# Enzymatic and Bio-molecular approaches towards Quorum Quenching

Thesis Submitted to **AcSIR**

*For the Award of the Degree of*
DOCTOR OF PHILOSOPHY
*In*

BIOLOGY


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# CERTIFICATE

This is to certify that the work incorporated in this thesis entitled, **"Enzymatic and Bio-molecular approaches towards quorum quenching"** submitted by **Ms. Ruchira Mukherji** was carried out by the candidate under my supervision. The thesis contains original work and any data obtained from other sources has been duly acknowledged throughout the thesis.

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# DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled "**Enzymatic and Bio-molecular approaches towards quorum quenching**" is completely genuine and the work towards this thesis was carried out by me under the kind supervision of **Dr. (Mrs.) Asmita Prabhune** at the Division of Biochemical Sciences, CSIR- National Chemical Laboratory (NCL), Pune, India. The thesis is being submitted by me to AcSIR for the degree of Doctor of Philosophy in Biology.

I further affirm that the subject matter of this thesis has not formed the basis for award of any other degree, diploma, associate-ship, and fellowship, in this or any other University or other institution of higher learning. The material obtained from other sources has been duly acknowledged throughout the thesis.

DATE: 23.09.2014
Pune

Ruchira Mukherji
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This Thesis is dedicated to...

My Maa, Babba, Bhai,

Didaa...and My Supervisor...

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# Acknowledgements

*The road to a PhD is an expedition that is incomplete without the encouragement and support of countless number of people who consciously or unconsciously make this road worth travelling. I would like to take this opportunity to show my appreciation for all those who really matter.*

*First and foremost I would like to thank my guide and my mentor (in the truest sense)* ***Dr. (Mrs.) Asmita Prabhune.*** *She has been the driving force behind my success as a PhD student. She has helped me grow, under her kind benevolence, not only as a researcher but also as an individual. I have always craved freedom in my scientific forays and freedom is what I got while working under Dr. Prabhune in her lab. She always promoted us to maintain the goodness of our hearts because only a good human being can do great science. She has been like a mother figure to me constantly encouraging me with her love and support. Since the day I joined NCL, Dr. Prabhune has slowly and steadily taught me the ways of the scientific world. Her patience and persistence with her students is commendable and she is always open to questioning and discussions. I have always looked up to her as an excellent example of a successful woman scientist and a marvelous teacher. Ma'am, you will never be forgotten as you have a very special place in my heart and my mind.*

*Next I would like to express my gratitude towards* ***Dr. R. Nandini Devi.*** *I am so glad to have worked with her during my PhD tenure. Without her constant co-operation and diligence, the work involving surface functionalization of gold-cluster with bacterial quorum sensing signal molecules, would not have materialized. My Doctoral assessment committee (DAC) members,* ***Dr. BLV Prasad, Dr. RV Gadre*** *and* ***Dr. JM Khire,*** *must be receiving special thanks. I admire them and acknowledge them for their timely suggestions and for monitoring my progress throughout my PhD Tenure. I would also like to thank* ***Dr. CG Suresh*** *for his guidance during my efforts to study KcPGA's AHL degradation capability. He has also been very patient with me during my synopsis and Thesis submission process. I thank him for answering all my queries about the ACSIR submission process and for his constant kind support.*

*Further I would like to acknowledge* ***Dr. (Mrs.) Archana Pundle*** *for all her help and* ***Dr. Suresh Kumar Ramaswamy*** *for his assistance in initiating the molecular cloning work.*

*Past and present lab-mates from Dr. Prabhune's lab, made my stay here at NCL very enjoyable. So to begin with I would like to express gratitude towards my seniors, Dr. Kasturi Joshi, Vrushali Dengle, Dr. Jayshree Kantak , Dr. Reetika Gupta and Dr. Debanjan Guin (Debu Bhaiyaa),I have learnt so much from all of you. I would also like to thank my present labmates : Hrishikesh Mungi, Pushparani Devi, Avinash, Pooja Singh, Parul Dubey, Pradeep Kumar Singh, Priti Darne, Mihir Mehta, Pooja Ghushe and Amrita Patil.*

*It is very vital to show my gratitude to Dr. Nishant Kumar Varshney for helping me with KcPGA enzyme kinetics, Priyabrata Panigrahi for the Molecular Modeling work in case of KcPGA and Ranu Sharma for her help with xylitol molecular docking study. Special thanks to Dr. Santosh Tupe and Smriti Sanghi for all their help with imaging using Light microscopy.*

*I would like express my special appreciation for my friends and seniors from outside and within NCL, without their love and encouragement; my life here in Pune and NCL would be so incomplete. Firstly I would like to thank Somak Chowdhury who has been my best friend and my greatest critic since the longest time. He has shown me the importance to being practical and precise in life and has been my support through the darkest hours. Next I would like to honorably mention two of my seniors, Neeru Dhamija and Pratima Rawat. Neeru has always been like a big sister to me. Her caring and compassionate nature is what I miss the most. She is one of the most, well-organised people I have ever met and I have always tried hard to ape her working style. Pratima is the most hardworking, practical and patient person I know. And she has been a strong source of inspiration for me and I really miss the times I spent with her when we were roommates. I thank both of them (Pratima and Neeru) for being a part of my life. I also need to acknowledge my senior Sudeep Sabde, for showing me ways to achieve maximum productivity within the official working hours of any lab. Further I would like to thank my childhood friend, Parth Phalke, for being an awesome human being, I am so proud of him.*

*A big thank you to all my buddies in NCL: Ashish Deshpande (for being my witty friend and for smart suggestions and tips in molecular cloning), Hemangi (for being an awesomely stylish person and for our long discussions on Fabrics of India and so many other topics), Sayali Dalal (for being a true Aquarian and my constant and wonderful friend), Sheon Mary (for her amazing cooking and for being my cute and funny friend) , Hrishikesh Mungi (for being the most honest and trustworthy person in the lab), Deepak Chand (for being my crazy time-pass buddy) and Sonali (for simply being amazing). Pranjali, Gauri and Amey Bhide thank you for being my lunch buds. I want to thank Deepanjan, Manu, Priya Yadav, Arati and Rubina for our fun meetings and chit chat in the department corridor.*

*I wish to thank the Head, Division of Biochemical Sciences (Dr. Vidya Gupta) and the Director of National Chemical Laboratory (Dr. Saurav Pal) for giving me an opportunity to be a part of this department and NCL family, and University grants commission, New Delhi for the award of PhD fellowship.*

*I feel the need to acknowledge Indira Maam, Sheetal and Yashashsree for being very accommodating and helpful with all the administrative paperwork and Trehan Chacha, Giri ji, Ramakant ji, and Jagtap Kaka for all their assistance. Members of the Students Academic Office: Pavithran Sir, Kolhe Maam, Deepa and Priyanka: I thank you all for your co-operation.*

*Before I finish I would like to thank my family members. I want to deeply acknowledge my Mother for being my all-weather companion and for understanding my moods, dealing with my tantrums and giving me the space to grow as an individual. I thank my father for teaching me the importance of being disciplined from an early age. My brother for being the wise one, and for making me laugh with his antics, whenever I wanted to.I wish to thank my grandmother for that extra bit of pampering.*

*Finally, I thank GOD, the almighty for bestowing me with- strength, perseverance, a wonderful PhD supervisor, an amazing family, and loads of amazing friends*

## List of Abbreviations

| | |
|---|---|
| QS | Quorum Sensing |
| AHL | Acyl Homoserine Lactone |
| QQ | Quorum Quenching |
| AIP | Autoinducing peptide |
| AuNC | Gold Nanocluster |
| QSI | Quorum sensing inhibition/inhibitor |
| DSF | Diffusible signal factor |
| AI | Autoinducer |
| DPD | 4,5-dihydroxy-2,3-pentandione |
| Acyl CoA | Acyl co-enzyme A |
| SAM | S-adenosyl methionine |
| MTR | Methyl thioribose |
| *Kc*PGA | *Kluyvera citrophila* Penicillin G acylase |
| *Ec*PGA | *Escherichia coli* Penicillin G acylase |
| C7 HSL | N-Heptanoyl-L-Homoserine Lactone |
| C6 HSL | N-Hexanoyl-L-Homoserine Lactone |
| HSL | Homoserine Lactone |
| Ntn | N-terminal Nucleophile |
| BSH | Bile Salt Hydrolase |
| CV026 | *Chromobacterium violaceum* mutant strain 026 |
| OPA | *O*- Phthaldialdehyde |
| DTT | Dithiothreitol |
| PenG | Penicillin G (Benzyl Penicillin) |
| rpm | revolutions per minute |
| G-LIN | Glycomonoterpenol derived from Linalool |
| G-TER | Glycomonoterpenol derived from Alpha terpineol |
| MTA | Monoterpene Alcohol |
| HPLC | High performance liquid chromatography |
| FTIR | Fourier Transform Infra-Red Spectroscopy |
| TLC | Thin Layer Chromatography |
| NMR | Nuclear Magnetic Resonance |
| SEM | Scanning Electron Microscopy |
| TEM | Transmission Electron Microscope |
| XRD | X-ray Diffraction |
| SDS-PAGE | Sodium dodecyl-sulphate - Poly acrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| Ni-NTA | Nickel-nitrilotriacetic acid |
| EO | Essential oil |
| EOSL | Essential oil derived glycolipid |
| OA | Oleic acid |
| SL | Sophorolipid |
| $K_m$ | Half-maximum velocity, Michaelis constant |
| $V_{max}$ | The maximum velocity of enzyme reaction |
| µg | Micro gram |
| kDa | Kilo dalton |
| mM | Milli molar |
| m/z | Mass to charge ratio |
| LB | Luria bertani |
| MGYP | Malt extract-glucose-yeast extract-peptone |
| NCIM | National Collection of Industrial Micro-organisms. |
| MTCC | Microbial type culture collection |
| ATCC | American type culture collection |
| IPTG | Isopropyl-□-D-1-thiogalactopyranoside |

# Abstract of Thesis


**Chapter 1. Introduction**

This chapter provides a broad overview of bacterial cell to cell communication spelling out the details of gram negative and gram positive Quorum sensing. Since the present thesis specifically deals with gram negative Quorum Sensing mediated by Acyl homoserine lactone class of signal molecules this topic has been explained more vividly. The chapter further describes the various phenotype regulated by bacterial Quorum sensing and tools used to visualize this sort of cell to cell communication *in vivo* and *in vitro*. Additionally this chapter discusses the importance of bacterial QS as a viable drug target for development of sustainable anti-infective therapy. Both enzymatic and small molecule based inhibition of QS has been described. The chapter ends with a brief account of application of quorum sensing inhibition strategies in biotechnology and medicine.

**Chapter 2. A new role for penicillin acylases: Degradation of Acyl homoserine lactone quorum sensing signals by *Kluyvera citrophila* penicillin G acylase**

Use of penicillin acylases for the production of semi-synthetic penicillins is well-recognized and *Escherichia coli* penicillin G acylase (*Ec*PGA) has been extensively used for this purpose since many years. However, in the recent past *Kluyvera citrophila* penicillin G acylase (*Kc*PGA) is assumed to be a better substitute, owing to its increased resilience to extreme pH conditions and ease of immobilization. The present chapter describes a new dimension for the amidase activity of *Kc*PGA by demonstrating its ability to cleave bacterial quorum sensing signal molecules, acyl homoserine lactones (AHL) with acyl chain length of 6–8 with or without oxo-substitution at third carbon position. Initial evidence of AHL degrading capability of KcPGA was obtained using *Chromobacterium violaceum* CV026 based bioassay method. Kinetic studies performed under previously optimized conditions of pH 8.0 and 50$^{o}$C revealed 3-oxo-C6 HSL to be the best substrate for the enzyme with $V_{max}$ and $K_m$ values of 21.37 + 0.85 mM/h/mg of protein and 0.1

+ 0.01 mM, respectively. C6 HSL was found to be the second best substrate with $V_{max}$ and $K_m$ value of 10.06 + 0.27 mM/h/mg of protein and 0.28 + 0.02 mM, respectively. Molecular modeling and docking studies performed on the active site of the enzyme supported these findings by showing that the AHLs fit within the hydrophobic pocket of the enzyme active site. Additionally AHL degradative capacity of *Kc*PGA was compared to that of *Ec*PGA using experimental data, and *Ec*PGA was found to be a poor quorum quencher. Biofilm disruptive capability of *K. citrophila* PGA was also demonstrated, thus further broadening the scope of application of this enzyme.

**Chapter 3. Isolation of a novel strain of *Staphylococcus epidermidis* with potent Bile Salt Hydrolase and AHL cleavage potential**

The aim of the present chapter was to isolate a bile salt hydrolase (BSH) producer from fermented soy curd and explore the ability of BSH to cleave bacterial quorum sensing signals. Bacterial isolates with possible ability to de-conjugate bile salts were enriched and isolated on MRS medium containing 0.2% bile salts. BSH producing positive isolate with orangish pink pigmented colonies was identified as a strain of *Staphylococcus epidermidis* using biochemical and phylogenetic tools. *S. epidermidis* RM1 was shown to possess both potent BSH and N-Acyl homoserine lactone (AHL) cleavage activity. Genetic basis of this dual enzyme activity was explored by means of specific primers designed using *S.epidermidis* ATCC 12228 genome as template. It was observed that a single enzyme was not responsible for both the activity. Two different genetic elements corresponding to each of the enzymatic activity were successfully amplified from the genomic DNA of the isolate.

**Chapter 4A. Novel Species of *Bacillus* isolated from Fenugreek root nodule rhizosphere producing a quorum quenching enzyme**

This aim of this chapter was to isolate potential quorum quenching bacteria from novel rhizospheric environments. With this in mind Fenugreek root nodule rhizospheric soil was used. A potent quorum quencher isolated from this soil sample was identified using 16S rDNA sequencing and was found to be belonging to genus *Bacillus*. The isolate was

named as *Bacillus sp.* RM1 and it was found to produce an AHL lactonase. The QQ enzyme was then purified from the wild type bacterium and optimum pH and temperature for maximum enzyme activity was then determined. The purified enzyme was subsequently kinetically characterized. Further ability of this AHL lactonase to disrupt quorum sensing mediated biofilm formation in *Vibrio cholerae* was also demonstrated.

**Chapter 4B. A rapid iodometric method for qualitative detection of AHL Lactonases**

This sub-chapter describes a very simple and rapid qualitative assay to determine the presence of AHL lactonase in any given sample. It is based on a simple principle that penicilloic acid formed upon the action of AHL lactonase on Penicillin G molecule can quench upto 6-9 equivalent of added iodine reagent. Since quenched iodine cannot react with starch indicator present in the reaction mixture absence of blue color indicates presence of AHL lactonase activity. This assay could detect as less as 20µg of AHL lactonase present.

**Chapter 4C. Cloning and over expression of AHL lactonase from *Bacillus sp*.RM1**

In this chapter the *aiiA* AHL Lactonase gene from *Bacillus sp*. RM1 was amplified from the genomic DNA of the culture with the help of specific primers. The amplicon was successfully cloned into a TOPO TA expression vector and correctly oriented, sequence checked vector containing insert, was then used to transform BL21 DE3 cells for protein expression studies. Protein was expressed using an IPTG induction protocol and fractions containing the protein of interest were run on 15% SDS PAGE to determine its molecular weight. Biological activity of heterologously expressed AHL lactonase was then checked using *Chromobacterium violaceum* CV026 bioassay and recombinant protein was shown to retain its AHL degradative potential.

**Chapter 5A. Novel glycolipids synthesized using plant essential oils and their application in Quorum Sensing Inhibition and as anti-biofilm and anti-fungal agents**

Essential oils (EOs) form an important part of traditional medicine so their anti-microbial and, in the recent past, anti-quorum sensing activity has been well studied. However it is likely that due to their hydrophobic nature and reduced solubility in aqueous environments full potential of their activity cannot be realized. Hence it is only rational to formulate a process to make these molecules more hydrophilic in nature. The present chapter describes synthesis of glycolipids using 12 different essential oils as substrates, thus providing surfactant-like properties to these EOs. The synthesis protocol makes the use of *Candida bombicola* ATCC 22214 as producer organism. The production process required 7 days of incubation at 28$^{o}$C and 180 rpm. Preliminary characterization of the synthesized essential oil sophorolipids (EOSLs) was performed using thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR). Additionally, essential oils that were incapable of mediating quorum sensing inhibition (QSI) on their own became potent quorum sensing inhibitors upon conversion into their corresponding EOSLs. Anti-biofilm and anti-fungal activity of selected EOSLs was also demonstrated using *V. cholerae* and *Candida albicans* as test organisms respectively.

**Chapter 5B. A New class of bacterial quorum sensing inhibitors: Glycomonoterpenols**

With increasing burden of antibiotic resistant microorganism search for newer drug targets and potent drug molecules is a never ending scenario. Quorum sensing (QS), the phenomenon of bacterial cross-talk, is one such target that has captured the attention of many and has been touted of be the future of new age antimicrobials. Quorum sensing has the potential to regulate a plethora of bacterial virulence phenotypes and search of molecules with powerful quorum sensing inhibitory capacity are underway. Monoterpene alcohols (MTAs) like Linalool and Alpha terpineol have been shown to possess antimicrobial and anti-biofilm activity. However in this chapter an attempt was made to bring forth a new class of compounds, Glycomonoterpenols derived from these monoterpene alcohols. These Glycomonoterpenols were synthesized using *Candida bombicola* ATCC 22214 by feeding the cells with Linalool and Alpha terpineol

respectively as substrates in 10% glucose, production medium. The advantage of these molecules over their parent compound is their increased solubility, surfactant like properties, and additionally enhanced quorum sensing inhibitory (QSI) potential. A variety of gram negative bacteria capable of quorum sensing were selected namely, *Chromobacterium violaceum* CV026, *Pseudomonas aeruginosa* and *Vibrio cholerae*. Both these glycomonoterpenoid derivatives were found to possess strong ability to inhibit QS mediated phenotypes all the three gram negative bacteria.

**Chapter 6. *Pichia caribbica* synthesized xylitol as an inhibitor of AHL mediated gram negative quorum sensing**

Xylitol, a sugar alcohol, is fast gaining ground over other artificial sugar substitutes owing to its advantageous properties. Xylitol is a safer alternative for diabetics because of insulin-independent metabolism. It has many beneficial properties making it suitable to form an important part of odontological formulations. Conventionally, commercial production of xylitol involves harsh chemical method which operates at high temperature and pressure. Thus, microbial production of xylitol is preferred over chemical method, and yeasts have been extensively exploited for this purpose. In the present chapter, quantitative production of xylitol from D-xylose with the yield of 0.852 gm/gm and volumetric productivity of 1.83 gm/l/h in crystalline form, using novel yeast *Pichia caribbica* is reported. Also, a mild, safe procedure for product extraction has been described. The ability of xylitol to act as a quorum sensing antagonist in gram-negative marker strain *Chromobacterium violaceum* CV026 has been demonstrated here for the first time. Further QS receptor antagonism mediated by *Pichia caribbica* synthesized xylitol was also validated using experimental and in-silico tools.

**Chapter 7. Early Detection of bacteria capable of Quorum sensing using fluorescent Au nano-cluster probes surface functionalized with QS signal molecules**

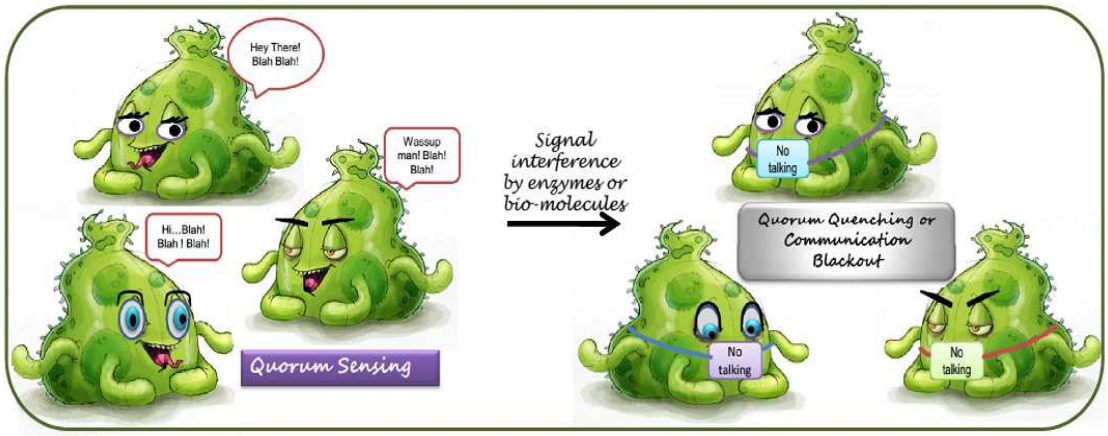
This chapter describes synthesis of fluorescent ultra-small gold clusters decorated with bacterial quorum sensing signal molecules, acyl homoserine lactone. These fluorescent probes were found to have emission in the near-infrared spectral region which is highly advantageous for bio-imaging studies. Imaging studies using different strains of bacteria

with and without acyl homoserine lactone (AHL) receptors with the aid of confocal microscopy showed that the probe interacts preferentially with cells possessing these receptors. This indicates that, with appropriate surface functionalization, the Au nano-clusters can be used for receptor specific detection of live cells capable of QS with enhanced selectivity. Such a system will further help in successful application of various quorum quenching strategies that have been highlighted in this thesis.

# Chapter 1

*Introduction*

# 1: Introduction




## 1.1 History: Discovery of bacterial cell to cell communication

For many years bacteria were considered to be simple organism capable of only singular/individual existence incapable of complex interactions. The only interaction that they were deemed to be capable of was simple mutualism and /or symbiosis with members of other species in any given metabolic consortia. This line of thinking was subjected to furthur inquiry when in late 1970's Nealson and Hastings [1-3] started the discussion about existence of intra-species communication, amongst a population of *Photobacterium fischeri* under study. In their paper in 1977 [2] they said 'The synthesis of the luminous system of the marine luminous bacterium *Photobacterium fischeri* is subject to a complex, self-regulated control system called auto-induction. The bacteria produce an autoinducer which accumulates in the medium at a constant rate (as a function of cell growth). When autoinducer reaches a critical concentration it stimulates, at the level of transcription, the synthesis of the luminous system. Auto-induction is thus viewed as an environmental sensing mechanism, which curtails the synthesis of the luminous system under dilute conditions' [2].

After its discovery in late 1970's the phenomenon of auto-induction received much attention and criticism. The *P. fischeri* system was studied further by different groups, to understand the molecular nature of the autoinducer and various regulatory elements underlying this phenomenon. It was actually more than a decade later that this

phenomenon was formally named as Quorum Sensing, in a mini-review by Fuqua *et al.* (1994) [4]. In their review titled "Quorum Sensing in Bacteria: the LuxR-LuxI Family of Cell Density-Responsive Transcriptional Regulators". Fuqua *et al.* [4] described the phenomenon of QS based regulation of bioluminescence in *Vibrio fischeri* as '…Certain bacterial behaviors can be performed efficiently only by a sufficiently large population of bacteria. We describe this minimum behavioral unit as a quorum of bacteria…this environmental sensing system allows bacteria to monitor their own population density. The bacteria produce a diffusible compound termed autoinducer which accumulates in the surrounding environment during growth. At low cell densities this substance is in low concentration, while at high cell densities this substance accumulates to the critical concentration required for activation of luminescence genes'[4].

Since then umpteen amount of work has gone into understanding this phenomenon of bacterial communication and the phenotypes under its transcriptional control. Studies on *Photobacterium fischeri* (now *Vibrio fischeri*) forms the paradigm of Quorum sensing (QS) mediated control of gene regulation. Also existence of cell density dependent regulatory proteins LuxR (quorum sensing Receptor) and LuxI (autoinducer synthesis gene) were first identified in *P.fischeri*, but ever since many homologs of these two regulatory proteins have been identified in different systems. Quorum sensing is now thought to be a common attribute of many bacterial species, and new QS systems and signal molecules are being identified at a rapid pace.

## 1.2 QS Circuit in *Photobacterium fischeri* (now *Vibrio fischeri*)

Quorum sensing based gene regulation was first identified in *V. fischeri*. *Vibrio fischeri* or *Photobacterium fischeri* is a bacterium of marine origin and it is found in a symbiotic relationship within the light organs of many sea creatures like the bobtail squid and certain fishes. Since the phenomenon of quorum sensing or autoinduction was first identified in *V.fischeri* hence this is one of the most well studied QS regulons.

*V. fisheri* is found living freely in sea water however at a low population density of $10^2$cells/ml. At this low cell number the bacterium is incapable of 'shining' since the concentration of autoinducing molecule in the extracellular environment is very low. Upon associating symbiotically with light organs of certain fishes or other marine

organism, bacteria easily attain cell numbers in the range of $10^{10}$-$10^{11}$cells/ml [2]. At this high cell number the autoinducing substance produced and secreted by the bacterium can cross the required threshold concentration, activating bacterial luciferase genes and we see the phenomenon of bacterial bioluminescence.

The molecular identity of *Vibrio fischeri* autoinducer was put forth in 1981 by Eberhard *et al.* [5]. It was found to be special acylated derivative of a lactonized homoserine molecule: N-3-oxo-hexanoyl homoserine lactone. Subsequently by 1983 the genes coding for the autoinducer synthase (Lux I), QS signal receptor (Lux R) and entire Lux gene operon were identified on a single DNA fragment by Engebrecht *et al.* [6].Further the same group also showed that the operon is regulated in similar manner in both *Escherichia coli* and *V. fischeri* by cloning the DNA fragment that encoding the Lux operon in *E.coli* [6]. By the end of 1980's and early 1990's several homologs of LuxI, LuxR and structurally different AHL (acylated homoserine lactone)-like signal molecules were identified [7, 8].

### *1.2.1 Autoinduction of bacterial luminescence:*

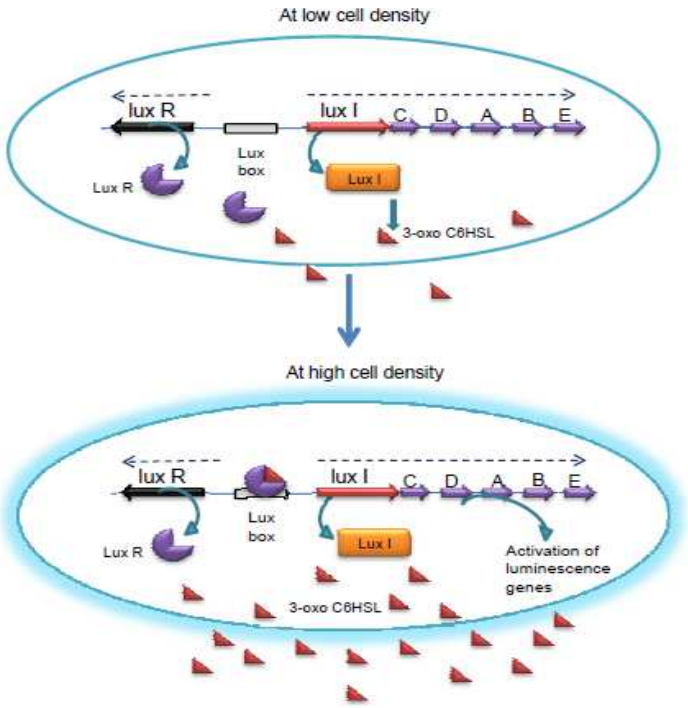



***Figure 1.1****: Vibrio fischeri QS circuit*

The *V. fischeri* QS signal molecule or autoinducer molecules was identified to be is 3-oxo-N-(tetrahydro-2-oxo-3-furanyl) hexanamide or more commonly N-3-(oxohexanoyl) homoserine lactone [5]. Bacterial cell membrane is permeable to the signal molecule, and thus, it diffuses out of the membrane into the extracellular environment. At low cell densities, QS signal molecule passively diffuses out of cells down a concentration gradient, while at high cell densities, it accumulates extracellularly (at which point ,intracellular concentration is equivalent to the extracellular concentration) (Fig.1.1). A concentration of the order of 10 nM is sufficient to activate transcription of the luminescence genes in *V. fischeri* [4]. The luminescence genes are organized in two distinct transcription units whose start sites are about 150 bp apart. One unit contains *luxR,*gene which encodes the 250-amino-acid LuxR protein, the transcriptional activator of luminescence genes [7]. The other unit is an operon, *luxICDABEG*, and is activated by LuxR in the presence of 3-oxoC6 HSL. A sequence having a dyad symmetry is found located at about -40 bp upstream from the start of *luxI CDABEG* transcription site and mutational analysis of this sequence demonstrated that it was required for *lux* operon activation by LuxR. The *luxI* gene encodes a 193-amino-acid protein and it is known as the autoinducer synthase gene [8]. The other genes in the *lux* operon play mechanistic roles in light production. *luxR* and *luxI* mediate cell density-dependent control of *lux* operon transcription. At low cell densities, *luxI* is transcribed at a basal level and 3-oxoC6 HSL accumulates slowly in the growth medium.At a sufficiently high 3-oxoC6 HSL concentration, this signal compound interacts with LuxR, which then activates transcription of the luminescence genes.

## 1.3 Quorum sensing phenomenon in different organisms

Quorum sensing is now thought to be a universal phenomenon among bacteria. To date numerous Quorum sensing systems modulated by a variety of structurally different signal molecules has been discovered [9]. Some presumed similarities exists in these systems because the ability to communicate is fundamental to all bacteria. Differences in the systems probably exist because each system has been optimized to endorse survival of the organism in the specialized niche which the particular species of bacteria inhabits [9, 10]. Thus, the types of signals, receptors, mechanisms of signal transduction, and target outputs of each quorum-sensing system reflects the unique lifestyle of a particular

bacterial species. Also the size of the quorum, for example, is not fixed but varies according to the relative rates of production and loss of signal molecule, which in turn is dependent on the prevailing local environmental conditions. Thus, quorum sensing is an integral component of the global gene regulatory networks which are responsible for facilitating bacterial adaptation to environmental stress [11]. Following is a general overview of quorum sensing systems in Gram negative and Gram positive bacteria.

***1.3.1 Quorum sensing in gram negative bacteria***:

Quorum sensing in gram negative bacteria is mediated by several chemically distinct classes of signal molecules which include the N-Acyl homoserine lactones (AHLs), 2-alkyl-4-quinolones, furanones, long-chain fatty acid derivatives (DSF), fatty acid methyl esters and 4,5-dihydroxy-2,3-pentandione (DPD) derivatives,and they are collectively referred to as autoinducers (AI) [11,12]. Of all the signal molecules mentioned N-Acyl homoserine lactone mediated quorum sensing has received most attention and AHLs are biggest and the most well characterized class of signal molecules. These gram negative signal molecules are mostly hydrophobic in nature hence they can easily diffuse in and out of the cell membrane and their active transport is not required [12]. Quorum sensing circuit described in *V. fischeri* that regulates bioluminescence gene expression in this bacterium forms the archetypical model of AHL mediated gram negative QS [13]. Lux I mediated synthesis of signal molecule (AHL) and Lux R mediated reception of signal and subsequent transcriptional regulation of specific genes is what forms the foundation of most AHL based gram negative QS circuits.

Another well studied organism capable of AHL mediated quorum sensing is *Pseudomonas aeruginosa*. This organism has two LuxR-LuxI quorum sensing system that account for global regulation of approximately 2-5% of its genes [14]. *P.aeruginosa* QS controlled phenotypes range from exo-enzyme production to secondary metabolite production virulence gene regulation and biofilm formation. It uses two QS systems: the 3oxoC12 HSL based LasR-LasI system and C4-HSL based RhlI-RhlI system [15,16]. The LasR/I system was originally identified to be regulating Elastase exo-enzyme production but is now known to be modulating expression of a variety of genes. RhlR/I system is involved in regulation of rhamnolipid biosurfactant production but is now

recognized to be having additional functions [17]. LasR/I system is required for activation of RhlR/I system thus creating an elegant QS regulatory circuit hierarchy in *P.aeruginosa* (Fig. 1.2).

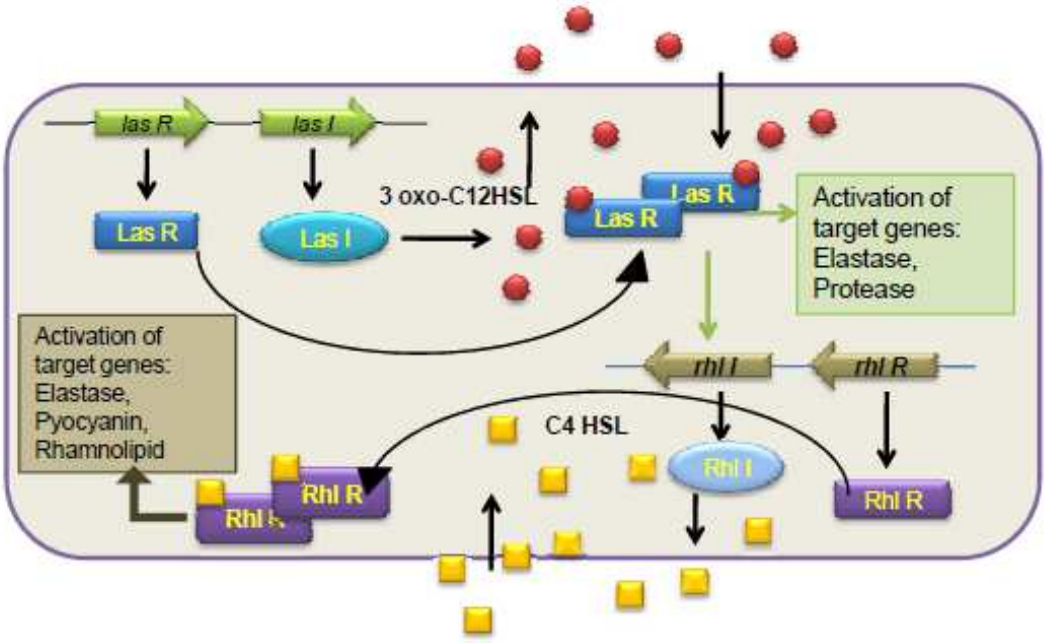



***Figure 1.2**: Hierarchical QS circuit in P.aeruginosa*

A different but interesting example of a gram negative bacterium capable of QS using signal molecules other than AHLs is *Xanthomonas campestris*. This organism causes black rot disease in a variety of cruciferous plants such as cabbages etc [18]. The bacterium regulates the production of extracellular enzymes such as proteases, pectinases and cellulases using quorum sensing system but the signal molecule used in this case is not AHL. The expression of the different phenotypes like exo-enzyme production, biofim formation, toxin production are all modulated by production of a small Diffusible signal factor (DSF) [18].It is a long chain fatty acid chemically characterized as cis-11- methyl-2-dodecenoic acid. The *rpf* group genes are responsible for DSF mediated QS: RpfF – signal synthesis, RpfC – signal detection and RpfG - signal transduction (Fig.1.3) [19]. Interestingly, RpfG is a phospho-diesterase that cleaves the intracellular second messenger, cyclic di-guanosine monophosphate (c-di-GMP) [18].DSF mediated

intercellular signaling is therefore coupled to intracellular signaling via c-di-GMP. DSF is not unique to *X. campestris* and is also produced by some other gram negative bacteria.

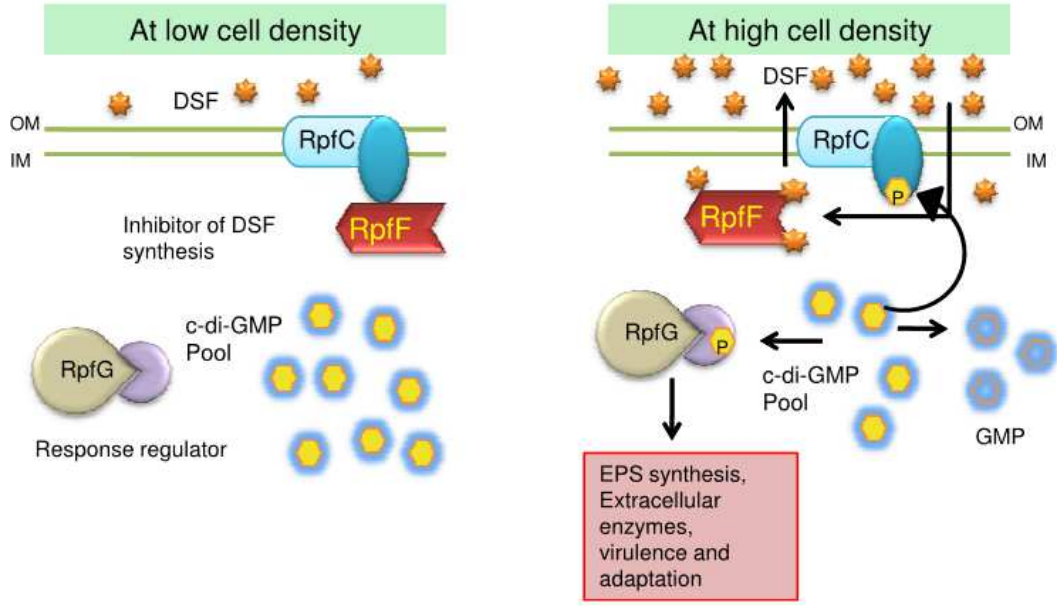



***Figure 1.3:*** *Quorum sensing in Xanthomonas campestris is mediated by DSF*

### *1.3.2 Quorum sensing in gram positive bacteria*:

Gram-positive bacteria communicate via modified cyclic- oligo-peptide signals and relay these signals using membrane-bound sensor histidine kinases receptors. Signal is transferred further by a phosphorylation cascade that influences the activity of a DNA-binding transcriptional regulatory protein termed as a response regulator. Each gram-positive bacterium recognizes a specific signal that is different form ones used by other bacteria; in other words, each transmembrane receptor recognizes only its specific cognate signal molecule. Gram positive peptide signals are not diffusible across the membrane; hence signal release is mediated by dedicated oligopeptide exporters [11, 13]. In most cases, signal is released with simultaneous signal processing and modification. While the biochemistry underlying these events is poorly understood, it is known that most peptide quorum-sensing signals are cleaved from larger precursor peptides, range in size from 5 to 34 amino acids in length and typically contain unusual chemical architectures like Lactone and thiolactone rings, lanthionines, and isoprenyl groups [10, 13]. Based on their structural uniqueness three different families of Autoinducing

peptides (AIPs) are known to date [10]: (i) the oligopeptide lantibiotics, exemplified by the lactococcal nisin, (ii) the16-membered thiolactone peptides, represented by the staphylococcal autoinducing peptide 1(AIP-1) (Fig.1.4) (iii) the isoprenylated tryptophan peptides, which includes ComX and its variants from Bacillus subtilis and other Bacillus species [10].

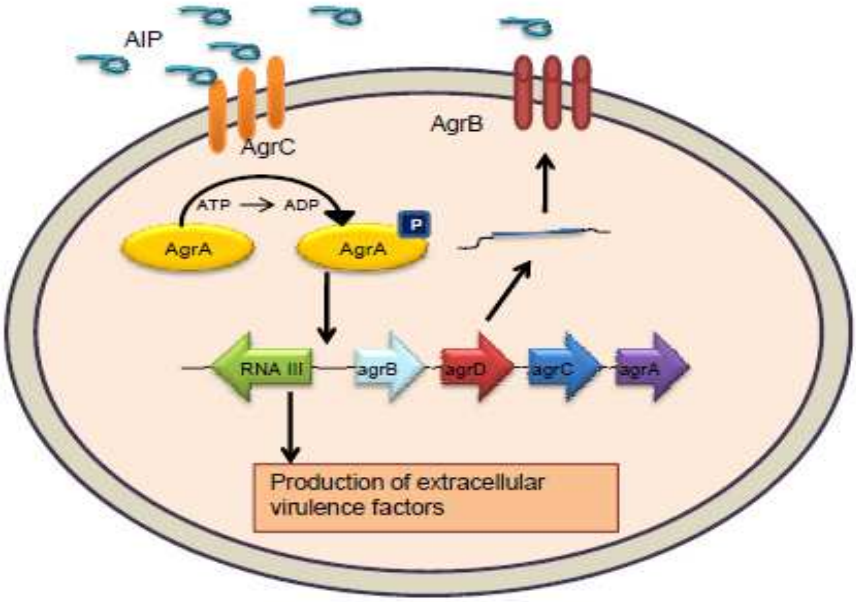



***Figure 1.4:*** *Quorum Sensing in Staphylococcus aureus is mediated by cyclic peptides*

Quorum sensing mediated via thio-lactone peptides is the most extensively studied gram positive system. This family of AIPs is structurally characterized by a 16-membered side chain-to-tail macrocyclic peptide to which a short linear peptide is attached N-terminally [10,12]. The prototypical member of this family is the modified octapeptide called AIP-1 which is employed by *S. aureus* as its QS signal molecule. The staphylococcal AIPs are derived from a operon, agrBDCA that comprises the genes required for AIP synthesis (agrBD) and AIP response (agrAC). Functionally, the AIPs are sensed by the two component signal transduction system (TCSTS) comprising of AgrC, as a transmembrane sensor histidine kinase, and AgrA, as a response regulatory protein. Interaction of the AIP with its cognate AgrC results in activation of the TCSTS, thus resulting in upregulation of the agr-mediated quorum sensing system. The effector of the S. aureus quorum sensing system is RNAIII, which has the capacity to initiate the transcription of genes that encode

a variety of exoproteins, like a-haemolysin, enterotoxin B, serine proteases etc. (Fig. 1.4) [12,13]. Thus, the agr regulon maintains the equilibrium of virulence factor expression during colonization and invasion phases of the staphylococcal infection. This family of small-to-medium sized thiolactone/ lactone peptides clearly represents the principal chemical architecture utilized by Gram-positive bacteria to mediate quorum sensing.

### *1.3.3 AI-2 mediated interspecies communication in bacteria:*

AHLs and peptides represent the two major classes of bacterial quorum sensing molecules, used by Gram-negative and Gram-positive bacteria, respectively, for intra-species communication. Recently, a family of molecules generically termed autoinducer-2 (AI-2) has been discovered .It has been proposed that AI-2 is a non species-specific autoinducer that mediates intra- and interspecies communication among Gram-negative and Gram-positive bacteria. AI-2-based quorum sensing was first identified in the early1990s in the Gram-negative bacterium *V. harveyi* [20,21]. It was observed that an AHL-deficient strain of the bacterium remained capable of producing bioluminescence even in the absence of the innate AHL autoinducer . This suggested that a second quorum sensing pathway, employing a different signaling molecule, was in use. This novel autoinducer, whose structure at the time was unknown, was termed AI-2. It was subsequently shown that cell free culture fluids from a number of bacterial species were capable of stimulating activity in a *V. harveyi*AI-2 reporter strain suggesting that the AI-2 signal may be produced by numerous bacterial species [22]. Gene responsible for AI-2 was designated as *luxS*, and has been found in over 70 bacterial species since its discovery [20-22].Structure of some common Quorum sensing signal molecules have been given below (Fig. 1.5).

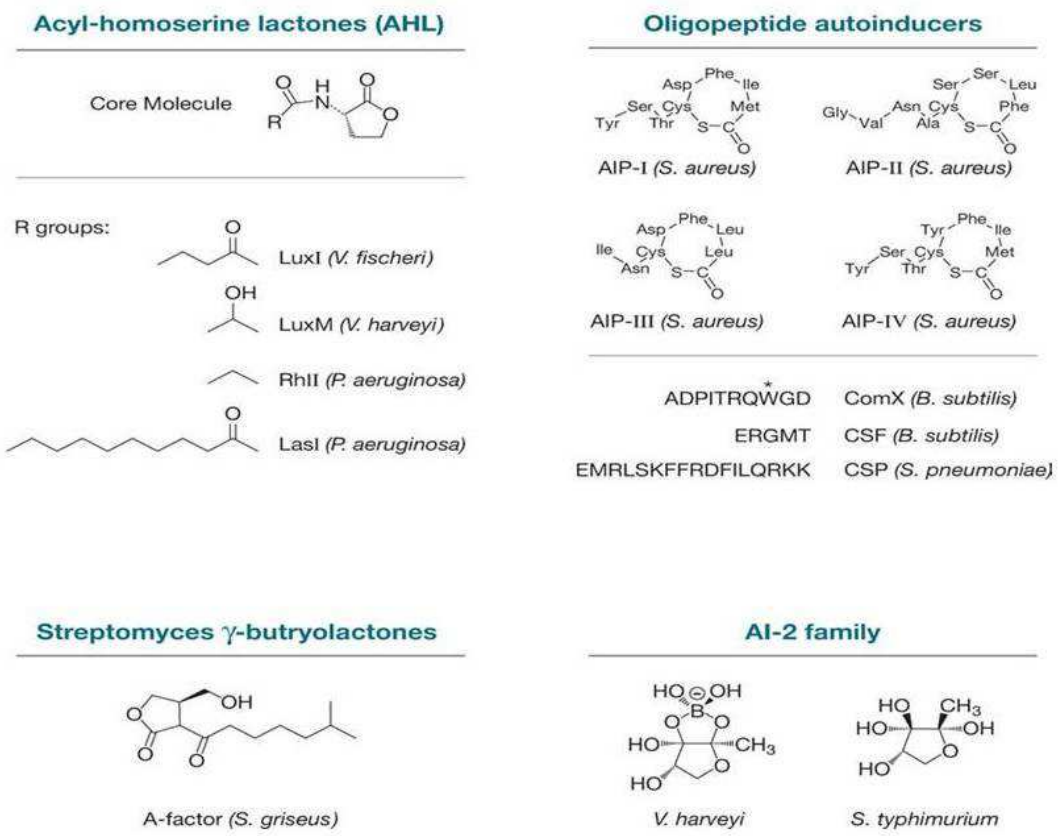



***Figure 1.5:*** *Structure of different Quorum Sensing signal molecules (from Reference [13])*

## 1.4 AHL Based Quorum Sensing in Gram negative bacteria- Detailed description of key players

The quorum sensing circuit outlined in Figure 1(*V. fischeri* system) represents the basic model for AHL-mediated QS. Regulatory proteins involved in all the AHL-based quorum sensing systems discovered subsequent to the *V. fischeri* circuit have been termed LuxI-type synthases and LuxR-type receptors [22-24]. Thus, a LuxI-type protein produces a diffusible AHL ligand which above a certain threshold concentration, productively binds to its cognate cytoplasmic receptor (a LuxR-type protein). This AHL-LuxR-type protein complex then modulates the expression of target genes that are involved in population wide response. Homologs of LuxI and LuxR have been identified in a large number of bacterial genomes with a variety of different AHLs regulating a range of physiological functions [24, 25]. In general, each bacterial species responds specifically to its own

unique AHL autoinducer/s. All AHLs have the same general structure only the length and functionality of the acyl chain varies [26].

### *1.4.1 Signal generators: AHL synthases or LuxI homologs:*

Three AHL synthase families have been identified so far, and these include the LuxI, HdtS, and LuxM families [23, 24]. Among the three the LuxI family is the best studied .LuxI homologs have been described in a large number of Gram-negative bacteria [25]. Biochemical studies, have demonstrated that enzymes in the LuxI family use S-adenosyl-methionine(SAM) and acyl-acyl-carrier-protein (acyl-ACP) as substrates to produce AHL molecule (Fig.1.6) [26]. These studies have identified seven-ten residues that are conserved in LuxI-family of proteins and are proposed to be involved in catalysis and SAM binding. All the conserved residues have been mapped to an N-terminal region of the protein [27, 28]. The C-terminal region of these proteins is less conserved and is proposed to recognize the acyl-ACP substrate, which is variable for different AHL synthases. To date crystal structures of three LuxI-family proteins have been reported: EsaI from plant pathogen *Pantoea stewartii*, LasI from *P. aeruginosa*, and TofI from the plant pathogen *Burkholderia glumae* [29]. The products of these three enzymes are 3-oxo-C6-HSL, 3-oxo-C12-HSL, and C8-HSL, respectively. EsaI, LasI, and TofI are all around 200 residues in length and share less than 20% sequence identity, but are structurally similar [29, 30]. LuxI-type proteins are responsible for the catalysis of an amide bond between the appropriately charged acyl–acyl carrier protein (acyl-CoA derivatives can also be substrates), the source of the fatty acyl side chain, and *S*-adenosyl-methionine (SAM), the source of the homoserine lactone ring (methionine moiety from *S*-adenosyl methionine (SAM) is lactonized to give homoserine lactone ring) [30,31] (Fig 1.6).The byproduct of the reaction, methyl thioadenosine is toxic (Fig.1.6). The nucleosidase enzyme, Pfs, cleaves MTA to two nontoxic products, adenine and methyl-thioribose (MTR) [23, 30, 31]. A diverse set of fatty acyl side chains of varying length, backbone saturation, and side-chain substitutions are incorporated into AHL signals; these differences are crucial for signaling specificity. Each LuxI protein produces the correct signal molecule with high fidelity.

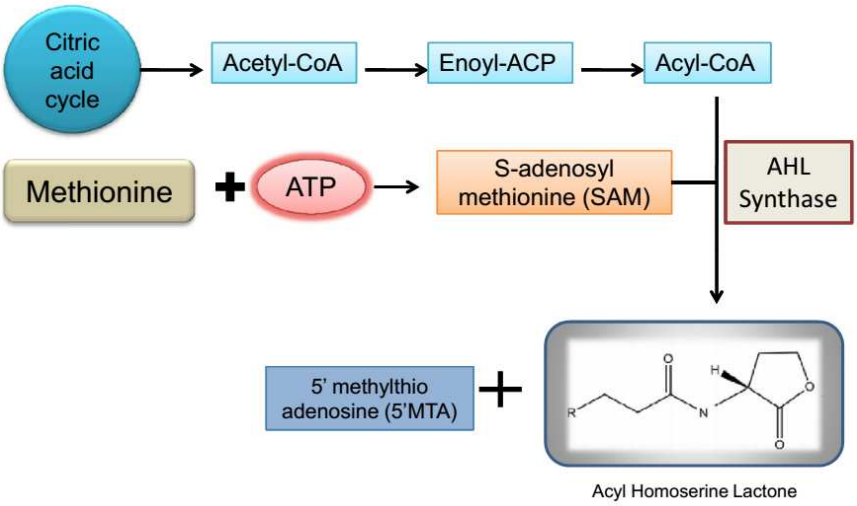



***Figure 1.6**: Pathway involved in synthesis of Gram negative QS signal molecule: N-Acyl Homoserine Lactones.*

### *1.4.2 Diversity amongst synthesized AHL signals:*

AHLs or Acyl HSLs have been identified in different members of Proteobacteria. General structure of these molecules is the same, that is, there is a fatty acid chain amide bonded to a lactonized homoserine moiety. However there is considerable structural variety in AHLs from different bacterium and also different AHLs synthesized by the same bacterium. The Acyl-carbon chain length of Acyl-HSLs varies considerable, and falls in the range of in the range of C4-C18.The chain increases by two carbon atoms at a time, and the longest chain length AHLs (C16 and C18) have been recently identified [32]. The third position of the Acyl carbon chain also shows some chemical modification. The third carbon the chain may be fully reduced or oxidized to form carbonyl carbon or may be attached to a hydroxyl group. All the chemical substitution and the variable length of the carbon chain of Acyl-HSLs determine their specificity and hence biological activity [27, 28].

### *1.4.3 Signal Receptors and Transducers: LuxR protein and its homologs:*

N-acyl homoserine lactone (AHL) quorum sensing signal molecules are recognized by LuxR-type receptors, which constitute a class of transcription factors that possess an amino-terminal AHL-binding domain and a carboxy-terminal DNA-binding domain. Most characterized LuxR-type receptors are transcriptional activators that are positively regulated by cognate AHL molecules, however there are some reports of LuxR type repressors [22, 28]. An example positive gene regulation by LuxR is, the binding of 3-oxo-C6-HSL to LuxR protein from *V. Fischeri* which triggers receptor binding to the promoter of target genes to activate gene expression. The expression of the AHL synthase (LuxI) is also upregulated by LuxR, resulting in a positive feedback loop [26]. LuxR-type AHL receptors that function as transcriptional repressors have also been reported, and the best studied example being EsaR from *P. stewartii* [23, 24]. It has been suggested that LuxR type proteins reside in the cytoplasm or are found loosely associated with the inner leaflet of the cytoplasmic membrane [23]. Ligand binding initiates a conformation change in the receptor monomer and promotes dimerization. LuxR dimers thus formed can interact with conserved DNA sequences upstream of target gene promoters [23, 26].

The understanding of the mechanism of signal recognition by the LuxR-type AHL receptors has been largely been based on structural studies. The crystal structures of five LuxR-type AHL receptors have been reported, including $TraR_{At}$ from *Agrobacterium tumefaciens,* $TraR_{NGR}$ from *Rhizobium sp. NGR,* LasR and QscR from *P. aeruginosa,* and CviR from *Chromobacterium violaceum* [33]. The full-length structures show that LuxR proteins are homodimers composed of two domains, a large N-terminal domain (~170residues) and a small C-terminal domain (~65 residues) which are connected through a highly flexible linker loop (~10 residues) [33]. LuxR proteins have been shown to homo-dimerize in the presence of cognate signal and the extent of dimerization is dependent on the concentration of the ligand. X-ray structural studies revealed that each monomer contained one deeply buried ligand binding domain the size of which varies with the cognate signal molecule. For example, the AHL-binding pocket of LasR is larger than that of TraR since it has to accommodate a longer acyl side chain of C12 instead of C8 [33]. LuxR proteins bind to DNA targets through the C-terminal domain. The DNA targets of LuxR proteins, termed lux boxes, are typically perfect of imperfect inverted

repeats that lie in close proximity to the promoter region of the target genes [23, 24]. For LuxR proteins to act as transcription activators, binding to the lux box with their helix-turn-helix motif (HTH) in their C-terminal domain and interaction with the sigma subunit of RNA polymerase (RNAP) is very important. Depending on the organism, these related motifs have been called lux boxes (*V. fischeri*), las boxes (*P. aeruginosa*) or tra boxes (*Agrobacterium tumefaciens)* [26].Available data indicates that the C-terminal region is sufficient for DNA binding and transcriptional activation and strongly suggests that the amino-terminal half of the protein plays some inhibitory role and that the autoinducer is the neutralizer of this repression [33]. Although LuxR proteins are mostly highly AHL specific, many can be activated to different extents by closely related compounds, which explain their utility as components of AHL biosensors. AHL biosensors are based on a given LuxR protein and the promoter of a target gene fused to a reporter such as *lux* [23].

