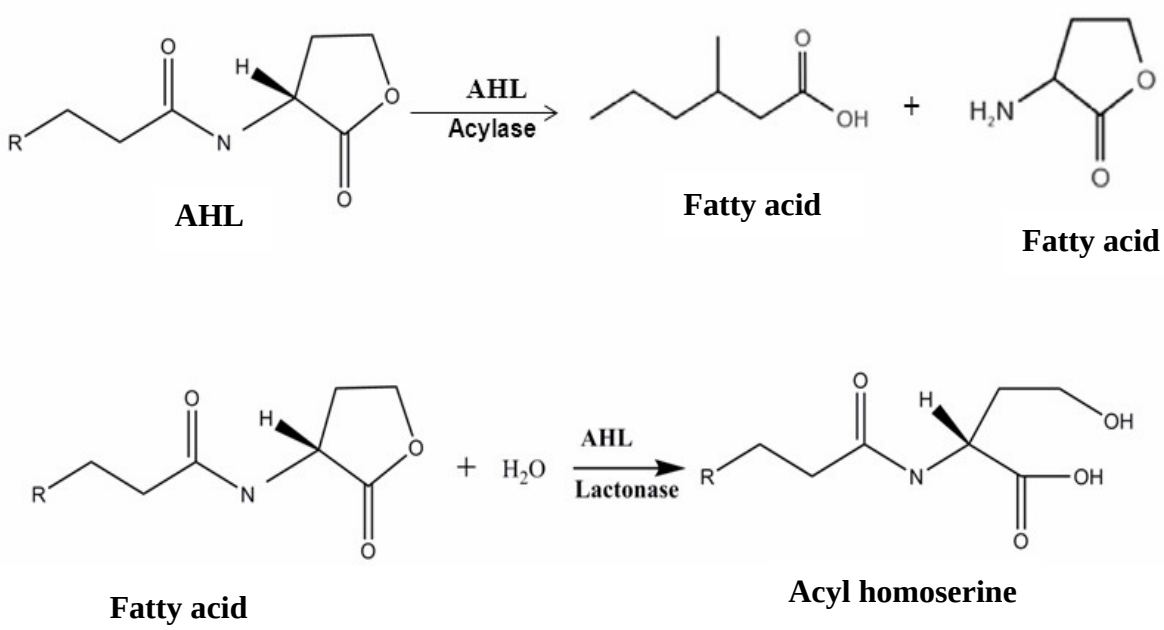


Fig.8 Anti-biofilm activity of *Bacillus sp.* RM1 AHL lactonase against *V. cholerae*. 8 hours old *Vibrio cholerae* biofilm was grown in the presence and absence of concentrated *Bacillus sp.* RM1 AHL lactonase. Significant inhibition of biofilm formation was observed in TEST1 when compared to CONTROL.



Scheme.1 Reaction catalyzed by an AHL acylase and an AHL lactonase



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