Identification and Characterization of the Quorum Quenching Enzymes in Bacterial Strains Isolated from Sludge of Membrane Bioreactor

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Supervised By

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THESIS ACCEPTANCE CERTIFICATE

It is certified that the contents and form of thesis entitled **"Identification and Characterization of the Quorum Quenching Enzymes in the Bacterial Strains Isolated from Sludge of Membrane Bioreactor"** submitted by Syeda Javariya Khalid have been found satisfactory for the requirement of the degree.

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DECLARATION

I, **Syeda Javariya Khalid,** declare that all work presented in this thesis is the result of my own work. Where information has been derived from other sources, I confirm that this has been mentioned in the thesis. The work here in was carried out while I was postgraduate student at Atta-ur-Rahman chool of Applied Biosciences NUST under the supervision of Dr. Fazal Adnan.

_____

Syeda Javariya Khalid

This thesis is dedicated to my parents who have always stood by my side and supported every decision I've ever made.

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ABSTRACT

Antibiotic resistance has led to the emergence of `superbugs` that are bacteria resistant to multiple drugs commonly prescribed for treatment of bacterial infections. Currently we are left with very limited options to combat these infections. Many novel antibacterial approaches are under investigation. Quorum Sensing has been reported to be extensively involved in causing bacterial infections. It includes synthesis of signaling molecules for intercellular communication of bacteria. Identification of Quorum Sensing signaling molecules has led to the discovery of new targets to inhibit infections. These signaling molecules can be targeted at their production, after production or at their recognition site by receptor protein. This process is known as Quorum Quenching and can serve as a powerful tool to combat multidrug resistant infections without imposing selection pressure on bacteria thus eliminating the risk of antimicrobial resistance. In the present study, bacteria responsible for degradation of signal molecules in the bacterial pathogens have been isolated from sludge of membrane bioreactor. Four of the isolated strains showed maximum growth on minimal media with Acyl Homoserine Lactones (AHLs), signaling molecules in bacteria, as their sole Carbon and Nitrogen source. These strains were characterized for their ability to degrade AHLs and were checked for their inhibition effect on biofilms formed by the pathogenic *Pseudomonas aeruginosa*. After 16S rDNA sequencing, these four strains were verified at the species level as *Bacillus cereus* strain QSP03, *Bacillus subtilis* strain QSP10, *Pseudomonas putida* strain QQ3 and *Pseudomonas aeruginosa* strain QSP01. Presence of three Quorum Quenching enzymes (AHL lactonases and AHL acylases) producing genes, *AiiA*, *PvdQ* and *QuiP* was confirmed via respective primers and sequencing. The AHL degradation capability and identification of respective enzyme producing genes indicates the current bacterial isolates to be potential Quorum Quenching strains.

Chapter 1

INTRODUCTION

A huge number of economic and personal losses are being faced all over the world because of growing number of plant, animal and human pathogens. Mankind was helpless against these severe problems until the foundation of Germ Theory which identified microbes as the agent for transmission and spread of infections (Morens, Folkers et al. 2004). This led to the formulation of antibiotics and further improvements proved to be the revival of history of medicine. This allowed the treatment of those infections and diseases which were once considered catastrophic. However this optimistic approach did not go long in treating these dreadful infections as many pathogens developed resistance against antibiotics and microbial infections continue to be the major cause of large number of illness and deaths all over the world (Livermore 2004). Antibiotic resistance in microbes has caused the rise of `superbugs` that show resistance to many frequently used antibiotics. This whole scenario calls the need to find out some new strategies for treatment against these infectious diseases (Williams 2002).

Traditional antibiotics kill pathogens by obstruction of DNA replication, RNA and protein synthesis so basically interfere with housekeeping genes which put a life and death stress on microorganism. They undergo selection pressure and ultimately become resistant to antibiotics. So to better cope with this problem we not only need the improvements in use and prescription of antibiotics, but in fact novel approaches must be introduced to treat infections. A perfect antibacterial strategy should not impose a life or death stress on bacteria that results in selection of resistant mutants. Quorum Quenching (QQ) is one of such promising strategy that has the potential to be effective treatment against infections (Hentzer and Givskov 2003; Zhang 2003).