## 1.5 Some interesting Quorum Sensing mediated phenotypes

### *1.5.1 QS and biofilm formation*

Bacteria often tend to attach to surfaces and form communities trapped in an extracellular polysaccharide matrix. These communities are called a biofilms. A mature biofilm is actually a collection of diverse bacterial genera and species having different metabolic backgrounds, however there is always one key player co-ordinating metabolic activities in all biofilm communities [34-36]. *P. aeruginosa* is often found in most naturally occurring biofilms [37]. Under the right conditions in the laboratory, *P. aeruginosa* forms characteristic biofilms whose thickness can be in the range of several hundred micrometers [37]. Development of a mature biofilm proceeds through a programmed series of events associated with a succession of different bacterial genera. After attachment, cells multiply to form a layer on a solid surface. This initial attached to surfaces is facilitated by flagella and pili [36]. Individuals in the layer then exhibit a surface motility called twitching. Twitching depends on type IV pili. As a result of twitching motility, small groups of cells called microcolonies form (Fig.1.7) [35, 36]. Microcolonies then differentiate to form a mature biofilm after receiving appropriate signals. Quorum sensing signal, specifically AHL signals, have been shown to play are

very crucial role in establishment of mature biofilm architecture [34]. Microcolonies in a mature biofilm may attain different shapes like tower and/or mushroom-shaped architectures (Fig.1.7). The cells in these structures are encased in an extracellular polysaccharide matrix [38]. Fine water channels that allow the flow of nutrients into and waste products out of the biofilm innervate these structures [36,38]. There is a significant physiological heterogeneity within biofilms. In *P. aeruginosa* biofilms there is a steep oxygen gradient with significant amount of oxygen present in the peripheral region but lesser amount present in the centre [37, 35]. Apparently similar gradients exist for other nutrients and pH.In mixed bacterial biolfilms existence of these gradients dictate physiological variability among individual cells in the biofilm, with slower-growing cells present deeper within the biofilm and more actively growing cells at the periphery [34, 37]. Bacteria in these mature biofilms are typically upto 1000 times more resistant to anti-microbial agents, including antibiotics as compared to planktonic cells [35]. Thus bacteria residing within these biofilms can cause many different types of chronic or persistent bacterial infections.

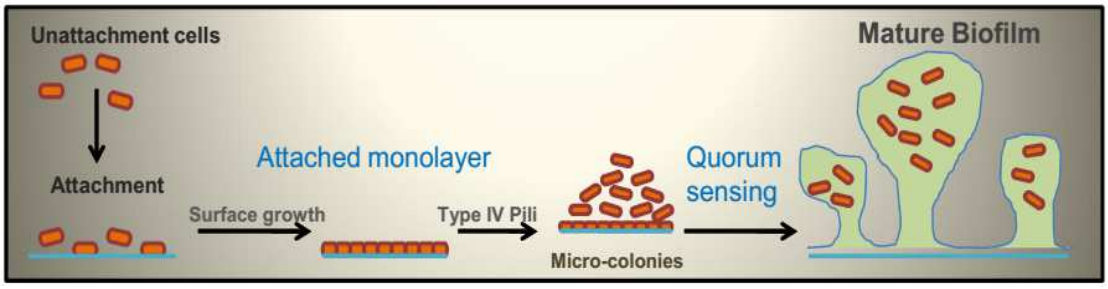



***Figure 1.7****: Schematic representation of various stages involved in formation of a mature biofilm*

### *1.5.2 QS based extracellular virulence factors production in bacteria*

The opportunistic pathogen *P. aeruginosa* can cause a wide range of infections and infirmities. It can cause invasive infections at the site of injured tissues and burn wounds, and can cause cystic fibrosis like chronic lung infections in immuno-compromised individuals as well [39, 40]. *Pseudomonas* infection involves an array of extracellular virulence factors including the elastin-specific proteases LasB and LasA. LasR (the QS transcriptional regulator similar to LuxR) has been found to regulate expression of *lasA* (Protease), *aprA* (alkaline protease A), and *toxA* (exotoxin A) (Fig. 2).A second gene

(*lasI*), which encodes a polypeptide homologous with LuxI and was found immediately downstream of lasR [41]. LasI directs the synthesis of N-(3-oxododecanoyl)-L-homoserine lactone. LasR also activates transcription of *lasB* elastase gene in the presence of *P.aeruginosa* QS signals [40, 41]. The expression of an extracellular protease specifically at high cell density is quite reasonable, because at low cell densities, the enzyme would exist at a decidedly low concentration as a result of diffusion, and proteolytic degradation would not be sufficient to benefit *P. aeruginosa* [25,41].

*Erwinia carotovorum* is a bacterial pathogen that causes the post-harvest soft rot infections of many vegetable crops including potato, carrot and green pepper [42, 43]. The virulence of this plant pathogen depends on the synthesis and secretion of a number of exo-enzymes including pectinases, cellulases and proteases. Expression of the genes coding for these plant cell wall degrading enzymes is under QS control [44]. *E. carotovora carI* (AHL Synthase gene) mutants are not only carbapenem-negative but are also have decreased exoenzyme production and their virulence is severely attenuated *in planta* [43]. Both virulence and antibiotic production can be restored by the exogenous supply of 3-oxo-C6-HSL. In contrast, mutation of *carR* (The *luxR* homolog, QS regulator) has no impact on exo-enzyme synthesis, indicating that *E. carotovora* has additional LuxR-type proteins [42]. A different *luxR* gene, *virR*, is required for exo-enzyme production [42,44]. There are many other phenotypes whose expression is modulated by LuxI/R type regulatory elements. Some of them have been enlisted in table below.

***Table 1.1:*** *Some examples of gram negative quorum sensing systems regulated by different LuxI/R homologs and their cognate AHL QS signals(Table from Reference [10]).*

| organism | major AHL(s) | LuxR | LuxI | phenotypes |
|---|---|---|---|---|
| *Aeromonas hydrophila* | C4-HSL | AhyR | AhyI | biofilms, exoproteases |
| *Aeromonas salmonicida* | C4-HSL | AsaR | AsaI | exoprotease |
| *Agrobacterium tumefaciens* | 3-oxo-C8-HSL | TraR | TraI | plasmid conjugation |
| *Agrobacterium vitiae* | C14:1-HSL, 3-oxo-C16:1-HSL | AvsR | AvsI | virulence |
| *Burkholderia cenocepacia* | C6-HSL, C8-HSL | CepR, CciR | CepI, CciI | exoenzymes, biofilm formation, swarming motility, siderophore, virulence |
| *Burkholderia pseudomallei* | C8-HSL, C10-HSL, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, 3-hydroxy-C14-HSL | PmlIR1, BpmR2, BpmR3 | PmlI1, PmlI2, PmlI3 | virulence, exoprotease |
| *Burkholderia mallei* | C8-HSL, C10-HSL | BmaR1, BmaR3, BmaR4, BmaR5 | BmaI1, BmaI3 | Virulence |
| *Chromobacterium violaceum* | C6-HSL | CviR | CviI | exoenzymes, cyanide, pigment |
| *Erwinia carotovora* ssp. *carotovora* | 3-oxo-C6-HSL | ExpR/CarR | CarI (ExpI) | carbapenem, exoenzymes, virulence |
| *Pantoea* (*Erwinia*) *stewartii* | 3-oxo-C6-HSL | EsaR | EsaI | exopolysaccharide |
| *Pseudomonas aeruginosa* | C4-HSL; 3-oxo-C12-HSL | LasR, RhlR, QscR, VqsR | LasI, RhlI | exoenzymes, secretion, HCN, biofilms |
| *Pseudomonas aureofaciens* | C6-HSL | PhzR, CsaR | PhzI, CsaI | phenazines, protease, colony morphology, aggregation |
| *Pseudomonas putida* | 3-oxo-C10-HSL, 3-oxo-C12-HSL | PpuR | PpuI | biofilm formation |
| *Pseudomonas chlororaphis* | C6-HSL | PhzR | PhzI | phenazine-1-caboxamide |
| *Pseudomonas syringae* | 3-oxo-C6-HSL | AhlR | AhlI | exopolysaccharide, swimming motility, virulence |
| *Rhizobium leguminosarum* bv *viciae* | 7-*cis*-C14-HSL/C6-HSL/C7-HSL/C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL | CinR, RhiR, RaiR, TraR, BisR, TriR | CinI, RhiI, RaiI | root nodulation/symbiosis, plasmid transfer, growth inhibition; stationary phase adaptation |
| *Rhodobacter sphaeroides* | 7-*cis*-C14-HSL | CerR | CerI | aggregation |
| *Serratia* spp. ATCC 39006 | C4-HSL | SmaR | SmaI | antibiotic, pigment, exoenzymes |
| *Serratia liquefaciens* MG1 | C4-HSL | SwrR | SwrI | swarming motility, exoprotease, biofilm development, biosurfactant |
| *Serratia marcescens* SS-1 | C6-HSL, 3-oxo-C6-HSL | SpnR | SpnI | sliding motility, biosurfactant, pigment, nuclease, transposition frequency |
| *Serratia proteamaculans* B5a | 3-oxo-C6-HSL | SprR | SprI | exoenzymes |
| *Sinorhizobium meliloti* | C8-HSL, C12-HSL, 3-oxo-C14-HSL, 3-oxo-C16:1-HSL, C16:1-HSL, C18-HSL | SinR, ExpR, TraR | SinI | nodulation/symbiosis |
| *Vibrio fischeri* | 3-oxo-C6-HSL | LuxR | LuxI | bioluminescence |
| *Yersinia enterocolitica* | C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL | YenR, YenR2 | YenI | swimming and swarming motility |
| *Yersinia pseudotuberculosis* | C6-HSL, 3-oxo-C6-HSL, C8-HSL | YpsR, YtbR | YpsI, YtbI | motility, aggregation |

## 1.6 Why cell density dependency? Musings from an evolutionary biology standpoint

Social behavior in animals and higher mammals has always been a fascinating subject of study for an evolutionary biologist however it is only recently that social behaviour in micro-organisms is beginning to be considered with respect to the evolutionary theory. Bacteria exhibit remarkable social behaviors, which some workers have suggested are analogous to those performed by insects, vertebrates and humans [45,46]. An interesting example in this respect is that of *Myxococcus xanthus*. Cells of *M. Xanthus* exhibit socially dependent swarming across surfaces which allows the given population to seek out bacterial prey in a manner that has been likened to hunting wolf packs [45]. It is now known that bacteria can communicate using small diffusible signal molecules to coordinate expression of specific target genes and produce factors that are then secreted outside of the cells in a process known as quorum sensing (QS). The underlying assumption made to explain QS is that the secretion of these extracellular factors is more beneficial at higher cell densities. It has indeed been demonstrated that the benefit of QS is relatively greater at higher population densities, and this is because all of the energy that is spent in communicating and producing goods in bulk proves to be fruitful [47]. In other words QS is used to coordinate the switching on of social behaviors at high densities because under these conditions such behaviors are more efficient and will provide the greatest benefit. Rationale behind this being that at low densities, the action of extracellular factor production would be relatively inefficient because they would diffuse away before they could be used. Whereas, at higher cell densities, a greater proportion of the products produced and secreted extracellular can be assimilated providing community wide benefits. Since it is widely understood that signaling is favored to coordinate behavior at the population level, it can be presumed that QS shares conceptual links with other forms of signaling, such as alarm or food calls in birds and mammals [48]. Quorum sensing provides unusual strength to a given population of bacterium to overcome hazardous circumstances, in which it would have been difficult for an individual bacterium to survive, i.e. in case of overcoming host defenses. Thus, this social nature of QS is not only of interest from a pure evolutionary perspective, but also because it can be exploited as a novel medical intervention strategy [46]. An

interesting amalgamation of knowledge from evolutionary biology study and molecular methods can be achieved, if cheaters or free-loaders, who do not either produce or respond to the QS signal molecules, in a given quorum sensing population are used as tools [45-48] . These cheaters could be used to reduce population size and virulence, or even introduce medically beneficial alleles into infective populations [45]. Speculations into the amount of energy invested in these kind of social interactions has made us understand that because of the natural history of micro-organisms many of these social interactions are actually between relatives, in which case signaling and cooperation will be favored by kin-selection [48]. In contrast, it is supposed that most cases of QS based interaction between bacterial species or across kingdoms however are truly cases of cues or coercion [49]. Signaling and cooperation can sometimes be favored between species and across kingdoms; however this will require unique environmental conditions that are likely to be met only rarely [49].

## 1.7 Visualizing Gram negative Quorum Sensing using Biosensor strains

To date, all the AHL biosensors bacterial strains that have been described are based on either *lux, lacZ,* or *gfp* reporter gene fusion systems or simple pigment induction [50]. These biosensor strains have been developed in such way that they can be used to detect the presence of a broad range of AHLs produced by Gram-negative bacteria. They are a sensitive and convenient method for detection of AHLs. Biosensors strains contain quorum sensing regulatory promoters fused to *lux* or *lacZ* operon and usually have inactivated AHL synthase gene [50, 51]. So these biosensor strains cannot produce their own QS signal molecule but their promoter gene gets activated by exogenously supplied quorum sensing signals. LuxR homologs present in these biosensor bacteria interact with its cognate signal molecule and initiate expression of target reporter gene. The reporter gene encode an easily detectable phenotypes like, violacein pigment production by *C. violaceum* CV026, green fluorescent protein production by *V. fischeri* pJBA88, bioluminescence by *E.coli* pSB401 etc [50]. Since each of these strains can detect a narrow range of AHLs and thus more than one kind of such biosensors are required to test the wide range of AHLs [51]. These biosensors are employed for detection of both

native AHL molecules as well as detection of, AHL based QS signal agonists and antagonists. The AHL detection bioassays are most frequently performed by overlay method while quantitative assays are performed by liquid cultures [51]. The most commonly used biosensor strain for detection of long chain AHLs is *Agrobacterium tumefaciens* NT1 (traR, tra::lacZ749) contains a lacZ fusion in the traR gene and produces blue colour from the hydrolysis of 5-bromo-4-chloro-3-indolyl-$\beta$-Dgalactopyranoside by the $\beta$-galactosidase activity [52] (Table 1.2). For detection of medium-chain length AHLs with acyl chains of C4 toC8 *Chromobaterium violaceum* CV026 is most regularly used [52, 53]. It is mini-Tn5 mutant of *C. violaceum* ATCC 31532 containing LuxR homolog CviR regulating the production of violacein, which is a purple colored pigment produced by the bacterium in response to AHLs [53] (Table 1.2).

***Table 1.2**: Biosensor strains used in Quorum sensing and Quorum Quenching assays* (Table adapted from [50])

| ***Biosensor strain*** | **AHLs detected** | **Detectable phenotype** |
|---|---|---|
| *A. tumefaciens* strain NT1 (pDCI41E33) | AHLs with 3-oxo-, 3-hydroxy-, and 3-unsubstituted side chains of all lengths from C6-C14HSL. | β-galactosidase activity |
| *Chromobacterium violaceum* strain CV026–CviR receptor | Medium chain length AHLs | Violacein pigment production |
| *E. coli* (pSB403) plasmid carrying a lux CDABE cassette activated by AhyR receptor of *A. hydrophila* | C4HSL | Bioluminescence |
| *E. coli* JM109(pSB401) plasmid carrying a lux CDABE cassette activated by LuxR receptor of *Vibrio fischeri* | C6HSL<br>3-Oxo-C8-HSL<br>C8-HSL | Bioluminescence |
| *Pseudomonas putida*117(pAS-C8)-CepR receptor | C8HSL | Green Fluorescent Protein |
| *P. putida* IsoF⁄gfp<br>*S. liquefaciens* strain PL10—LuxAB reporter | 3OC12HSL<br>C4HSL | Fluorescence<br>Bioluminescent |
| *Sinorhizobium meliloti* Rm41 (pJNSinR) | C16–C20HSL | β-galactosidase activity |
| *C. violaceum*VIR24 (CviI receptor) | 3-Oxo-C6-HSL, C6HSL, C7HSL,3-oxo-C8HSL, C8HSL, C10HSL, C12HSL, and C14HSL | Violacein pigmentation |

## 1.8 Imaging Quorum Sensing Receptors in live bacteria: monitoring pathogenic bacteria capable of QS in real time

It is only recently, that the need to monitor pathogenic Gram-negative bacteria, capable of AHL based quorum sensing, has been identified. Real time tracking of bacteria possessing QS receptors during infection can provide better understanding of the host–pathogen interaction. In principle, the presence of exogenous AHLs can be detected by a reporter gene fused to any QS target gene maintained in a bacterial cell having an inactivated signal generator (I) [50].This is the exact principle on which bacterial biosensors work, however the major disadvantage of these biosensors is the time taken by them to respond to these AHL signals (because it involves growth of bacteria). Detection of QS in live cells has proved to be challenging however in the past few years three reports (including one from our laboratory [54]) has shown that such a scenario is indeed possible. These findings open up a strong possibility for development of a chip-based biosensor for quick detection of pathogenic bacteria capable of QS in any given sample. First report by Gomes *et al.* talk about the use of labeled ligands (AHL molecules) with a fluorescent tag as the simplest tools for detection of QS receptors in live cells [55]. Such an approach of labeling native Ligand (AHL molecules) is often accompanied by insufficient binding affinity towards the receptor and competition problems with the natural agonists [55]. However the synthetically labeled molecule was found to mimic the activity of the natural agonist throughout a large concentration range, and the group reported excellent labeling of bacterial quorum-sensing receptors in live cells [55]. This operationally simple, fast and inexpensive method was then successfully applied to the selective labeling of the *Burkholderia cenocepacia* quorum sensing receptor CepR. Furthermore, selective labeling was achieved in mixed bacterial cultures, demonstrating the potential of this approach as a very powerful tool to visualize quorum sensing in bacteria in their natural habitat [55]. In the second report Meijler and co-workers, have reported an aniline-catalyzed two-step labeling strategy for the visualization of the *Pseudomonas aeruginosa* LasR QS receptor in live cells by using a selective bio-orthogonal ligation [56]. This method is based on the selective covalent binding of the iso-thiocyanate functionalized AHL to LasR and good labeling quality was achieved [56].

However, this approach is restricted to the absence of native AHLs and cannot be applied for the investigation of bacteria naturally producing AHLs.

## 1.9 Quorum Quenching: Signal depletion and communication confusion

As discussed previously in great detail Quorum sensing is a cell density-dependent signaling mechanism used by wide variety of bacteria for co-coordinating a population wide response in regulating phenotypes, such as expression of virulence genes, antibiotic resistance and bio-film formation. In this light, it is significantly evident that disruption of this kind of bacterial communication can serve as a fruitful anti-virulence strategy with enormous therapeutic potential [57]. Any attempt to impede or hamper cell to cell communication in bacteria is known as Quorum Quenching (QQ) or signal interference [58]. With the ever increasing burden of antibiotic resistance and incessant increase in MDR strains need for an alternative drug target with the scope of development of sustainable anti-infective therapy is utmost essential. The quorum quenching therapeutic approach is one such strategy that promises a lower risk of resistance development [58, 59]. Interference with QS (and thus virulence gene expression) generally does not affect the growth and fitness of the bacteria and, hence, does not exert an associated selection pressure for development of drug-resistant strains [59]. Over the years excessive amount of research has gone into better understanding the mechanisms of existing bacterial communication networks and search for newer QS networks. All this has led to the development of many innovative QS interference strategies based on the fundamental principle of impeding the interaction between a quorum molecule and its cognate receptor [60]. Since Gram-negative bacteria are an important group of pathogens, with high rate of prevalence of drug resistance and responsible for the majority of hospital-acquired infections, a greater part of QS inhibitory strategies that have been developed, are against this group of pathogenic bacteria [60].

There are three essential steps in the bacterial quorum sensing pathway:

1. Signal molecule synthesis,
2. Extracellular accumulation of quorum sensing signal molecules
3. QS molecule (Ligand) /receptor interaction and activation of QS phenotypes

Thus based on the above mentioned steps interference in the QS mechanism can be achieved in a variety of ways. Following table highlights the steps in the QS circuit can be or have been potential targets for various inhibition strategies (Table 1.3).

***Table 1.3**: Key steps involved in bacterial QS and their proposed inhibition strategy:*

| *Quorum sensing process* | *Cellular components involved* | *Potential QQ strategy* |
|---|---|---|
| **QS signal generation** | AHL Synthase or Lux I homolog protein; cellular precursor molecules SAM and Acyl-ACP | LuxI protien inhibitors; structural analogues of precursor molecules. |
| **QS signal accumulation** | Active or passive transport of signal molecules across cell membrane | Quorum quenching enzymes that degrade QS signal molecules |
| **QS signal reception** | Signal receptors or LuxR protein homologs | LuxR protein inhibitors; structural analogs of signal molecules |

The following figure provides a more graphical interpretation of information contained in Table 1.3.

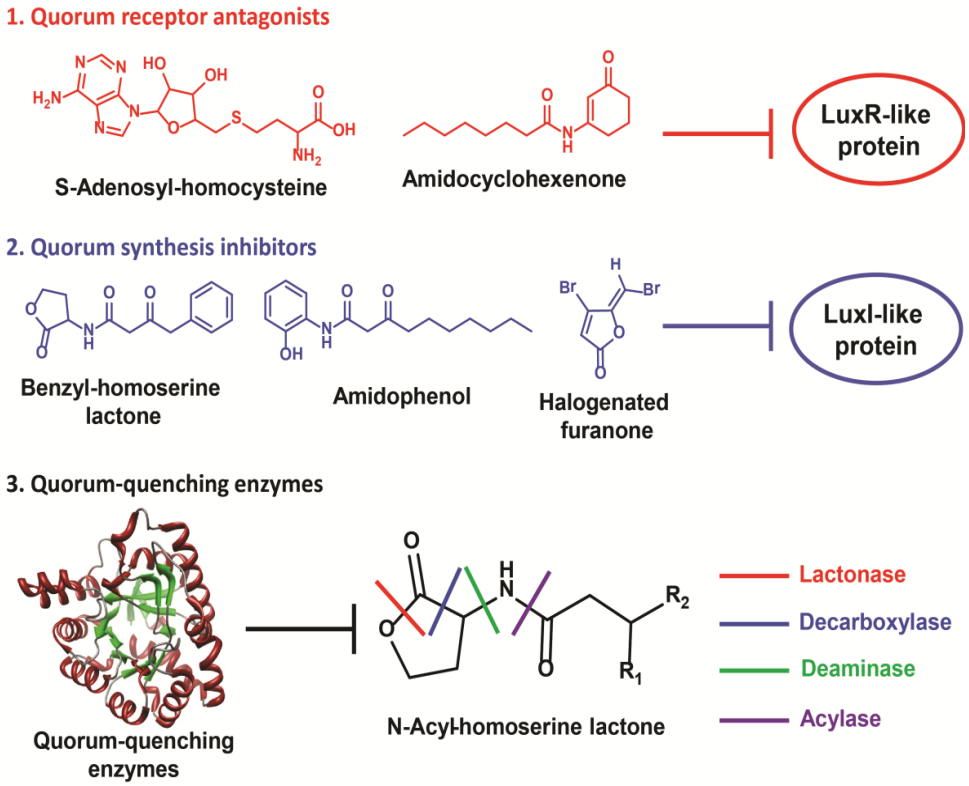



***Figure 1.8:** Strategies involved in inhibiting gram negative Quorum Sensing (Adapted from [61])*

### *1.9.1 Inhibition of signal synthesis:*

The AHL synthases or LuxI homologs use *S*-adenosyl methionine (SAM) as the amino donor for generation of the homoserine lactone ring moiety, and an appropriately charged acyl carrier protein (ACP) as the precursor for the acyl side chain of the AHL molecule and it is well accepted that LuxI-type protein is required and sufficient for production of AHL signals [30,31].SAM and acyl-ACP from important parts of, the fundamental, amino acid synthesis and fatty acid metabolism pathways respectively [62]. Howver since the reaction chemistry of AHL synthase with SAM appears to be unique, even though SAM is a necessary and common intermediate in many prokaryotic and eukaryotic pathways SAM analogs could be used as specific inhibitors of quorum-sensing signal generation, without affecting eukaryotic enzymes that use SAM [63]. Thus far, analogs of SAM have been found to be potent inhibitors of the RhlI synthetase from *P. aeruginosa*, in vitro systems [63, 64]. The compounds include L/D-*S*-adenosyl homocysteine, sinefungin, butyryl-SAM, and the most effective L-*S*-adenosyl cysteine that was able to reduce the activity of RhlI by 97% [64].

### *1.9.2 Inhibition of signal accumulation:*

Quorum sensing or bacterial cell-to-cell communication can also be inhibited by a decrease in the extracellular concentration of active signal molecule [65]. AHL decay might be a consequence of either an enzymatic or a non-enzymatic reaction. AHL signals undergo spontaneous lactonolysis/inactivation at high pH values and are also degraded at very high temperatures [64]. Enzymatic degradation of AHL molecules have been of great interest and enzymes capable of inactivating AHLs have received increasing amounts of attention since the past few years [65, 66]. Most studied AHL-degrading enzymes thus far are either AHL lactonases that hydrolyze the ester bond in the lactone ring or AHL acylases that hydrolyze the amide bond that holds the acyl chain and the homoserine lactone together [65-69]. However, there a few other enzymes, that have shown the ability to inactivate AHLs, either by oxidizing the ω-end of the acyl chain (De-aminases) or by reducing the beta-keto carbonyl of 3-oxo-acyl-homoserine lactones (Decarboxylases) [64, 68].

1.9.2.1 Enzymatic degradation of signal molecules by **AHL Lactonase**:

One of the most well studied class of quorum quenching enzyme is the **AHL lactonase**. The AHL lactonase family of enzyme possesses hydrolytic activity toward a broad spectrum of AHLs, regardless of the acyl chain length and the oxidation state at the C3 position of the acyl chain [68]. These enzymes are highly specific toward AHLs with little to no activity toward non-acylated homoserine lactones. End products of AHL lactonases mediated enzyme reaction is an acyl-homoserine, however this reaction is reversible, and at acidic pH (pH<2) biologically active AHLs are regenerated [67,68]. Dong *et al.* in 2000 [70] found that a *Bacillus* species (*Bacillus sp*. 240B1) could produce an enzyme, termed AiiA that catalyzed the hydrolysis of AHL molecules by cleaving the lactone ring. The *aiiA* gene encoded a gene product, a 250 amino acid protein, which was later characterized as a lactonase, catalyzing the hydrolysis of the ester bond in the lactone ring of a wide variety of AHLs [70]. The enzyme was characterized to be belonging to metallo beta-lactamase superfamily and was more specifically a zinc metalloprotease [59,70]. Over-expression of the *aiiA* gene in the plant pathogen *Erwinia carotovora* has been reported to result in significantly reduced accumulation of AHL signals, decreased extracellular enzyme activity, and hence attenuated soft rot disease symptoms *in planta* [71]. Moreover, transgenic plants expressing AiiA were shown to be significantly less susceptible to infection by *E. carotovora* [68,70, 71]. Since this initial report of the discovery of a Lactonase from *Bacillus sp*. 240B1, many similar AiiA like enzymes were isolated from different species and sub-species of *Bacillus* (*B. thuringiensis, B. cereus, B. anthracis, B. mycoides* etc.), sharing more that 90% -95% sequence homology in most cases indicating that all *aiiA* enzymes in *Bacillus* sp. may have evolved from the same ancestral gene [68,59].

Several other family of AHL Lactonase has been reported, other than the AiiA type of Lactonases found in *Bacillus* sp. These include *Agrobacterium tumefaciens* (*attM*), *Klebsiella pneumoniae* (*ahlK*), and *Arthrobacter* sp. (*ahlD*), however since these enzymes also contain the conserved zinc-binding motif; they have been predicted to share a similar catalytic mechanism as AiiA. A different AHL lactonase, encoded by *aiiB*, was identified in *A. tumefaciens* by Carlier *et al.*2003 [72]. However this enzyme shares only 28% amino acid sequence identity with AiiA240B1 from *Bacillus* sp., excluding it from

the AiiA family. AiiB prefers substrates with longer acyl chain compared to AiiA and has considerably reduces activity towards oxo-substituted molecules [72].

One of the most divergent class of AHL lactonases was identified from *Rhodococcus erythropolis*. The 323 amino acid enzyme encoded by *qsdA* (for quorum sensing signal degradation) is a member of the phosphotriesterase (PTE) superfamily and is unique to *Rhodococcus sp.*[73]. PTEs are zinc metalloproteases that were originally identified for their ability to hydrolyze phosphotriester-containing organophosphorus compounds, but recently more members of this family were found to possess lactonase like activity as well [64,68].

1.9.2.2 Enzymatic degradation of signal molecules by **AHL Acylase**:

The second major cluster of AHL-degrading enzymes, is **the AHL acylases**, that target the amide bond holding the fatty acyl chain to the homoserine lactone moiety. Compared to the AHL Lactonase, Acylases are a less studied cluster. AHL Acylases or amido-hydrolases were first identified by Leadbetter and Greenberg [74], who discovered a strain of *Variovorax paradoxus* capable of utilizing AHLs as a sole source of energy and nitrogen. This strain does not produce AHL signal molecules of its own but can grow on a variety of AHLs varying in acyl chain length from 4 to 12 carbons long as well as oxo-substituted derivatives [74, 67]. Further examination into its ability to grow on AHLs of varying chain length as a sole source of carbon and nitrogen suggested that the acyl group and not the lactone ring was the energy source. Additionally, disappearance of HSL from the culture medium suggested that other enzymes may help utilize HSL as a nitrogen source [74]. First report of a bona fide AHL acylase was from a *Ralstonia sp*.[75]. The AHL acylase isolated from *Ralstonia strain XJ12B* [75] was named AiiD and it represents a distinct family of AHL acylases. The AiiD enzyme from Ralstonia shares significant animo acid sequence homology with members of the N-terminal nucleophile (Ntn) hydrolase superfamily, like, cephalosporin acylase and Penicillin acylases [75, 67]. Ntn-hydrolase is a super-family of enzymes whose members undergo post-translational auto-proteolyitc cleavage to yield two subunits (alpha and beta) that constitutes the conformationally active enzyme and they have the typical $\alpha\beta\beta\alpha$ fold structure [75,77]. Over-expression of recombinant AiiD in *P.aeruginosa* prevented AHL accumulation in

the culture medium, thus reducing virulence, and impairing swarming motility as well as killing of *C. elegans* [76,59].

*Pseudomonas aeruginosa* genome has been predicted to code for multiple AHL acylases [77]. One of the first homolog of an AHL acylase from *P.aeruginosa*, was found to be encoded by PA2385, previously designated *pVdQ* (for its role in the biosynthesis of the siderophore pyoverdine). PvdQ is one of the most extensively studied member of its class [77] . PvdQ is responsible for de-amidation of AHLs as a result of which HSL is accumulated as the degradation product. Over-expression of *pVdQ* in *P.aeruginosa* PAO1 inhibited accumulation of 3-oxo-C12-HSL. However, a *pVdQ* knockout strain was still able to utilize AHLs as a sole source of carbon, implying that another enzyme may be involved in the AHL degradation process [78]. Indeed, a second AHL acylase was found in *P.aeruginosa* and was named *quiP* for quorum signal utilization and inactivation [77]. QuiP shares 21% a.a. sequence homology with PvdQ and 23% homology with AiiD from *Ralstoni*a sp., and it is another member of the Ntn-hydrolase family, with substrate preference for long-chain AHLs. The recently solved crystal structure of PvdQ [78] led to its characterization as a member of the Ntn-hydrolase family, with a slightly larger hydrophobic active site pocket. PvdQ is first expressed as a proenzyme with a αββα-fold structure common to all Ntn-hydrolases. Autoproteolytic cleavage yields the formation of a catlytically active enzyme. Following the elucidation of the crystal structure, a mechanism for the acylation reaction was proposed by Bokhove *et al.* [78] where the N-terminal serine, located in the bottom of the hydrophobic binding pocket, acts as a nucleophile which then mediates cleavage of amide bond. This proposed mechanism is similar to that proposed for Penicillin G acylase (an important member of Ntn-hydrolase structural superfamily). However it is interesting to note that to date, there are no reports of an authentic Penicllin G acylase acting as an AHL acylase other than the one report from our lab (See Chapter 2).

A significantly different AHL acylase was isolated from *Anabaena* sp. strain PCC7120 and it was named as AiiC [79]. This enzyme shares 29% a.a. sequence homology with QuiP of *P.aeruginosa* and is considered another member of the Ntn-hydrolase family, although it differs from the other reported AHL acylases by its broad substrate specificity

and its ability to hydrolyze AHLs with 4-16 carbons in the acyl chain, with or without the 3-oxo substitution [79]. Another similar AHL acylase was found in *Comamonas* strain D1, which shows a very wide specificity for different AHLs, although it was reported that *Comamonas* could not grow on AHLs as a sole source of carbon or nitrogen [64,59]. Apart from the above mentioned reports a number of other bacterial genera have been shown to possessing AHL acylase activity and some of them have been listed in table below.

***Table 1.4**: Some reported AHL acylases and AHL Lactonases [Adapted from [62 and 82]*

| Source of QQ enzyme | Type of Enzyme | Name of enzyme | AHLs degraded |
|---|---|---|---|
| *Bacillus sp .strain 240B1* | AHL Lactonase | AiiA | 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL |
| *Bacillus thuringiensis* | AHL Lactonase | AiiA | C6HSL,3-oxo-C6-HSL, 3-oxo-C8-HSL, C8HSL,C7HSL,C10HSL |
| *Agrobacterium tumefaciens* | AHL Lactonase | AttM | 3-oxo-C8-HSL, C6-HSL |
| *Ochrobactrum sp.T63* | AHL Lactonase | AidH | C4-HSL, C6-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL, C10HSL |
| *Rhodococcus erythropolis W2* | AHL Lactonase (PTE superfamily ) | QsdA | AHLs with or without substitution on carbon 3 an acyl chain ranging from 6 to 14 carbons |
| *Microbacterium testaceum StLB037* | AHL Lactonase | AiiM | 3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, C10-HSL |
| *Pseudomonas aeruginosa* PAO1 | AHL Acylase | PvdQ | AHLs with or without substitution on carbon 3 and acyl chain ranging from 10 to 14 carbons |

***Table 1.4 Continued.***

| Source of QQ enzyme | Type of Enzyme | Name of enzyme | AHLs degraded |
|---|---|---|---|
| *Pseudomonas aeruginosa* PAO1 | AHL Acylase | QuiP | AHLs with or without substitution on carbon 3 acyl chain ranging from 7 to 14 carbon |
| *Anabaena sp.* PCC 7120 | AHL Acylase | AiiC | AHLs with or without substitution on carbon 3 and with an acyl chain ranging from 4 to 14 carbons |
| *Pseudomonas syringae* strain B728a | AHL Acylase | HacB | AHLs with or without substitution on carbon 3 and with an acyl chain ranging from 6 to 12 carbons |
| *Shewanella sp.* strain MIB015 | AHL Acylase | Aac | C8-HSL, C10-HSL and C12-HSL |
| *Streptomyces sp.* strain M664 | AHL Acylase | AhlM | C8-HSL, C10-HSL, 3-oxo-C12-HSL |
| *Tenacibaculum Maritimum* strain NCIM B2154(T) | AHL Acylase | - | C10-HSL |
| Variovorax paradoxus VAI-C | AHL amino-acylase | - | C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL |

### *1.9.3 Inhibition of AHL signal reception:*

Blocking of quorum-sensing signal transduction can be achieved by an antagonist molecule capable of competing or interfering with the native AHL signal for binding to the LuxR-type receptor [64,80] . Competitive inhibitors are generally structurally similar to the native AHL signal, in order to bind to and occupy the AHL-binding site but fail to activate the LuxR-type receptor [81]. Noncompetitive inhibitors may show little or no structural similarity to AHL signals, as these molecules bind to different sites on the receptor protein [82]. Several workers have generated substantial knowledge about the

structure-function relationships of AHL signals, which is of great value for the continued search for potent quorum-sensing inhibitors [64, 80-83]. Numerous synthetic AHL analogs have been described and they can be substituted in either the side chain or the ring moiety of the original AHL molecule.

1.9.3.1 Substitutions on the side chain:

The acyl side chain has been modified in several ways, and it has been shown that the length is crucial to activity [64]. Interestingly, AHL analogs with a longer side chain than the native AHL generally appear to be more efficient inhibitors than AHL analogs with a shorter side chain [64, 83, 63]. This observation might suggest that a minimum acyl side chain length, as determined by the native AHL signal, is required for binding to LuxR homologs and that longer acyl chains cannot be accommodated in the AHL-binding site of LuxR-type receptors [82, 83].The flexibility of the acyl side chain also appears to be important for binding to LuxR-type proteins. For example, reduction of the chain rotation by introduction of an double bond close to the amide linkage almost completely abolishes binding to the receptor [63, 64, 84]. Also if the C-3 atom in the side chain is replaced by a sulfur atom, it produces a potent inhibitor of both the *lux* and *las* systems as described by Persson *et al.* (2005) [85]. Likewise, if the C-1 atom is replaced by a sulfonyl group, a QSI is also generated [64, 84, 86].Another strategy to modify the AHL signal molecules is to place atoms or groups at the end of the side chain. Substituting secondary aryl groups at the C6 atom (last carbon atom of the acyl chain) of 3-oxo-C6 HSL gives rise to an antagonist [64,86]. Although the size difference between the native molecule and this aryl substituted derivative is negligible, the differences in activity are probably due to the ability of the aryl compounds to interact with other aromatic residues in the protein [82]. If the size of the substituent is increased further to include tertiary alkyl derivatives or even larger alkyl and aryl moieties they become potent antagonists demonstrating that bulky molecules cannot enter the AHL binding site in the receptor protein [84].

1.9.3.2 Substitutions on the HSL ring:

The homoserine lactone moiety is generally very sensitive to modifications, and the chirality is crucial to biological activity [64]. Natural AHL signals are L-isomers, whereas D-isomers are generally devoid of biological activity [83]. The acyl side chain

appears essential for activity, as exemplified in *E. carotovora*, in which homoserine lactone ring without the acyl chain fails to activate the quorum-sensing system [85,86]. Most compounds with a keto-oxygen in the ring or an extra carbon expanding the ring have exhibited little or no binding to the LuxR protein (Schaefer *et al.* 1996) [87].Compounds having acyl alcohols or acyl amides attached on the C3 carbon atom of the ring are still able to function as agonists of LuxR. On the other hand, if the substituents are placed on the C4 atom, the compounds are not able to interact with the LuxR receptor. This indicates that there is more "free space" around the C3 atom of the ring inside the AHL binding pocket (Olsen *et al.* 2002) [88]. Instead of single substitutions, the entire ring can be exchanged with another cyclic structure. In an attempt to look for compounds able to interfere with QS in *P. aeruginosa*, the side chains of 3-oxo-C12 HSL and C4 HSL were attached to amino-cyclo-alcohol and amino-cyclo-ketone with either five or six carbons in the ring [82, 84, 89]. The C12 amino cyclo hexanol compound was a strong activator of the LasR protein but inhibited RhlR, whereas the C4 keto compounds were the most potent agonists of the RlhR protein, but antagonized LasR [63, 64, 82]. This indicates even though there are very small differences in the binding cavity of the two QS receptors (LasR and TraR) they do not perceive the HSL moiety of AHL signal in the same manner [80, 64]. An even more potent inhibitor of LasR is 3-oxo-C12-(2-aminophenol) which is able to abolish production of pyocyanin and elastase; in addition, it can disrupt normal biofilm formation by *P. aeruginosa* [80-82]. This is interesting because as mentioned previously 3-oxo-C12-(2-amino cyclo hexanol) is an activator of LasR protein, however as a phenol group replaces the hexanol a potent inhibitor is created [63, 64, 82]. A similar situation is seen with analogs of 3-oxo-C6 HSL which regulates the expression of *lux* genes in *V. fischeri* system. If the HSL ring is replaced by a hexane ring, the ability to activate LuxR is retained. On the other hand, if the HSL ring is replaced by a benzyl group, an inhibitor of LuxR is generated [86, 87, 90].

### *1.9.4 Antibody based inhibition of QS:*

Another approach towards development of a successful anti-infective therapy is sequestering QS signals with the help of antibodies [94-96]. The 3O-C12-HSL a major QS molecule of gram negative pathogen *Pseudomonas aeruginosa* has been shown to

exert cytotoxic effects on mammalian cells [58, 95]. These findings support the development of new strategies to combat microbial infections by targeting the QS signaling molecules. Because AHLs and related signal molecules have low molecular weight and are non-proteinaceous in nature they cannot elicit an antibody-based immune response on their own so they need to be conjugated with a carrier protein [94, 95]. Monoclonal antibodies (mAbs) raised against the hapten conjugated 3O-C12-HSL demonstrated an affinity for 3O-C12-HSL molecule in vitro, and exhibited high specificity because they did not recognize any of the short-chain oxo-substituted AHL molecules [82, 96]. These mAbs exerted a clear inhibitory effect on the production of pyocyanin by P. aeruginosa, a QS-controlled virulence factor. Immunization of mice with 3O-C12HSL — BSA (protein) conjugate generated specific antibody in serum [82,83] . Intranasal challenge of mice with $3\times10^6$cfu *P. aeruginosa* PAO1 resulted in 36% survival of immunized mice up to day 4 in comparison to controls, where all of them died within 2 days after infection [82,83].

### *1.9.5 Quorum sensing inhibition by eukaryotes: natural compounds from Fungi, Algae, Plants and Animals causing communication blackout:*

AHLs and AHL-like signal molecules form the basis of quorum sensing mediated virulence factor production by pathogenic bacteria. Interestingly these signal molecules are also perceived by the eukaryotic hosts and consequently leads to production of defense molecules in response to this stimulation. Additionally many plant derived natural products, like essential oils, various phytochemicals, flavanoids and crude extracts derived from different plant parts have proven to be important source of QSIs. Enzymes that degrade QS signal molecules have also been identified in some other eukaryotic sources [82]. Among animals, mammalian paraoxonases (PONs) are known to perform hydrolytic activities on AHL lactone ring, hence PONs produced by human airway epithelial cells have been found to inactivate AHLs (3OC12HSL) produced by *P. aeruginosa* via lactonolysis [59,62]. Halogenated furanones produced by red alga *Delisea pulchra* were able to inhibit AHL-dependent carbapenem antibiotic synthesis and extracellular pectinitolytic enzyme production in *P. carotovorum* subsp. *Carotovorum* [97]. Some more examples of molecules from eukaryotic origin with QSI potential have been listed in table below.

***Table 1.5:*** *List of selected Quorum sensing inhibitors from eukaryotic origin (partially adapted from [82])*

| Source and Name of QSI | Active against (organism) and QS phenotype inhibited | Reference. |
|---|---|---|
| *Delisea pulchra*(Australian macroalga, Sea weed): Halogenated Furanone | *Vibrio harveyi* Toxin production and Luminescence | [97] |
| *Penicillium*: patulin and penicillic acid | *P. aeruginosa* Biofilm | [98] |
| *Allium sativum*(garlic) extract | *P. aeruginosa* Biofilm formation<br>*P. aeruginosa* strain IsoF/gfp Fluorescence | [99] |
| Garlic extract and p-coumaric acid | *P. aeruginosa* strain IsoF/gfp Fluorescence | [100] |
| *Ajoene*, a sulfur-rich molecule from garlic | *P. aeruginosa* virulence factors and Rhamnolipid production | [101] |
| *Allicin* | *Pseudomonas aeruginosa* biofilm | [102] |
| Cinnamaldehyde and cinnamaldehyde derivatives | *Vibrio spp* AI-2-mediated QS—bioluminescence, protease activity, pigment formation | [103] |
| Vanilla extract | *Chromobacterium violaceum* CV026 violacein pigment formation | [104] |
| Blueberry extracts , Grape extracts, Raspberry extracts | *C. violaceum* Violacein production | [105] |
| *Brassica oleracea*, Basil, thyme, rosemary, ginger and turmeric: extracts of these herbs and spices | *C. violaceum* Violacein production | [105] |
| Medicinal plant extracts:*C. erectus*(leaves),*C. viminalis* (leaves),*B. buceras*(leaves) | *P. aeruginosa* Protease, elastase, pyoverdin production and biofilm formation | [106] |
| *Manilkara zapota*<br>*Musa paradiciaca*<br>*Ocimum sanctum* | *C. violaceum* Violacein production<br>*P. aeruginosa*PAO1 Pyocyanin pigment,Elastase production and biofilm formation | [107] |

***Table 1.5 Continued.***

| Source and Name of QSI | Active against (organism) and QS phenotype inhibited | Reference |
|---|---|---|
| *Medicago sativa* seed exudates | *C. violaceum* Violacein production | [108] |
| L-Canavanine, an arginine Analog | *Sinorhizobium meliloti* Exopolysaccharide II (EPSII) | [108] |
| *Medicago truncatula*(seedling fractions 8–12 and 21–24) | E. coli LuxR reporter *C. violaceum* CviR reporter AhyR reporter | [109] |
| *Combretum albiflorum*(bark) | *P. aeruginosa* Biofilm formation and elastase. | [110] |
| Flavanone naringenin | Quorum sensing-controlled virulence factors in *Pseudomonas aeruginosa* | [111] |
| Wheat bran | *Escherichia coli* JB523, containing plasmid pJBA130, production green fluorescent protein | [112] |
| Carvacrol | *Chromobacterium violaceum* Violacein production | [113] |
| Clove oil | Biofilm of *Pseudomonas aeruginosa* and *Aeromonas hydrophila* | [114] |
| Iberin from horseradish | Inhibitor of *Pseudomonas aeruginosa* virulence factor | [115] |
| Broccoli extract | *Escherichia coli* O157:H7 | [116] |
| Honey (at low concentration) | *Escherichia coli* O157:H7 biofilm formation and virulence | [117] |
| Caffeine | *C. violaceum*CV026 pigment production and *P. aeruginosa* PAO1swarming motility | [118] |
| Plant essential oils | *C. violaceum*CV026 pigment production | [119] |
| Flavonoid-rich fraction from *Centella asiatica* | *P. aeruginosa*PAO1 | [120] |

***Table 1.5 Continued.***

| | | |
|---|---|---|
| Curcumin from *Curcuma longa* | QS inhibition in Urinary pathogens | [121] |
| Eugenol | *P. aeruginosa* PAO1 Elastase swarming and biofilm inhibtition *C. violaceum*CV026 pigment inhibition, β-galactosidase luminescence inhibition in *E. coli* | [122] |
| Paraoxonases | *Pseudomonas aeruginosa* Biofilm | [123] |

## 1.10 Application of Quorum Quenching in Biotechnology and Medicine

Since quorum sensing pathways are central to virulence of bacterial pathogens and omission of genes involved in such pathways affects microbial pathogenesis adversely, numerous ways to inhibit quorum sensing in bacterial pathogens have been investigated [124, 125]. The discovery of quorum quenching enzymes, in addition to numerous natural and synthetic quorum sensing inhibitors has helped assess the feasibility of this novel strategy [125,82] . The expression of a quorum quenching enzyme, in numerous pathogenic bacteria has shown to successfully attenuate their virulence [82,83] . Also transgenic plants expressing AHL-Lactonase can effectively quench bacterial QS signaling and in turn regulate virulence gene expression whereas untransformed control plants develop severe disease symptoms (Dong *et al.*, 2002) [70]. These results demonstrate that externally expressed AHL-degradation enzyme at physiological-relevant concentrations is sufficient in eliminating the QS signals and suppressing the QS-dependent virulence gene expression by pathogens. Novel quorum quenching enzymes with increased stability and broad substrate specificity might hold great promise for the genetic engineering of disease resist plants. QQ acylases from different bacteria have also demonstrated the ability to inhibit biofilm formed by mixed bacterial population. Porcine kidney acylase I, an enzyme that has been shown to possess AHL acylase activity, has proven to have tremendous potential to prevent membrane fouling [127,128].However is a little premature to discuss the potential implications of quorum quenching enzymes and molecules in the context of their actual pharmaceutical application, since a lot remains to be studied with regards to their delivery, stability, efficacy, toxicity and side effects.

Some other interesting applications of quorum sensing inhibitory strategies in the field of biotechnology have been enlisted in the table below.

***Table 1.6****: Some biotechnological application of Quorum quenching or Quorum sensing inhibition(adapted from reference [82]:*

| Quorum sensing inhibitor and source | Biotechnological application | Reference |
|---|---|---|
| AiiA lactonase from Bacillus sp. | Attenuates the virulence of Erwinia carotovora | [70] |
| Microbacterium testaceum StLB037 associated with potato leaf | AHL degrader—effective against Pectobacterium carotovorum subsp. carotovorum, which causes soft rot diseases in many plants | [126] |
| Porcine Kidney Acylase I | Biofilm disruption in aquaculture<br>Prevention of biofouling | [127]; [128] |
| Nano-filtration membrane immobilized with a QQ acylase | Suppress exopolysaccharides and inhibits biofilm formation | [129] |
| Kojic acid and other biomolecules from bacteria | Prevention of Marine biofouling | [130] |
| Natural and synthetic brominated furanones | Protect brine shrimp—Artemia franciscana from<br>Pathogenic Vibrio spp.<br>Protect rotifer—Brachionus plicatilis from V. harveyi | [131]<br>[132] |
| Microbes isolated from the guts of marine organisms such as Penaeus vannamei shrimp | Degrade Vibrio harveyi HAI-I in vitro and improve growth rates of rotifers<br>Cultures enhanced the survival of turbot larvae | [132]<br>[133] |
| Microflora (Shewanella sp. strain MIB010) from the intestine of Ayufish, Plecoglossus altivelis | Effective against QS regulated biofilm produced by fish pathogen,Vibrio anguillarum | [134,135] |
| Isolates from tobacco rhizosphere | Effective inhibit pectinolytic activity of Pectobacterium carotovorum | [136] |

## 1.11 Outline of the thesis:

The thesis is broadly divided into three parts and which is further divided into 6 individual chapters, as follows:

- Chapter 1: Introduction

**A. Enzymatic means of inhibition of Bacterial Quorum Sensing**.

- Chapter 2: *Kluyvera* citrophila Penicliin G acylase acting as an AHL acylase.
- Chapter 3: Possible correlation between Bile salt hydrolase and AHL deamidation
- Chapter 4: AHL Lactonase characterized from Novel *Bacillus* Species isolated from Fenugreek root nodule rhizosphere.

**B. Bio-molecular means of inhibition of Bacterial Quorum Sensing**

- Chapter 5:Plant Essential oils derived glycolipids as potent quorum sensing inhibitors and anti-biofilm agents
- Chapter 6: Glycomonoterpenols: A new class of bacterial quorum sensing antagonists

**C. Imaging Quorum Sensing: A diagnostic view**

- Chapter 7: Early imaging of bacteria capable of quorum sensing using Au nanoclusters surface functionalized using bacterial quorum sensing signal molecules.

**Chapter 1. Introduction**

This chapter provides us with overview of the thesis. Beginning with what is Quorum Sensing, the chapter goes on to describe how microbial communication takes place in different types of organism. Since the work done in the thesis revolves around gram negative organism special emphasis has been given to N-Acyl homoserine lactone mediated QS and phenotypes regulated by this kind of bacterial communication. The focus has then been shifted to why there is need to inhibit QS and various mechanism of Quorum quenching (QQ) (both enzymatic and bio-molecular) reported till date. Also various detection methods used to study quorum sensing and inhibition of QS in bioassay based systems has been described. A concise account of efforts gone into imaging quorum sensing in live bacteria has also been illustrated. The chapter ends with a brief but interesting account of importance of QQ strategies in biotechnology and medicine.

**Chapter 2. A new role for penicillin acylases: Degradation of Acyl homoserine lactone quorum sensing signals by *Kluyvera citrophila* penicillin G acylase**

In this chapter a fascinating new dimension for the amidase activity of *Kc*PGA has been demonstrated by describing its ability to cleave bacterial quorum sensing signal molecules, acyl homoserine lactones (AHLs). *Kc*PGA showed enhanced activity towards medium chain length AHLs i.e. molecules with acyl chain length of 6–8 with or without oxo-substitution at third carbon position. Initial evidence of AHL degrading capability of *Kc*PGA was obtained using CV026 based bioassay method. The enzymatic activity was kinetically characterized in terms of two of its best AHL substrates. Molecular basis of AHL cleavage by *Kc*PGA was also explored using docking studies. Also AHL cleavage activity of *Kc*PGA was compared to that of *Ec*PGA, one of its closest homologous enzymes. Further *Vibrio cholerae* biofilm disruption potential of *Kc*PGA was also demonstrated at the end of this chapter.

**Chapter 3. Isolation of a novel strain of *Staphylococcus epidermidis* with both bile salt hydrolase and AHL cleavage potential**

The chapter describes isolation of a very interesting organism from fermented soy curd capable of both AHL cleavage and bile salt de-conjugation. The isolate showed orangish pink pigmentation and was identified to be a strain of *Staphylococcus epidermidis* based on 16s rDNA sequencing. It was later named as *S.epidermidis* RM1 and characterization of its enzyme activity in terms of both AHL acylation and bile salt hydrolysis was performed. AHL cleavage was monitored by using HPLC as well CV026 based bioassay and BSH activity was estimated using Ninhydrin assay. Genetic basis of the dual activity shown by the isolate was investigated further revealing that it possesses separate genes en-coding for both the enzyme activity. Since the isolate, *S.epidermidis* RM1 showed both potent AHL deamidation and BSH activity it was touted to be an exciting candidate for possible probiotic application.

**Chapter 4A. Novel Species of *Bacillus* isolated from Fenugreek root nodule rhizosphere capable of producing a quorum quenching enzyme**

In this chapter isolation of a quorum quenching bacterium from Fenugreek root nodule rhizosphere has been reported. Root nodule rhizospheric soil was chosen as the source of

isolation because area near root nodules is rich and teeming with complex bacterial interactions including Quorum Sensing and Quorum Quenching. The isolate was identified to be a novel *Bacillus* sp. (named *Bacillus* sp. RM1) based on Phylogenetic analysis. The basis of AHL degradative capability of the isolate was studied and it was found to be a potent AHL lactonase producer. Purification of this enzyme from the wild type bacterium, its optimization and kinetic characterization has been described in this chapter, in addition to checking the biofilm inhibition potential of the wild type AHL lactonase against *Vibrio cholerae* biofilm.

**Chapter 4B. Development of a rapid iodometric method for qualitative detection of AHL lactonase activity**

This small sub-chapter describes a rapid but elegant assay to detect AHL Lactonases in any crude enzyme preparation or given column fraction. This assay allows high throughput detection of AHL Lactonases in multiple samples and is based on a very simple principle.

**Chapter 4C. Cloning and over expression of AHL lactonase from *Bacillus* sp.RM1**

The *aiiA* gene homolog coding for the AHL lactonase in *Bacillus sp.*RM1 Isolated from Methi (Fenugreek) root rhizosphere was cloned in an appropriate expression vector and over-expressed in BL21DE3 strain of *E.coli*. Biological activity of the recombinant enzyme was also studied using CV026 based bioassay.

**Chapter 5A. Novel Glycolipids synthesized using plant essential oils as potent quorum sensing inhibitors**

This chapter talks about interesting glycolipids like- molecules synthesized using plant essential oils as substrates. Conversion into their glycolipid like soluble forms has been shown to increase their quorum sensing inhibitory and biofilm-disrupting property. It is well known that essential oils (EOs) form an important part of traditional medicine, however it is likely that due to their hydrophobic nature and reduced solubility in aqueous environments full potential of their activity cannot be realized. Hence, 12 different essential oils (EOs) were used as substrates, and conversion into their glyco-forms made them more soluble and provided them surfactant-like properties. Preliminary characterization of the synthesized essential oil sophorolipids (EOSLs) was performed using thin layer chromatography (TLC) and Fourier transform infrared spectroscopy

(FTIR). Essential oils that were incapable of mediating quorum sensing inhibition (QSI) on their own became potent quorum sensing inhibitors upon conversion into their corresponding EOSLs. Anti-biofilm potential of these EOSLs was also demonstrated using *V. cholerae* as test organism. Additional with the aim of creating a topical formulation using these EOSLs, anti-fungal activity of selected EOSLs against *Candida albicans*, important yeast causing topical infections, was also demonstrated.

**Chapter 5B. Glycomonoterpenols: as a new class of bacterial quorum sensing antagonists**

In this chapter synthesis of a new class of bacterial quorum sensing inhibitory compounds, Glycomonoterpenols, derived from two monoterpene alcohols, namely Linalool and Alpha terpineol respectively has been described. These Glycomonoterpenols were synthesized using *Candida bombicola* ATCC 22214 by feeding the cells with Linalool and Alpha terpineol respectively as substrates in 10% glucose, production medium. The advantage of these molecules over their parent compound is their additional surfactant like properties, increased solubility and enhanced quorum sensing inhibitory (QSI) potential. A variety of gram negative bacteria (*Chromobacterium violaceum CV026, Pseudomonas aeruginosa and Vibrio cholerae*)capable of elaborating easily detectable quorum sensing mediated phenotypes (violacein production, LasA protease & Pyoverdine production, Biofilm formation respectively) have been selected. Both these glycomonoterpenoid derivatives have been shown to possess strong Anti-QS activity successfully inhibiting all QS regulated phenotypes.

**Chapter 6. *Pichia caribbica* synthesized xylitol as an inhibitor of AHL mediated gram negative quorum sensing**

In the present chapter, quantitative production of xylitol from D-xylose containing artificial medium using a novel yeast *Pichia caribbica* has been demonstrated. With xylitol yield of 0.852 gm/gm and volumetric productivity of 1.83 gm/l/h xylitol in crystalline form, this process is far superior than many others reported to date. Also, a mild, safe procedure for product extraction and purification has been described. Synthesized product (xylitol) was characterized using different analytical techniques: SEM, FTIR, NMR and HPLC and was compared with that of pure sigma xylitol. Additionally ability of xylitol to act as a quorum sensing antagonist in gram-negative

marker strain *Chromobacterium violaceum* CV026 has also been demonstrated. Molecular basis of AHL antagonism by *Pichia* synthesized xylitol has been investigated further using both experimental and *In silico* tools.

**Chapter 7. Early Detection of bacteria capable of Quorum sensing using fluorescent Au nano-cluster probes surface functionalized with QS signal molecules**

This chapter talks about synthesis of fluorescent ultra-small gold clusters decorated with bacterial quorum sensing signal molecules, acyl homoserine lactone (AHL). These fluorescent probes were found to have emission in the near-infrared spectral region which is an advantageous attribute for bioimaging. Imaging studies using different species of bacteria with AHL receptors (*E.coli* with SidA receptors) and without AHL receptors (*S.aureus*) through the aid of confocal microscopy have shown that the probe interacts preferentially with cells possessing these AHL based QS receptors. This probe targets the binding sites for QS molecules within bacterial cells and is not dependent on the concentration of signal molecules. This property makes these systems independent of cell density and can be used for imaging bacteria before they attain a virulent quorate state.

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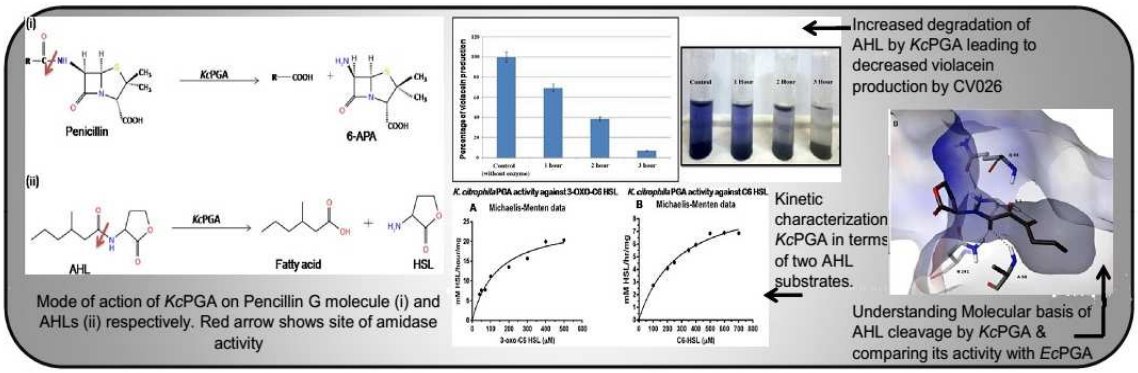
# Chapter 2

*A new role for penicillin acylases: Degradation of Acyl homoserine lactone quorum sensing signals by Kluyvera citrophila penicillin G acylase*

**This Chapter has been published as:**

**Mukherji R,** Varshney NK, Panigrahi P, Suresh CG, Prabhune A (2014), A new role for Penicillin Acylases: Quorum Quenching by Kluyvera citrophila Penicillin G Acylase, ***Enzyme Microb.Tech.***
**DOI: 10.1016/i.enzmictec.2013.12.010**

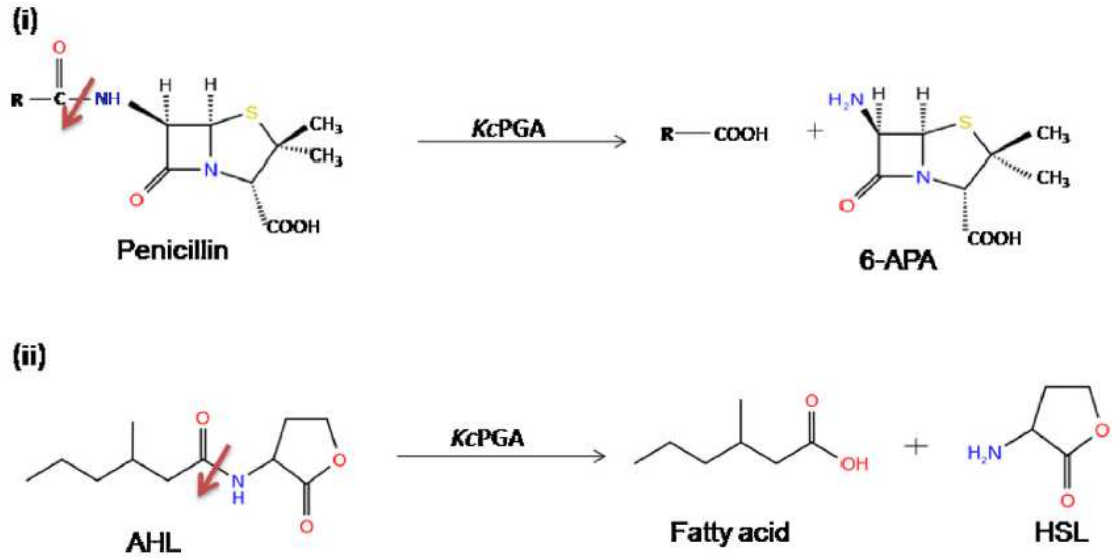
# 2. A new role for penicillin acylases: Degradation of Acyl homoserine lactone quorum sensing signals by *Kluyvera citrophila* penicillin G acylase




## 2.1. Introduction

Penicillin acylases (PAs) have been recognized as enzymes of tremendous industrial importance for more than 50 years now. PAs provide us with a greener route to obtain the essential beta-lactam (6-APA) nucleus using which various semi-synthetic penicillins are prepared [Scheme 2.1(i)]. Penicillin acylases belong to the structural super family of N-terminal nucleophilic hydrolases (Ntn-hydrolases). They are classified into different groups based on the substrate specificity shown by the enzyme. Many organisms have been implicated in the production of both penicillin G acylase and penicillin V acylase, including bacteria, fungi and actinomycetes. In the recent past penicillin G acylase produced by *Kluyvera citrophila* (*Kc*PGA) has received more attention compared to *Escherichia coli* PGA (EcPGA) due to its numerous industrial process-friendly properties, namely increased resilience to harsh conditions and ease of immobilization [1].Like other PGAs, *Kc*PGA is also produced as a pro-peptide which auto-proteolytically gets cleaved into an $\alpha$ and $\beta$ chain of 209 and 555 amino acid residues, respectively, which then eventually forms a heterodimer [2]. Although the mechanism of action of PGA for commercial use is widely understood, much uncertainty surrounds its in vivo role in the organism. It is proposed that PGA may function during the free-living mode of the organism to degrade aromatic molecules like phenyl acetylated compounds and generate carbon source. In the present study a novel and very fascinating property of

*Kc*PGA has been highlighted which may alter our present perception of the ultimate application of this enzyme and its tangible role in nature. The present report talks about the ability of this enzyme to cleave bacterial communication signal molecules known as acyl homoserine lactones (AHLs) with great efficiency [Scheme 2.1(ii)].Single cell bacteria have lately been shown to communicate with their own kind using small diffusible signal molecules in a density dependent manner and this phenomenon has been recognized as quorum sensing (QS) [3].Quorum sensing based transcription regulation has been shown to regulate a variety of important cellular functions like mating, virulence against the host, antibiotic production, competence development and range of other phenomenon. AHLs, the largest and the most well characterized class of signal molecules, mediate communication amongst Gram negative bacteria .A diminished accumulation of these molecules impedes intercellular communication, leading to a state of communication blackout. This forms the basis of the phenomenon known as quorum quenching (QQ) [4].




***Scheme 2.1****: Reaction catalyzed by KcPGA: the red arrow shows the site of action of enzyme. (i) A schematic representation of penicillin deacylation by KcPGA (ii) A schematic representation of the digestion of AHLs by KcPGA as reported in this manuscript.*

The quorum quenching approach aims at reducing the expression of quorum sensing mediated phenotypes without any other damaging influence on the producer organism. Thus, quorum quenching has been touted to be the future in the development of

sustainable antibacterial therapeutics, since there is a decidedly reduced propensity for development of bacterial resistance against this approach considering that it does not impose any 'life or death' selective pressure on the target organism [5].Of the many probable ways to quench quorum sensing, enzy-matic methods have received increasing amounts of attention since the year 2003 [6]. Two types of enzymes that have been shown to degrade acyl homoserine lactones are AHL lactonases and AHL acylases. Several AHL lactonases have been discovered and studied in detail, but AHL acylases in comparison have been a less studied cluster. AHL acylases show great degree of similarity with Ntn-hydrolase family of enzymes. PvdQ, one of the best characterized AHL acylase obtained from *Pseudomonas aeruginosa* and active only against long chain AHLs, shows 24% similarity with *Ec*PGA [7]. Analysis of crystal structure of PvdQ AHL acylase shows the presence of αββα core structure which is a characteristic of all members of Ntn-hydrolase structural superfamily, including *Kc*PGA. AHL acylases reported to date differ greatly in their substrate specificities acting exclusively against one type or group of similar types of molecules [8]. In the present report AHL degrading capability of a well-recognized penicillin G acylase, *Kc*PGA has been shown for the first time. *Kc*PGA specifically degrades medium chain length AHLs (C6–C8) efficiently, which has been validated by using chemical, biological, and in-silico approaches.

## 2.2. Materials and Methods:

### *2.2.1. Culture conditions used for the microorganisms*

*Chromobacterium violaceum* tn5 mutant CV026, obtained as kind gift from Dr. Paul Williams, University of Nottingham, was grown at 30◦C in Luria-Bertani broth supplemented with 100 µg/ml Ampicillin and 30 µg/ml Kanamycin.

### *2.2.2. Cloning, over expression and purification of enzyme KcPGA*

A 2562 bp gene fragment covering the region from 12 nucleotides upstream from the start codon and 12 nucleotide downstream of pac gene was amplified from chromosomal DNA of *K. citrophila* DMSZ 2660 (ATCC 21285) with the help of gene (Accession No-M15418) specific primers using components from KOD polymerase kit and generalized PCR conditions. The amplified PCR product with desired restriction site near the ends was ligated to expression vector pET26b (+) and protein was expressed in BL21

DE3pLysS. Purification of expressed recombinant C-terminal histidine tagged *Kc*PGA, was performed by affinity chromatography using $Ni^{+2}$ Sepharose beads followed by gel filtration on Sephacryl S-200.The fractions containing *Kc*PGA protein were checked for the presence of PGA activity using the modified method of Shewale *et al.*1987 [9] which is based on colorimetric assay proposed by Bombstein and Evans [10]. Purity of the positive fractions was checked on SDS PAGE. Details of cloning, overexpression, crystallization, and preliminary X-ray analysis of *Kc*PGA is reported [11]. (This work is not a part of the present thesis)

*2.2.3. Deacylation of N-acyl homoserine lactone by KcPGA (HSL-OPA assay)*

This assay was used to determine the amido-hydrolase activity of *Kc*PGA against AHLs. Free amino acid in the form of the HSL moiety released during the amido-hydrolysis of AHLs was estimated using o-phthaldialdehyde (OPA, Sigma–Aldrich, India) in 0.1 M Na-borate. OPA stock was prepared by adding 4 mg of OPA in 0.1 ml of ethanol with 5 mg of dithiothreitol (DTT) dissolved in 4.9 ml of 0.1 M Na-borate buffer pH 9.0. OPA (O-phthaldialdehyde) is a primary amine-reactive fluorescent detection reagent. Deacylation of AHL compounds with *Kc*PGA results in the formation of HSL, which can be detected following the reaction of its primary amine group with OPA. AHL-acylase degradation product was immediately mixed with 100 µl of O-phthaldialdehyde solution and then the mixture was incubated for 2 min at 25$^{o}$C to prepare the fluorescent derivative of the released HSL. The quantitative estimate of HSL released as a result of AHL degradation reaction is estimated by measuring the intensity of fluorescence at 340 nm, which is proportional to HSL released. A standard plot of pure HSL (Sigma) in the concentration range of 0.1–1 mM was plotted for calculating the amount of HSL released after enzymatic degradation.

*2.2.4. AHL degradation bioassay using CV026 (qualitative)*

This assay has been designed in such a way that a zone of violacein synthesis can be seen around the agar well with AHL containing reaction mixture. The amount of violacein produced is directly proportional to the amount of AHL remaining in the reaction mixture after enzymatic degradation. *Chromobacterium violaceum* mutant CV026 is used as the test organism which has the ability to respond to a variety of signal molecules [12]. The

QS signal molecule (namely C6-HSL or C7-HSL) at a concentration of 12.5 µM was added to 1 ml of reaction buffer (phosphate buffer pH 7.0) containing 20–40 µg of *Kc*PGA. The reaction mixture was kept at 35$^{o}$C for a period of 3 h. For the bioassay plate 75 µl overnight culture of CV026 was added to 10 ml of Luria-Bertani soft agar and mixed well. The soft agar was then overlayed onto basal LA plate, and a 4 mm diameter well was dug in the centre of the plate using a sterile cork borer after the overlay was set. To the agar well 50 µl of the reaction mixture containing AHL and *Kc*PGA was added at 0 h and after 3 h of reaction, the plates were incubated in upright position at 30$^{o}$C for 24 h. The diameter of zone of violacein synthesis was then measured and compared with appropriate controls in which enzyme was absent.

*2.2.5. Violacein inhibition assay (quantitative)*

This assay is based on a parallel principle to the one described above, wherein the presence of an AHL acylase or AHL degrading enzyme quantitatively decreases the production of the purple pigment violacein which can then be estimated colorimetrically [13].In the first step, a reaction mixture containing 0.5 mM AHL (C6 HSL) and 30 µg of enzyme in phosphate buffer pH 7.0 was incubated separately in a water bath set at 35◦C, for a period of 3 h, with samples aliquoted every hour. A blank reaction was also set which was devoid of any enzyme. Subsequently 75 µl of overnight culture of CV026 was inoculated in 10 ml LB broth and 20 µl of the above mentioned reaction mixture was added at each time interval respectively. The tubes (containing LB + CV026 + aliquoted reaction mixture) were incubated at 30$^{o}$C for 16–18 h under shaking conditions. The violacein produced was extracted from the culture broth by dissolution of the pigment in DMSO and separating it from the cell mass by centrifugation. Purple pigment production was quantitatively estimated by measuring the absorbance at 570 nm.

*2.2.6. Optimization of reaction conditions for AHL degradation by KcPGA*

To determine the optimum temperature of AHL degradation by *KcPGA*, the deamidase activity was measured at different temperatures in the range of 20–90$^{o}$C at pH 8.0 in 0.1 M phosphate buffer for 3 h .To ascertain the optimum pH of enzyme, deamidase activity was determined at 50◦C in the pH range 1.0–12.0using 0.1 M buffers. Appropriate controls for pH studies were used that contained substrate at each respective pH in the

absence of enzyme. HSL-OPA assay was used for monitoring AHL degradation. All experiments were performed in triplicates.

*2.2.7. Enzyme kinetics*

Kinetic characterization of AHL degradation by *Kc*PGA was carried out at pH 8.0 and 50$^{o}$C using 0.1 to 1 mM of substrate. The enzyme was incubated with various concentrations of the substrates and the $V_{max}$, $K_m$ values were calculated by fitting a linear regression curve to data points using Lineweaver–Burk plot. The experiments were performed in triplicates.

*2.2.8. Molecular modeling and binding studies*

In the absence of any reported 3D structure for *Kc*PGA, its tertiary structure was modeled using coordinates from a high resolution crystal structure of its homolog from *E. coli* (*Ec*PGA, PDB ID: 1GK9).This homology based modeling was carried out using the structure prediction module of Prime (version 3.1, Schrödinger, LLC, New York, NY, 2012). The resulting model was validated for its stereo-chemical and geometry quality by model validation pro-grams Procheck [14] Errat [15] and Verify3D [16]. To understand the specificity of *Kc*PGA for PenG and AHLs, the modeled enzyme structure was docked with PenG, C4-HSL, C6-HSL, C7-HSL, C8-HSL, C12-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL, and 3-oxo-C12-HSL. The shape and physical properties of the binding site was first represented in a grid, of dimension 30 × 30 × 30˚A, centered on the catalytic Ser _1 residue, using “Receptor Grid Generation” utility of Glide (version 5.8, Schrödinger, LLC, New York, NY, 2012). Initial 3D conformations of each ligand were generated and partial charges on each of the ligand atoms were assigned using Ligprep (version2.5, Schrödinger, LLC, New York, NY, 2012). During docking, the receptor was treated as rigid while the ligands were docked flexibly, exploring their rotational and translational degree of freedom. Extra precision (XP) mode of Glide was used for docking, which predicted the ligand poses based on their spatial fit and interaction complementarity with the receptor binding site residues. From the resulting receptor-ligand complex, a rough estimate of the binding affinity in terms of free energy of binding was calculated using an empirical scoring function of Glide called G-score. Substrate specificity was then

evaluated based on binding mode, affinity as well as interaction of ligand functional groups with the binding site residues.

*2.2.8. Comparison of AHL degradative activity of KcPGA with its close relative Escherichia coli PGA (EcPGA)*

Commercially available *E.coli* Penicillin G amidase preparation was used to check its AHL deamidase activity. *Kc*PGA shares 83% sequence homology with *Ec*PGA so it is only logical to think that *Ec*PGA might also have AHL degradative capacity. To analyze *Ec*PGA's AHL cleavage potential HSL-OPA assay was used , as described previously. The conditions used for checking AHL degradation by *Ec*PGA were similar to that used for *Kc*PGA and are described as follows: Enzyme concentration: 30µg, temperature of incubation: 50$^{o}$C, pH of incubation: 8.0, time of incubation: 3 hours and lastly AHL concentration used: 0.5 mM. Two specific AHL molecules were used namely C6HSL and C7HSL and activity of *Ec*PGA on these two substrates was compared with that of *Kc*PGA.

*2.2.9. Quantitative estimation of Vibrio cholerae biofilm inhibition by KcPGA*

Biofilm forming potential of *V. cholerae* has been recognized as an important part of the pathogenesis of this well known enteric pathogen. Biofilm formation is quorum sensing mediated phenotype so any enzyme that can degrade quorum sensing signal molecules may have the potential to inhibit biofilm formed by *Vibrio* Sp. In this assay 5µl of overnight culture of *V. cholerae* was added to 200µl of culture media with appropriate concentration of *Kc*PGA in a 96 well plate. In the control wells of the plate 5µl of *V.cholerae* culture was added to 200µl of growth medium alone. The plate was then incubated at 37$^{o}$C for 16-18 hours with gentle shaking. After incubation period all the spent media from the wells along with planktonic cells was discarded and the biofilms were gently washed twice with deionized water. The biofilm left in the wells was then allowed to air dry. It was subsequently stained with 0.1% crystal violet for 10 mins. After discarding excess of staining solution the biofilm was washed with deionized water twice. Stained and air dried biofilms were then treated with 200µl of 95% ethanol to dissolve all the dye absorbed in the biofilm. The content from each well was then transferred to a fresh appropriately labeled well respectively. Optical density (O.D.) was

measured at 595 nm using a Microtitre plate reader. The intensity of purple color obtained is directly proportional to the amount of biofilm formed in each well. Optical density of purple color from the TEST well was compared to that of control well to determine percentage inhibition of biofilm formed.

**2.3. Results and Discussion:**

Industrial applications of penicillin acylases have long been known and extensively studied. Use of these essential industrial enzymes for any other intention other than industrial synthesis has either never been considered or it may not have seemed promising. This chapter presents an unanticipated discovery of the ability of *K. citrophila* PGA to act as an AHL degrading enzyme. As mentioned previously AHLs are important part of the Gram negative bacterial world as these signal molecules play an important role in the phenomenon of quorum sensing which mediates cell density dependent gene regulation. Enzymes capable of degrading AHLs have received immense attention in the past few years because they present an interesting paradigm of infection control without posing the risk of development of antibiotic resistance in the target organism. AHL acylase is a type of AHL degrading enzyme that cleaves AHL molecules by hydrolyzing the amide linkage between the acyl side chain and HSL moiety in a manner that is very reminiscent of the mode of action of penicillin acylases. It has indeed been proven using detailed structural studies that AHL acylases belong to the same super family of Ntn-hydrolases as all other penicillin acylases. This logic allows musing upon the fact that maybe penicillin acylases and other Ntn-hydrolase super family enzymes possess the ability to cleave AHLs and vice versa. Following the same logical reasoning, Lin *et al.* 2003 [17] have tried to explore the ability of commercial preparations of *Ec*PGA and Porcine kidney acylase I to degrade AHLs with no success. But, contrary to these findings by Lin *et al.* 2003[17], porcine kidney acylase I has been shown to cleave bacterial quorum sensing signal molecules, namely C4 and C8 HSL, by Xu *et al.*2003 [18]. In the present chapter, ability of *Kc*PGA to cleave AHLs has been highlighted for the first time. This recombinant purified *Kc*PGA has been shown to degrade AHL molecules with acyl side chain of 8 carbons or less with or without oxo-substituent at the third carbon position of the chain. On the other hand, AHL acylases that have been identified till date have also been checked for their ability to degrade penicillin and other

beta lactam antibiotics [17, 19].All, except one, AHL acylase have been shown to be incapable of degrading penicillin or other beta lactam molecules. AhlM an AHL acylase identified from a Streptomyces sp. [20] was found to be able to degrade AHLs as well as PenG showing that the enzyme has relatively broad substrate specificity.

*2.3.1. Qualitative and quantitative estimation of AHL acylase activity of KcPGA*

AHL acylase activity of *Kc*PGA was initially checked by bioassay based approaches using biosensor strain *Chromobacterium violaceum* tn5 mutant CV026 to provide a preliminary inkling of AHL degradative capability of *Kc*PGA. It was observed that after 3 h incubation of AHLs with *Kc*PGA degradation of QS signal molecules was indicated by the decrease in the zone of violacein production by CV026 (Fig. 2.1). However, to determine the exact amount of AHL degraded or the percentage decrease in violacein production a more precise quantitative assay was performed. Quantitative estimation of decrease in violacein production with time was done using a spectrophotometer based assay following the method described by Choo *et al.* 2006 [13] with slight modification. It was observed that there was almost complete degradation of C6 HSL after incubation with *Kc*PGA over a period of three hours and percentage decrease in violacein production was plotted as a function of time (Fig. 2.2).

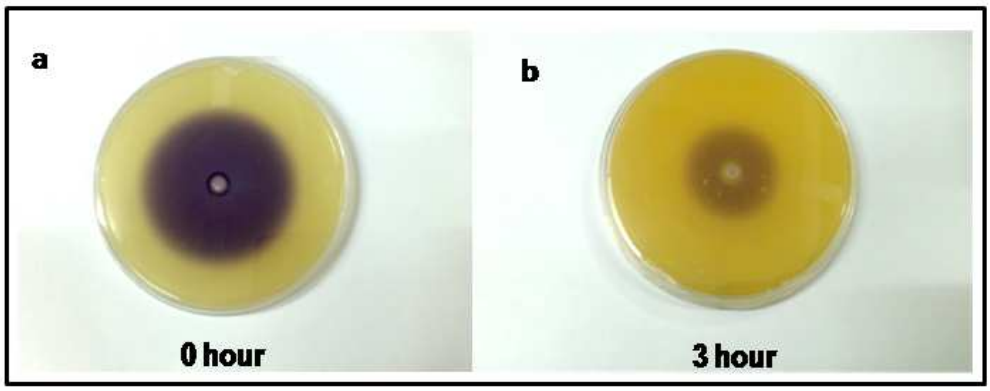



***Figure 2.1:*** *Qualitative depiction of violacein inhibition by KcPGA mediated AHL degradation- (a) Violacein production at 0hour mediated by C6 HSL; (b) Decreased violacein pigment production after 3 hour of C6 HSL degradation by KcPGA.*

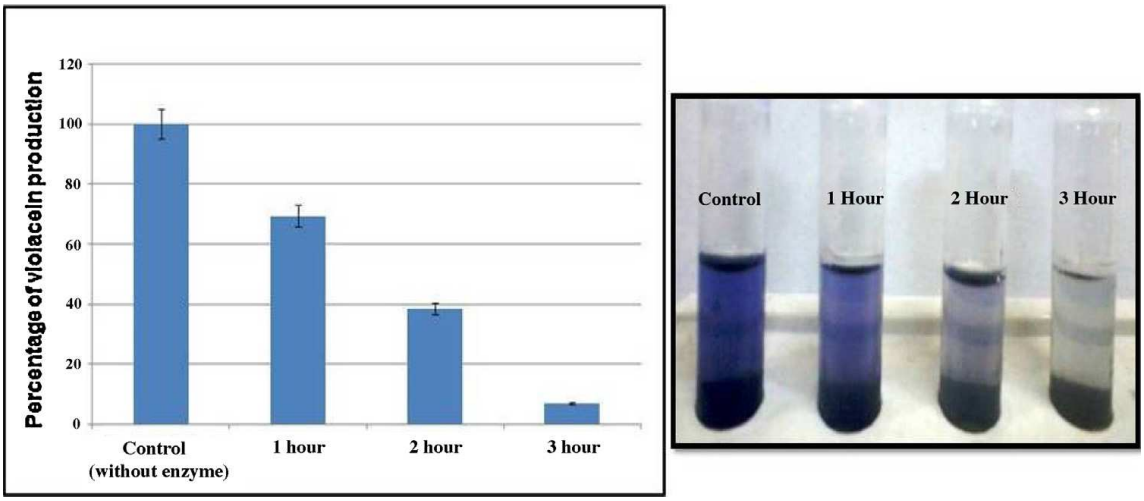



***Figure 2.2:*** *Quantitative estimation of decrease in violacein production by CV026 due to AHL degradation activity of KcPGA. Optical density of the purple pigment (violacein) produced at the end of incubation period was measured at 570 nm. 1, 2, and 3 h correspond to time of exposure of AHL to KcPGA acylase activity*

*2.3.2. HSL-OPA fluorimetric assay to monitor AHL cleavage by KcPGA and optimizing reaction parameters for kinetic characterization of the enzyme*

Cleavage of AHLs by *Kc*PGA was also confirmed by OPA based fluorimetric assay using a range of substrates. The optimum conditions for *Kc*PGA catalyzed AHL degradation were determined using the same assay before carrying out the kinetics study of this enzyme reaction. For determining optimum temperature of activity a range of temperatures were checked, from 20 to 90$^{o}$C. Deacylation activity of *Kc*PGA at different temperatures was compared amongst four acylated homoserine lactones (AHLs) for which the enzyme showed activity, however the concentrations of all the substrates was kept the same. For the highly active substrates the reaction would have been over in the specified time of 3 hours and within the temperature range of 30–50$^{o}$C. Only in the case of C8 HSL, which was found to be the least preferred substrate of all the AHLs tested, the completion of activity reached to close at 50$^{o}$C. Hence we see the saturation of activity (plateau region in the graph) at 30–50$^{o}$C for some of the substrates used (Fig. 2.3).

If different concentrations of the HSLs were chosen for one concentration of enzyme we would have seen similar curves, as seen in case of C8 HSL, for all the HSLs considered.

Nonetheless it was observed that maximum AHL degradation (when all substrates were considered) was obtained at 50°C. The pH optimum for AHL acylase activity of *Kc*PGA was found to be 8.0. Under these optimal conditions of pH and temperature the AHL deamidation reaction when continued for 3 h gave best results (Fig. 2.3).

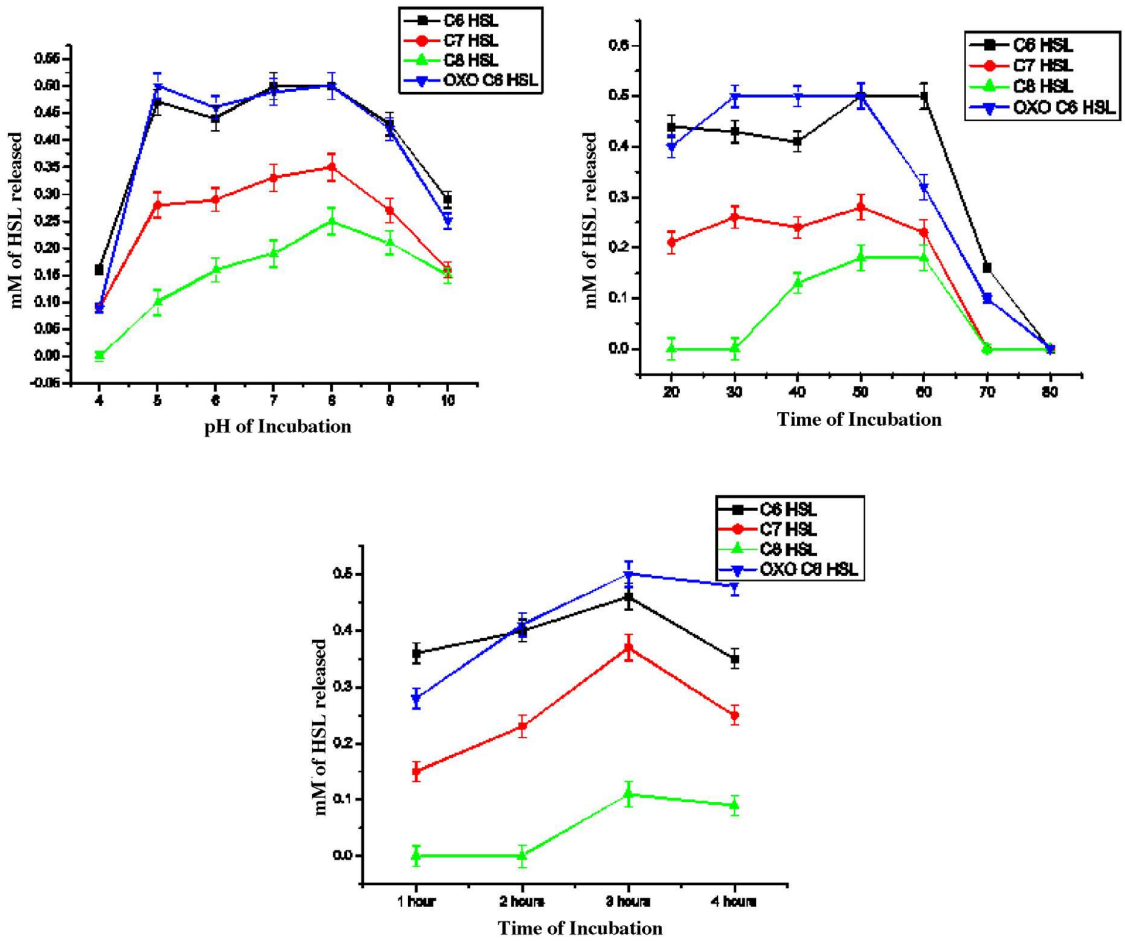



***Figure 2.3:*** *Optimization of reaction parameters (pH, temperature and time of incubation) for maximum deacylation of AHLs by KcPGA. Deacylation reaction was monitored by estimating the amount of OPA derivatized fluorescent active HSL formed. Reaction time for determining optimum pH and temperature was three hours.*

The enzyme was incubated with various concentrations of the substrate from 0.1 to 1.0 mM. The effect of increasing concentration of AHLs on acylase activity of *Kc*PGA was

observed by plotting the Michaelis–Menten Plot. The $V_{max}$, $K_m$ values were calculated by fitting a linear regression curve to data points using Line-weaver-Burk plot. Catalytic efficiency of *Kc*PGA calculated for two of its best substrates, namely 3-oxo-C6-HSL, and C6-HSL, yielded $Kc_{at}/K_m$ values of $0.651 \times 10^3 M^{-1}s^{-1}$ and $0.103 \times 10^3 M^{-1}s^{-1}$, respectively (Fig.2.4) (Table 2.1).Kinetics of enzyme reaction mediated by AHL acylases has not been studied in detail; so far very few reports are available showing catalytic efficiency values of the aforesaid enzyme reaction. Due to the above mentioned reason, in depth comparative analysis of our results with the already published data is not straightforward.

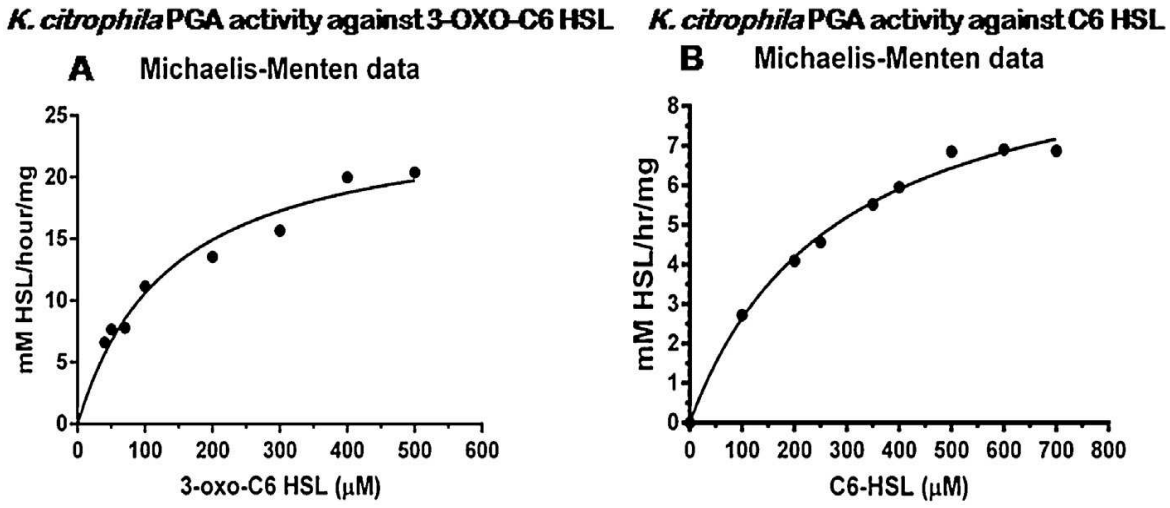



***Figure 2.4:*** *Kinetics of AHL degradation by KcPGA performed under previously optimized condition and monitored using HSL-OPA assay.(A) and (B) show Michaelis–Menten plot for 3-oxo-C6 and C6 HSL degradation by KcPGA, respectively.*

***Table 2.1:*** *Table containing available kinetics data of a known AHL acylases and KcPGA in terms of both AHLs and Penicillin G*

| Report | $K_m$ | $K_{cat}/K_m$ |
|---|---|---|
| **1.Feng Xu et.al (2003)** | $K_m$ = 81mM pH 9.0 and 23°C for C4 HSL | |
| **2. Wahjudi et.al (2011)** | $K_m$ = N.A. pH 7.5 and 30°C | $K_{cat}/K_m$ for C8HSL = 0.14 X $10^4$/M/sec $K_{cat}/K_m$ for 3oxo- C12 HSL 7.8 X $10^4$/M/sec |
| **3.Our data with *Kc*PGA for AHLs** | $K_m$ = 0.1 ± 0.01mM pH 8.0 and 50°C for 3oxo- C6 HSL | $K_{cat}/K_m$ = 0.651 X $10^3$/M/sec |
| | $K_m$ = 0.28± 0.02mM pH 8.0 and 50°C for C6 HSL. | $K_{cat}/K_m$ = 0.103 X $10^3$/M/sec |
| **4. Our data with *Kc*PGA for Penicillin G** | $K_m$ = 2.68± 0.3 mM pH 7.5 and 50°C | $K_{cat}/K_m$ = 0.033/M/sec |

PvdQ, an AHL acylase encoded within the *Pseudomonas aeruginosa* PA01 genome, is one of the best studied AHL acylase. But $K_m$ and $K_{cat}/K_m$ values even for this enzyme are not available. However, a new gene in P. aeruginosa PA01 genome, pa0305, has recently been identified by Wahjudi *et al.* 2011 [21]. It was initially predicted to encode a penicillin acylase but has now been shown to code for an AHL degrading enzyme. Kinetic studies using the purified AHL Acylase enzyme coded by pa0305, with N-octanoyl-L-homoserine lactone (C8-HSL) and N-(3-oxo-dodecanoyl)-L-homoserine lactone(3-oxo-C12-HSL) as substrates showed that the enzyme has robust activity towards these two AHLs, with $K_{cat}/K_m$ values of $0.14\times10^4 M^{-1}s^{-1}$ towards C8-HSL and $7.8\times10^4 M^{-1}s^{-1}$ towards 3-oxo-C12- HSL [21].The values of $K_m$ and $K_{cat}/K_m$ for *Kc*PGA activity on AHLs are comparatively less than that of pa0305 but considering the fact that AHLs are not the previously tested standard substrate for *Kc*PGA these values of catalytic efficiency appear to be of considerable significance. In addition, kinetics of both AHL and penicillin G degradation by *Kc*PGA was analyzed with the aim of comparing the efficiency of this enzyme in terms of these substrates.Comparison of $K_{cat}/K_m$ values, which tells us about the efficiency of an enzyme catalyzed reaction, revealed that penicillin G is still the preferred substrate ($K_m$ = 2.68 mM and $K_{cat}/K_m$ = 33.62 $mM^{-1}s^{-1}$) (Table2.1) but the enzyme can in addition also act on two AHL molecules C6HSL and 3-

oxo-C6 HSL, with some degree of preference, showing diversity of the enzyme with regards to its choice of substrate. Based on the results obtained during this study it was observed that *Kc*PGA shows more preference towards shorter chain length AHLs. Substrate preference for *Kc*PGA is as follows: 3-oxo-C6-HSL > C6-HSL > C7-HSL > C8-HSL. *Kc*PGA did not show any activity against C4 HSL or AHLs with chain length higher than C8, namely C10 HSL and C12 HSL.

*2.3.3. In-silico docking studies to identify the molecular basis of AHL acylase activity*

To identify the molecular basis of increased affinity of the enzyme for medium chain length AHLs, specifically 3-oxo-C6-HSL, molecular modeling and docking studies were carried out. *Kc*PGA sequence shares 83% identities with *Ec*PGA. At such a high sequence identity, modeling the 3D structure of *Kc*PGA using *Ec*PGA as template is reliable and indeed the built model was found to be of high quality by model validation programs. Main chain conformations of all the residues of the model were within the most favored or additionally allowed region in the Ramachandran plot and the overall model quality or G-factor was found to be −0.16, suggesting it to be a valid model. Similarly, the overall quality factor as estimated by the Errat was 92.8%, close to the value for a high quality structure. More than 95% of the residues were having Verify3D quality score above 0.2 indicating no local conformational error. Hence, the *Kc*PGA model structure was used further for docking study. Table 2.2 summarizes the results from the docking studies. It lists the free energy of binding of the best predicted pose of these ligands in terms of G-score.

***Table 2.2*** *List of free energy of binding of substrates under consideration measured in terms of Gscore including the distances and angles made by the atoms of amide bond of substrate with the catalytic residues Sβ1, Nβ24, Aβ69 and Qβ23.*

| Substrate | Gscore (Kcal /mol) | Distance[a] | | | | Angle[b] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Sβ1 (OG)-C | Nβ241 (ND2)-O | Aβ69(N)-O | Qβ23 (O)-N | N-C-Sβ1 (OG) | N-C-Nβ241 (ND2) | N-C-Aβ69 (N) | C-N-Qβ23 (O) |
| PenG | -7.64 | 2.87 | 3.33 | 3.28 | 2.79 | 120 | 104.3 | 147 | 97.3 |
| R63O | -5.37 | 2.86 | 2.94 | 3.15 | 3.15 | 122.6 | 107.2 | 149.6 | 97.3 |
| R6 | -4.48 | 2.98 | 2.84 | 3.16 | 3.33 | 121.2 | 107.8 | 151.9 | 97.2 |
| R7 | -4.97 | 3.56 | 3.24 | 4.67 | 2.81 | 119.5 | 132.5 | 172.6 | 91.9 |
| R8 | -4.73 | 3.57 | 3.25 | 4.7 | 2.81 | 119.5 | 133.1 | 172.9 | 91.6 |
| R4 | -3.45 | 6.57 | 5.18 | 7.98 | 4.83 | 70.5 | 95.4 | 75.3 | 125.4 |
| R83O | -4.13 | 4.45 | 4.66 | 6.69 | 3.03 | 122.7 | 167.3 | 138 | 90.1 |
| R12 | -1.68 | 6.48 | 5.1 | 7.76 | 6.66 | 147 | 104 | 114.5 | 17.1 |
| R123O | -4.57 | 6.49 | 5.22 | 8.04 | 6.66 | 152.3 | 114.8 | 119.8 | 29.5 |

[a] Distances are represented as Receptor atom-Ligand atom. C, O and N atom of Ligand corresponds to atoms of amide bond.

[b]Angles are represented as N -C-Receptor atom where N and C corresponds to atoms of ligands amide bond.

The docking results correlated well with the experimental findings. Among PenG and AHLs, PenG was found to be the most preferred substrate for *Kc*PGA, having highest free energy of binding −7.64 kcal/mol. *Kc*PGA has a distinct, small hydrophobic pocket (Fig. 2.5), ideally suitable for binding smaller hydrophobic groups of the substrates. In case of PenG, the hydrophobic phenyl group occupies this pocket whereas, in AHLs the hydrophobic acyl side chain occupies the pocket. In case of AHLs, the Acyl side chain groups of length 6, 7, and 8 carbon atoms fit in the hydrophobic pocket such that their amide group maintains geometry in the vicinity of the active site residues (Sβ1, Qβ23, Aβ69, and Nβ241) favorable for the cleavage. Although the binding free energy value shows favorable binding for C4 HSL, C12 HSL, 3-oxo-C8 HSL, and 3-oxo-C12 HSL, however, a large distance between their amide carbon atom and the nucleophile atom OG of Sβ1 residue (Table 2.2) i.e., large nucleophillic attack distance does not permit the cleavage of the amide bond.

Among the favorable acyl chain lengths of AHLs, *Kc*PGA prefers 3-oxo-C6 HSL followed by C6 HSL, C7 HSL, and C8 HSL, which coincide with the experimental

findings. The geometry of the amide bond in 3-oxo-C6 HSL and C6 HSL with the functional groups in the catalytic sites is very close to the ideal geometry as shown by PenG (Table 2.2) with the exception of the loss of hydrogen bond with Qβ23, which results in decrease of their free energy of binding compared to PenG. Further addition of any methylene group in the side chain (C7 HSL and C8 HSL) moves the amide bond away from the hydrophobic pocket leading to variations in the distance of Aβ69(N)-amide(O) and the angles amide(C)-amide(N)-Nβ241(ND2), and amide(C)-amide(N)-Aβ69(N) (Table 2.2). Due to these variations, in case of C7 HSL and C8 HSL, the hydrogen bond with residue Nβ241 and Aβ69 is lost, while maintaining the hydrogen bond with the residue Qβ23.

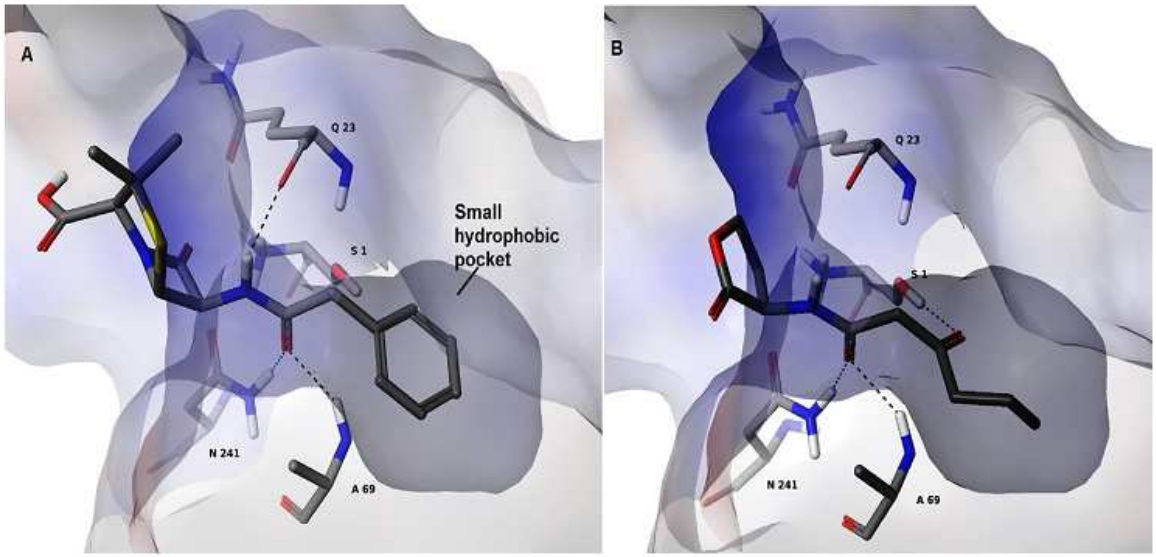



***Figure 2.5:*** *Predicted binding modes of (A) PenG and (B) 3-oxo-C6-HSL.The hydrophobic phenyl chain of PenG and the acyl side chain of 3-oxo-C6-HSL are accommodated in the hydrophobic pocket of binding site*

*2.3.4. Comparison of AHL degradative capacity of EcPGA with KcPGA*

*Ec*PGA is a close relative of *Kc*PGA sharing 83% homology hence it was not wrong to assume that *Ec*PGA may also possess the ability to degrade bacterial quorum sensing signal molecules. However upon comparing the AHL degradation potential of both these enzymes in terms of two substrates (C6 and C7HSL) it became clear that *Ec*PGA is not a potent quorum quencher. Figure 2.6 shows relative activity of *Kc*PGA and *Ec*PGA on C6 and C7HSL respectively.

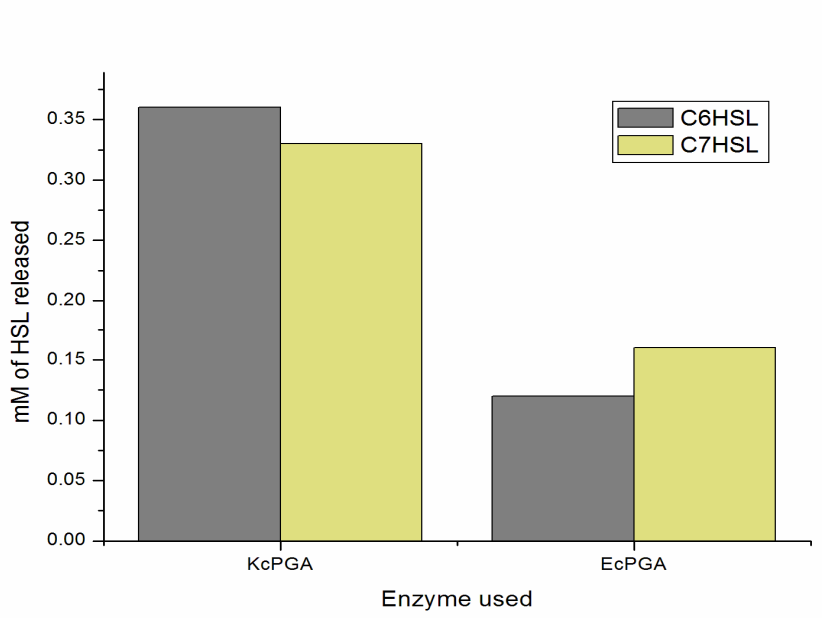



***Figure 2.6:*** *Comparison of AHL degradative capacity of KcPGA and EcPGA in terms of two AHL substrates.*

*2.3.5. Anti-biofilm activity of Kluyvera citrophila PGA against Vibrio cholerae*

*Vibrio cholerae* is an important human gastro-intestinal pathogen which relies on quorum sensing regulons to elaborate many of its virulent phenotypes [22]. Ability to form biofilm on the intestinal epithelium is an important part of *V. cholerae* pathogenesis and any molecule that has the capacity to inhibit quorum sensing can disrupt biofilm formation by this pathogen to some extent. *Kluyvera citrophila* Penicillin G acylase was shown to inhibit *V. cholerae* 18 hours old biofilm upto 60% and the concentration of enzyme required to mediate this activity was 140µg (Fig.2.7).*Escherichia coli* PGA was not tested for anti-biofilm activity owing to its inherently feeble quorum quenching activity.