This new strategy became important after the realization of `Quorum Sensing` (QS) in bacteria. QS is a communication mechanism in microbes in which microbial cells communicate with each other through signaling molecules. They regulate and monitor changes in population density and trigger the expression of virulent genes and other infection related phenotypes in response to signals which are mostly small molecules such as oligopeptides, fatty acid derivatives or furanones (Zhang and Dong 2004).

Quorum sensing is an important process in bacteria and diligently involved in regulation of virulent phenotypes. A large number of biologically important functions such as antibiotic production, motility, luminescence, plasmid transfer, biofilm formation, virulence factors and many others are controlled by quorum sensing (Whitehead, Barnard et al. 2001). These exciting discoveries about quorum sensing regulated phenotypes especially those related to virulence and biofilm formation and increasing antibiotic resistance issue has caused the scientific community to actively engage in finding some novel approaches to disrupt this communication process. These approaches are collectively known as `Quorum Quenching` (Hentzer and Givskov 2003).

First case of quorum sensing was reported about three decades ago in *Vibrio fischeri* after the detection of a substance that caused bioluminescence in this bacteria even in early growth stage. While normally it induced bioluminescence in this microorganism after entering mid-exponential stage (Nealson, Platt et al. 1970). It was afterwards identified as Acyl Homoserine Lactone (AHL) (Eberhard, Burlingame et al. 1981). These signaling molecules have been identified mainly in Gram negative bacteria. These molecules vary in length and at substitution position in acyl side chain (Fuqua and Greenberg 2002). Bacteria use these molecules to monitor and synchronize their population density and trigger the expression of target genes. AHL are secreted by bacteria themselves in environment and their presence is perceived by cells at a certain threshold and so

response is generated by activation of target gene (Shih and Huang 2002). AHL-based quorum sensing is a well characterized and best identified communication process of bacteria. About 70 species of bacteria are reported to be containing AHL-mediated quorum sensing involving many clinically important pathogens such as *Pseudomonas aeruginosa* (Passador, Cook et al. 1993; Williams, Winzer et al. 2007).

P. aeruginosa is an opportunistic pathogen and responsible for severe infections such as pneumonia in immunocompromised individuals. Its quorum sensing mechanism is well defined and characterized among Gram negative bacteria (Deep, Chaudhary et al. 2011). *P. aeruginosa* infection involves colonization of bacterial cells in upper portion of respiratory tract in patients. This colonization pattern of QS bacteria require a sessile surface for attachment of cells called biofilm (Costerton, Stewart et al. 1999).

Biofilms are cellular communities attached to a substrate and bounded by extracellular matrix which comprises of lipids, nucleic acids, proteins, macromolecules, polysaccharides and many other chemicals (O'Toole 2003; Wei and Ma 2013). Polysaccharides are the main component of matrix that promote attachment to the substrate, maintain the structure of biofilms and protect the cells from environmental stresses such as antimicrobial agents and defense strategies of the host (Tolker-Nielsen 2014).

Biofilms are the key component of dreadful infections caused by many pathogens especially *P. aeruginosa* (Byrd, Pang et al. 2011; von Rosenvinge, O'May et al. 2013). Biofilms help bacteria to survive persistently and also reduce their sensitivity to antimicrobial agents. There has been reports about the possibility of quorum sensing regulated genes conferring resistance against antibiotics to cells in biofilms. Quorum sensing deficient strains of *P. aeruginosa* JP1 and JP2 showed increased susceptibility when treated with antibiotics. This increased sensitivity could be

linked to less Exopolysaccharide (EPS) production and thin biofilms because of quorum sensing deficiency (Shih and Huang 2002).

So quenching the quorum of bacteria has the potential to obstruct the pathogen`s ability to synchronize its cell population and virulence factors. It ensures the time for host to combat infection naturally through immune system (Chen, Gao et al. 2013).

As AHL-based Quorum sensing system is one of the best described communication process of bacteria involving production, secretion and recognition of AHL autoinducers, any strategy to suppress their production could be promising in dealing with life threatening ailments (Ozer, Pezzulo et al. 2005).