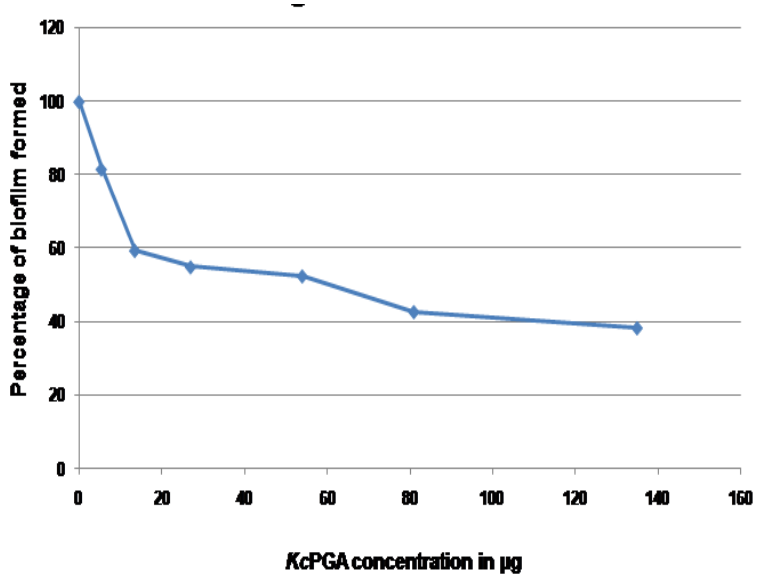



***Figure 2.7:*** *Percentage inhibition of biofilm formed by Vibrio cholerae, mediated by K.citrophila PGA.*

## 2.4. Conclusion:

The present chapter highlights the ability of *Kc*PGA to act as an AHL acylase specifically against medium chain length AHLs. AHL degradation capability of penicillin G acylases has not been shown before and this is the first time that a well-known Penicllin G acylase has been shown to also act as an AHL acylase. Deamidation of AHLs by *Kc*PGA was validated using biological and chemical assays in addition to in-silico tools and this adds a new facet to the range of biological activity of this enzyme. Additionally comparison of *Kc*PGA's AHL degradative potential was done with its close relative *Ec*PGA, however AHL acylase activity oh *Kc*PGA was found to be far superior. *Kc*PGA was also demonstrated to inhibit *V. cholerae* biofilm formation up to 60% which puts forth prospective use of this enzyme for biofilm disruption in aquaculture.

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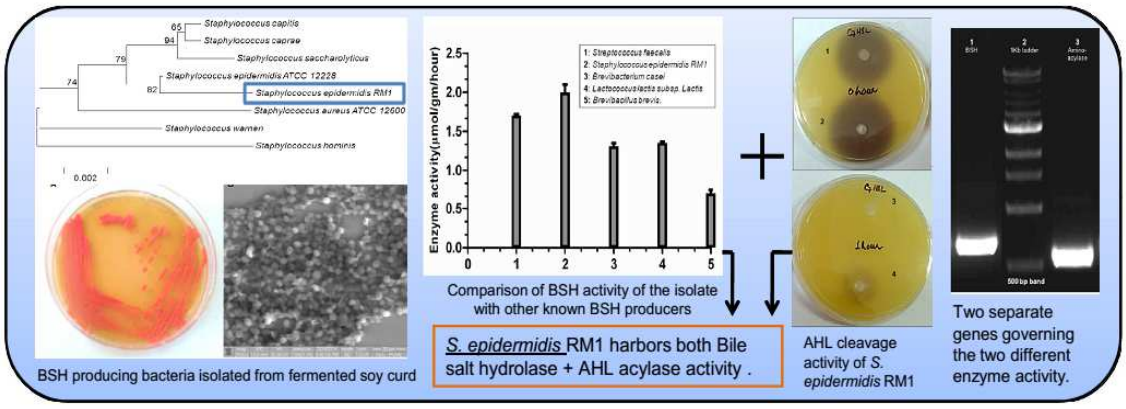
# Chapter 3

## *Isolation of a novel strain of Staphylococcus epidermidis with potent Bile Salt Hydrolase and AHL cleavage potential*

**This Chapter has been communicated as:**

**Mukherji R,** Prabhune A. Possible correlation between Bile salt hydrolysis and AHL Deamidation: *Staphylococcus epidermidis* RM1 a potent quorum quencher and bile salt hydrolase producer. (**Communicated Manuscript**)

# 3. Isolation of a novel strain of *Staphylococcus epidermidis* with potent Bile Salt Hydrolase and AHL cleavage potential




### 3.1. Introduction:

Bile salt hydrolysis is an important biological reaction as it has widespread implication in the host environment [1]. Additionally capability to de-conjugate bile salts offers tremendous physiological advantage to organisms inhabiting or hoping to inhabit the human gastrointestinal epithelium. Persistence in the GI tract and tolerance to the surface active properties of bile is facilitated by the enzyme bile salt hydrolase [2]. This enzyme is a typical member of Ntn hydrolase super family, showing all the hallmark characteristics, especially its mode of deamidation of bile salts [3]. A variety of gram positive bacteria possess this enzymatic activity including different species of *Lactobacillus*, *Clostridium*, *Bifidobacterium* and *Staphylococcus* [4]. A number of pathogenic bacteria have also acquired BSH activity via horizontal transfer of BSH genes [5]. Any organism to be deemed fit to serve as a prospective probiotic strain must possess potent BSH activity as a prerequisite [1].

N-Acyl Homoserine Lactones (AHLs) are molecules that serve as communication signals amongst many gram negative bacteria, some of which include clinically important pathogens [5, 6]. Quorum sensing (or the phenomenon of bacterial communication) mediated by these AHL molecules can be quenched or impeded by a class of enzymes known as AHL acylases [7, 8]. This strategy has been appreciated as a possible next

generation antimicrobial therapy with reduced predisposition towards resistance development [9]. AHL acylases have been a less studied cluster of enzymes capable of mediating quorum quenching. PvdQ is one of the well studied AHL acylases isolated from *Pseudomonas aeruginosa* PAO1 [10]. Detailed structural characterization of the enzyme has revealed that it also is a member of Ntn hydrolase super family, showing the αββα core and typical amido-hydrolytic activity [11].

To date no real connection has been established between AHL acylases and Bile salt hydrolase although both belong to the same class of enzymes showing similar mode of action. Moreover there are also no reports of an organism, which has potent BSH activity, to be also an AHL degrader, or vice versa. In, the present article, bile de-conjugation activity of a new strain of *Staphylococcus epidermidis, S. epidermidis* RM1 has been put forth. Very intriguingly it has been observed that the organism also possesses potent AHL inactivation activity via deamidation of the signal molecules. This is the first report of its kind, where these two different activities are being harbored within the same organism, and it indeed unlocks a plethora of possible advantages that this strain may have over other conventionally used probiotic organisms.

## 3.2. Materials and Methods

### *3.2.1. Microorganism used and Culture conditions*

*Chromobacterium violaceum* CV026 (a mini Tn5 mutant of wild-type *Chromobacterium violaceum*) obtained as a kind gift from Dr. Paul Williams, University of Nottingham, was used as a biosensor strain in this study. The culture was grown in Luria bertani medium supplemented with antibiotics, 30µg $ml^{-1}$ Kanamycin and 100µg $ml^{-1}$ Ampicillin.CV026 is incapable of synthesizing AHLs of its own but responds to exogenously supplied synthetic AHL molecules by violacein synthesis. Standard bile salt hydrolase producing cultures were obtained from different culture collection centres and grown on appropriate media without antibiotics. *Lactococcus lactis subsp. lactis* MTCC 440 in Brain-heart Infusion medium, *Brevibacillus brevis* MTCC 7539 in Nutrient broth, *Brevibacterium casei* MTCC 1530 in Casein-Peptone broth and *Streptococcus faecalis* NCIM 2403 in MRS medium.

*3.2.2. Isolation of BSH producer from fermented soy curd*

200 ml of unflavored unsweetened soy milk from a sterile tetra-pack was transferred to a 250ml Erlenmeyer flask aseptically and incubated at 30$^{o}$C under static conditions for a week. Isolation of bile tolerant organisms from the curdled soy milk was done using MRS Broth containing 0.2% w/v bile salts. Fermented soy milk sample was serially diluted 1000 times and 1 ml of sample from each of the last two highest dilutions were transferred to 10 ml MRS broth with 0.2 % w/v bile salts respectively. Tubes were incubated at 35$^{o}$C for 1 week. Growth obtained in the tubes was pelleted down by centrifugation at 8000 rpm for 2mins. The pellet was re-suspended in saline and was then plated on MRS agar containing 0.2% w/v bile salts. The plates were incubated at 35$^{o}$C for 7 days. After 7 days orangish pink colored colonies were obtained predominantly on the agar plates which were further purified by streaking on the same media. Pure culture obtained was then used for isolation of genomic DNA and other chemical and biological assays.

*3.2.3. Genomic DNA isolation and Identification of Isolate via 16S rRNA sequencing*

DNA was isolated according to the modified method reported by Sambrook *et al.* 1987 [12].The extracted genomic DNA was quantified and the 16S rRNA gene was amplified using universal primers 8F and 1525R [13] 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 obtained was deposited in the GenBank database under the accession number KJ571489. 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 [14].Phylogenetic tree was computed in MEGA 4 using the neighbor joining method [15].

*3.2.4. Bile salt hydrolase activity*

Bile salt hydrolase activity was determined using Ninhydrin reagent (Sigma-Aldrich, INDIA Ltd.) which allows estimation of the amount of free amino acids (Taurine /Glycine) released upon degradation of substrate (Sodium Taurocholate / Sodium Deoxycholate) by the cell bound enzyme at 40 °C in 0.1 M sodium phosphate, pH 6.7 within one hour. After incubating 20 mg of thoroughly washed 48 hours old, cells of S. *epidermidis* RM1 with selected substrate for 60 minutes, reaction was stopped by addition of 15% (w/v) trichloroacetic acid. Appropriate controls were taken by incubating cells without substrate. The reaction mixture was then centrifuged at 10000 rpm for 2 min and 20µl of supernatant was mixed with an equal volume of 2% Ninhydrin solution before boiling for 15 min. 2ml of de-ionized water was then added to the assay tubes and the absorbance was recorded at 570nm to determine the amount of free amino acids released from the substrate. One unit of BSH activity is defined as the amount of enzyme that liberates 1micromole of the free amino acid from the substrate per unit time. A standard plot of glycine estimation using Ninhydrin reagent was established using glycine concentration of 0.1 – 1.0 micromole.

Bile salt hydrolase activity of standard BSH producing organisms was also estimated using the same assay. However it is important to note that cells from each of the standard cultures were harvested at the time of maximum BSH activity.

*3.2.5. Whole cell AHL degradation activity*

Thoroughly washed 20 mg cells of *Staphylococcus epidermidis* RM1 were suspended in 200 µl of phosphate buffer pH 6.7 and 0.125, 0.25, 0.5, 0.625 and 0.75mM of C6HSL or C7HSL was added to the reaction mixtures respectively. At 0 hour and after 1hour of incubation at 40$^{o}$C, 20 µl of reaction mixture was taken respectively, 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 amount 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. No

spontaneous degradation of C6 HSL/C7 HSL molecule was observed at the said pH of reaction. Additionally it was important to check whether the enzymatic degradation of QS signal molecules is due to an AHL lactonase or AHL acylase. To verify that, reaction tubes containing appropriate amount of AHL and 20 mg washed cells were incubated at 40$^{o}$C for more than one hour to allow compete degradation of provided AHL .The reaction mixture was acidified to pH 2.0 and the tubes were further incubated at room temperature for two hours. Re-lactonization of the degraded AHLs, as evidence by ability of CV026 to perceive them and in-turn synthesize violacein, indicates AHL lactonase activity. Inability of degraded AHLs to regain their biological activity even after acidification of reaction mixture indicates AHL amido-hydrolase activity.

*3.2.6. HPLC identification of AHL acylase activity*

After confirming that the enzyme activity is due to an AHL acylase and not Lactonase, analysis of degradation products was done using HPLC. Cleavage of an AHL molecule via an acylase will release free HSL moiety which can then be derivatized for easy detection. Derivatization of HSL molecule using Dansyl chloride has been reported previously which increases its hydrophobicity (hence retention) and makes it fluorescent; however Dansyl chloride does not react with intact AHL molecule [16]. Hitachi Chromeline HPLC system with Thermo Scientific C18 reverse phase HPLC column (250 X 4.6 mm) was used in this study. Solvent system used was Methanol: Water (50:50) at a flow rate of 1ml/minute. Intact AHL and derivatized HSL were detected using UV detector set at 219 nm. Dansylation of HSL alone and the reaction mixture was done as per method reported by Zhang *et al.* 2007 [16].

*3.2.7. Determining genetic basis of the dual enzyme activity of S.epidermidis RM1*

Genomic DNA from *S.epidermidis* RM1 was isolated as described previously (see section 3.2.3). *Staphylococcus epidermidis* ATCC 12228 whole genome sequence was used as reference genome. Sequence for a gene putatively encoding for a Penicillin V amidase (orthologously known as choloylglycine hydrolase) was used as a template to design primers for detection of gene coding for a bile salt hydrolase in *S.epidermidis* RM1. Since there existed no annotation for a gene coding for N-acyl homoserine lactone

acylase in the *Staphylococcus epidermidis* ATCC 12228 genome, sequence for a gene encoding an amino-acylase was used a template for primer designing. Expected amplicon size to be obtained with BSH specific primers was 666bp and that with amino-acylase specific primers was 553 bp. Appended below are the primers used to amplify genes coding for a BSH and amino-acylase respectively.

Primers used to detect the **BSH coding gene** in *S .epidermidis* RM1:

**BSH_for C G A T G G A C T TT G C A T TT G A A**
**BSH_rev G G C A T C G A T A A C T G C A C C T T**

Primers used to detect the **Amino acylase coding gene** in *S .epidermidis* RM1:

**Ama_For G G C T C A T C CCC A C A T A C A T C**
**Ama_Rev C C A C T G C C T C T G C A A C T A C A**

### 3.3. Results and Discussion

*3.3.1. Isolation and Identification of Isolate obtained from fermented soy curd*

BSH producer was isolated from fermented soy curd using MRS broth and agar containing 0.2% bile salts. Bile salt hydrolase producers have been isolated from a variety of sources; however use of fermented soy milk as a source of BSH producer has not been attempted before. The isolate obtained during the course of this study was found to be a new orange-pink pigmented strain (Fig. 3.1A) of *Staphylococcus epidermidis* showing typical cocci-in-cluster type morphology under scanning electron microscopy (Fig. 3.1B) .16s rRNA based phylogenetic analysis showed that the isolate *S.epidermidis* RM1 cladded with *Staphylococcus epidermidis* ATCC 12228,albeit with some evolutionary distance (Fig. 3.2).Biochemical characterization of the isolate, *S.epidermidis* RM1, showed that it had slightly different sugar fermentation pattern (in peptone water base containing 0.5% sugar) when compared to *Staphylococcus epidermidis* ATCC 12228 (Table 3.1).

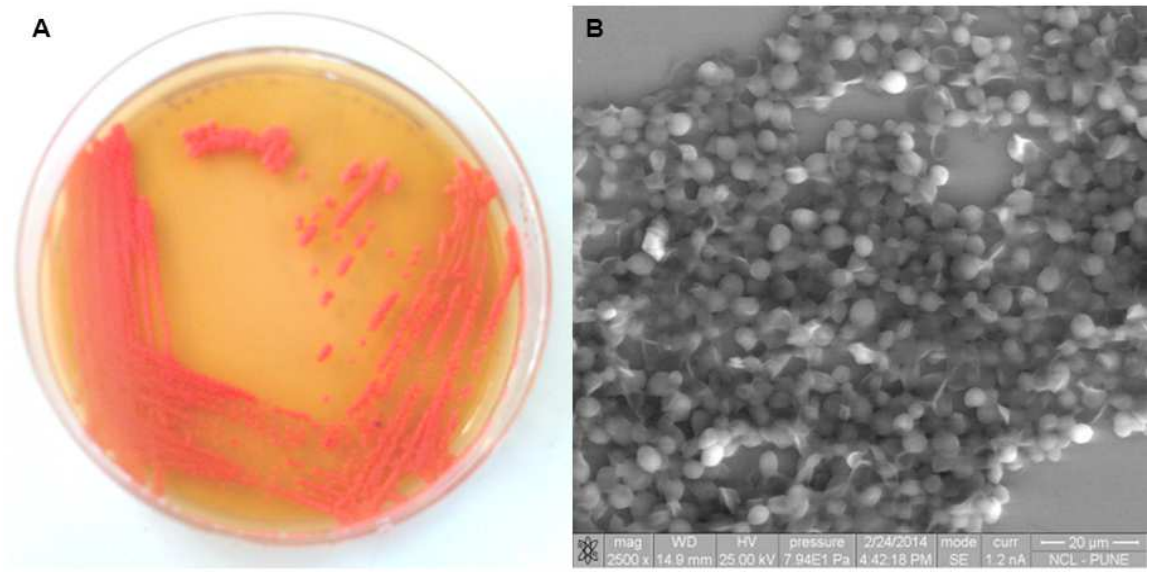



***Figure 3.1*** *MRS agar plate with 0.2% w/v bile showing luxuriant growth of Staphylococcus epidermidis RM1 (**A**).Scanning electron micrograph of the S. epidermidis RM1 showing typical cocci in clusters morphology (**B**).*

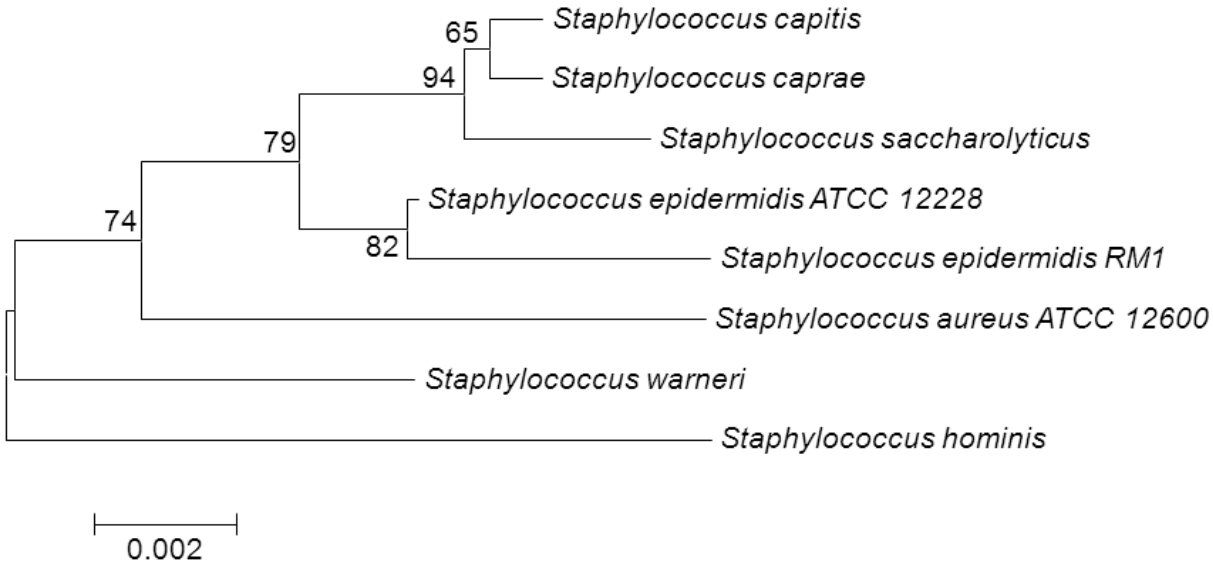



***Figure 3.2:*** *Phylogenetic identification of Staphylococcus epidermidis RM1: Unrooted phylogenetic tree obtained from 16S rRNA gene sequence of S. epidermidis RM1.The neighbor joining tree shows that S. epidermidis RM1 is closely related to S.epidermidis ATCC 12228 albeit with slight evolutionary distance.*

***Table 3.1**: Comparison of results for sugar fermentation and other biochemical tests between S. epidermidis RM1 and S. epidermidis ATCC 12228.*

| **1.Sugar fermentation** | ***Staphylococcus epidermidis* RM1** | ***Staphylococcus epidermidis* ATCC 12228** |
|---|---|---|
| **i. Glucose (anaerobic)** | +++ | +++ |
| **ii. Glucose (aerobic)** | +++ | +++ |
| **iii. Sucrose** | +++ | +++ |
| **iv. Mannose** | +++ | ++ |
| **v. Fructose** | +++ | +++ |
| **vi. Galactose** | ++ | ++ |
| **vii. Glycerol** | - | ++ |
| **viii. Lactose** | + | ++ |
| **ix. Maltose** | - | +++ |
| **x. Mannitol** | - | - |
| **xi. Xylose** | - | - |
| **2.Catalase Test** | Positive | Positive |
| **3.Nitrate Reduction test** | Positive | Positive |

*3.3.2. Estimation of Bile Salt de-conjugation capacity of S. epidermidis RM1*

Evidence of BSH activity has been found in many gram positive organisms previously, including genera *Staphylococcus* [17, 18].Hence BSH activity shown by the isolate obtained in this study, *S. epidermidis* RM1, was not surprising. Bile salt hydrolase activity of the isolate was determined using Ninhydrin assay and its activity was compared with those of standard BSH producing cultures obtained from different culture collections of the country. All the cultures, namely *Lactococcus lactis subsp. lactis* MTCC 440, *Brevibacillus brevis* MTCC 7539, *Brevibacterium casei* MTCC 1530 along with *Streptococcus faecalis* NCIM 2403, showed significant bile salt hydrolysis and *S.epidermidis* RM1 also exhibited comparable activity (Fig. 3.3). *Staphylococcus*

*epidermidis* RM1 showed maximum BSH activity within 48 hours, and showed strong affinity towards Glycodeoxycholic acid (GDCA) as substrate, compared to all other bile salts tested (Taurodeoxycholic acid (TDCA), Taurocholic acid (TCA) and Glycocholic acid (GCA), Fig. 3.4).

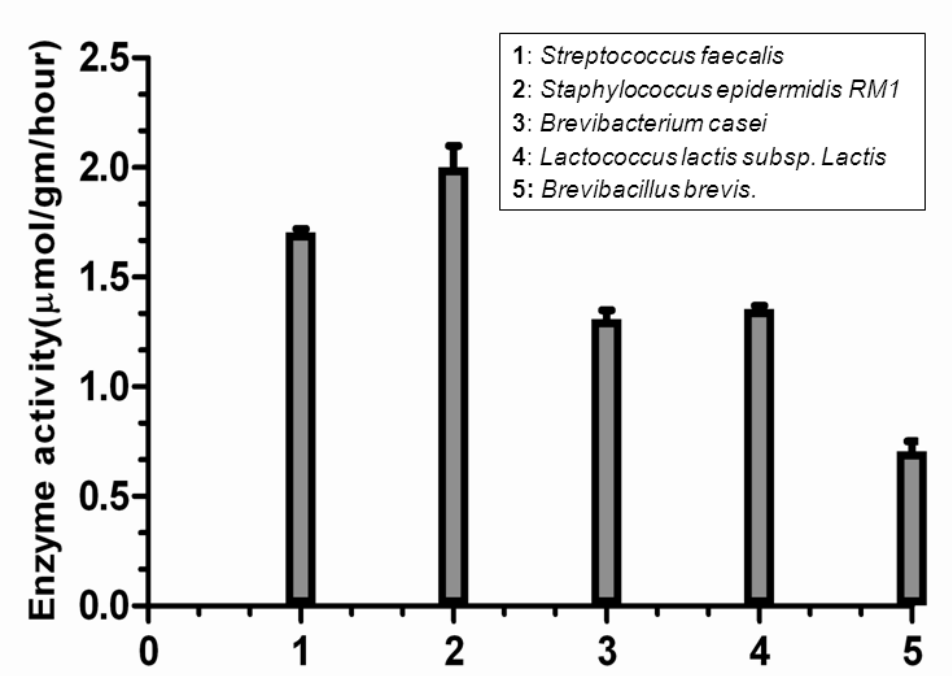



***Figure 3.3:*** *Comparison of Bile Salt Hydrolase Activity. Staphylococcus epidermidis RM1 and four other lactic acid bacteria capable of producing bile salt hydrolase were selected and their enzyme activity was compared using Ninhydrin assay using GDCA as substrate.*

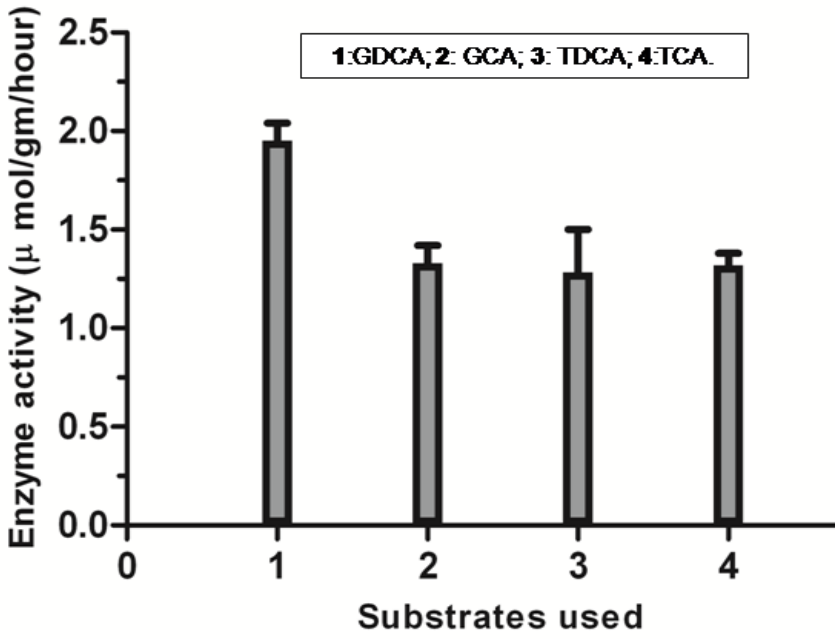



***Figure 3.4:*** *Substrate Specificity of Staphylococcus epidermidis RM1.Four different Bile salts from Sigma-Aldrich, INDIA, were used to be the preferred substrate for S.epidermidis RM1.THE reaction was carried out at 37oC for 1 hour and the activity was determined using Ninhydrin Assay. As seen from the figure highest activity was seen with GDCA.*

### *3.3.3. Determining the ability of S. epidermidis RM1 to degrade bacterial QS signal molecules*

Interestingly, although *S.epidermidis* RM1 did not show any Penicillin G acylase or Penicillin V acylase activity, it showed potent AHL acylase activity against N-hexanoyl homoserine lactone (C6-HSL) and N-heptanoyl homoserine lactone (C7-HSL).Preliminary identification of its AHL degradative capacity was obtained using *Chromobacterium violaceum* CV026 based bioassay. It was observed that the culture could completely degrade up to 0.75 mM of substrate C6 HSL (Fig. 3.5) and up to 0.5mM of C7 HSL (Fig. 3.6) within one hour of incubation at 40$^{o}$C in 0.1 M phosphate buffer pH 6.7. However to determine whether the enzymatic basis of AHL degradation was lactonolysis or deamidation, acidification of the reaction mixture to pH 2.0 was carried out at the end of the reaction period. Absence of reactivation of AHL molecules in the acidified reaction mixture indicated AHL degradation by amido-hydrolysis and not by action of a lactonase.


***Figure 3.5:*** *AHL degradation by Staphylococcus epidermidis RM1. 20mg washed cells of S. epidermidis RM1 grown in two different media (MRS and Trypticase soy agar) were incubated with 0.75mM C6-HSL at 37°C for 1 hour in buffer of pH 6.7 respectively (Well 1 and 2) .Complete degradation of the provided AHL was observed within that hour (Well 3 & 4).*


***Figure 3.6:*** *Detection of AHL degradation by whole cells of S. epidermidis RM1: CV026 based bioassay system was used to monitor C7 HSL cleavage by S. epidermidis RM1.Washed and appropriately weighed cells of the culture were incubated with 0.5mM and 0.75mM of C7 HSL (wells 1 and 2 respectively) in buffer of pH 6.7 for 1 hour at 40°C.After one hour complete degradation 0.5mM C7 HSL (well 3) and 90% degradation of 0.75mM C7 HSL (well 4) can be observed.*

*3.3.4. HPLC based confirmation of AHL acylase activity of S. epidermidis RM1*

AHL acylase cleavage product was also confirmed by HPLC. Free HSL (Homoserine lactone) moiety released from the substrate C7 HSL was derivatized using dansyl chloride in the reaction mixture**.** Fig. 3.7 shows retention times for substrate (C7 HSL) alone, dansylated HSL alone and reaction mixture derivatized with dansyl chloride showing peaks for both substrate and dansylated HSL at their respective retention times.

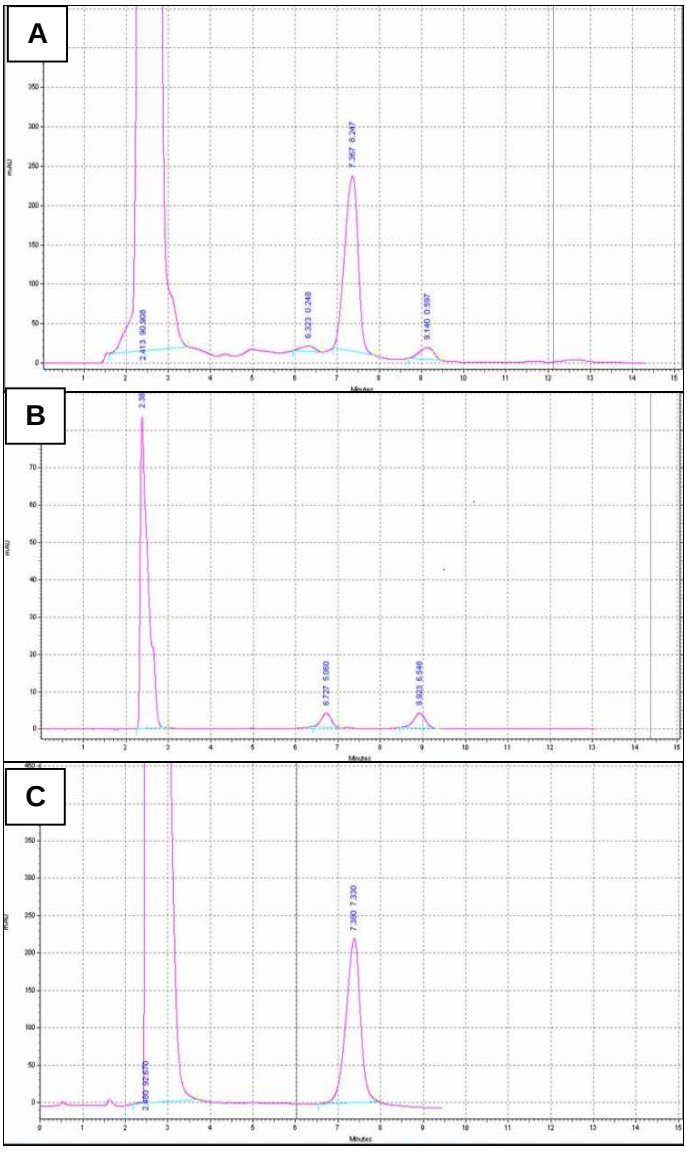



***Figure 3.7:*** *HPLC identification of S. epidermidis RM1 AHL acylase degradation product: Retention of C7 HSL alone can be seen at 7.38 minutes (Panel* ***C****) and retention time of dansylated HSL can be seen in two peaks one at 6.7 minutes and 8.9 minutes in (Panel* ***B****). Dansylation of reaction mixture containing C7 HSL degraded by whole cells of S. epidermidis RM1 shows three peaks (Panel* ***A****)one corresponding to substrate ( Retention time 7.36 minutes) and other two corresponding to dansylated HSL (Retention times 6.32 and 9.1 minutes).*

*3.3.5. Investigation into understanding the mechanism of dual enzyme activity shown by S. epidermidis RM1*

Now since *S. epidermidis* RM1 demonstrated both BSH and AHL acylase activity, it is only logical to think that the BSH may be responsible for AHL cleavage also. The rationale behind this musing being that, both AHL acylase and BSH are amido-hydrolases belong to Ntn hydrolase super-family of enzymes and previously another member of the same super-family have been revealed to hold AHL cleavage potential [19].So an attempt was made to investigate this aspect further. Prior to this there is only one report of a bile salt hydrolase producing organism , *Lactobacillus plantarum* WCFS1, also capable of Acyl homoserine lactone (AHL) cleavage, however the activity was found to be very feeble [18].All the above findings solicited investigation into the possibility of bile salt hydrolase produced by *S.epidermidis* RM1 to be acting as an AHL acylase.This was done by further studying the genetic basis of this very interesting dual enzyme activity shown by *S.epidermidis* RM1.Genome of a closely related organism, *S. epidermidis* ATCC 12228, was used as template to design primers for amplifying a probable BSH and AHL acylase gene present in the genome of *S.epidermidis* RM1. A putative Penicillin V acylase sequence served as a template for designing primers for BSH gene detection, the premise being, many putative Penicillin V amidases have turned out to be biologically active BSHs and vice versa [20].Both these enzymes being evolutionarily closely related show interchangeable enzyme activity and substrate specificity. Sequence for a putative penicillin V amidase annotated within the genome of *S. epidermidis* ATCC 12228 was used as template to design primers for BSH gene amplification from *S.epidermidis* RM1. Amplicon obtained after the PCR reaction was of the expected size of 666 bp (Fig. 3.8 Lane1) and upon sequence analysis showed a 98% match with the template sequence.

Since there were no annotations corresponding to an Acyl homoserine lactone acylase in the genome of *S.epidermidis* ATCC 12228, a gene coding for an aminoacylase was used as template for primer designing.The rationale behind using an aminoacylase sequence as template was that, there have been previous reports of quorum quenching bacteria using amino-acylases to cleave AHL molecules [21]. Primers pair designed on the basis of this template could in fact amplify a region in the *S.epidermidis* RM1 genome, and the

amplicon obtained was of the expected size of 553 bp (Fig.3.8 Lane 3) .Upon BLAST analyses of the sequences the amplicon showed 99% homology with the template sequence.

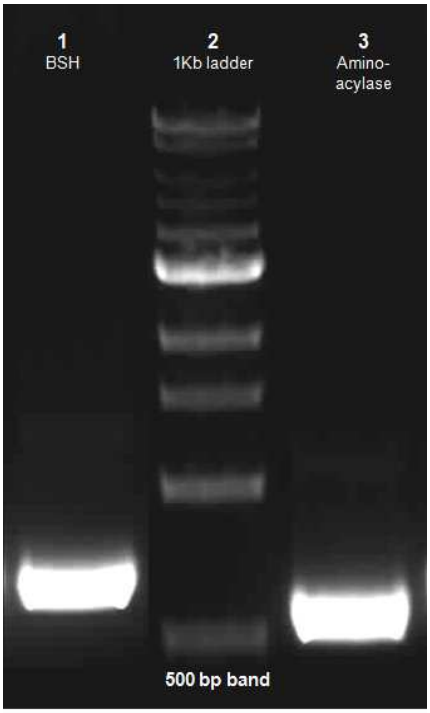



***Figure 3.8:*** *1% agarose gel showing amplicons of expected size amplified using two different set of primers from genomic DNA of S.epidermidis RM1.*

**Amplicon Sequence with BSH specific primers:**

TTCATTAAATTTTCATCTTCATTATGGGAACAGCGTAGTTGGTGTCTTAAATATGTAGCGCGTA
TAAAACGTTCTGTTGACGTATAACCACCCGGTAAGCCATTTGTTCCTGCTTCACAGCCCATTGA
TCTTACTAGCACTTTACCTATTAATTGATTGGTTGATTTCTGTGGCGTTAAAAAAGCGTAATTT
CTTAAGTTAGATAGATGCCAATCTAATTTAGGTTCATTTGTTAAGGTATGAACATAATTATCTT
TAACTATTAATAAGCCATTGTGAGGTTCTATGGCTACGGTATGTCCTGTTTCATCAGTGACCAT
GAAATGTAAAGGAGGAACGATATTCAAAGTCGTATTTTTTTCATTCATAATATTGATTTTCTTA
ACCTTTTGTTTTAATTCGCTAATACTTTTATTAAAACCTAAAACCCAAACAATAAACTCCTCAG
GTGCTAAGTTAAAATAACCATAACGTTTATGGGTACTGTATGAGGCTTCACCAGTGAAGTAAT
GGTTCGAAATAGCTAAACCTTTTTCGTTTATACCATCACCAAATCTATAACGTCCTACTTTTAA
ATTAGTTCCAACAAAACCATATTCAAGACGCATGTCTGAATCTAGATCGAATTGGTAGTGATA
ATGGCGTG

**Amplicon Sequence with Amino Acylase specific primers:**

TAAACTTAGGATGATGGTCGAGGGTACATAGGTCCATCTTCTGGTGCTGCACCCGAATAAATA
AATGTTGAAGGTCTTTCTAACGCATAAAACGCGAAATCTTCTGAGGGTGGCTGCGGCTCACAA
ATATCTATTGCTTTTATGTCATCCAGCTTAGCATTTTTTAAAGTTGTTGCTACATAAGAAGTAA
ATTCAGGATCATTGTAAAGAGCTGGATAATCCTTTTTAAATTCAAAATCACAAATCACTCCGA
ACATCTCTTCTAATCCTCTGACTAGTCGTGTCATTTCATTCTGAATATTGTCTCTTGTATCATCA
GTCAGTGCACGTACATCACCTTCGATTGTTATTGTATCTTTAATCACATTGAATTGTCCTTTCCC
GTCGAAGGAACCTATTGTGACTACACCCGTTTCAAACGGATTTAAGCGTCTTGAAACAATGGT
CTGTGCGGTCGTTACAAAATGCGCACCTGCTACAATAGCGTC

Based on the results obtained from the above mentioned exercise it could be clearly said that the AHL deamidation and bile salt deconjugation activity exhibited by *S.epidermidis* RM1 is governed by two independent gene sets. So there is indeed no correlation between Bile salt hydrolysis and AHL acylase activity of the isolate.

### 3.4. Conclusion:

For various bacterial pathogens, QS has been proposed to facilitate establishment of disease pathogenesis by allowing well-timed expression of required virulence factors so that they can easily escape the host immune system [22]. AHL mediated QS has been hypothesized to facilitate virulence gene expression, adhesion to gut epithelium and eventual establishment of *Enterohemorrhagic E. coli* (EHEC), *Vibrio parahaemolyticus, Yersinia sp.* etc. [23, 24].Any organism capable of cleaving AHLs in this niche environment can help impede expression of these injurious AHL mediated QS phenotypes. All the findings highlight the uniqueness of the isolate especially in terms of a potential probiotic organism. Since *S. epidermidis* RM1 possess both AHL acylase and Bile salt hydrolase activity its possible superiority over available probiotic strains is tremendous.

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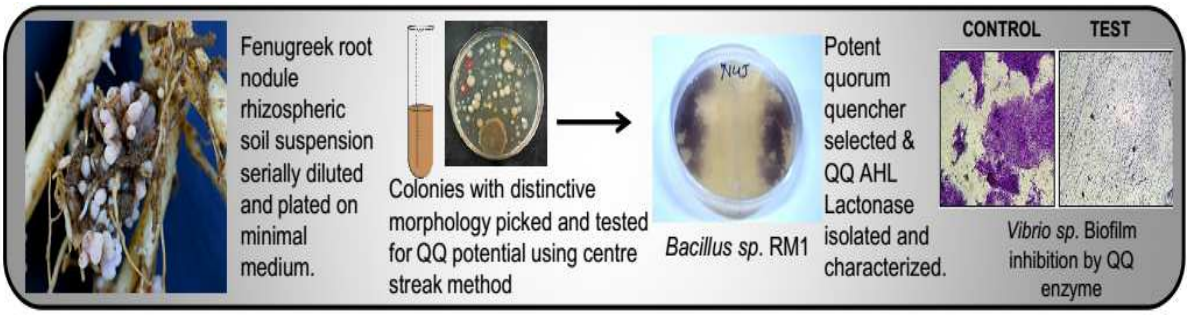
# Chapter 4A

## *Novel Species of Bacillus isolated from Fenugreek root nodule rhizosphere producing a quorum quenching enzyme*

**This Chapter has been communicated as:**

**Mukherji R,** Prabhune A. Enzyme purification and kinetic characterization of AHL lactonase from *Bacillus sp*.RM1 a novel and potent quorum quencher isolated from Fenugreek root nodule rhizosphere. (Communicated Manuscript)

# 4*A*. Novel Species of *Bacillus* isolated from Fenugreek root nodule rhizosphere producing a quorum quenching enzyme




**4A.1. Introduction:**

This chapter talks about 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. 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 [1]. 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 [1]. Numerous bacterial phenotypes are regulated via AHL mediated QS like biofilm formation and virulence genes expression [2]. In soil environments where competition is inevitable any organism capable of quenching quorum sensing mediated gene regulation 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 [3]. 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[4].Thus quorum quenching (QQ) capability is widespread in soil ecosystems and effectors include both enzymes and bio-molecules. AHL lactonases, hydrolases that cleave the lactone ring of AHLs rendering them biologically inactive, have been reported in numerous members of the firmicute clad especially *Bacillus sp.*, an organism that is found most commonly in soil [5]. 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* [6,7].

First report of an AHL lactonase from genus *Bacillus* was by Dong et al. in 2000 [8]. 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 [9]. AHL lactonases have also been described in other Firmicute like genus *Arthrobacter* and in Actinobacteria (*R. erythropolis*) etc.[10-14]. The 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.

## 4A.2. Materials and methods:

### *4A.2.1. 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.

*4A.2.2. 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 (see appendix for composition). 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.

*4A .2.3. 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 in 2004 [15] 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.

*4A.2.4. 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 [16].The extracted genomic DNA was quantified and the 16S rRNA gene was amplified using universal primers 8F and 1525R [17] 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 (Thompson *et al*., 1994). Phylogenetic tree was computed in MEGA 4 using the neighbor joining method [18].

*4A.2.5. 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.

*4A.2.6. Growth of Bacillus sp. RM1 in minimal medium containing AHL as sole source of carbon*

KG Chan *et al*., in 2009 [19] 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 [20].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.

*4A.2.7. Purification of QQ enzyme (AHL lactonase) from Bacillus sp RM1*

4A.2.7.1. 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 protien preparation was then dialyzed overnight in 100X volume of 10mM phosphate buffer pH 7.0 and purified further using Q-sepharose column chromatography.

4A.2.7.2. 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.

4A.2.7.3. 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.

*4A.2.8. 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.

*4A.2.9. 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.

*4A.2.10. 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 [21] 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.

**4A.3. Results and Discussion:**

*4A.3.1. 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 [22]. 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 chapter 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* [15] (See section *4A.2.3*). AHL degradation activity of selected isolates can be seen in Figure 4A.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.

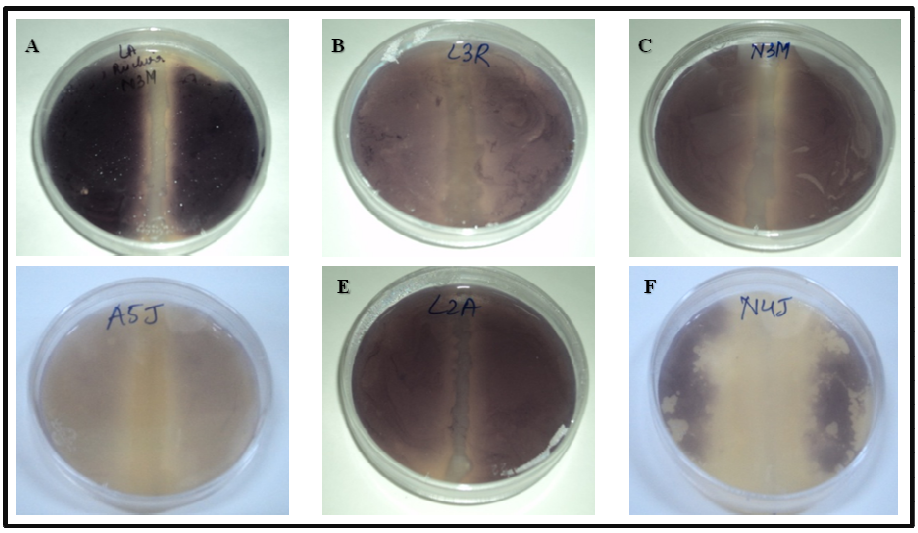



***Figure 4A.1****: AHL degradation activity of selected isolates as determined by centre streak method.*

*4A.3.2. 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:

CATAACGCCGAATGGGATTAAAGAGCTTGGCTCTTATGAAGTTAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTGCCCATAAAACTGGGATAACTCCGGGAAACCGGGGC
TAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTG
TCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCAACGATGCGTACCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACAAAAGTC
TGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTT
AGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCC
CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGA
AAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTG
GCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGA
GGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTAC
GGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAA

ACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATC
TTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCT
ACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAAC
CGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
TCACACCACGAGAGTTTGTACACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGC
CGCCTAAGTGGGACAGATGATTGGGGTGAAGTCGTAACAGGTAGCCGTATCGGAAG
GTGCGGCTGGGATCCACCTCCTCAG

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. 4A.2). As mentioned before AiiA homologs are widespread among *Bacillus sp*. [6] and AHL lactonase from *Bacillus thuringiensis* has already been crystallized [23]. Consequently AHL lactonase activity demonstrated by our isolate, *Bacillus sp*. RM1, obtained from rhizospheric soil sample is not unanticipated.

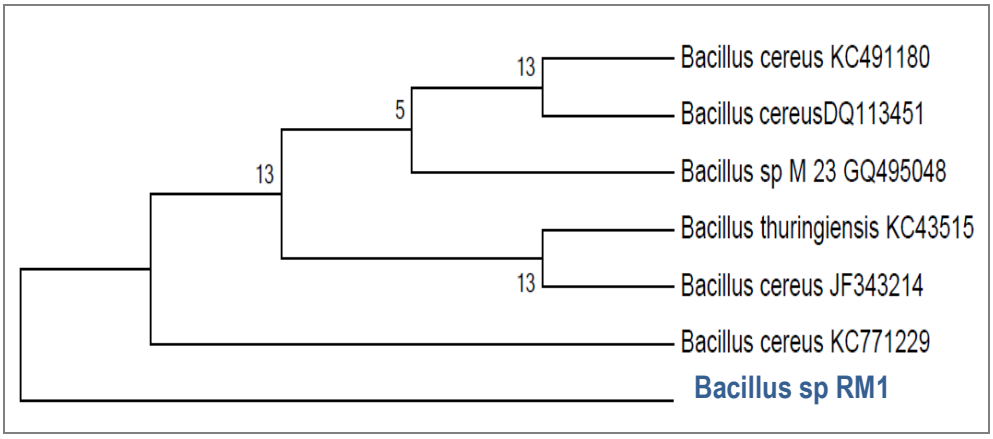
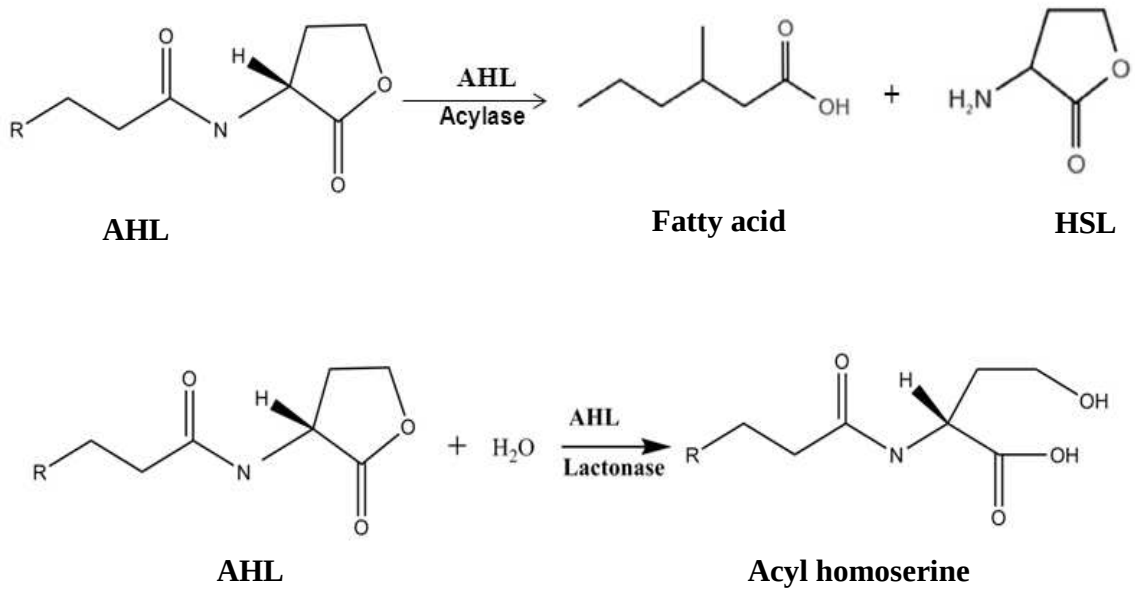



***Figure 4A.2**: An unrooted phylogenetic tree describes the evolutionary relatedness of isolate under study.*

*4A.3.3. 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 4A.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. 4A.3)




***Scheme 4A.1****: Reaction catalyzed by an AHL acylase and an AHL lactonase*

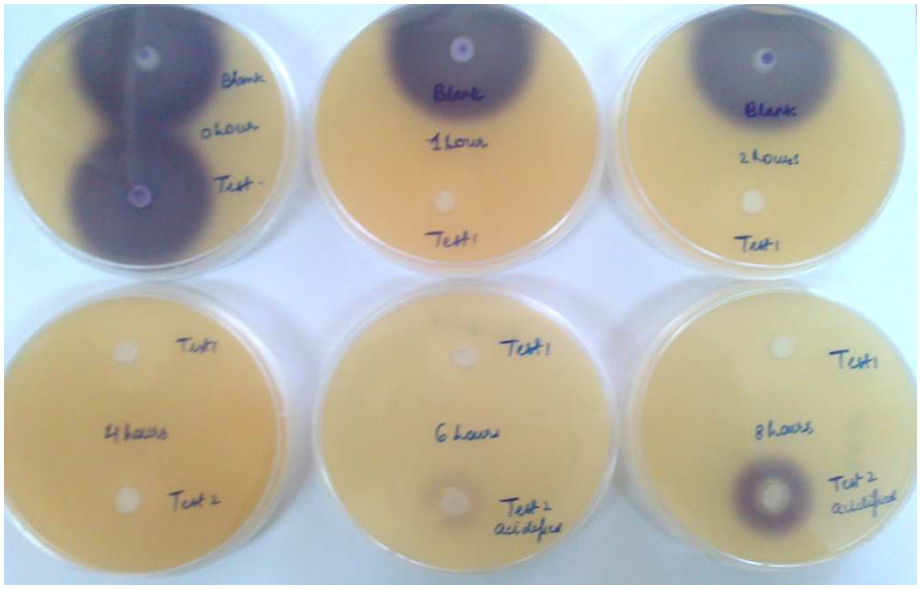



***Figure 4A.3****: AHL lactonase activity of the isolate confirmed by CV026 based bioassay.*

*4A.3.4. 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*. in 2009 [19].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.4A.4). No growth was obtained in the control tubes (See section 4A.2.6 for details about CONTROL). This confirms the ability of *Bacillus sp*. RM1 to synthesize an AHL lactonase with broad substrate specificity which is in accordance with previous reports.

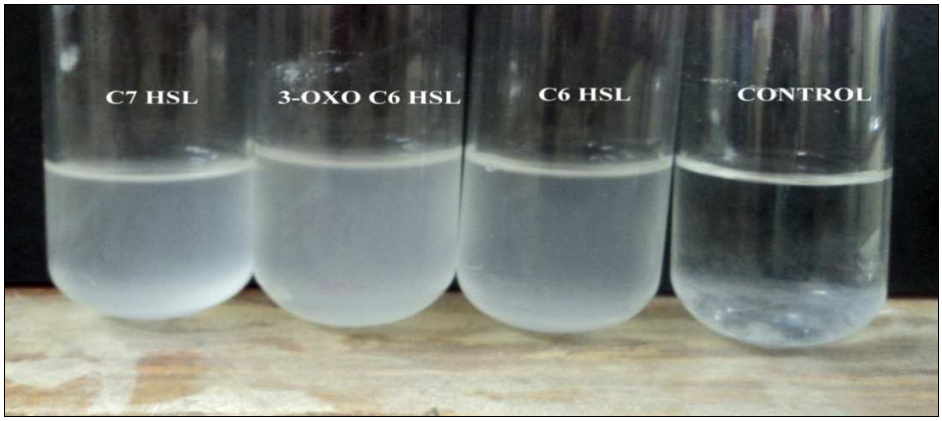



***Figure 4A.4****: Growth of Bacillus sp. RM1 in KG medium, with AHLs as sole source of carbon*

*4A.3.5. 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. 4A.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.4A.6). The protein showed band at the expected size of ~28 kDa, which is similar to previous reports [5-8] (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.