There are so many ways to interfere with QS mechanism. For example, many natural substances can imitate AHLs and block their recognition by receptors. These AHL imitates generally block the protein that works as AHL receptor and initiates the expression of target genes (Manefield, de Nys et al. 1999). Some higher plants also produce metabolites that disrupt QS system in microbes. Some plants secret the compounds from their roots that mimic bacterial signaling molecules affecting the gene expression pattern of rhizosphere bacteria (Gao, Teplitski et al. 2003). Inhibitory effect on *P. aeruginosa* QS system and suppression of virulence factors by plant extracts has been reported too (Adonizio, Kong et al. 2008). Synthetic QS inhibitors have also been reported (Reverchon, Chantegrel et al. 2002).

Any factor that disrupts the QS system in bacteria has good potential to be used as a treatment strategies. However AHL degradation achieved through enzymes produced by many other bacteria described as `Quorum Quenching bacteria` is one of the most potent strategy for QS inhibition. Many bacterial species from different genera have been reported for AHL degradation activity. Chemical structure of AHLs suggest four possible ways of their degradation. Enzyme lactonase

and decarboxylase lead to hydrolysis of lactone ring in its structure whereas enzyme acylase and deaminase cleave the molecule in free fatty acids and lactone ring. Two quorum quenching enzymes named AHL lactonase and AHL acylase have been well characterized of all (Dong and Zhang 2005). These enzymes degrade AHLs by using them as substrate. Bacteria producing these enzymes are termed as `Quorum Quenching Bacteria`. These AHL degrading bacteria are usually found in soil or rhizosphere (Yoon, Lee et al. 2006). The availability of oxygen, water, ions and nutrients is highly variable in such wide habitat. Both AHL lactonase and AHL acylase are cytoplasmic in nature and the only extracellular QQ enzyme reported yet is AhlM acylase in *Streptomyces* sp. (Park, Kang et al. 2005).

Why bacteria produce such enzymes which degrade signaling molecules in other bacteria is interesting aspect. Some data suggest that AHLs serve as the source of carbon and nitrogen in QQ bacteria thus acting as energy reservoirs (Chai, Tsai et al. 2007). V*ariovorax paradoxus* is reported for such activity as it uses fatty acids obtained as result of AHL degradation (Yang, Han et al. 2006). *Nocardioides kongjuensis* is a soil bacterium and utilizes AHL as their carbon source (Yoon, Lee et al. 2006).

Expression of such enzymes also ensure the survival of bacterial species in competition for limited resources in environment as in the case of *Bacillus thurigiensis* and *Pectobacterium carotovorum. B. thurigiensis* does not affect the growth of *P. carotovorum* but limits its adaptability because of quorum quenching enzyme synthesis (Dong, Zhang et al. 2004). So these AHL degraders can switch quorum sensing on and off and can play important role in mutual interactions i.e. both pathogenic and symbiotic.

Chapter 2

REVIEW OF LITERATURE

2.1 Quorum Sensing Bacteria:-

QS Regulate the expression of genes that control activities useful when performed by group of bacteria. There is a difference in QS systems of Gram positive and Gram negative bacteria. Gram negative bacterial QS system involves the production of small molecules called autoinducers. These may be the S-adenosylemethionine (SAM) dependent molecules or Acyl Homoserine lactones (AHLs). Once produced in the cell they can freely move through inner and outer membranes and at threshold level which occurs at high cell density, they bind to cytoplasmic transcription factor working as receptors. So the expression of genes in QS regulon is regulated through AHL bound transcription factors (Rutherford and Bassler 2012).

2.1.1 Quorum Sensing in Gram Positive Bacteria:

In Gram positive bacteria, small molecules called auto-inducing peptides (AIPs) are produced. They undergo processing after production in bacterial cells and secreted. When their concentration reaches at a threshold, which usually occurs at high cell density, they bind to an associated receptor protein histidine kinase. It's a membrane bounded receptor. This binding initiates kinase activity which autophophorylates and transfer this phosphate to associated cytoplasmic regulator to generate response. This phosphorylated regulator generates response by activation of target genes in QS regulated system (Rutherford and Bassler 2012).