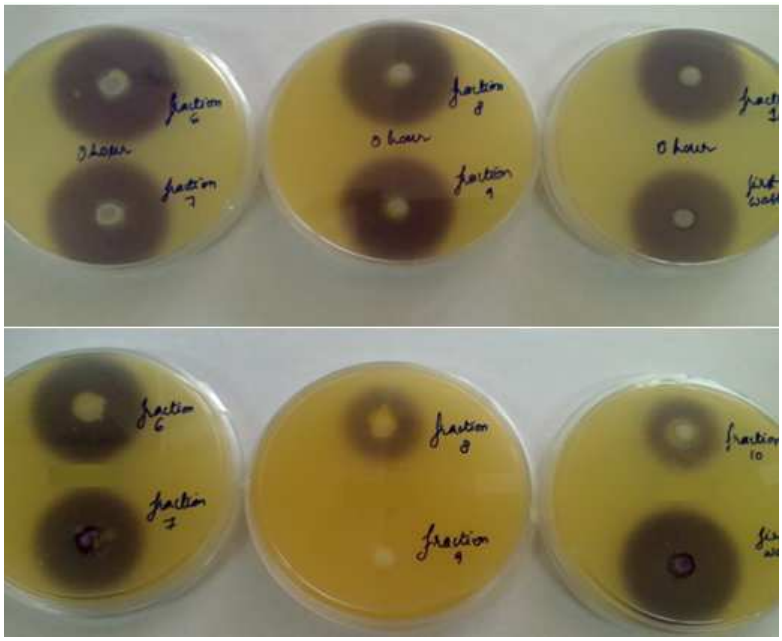


***Figure 4A.5****: Bioassay based identification of column fractions containing enzyme of interest (AHL lactonase).*

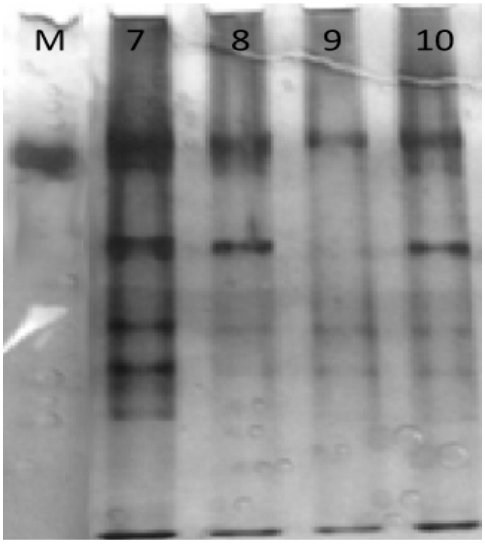



***Figure 4A.6:*** *Native poly-acrylamide gel electrophoresis showing protein of interest. From left to right- Molecular weight marker: Carbonic Anhydrase 29 kDa (Sigma-Aldrich, India), Column Fraction 7, 8, 9 and 10 containing Bacillus sp. RM1*

Optimum parameters to get maximum AHL degradation by *Bacillus sp.* RM1 AHL lactonase were then determined. Of all the pH and temperature values tested (see section *4A.2.8* for more details) pH 7.0 and temperature of 50$^{o}$C (Fig.4A.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.4A.7 and Table 4A.1) respectively.Kinetic characterization of some other AHL lactonases can be found elsewhere (Table 4A.1) [24,25]. 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.

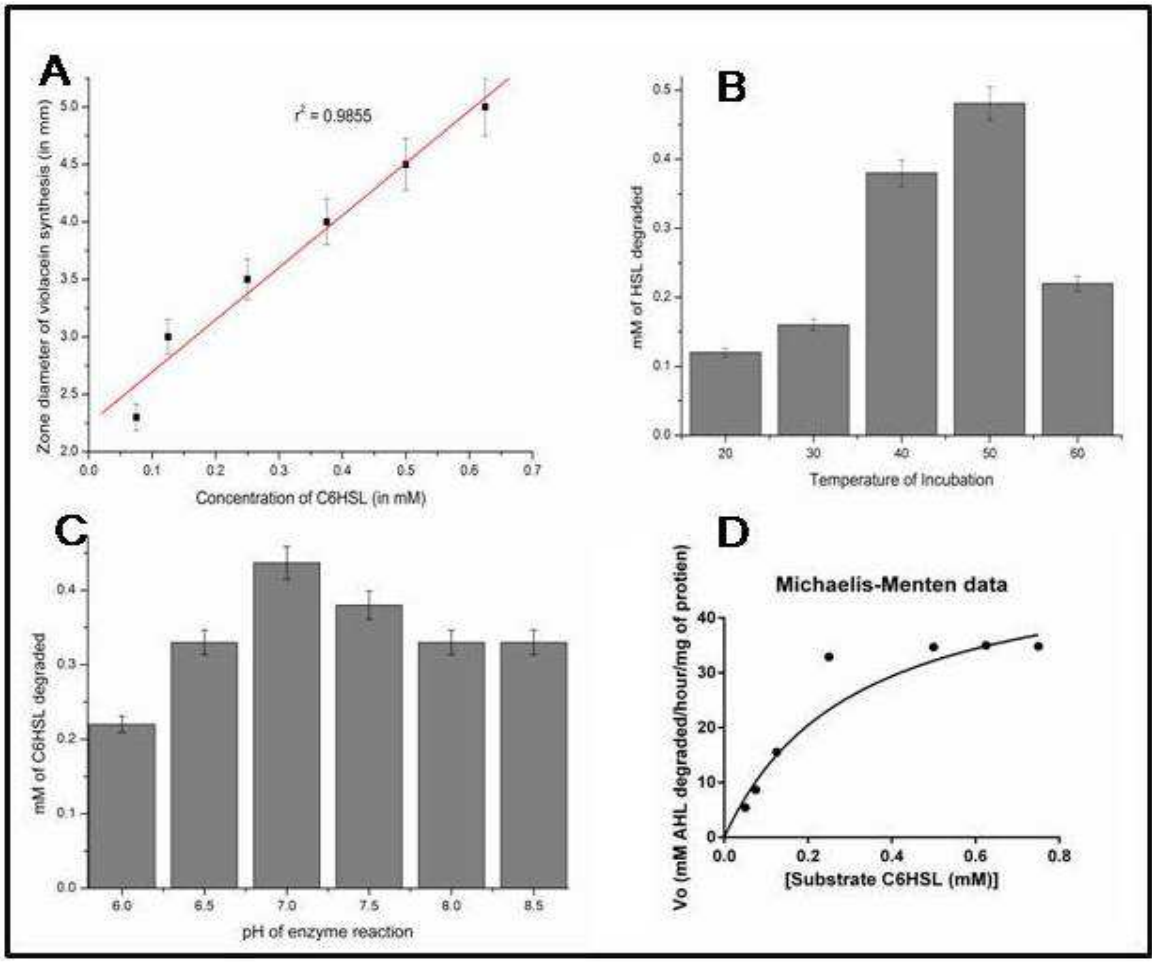



***Figure 4A.7****: From top left: (A) Standard plot showing linear correlation between amount of violacein synthezized and mM of substrate(C6HSL) required; (B) and (C) Optimum temperature and optimum pH of the enzyme catalyzed reaction;(D) Kinetics of the C6HSL clevage by Bacillus p. RM1 AHL lactonase as determined by Michaelis Menten plot.*

***Table 4A.1****: Comparison of kinetic characteristics of some selected wild type and mutant AHL lactonase*

| | AHL Substrate used | Origin of protien | 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 |

*4A.3.6. 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. 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. Inhibition of *Vibrio* sp. biofilm by AiiA class of AHL lactonases has also been described previously by Augustine *et al*.,2010 [21] 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 [26].

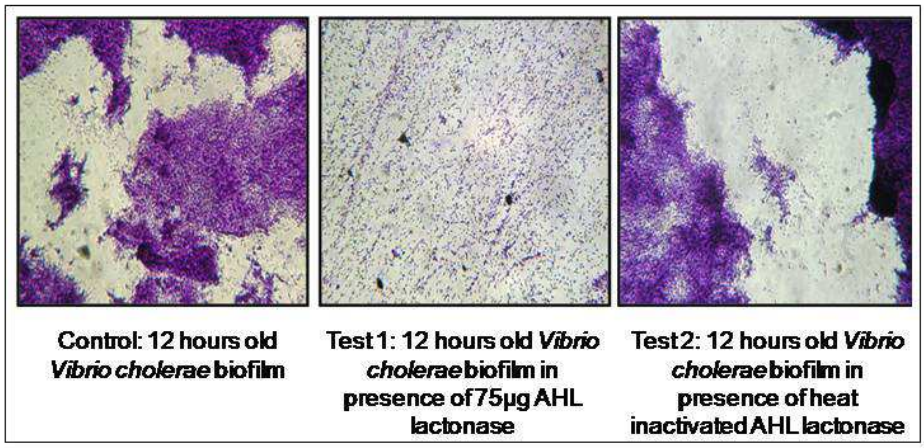



***Figure 4A.8****: Anti-biofilm activity of Bacillus sp. RM1 AHL lactonase against V. cholerae.*

## 4A.4. Conclusion:

This chapter 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.

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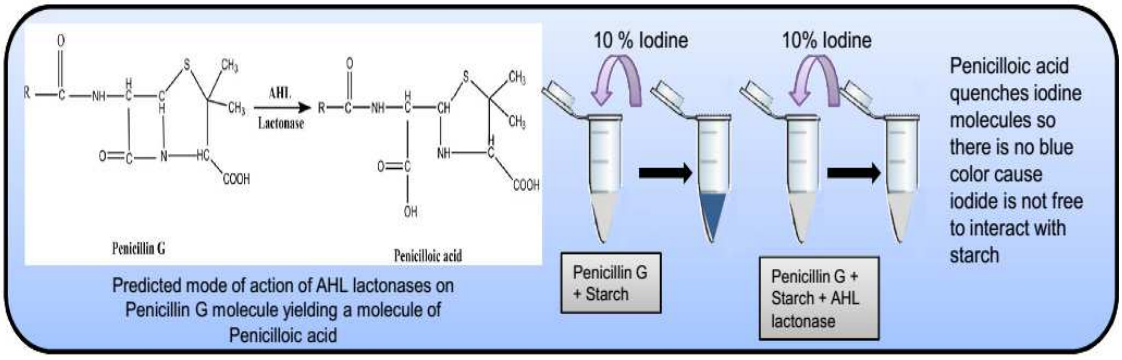
# Chapter 4B

## *A rapid Iodometric method for qualitative detection of AHL Lactonases*

**This Chapter has been published as:**

**Mukherji R,** Prabhune A (2014), A rapid iodometric method for qualitative detection of AHL Lactonases, World Journal of Pharmaceutical research. 3:1126-1132.

# 4*B*. A rapid iodometric method for qualitative detection of AHL Lactonases




**4B.1. Introduction:**

Quorum sensing (QS) is now an accepted means by which a given bacterial population interacts socially [1]. Moreover it is now well established that it is a major mechanism by which bacterial pathogens regulate their virulence gene expression [2]. Bacteria, with thehelp of their quorum sensing signals and their cognate receptors, are able to take full advantage of the available environment and evade the deleterious host mechanisms. This population density dependent phenomenon allows bacterial community to come together and function as a single entity, thus increasing the strength and the persistence of the invading pathogens [1, 3]. Quorum Sensing has been studied in both gram positive and gram negative bacterium, and the most well characterized class of signal molecules in case of gram negative bacterium is Acyl homoserine lactone [2].Expression of a diverse range of phenotypes is mediated by QS based regulons like, antibiotic production, pigment formation, biofilm formation ,expression of exo-enzymes and virulence factors, like, proteases, lytic enzymes, exotoxins etc. [1-3].In this light, impeding quorum sensing in any way will weaken the bacterial pathogen and thus allowing anti-infective therapies a chance to destroy the invader. This concept is better known as Quorum Quenching (QQ). Quorum Quenching has been predicted to be the future of antimicrobial therapy because it offers the least possible opportunity of development of resistance, by imposing no life or death selective pressure upon the pathogenic bacteria [4]. A variety of mechanisms have been proposed which can aid in quenching, quorum sensing, in gram

negative systems, and the enzymatic means of QQ has been proven to be the most promising. Of the different classes of enzymes capable of quenching AHL mediated quorum sensing, two major classes of enzymes have received significant attention, namely AHL acylase and AHL lactonase [5].

To date AHL Lactonases have been identified and studied in varied organisms. The first report of an authentic AHL Lactonase was by Dong et al. (2000), wherein they identified a gene *aiiA* from *Bacillus sp*. 240B1 which was subsequently shown to code for a potent AHL degrading lactonase [6].Since then AHL lactonase producing capability has been recognized in many different genera and in fact in different species and sub-species of *Bacillus* [7].AHL lactonase acts on the lactone ring of the quorum sensing signal molecule, hydrolyzing it and inactivating the QS signal molecule (Fig.4B.1A).Assay systems are well in place that allow detection of these AHL lactonases. Bioassay based approach utilizes a biosensor strain capable of responding to AHL signals by elaborating an easily detectable phenotype [8].However these assays can be time taking, when test sample numbers are large, and may give false positive results if ample amount of care is not taken and appropriate controls are not available. Chemical assays used to detect AHL lactonase activity are based on the premise that hydrolysis of AHLs yields a lactone ring-opened product along with, release of one H+ ion and this production of H+ ion can be detected by using a continuous spectrophotometric pH indicator assay [9]. However this system suffers from the requirement of very high substrate (AHL) concentration and very pure enzyme preparations. Also finding the perfect buffers and indicators pairs to match the reaction pH and indicator p*Ka* can be very tricky. In this chapter a facile qualitative detection system, to detect AHL Lactonase activity in even crude enzyme preparations using Penicillin G as substrate has been proposed (Fig. 4B.1B).

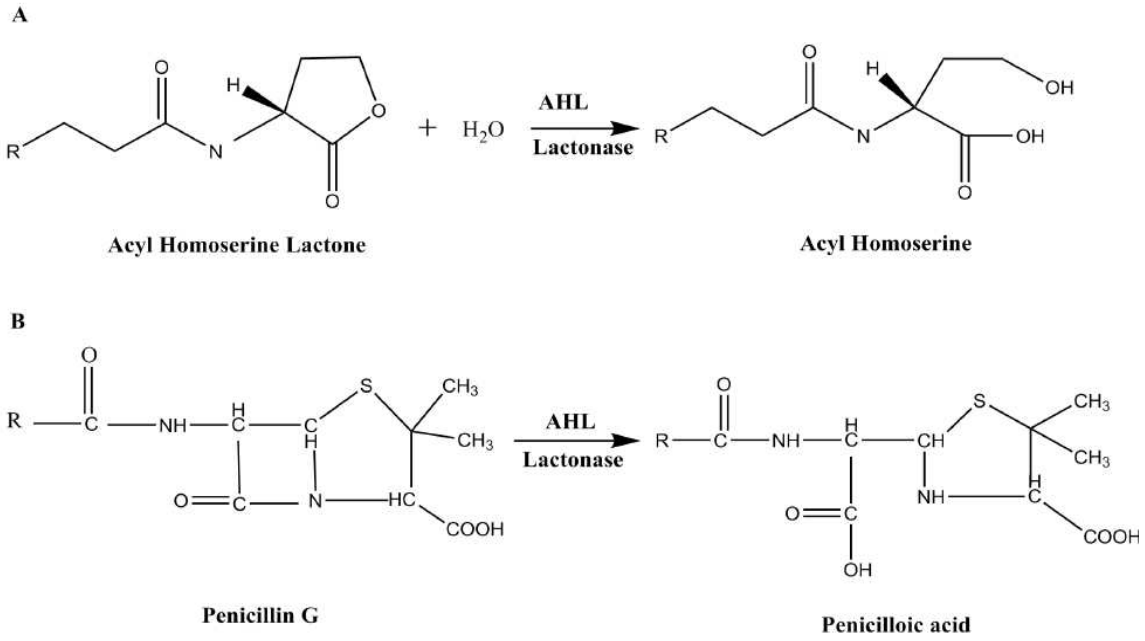



***Figure 4B.1****: Known mode of action of AHL Lactonase on Acyl homoserine lactone molecule (**A**). Predicted activity of AHL Lactonases on Penicillin G molecule (**B**)*

## 4B.2. Materials and Methods

### *4B.2.1. AHL Lactonase enzyme preparation*

Crude dialyzed and partially purified enzyme preparations of AHL Lactonase were obtained from novel *Bacillus sp*. RM1 as described previously in Chapter 4A.Concentration of both crude dialyzed enzyme and partially purified enzyme was set at 1mg/ml before use in this iodometric assay.

### *4B.2.2. Reagents and chemicals*

Penicillin G potassium salt was procured from SIGMA-Aldrich, India. Soluble Starch and Iodine crystals of high purity were obtained from HiMedia, India.

*4B.2.3. Iodometric assay for detecting AHL Lactonase activity*

This assay is based on the foundation that since AHL lactonase belongs to beta-lactamase superfamily of enzymes it possesses the capability to act on intact Penicillin molecule and hydrolyzing it to Penicilloic acid. Penicillin G as such is inert to iodine in neutral aqueous solution while penicilloic acid, the inactivation product derived from Penicillin quenches 6-9 equivalents of added iodine, thus iodine (the colorimetric reagent)is not free to interact with 0.1% starch (already present in the reaction mixture) and gives no blue color [10]. However in Penicillin only control (without enzyme) since there is absence of penicilloic acid all the added iodine reagent is free to interact with the starch already present in the reaction mixture thus giving blue color to the mixture. The hydrolyzing activity of AHL lactonase on Penicillin G was estimated by incubating the reaction mixture at 40$^{o}$C for 15 minutes. Presence or absence of blue color, which develops as a result of interaction of free/unquenched iodine with 0.1% starch solution, was noted within 10 seconds of addition of Iodine reagent . Appropriate controls were used to eliminate confusing observations.

**4B.3. Results and Discussion**

Both biological and chemical methods are available for detection of AHL lactonase activity; however each of them suffers from their own drawbacks. The present chapter reports a rapid method of detection of AHL lactonase activity based on a very simple principle. AHL lactonase belong to beta-lactamase super-family [7]. Members of beta lactamase super-family are known to act on beta-lactam ring of Penicillin and cephalosporin molecules, causing a ring opening hydrolysis reaction. The above two facts led to the musing that AHL lactonases may also possess the ability to hydrolyze penicillin molecule leading to the formation of Penicilloic acid. This forms the basis of our qualitative assay. Penicilloic acid thus formed, after Penicillin G inactivation, could then easily be detected using starch-iodine detector-indicator system, which is already a well-established method for penicilloic acid detection first reported by Cole *et al.* in 1973[10]. An important fact that should be given some consideration here is that there is slight amount of spontaneously degradation of Penicillin molecule at the required incubation

temperature of 40°C, hence the Penicillin only control (tube2, Fig. 4B.2) is bound to show a slight decrease in blue color when compared with starch only control (tube 1, Fig.4B.2). To minimize this spontaneously degradation of substrate, incubation time and temperature parameters must be modified in order to keep all ambiguities at bay. The assay was performed using different concentrations of partially purified AHL lactonase preparations from a novel *Bacillus sp*.RM1 namely, 20, 100 and 200µg of enzyme and a scheme showing how different reactions were set up along with controls can be seen in Figure 4B.2.It was found that the system could detect degradation of Penicillin G with as less as 20µg of enzyme (Fig. 4B.3).

| | Contents of the Tube (600ul of reaction Mixture) | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Penicillin G (20mg/ml) | - | 200 µl | 200 µl | 200 µl |
| Starch Indicator (0.1%) | 200 µl | 200 µl | 200 µl | - |
| Lactonase enzyme preparation (1mg/ml) | - | - | 200 µl | - |
| Milli-Q water | 200 µl | 200 µl | - | 200 µl |

**Incubate at 40°C for 15 minutes & add 20µl of 10% Iodine Solution.**

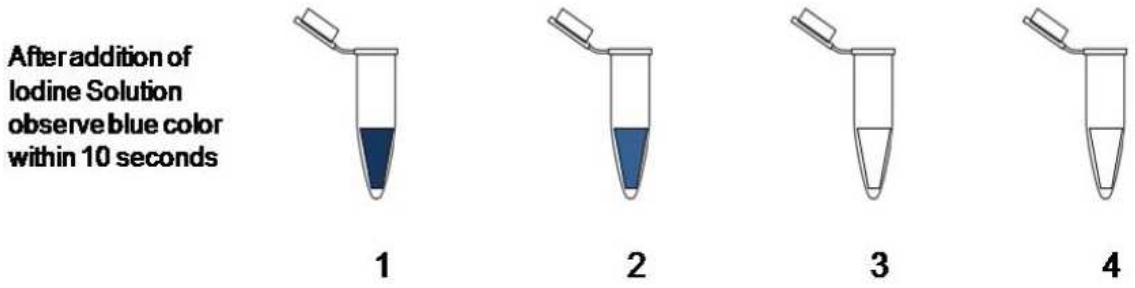



***Figure 4B.2:*** *Schematic representation of the basic protocol used to determine hydrolyzing activity of AHL lactonases.*

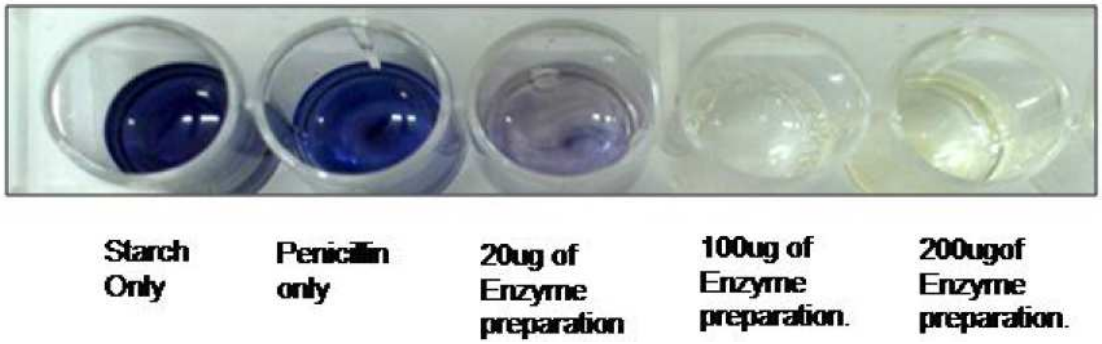



***Figure 4B.3****: Photographic evidence of AHL lactonase activity on Penicillin G molecule. With increasing concentration of enzyme preparation more amount of penicilloic acid is produced hence the decrease in blue color.*

It should be also noted that Penicilloic acid formed after hydrolysis of Beta-lactum ring of Penicillin G quenches iodine very quickly hence color development should be observed within 10secs of addition of10% iodine solution. The biggest advantage that this assay system offers is in terms of time and economy. The color development can be observed within seconds, does not require high concentration of AHLs and this assay can easily be performed in a 96 well format to screen large number of enzyme fraction within minutes.

**4B.4. Conclusion:**

A straightforward method to detect presence of AHL lactonase in any given crude or partially purified enzyme preparation was put forward. The present method has the potential to be developed into a high- throughput assay for detecting AHL lactonases. AHL lactonase are important quorum quenching enzymes and have tremendous biotechnological potential, therefore a process that allows easy detection of this enzyme will fast gain popularity among the scientific community.

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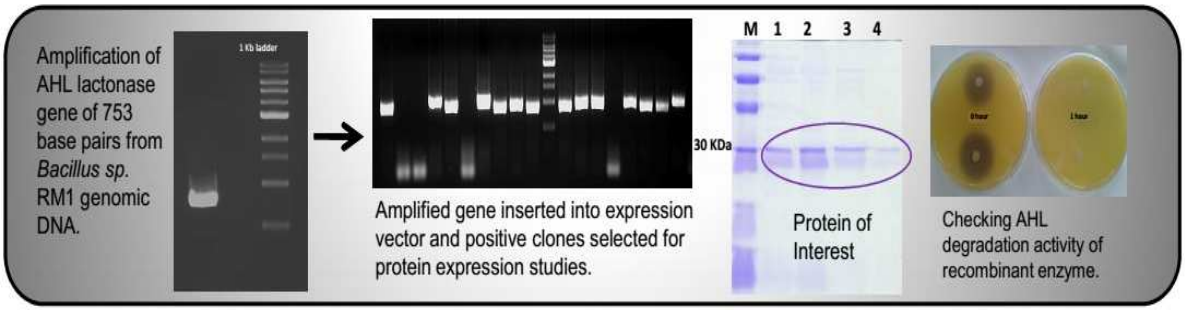
# Chapter 4C

*Cloning and over expression of AHL lactonase from Bacillus sp.RM1*

# 4*C*: Cloning and over expression of AHL lactonase from *Bacillus sp*.RM1




**4C.1. Introduction:**

Quorum-quenching enzymes have been proven to be extremely valuable as biochemical tools,to better comprehend the QS phenomenon, and as possible therapeutic proteins. One of the best-characterized families of QQ enzymes is the AHL (Acyl-homoserine lactone) lactonases, which hydrolytically cleave the autoinducer's or QS signal molecule's lactone ring thus inactivating cell to cell signaling [1]. An improved understanding of how this enzyme works can help design more selective and competent biomolecules with increased QQ potential. To facilitate these studies, heterologous expression of this enzyme in different systems has been attempted previously [2]. These procedures have been predicted to facilitate production of more useful quorum-quenching reagents with enhanced mechanistic characteristics which can be useful in both biochemical studies and possible therapeutic applications [2]. The different expression systems that have been used for heterologous expression of AHL lactonase are either *E.coli* based expression vectors like BL21 DE3 pLysS [3-5] or *Pichia pastoris* [6] based high yielding expression system. The recombinant over-expressed enzyme has been successfully purified and has been used for directed evolution efforts [7, 8] and infection inhibition studies in real life in-vivo models [9].

Many plant and animal pathogens have been genetically modified to express AHL lactonases and this has led to a decreased intracellular accumulation of cognate signal molecules thus leading to a diminished virulence. For example genetically-modified

*Erwinia carotovora* [10, 11] and *Pseudomonas aeruginosa* expressing AHL-lactonase [12] showed decidedly reduced pathogenecity. The first report of an AHL-lactonase coding-gene (*aiiA*) from *Bacillus sp*. 240B1 was by Dong et al. [13].Cloning and over expression of this gene in the plant pathogen *Erwinia carotovora* attenuated *E. carotovora* pathogenecity by reducing intra & extracellular AHL accumulation [10]. To date, AHL-lactonases genes from numerous *Bacillus sp*. have been successfully recombinantly expressed which has been shown to attenuate many disease causing bacteria (*i.e.* plant pathogen *E. carotovora* ,human pathogen *Pseudomonas aeruginosa*) [14-16] and plants pathogens [17]. *E. carotovora* is a well known plant pathogen that causes soft rot disease in plants, like potato, eggplant, Chinese cabbage, carrot, celery, cauliflower, and tobacco. Transgenic plants expressing AHL lactonase exhibit significantly enhanced resistance to *E. carotovora* infection and delayed development of soft rot symptoms [18-20].

The present chapter reports cloning and expression of AHL lactonase from *Bacillus sp*. RM1 in *E.coli* expression system BL21 DE3.The his-tagged recombinant enzyme was purified using affinity chromatography and activity of the purified enzyme was checked using CV026 based bioassay and highest activity was obtained at pH 7.0 and at 50$^{o}$C, which was identical to that of wild type enzyme.

**4C.2. Materials and Methods:**

*4C.2.1. Genomic DNA extraction from Bacillus sp. RM1and Primer designing*

For extraction of genomic DNA the culture was grown in 10 ml Trypticase soy broth for 18 hours at 28$^{o}$C and 180 rpm. Cell pellet was then harvested to be processed further. Genomic DNA was then isolated according to the modified method reported by Sambrook *et al*. 1987.Primers for amplifying the *aiiA* AHL lactonase gene homolog from *Bacillus sp*. RM1 were designed using *Bacillus cereus* strain MBG25 *aiiA* gene sequence as template. The primers were designed keeping in mind that the expression system to be used was pEXP5-CT/TOPO®TA expression vector from Invitrogen. Appended below is the *aiiA* Lactonase gene sequence from *Bacillus cereus* strain MBG25 (accession number: JF501512) followed by the primers used for amplifying the gene of interest from the culture under study.

ATGACAGTAAAGAAGCTTTATTTCATCCCAGCAGGTCGTTGTATGTTAGATCATTCTTCTGTTA
ATAGTACACTCGCGCCGGGGAATTTATTGAACTTACCTGTATGGTGTTATCTTTTGGAGACAG
AAGAGGGGCCTATTTTAGTAGATACAGGTATGCCAGAAAGTGCAGTTAATAATGAAGGGCTT
TTTAACGGTACATTTGTTGAAGGGCAGATTTTACCGAAAATGACTGAAGAAGATAGAATCGTA
AATATATTAAAGCGTGTAGGGTATGAGCCGGACGACCTTTTATATATTGTTAGTTCTCACTTAC
ATTTTGATCATGCAGGAGGAAACGGTGCTTTTACAAATACACCGATTATTGTGCAACGAAAGG
AATATGAGGCAGCACTTCATAGAGAAGAATATATGAAAGAATGTATATTACCGCATTTGAACT
ACAAAATTATTGAAGGGGATTATGAAGTGGTACCAGGTGTTCAATTATTGTATACGCCAGGTC
ATTCTCCAGGCCATCAGTCGCTATTAATTGAGACAGAAAAATCCGGTCCTGTATTATTAACGA
TTGATGCATCTTATACGAAAGAAAATTTTGAAGATGAAGTGCCGTTCGCGGGATTTGATTCGG
AATTAGCTTTATCTTCCATTAAACGTTTAAAAGAAGTTGTGGCGAAAGAGAAGCCAATTATTT
TCTTTGGTCATGATATAGAGCACGAAAAGGGTTGTAAAGTGTTCCCTGAATATATA

**Primer pairs used for amplification of gene of interest:**

**pEX5CT** For- ATGACAGTAAAGAAGCTTTATTTC

**pEX5CT** Rev- TATATATTCAGGGAACACTTTACAAC

*4C.2.2. PCR amplification of target gene and cloning in expression vector*

The *aiiA* gene homolog was amplified from the genomic DNA of *Bacillus sp.* RM1 using the above mentioned primer set. The PCR reaction mixture contained 50ng of genomic DNA and 2 units of HiMedia Taq polymerase along with Taq polymerase buffer with $MgCl_2$, 1mM dNTPs, and gene specific primer pairs. The PCR amplification conditions used were as follows: denaturation at 95$^{o}$C for 5 minutes, followed by 25 cycles of, 95$^{o}$C for 45 secs, annealing at 57$^{o}$C for 45 secs then 72$^{o}$C for 1 minute, with subsequent elongation step at 72$^{o}$C for 2 minutes. The amplified product was checked on 1% agarose gel along with 1Kb DNA ladder (New England Biolabs Ltd.) to determine the size of the product.

After obtaining amplicon of desired size, sufficient quantity of amplified product was collected by setting up multiple PCR reactions. The product was immediately PCR purified to prevent the delicate TA overhangs from falling off the product.PCR purified product was then cloned into pEXP5-CT/TOPO®TA expression vector from Invitrogen. The advantage of using this expression system is that it is a one-step cloning method for the direct insertion of Taq polymerase-amplified PCR products into the expresssion

vector for T7-based, high-level expression of recombinant fusion proteins. No ligase,restriction enzymes and PCR primers containing special restriction sites are required to generate the expression construct. The expression vector backbone contains C-terminal fusion tag for detection and purification of recombinant proteins and TOPO-TA cloning site for rapid and efficient cloning of Taq-amplified PCR products. However while using this expression system it is important to remember that the insert might get incorporated into the vector backbone into either the correct or reverse orientation. So clones must be checked for the correct orientation of insert.

The expression vector containing the insert was then used to transform chemically competent *E.coli* cells (TOP10) and the transformed cells were plated on Luria bertani agar containing 100µg/ml Ampicillin. The plates were incubated at 37$^{o}$C for 16-18 hours or till discernable transformed colonies were obtained.

*4C.2.3. Selecting positive clones with correct orientation of insert using colony PCR*

Transformed colonies of TOP10 cells containing vector backbone with insert were then checked for the orientation of inserted gene of interest. This was done using colony PCR with two sets of primers one vector specific primers and other insert specific primers, and these two primers set were used to make two different types of PCR reaction mixtures. Briefly, colony PCR reaction mixture containing vector specific forward primer and insert specific reverse primer would amplify an insert which was in correct orientation. Whereas a reaction mixture containing both vector specific and insert specific forward primer would amplify an insert which was in reverse orientation. Thus 20 clones were picked up and each was subjected to amplification with the two different PCR reaction mixtures. Clones showing correct orientation of insert were then selected and studied further.

*4C.2.4. Plasmid extraction from selected clones and transforming BL21 DE3 competent cells for protein expression studies*

Plasmid DNA from clones with correct orientation of insert was extracted using SIGMA Gene-Elute Plasmid Mini-prep Kit. Plasmid DNA obtained from 50 ml culture of each clone was quantified and diluted samples (containing 30-40ng/ul of DNA) were then sent

for sequencing to get final confirmation of orientation of insert and in-frame His- tag. Sequence checked plasmid DNA was then used to transform BL21 DE3 cells for protein expression studies.10-20 ng DNA was used for transformation experiment and the transformed cells were plated on Luria bertani agar containing 100µg/ml Ampicillin. The plates were incubated at 37$^{o}$C for no longer than 16 hours to avoid overgrowth of transformed colonies.

*4C.2.5. Selecting positive BL21 DE3 clones and purifying recombinant AHL lactonase under optimized conditions*

Colonies of BL21 DE3 cells were picked up from the transformed ampicillin containing agar plate and checked for the presence of vector containing insert using colony PCR before actually proceeding for protein expression studies.

For induction of recombinant protein, selected clone was initially inoculated in 10 ml of Luria bertani broth containing 100µg/ml Ampicillin and incubated at 37$^{o}$C and 180rpm shaker overnight. 1% of inoculum (from the overnight culture) was then added to 100 ml of Luria bertani broth with100µg/ml Ampicillin in a 250 ml Erlenmeyer flask. The flask was incubated at 37$^{o}$C and 185rpm for 90 minutes or till culture reached an optical density of 0.6.After reaching the required OD the culture was induced with 0.15 mM of IPTG and the flask was then incubated at 15$^{o}$C and 150 rpm for a period of 6 hours. Induced cells were harvested by centrifugation at 4000 rpm for 10 minutes at 4$^{o}$C. The cell pellet obtained was suspended in ice-cold lysis buffer (0.1 M phosphate buffer pH 7.0 with 20 mM imidazole ) and was then sonicated at 40 amplitude for 3 minutes with 10 secs ON and 10 secs OFF cycle. Sonicated cell suspension was then centrifuged at maximum speed for half an hour in a pre-cooled rotor to get clear cell lysate.

The clarified cell lysate was then used to purify protein of interest. The lysate was mixed with Ni-NTA affinity purification matrix and kept on a rocker with gentle shaking for half an hour. The mixture of cell lysate and matrix was then added to a clean column and the matrix was allowed to settle under gravity. The cell lysate was then allowed to flow through the matrix and it was collected. The column was then washed with 30 ml of Wash buffer (0.1 M phosphate buffer pH 7.0 with 70 mM imidazole), to remove all the

non specific and unbound proteins. Protein of interest was then eluted using minimum volume of elution buffer (0.1 M phosphate buffer pH 7.0 with 250 mM imidazole) to get concentrated protein fractions. Eluted fractions, along with sonicated supernatant and column flow-through and wash samples were then checked on 15% SDS PAGE to detect the presence of target protein.

*4C.2.6. Checking biological activity of recombinant AHL lactonase*

Purified protein was then tested for its ability to degrade QS signal molecules using *C. violaceum* CV026 based bioassay (method previously described in Chapter 4A section 4A.2.5). Known amounts of recombinant AHL lactonase (6, 12, 24 or 36 µg of enzyme) was taken and 0.25 mM of C6 HSL and C7HSL were added to two different reaction mixtures respectively. The reaction was incubated at $50^{o}$C for 1 hour. Degradation of AHL by the purified AHL Lactonase can be evidenced by decrease in the zone of violacein synthesis by biosensor strain CV026.

**4C.3. Results and Discussion:**

AHL Lactonase from *Bacillus sp*. RM1 was cloned using a simple one step cloning strategy. Expressed enzyme was purified using affinity chromatography and its biological activity was confirmed. Detailed description and discussion of the results obtained is as follows:

*4C.3.1. Cloning and over expression of AHL Lactonase from Bacillus sp. RM1*

Gene encoding an *aiiA* homolog from *Bacillus sp*.RM1 genome was amplified via primers that were designed using *aiiA* Lactonase gene sequence from *Bacillus cereus* strain MBG25 as template. Amplicon of the expected size of 753 bp was obtained at the end of PCR optimization studies (Fig. 4C.1). *aiiA* gene of same size has also been amplified from genome of other *Bacillus sp.* as reported previously [3,5].

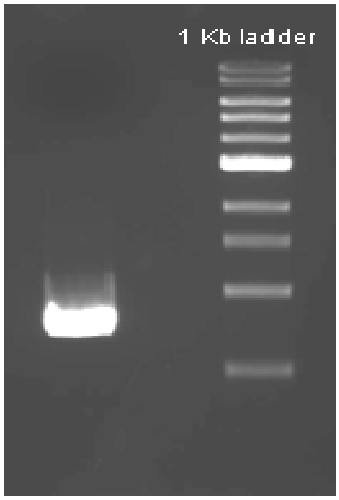



***Figure 4C.1****:1% agarose gel showing aiiA gene homolog of 753 base pairs amplified from isolate under study.*

The amplified gene of interest was then cloned into pEXP5-CT/TOPO®TA expression vector and clone with correct orientation of insert were then selected based on Colony PCR results (Fig. 4C. 2). Plasmid from selected clones showing correct orientation of insert was then extracted and sent for sequencing. Sequence confirmed clones showing presence of in-frame His-tag were used for protein expression studies. Appended below is the sequence of a selected positive clone showing in frame C-terminal His tag (highlighted in yellow):

ATCATTCTTCTGTTAATGGTACACTCGCGCCGGGGAATTTATTGAACTTACCTGAAGGGT
GTTATCTTTTGGAGACAGAAGAGGGGCCTATTTTAGTAGATACAGGTATGCCAGAAAGTG
CAGTTAATAATGAAGGGCTTTTTAACGGTACATTTGTTGAAGGACAGATTTTACCGAAAA
TGACTGAAGAAGATAGAATCGTGAATATATTAAAGCGTGTAGGGTATGAGCCGGACGACC
TTTTATATATTATTAGTTCTCACTTACATTTTGATCATGCAGGAGGAAACGGTGCTTTTA
CAAATACACCGATTATTGTGCAGCGAACGGAATATGAGGCAGCACTTCATAGAGAAGAAT
ATATGAAAGAATGTATATTACCGCATTTGAACTACAAAATTATTGAAGGGGATTATGAAG
TGGTACCAGGTGTTCAATTATTGTATACGCCAGGTCATTCTCCAGGCCATCAGTCGTTAT
TCATTGAGACGGAGCAATCCGGTTCAGTTTTATTAACAATTGATGCATCGTACACGAAAG
AGAATTTTGAAGATGAAGTGCCGTTCGCAGGATTTGATCCAGAATTAGCTTTATCTTCAA
TTAAACGTTTAAAAGGAGTTGTGGCGAAAGAGAAACCAATTGTTTTCTTTGGTCATGATA
TAAAGCAGGAAAAGGGTTGTAAAGTGTTCCCTGAATATATAAAGGGTCATCATCACCATC
ACCATTGAGTTTA

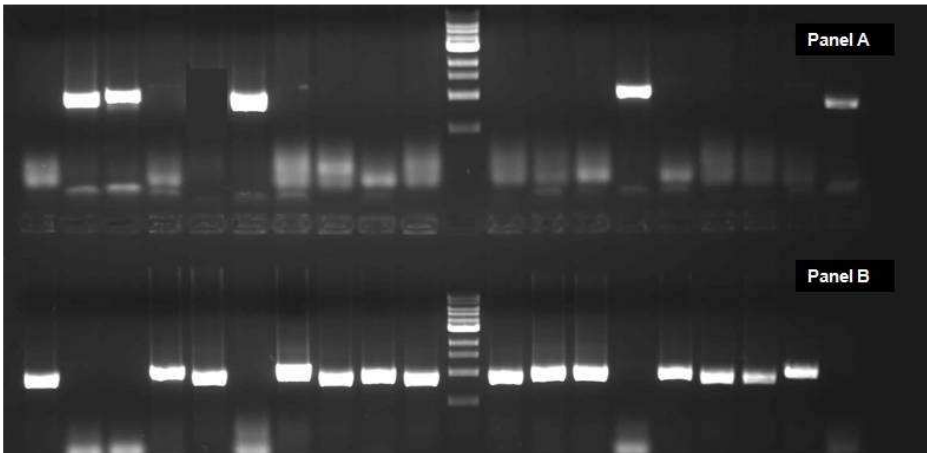



***Figure 4C.2:*** *Finding clones having insert in the correct orientation. Panel* ***A (clones with inversely oriented insert)*** *shows colony PCR results with Vector Forward and Insert Forward primers in the reaction mixture. Panel B (****clones with correct orientation of insert****) shows colony PCR results with Vector Forward and Insert Reverse primers in the reaction mixture.*

Protein was expressed using IPTG induction protocol. C-terminal His- tagged protein was then purified by Ni-NTA affinity chromatography. 15% SDS-PAGE was then used to check the molecular weight of the purified protein which was found to be around 30kDa which is a little higher than wildtype due to addition of His-Tag (Fig. 4C.3). Similar increase in molecular weight of heterologously expression AiiA lactonase has been reported by others [2, 6].

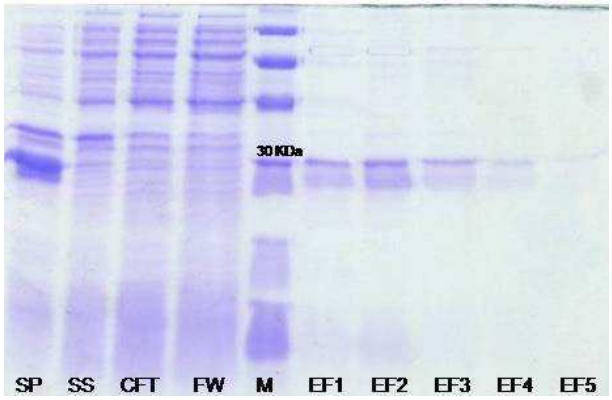



***Figure 4C.3:*** *15% SDS PAGE showing protein of interest: SP- Sonicated cell pellet; SS- Sonicated supernatant; CFT- column flow through; FW- column first wash; M- SDS PAGE marker;EF1-5 are eluted fraction 1-5 which show protein of interest of 31 KDa.*

*4C.3.2. Checking biological activity of recombinant AHL Lactonase using CV026 Bioassay*

Purified AHL Lactonase after desalting and concentration was used to check its AHL cleavage activity.24µg of AHL Lactonase was the minimum amount of protein that could easily degrade 0.25 mM of two different AHLs (C6HSL and C7HSL).This proves that heterologous expression of this protein in *E.coli* system did not in any way adversely affect its biological activity.

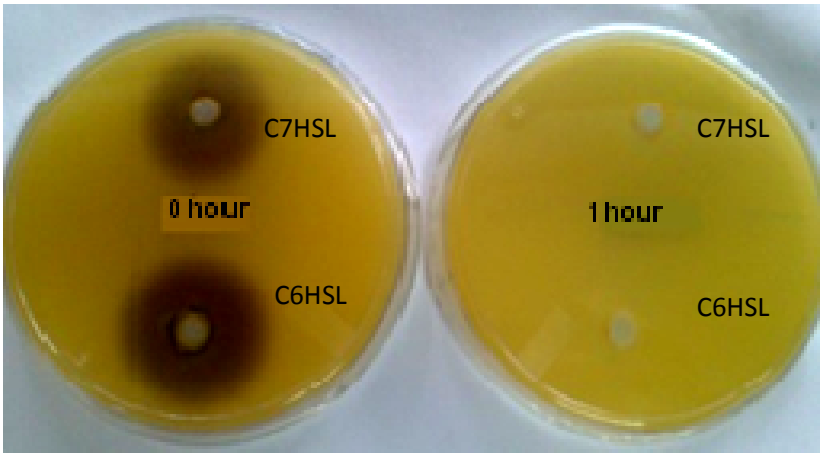



***Figure 4C.4:*** *AHL degradation activity of recombinant AHL lactonase as determined using CV026 based bioassay.*

**4C.4. Conclusion**:

In conclusion, an attempt was made to amplify and over-express AHL lactonase from *Bacillus sp.* RM1. Heterologous expression of AHL lactonase in a gram negative expression system did not alter its biological activity. The expression vector used to express the protein of interest is unique to this study and allowed one step cloning of the amplicon. The recombinant protein showed band at the expected size in 15% SDS PAGE. Over-expressed enzyme was found to degrade AHLs with great efficiency. Similar approaches can be used to heterologous express AHL lactonase from *Bacillus sp.*RM1 in *in-vivo* animal and plant models to observe attenuation of QS mediated virulence in infecting pathogenic organisms.

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# Chapter 5A

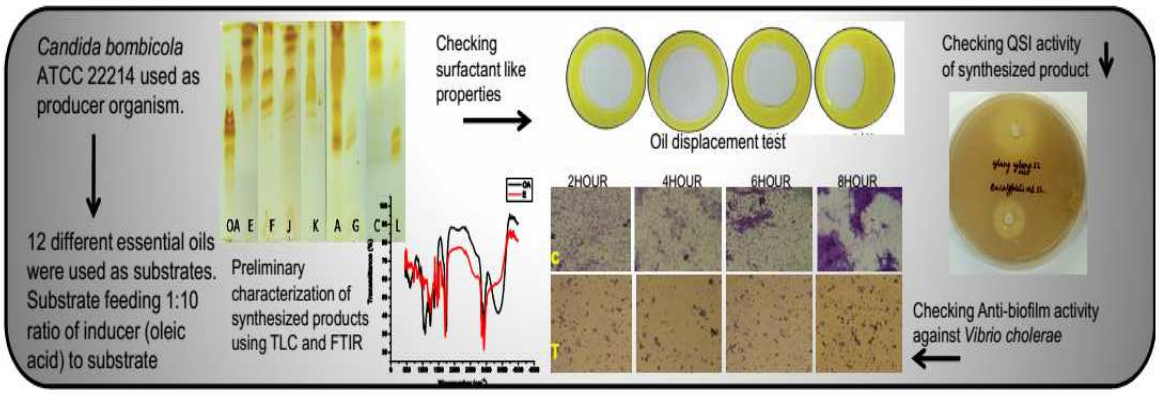
## *Novel glycolipids synthesized using plant essential oils and their application in Quorum Sensing Inhibition and as anti-biofilm and anti-fungal agents*

**This Chapter has been published as:**

**Mukherji R,** Prabhune A (2014), Novel glycolipids synthesized using plant essential oils and their application in quorum sensing inhibition and as anti-biofilm agents, **The Scientific World Journal. DOI: 10.1155/2014/890709**

**Abstract Accepted** based on this Chapter in **International Union of Microbiological Societies (IUMS 2014)** –XIV$^{th}$ International Congress of Bacteriology and Applied Microbiology.

# 5A. Novel glycolipids synthesized using plant essential oils and their application in Quorum Sensing Inhibition and as anti-biofilm and anti-fungal agents




**5A.1. Introduction:**

Essential oils (EOs) have always garnered an important place in traditional medicine and amongst alternative healing practioners. With their multitude of immunomodulatory and antimicrobial activities they have been used since many years in treatment of variety of conditions [1]. Essential oils are a mixture of numerous volatile components that are produced as a result of plant secondary metabolism. EO components can be differentiated in two different but biochemically related groups. The two main groups are compounds are molecules of terpene and terpenoid origin and secondly aromatic and aliphatic components [2].EOs are extracted from aromatic and medicinal plants using varietyof different methods, including water or steam distillation of different plant parts.

Antibacterial activity of various EOs has been studied before by different groups around the world [3-6].In the recent past Quorum sensing inhibition mediated by essential oils and its components has also been analyzed [7-12]. Quorum sensing (QS) is the form of bacterial communication that allows individual bacterial cells to come together and function as a single entity protecting them from diverse deleterious conditions [13, 14]. A

variety of genes are under the quorum sensing control regulon many of which modulate bacterial virulence including genes involved in exo-enzyme production and biofilm formation[15,16.17]. In this light, inhibition of quorum sensing has been envisioned to be the new target for developing sustainable anti-infective therapies because hindrance in this form of communication amongst bacteria weakens their might making them more susceptible to the applied mode of treatment [18].

Essential oil glycolipids have never been synthesized before. Rationale behind synthesizing such molecules was that upon conversion into their corresponding glyco-forms, the component essential oils may become more soluble in aqueous medium helping them acquire some additional property which has been unforeseen previously. This was indeed observed when some of the EOs reported in this study, did not show QSI potential on their own, but became potent QS inhibitors upon conversion into corresponding EOSLs. They are no reports that mention inhibition of quorum sensing by Tea Tree oil, Bergamot oil ,Basil oil, Ylang Ylang oil and Frankincense oil and although the individual oils have no QSI activity, after conversion into their corresponding glycolipic forms they have been shown be potent inhibitors of bacterial quorum sensing. The present chapter thus describes a method of preparation of these Essential oil glycolipids (EOSLs) which possess both QSI and bio-surfactant like property. In addition few selected synthesized EOSLs have also been shown to possess various degrees of anti-biofilm activity against *V.cholerae* biofilm and anti-fungal activity against pathogenic yeast *Candida albicans.*

**5A.2. Material and methods:**

*5A.2.1. Culture conditions and maintenance of microorganisms*

*Candida bombicola* ATCC 22214 used for EOSL synthesis was maintained on MGYP agar slants (malt extract, 0.3 g%; glucose, 2 g%; yeast extract, 0.3 g%; peptone, 0.5 g%; and agar, 2.0 g%). The microorganism was sub-cultured every 4 weeks and maintained at 4 °C in a refrigerator. *Chromobacterium violaceum* CV026 (Kindly provided by Dr. Paul Williams, University of Nottingham) was grown in Luria Bertani broth supplemented with 100 μg/ml Ampicillin and 30 μg/ml Kanamycin. Culture was preserved in the form of glycerol stock and was revived whenever required. *Vibrio cholerae* MTCC0139 used

in the anti-biofilm assays was grown in Luria Bertani broth without antibiotics and maintained in the form of glycerol stock kept at -70$^{o}$C.*Candida albicans* NCIM 3102 was grown in Sabourauds' broth aand was maintained on Sabourauds's agar slants at 4$^{o}$C.

*5A.2.2. Inoculum development and Production of EOSLs*

10ml of MGYP broth was inoculated with *Candida bombicola*. After 24 h incubation at 28 °C, 180 rpm, it was added to 90 ml MGYP broth, and was incubated further for another 48 h. After 48 hours of growth cells were harvested by centrifugation at 5000 rpm for 20 mins. The pellet was then re-dispersed in production medium of 10% glucose [21]. Substrates i.e. oleic acid (the inducer procured from SIGMA Aldrich,INDIA) and selected Essential oils (purchased from Soulflower INDIA Ltd.) in different ratios of ratios 1:20, 1:10, 1:5, 1:2 were then added to the production medium and the flask was incubated at 28$^{o}$C, 180 rpm for a period of 7 days. After the production period cells were removed by centrifugation at 5000rpm for 20 mins. The culture supernatant containing the product was extracted thrice with equal volume of ethyl acetate. The aqueous layer was then separated from the solvent layer which contained the product. Rotary evaporation of the solvent layer yielded the synthesized EOSLs. The product was collected in a clean dry glass vial and purged to remove any traces of ethyl acetate. Different EOs gave different yield of resultant product.

*5A.2.3. TLC analysis of the synthesized EOSLs*

Newly synthesized EOSLs were compared against previously synthesized OASL using thin layer chromatography (TLC). TLC was performed on commercially available silica gel coated aluminum sheets (Merck Aluminum TLC Silica Gel Plates 60 F 254). The solvent system used was 65:15:2 Chloroform: Methanol: Water (v/v/v).The bands on the gel after completion of the run were visualized using iodine vapors. Yellowish brown bands appear on a white background after incubation with iodine vapors for a period of 2 minutes. The band migration pattern obtained with OASL was compared with each of the synthesized EOSLs by correlating the retention value (Rf values) of each band.

*5A.2.4 .Oil Displacement activity of Synthesized EOSLs*

The oil displacement test is a method used to measure the diameter of the clear zone of displaced oil, which occurs after addition of a surfactant-containing solution to an oil-water interphase. The oil displacement test was done by adding 20 ml of distilled water and 3 ml of Jatropha Oil to a 90 mm Petri dish. 30 µl of 5mg/ml solution of synthesized EOSLs was dropped onto the oil- water interface. The diameter of the clear halo formed after displacement of oil was visualized under visible light and was measured within 10 s.

*5A.2.5. FTIR analysis of the EOSLs*

FTIR spectroscopic analysis of the synthesized EOSLs and Oleic acid sophorolipid (OASL) alone was performed to compare and analyze the similarities and differences in the newly formed EOSLs and original OASL. It was performed using Perkin Elmer FTIR system Spectrum BX over the spectral range of 400-4000cm.EOSL samples were prepared by making a pellet in Potassium bromide (KBr). Data from 17 consecutive scans was collected. Spectral data obtained was plotted on a graph of Transmittance (%) vs. Wave number ($cm^{-1}$).

*5A.2.6. Quorum sensing inhibition by EOSLs*

Anti-Quorum Sensing activity of essential oils alone, Oleic acid sophorolipid alone and the newly synthesized EOSLs was performed using method described elsewhere with slight modifications. Briefly 50 µl of overnight broth culture of CV026 was added to 10 ml of molten cooled Luria Bertani soft agar along with 0.25 µl (corresponds to 1.25 µM) of QS signal molecule,C6-HSL and the mixture was overlayed onto Luria Bertani Agar plates. After the overlay solidified wells of 6mm diameter were dug using a sterile corkborer.50 µl of different samples was added to the wells and the plates were incubated overnight at 28$^{o}$C.EOs and EOSLs with capacity to inhibit QS showed a colorless zone around the well in a purple mat of violacein produced by test culture CV026 [19].All experiments were carried out in triplicates for the sake of reproducibility.

*5A.2.7. Anti-biofilm activity of the synthesized EOSLs*

Biofilm formation is another phenotype governed by quorum sensing. Any compound/molecule capable of impeding this form of bacterial communication will also in-turn disrupt bacterial ability to form biofilms. To observe anti-biofilm activity of EOSLs 10ul of overnight culture of *Vibrio cholerae* was added to 2 ml of sterile Luria Bertani medium in a 30mm petri dish containing a sterile coverslip. 10 µl of 10 % EOSL solution was added to each test plate respectively and the plates were incubated at 30$^{o}$C for 2, 4, 6 and 8 hours. Control plates without EOSLs served as system for monitoring un-interrupted biofilm formation by test organism *V.cholerae*. Biofilm formed after each incubation period was visualized using crystal violet staining method. Briefly, spent medium was discarded after completion of incubation period and the cover slips were rinsed twice with Milli-Q water to remove un-adhered bacterial cells. The biofilm was then stained with 0.4% crystal violet solution for 5 mins, after which the staining solution was removed and the biofilm was gently washed twice with Milli-Q water and was allowed to air dry [20].Stained biofilm was then visualized under light microscope at 40X magnification.

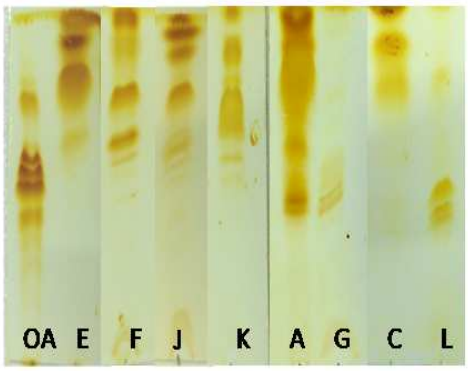
*5A.2.8. Antifungal activity of EOSLs against Candida albicans*

With the aim of producing a topical formulation with the synthesized EOSLs its anti-microbial activity against, *Candida albicans*, a well known opportunistic pathogenic yeast capable of causing severe topical infections, was carried out. Briefly, *Candida albicans* was grown in sterile 10 ml Sabourauds broth for 48 hours at 37$^{o}$C.After incubation period cells were harvested by centrifuging 2 ml culture medium. Cells were then suspended in sterile saline and optical density was adjusted to 0.1 at 600nm and saline suspension of *C. albicans* was then diluted 100 times. This was the working cell suspension for the antifungal studies with selected EOSLs. EOSL concentration of 50-500µg in 1ml of cell suspension was used and the TEST tubes were incubated at 37$^{o}$C with constant shaking.50µl of sample was withdrawn from each of the TEST tubes at every two hourly intervals and was plated on sterile Sabouraud's agar.MIC values of each EOSL against *Candida albicans* was then calculated after incubating the plates for 48 hours at 37$^{o}$C.

**5A.3. Result and Discussion:**

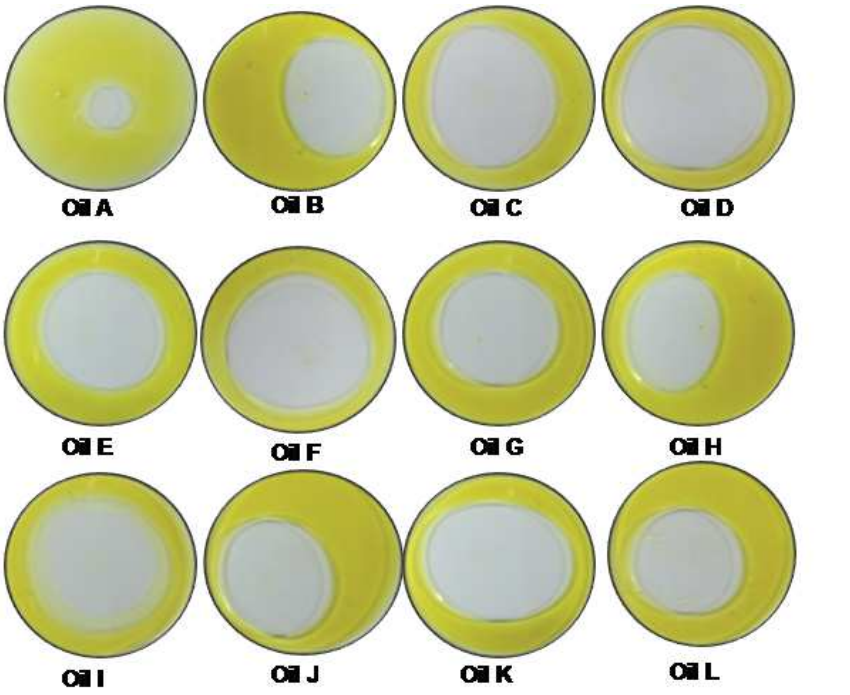
*5A.3.1. Synthesis of EOSLs using Candida bombicola ATCC 22214*

For synthesizing these EOSLs, *Candida bombicola* ATCC 22214 was used as the producer organism. *C.bombicola* ATCC 22214 mediated SL synthesis protocol has already been well established in our group [21], but use of plant EOs as substrates for glycolipid synthesis has not been reported before. Initially EOs alone were added to the production medium (10% glucose) for SL synthesis but this led to partial cell death and after the 7 day incubation period there was very little accumulation of synthesized product, which was unrecoverable. Hence Oleic acid (OA) was used as an inducer molecule so that the organism adapts better to the newly added substrate and synthesis takes place more efficiently. It is hypothesized that SL allows easy emulsification of the added essential oil and thus aids in incorporation of the EO in final product. OA was used as an inducer in varying ratios of OA: EO, starting from low concentration of OA to progressively higher, namely 1:20, 1:10, 1:5 and 1: 2.However of all the ratio of OA: EO tested, 1:10 (OA: EO) was found to work well. All the synthesized EOSLs were analyzed by TLC and they were compared with an OASL control. All, except three, synthesized EOSLs, showed bands differing from that of OASL molecule (Fig.5A.1).Also, as expected, all the EOSLs were able to displace oil at the oil-water interface to varying degrees due to their newly acquired surfactant like properties (Fig. 5A.2).




**OA : Oleic acid sophorolipid; A: Lemongrass oil SL; C: Cinnamon oil SL; E: Basil oil SL; F: Bergamot oil SL; G: Eucalyptus oil; J: Tea Tree oil SL; K: Ylang Ylang oil SL; L: Frankincence oil SL.**

***Figure 5A.1****: TLC analysis of synthesized EOSLs*




**A: Lemongrass oil SL; B:Peppermint oil SL; C:Cinnamon oil SL; D:Rosemary oil SL ;E: Basil oil SL ; F: Bergamot oil SL G: Eucalyptus oil SL ;H: Orange oil SL; I: Citronella oil SL; J: Tea Tree oil SL; K: Ylang Ylang oil SL; L: Frankincense oil SL.**

***Figure 5A.2****: Oil Displacement activity of the synthesized EOSLs*

*5A.3.2. Quorum sensing inhibitory potential of EO alone and their glycolipid derivatives*

Investigation into quorum sensing inhibition by essential oils obtained from different sources has been taken up in various laboratories and anti-QS property of essential oils is coming to light. In our report anti-QS potential of 12 oils has been studied and the data has been presented in Table1. Majority of oils used in this study, when tested alone, showed marginalQSI potential mostly because of their reduced solubility in the growth medium. Keeping this in mind the assay protocol was modified to include an emulsifying agent that could increase the solubility of oils in the growth medium thus making them more effective than before. Oleic acid SL at a final concentration of 10mg/ml (no inherent QSI activity of OASL at this concentration) helped to emulsify the oils completely before addition to the agar wells in the test plate. In case of Lemongrass oil+ OASL, approximately 50% increase in zone of inhibition was observed and other oils that

showed no QSI activity before became active when added in combination with OASL namely, Basil oil+OASL showed an 11% increase in zone size,YlangYalng oil + OASL 12% increase in activity and Peppermint Oil +OASL 16% increase in QSI activity(Table 5A.1).These observations led to the musing that, if the EOs could be converted into their corresponding EOSLs they might acquire indigenous QS inhibition property.

***Table 5A.1****: Tabulated data showing quorum sensing inhibitory (QSI) activity of Essential oils used in this study, alone and in combination with 10mg/ml of OASL. Also QSI activity of all synthesized EOSLs.*

| Plant essential oil (common name) | Plant essential oil (scientific name) | Major component | Anti QSI activity of essential oil alone (20% oil)against CV026 (inhibition zone size in mm) | Anti QSI activity of EO + OASL against CV026 (inhibition zone size in mm) [a] | Anti QSI activity of EOSL against CV026 (inhibition zone size in mm) [b] |
|---|---|---|---|---|---|
| Lemongrass Oil | *Cymbopogon citratus* | Citral | 15 | 28 | 25 |
| Peppermint Oil | *Mentha piperita* | Menthol | - | 16 | 13 |
| Cinnamon Oil | *Cinnamomum verum* | Cinnamaldehyde | 15 (10% EO) | 20 (10% EO + OASL at 10mg/ml) | Growth inhibition at 10mg/ml EOSL. |
| Rosemary Oil | *Rosmarinus officinalis* | (+) Alpha Pinene | - | - | 13 |
| Basil Oil | *Ocimum basilicum* | L-Linalool | - | 11 | 30 |
| Bergamot Oil | *Citrus bergamia* | L-Linalool | - | - | 17 |
| Eucalyptus Oil | *Eucalyptus Sp.* | 1,8 - Cineole | - | - | 23 |
| Orange Oil | *Citrus sinensis* | Limonene | - | - | 13 |
| Citronella Oil | *Cymbopogon nardus* | Citronellal | - | - | 12 |
| Tea Tree Oil | *Melaleuca alternifolia* | Alpha terpineol | - | - | 26 |
| Ylang Ylang Oil | *Cananga odorata* | L-Linalool | - | 12 | 33 |
| Frankincense Oil | *Boswellia carteri* | (+) Alpha Pinene | - | - | 17 |

[a] 1 ml reaction mixture contained 20% EO and OASL at a concentration of 10mg/ml. Also OASL alone used at a concentration of 10 mg/ml had no QSI activity.

[b] EOSL concentration used 20 mg/ml.

Interestingly it was observed that oils that were not showing any QSI activity before, either alone or in combination with OASL, became potent inhibitors of quorum sensing mediated phenotypes upon transformation into their corresponding EOSLs (Table 5A.1).Orange Oil SL, Citronella Oil SL and Rosemary Oil SL, showed smaller zones of quorum sensing inhibition when compared to other EOSLs (Table 5A.1) however the individual oils showed no QSI activity. Also it was intriguing to note that, all three EOSLs mentioned before with lesser QSI activity (Orange Oil SL, Citronella Oil SL and Rosemary Oil SL) were found to be very similar in composition to OASL. YlangYlang oil whose QSI potential has never been explored was used in this study for the first time and its EOSL has been shown to a very powerful inhibitor of QS mediated phenotype. Ylang-ylang oil is extracted from fresh flowers of the tree of the same name, by water or steam distillation. It has many therapeutic properties like antidepressant, antiseptic, hypotensive and EOSL of this oil with potent anti-QS activity will definitely have a broader range of medical application.Also other EOs used in this study whose QSI potential is being explored for the first time include: Bergamot oil, Frankincense oil, Basil oil and Tea Tree oil. All these oils when used alone showed no QSI activity however their EOSLs could very well inhibit QS mediated phenotypes. The order of QSI potential of EOSLs of the above mentioned EOs is as follows Basil oil > Tea tree oil >Frankincense oil = Bergamot oil (Table 5A.1).

*5A.3.3. Preliminary characterization of EOSLs using FTIR*

FTIR analysis demonstrated that synthesized EOSLs show certain peaks similar to OASL however, certain new peaks could be seen in the spectra of the synthesized EOSLs. Aromatic ring structure C=C stretch around 1520-1515 $cm^{-1}$, which may be arising, from certain aromatic components of each essential oils was observed in the spectra for Basil oil SL, Tea Tree Oil SL, Bergamot Oil SL, Eucalyptus oil SL and Frankincense oil SL(Fig. 5A.3a). Peaks around 1760- 1670 $cm^{-1}$arising from C=O of aldehydic, ketonic ester or carboxylic residues was seen in the spectra of Lemongrass oil SL, Cinnamon oil SL , Basil , Bergamot and YlangYlang oil SL. Vibrational peaks specific to methylene groups (2850 and 2925 $cm^{-1}$) and aromatic C-H bond(700- 750 $cm^{-1}$)were seen in the spectra of all oils however the former peaks (specific to methylene groups) were also visible in OASL spectra(Fig. 5A.3a). C-C bond conjugated with benzene ring stretch

(1600 –1625 $cm^{-1}$)was observed in the spectra of Basil oil SL, Eucalyptus oil SL and YlangYlang oil SL.

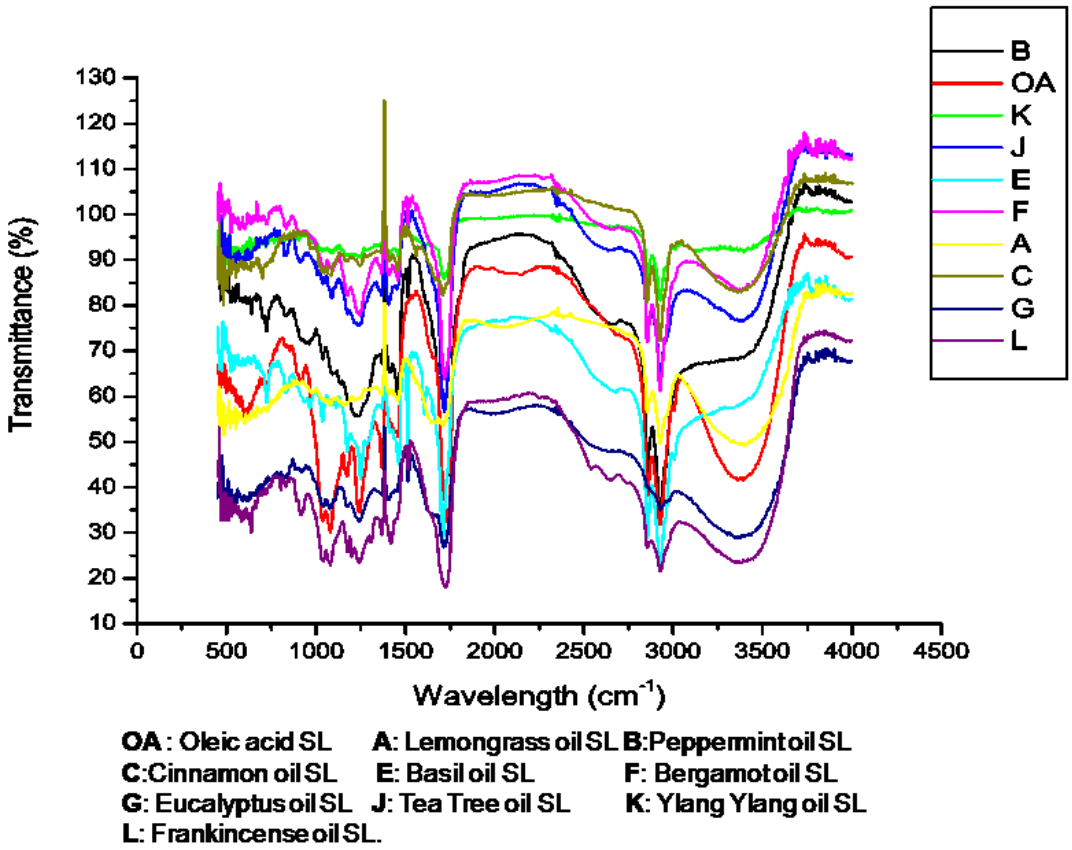



***Figure 5A.3a:*** *FTIR analysis of the synthesized EOSLs along with OASL*

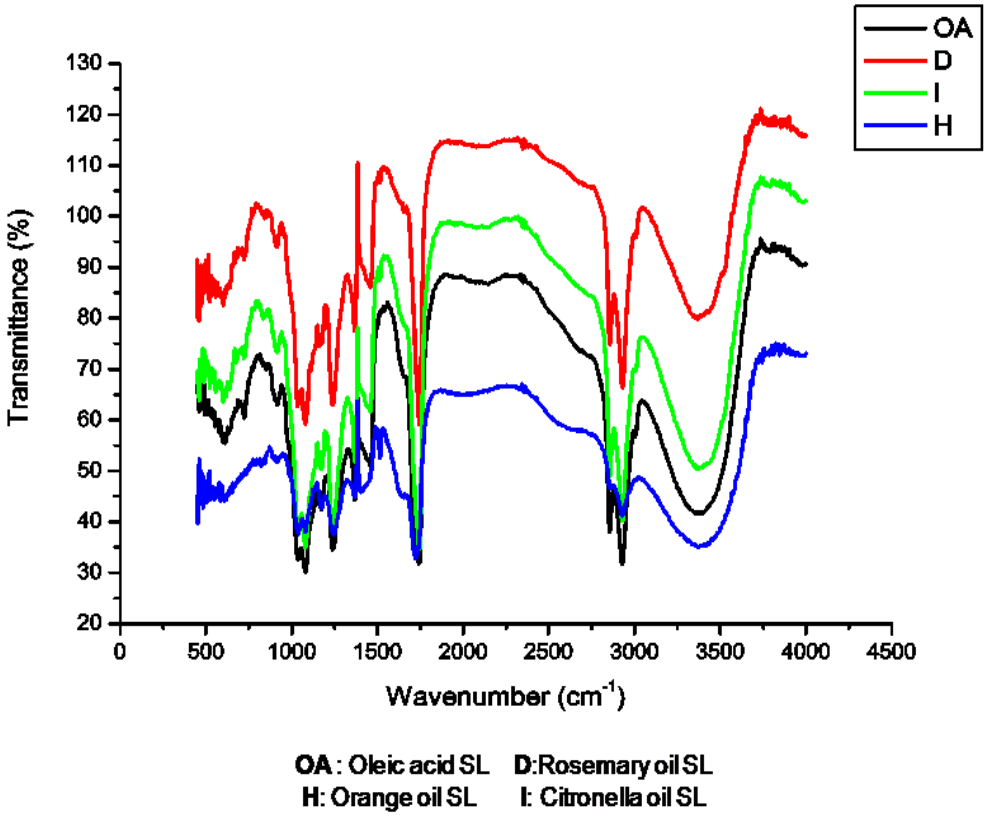


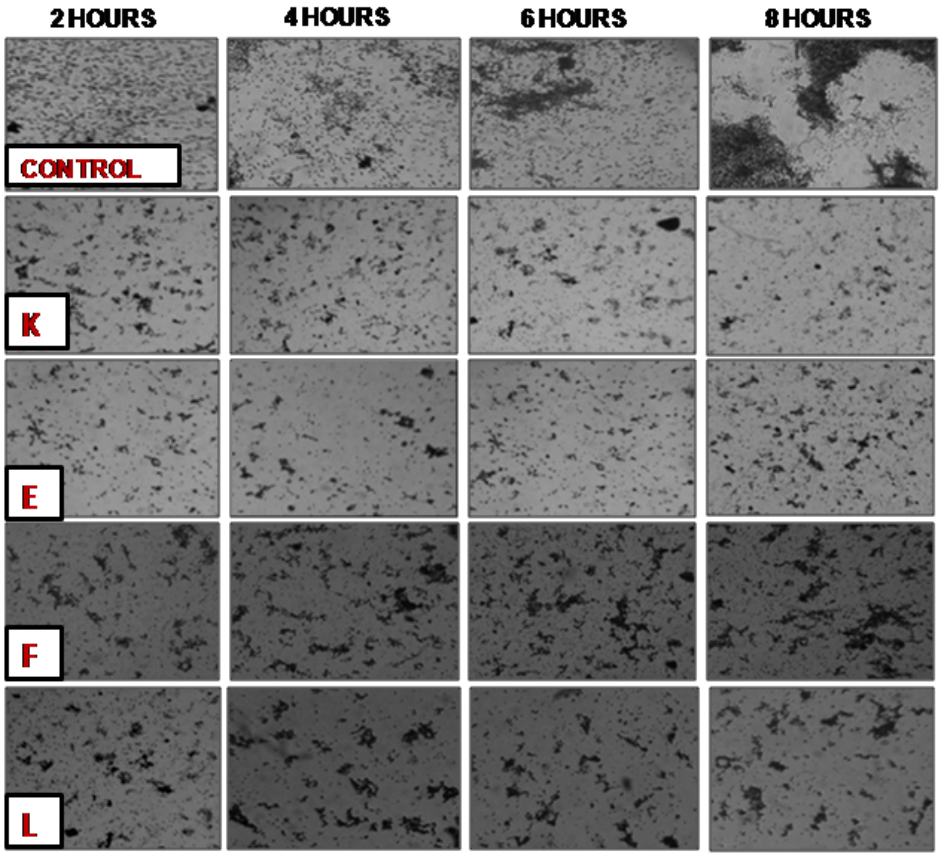

***Figure 5A.3b:*** *FTIR spectra of EOSLs with very high degree of similarity to OASL.*

Citronella, Rosemary and Orange oil SLs showed spectra very similar to that of OASL, and likewise their QSI activity was also not very pronounced (Fig. 5A.3b).

*5A.3.4. Anti-biofilm activity of EOSLs against Vibrio cholerae*

Essential oils of Lavender, Tea tree and Lemon balm have been shown to have anti-biofilm activity against *Staphylococcus aureus* and *Escherichia coli* as test organism [22]. Also Stephano *et al.* (2008) [23] have shown that essential oils from two *Bowellia sp.*(Frankincense oil) have the ability to inhibit biofilm formed by two species of *Staphylococcus* and *Candida albicans*. Peppermint oil has also been shown to inhibit biofilm formed by *C.albicans* [24].Adukwu et al. [25] have shown anti-biofilm activity of Lemongrass EO and grapefruit EO against five strains of *Staphylococcus aureus.* In our study anti biofilm activity of not EOs but selected EOSLs have been analyzed as they are expected to have improved ability to inhibit initial adhesion of microorganisms to solid surface due to their biosurfactant like property in addition to their antimicrobial and quorum sensing inhibitory property. Selected EOSLs were able to inhibit adhesion of

microorganism to the glass surface and arrest biofilm formation in the initial stages itself (Fig. 5A.4).QSI potential of these EOSLs has been established before, and that, may be responsible for the obvious decrease in bacterial biofilm, because genes required for exopylysacchride (EPS) production (EPS is the essential component for establishment of biofilm architecture and maturation [10]) are under QS control. YlangYlang oil SL and Basil oil SL both showed potent anti-biofilm activity as observed microscopically. Moreover test organism used in our study was *V.cholerae* which has added significance from an Indian subcontinent perspective because cholera is an endemic problem and biofilm formed by *V.cholerae* is an important part of its pathogenesis and disease establishment.




**CONTROL: *Vibrio cholerae* growing in the absence of EOSLs.**
**K: Ylang Ylang oil SL**
**E : Basil oil SL**
**F: Bergamot oil SL**
**L : Frankincense oil SL**

***Figure 5A.4**: Anti-biofilm activity of selected EOSLs against Vibrio cholerae*

*5A.3.5. Anti-fungal activity of EOSLs against Candida albicans*

*Candida albicans* is known to cause debilitating infections in immune-compromised patients like oral candidiasis, oesophageal candidiasis and vaginal candidiasis. Incidences of drug resistant *Candida albicans* infection have come to the fore hence alternative means of treating these infections are the need of the hour. Essential oils have been shown to possess powerful anti-fungal activity especially against *Candida albicans*. Hence it is with the aim of developing a potent anti-*Candida* formulation fit for topical application that these EOSLs were investigated further. Minimum inhibitory concentration required to successfully inhibit visible growth of *C.albicans* was found to be very low for the sleeted EOSLs and the values have been listed in Table 5A.2.Thus EOSLs with their remarkable anti-fungal and surfactant like property can be easily used to formulate highly effective hand wash or feminine wash.

***Table 5A.2****: Anti-Candida albicans activity of selected EOSLs with their corresponding minimum inhibitory concentration*

| Sample name | MIC value |
|---|---|
| Tea Tree oil SL | 400µg/ml |
| Bergamot oil SL | 250µg/ml |
| Ylang Ylang oil SL | 200 µg/ml |
| Basil oil SL | 200 µg/ml |
| Peppermint oil SL | 500 µg/ml |

**5A.4. Conclusion:**

This article highlights the advantage of conversion of EOs to EOSLs because this conversion reaction bestows additional chemical and physical properties to the component EOs making them more soluble, better quorum sensing inhibitors and powerful anti-biofilm and anti-fungal agents. Moreover the chapter reports use of some EOs whose ability to inhibit quorum sensing has not been explored before namely, Ylang Ylang oil, Frankincense oil, Basil oil, Bergamot oil ,Tea tree oil, and successful production of their EOSLs with enhanced QSI activity. Detailed characterization of all the synthesized EOSLs was undertaken but proved to be highly cumbersome due to the enormous number of components that are a part of each EO.Further due to enhanced quorum sensing inhibitory, anti-fungal and biosurfactant like property, warrants the use of these EOSLs in topical formulation like hand washes or feminine washes which would aim to prevent spread of various fungal and bacterial infections.

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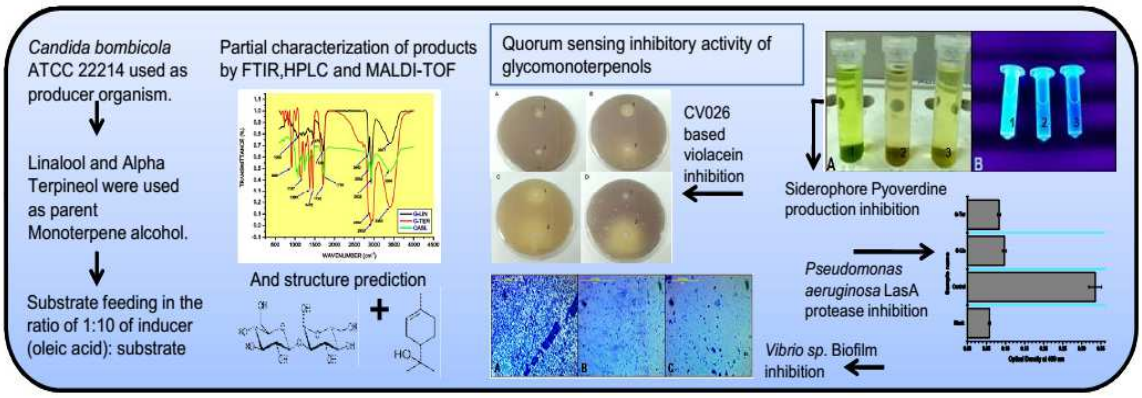
# Chapter 5B

*A New class of bacterial quorum sensing inhibitors: Glycomonoterpenols synthesized using Linalool and Alpha terpineol as substrates*

**This Chapter has been communicated as:**

**Mukherji R** , Prabhune A (2014), A new class of bacterial quorum sensing antagonists: Glycomonoterpenols synthesized using linalool and alpha terpineol. (Communicated Manuscript)

# 5*B*. A New class of bacterial quorum sensing inhibitors: Glycomonoterpenols




**5B.1. Introduction:**

Over the last two decades, the notion that individual bacterial cells operate as autonomous units has been outmoded with the insight that social interactions are common throughout the prokaryotic world. This kind of intercellular communication allows bacteria to come together and function as a single entity not unlike a multi-cellular organism. The term Quorum Sensing was introduced in scientific literature in 1994 by Fuqua *et al.* [1].Today the term quorum sensing is used irreplaceably to describe the phenomenon of bacterial communication [2]. It is a population density dependent phenomenon which provides bacteria capable of QS, with a definite competitive advantage in harsh and otherwise deleterious conditions. The phenomenon of quorum sensing occurs in both gram negative and gram positive bacterium. It is mediated by small molecular weight easily diffusible signal molecules often known as autoinducers [3].Acyl homoserine lactones (AHLs) are the most well characterized class of signal molecules (autoinducers) in case of gram negative bacteria. A range of phenotypes are regulated through QS based regulon including: pigment formation, exo-polysaccharide secretion and biofilm formation, antibiotic production, exo-enzyme production etc. [4]. Gram negative pathogens of much clinical importance, for example *Pseudomonas aeruginosa*, *Vibrio sp.* often use QS to modulate their virulence gene expression [5].

Consequently the possibility of inhibiting the numerous virulent phenotypes of the said pathogens, simple by impeding QS, seems like a very appealing scenario for development of next generation of antimicrobials. Additionally with the ever increasing surge of drug resistant organism newer drug targets with significantly decreased propensity of development of resistance are the need of the hour and quorum sensing fits this prerequisite perfectly.

Quorum sensing can be quenched either via enzymatic degradation of signal molecules or via the aid of small molecule inhibitors [6]. To date many such small molecular weight natural and synthetic quorum sensing inhibitors have come to the fore [7-9].In this article we report use of two Monoterpene alcohols namely, linalool and alpha terpineol, derived Glycomonoterpenols as potent quorum sensing inhibitory compounds. Monoterpene alcohols are popular volatile components distributed widely in aromatic plants and are responsible for different flavors and fragrances. Alpha terpineol, a volatile monoterpenoid alcohol, is a major component of the essential oil of many plants and has been reported to enhance the permeability of skin to lipid soluble compounds [10]. It has also been shown to inhibit proliferation of human erythroleukaemic cells. Alpha terpineol has also been described to have anti-inflammatory, antibacterial and antifungal activities [10].Linalool is also a monoterpene alcohol and is a major component of essential oil of many aromatic plants. It is commonly used as an additive in food processing and beverage industry [11].Additionally it has also found widespread application in cosmetics toiletries and household detergents because it is not a skin irritant [12]. Linalool has been described to have antibacterial, antiviral, anti-inflammatory, analgesic and anti-diabetic activity [11].Reports on bacterial quorum sensing inhibitory activity of Linalool and alpha terpineol do not exist to date. However, we have shown that, conversion of these molecules into their glycomonoterpenol derivatives bestows tremendous QSI potential. In the present chapter quorum sensing inhibition by this new class of molecules has been substantiated using three different gram negative systems capable of producing easily detectable QS regulated phenotypes.

**5B.2. Materials and Methods:**

*5B.2.1.Microorganisms used and their culture conditions*

*Candida bombicola* ATCC 22214 used for glycomonoterpenol synthesis was grown on MGYP medium and maintained at 4 °C in a refrigerator. *Chromobacterium violaceum CV026* (A kind gift from Dr. Paul Williams, University of Nottingham) a mini tn5 mutant of wild type *Chromobacterium violaceum* was used as a biosensor strain in the quorum sensing inhibition assays. The culture was grown in Luria bertani medium supplemented with 100µg/ml ampicillin and 30µg/ml Kanamycin at 30$^{o}$C.*Pseudomonas aeruginosa* NCIM 5029 was routinely cultured in Luria Bertani medium without antibiotics. For pyoverdine production and inhibition assay *P.aeruginosa* was grown in a chemically defined medium at 35$^{o}$C.*Vibrio cholerae* MTCC 0139 was used as a test organism in the anti-biofilm assay and was grown and maintained in Luria bertani broth and agar medium respectively.

*5B.2.2. Production of Monoterpene alcohol derived Glycomonoterpenols*

10ml of MGYP broth was initially inoculated with *Candida bombicola* ATCC 22214. After 24 hours of incubation at 28 °C and 180 rpm, the culture was added to 90 ml MGYP broth, and was incubated further for another 48 hours. After 48 hours of growth cells were harvested by centrifugation at 5000 rpm for 20 mins. The pellet was then re-dispersed in production medium of 10% glucose. Substrates i.e. selected Monoterpene alcohols (MTAs), Linalool and Alpha-terpineol (purchased from Sigma-Aldrich, India Ltd.) and oleic acid (as an inducer), were then added to the production medium respectively in the ratio of 10:1 and the flasks were incubated at 28$^{o}$C and180 rpm for a period of 7 days [13]. After the production period cells were removed from the production medium by centrifugation at 5000rpm for 20 mins. The culture supernatant from individual flasks containing the product was extracted thrice with equal volume of ethyl acetate. The solvent layer was rotary evaporated to obtain the product which was then collected in a clean dry glass vial and purged to remove any traces of solvent. Synthesized products were then characterized using different analytical techniques.

*5B.2.3. Characterization of synthesized product*

5B.2.3.1. FTIR analysis of the synthesized Glycomonoterpenols

Fourier transform infra-red (FTIR) spectroscopic analysis of the synthesized products, Glycomonoterpenol derived from Linalool (G-LIN) and Glycomonoterpenol derived from Alpha terpineol (G-TER), along with Oleic acid sophorolipid (OASL) were performed separately to compare and analyze the similarities and differences in the newly formed glycomonoterpenols and OASL. FTIR was carried out using Bruker FTIR Spectrophotometer Tensor 27 over the spectral range of 400-4000cm. Samples were prepared by making a pellet in Potassium bromide (KBr). Data from 100 consecutive scans was collected. Spectral data obtained was plotted on a graph of Transmittance (%) vs. Wave number ($cm^{-1}$).

5B.2.3.2. HPLC analysis of synthesized product

Pure Linalool and Alpha terpineol and the Glycomonoterpenols derived from them were analyzed on Hitachi Chromeline HPLC system using Thermo Scientific C18 reverse phase HPLC column 250 X 4.6 mm. The mobile phase used was mixture of Acetonitrile and Deionized water (with 0.012% TFA) in the ratio of 55:45. The flow rate was maintained at 1.0 ml per minute, and column temperature was maintained at 40 °C. The parent Monoterpene alcohols and the synthesized Glycomonoterpenols (G-LIN and G-TER) were detected using UV-detector system at 210 nm. Concentration of all samples used for analysis was 1mg/ml and the sample was prepared in methanol.

5B.2.3.3. MALDI-TOF MS analysis

1mg of each sample (Parent monoterpene alcohols, G-LIN and G-TER) was dissolved in 1ml ACN respectively. 4µl of sample thus prepared was mixed with 8 µl of CHCA matrix (10mg/ml) and spotted on the MALDI plate. MALDI-MS study was done on AB SCIEX TOF/TOF 5800.

*5B.2.4. Chromobacterium violaceum CV026 based Violacein inhibition assay*

QSI potential of two Monoterpene alcohols and Oleic acid sophorolipid alone and in combination with each other along with the newly synthesized glycomonoterpenols was

performed using method described elsewhere with slight modifications. Briefly 50 µl of overnight broth culture of *CV026* was added to 10 ml of molten cooled Luria Bertani soft agar along with 1.25 µM of QS signal molecule, C6-HSL and the mixture was overlayed onto Luria Bertani Agar plates. After the overlay solidified wells of 6mm diameter were dug using a sterile corkborer.50 µl of each test sample (Listed in Table 1) was added to respective wells and the plates were incubated overnight at 30$^{o}$C in upright position [14].Samples with capacity to inhibit QS showed a colorless zone around the wells in a purple mat of violacein produced by test culture *CV026* (Table1). All experiments were carried out in triplicates.

*5B.2.5. Anti-biofilm activity using Vibrio cholerae and Pseudomonas aeruginosa as test organism*

Biofilm formation is another phenotype governed by quorum sensing which helps in establishment and persistence of disease causing entities. Any compound/molecule capable of impeding this form of bacterial communication will also in-turn disrupt bacterial ability to form biofilms. To observe anti-biofilm activity the MTA derived Glycomonoterpenols 10ul of overnight culture of *Vibrio cholerae* and *Pseudomonas aeruginosa* grown in Luria bertani medium was added to 2 ml of sterile Luria Bertani medium in a 30mm petri dish containing a sterile coverslip. 0.5 -3.0 mg of G-LIN and G-TER was added to test plates respectively and the plates were incubated at 35$^{o}$C for 8 hours. Control plates without test compound served as a system for monitoring un-interrupted biofilm formation by the test organism. Biofilm formed after the incubation period was visualized using crystal violet staining method. In brief, spent medium was discarded after completion of incubation period and the cover slips were gently rinsed twice with Milli-Q water to remove un-adhered bacterial cells. The biofilm was then stained with 0.1% crystal violet solution for 10 mins, after which the staining solution was removed and the biofilm was gently washed twice with Milli-Q water and was allowed to air dry. Stained biofilm was then visualized under light microscope at 400X magnification [15].

*5B.2.6. Inhibition of Pyoverdine production by P.aeruginosa*

*Pseudomonas aeruginosa* is a well-known for possessing an arsenal of virulence factors. One such factor is Pyoverdine which is often produced in combination with pyocyanin (the water soluble blue pigment of *Pseudomonas*). This is also a water soluble yellowish green strongly fluorescent pigment which functions as a siderophore and its production is maintained under QS control. Any molecule that disrupts quorum sensing will have the capacity to stop this pigment production.10 ml of King's medium B was used which enhanced pyoverdine production. TEST tubes containing a range of concentration (from 0.5-3mg) of MTA derived Glycomonoterpenols and the 10ul of overnight broth culture of *P.aeruginosa* grown in Luria Bertani medium were incubated at 35$^{o}$C for 24 hours. The CONTROL tube was devoid of any QS inhibitory agent and hence exhibited good production of pyoverdine. Amount of pyoverdine produced in the samples was measured by recording the optical density of cell free supernatant at 405nm.

*5B.2.7. Inhibition of Pseudomonas aeruginosa LasA protease activity*

Another one of important virulence factor produced by opportunistic pathogen *P. aeruginosa* is Protease enzyme production. This enzyme assists in invasive infections of *Pseudomonas* as it helps degrade infected host tissues. Its production is under tight control of Las I/R quorum sensing regulatory circuit and any molecule that disrupts this system inhibits LasA protease production by *P.aeruginosa*. The assay used to detect proteolyitc activity of *P.aeruginosa* was performed based on the method reported previously, albeit with slight modification. Briefly, 150 µl of culture supernatent of *P.aeruginosa*, grown in 10ml Luria Bertani medium with or without QSI compound at 35$^{o}$C for 24 hours, was added to 500 µl of 0.6% azocasein (Sigma Aldrich,India) in 0.05 M TrisHC1 and 0.5 mM $CaCl_2$ (pH 7.5), and incubated at 37°C for 30 mins. The reaction was stopped by the addition of trichloroacetic acid (l5%, 500 µl) followed by incubation at 4$^{o}$C for 10 minutes and then centrifugation at 10,000 rpm for 10 minutes. Absorbance of supernatant free of any particulate matter was measured at 400 nm in a UV–visible spectrophotometer. Control tubes devoid of any QSI compound were set-up, which allowed uninhibited production of *P.aeruginosa* LasA Protease, and the proteolytic activity on azocasein was tested using the protocol described above. Samples containing significant proteolyitc activity will release the azo-dye conjugated with casein and thus

will impart a residual orangish yellow color to the supernatant after the said reaction period; however samples with no ability to degrade protein will have colorless supernatant. Appropriate controls were used wherever required to eliminate false positives.

**5B.3. Results and Discussion:**

Being the phenomenon driving the virulence gene expression machinery of variety of pathogens, quorum sensing has received a lot of attention as a very viable drug target. A halogenated furanone isolated from marine macroalgae *Delisea pulchra* was the first identified small molecule inhibitor of quorum sensing mediated phenotypes [16].Since then a plethora of quorum sensing inhibitory compounds have been isolated from a variety of sources, including fungi, plants, animals, bacteria, dietary phytochemicals, medicinal plants etc. [17-20].Linalool and Alpha terpineol are phytochemicals specifically belonging to a class of molecules called monoterpene alcohol and they from a major component of many plant essential oils like Basil, Lavender, and Juniper and Tea Tree oil. Antimicrobial activity of both theses monoterpene alcohol has been studied before [21].Anti-biofilm potential of linalool especially in case of *Candida albicans* has been described previously [22]. However activity of these two molecules in perspective of inhibition of bacterial quorum sensing has not been explored previously. These MTAs on their own do not possess any quorum sensing inhibitory activity, however conversion of these MTAs into their respective glycomonoterpenol derivatives offers significant addition to their existing properties and makes them powerful quorum sensing inhibitors.

*5B.3.1.Initial stuies on QSI potential of parent MTAs and Synthesis of Glycomonoterpenols using Linalool and Alpha terpineol as parent molecules*

Initial studies on Quorum sensing inhibitory potential of linalool and alpha-terpineol respectively were performed using *CV026* based bioassay. *Chromobacterium violaceum* mutant strain *CV026* is a biosensor strain incapable of synthesizing AHL molecules of its own but can respond to exogenously supplied AHLs via production of purple pigment violacein. Anti-QS bioassay performed using *CV026* allows easy visualization of QSI potential of the test compound, by studying the colorless halo in the mat of purple pigment. During this study it was understood that, on their own , the two MTAs do not

posses any promise as a QS inhibitor (Table 5B.1). However mixing of both linalool and alpha terpineol with oleic acid sophorolipid (OASL) respectively (OASL has no QSI potential of its own even at 100mg/ml) showed a marginal increase in their solubility and ability to inhibit QS mediated violacein production (Table 5B.1). These very intriguing observations led us to believe that if these MTAs could somehow be derivatized or transformed into a more amphiphilic form it would help enhance their QSI activity. Indeed conversion of linalool and alpha terpineol into their glycomonoterpenols, (G-LIN and G-TER) conferred tremendous ability to inhibit various QS mediated phenotypes (Table 5B.1 and Fig.5B.1) in addition to bestowing increased solubility and surfactant like property. G-LIN and G-TER were synthesized using *Candida bombicola* ATCC 22214 based production protocol and the products (Glycomonoterpenols) were harvested after a 7 day incubation period respectively.

***Table 5B.1*** *Determination of anti-quorum sensing activity of MTAs only, MTA+ OASL and synthesized Glycomonoterpenols using Chromobacterium violaceum CV026 based bioassay system*

| Sample Name ⟶ | Linalool only (10 mg/ml) | Alpha terpineol only (10mg/ml) | 10 mg/ml Linalool +10mg /ml OASL | 10 mg/ml Alpha terpineol + 10mg/ml OASL | 10 mg/ml solution of G-LIN | 10 mg/ml solution of G-TER |
|---|---|---|---|---|---|---|
| Diameter of zone of inhibition of violacein synthesis (in mm) | 0 | 12 | 11 | 0 | 35 | 37 |

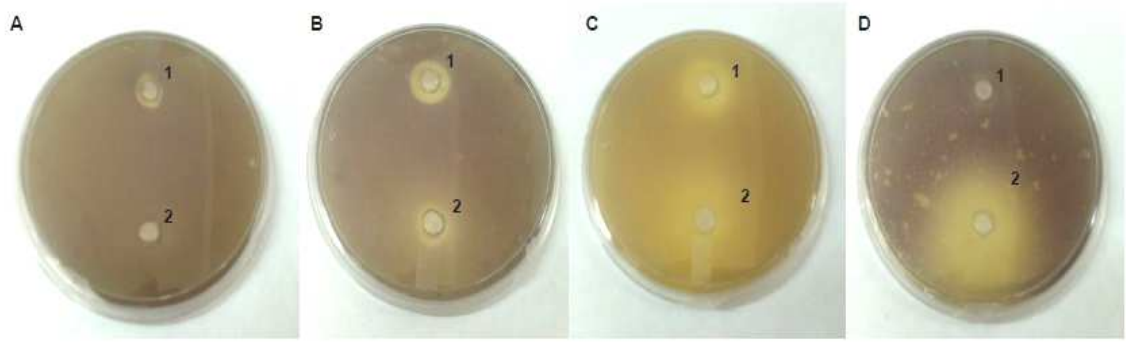



***Figure 5B.1****: Quorum sensing inhibitory potential of synthesized Glycomonoterpenols determined using CV026 based bioassay*
*Panel A: well 1 (Linalool only); well 2 (Alpha terpineol only)*
*Panel B: well 1 (Linalool +OASL) well 2 (Alpha terpineol + OASL).*
*Panel C: well 1 (Linalool +OASL) well 2 (G-LIN)*
*Panel D: well 1 (Alpha terpineol + OASL) well 2 (G-TER)*

### *5B.3.2. Partial Characterization of synthesized Glycomonoterpenols G-LIN and G-TER using FTIR, HPLC and MALDI-TOF MS*

The synthesized product was initially characterized using FTIR. Further characterization of synthesized product was done using HPLC and MALDI-TOF MS.Analysis of the FTIR spectrum of both G-LIN and G-TER revealed clear evidences of incorporation of parent MTAs into the final product (Fig. 5B.2). Methyl groups which are a part of the original MTA molecule could also be seen in the Glycomonoterpenols derived from them (1380 $cm^{-1}$). Presence of methylene groups could be substantiated by peak at 1470 $cm^{-1}$ in both G-LIN and G-TER spectra. Also a broad stretch in the 3200-3400 $cm^{-1}$ region corresponding to the O–H stretch frequency in the glucose moiety can be observed in all three spectra. The asymmetrical and symmetrical stretch modes of methylene ($CH_2$) groups of glucose and sophorose occur at 2932 and 2852 cm−1, respectively and this was observed in both G-LIN and G-TER spectrum (Fig. 5B.2). A sharp peak at 905 $cm^{-1}$ in the G-TER spectra was indicative of aromatic benzene nucleus of alpha terpineol structure. C-O stretching from primary alcohols was observed in the G-LIN spectra (1060 $cm^{-1}$). Moreover, peak corresponding to C=O stretch of esters/lactones (1745 $cm^{-1}$) could be seen in case of OASL and presence of C=O stretch of saturated aliphatic cyclic 6

membered ring of glucose (1720 $cm^{-1}$) was evident in the spectrum of G-LIN and G-TER (Fig. 5B.2).

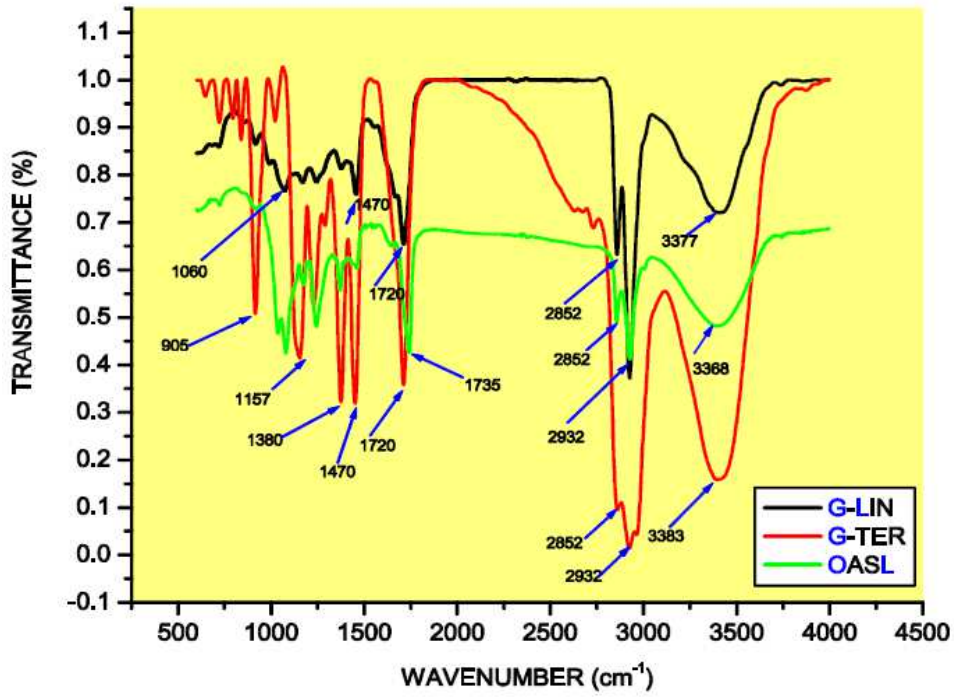



***Figure 5B.2 :*** *Fourier transform Infrared Spectroscopic analysis of the synthesized Glycomonoterpenols. Specific signatures in the spectra have been indicated with arrows*

HPLC analysis was done for both pure MTAs and MTA derived glycomonoterpenols. In the spectra of both G-LIN and G-TER appearance of new peaks in latter part of the spectrum indicated existence of newly synthesized product. Additionally a small peak corresponding to the original unconverted MTA could also be seen in the G-LIN and G-TER spectra. (Fig. 5B.3).

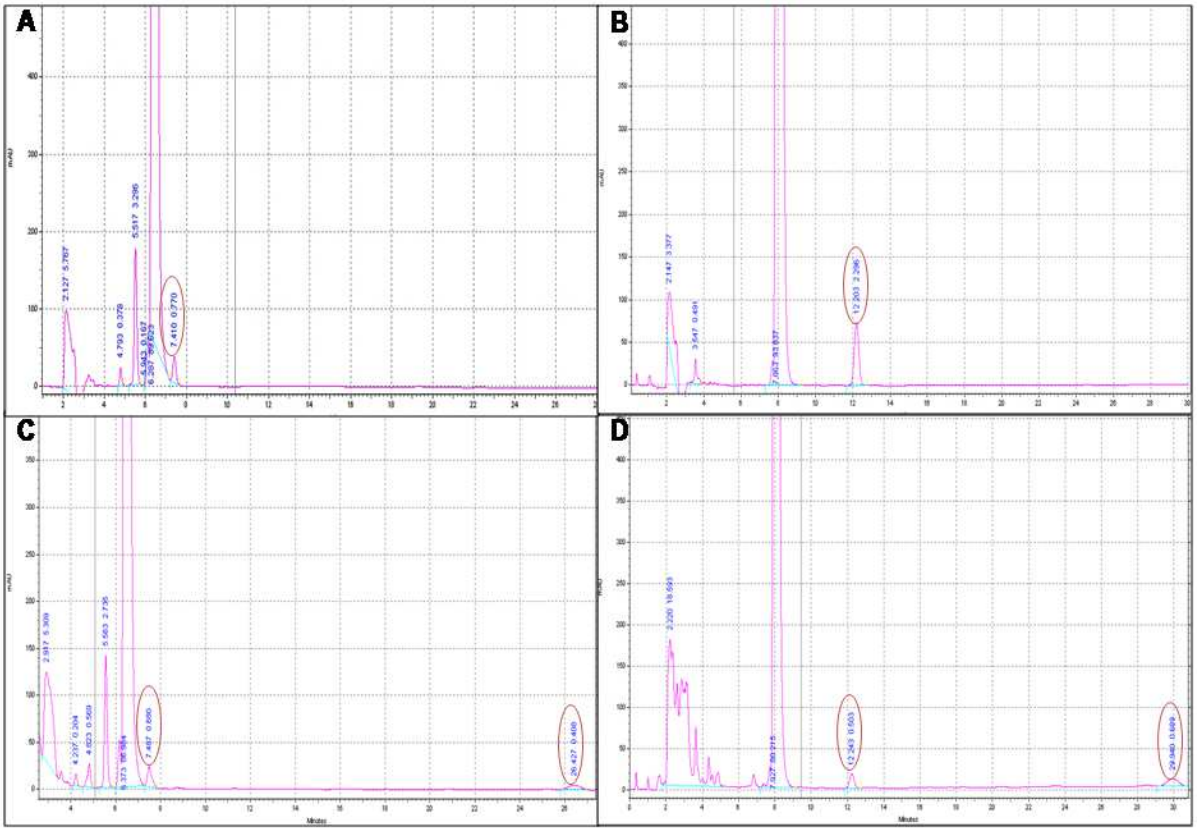



***Figure 5B.3:*** *HPLC analysis of parent MTAs and synthesized Glycomonoterpenols at 210 nm using C18 column. Panel A: Alpha terpineol only; B: Linalool only ; C: G-TER and D: G –LIN*

Furthermore synthesized product was also analyzed by MALDI TOF MS analysis (Fig.5B.4). Theoretically conjugation of a Sophorose head group (molecular weight 342.2) to the original MTA molecule (both Linalool and Alpha terpineol having same molecular weight of 154.24) was predicted to yield a product of molecular weight 494.5.This was validated experimentally when a peak corresponding to *m/z* of 496.1 $[M + 2H]^+$ was observed in the MALDI TOF MS spectra of the synthesized product. Mono-acetylation of this product and further adduct formation was estimated to give a product peak of higher *m/z* value. Peak corresponding to *m/z* value of 578.5 was observed in the spectra of both G-LIN and G-TER respectively (Fig. 5B.4). This *m/z* corresponds to a single potassium ion adduct of the mono-acetylated sophorose head containing product $[M+ K+ 2H+Acetyl]^+$ (Table 5B.2). Moreover with addition of one single glucose head group to the existing MTA molecule would theoretically yield a molecule of mass of

approximately 332, and interestingly adducts corresponding to this molecular mass could be identified in the spectra of G-TER but not G-LIN. Peak with *m/z* value of 437.5 correlates to sodium and potassium adduct of the mono-acetylated single glucose head group product [M+ H+ Na+ K+ Acetyl] $^{+}$ (Fig. 5B.4).Molecular formula of synthesized products were predicted based on this analysis and they have been listed in Table 5B.2.