The gram positive quorum sensing system differs from that of gram negative and to date no gram positive bacteria has been reported to produce AHLs as signaling molecules. The gram positive

bacteria regulate gene expression by making use of signaling molecules called oligopeptides which are small post-translationally processed peptide signaling molecules. These signaling molecules are secreted and then are either reinternalized via an oligopeptide transport system or detected at the bacterial surface by two-component systems (Monnet and Gardan 2015).

2.1.2 Quorum Sensing in Gram Negative Bacteria:

LuxI/ LuxR type Quorum Sensing system exists in Gram negative bacteria (Hastings and Greenberg 1999). The main Gram Negative bacterial species belonging to proteobacteria contain AHL- mediated Quorum Sensing system (Hardman, Stewart et al. 1998). AHLs are primary signaling molecules in Gram negative bacteria. They are small, neutral lipids and amphipathic in nature. AHL synthesis has been discovered in about 90 species of bacteria. Total three enzymes have been reported yet to produce AHLs. They belong to LuxM, HdtS and LuxI families (Waters and Bassler 2005). The typical example of LuxI type AHL synthase was first described in *Vibrio fischeri.* S-adenosyl Methionine (SAM) and acyl-acyl Carrier protein (acyl-ACP) serve as substrate for production of AHLs in LuxI type AHL synthase (Hanzelka and Greenberg 1996). In Vitro studies have confirmed the requirement of SAM and acyl-ACP substrates for AHL production (More, Finger et al. 1996).

The most well characterized and most widespread class of AHL synthase is LuxI type. Its homologues have been found in about 150 bacterial species of proteobacteria belonging to alpha, beta and gamma classes. AHL synthase vary greatly in AHL synthesis. However they are somehow grouped on the basis of their structural and phylogenetic relationship. A domain of about 205 amino acid residues exist in these enzymes. More than 20% identity and 40% similarity is conserved between any two members of these classes (Watson, Minogue et al. 2002). *Pseudomonas aeruginosa* is a member of class gamma and its LuxI synthase is more similar to

AHL synthase enzymes of alpha and beta classes of proteobacteria than to gamma class AHL synthase enzyme Esa1. In spite of these differences, there is a stretch of completely conserved amino acids that describes the AHL synthase family belonging to LuxI-type (Hanzelka, Stevens et al. 1997; Parsek, Schaefer et al. 1997).

A broad range of AHLs is produced by different bacterial species. They range from C_4-HSL to C_{18}-HSL produced by *Pseudomonas aeruginosa* and *Sinorhizobium meliloti*. They differ in length of acyl chain (Pearson, Pesci et al. 1997; Marketon, Gronquist et al. 2002). They also vary in oxidation state at C3 position (Watson, Minogue et al. 2002).

As AHLs serve as signaling molecules and control a wide spectrum of QS dependent phenotypes. Better understanding of AHL-mediated QS can help us to effectively use natural and synthetic inhibitors to block AHL signaling and control of diseases (Leadbetter and Greenberg 2000; Smith, Bu et al. 2003).