***Table 5B.2:*** *Predicted structure and molecular formula of synthesized products:*

| m/z | Form | Derivative | Formulae | Calculated Mass [M] |
|---|---|---|---|---|
| 496.1 [M+2H]+ | Acidic Product | Sophorose head group | C22H38O12 | 494.55 |
| 578.5 [M+K+2H]+ | Acidic Product | Sophorose Head group +mono acetylation | C24H40O13 | 536.59 |
| 437.5 [M+K+Na+H]+ | Acidic Product | Glucose head group + mono acetylation | C18H30O8 | 374.29 |
| 178.2 [M+Na+H]+ | Parent molecule | No change | C10H18O | 154.24 |

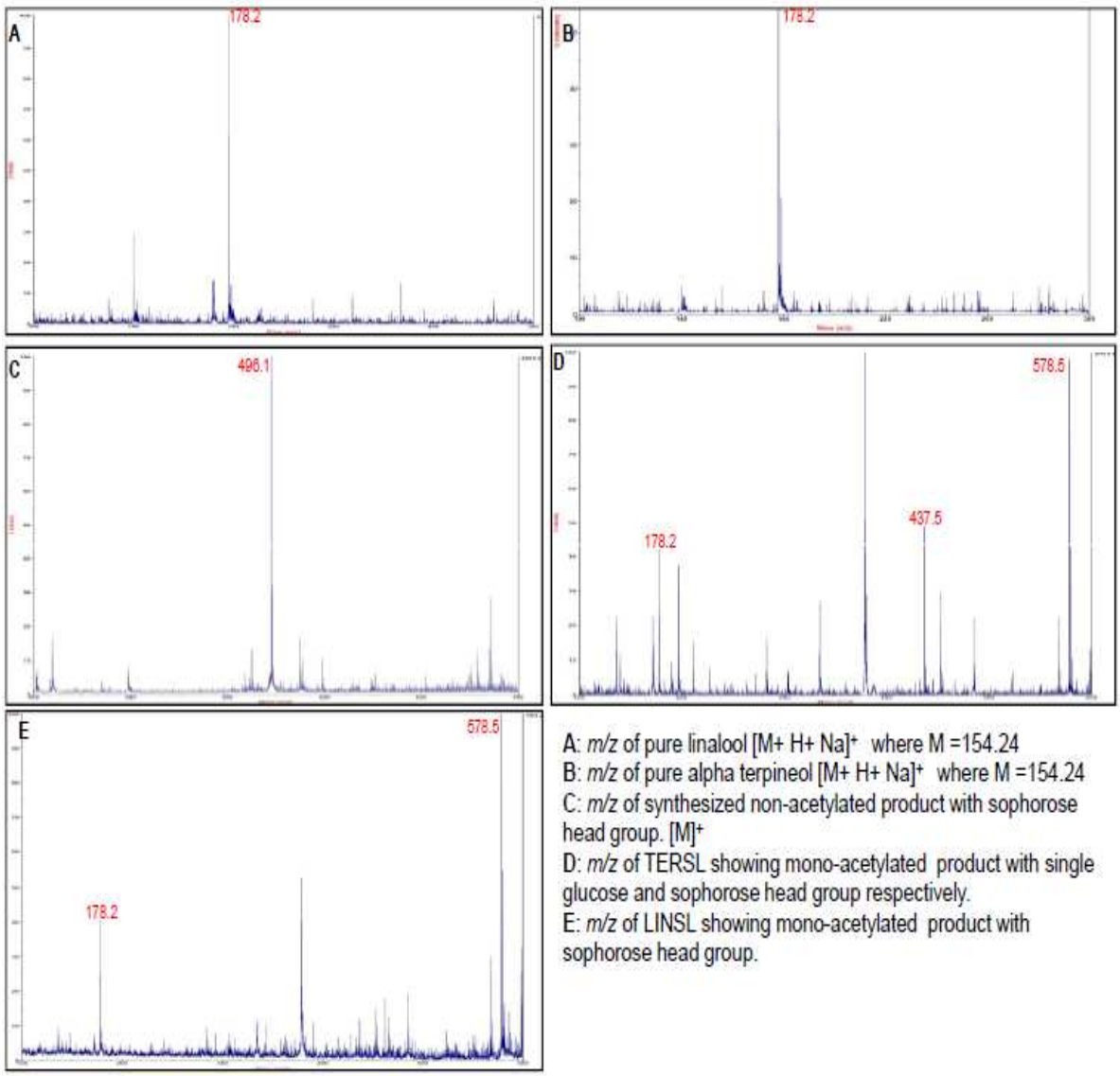



***Figure 5B.4:*** *MALDI-TOF MS analysis of the parent monoterpene alcohols and synthesized Glycomonoterpenols. Samples were analyzed using CHCA matrix and matrix peak can be seen in the spectra of panel D and E. Panel A: Linalool only; B: Alpha terpineol only; C and E: G-LIN and Panel D: G-TER*

*5B.3.3.FurtheriInvestigation into quorum sensing inhibitory potential of G-LIN and G-TER*

For further assessment of the quorum sensing inhibitory potential of these newly synthesized Glycomonoterpenols two other well established gram negative systems capable of elaborating easily detectable quorum sensing dependent phenotypes were chosen.

*Vibrio cholerae* is the causative agent of cholera a disease that is endemic to South East Asia and Africa. Biofilm formation is an essential part of *V.cholerae* pathogenesis and disease establishment [23].Biofilm formation is another phenotype that is modulated by quorum sensing. , because genes required for exopylysacchride (EPS) production (EPS is the essential component for establishment of biofilm architecture and maturation) are under QS control. Any molecule capable of disrupting the biofilm forming potential of *V.cholerae* has remarkable potential to serve as a drug candidate against cholera. Both G-LIN and G-TER respectively could disrupt biofilm formed by *V.cholerae.* Of all different the concentrations tested 2.5 mg and 2.0 mg of G-LIN and G-TER respectively could successfully inhibit biofilm formation by *V.cholerae* (Fig. 5B.5)

*Pseudomonas aeruginosa* is another one of the dreaded gram negative pathogens capable of causing a list of infirmities and most of its virulence genes are under QS control [24]. Pyoverdine, the yellowish green colored fluorescent siderophore pigment produced by *Pseudomonas aeruginosa* is an important part of its pathogenesis, and its production is regulated by QS [25]. This siderophore allows the bacterium to thrive in otherwise iron limiting conditions within the host body, and it competes with lactoferrin and transferrin for the available iron in its immediate environment. Pyoverdine has also been found to be important in biofilm development by the bacterium [25]. A number of reports exist wherein both synthetic and naturally occurring compounds have been used with the aim to disrupt QS mediated virulence factor production and biofilm formation by *P.aeruginosa* [26-29]. Hence with this in mind anti-biofilm activity and Pyoverdine synthesis inhibition of G-LIN and G-TER were also tested. It was observed that 0.5mg and 0.75 mg of G-LIN and G-TER respectively, could inhibit up to 90% of Pyoverdine production by the test organism, without causing any apparent growth inhibition (Fig. 5B.6). Anti-biofilm activity of G-LIN and G-TER against *P.aeruginosa* was also found to

be very impressive, with 1mg of each compound inhibiting 85-90% (approximation) of biofilm formed (Fig. 5B.7). Lastly proteolyitc activity of *P.aeruginosa* is an important part of its virulence repertoire and is used by the pathogen to destroy host tissue [28]. The synthesized Glycomonoterpenols, G-LIN and G-TER, could inhibit LasA protease production up to 72% and 75% respectively (Fig. 5B.8). Thus, significant inhibition of *Pseudomonas* pyoverdine production, biofilm formation and LasA protease production by G-LIN and G-TER, exemplifies their QSI activity and elaborates the possibility of developing these Glycomonoterpenols as molecules for treatment of *Pseudomonas aeruginosa* infection.

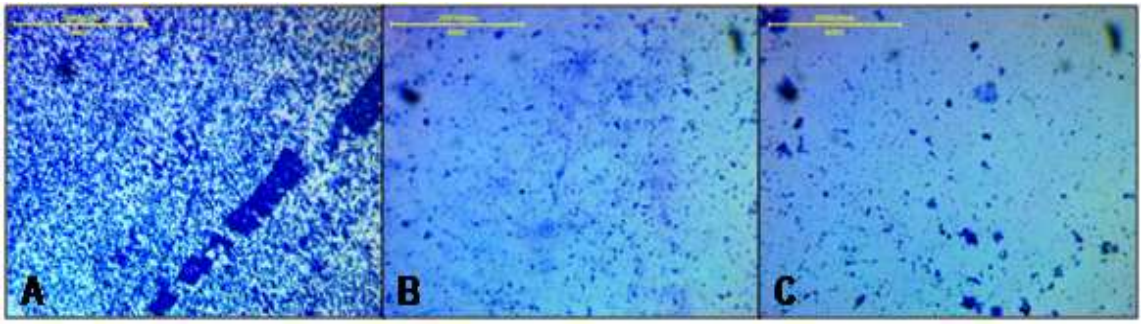



***Figure 5B.5****: Anti-biofilm activity of Glycomonoterpenols against Vibrio cholerae after 8 hours of incubation.* ***Panel A****: Control without any test compound.* ***Panel B****: G-LIN and* ***Panel C****: G-TER*

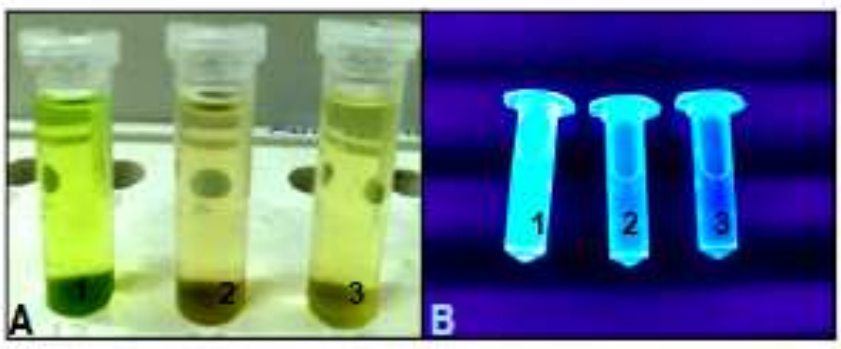



***Figure 5B.6:*** *Pyoverdine inhibition by Glycomonoterpenols. Tube 1,2,3 correspond to Control tube without glycomonoterpenol, G-LIN and G-TER treated culture respectively in both panel A and B. Panel A: visual detection of decrease in pyoverdine production. Panel B: Decrease in pyoverdine production detected under UV light, decreased florescence in test tubes 2 and 3 compared to control tube 1 is apparent.*

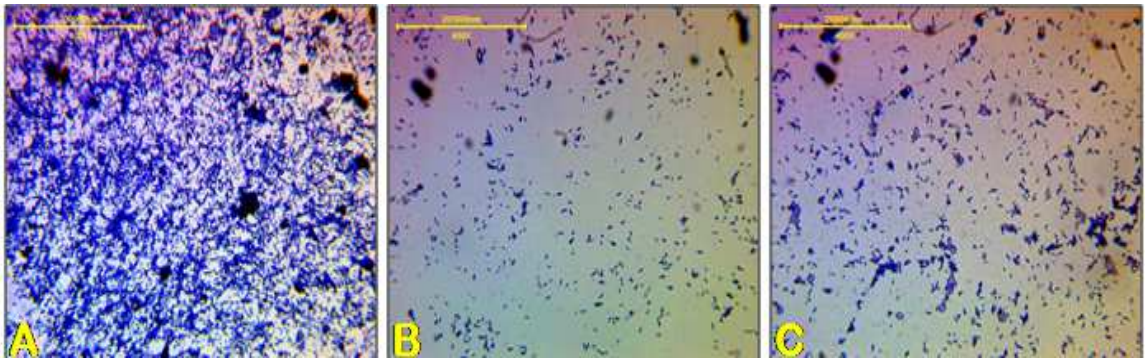



***Figure 5B.7:*** *Anti-biofilm activity of Glycomonoterpenols against P. aeruginosa after 8 hours of incubation. Panel A: Control without any test compound. Panel B: G-LIN and Panel C: G-TER*

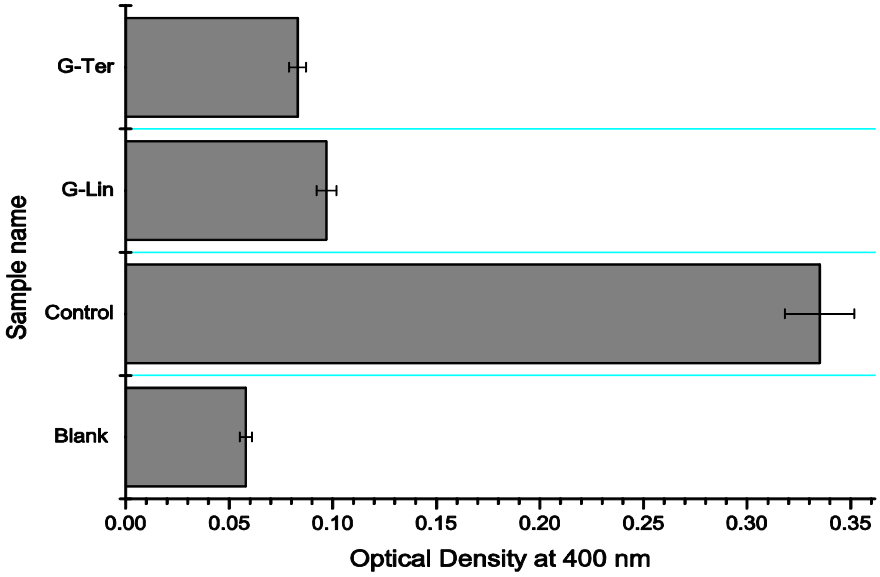



***Figure 5B.8:*** *Inhibition of Pseudomonas aeruginosa LasA Protease activity on azo-casein by G-LIN and G-TER.*

## 5B.4. Conclusion:

In conclusion, this chapter highlights synthesis and partial characterization of a new class of compound that shows great promise as a quorum sensing inhibitor. Glycolipid synthesis using monoterpene alcohol has never been attempted previously and such aprocess is being reported here for the first time. Conversion into their glyco-derivatives (named-Glycomonoterpenols) has made the parent MTAs (Linalool and Alpha terpineol) more soluble and enhanced their biological activity or more specifically their ability to inhibit quorum sensing mediated phenotypes. The synthesized products G-LIN and G-TER, have been partially characterized using, HPLC, FTIR and MALDI-TOF MS. Estimation of their QSI potential was initially made using CV026 based bioassay system, where significant inhibition of violacein production could be mediated by the synthesized Glycomonoterpenols. Further remarkable inhibition of QS mediated phenotypes in two other important gram negative pathogens, *Vibrio cholerae* and *Pseudomonas aeruginosa* was also demonstrated using G-LIN and G-TER respectively. Elucidation of the exact chemical structure and mechanism of formation of these Glycomonoterpenols are topics that need further probing and efforts in this direction are underway.

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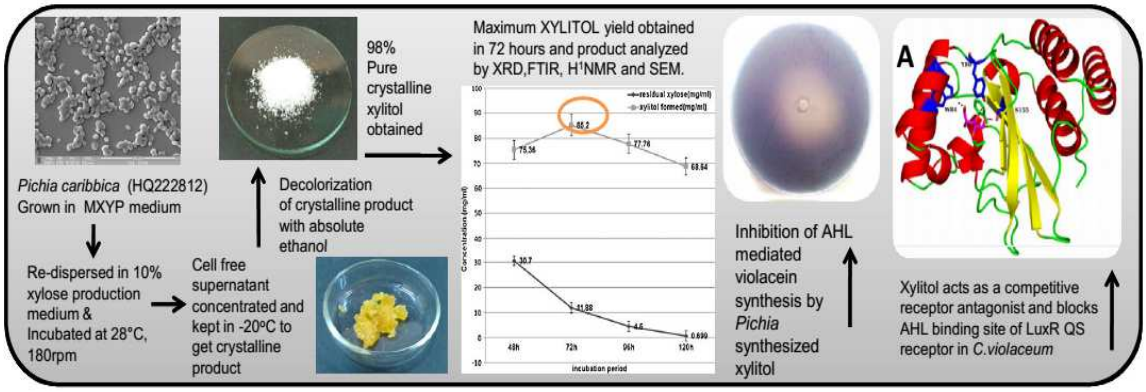
# Chapter 6

## *Pichia caribbica synthesized xylitol as an inhibitor of AHL mediated gram negative quorum sensing.*

**This Chapter has been published as:**

**Mukherji R**, Joshi-Navare K, Prabhune A (2013),Crystalline Xylitol Production by a Novel Yeast, *Pichia caribbica* (HQ222812), and Its Application for Quorum Sensing Inhibition in Gram-Negative Marker Strain *Chromobacterium violaceum* CV026, ***Appl. Biochem. Biotechnol.***
**DOI: 10.1007/s12010-012-0039-4**

# 6. *Pichia caribbica* synthesized xylitol as an inhibitor of AHL mediated gram negative quorum sensing




**6.1. Introduction:**

Xylitol is a five-carbon sugar alcohol belonging to the polyol group which has been widely used as an ideal sweetener for diabetic patients because of its insulin-independent metabolism and low glycemic index. The value of xylitol market reached $340 million with the global consumption 43,000 tons in 2005 [1]. The global market demand for polyols is expecting annual 2.7 % increase. The advantageous physical and chemical properties of xylitol make it a highly sought after compound for pharmaceutical, odontological, and food industries. A variety of interesting applications have been reported for xylitol especially in the field of odontology as an important component of toothpastes, mouthwashes, and sugar-free chewing gums [2]. Due to the anti-biofilm activity and negative heat of solution associated with it, xylitol forms an important part of odontologial formulations. It has been shown to prevent tooth decay and cause tooth re-hardening and remineralisation [3].There are several pharmaceutical applications of xylitol. It finds use in post-traumatic or post-operative conditions when efficient glucose utilization is inhibited, due to the induced resistance to insulin by excessively secreted stress hormones. Catabolic disorders can also be corrected due to the anabolic effects produced by xylitol. Xylitol, when used regularly in diet, limits obesity. It is used for

parenteral nutrition in infusion therapy as it is inert to amino acids. It is also used in treatments for lipid metabolism disorders [4].

In nature, xylitol is abundantly found in fruits and vegetables such as berries, corn husks, oats, lettuces, cauliflowers, and mushrooms, but its extraction from them is easier said than done, owing to the fact that the resultant yield is very low [5]. Xylitol is industrially produced by chemical hydrogenation of pure synthetic xylose or from the xylan fraction obtained after hemicellulose degradation at high temperature and under high pressure of about 50 atm using raney nickel as catalyst [6]. However, the limited product yield of about 50–60 % associated with this process, and the expensive downstream processing steps involved in product extraction and purification makes it an unsuitable method of choice [7]. Hence, alternative methods involving fermentation by microorganisms and also enzymatic conversion of D-xylose to xylitol operating at ambient conditions are currently being explored.

Fermentative conversion of lignocellulosic biomass into xylitol has gained immense popularity, despite it being a time-consuming process involving optimization of several variables [8]. Yeasts have been touted to be the best xylitol producers from hemicellulosic or other xylan-rich biomass among all the microorganisms investigated. Yeast of genera *Candida*, *Debaryomyces*, and *Pichia* have been reported to give good xylitol yield from their fermentation medium [9]. Microbially, xylitol is produced in a one-step process by reduction of xylose using the enzyme xylose reductase in the presence of NADPH. Enzymatic conversion of xylose to xylitol using this enzyme xylose reductase has been proposed to be a far superior process in terms of productivity for large-scale production of xylitol [10]. Nevertheless, microbial conversion of renewable lignocellulosic biomass or xylose-containing synthetic media into xylitol is the most preferred method of choice, more so because the fermentation process can be easily controlled. In this chapter the production of xylitol with 98-99 % purity from a simple D-xylose-containing artificial medium by a previously unreported organism *Pichia caribbica* with simple cost friendly recovery process has been described. The *Pichia* synthesized xylitol has also been characterized in detail with different analytical techniques namely X-ray diffraction (XRD), nuclear magnetic resonance (NMR),

Fourier-transform infrared (FTIR), high-performance liquid chromatography (HPLC), and scanning electron microscopy (SEM).

Apart from the odontological activity reported so far, xylitol is also known to possess antimicrobial activity. It averts ear and upper respiratory tract infections caused by *Streptococcus pneumoniae*, which is responsible for 30 % or more of such attacks.It also suppresses infections by *Haemophilus influenzae*, another important pathogen implicated in these kinds of infirmities.A notable anti-biofilm effect of xylitol has been previously recognized against *Streptococcus mutans*, the organism associated with dental decay. The polysaccharide capsule of *S. mutans* plays major role in tooth enamel adherence and subsequent formation of biofilm in the form of dental plaque. The capsule synthesis occurs through the utilization of dietary sugars like sucrose. D-Xylitol cannot be utilized by *S. mutans*, and thus, capsule synthesis and further consequences are avoided [5]. Most reports published to date talk about anti-pathogenic effect of xylitol against gram-positive organisms; however, none of them mention about xylitol acting as a quorum sensing inhibitor in gram-negative systems. In this chapter, anti-AHL (anti-acyl homoserine lactone) activity of xylitol has been reported using *Chromobacterium violaceum* CV026 as the test organism since it serves as a representative gram-negative system capable of responding to many natural and synthetic AHL molecules. Xylitol produced by *P. caribbica* reported here could inhibit quorum sensing by three distinct molecules namely C6-HSL, C8-HSL, and 3-oxo-C6-HSL, all of which serve as important signalling molecules in virulence gene regulation of various pathogenic gram-negative bacteria.

## 6.2. Materials and Methods:

### *6.2.1. Reagents and Media*

All the media ingredients used were of analytical grade supplied by either Hi-media pvt. Ltd., India, or Merck India Ltd. Acyl homoserine lactones were purchased from Cayman chemicals, USA. Standard xylitol was purchased from Sigma Aldrich,INDIA.

### *6.2.2. Culture Condition/Maintenance of Microbe*

The culture *P. caribbica* (accession number HQ222812) [11] was maintained on MXYP agarslants (malt extract, 0.3 g%; xylose, 2 g%; yeast extract, 0.3 g%; peptone, 0.5 g%;

and agar, 2.0 g%) slants. The microorganism was sub-cultured in every 4 weeks and maintained at 4 °C in a refrigerator. *C. violaceum* CV026 was grown in Luria Bertani Medium supplemented with100µg/ml Ampicillin and 30µg/ml Kanamycin. Culture was preserved in the form of glycerol stock and was revived whenever required.

*6.2.3. Inoculum Development for P. caribbica*

Ten milliliters of MXYP broth (0.3 g% malt extract, 0.3 g% yeast extract, 0.6 g% mycological peptone, 2 g% D-xylose) was inoculated with *Pichia* strain BY2 (*P. caribbica*). After 24 h incubation at 28 °C, 180 rpm, it was added to 90 ml MXYP broth, and incubation was continued further for 48 h.

*6.2.4. Fermentation Procedure for Conversion of D-Xylose to Xylitol*

The cells were harvested from MXYP broth after 48 h by centrifugation at 5,000 rpm, 20 min, 10 °C. These cells (wet weight 4.0 g dry weight approximately 1.0 g) were then redispersed in100ml of 10 % xylose solution. And the incubation was continued further till 120 h, with periodic monitoring of xylitol and residual xylose from fermentation broth using HPLC system of Chromeline-Hitachi. The column used was Waters Sugar Pak 6.5× 300 mm. The mobile phase used was Milli-Q water with 100µM EDTA and 200µM $CaCl_2$. The flow rate was maintained at

0.4 ml per minute, and column temperature was maintained at 70 °C. The sugar and sugar alcohol were detected with the help of Chromeline L-2490 refractive index detector [1].

*6.2.5. Product Harvesting*

After 72 h of incubation, the cells were separated by centrifugation at 5,000 rpm, 20 min, 10 °C, and the yellow-colored supernatant was processed further to separate the synthesized xylitol. The cells obtained after fermentation can be reused for up to three times without substantial loss of efficiency.

6.2.5.1.Concentration of Product

The supernatant broth was subjected to rotary vacuum evaporation at 85 °C to get a viscous fluid which was further concentrated by Centrivac (LABCONCO Centrivap

concentrator) system. Further crystallization was induced by storing the product in refrigerator at−20 °C.

6.2.5.2. De-colorization of Product

De-colorization was done using absolute ethanol. Five milliliters of absolute ethanol was added to 2 g of semi-crystalline yellow-colored material. Over the period of 24 h, the material was washed thrice with absolute ethanol and after overnight contact; powder was again washed with the solvent. The intense yellow color got eluted from the crystals into the solvent (ethanol) layer. The solvent was discarded, and residual solvent was allowed to evaporate at room temperature to obtain complete dry white crystalline powder.

*6.2.6. Characterization of Xylitol*

Complete characterization of the synthesized product was done with the help of XRD, $^{1}$H NMR, FTIR, HPLC, and SEM. Purity of the compound was specifically determined by HPLC and $^{1}$H NMR.

6.2.6.1. X-ray Diffraction Analysis

To further confirm the identity of crystal, XRD (Philips X'Pert PRO) was done. For XRD analysis, the crystalline sample was crushed to a very fine powder in a mortar and pestle. It was then filled in a 1 cm by 1 cm in size and 1 mm-Deep Square etched on a glass slide. The glass slide was carefully placed in an empty Petri dish and taken to the X-ray diffractometer, and its spectra were recorded. The diffraction pattern obtained was then matched against the standard JCPDS-PDF database to confirm that the crystals were that of xylitol.

6.2.6.2. $^{1}$H NMR Analysis

Two milligrams of samples namely *Pichia* synthesized xylitol and SIGMA xylitol were dissolved in 0.5 mL of deuterated methanol. 1H NMR (200 MHz) spectra was recorded by Bruker AC200 at 25 °C. Chemical shift was expressed in parts per million. TMS was used as an internal standard.

6.2.6.3. FTIR Analysis

After separating the white crystalline compound from the spent broth, it was subjected to preliminary identification by FTIR. The crystals were crushed with KBr, pelleted, and the FTIR spectra were recorded on a Perkin-Elmer Spectrum One in the frequency range of 4,000 to 500 $cm^{-1}$.Additionally, FTIR spectra of pure xylitol from SIGMA and pure xylose were also recorded.

6.2.6.4. HPLC Analysis

The HPLC method as described earlier was followed. The xylitol separated from fermentation broth and commercial xylitol were subjected to HPLC analysis to know about the purity of synthesized xylitol.

6.2.6.5. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (FEI Quanta 200 3D) was used to visualize the crystal morphology. The powdered crystalline sample was loaded onto silicon wafers and visualized.

*6.2.7. Violacein Inhibition Assay (Qualitative) Using Pichia-Synthesized Xylitol*

This assay has been designed in such a way that a zone of inhibition of violacein synthesis can be seen around the agar well containing the probable QS Inhibitor. *C. violaceum* mutant CV026 is used as the test organism which has the ability to respond to a variety of signal molecules [12]. The QS signal molecule (namely C6-HSL, C8-HSL, or 3-oxo-C6-HSL) at a concentration of 12.5µM is added to 10 ml of Luria Bertani (LB) soft agar containing 100µl of overnight culture of CV026. The soft agar is then overlaid onto basal LA plate, and a 4 mm diameter well is dug in the center of the plate using a sterile cork borer after the overlay is set. To the agar well, 75µl of appropriate concentration of the compound under investigation was added, and the plates were incubated in upright position at 30 °C for 24 h. The diameter of zone of violacein inhibition was then measured. Pure xylitol from SIGMA was used for comparative analysis.

*6.2.8. Violacein Inhibition Assay (Quantitative)*

This assay is based on a similar principle to the one described above, that is, the presence of any quorum sensing inhibitor will quantitatively decrease the production of the purple pigment violacein which can then be estimated colorimetrically [13]. In this tube assay, 100µl of overnight grown culture of CV026 was inoculated in 10 ml LB broth containing the QS signal molecule (namely C6-HSL) at appropriate concentrations. The test compound, *P. caribbica*, synthesized xylitol, was added at increasing concentrations in a series of test tubes. The violacein produced is extracted from the culture broth by dissolution of the pigment in DMSO and separating it from the cell mass by centrifugation. The amount of xylitol required to inhibit or substantially decrease the purple pigment production could then be determined by quantitatively estimating the optical density of purple pigment extracted at 570 nm. Appropriate positive and negative controls were used. All experiments were done in triplicate for confirming reproducibility.

*6.2.9. Competitive Receptor antagonism assay and docking studies as tools to understanding molecular mechanism of QS inhibition by Xylitol*

To determine whether xylitol acts as a competitive receptor antagonist a simple experimental design was followed. Highest concentration of xylitol shown to mediate complete inhibition of violacein synthesis was used for this study and this concentration was kept constant. The concentration of native ligand of the LuxR receptor of *C. violaceum*, C6HSL, was gradually increased. Alleviation of inhibition of violacein synthesis mediated by xylitol, upon increasing C6HSL concentration would prove that it indeed binds to the ligand binding site of the CviR (LuxR homolog in *C. violaceum*) [14] thus inhibiting signal reception by native QS signal molecule.

Docking studies were also performed to further validate this finding. Structure of CviR the cognate LuxR receptor of *Chromobacterium violaceum* bound to its native ligand C6HSL was downloaded from PDB (PDB ID 3QP5).Docking studies were then performed using GLIDE (version 5.8, Schrödinger, LLC, New York, NY, 2012) at the ligand biding site of the receptor.3D structure of xylitol and C6HSL were also downloaded from PubChem and all ligands were prepared by LigPrep before beginning

of docking studies. The CviR receptor was treated as rigid while xylitol (Antagonist) and C6HSL (agonist) were docked flexibly, exploring their rotational and translational degree of freedom. Extra precision (XP) mode of Glide was used for docking. From the resulting receptor-ligand complex, a rough estimate of the binding affinity in terms of free energy of binding was calculated using an empirical scoring function of Glide called G-score.

## 6.3. Results and Discussion:

### *6.3.1. Product Harvesting, Concentration, Decolorization, and Yield Calculation*

Microbial sources have been exploited extensively for xylitol production; among them, yeasts have been found to be the most promising. Yeasts are especially favored because they are very robust organisms and can easily grow on both chemically defined media as well as inexpensive renewable resources like hemicellulose. In the present chapter, xylitol synthesis has been achieved through fermentative conversion of D-xylose. The popular *Candida* species employed for D-xylose to xylitol conversion are *Candida tropicalis*, *Candida guilliermondi*, *Candida parapsilosis* [9], and *Candida peltata*[15]. A chemically defined medium was used in case of *C. tropicalis* and xylitol yield obtained was 0.87 g/g[16]. However xylitol production was associated with longer incubation period, i.e., 120 h, thus effectively reduced the value of volumetric productivity. *Debaryomyces hansenii* has also been another organism of choice for xylitol production from D-xylose with the yield values falling in the range 0.76–0.81 g/g. However, this was achieved at 35–37 °C of incubation [1, 17, 18]. *Pichia stipitis* has also been reported for xylitol production [9]. In this chapter a new species of *Pichia* namely *P. caribbica* has been reported for the first time which is capable of quantitative conversion of pure xylose into xylitol fermentatively. The theoretical yield value of 93.61 % in present case is specifically remarkable as the theoretical yield values reported so far for xylitol production from D-xylose are in a range of 65–85 % of the theoretical value [19]. Additionally, the high yield has been achieved without any genetic engineering and optimization of pH, temperature conditions unlike other reports involving extensive optimization of fermentation conditions and genetic manipulation [20, 21]. The *P. caribbica* xylitol production was monitored at periodic intervals with HPLC. The trend

observed is depicted in Fig.6.1. Maximum xylitol content was observed after 72 h of incubation, and then there was a decrease which can be attributed to its utilization for cell maintenance purpose. The maximum xylitol produced by free cells was 85.2 g per 100 g/L of xylose at ambient pH and temperature with the yield of 0.852 gm/gm and volumetric productivity of 1.83 gm/l/h. Also described herein is a novel technique of separation of xylitol from its fermentation broth in almost pure crystalline form which reduces the cost and recovery associated with the downstream processing. The process involves simple rotary evaporation and concentration of the spent broth to achieve almost quantitative yield of synthesized xylitol, followed by low temperature storage for 48 h to induce crystal growth. The crystals obtained in this case were associated with yellow pigment which was completely removed by treatment with only 100 % ethanol, lending it safe for food and pharmaceutical applications. Use of low-cost hemicellulosic raw material is preferred over pure D-xylose for xylitol production on basis of process economics. However, the cost and time associated with present production, extraction, and decolorization method could compensate for the higher cost of D-xylose as starting material. The decolorization procedures for xylitol obtained using hydrolyzed hemicellulosic intermediates involve the use of activated charcoal or other adsorbents [22]. The use of adsorbents causes around 20 % loss of xylitol and makes the crystallization difficult and time-consuming [23], thus complicating the downstream processing and eventual product purification [24].

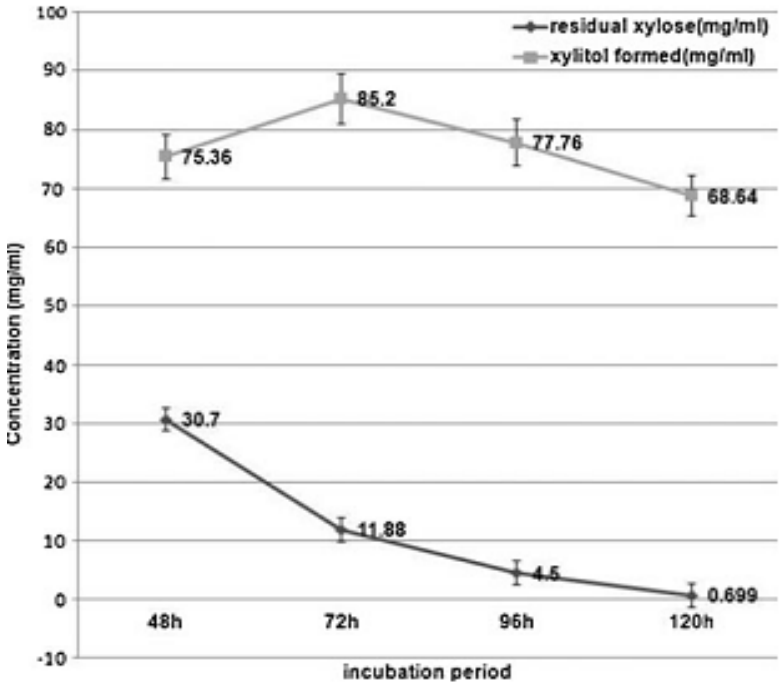



***Figure 6.1**: Monitoring Xylitol synthesis from D-xylose by P. caribbica using HPLC*

*6.3.2. Characterization of Pichia caribbica synthesized Xylitol*

XRD spectra of *Pichia* synthesized crystalline xylitol was recorded, and the data was analyzed. On comparing the experimental spectra against that of standard xylitol in the database, it was evident from Fig.6.2 that the white crystalline compound was indeed xylitol as more than 98 % of spectral peaks were overlapping with each other (JCPDS PDF no.34-1802).$^{1}$H NMR spectra recorded using deuterated methanol as solvent and TMS as an internal standard revealed that in both Sigma xylitol and *Pichia* synthesized xylitol similar spectral peaks ; hence, they were essentially the same compound (Fig.6.3). The FTIR spectra of *Pichia* xylitol and pure sigma xylitol were recorded and are presented in Fig.6.4. Upon analyzing the FTIR data, it was observed that the IR spectra represented a broad stretching around 3,200–3,400 $cm^{-1}$ which is characteristic of hydroxyl group present in this sugar alcohol and a weak C–H stretching band at around 2,932 $cm^{-1}$ for both experimental and sigma xylitol. The absorption band from 1,300 to 800 $cm^{-1}$, is called “finger print” region, and is related to conformation and surface structure of molecule. These bands have always been very hard to explain; however, in

the spectra, it can be seen that both *Pichia* and sigma xylitol show essentially similar peaks in the fingerprint region also. There was a strong characteristic peak around 1,410 and 2,931 $cm^{-1}$ which is typical of methylene groups present in the molecule. Thus, on comparison of both the spectra, the compound was confirmed to be xylitol. HPLC analysis revealed that *Pichia* synthesized xylitol showed similar retention time as that of standard Sigma xylitol under identical conditions. Also, after analyzing the spent culture supernatant for residual xylose content, it was observed that almost 80 % of the xylose in the medium had been fermentatively converted into xylitol within 72 h which is indicative of quantitative xylitol production by this organism. The crystal morphology seen under the scanning electron microscope (Fig.6.5) was indicative of the purity of the compound and was similar to that reported previously [25]. The shape of the crystals appeared rounded, however there was agglomeration in some areas which can be neglected.

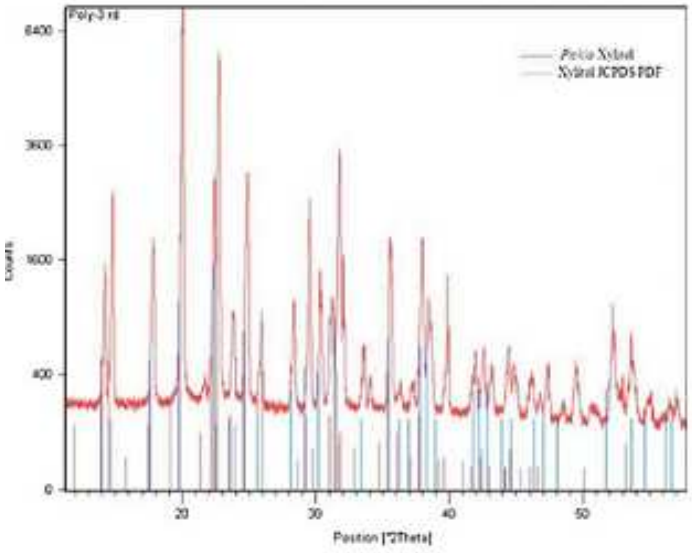



***Figure 6.2****: Powder XRD spectra of P. caribbica synthesized xylitol (red)compared with spectra of standard xylitol from JCPDS-PDF database (blue).*

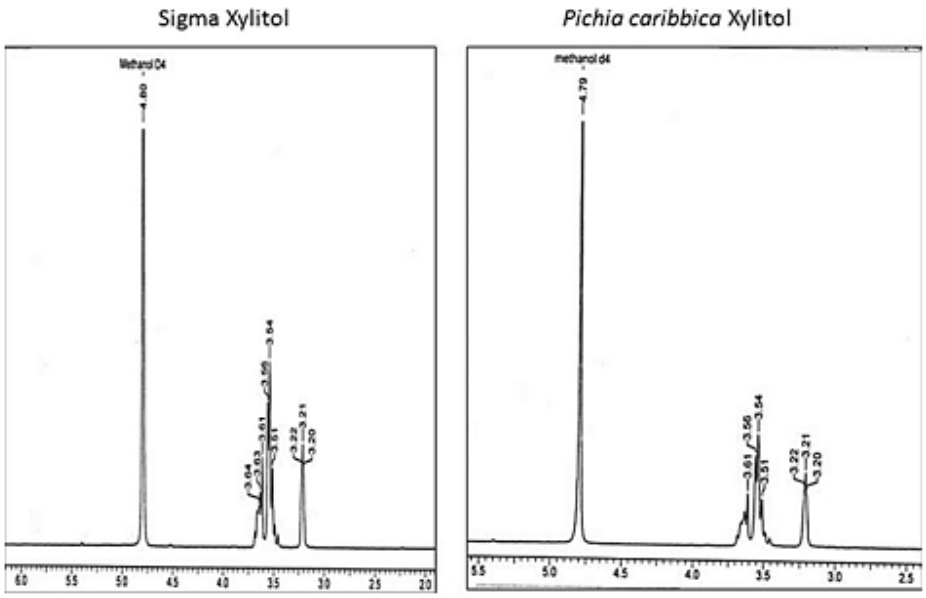



***Figure 6.3**: Comparison of $^{1}H$ NMR Spectra of P. caribbica synthesized xylitol and SIGMA xylitol show that they are essentially the same.*

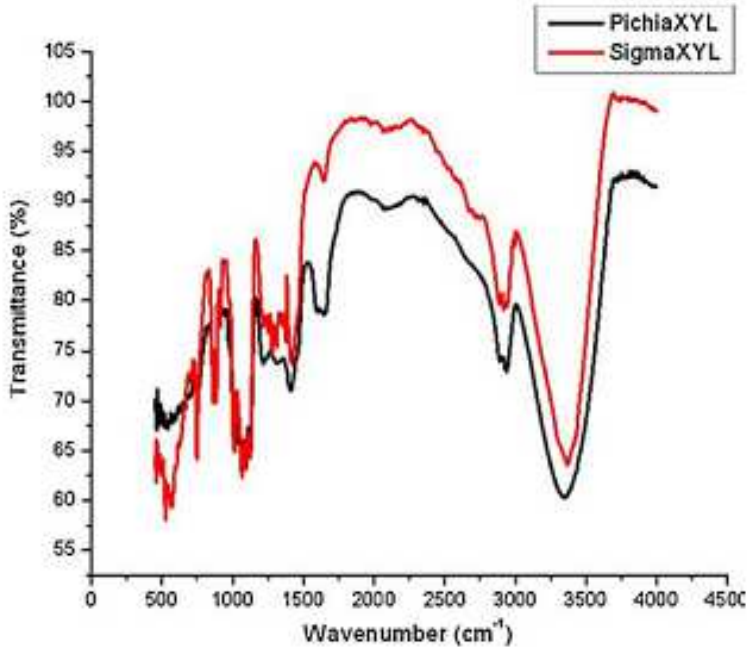



***Figure 6.4**: FTIR spectra in transmission mode recorded for both Sigma xylitol and P. caribbica synthesized xylitol, showing high degree of similarity between the two.*

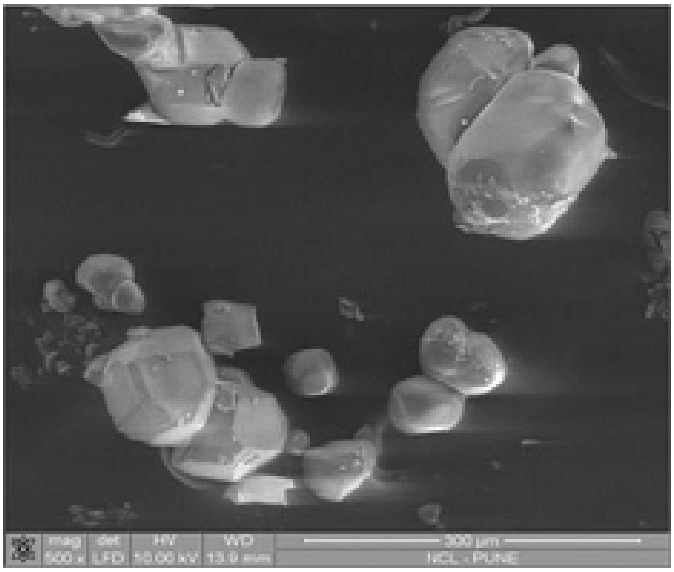


***Figure 6.5:*** *Scanning electron micrograph of P. caribbica synthesized xylitol revealed typical crystal morphology (magnification ×500)*

*6.3.3. Violacein Inhibition Assay (Qualitative and Quantitative)*

Due to a plethora of interesting characteristics, xylitol has become a pharmaceutically important compound. Xylitol has been shown to be an inhibitor of biofilm formation in gram-positive dental pathogen *S. mutans* , making it an indispensible addition to dental hygiene product. It has also been reported to avert middle ear infection caused by *S. pneumonia* which is an important gram positive bacterial pathogen. But ability of xylitol to impede quorum sensing-associated phenotype in gram negative organisms has not been explored before. The present work investigates the ability of *Pichia*-synthesized xylitol to inhibit gram-negative quorum sensing signal (acyl homoserine lactone) reception. Acyl homoserine lactones are the largest class QS signals which play a crucial role in gram-negative cell to cell signaling and virulence gene expression. Pichia-synthesized xylitol was able to antagonize not one but three different AHL
signal (C6-HSL, C8-HSL, and 3-oxoC6-HSL) and receptor interactions as determined using CV026-based AHL antagonism assay.Violacein synthesis in *C. violaceum* is a quorum sensing-associated phenotype, the signal molecule in this case being C6-HSL. Inhibition of quorum sensing or quorum sensing signal antagonism will not result in any

violacein synthesis which is a powerful indicator as it helps to screen for QSIs with efficiency. In both qualitative (Fig.6.6) and quantitative assays (Fig.6.7), it was observed that xylitol could impede QS and hence inhibit expression of QS associated phenotype in CV026. From Fig.6.7, we can see that *Pichia* synthesized xylitol could quantitatively inhibit violacein production induced by the signal molecule C6-HSL in CV026. It was seen that increasing the concentration of xylitol was not inhibitory to the growth of the organism, but it severely impaired quorum sensing signal reception. Similar results were obtained with standard xylitol from Sigma. It is interesting to note that all of three signal molecules, C6-HSL, C8-HSL, and 3-oxoC6-HSL that are antagonized by xylitol are important signaling components for virulence gene activation in major gram negative pathogens. This finding is very crucial since it broadens the scope of application of xylitol in various bio-therapeutics.

***Figure 6.6:*** *Qualitative violacein synthesis inhibition by agar well diffusion method shows ability Of Pichia xylitol to antagonize C6-HSL, quorum sensing signal reception in C. violaceum CV026.*

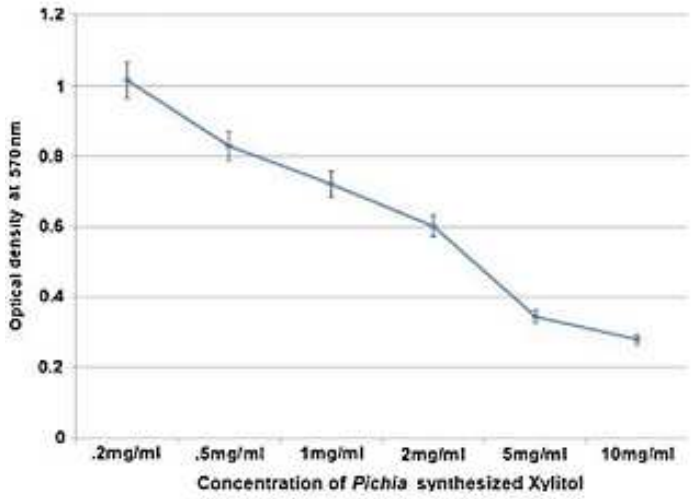



***Figure 6.7:*** *Quantitative inhibition of violacein synthesis in the presence of increasing concentration of Pichia-synthesized xylitol.*

*6.3.4 Proof of receptor antagonistic activity of xylitol using experimental and In silico tools:*

Biological receptors are protein molecules that are activated upon binding to specific ligand. These receptors can be membrane-bound, occurring on the cell membrane, or intracellular/ cytoplasmic. Receptor activation occurs as a result of non-covalent interaction between the receptor and its ligand, at the binding site on the receptor. Competitive antagonists reversibly bind to the receptor at the same binding site as the cognate ligand or agonist, but fail to activate the receptor. Agonists and antagonists "compete" for the same binding site on the receptor. Once bound, an antagonist blocks binding of agonist to the receptor. The level of activity of the receptor will be determined by the relative affinity of each molecule for the site and their relative concentrations. Increasing the concentration of agonist will increase the proportion of receptors with agonist bound to it, hence higher concentrations of the antagonist will be required to obtain the same degree of binding site occupancy. Competitive receptor antagonism can be overcome by increasing the concentration of agonist.

To confirm the fact that xylitol indeed acts as a receptor antagonist blocking the ligand binding site of CviR (LuxR homolog in *C.violaceum*) thus inhibiting violacein synthesis,

a simple approach was taken. In a small experiment concentration of the antagonist xylitol was kept constant at 10mg/ml (because at this concentration almost complete inhibition of violacein synthesis was observed, Fig.6.7) and concentration QS signal molecule C6HSL (agonist) was increased progressively. Increase in C6HSL concentration in the growth medium was accompanied by increase in violacein synthesis, showing alleviation of xylitol mediated inhibition of violacein synthesis (Fig. 6.8). Docking studies performed at the Ligand binding site of CviR also supports this finding (Fig. 6.9). Xylitol fits well within the AHL binding pocket of CviR, however the free energy of binding is decidedly less when compared to that C6HSL (the native agonist).So an increase in concentration of C6HSL could easily displace inhibitor/antagonist xylitol from the AHL binding pocket because the receptor has significantly more affinity for its native ligand C6HSL.

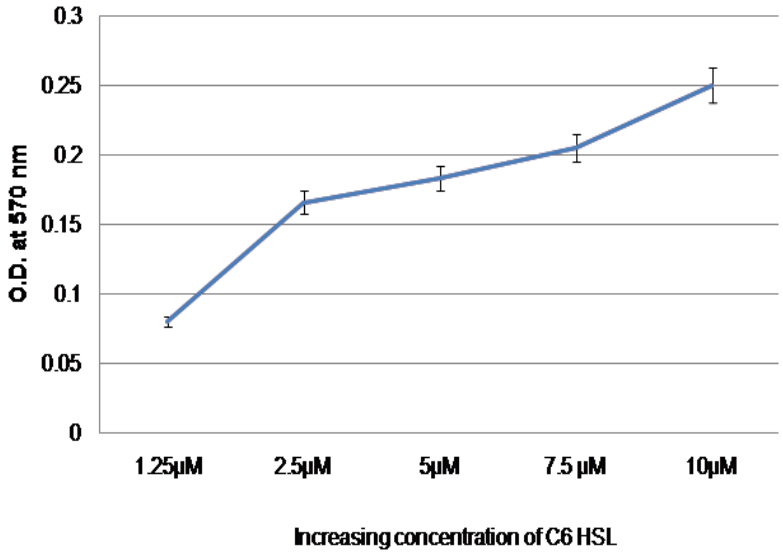



***Figure 6.8***: *Graph showing alleviation of Pichia xylitol mediated inhibition of Violacein synthesis by addition of increasing concentration of C6HSL.*

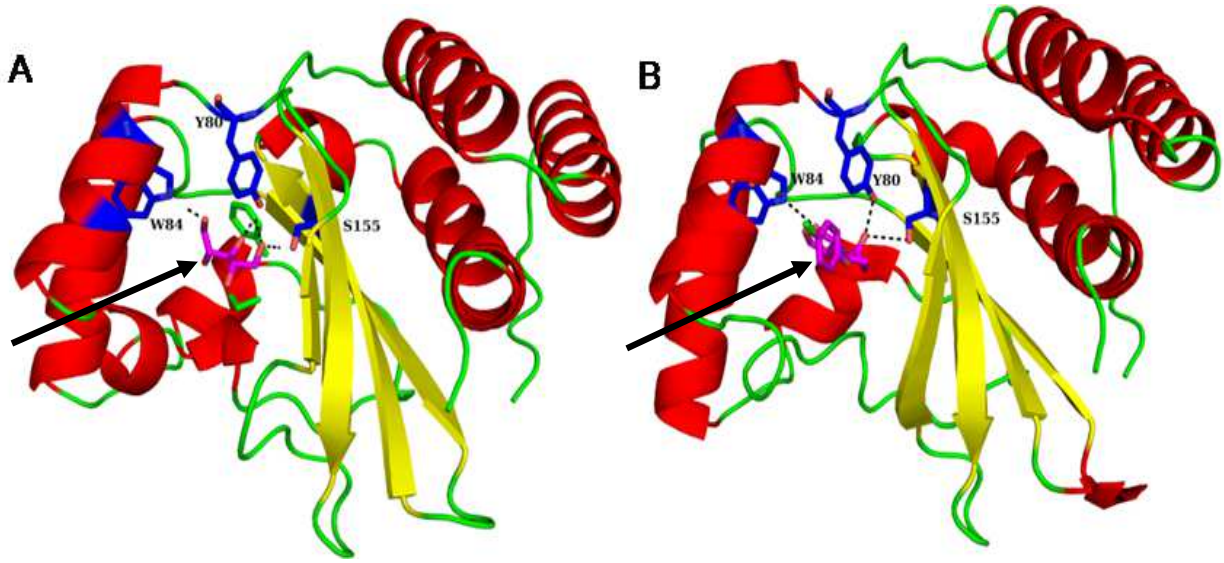



***Figure 6.9:*** *Molecular docking experiment revealed that xylitol (in pink, indicated by arrow) fits perfectly within the Ligand binding pocket of Lux R homolog, CviR, of C. violaceum (Panel A), albeit with reduced binding affinity as compared to that of cognate ligand C6HSL (Panel B, in pink, indicated by arrow)*

## 6.4. Conclusion:

Present work investigated the ability of newly isolated yeast *P. caribbica* to synthesize xylitol from D-xylose containing medium, and it was found to give high yield of 85.2 g/g of xylitol per gram of xylose with resting cell method. Also, the synthesized xylitol could be extracted using a fast, simple procedure that is cost-effective and acceptable for human consumption. The extracted xylitol was more than 98 % pure and had the typical crystal morphology. Furthermore, it was seen that xylitol had the ability to inhibit quorum sensing mediated phenotype of violacein pigment production in gram-negative marker strain *C. violaceum* CV026.Investigation into the mechanism of quorum sensing inhibition of xylitol revealed that it acts as a competitive receptor antagonist blocking signal reception and thus impeding expression of quorum sensing mediated phenotypes.