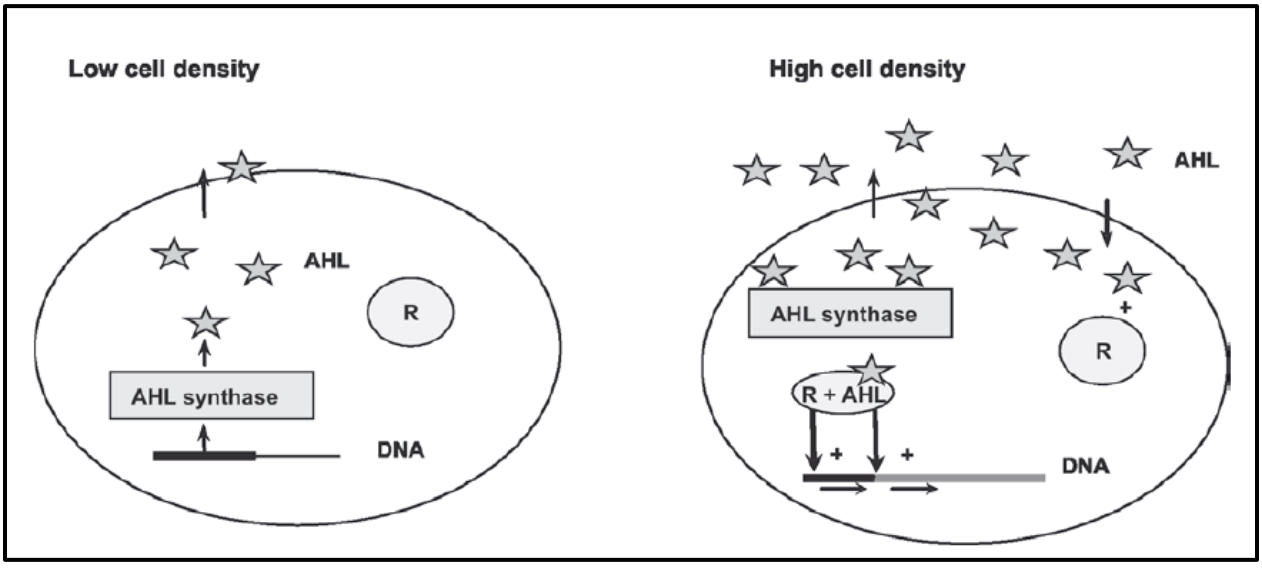


Figure 1. AHLs produced by bacterial cell can diffuse passively or actively in and out of cell. At low cell density (Left side), they are not detected by receptor protein. However, at higher cell density (Right side) they accumulate and when threshold reached, they are detected by regulatory protein which is mostly a transcription factor. This complex of AHL and protein bind to promotor region of target gene and activate it. Adopted from (Czajkowski and Jafra 2009).

So, the key steps involved in Quorum Sensing mechanism of Gram Negative bacteria are

- Signal generation i.e. AHL
- A buildup of this signal
- Detection of signal by receptor protein
- Target gene expression

2.2 Antibiotic Resistance and Emergence of Superbugs:-

The term superbugs refer to microbes endowed with high levels of resistance to antibiotics specified for their treatment due to multiple mutations. This evolution of multi drug resistant pathogens such as Methicillin Resistant *Staphylococcus aureus* (MRSA) and multidrug resistant (MDR) *Mycobacterium tuberculosis* emerged subsequent to the wide and frequent use of antibiotics. Other resistant pathogens associated with nosocomial infections include with *Acinetobacter baumannii, Burkholderia cepacia, Campylobacter jejuni, Citrobacter freundii, Clostridium difficile, Enterobacter spp., Enterococcus faecium, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella spp., Serratia spp., Staphylococcus aureus, Staphylococcus epidermidis, Stenotrophomonas maltophilia, and Streptococcus pneumoniae.* Essentially antibiotic resistance can also be considered a virulence factor since often the super resistant strains have also acquired enhanced transmissibility and increased virulence (Davies and Davies 2010).

The predominant role of humans in the generation of environmental reservoirs of antibiotic resistance cannot be denied. Since little selection pressure is contributed by the naturally occurring antibiotic strains in their environment (Gottlieb 1976), it is safe to assume that the commercial

production is largely responsible for the mass release of these antibiotics into the biosphere (Davies and Davies 2010).

2.3 Biofilms Resist Antimicrobial Agents:-

During the cascade of environmental stresses, most of bacterial cells dwell in the biofilms. These are substrate attached colonies of bacterial cells and provide protection in case of harsh environment (Bayramov and Neff 2017). Biofilms are produced as a result of intercellular communication process of bacteria i.e. Quorum Sensing. Biofilm formation leads to enhanced antibiotic resistance (Shih and Huang 2002). Antibiotics are currently the main strategy to treat biofilms. However resistance to antimicrobial reagents is well known in the case of biofilm forming bacteria. Physiological adaptations to the environment by biofilms is the main strategy for their resistance (Brown, Allison et al. 1988). Role of quorum sensing in biofilm formation is well reported in *P. aeruginosa* (Davies, Parsek et al. 1998). N-(3-oxo-dodecanoyl)-L-Homoserinelactone (3-oxo-C_{12}-HSL) and N-butanoyl-L-Homoserinelacton (C_4-HSL) are main quorum sensing signaling molecules identified in *P. aeruginosa* (Pearson, Passador et al. 1995).