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# Chapter 7

*Early Detection of bacteria capable of Quorum sensing using fluorescent Au nano-cluster probes surface functionalized with QS signal molecules.*

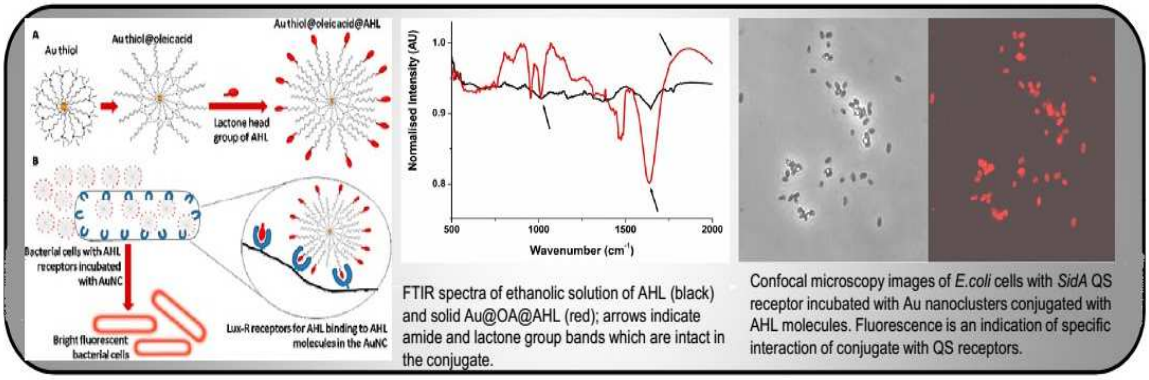
**This Chapter has been published as**:

**Mukherji R**, Samanta A, Illathvalappil R, Chowdhury S, Prabhune A, R Nandini Devi (2013), Selective Imaging of Quorum Sensing Receptors in Bacteria Using Fluorescent Au Nanocluster Probes Surface Functionalized with Signal Molecules, ***ACS Appl. Mater. Inter***. **DOI: 10.1021/am404093m**

**Also presented in the form of a poster:**

**BEST POSTER** Award for **POSTER PRESENTATION** in **Second Conference on Nano-Bio-Med-2013, held in ICTP (**Trieste, **ITALY**) from 14 - 18 October 2013.
Poster Title: ***Imaging quorum sensing receptors in bacteria using fluorescent Au Nanocluster probes surface functionalized with signal molecules***

# Chapter 7. Early Detection of bacteria capable of Quorum sensing using fluorescent Au nano-cluster probes surface functionalized with QS signal molecules




FTIR spectra of ethanolic solution of AHL (black) and solid Au@OA@AHL (red); arrows indicate amide and lactone group bands which are intact in the conjugate.

Confocal microscopy images of *E.coli* cells with *SidA* QS receptor incubated with Au nanoclusters conjugated with AHL molecules. Fluorescence is an indication of specific interaction of conjugate with QS receptors.

### 7.1. Introduction:

Quorum sensing (QS) or the phenomenon of bacterial communication has been identified and studied in detail for more than a decade now[1-3]. Such kind of intercellular communication allows bacteria to come together and function as a single entity more or less like a multi-cellular organism[4]. Quorum sensing is suggested to be the mechanism by which biofilm formation takes place, which enhances the virulence and drug resistance, of various bacterial pathogens. QS is mediated by small easily diffusible signal molecules which regulate target gene expression and is highly dependent on the density of bacterial cells in the medium. Structurally different categories of molecules mediate quorum sensing in different genera of bacteria and all these molecules have been loosely termed as autoinducers [5]. Gram positive bacteria depends on autoinduction by small peptides and in gram negative bacteria, QS is mediated by acylated homoserine lactones (AHL) which the largest and one of the most well characterized class of QS signal molecules[6,7]. The autoinducers are perceived by their complementary receptors, and this process is highly selective with respect to the signal molecule and receptors. Binding of autoinducers to the specific receptor sites triggers a cascade which regulates a wide range of phenotypes often detrimental to the host organisms[8-10]. In case of AHL

autoinducers, receptors belong to Lux-R family of transcription regulators and they are usually located intracellularly or may be found attached to the inner leaflet of bacterial cell membrane[11]. Even though lot of biochemical information regarding the mechanistic aspects of quorum sensing has been brought to light in recent years, attempts to visualize this phenomenon have been rare.

Recently, imaging the phenomenon of quorum sensing in gram negative organisms, specifically *Pseudomonas* aeruginosa (PAO1) has been attempted, employing in vivo approaches whereby a quorum sensing biosensor strain was used as a probe to detect the signal [12]. However, this method is dependent on growth of both the test strain and the biosensor. Such growth associated methods are time-consuming and carry the risk of a full blown infection of the host in the meantime. Hence such methods are not appealing enough when urgent detection of bacterial pathogens is required. Imaging and identifying bacterial pathogens at low cell numbers, before they reach a quorate state and cause havoc, seems highly desirable. Such an early detection system can be envisaged if we target the receptors which are present in the bacterial cells even before the virulent quorate phase.

Very recently, CepR quorum sensing receptors in live cells of Burkholderia cenocepacia have been tagged using signal molecules which are terminally labeled with fluorescent organic moieties [13]. However, inorganic nanomaterial based fluorescent probes emitting in near-infrared (NIR) spectral region are understood to be superior to organic dyes because of their higher photostability, reduced background fluorescence etc. [14]. Imaging quorum sensing by such inorganic nanomaterial based fluorescent probes is as of yet unexplored. In this scenario, a simple, photostable and selective fluorescence imaging of quorum sensing , based on QS receptors, facilitating early detection of bacteria will be advantageous. This chapter describes synthesis of an inorganic fluorescent gold nanocluster based imaging probe selective to bacteria possessing AHL receptors has been developed. *E. coli* is a gram negative bacterium which perceives AHL signal molecule through LuxR family of receptors SdiA [15] but does not produce the signal molecules. This method seems to have potential for species specific selective imaging of quorum sensing in vivo with the added advantage of being capable of detecting bacterial cells much before the virulent stage and at lesser populations.

## 7.2. Materials and Methods:

### *7.2.1. Synthesis of AuNC and AHL conjugate*

Synthesis of Au nanoclusters is reported elsewhere [16] (This work is a part of another thesis). Briefly, 8.5 mL of a stock solution of Aurochloric acid (99.9%, Aldrich) in acetonitrile (10 mM) was mixed with 20 mL of acetonitrile. Thiol (0.593 g, 1.6 mmol) in 2 mL of methanol was added to this mixture. The mixture was stirred for 30 min. One hundred microliters of sodium borohydrate solution (1.58 M) in methanol was added and stirring was continued for 45 min. All the steps were carried out in ice-cold conditions. Thirty milliliters of water was added to gold nanocluster solution and acetonitrile was removed in vacuum. Aqueous solution of gold nanocluster was purified by dialysis using cellulose membrane (12 kDa MWC Sigma Aldrich). The concentration of this final solution was found to be 2 mM of Au by elemental analysis. Thirty microliters of a stock solution of oleic acid in methanol (0.6 mM) was mixed with 1 mL of the above gold nanocluster solution. Ten microliters of AHL (99%, Cayman Chemicals) from a 25mM stock solution dissolved in 500μL of ethanol was added to the above mixture and stirred for 4 h. This was considered as the working solution of the composite to be used for incubation with bacterial cells. For intermediate nanoclusters, same volume of either AHL or oleic acid was added to 1 mL Au−thiol solution.

### *7.2.2. Characterization of synthesized Au cluster and Au@OA@AHL cluster*

FEI Tecnai TF-30 electron microscope, operating at 300 kV was used for high resolution transmission electron microscopy (HRTEM). Samples for HRTEM were prepared by evaporating a droplet of solution onto a carbon coated copper mesh 200 grid. UV−vis spectra were recorded on a Cary 5000 UV−vis−NIR spectrophotometer. Fluorescence emission spectra were obtained on a Photon Technology International Fluorescence Instrument. Samples were taken in 3.5 mL quartz cells of 10 mm path length for fluorescence measurements. Two detectors were used: (1) R2658 PMT in PTI cooled housing with a single em mono, 1200 L/mm, 400-nm blazed grating; (2) In GaAs diode with chopper and locking and a single em mono, 600 L/mm, 1250-nm blazed grating. The spectra were corrected for the sensitivity of the respective emission channel and normalized. Quantum yield was calculated based on experiment in a small integrating

sphere and was done using a double/single (excitation/emission) mono-chromator format. The lamp used was a xenon arc lamp with a chopper set for 27 Hz. The detector was a 1700 nm In GaAs. The grating used was 600 L/ mm, 1.25μm blaze angle. The band pass was 12 nm for excitation and48 nm for emission. FTIR spectra were recorded on a Perkin-Elmer FT-IR spectrum GX instrument. KBr crystals were used as the matrix for preparing samples.

*7.2.3. Sample preparation and Imaging using Confocal Microscopy*

Olympus Fluoview Laser Scanning Microscope was used to observe the stained bacterial cells. Initially, bacterial cells were suspended in saline and incubated with the Au@OA@AHL conjugates for 2 h with gentle shaking. After incubation cells were pelleted down, washed with saline and re-suspended in fresh saline so that any unbound fluorescent clusters would not interfere with the imaging.Confocal microscopy slides were prepared using 70% glycerol as mounting medium and this did not hamper with the fluorescence of the sample. Cover-slips containing the sample and mounting medium were sealed at the sides to prevent drying by evaporation and slides were observed within 3−4 h of sample preparation.

**7.3. Results and Discussion:**

Inorganic semiconductor quantum dots like CdSe, HgSe, etc., and Au nanoclusters (NCs) are reported to be advantageous with emissions in NIR region thereby minimizing interference from endogeneous fluorescence of the sample media [17-19]. In case of Au nanomaterials, as the particle size decreases to <2 nm forming ultra small clusters, the electronic properties deviate from those of the bulk material and start exhibiting molecule like orbital characteristics which manifest as unique physical properties like fluorescence [20]. NIR fluorescence emissions of these materials render them ideal for bioimaging. The major drawback of Au NCs is their low quantum yield (QY); however, Lin *et al.* has demonstrated the capability of low QY AuNCs as fluorescence probes for specifically targeting human hepatoma cells [21]. Hence, with appropriate surface functionalizations, a widespread application of AuNCs in the field of sensing and imaging of a variety of biologically important systems can be envisaged. The fluorescent probe under study

consists of water dispersible AuNC with size <2 nm initially layered with oleic acid (OA) and subsequently coated with AHLs of chain length C6 and C8.HRTEM image of the Au cluster surface functionalized with OA and AHL, and its fluorescence spectra in comparison with AuNC alone is shown in Figure 7.1 and 7.2 respectively.

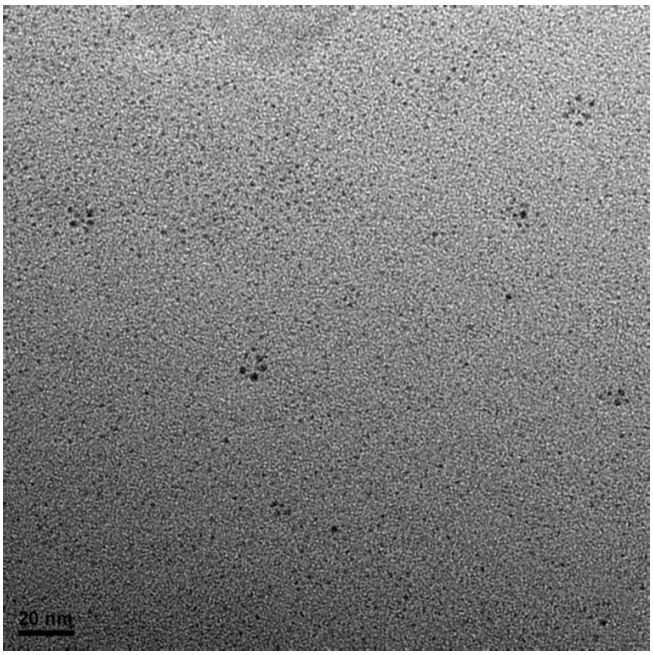



***Figure 7.1:*** *HRTEM images of Au @OA@C6-AHL*

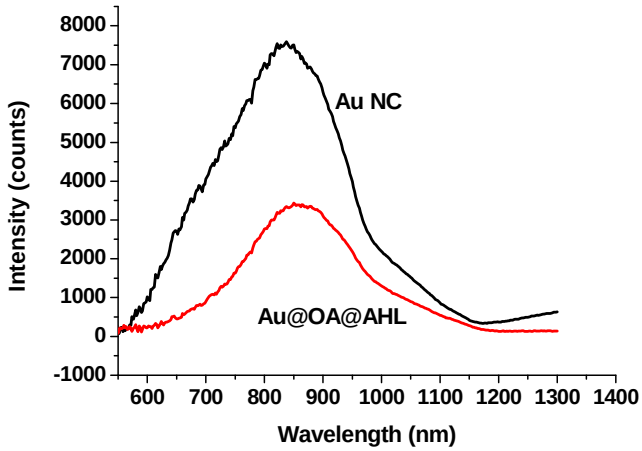



***Figure 7.2****: Fluorescence spectra of pristine Au-thiol nanoclusters and Au@OA@AHL composites (Excitation maximum at 646 nm)*

*Chromobacterium violaceum* mutant strain (CV026) based bioassay confirmed that the biological activity of AHL signal molecules is not lost despite being directly coated over a nanoparticle. This is the first report of such retention of activity by AHL molecules. CV026 is a biosensor organism used in QS studies because of its ability to respond to a variety of synthetic AHL molecules. Purple pigment (Violacein) production in CV026 is a QS-mediated phenotype and exogenously supplied AHLs can induce its production. When the Au NC composite was incubated with CV026, violet pigment production was observed indicating that chemistry used in synthesizing this AuNC-AHL conjugate did not in any way destroy the inherent QS biological activity of the AHL molecules (Fig. 7.3). Additionally, no enzymatic or lipolytic activity inherent to the mutant biosensor strain CV026 was responsible for release of AHL molecules from the Au@OA@AHL conjugate leading to eventual violacein production. This was confirmed by growing the culture in basal mineral medium with oleic acid as the sole source of carbon at a concentration of 0.1%. No growth was observed after incubating the culture for 48 h at 30°C under shaking conditions. This shows that the violacein is produced upon perception of the conjugate as a whole and not AHL alone that has been removed from the conjugate by enzymatic cleavage.

***Figure7. 3:*** *Violacein (purple pigment) produced by CV026 in response to the test Au@OA@C8-AHL*

The strategy employed here involves surface functionalizing fluorescent Au NCs with AHL in such a way that ability of the signal molecules to bind to specific receptors is not compromised. Initially, fluorescent Au nanoclusters are synthesized using an alkyl thiol ligand, N,N,N-tripropyl (11 mercaptoundecyl)ammonium chloride with a cationic ammonium headgroup to facilitate dispersibility in water, the most conducive solvent for biological systems [16]. The AHL signal molecules consist of lactone head group and a long chain alkyl group of varying lengths (structures of AHL molecules under study, C6-AHL and C8-AHL, with six and eight C alkyl chains are given below Fig.7.4).

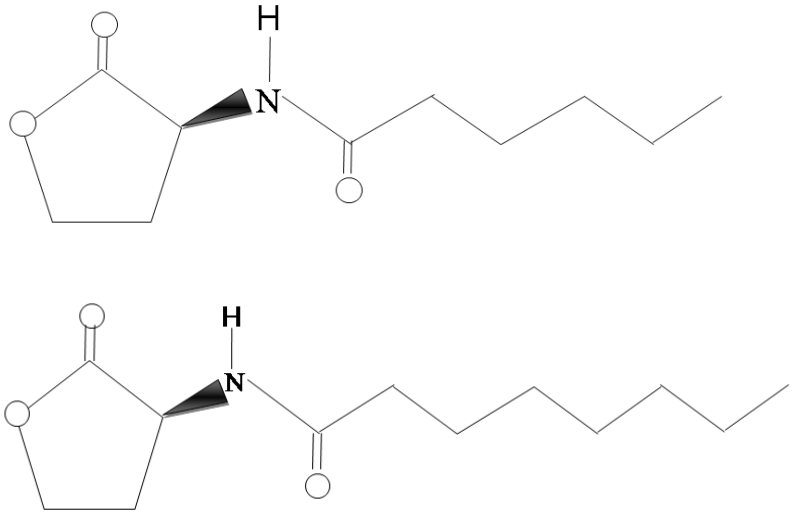
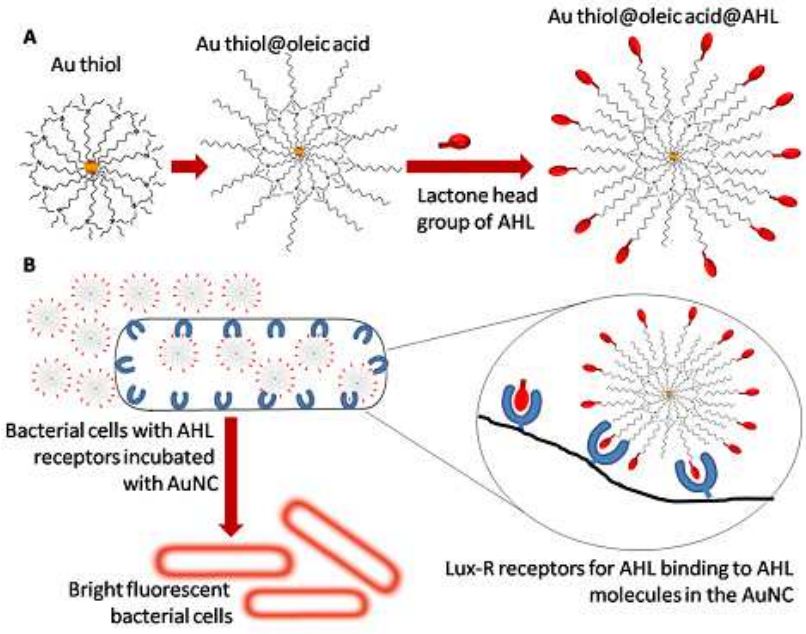



***Figure 4:*** *Chemical Structures of Acyl homoserine lactone molecules under study (top) C6 HSL and (bottom) C8HSL*

It is understood that ring carbonyl and 1-carbonyl groups of cognate AHL molecules bind to the N-terminal receptor sites of Lux R proteins through hydrogen bonding [22]. Hence it is imperative to leave the lactone ring and amide moieties intact and free to interact with the receptors. However, if pristine Au-thiolate cluster is added directly, there is a possibility of interaction between cationic head groups of the nano-cluster and the lactone moiety of AHL. Hence, further surface functionalization of the nano-clusters is necessary to avoid any cooperative interaction between lactone and cationic headgroup of AuNCs. A composite was designed such that outer surface of the nano-cluster would form a hydrophobic region which would then interact with the alkyl chains of AHL molecules (Scheme 7.1A). Accordingly, NCs were initially treated with oleic acid so that electrostatic attraction

between cationic head groups and COOH groups was facilitated. Such a binding would allow the hydrophobic alkyl chains of oleic acid to decorate the outer surface of NCs rendering them ideal for interaction with the long chain alkyl groups of AHL. In this way, the lactone would be free to attach to the receptors in bacterial cells (Scheme 7.1B, shown below).




[a]Binding sites shown schematically; actual location is not known.

***Scheme 7.1**: **A** and **B***

The average number of thiol molecules attached to the Au clusters was roughly estimated based on number of surface Au atoms and a methanol solution of oleic acid of equimolar concentration was added. Subsequently, lactones with varying chain lengths, namely, C6 and C8 were mixed with the above Au-thiol@oleic acid composite. Transmission electron microscopy revealed ultrasmall clusters of size <2 nm, functionalization not affecting the particle size (Fig. 7.1). The Au NCs were found to be fluorescent in NIR region with an emission maximum at 840 nm and quantum yield of 1.6% (Fig. 7.2).

Interactions between various functionalizing molecules were ascertained by solid state FTIR spectroscopy. Electrostatic interaction between carboxylic acid group and ammonium head group based on carboxylic C-O functionality was followed in

comparison to pristine oleic acid. It is known that this acid C-O stretch which is observed in 1710 $cm^{-1}$ in pristine sample disappears in bound states with appearance of bands near 1640 and 1540 $cm^{-1}$ corresponding to symmetric and asymmetric −COO vibrations (Fig.7.5a) [23]. We could also observe a strong band at 1636 $cm^{-1}$ corresponding to symmetric and a very weak band at 1523 $cm^{-1}$ corresponding to asymmetric −COO stretch (Fig.7.5a).This indicates a possible configuration of the cluster with COO group interacting with ammonium group thereby deploying the alkyl functionality on the outside. IR spectrum of the composite after addition of C6-AHL in comparison to methanolic solution of AHL is given in Figure 7.5b. It can be seen that amide group bands are more prominent than lactone bands in both [24,25]. In case of pure AHL, amide group bands consist of a strong C-O stretch band at 1646 $cm^{-1}$ and weak N−H bend at 1520 $cm^{-1}$. Possible lactone C-O stretch could be seen at 1772 $cm^{-1}$ and C−O stretch at 1167 and 1014 $cm^{-1}$ corresponding to O−CO and O−$CH_2$ vibrations (Fig.7.5b). After addition of AHL, the lactone group bands were intact at 1772 and 1011 $cm^{-1}$ indicating that the bioactive region is free to interact with the receptor sites (Fig.7.5b). O−C-O vibrations cannot be unambiguously assigned due to overlap with Au-OA bands. With these evidence it could be tentatively concluded that the composite has the structure Au-thiol@ oleicacid@AHL (Au@OA@AHL) with the lactone moiety on the outside of the cluster. Moreover, the surface charge was found to be negative with a zeta potential value of −38 mV indicating that the conjugate is highly stable.

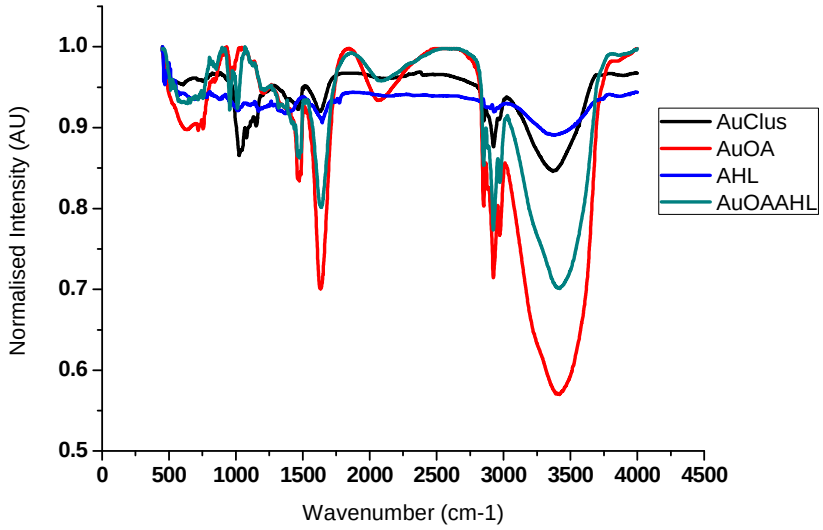



***Figure 7.5a**: FTIR analysis of all the compoment that make up the Au@OA@AHL conjugate*

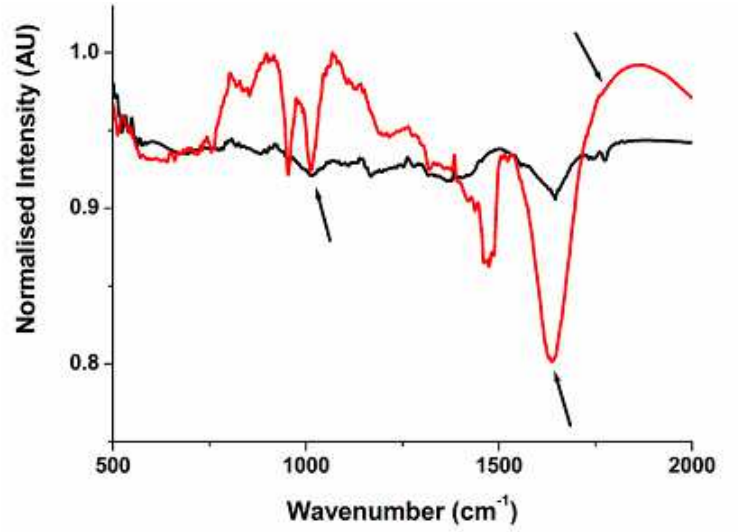



***Figure 7.5b:** FTIR spectra of ethanolic solution of C6-AHL (black) and solid Au@OA@AHL (red); arrows indicate amide and lactone group bands which are intact in the composite.*

Representative bacteria from two distinct groups, one gram negative (*Escherichia coli*) and one gram positive (*Staphylococcus aureus*) were selected for further fluorescence imaging. Quorum sensing receptors in *E. coli* are of the Lux-R family but it is interesting to note that this strain does not produce AHL molecules of its own and *S. aureus*, being gram positive does not contain the AHL receptors. All experiments were carried out

using low cell numbers of the order of $10^6$ cells/ mL, which is well below the quorate number of any bacterium. These cells were observed under confocal microscope at preset excitation and emission wavelengths of 547 and 567 nm respectively. In case of *E. coli*, Au clusters decorated with both C6 (Fig. 7.6) and C8-AHL (Fig. 7.7) showed bright fluorescent emission and cell contours could be easily identified. Supernatant solution without the cells also showed emission, however, in irregular agglomerated shapes pointing to unbound clusters (Fig.7.8). Since AHLs are specifically produced by gram negative bacteria, rationally the Au NCs decorated with AHL molecules should only interact with cells of *Escherichia coli* and not with *Staphylococcus aureus*, the gram positive bacterium with a different receptor system.

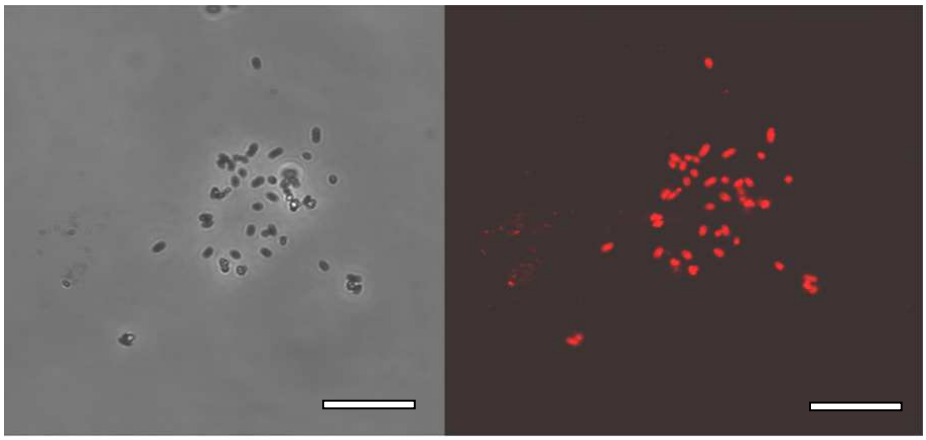


***Figure 7.6:*** *Confocal microscopy images of E. coli incubated with Au@ OA@C6-AHL: (left) phase contrast image and (right) fluorescence image of the same region. Scale bar is 10 μm.*

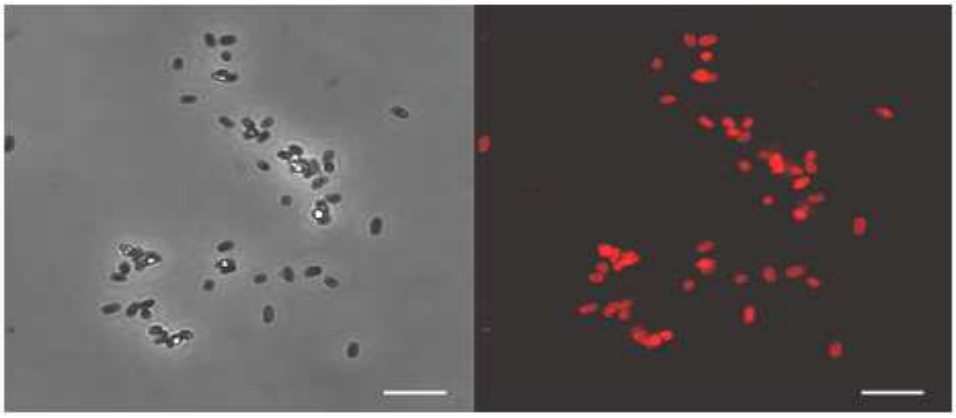


***Figure 7.7****: Confocal microscopy images of E. coli incubated with Au@ OA@C8-AHL: (left) phase contrast image and (right) fluorescence image of the same region. Scale bar is 5 μm.*

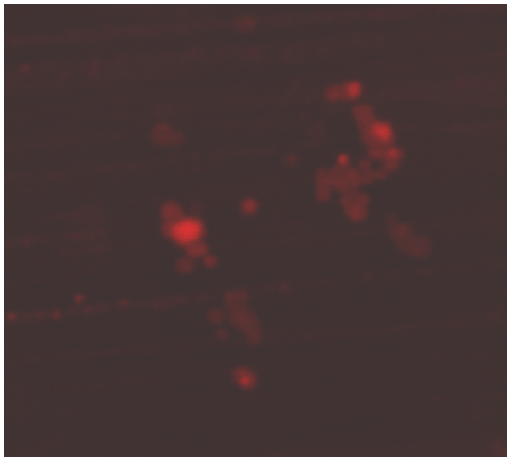


***Figure 7.8:*** *Confocal images of supernatant solution without bacterial cells after incubation of E.coli with Au@OA@C8-AHL for 4 h*

To establish this hypothesis, in a separate experiment, cells of *S. aureus* were also incubated with the conjugate (Au@OA@AHL) and observed under same conditions as *E. coli*. As expected, *S. aureus* did not show fluorescence indicating that it failed to interact with Au@OA@AHL as displayed in Figure 7.9. This shows the inherent specificity of this conjugate to interact with only those cells which possess receptors for AHLs. This indicates that the probe conjugates are capable of accessing the receptor sites within the bacterial cells. Moreover, the negative charge of the probe rules out any electrostatic interaction with the bacterial walls.

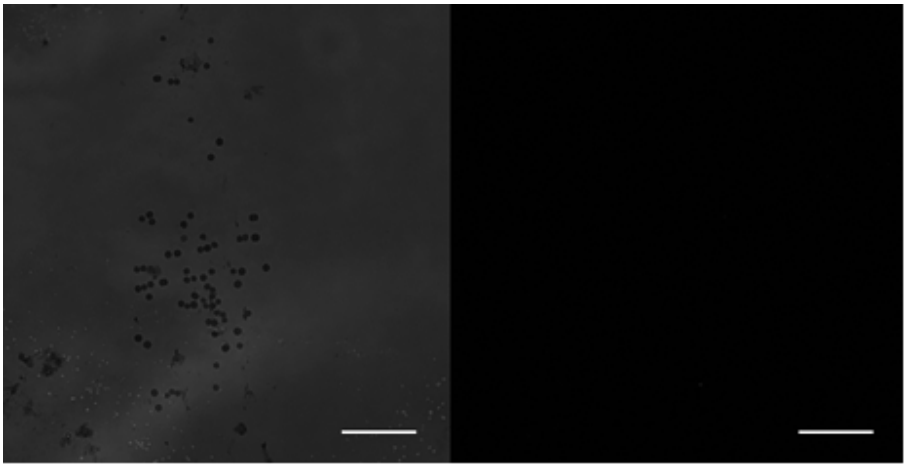


***Figure 7.9****: Confocal microscopy images of S. aureus incubated with Au@OA@C8-AHL: (left) phase contrast image and (right) fluorescence image of the same region. Scale bar is 5µm.*

To further test our hypothesis regarding the structure of the composite and mode of binding, *E. coli* cells were also incubated/treated with two intermediate NCs, namely, Au-thiol@AHL and Au-thiol@oleic acid. These NCs were synthesized in such a way that concentrations of Au-thiol as well as AHL and oleic acid were same as that of the Au@OA@AHL conjugate. Former sample represents the situation where outer surface of Au NCs is not hydrophobic so that lactone moiety may interact directly with cationic ammonium head group of the thiolate layer. In such a case, we envisage non-availability of the lactone head group of the signal molecule for binding to the receptors and hence there is no fluorescent detection of the bacterial cells. The latter indicates a similar case whereby a hydrophobic Au NC without the signal molecule is used. It is possible that the fluorescent Au NCs can indiscriminately access the receptors even without the signal molecules, in which case Au-thiol@ oleic acid should have the ability to be retained within the bacterial cells. Interestingly, no fluorescence could be seen (Fig.7.10) in either of these cases giving credence to the proposed structure model.

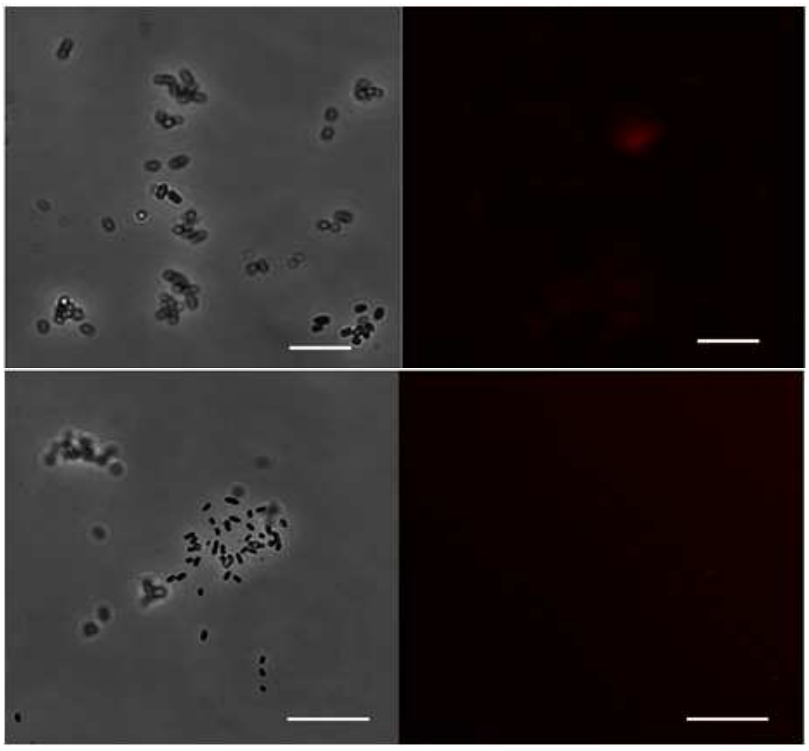


***Figure 7.10:*** *Confocal images of E. coli incubated with Au-thiol@C8-AHL (top) and Au thiol@oleic acid (bottom): (left) phase contrast image and (right)fluorescence image of the same region. Scale bars measure 10μm*

Very fascinatingly it was also found that in a mixed population of *E. coli* and *S. aureus*, gram negative bacterial strain *E. coli* could be exclusively observed during fluorescence imaging (Fig. 7.11). This could be identified due to the shape difference between the two type of bacteria from the phase contrast image, emphasizing the enhanced specificity of the probe. An interesting point worth highlighting is that *E. coli* cells do not produce AHLs but are able to detect and respond to these QS signal molecules by eventual gene regulation. Specific detection of *E. coli* by our probe system further highlights the fact it is the possession of AHL receptor that is imperative and not the growth stage of cell or population density of the cells for this system to function as a potential biosensor.

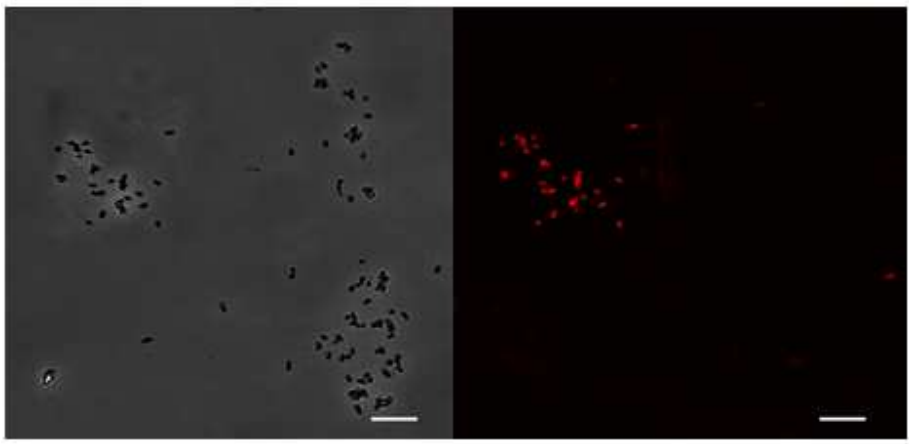


***Figure 7.11****: Confocal images of mixed population of E. coli and S. aureus incubated with Au@OA@C8-AHL: (left) phase contrast image and (right)fluorescence image of the same region. Scale bar is 10μm.*

To ascertain the interaction mechanism, another gram negative bacteria *Chromobacterium violaceum* (wild type) which is a natural producer of AHL molecules was selected. The logic here is that if the receptor sites are already interacting with cognate AHL in the system after the threshold concentration of signal molecules is reached, this will prevent the interaction of the fluorescent probe to these sites. In such a case, bacterial cells incubated with the conjugate should not show any fluorescence and indeed this was observed (Fig.7.12). Also, another test was carried out in which *E. coli* cells were first incubated with excess of free AHL so that the receptor sites are saturated and then this system was incubated with Au@OA@AHL conjugates. The confocal image does not show any indication of interaction by the probe to the bacterial cells (Fig. 7.13).

This indicates that the receptor sites were not available for binding to Au@OA@AHL conjugate due to their saturation with excess free AHL initially added.

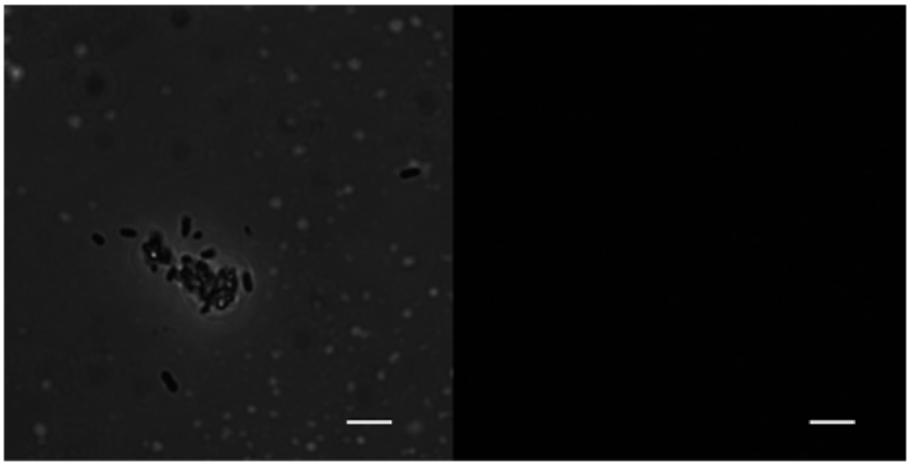


***Figure 7.12**: Confocal images of Chromobacterium violaceum (wild type), which produces AHL incubated with Au@OA@AHL: (left) phase contrast image and (right) fluorescence image of the same region. Scale bar is 2μm*

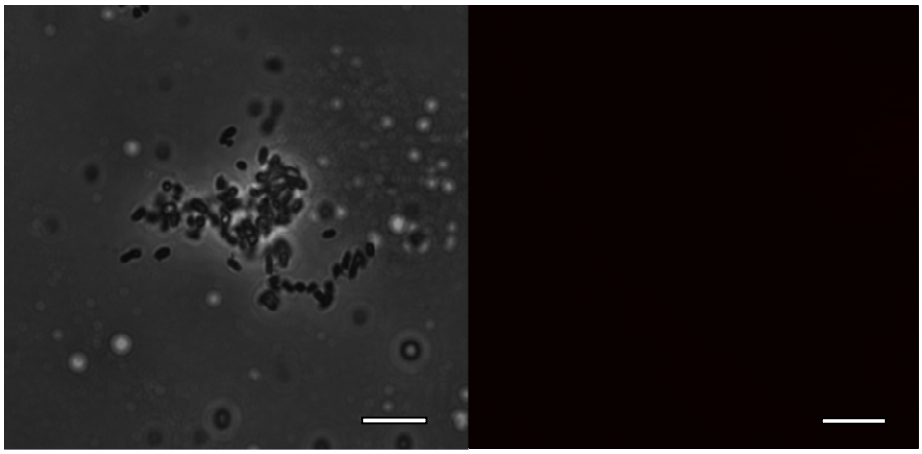


***Figure 7.13**: Escherichia coli + Au cluster + free excess C6-AHL. Free Au cluster even in the presence of C6-AHL does not interact with E.coli cells, hence no fluorescence is observed.*

These two observations prove that the AHL component of the conjugate interacts with the receptor sites specifically and also that these AHL molecules are strongly bound to the fluorescent Au-thiol nanocluster. However, the latter observation may point to a limitation of this material in detecting bacterial strains which produce AHLs at a fully quorate state because of preferential binding of free AHLs compared to those conjugated to the AuNC system.

**7.4. Conclusion**:

In summary, a novel inorganic fluorescent probe capable of selectively binding to receptor sites involved in quorum sensing is developed. The material is based on Au nanoclusters decorated with quorum sensing signal molecules for gram negative bacterial strains, acyl homoserine lactones. The structure was designed such that the bioactivity of the signal molecule, vis-á -vis their lactone and amide groups, is intact after interacting with the fluorescent Au nanoclusters. This probe targets the binding sites for QS molecules within bacterial cells. This property makes these systems independent of cell density and can be used before the bacteria attain a virulent quorate state. This is the first time such inorganic fluorescent probes are used for bacterial detection before quorate state and the excellent specificity for binding sites renders this system indeed ideal for targeted biosensors. The simple synthetic method employed would make it easy to design other such receptor specific fluorescent probes which can be employed in complex biological samples after appropriate sample processing. This technique has the potential to be used in detection of pathogenic as well as environmental bacteria. Further exploration into widening the detection species and understanding of specific binding mechanisms will pave way to more efficient early detection techniques

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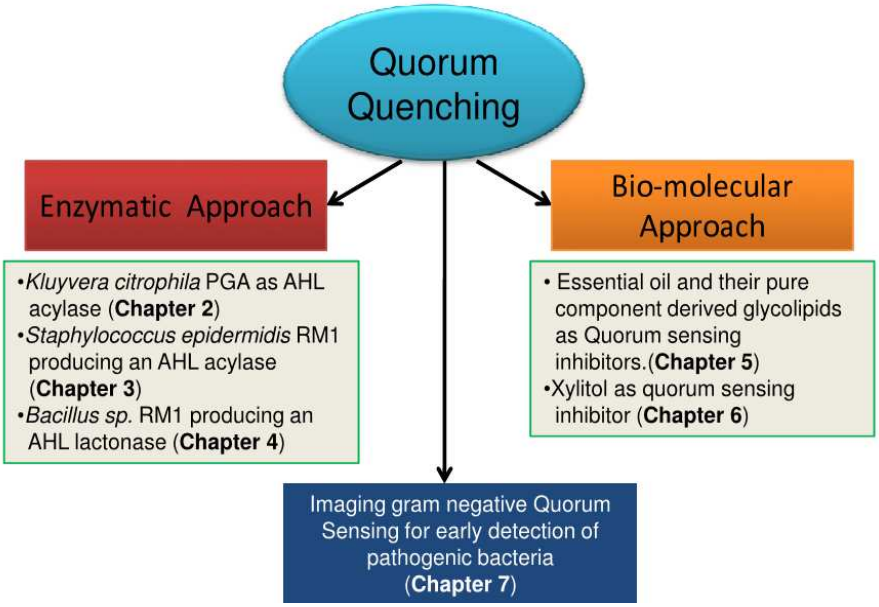
# Summary of thesis




Quorum Sensing or the phenomenon of bacterial communication has been studied in great detail by multiple groups around the world. Moreover with increasing knowledge about this kind of social interaction within the prokaryotic world, it has now become a unanimously accepted fact that targeting QS is a very viable option for development of next generation anti-infective therapeutics. With this aim in mind, the present thesis puts forth some very interesting approaches towards inhibition of quorum sensing in bacteria. Since gram negative pathogens are responsible for a wide variety of hospital-acquired, drug resistant, biofilm associated infections and their virulence genes expression is closely tied to their QS circuits, this thesis focuses on quenching QS specifically in gram negative systems. Both enzymes and small molecules have been investigated for their potential application as a quorum quenching strategy.

Chapter 2 describes a new dimension to the already existing knowledge about the amido-hydrolytic activity of *Kluyvera citrophila* Penicillin G acylase. Ability of *Kc*PGA to cleave medium chain length AHLs and disrupt *Vibrio sp*. biofilm is fascinating and a formulation comprising of immobilized *Kc*PGA, as a quorum quenching acylase, could

well be used for inhibition of biofilm formation in aquaculture and shipping industry. Moreover a directed evolution approach can also be used to tailor this enzyme in such a way, so as to profitably alter its substrate specificity and molecular affinity.

The fortuitous discovery of *Kc*PGA's AHL acylase activity gave the required impetus to check for AHL cleavage potential of other members of Ntn hydrolase super-family. With this in mind, isolation of a BSH producer from a previously unreported source, fermented soy curd was attempted. Surprisingly the isolate, *Staphylococcus epidermidis* RM1, had the ability to degrade both bile salts and AHL molecules. Upon further investigation it became clear that BSH was not responsible for AHL cleavage, rather the organism had two separate enzymes governing the two different activities. However these findings bring forth the possibility of using this, or any such strain, which posses both BSH and quorum quenching AHL acylase activity, as potential probiotic organism. Such a strain would indeed provide us with myriad of possible health advantages.

Another enzyme capable of impeding gram negative AHL mediated quorum sensing is AHL lactonase. Since rhizospheric environments are teeming with quorum sensing and quorum quenching bacteria, an interesting rhizospheric environment was chosen for isolating a potential quorum quencher and has been described in Chapter 4. *Bacillus sp.* RM1 isolated from Fenugreek root nodule rhizosphere was shown to be a very potent producer of an AHL lactonase. Heterologous expression of such QQ enzymes *in planta* has been known to prevent transgenic plants from infecting gram negative pathogens and *aiiA* AHL lactonase gene from *Bacillus sp.* RM1could also find application in such studies.

Available literature to-date provides us with numerous examples of both natural and synthetic inhibitors of bacterial quorum sensing. Among the natural sources, plant derived products, like crude extracts of plant parts, secondary metabolites and essential oils form variety of sources, have received increasing amount of attention. This is probably because these compounds have the ability to inhibit QS mediated phenotypes in diverse gram negative pathogens. Based on these lines Chapter 5A and 5B describe inhibition gram negative QS using plant essential oil and their pure component derived

glycolipids respectively. Hydrophobic molecules like essential oils and their component terpenoids cannot attain full functionality in aqueous environments. In Chapter 5A synthesis of glycolipids, derived from 12 different plant essential oils, some of which have been previously un-characterized in terms of quorum sensing inhibition, has been described. It was very fascinating to see that plant EO that had no QSI activity before became very potent inhibitors of gram negative QS mediated phenotypes. The biggest problem was characterizing these plant EO derived glycolipids, since each EO is made up of more than 10 to 20 components. To alleviate this predicament, two pure monoterpene alcohols, namely Linalool and Alpha terpineol, which formed the major components of many EOs tested in chapter 5A, were used. Chapter 5B talks about a new class of compound, called Glycomonoterpenols. Tremendous increase in QQ potential was observed in Linalool and Alpha terpineol derived Glycomonoterpenols, when compared to the parent compound. These molecules were shown to inhibit important QS mediated phenotypes especially in case of the dreaded gram negative pathogen *P.aeruginosa*. Since essential oils and their components like Linalool are already well recognized for human use, eventual formulation comprising of glyco-derivatives of these molecules, with enhanced quorum sensing inhibitory activity, have a chance of easily getting accepted for potential use in therapeutics.

Another small molecule whose pharmaceutical application is wide spread is xylitol. Chapter 6 describes xylitol synthesis using novel yeast *Pichia caribbica* with very high yield values. Xylitol was shown to be a potent inhibitor of gram negative QS signal reception and was found to be a competitive QS-receptor antagonist using both experimental and *in-silico* tools. This finding definitely broadens the scope of application of this pharmaceutically well accepted molecule.

Application of all these quorum quenching strategies becomes easy if there are tools available to visualize gram negative QS in live bacteria. Chapter 7 talks about synthesis of gold-nanocluster surface functionalized with bacterial QS signal molecules, Acyl homoserine lactones. These probes could specifically interact with bacteria possessing AHL receptors in mixed bacterial population, thus this method has the potential of early detection of gram negative pathogens capable of QS in a given sample. Early detection of

pathogens capable of QS will allow time for the QQ strategies elaborated this thesis to take effect and hence increase the chances of elimination of infecting species.

# List of Publications:

1. **Mukherji, R**., Joshi-Navare, K., & Prabhune, A. (2013). Crystalline Xylitol Production by a Novel Yeast, *Pichia caribbica* (HQ222812), and Its Application for Quorum Sensing Inhibition in Gram-Negative Marker Strain *Chromobacterium violaceum* CV026. **Appl. Biochem. Biotechnol. DOI: 10.1007/s12010-012-0039-4**

2. Singh, P.K., **Mukherji, R**., Joshi, K., Banerjee, A., Gokhale, R.R., Nagane, S., Prabhune, A. & Ogale, S. (2013). Fluorescent Sophorolipid Molecular Assembly and its Magnetic Nanoparticle loading: A Pulsed Laser Process. **Green Chem**. **DOI: 10.1039/C3GC40108A**

3. Book Chapter Titled: *Bacterial Adrenergic Receptor Kinases: Tools for Host-Pathogen cross talk from the Bacterial Virulence Repertoire*" authored by **Mukherji and Prabhune** accepted to be published in Vol. no. 3 of the book series "**RECENT DEVELOPMENTS IN BIOTECHNOLOGY**" by **Studium Press (India) Pvt. Ltd.**

4. **Mukherji, R**., Bhand, A. & Prabhune, A.A. (2013). Production of penicillin V acylase from novel soil actinomycete: Identification of isolate and optimization of physico-chemical parameters. **World J. Biol. Biol. Sci**. Volume 1, 1-10.

5. **Mukherji, R.** & Prabhune. (2014) A. Novel glycolipids synthesized using plant essential oils and their application in quorum sensing inhibition and as anti-biofilm agents. **The Scientific World Journal. DOI: 10.1155/2014/890709**

6. **Mukherji, R.,** Varshney. N.K., Panigrahi, P., Suresh, C.G. & Prabhune, A. A new role for Penicillin Acylases: Quorum Quenching by Kluyvera citrophila Penicillin G Acylase. (2014). **Enzyme Microb. Tech. DOI: 10.1016/j.enzmictec.2013.12.010**

7. **Mukherji, R.**, Samanta, A., Illathvalappil, R., Chowdhury, S., Prabhune, A. &R. Nandini Devi. (2013). Selective Imaging of Quorum Sensing Receptors in Bacteria Using Fluorescent Au Nanocluster Probes Surface Functionalized with Signal Molecules. **ACS Appl. Mater. Inter**. **DOI:10.1021/am404093m**

8. Bhaskara Rao, B.V., **Mukherji, R.,** Shitre, G., Alam, F., Prabhune, A.A. & Kale, S.N. (2013).Controlled release of antimicrobial Cephalexin drug from silica microparticles. **Mat. Sci. Eng. C.DOI:10.1016/j.msec.2013.10.002**

9. **Mukherji, R.** & Prabhune, A. (2014). A rapid iodometric method for qualitative detection of AHL Lactonases. **World J. Pharm. Res**. Volume 3, 1126-1132.

10. **Mukherji, R.** & Prabhune, A. (2015) A new class of bacterial quorum sensing antagonists: Glycomonoterpenols synthesized using linalool and alpha terpineol. (Accepted Article: **World J. Microb. Biot.**)

11. **Mukherji, R.** & Prabhune, A. Possible correlation between Bile salt hydrolysis and AHL Deamidation: *Staphylococcus epidermidis* RM1 a potent quorum quencher and bile salt hydrolase producer. (Manuscript Under Review: **Appl. Biochem. Biotechol.**)

12. **Mukherji, R.**, Prabhune, A. (2015) Enzyme purification and kinetic characterization of AHL lactonase from Bacillus sp.RM1 a novel and potent quorum quencher isolated from Fenugreek root nodule rhizosphere. **Int.J.Curr.Microbiol.App.Sci.** Volume 4(1), 909-924.

## List of Patents:

1. Fluorescent, spherical sophorolipid mesostructures for imaging and therapeutic applications. WO 2013164758 A1

2. PROCESS FOR: Production of Crystalline xylitol using Pichia caribbica (HQ222812) and its application for quorum sensing inhibition in model gram negative bacterium.WO 2014013506 A1

# Appendix: Media Composition

Minimal Medium: (Kumar, A. et al. 2008, **Appl. Biochem. Biotechnol**.)

| Components | g/L |
|---|---|
| $Na_2HPO_4$ | 12.8 |
| $KH_2PO_4$ | 3.1 |
| NaCl | 1.0 |
| $MgSO_4$ | 0.2 |
| Glucose | 4.0 |
| $NaNO_3$ | 3.0 |
| pH | 7.0 |

Trypticase soy broth: (Murray, P. R. et al. 1995, **Manual of clinical microbiology**, 6th ed.)

| Components | g/L |
|---|---|
| Pancreatic digest of casein | 12.8 |
| Soyabean meal | 3.1 |
| NaCl | 1.0 |
| Dextrose | 0.2 |
| $K_2HPO_4$ | 4.0 |
| pH | 7.3 |

Peptone Water base for Carbohydrate fermentation test: (MacFaddin, J. 1980, **Biochemical Tests for Identification of Medical Bacteria**, 2nd ed.)

| Components | g/L |
|---|---|
| Peptic digest of animal tissue | 10.0 |
| NaCl | 5.0 |
| pH | 7.2 |
| 0.5% desired sugar/carbohydrate | |
| 1% Phenol red solution | |

Sabourauds Broth: (Murray, P. R. et al. 1995, **Manual of clinical microbiology**, 8th ed.)

| Components | g/L |
|---|---|
| Peptone mycological | 10.0 |
| Dextrose | 40.0 |
| pH | 5.6 |

King’s Medium B with 15% glycerol: (Murray, P. R. et al. 1995, **Manual of clinical microbiology**, 8th ed.)

| **Components** | **g/L** |
|---|---|
| Proteoses peptone | 20.0 |
| $K_2HPO_4$ | 1.5 |
| $MgSO_4.7H_2O$ | 1.5 |
| pH | 7.2 |