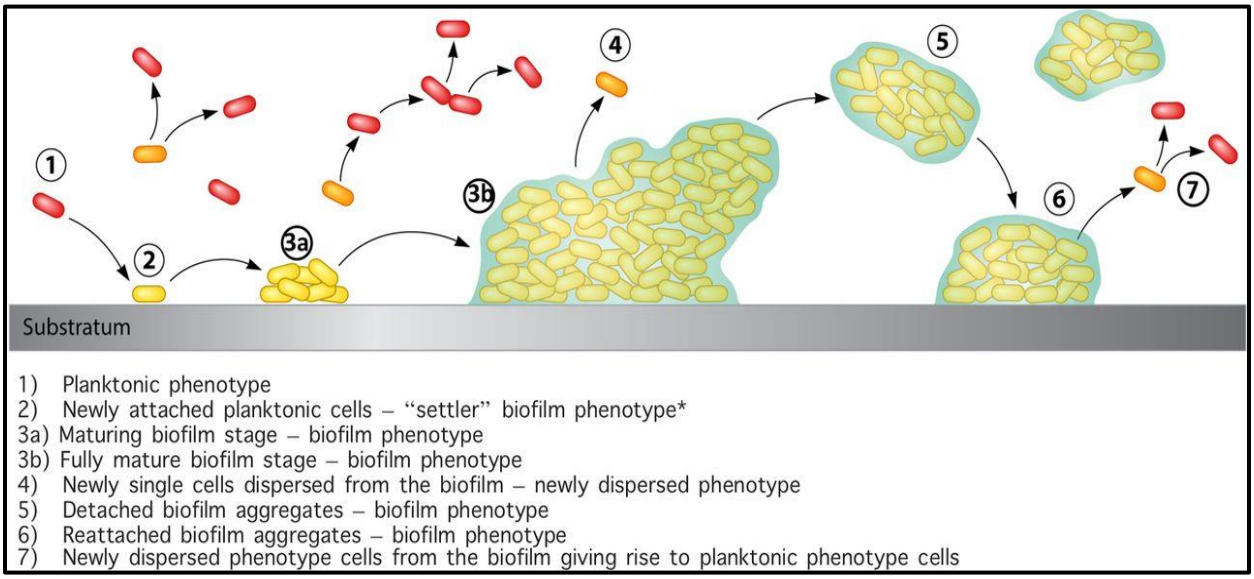


Figure 2. Various steps involved in *P. aeruginosa* biofilm formation as a result of Quorum Sensing. Adopted from (Cogan, Harro et al. 2016)

AHL signal generation initiates a number of biological activities in biofilm forming bacteria such as upregulation of virulence, adhesion, antibiotic resistance and many other metabolic changes. A protective glycocalyx is also formed in process (Khardori and Yassien 1995; Wu and Grainger 2006; Rodriguez-Rojas, Rodriguez-Beltran et al. 2013). Disrupting the bacterial adhesion process before the production of glycocalyx is a potential strategy to combat virulence in place of those antibacterial strategies which impose selection pressure on bacteria (Vasilev, Cook et al. 2009; Cavallaro, Taheri et al. 2014). Biofilms once formed make it very difficult to kill planktonic cells too. Protective glycocalyx shields the cells from antibacterial agents and a main factor in survival of infection (Costerton, Stewart et al. 1999).

Secretion of extracellular matrix in biofilms provide a protective niche to bacterial cells where they can grow, proliferate and acquire antibiotic resistance through transfer of genetic material among them (Arciola, Campoccia et al. 2005; Campoccia, Montanaro et al. 2010).

Glycocalyx is not the only factor in development of resistance in bacterial population, in fact the presence of heterogeneous subpopulations of bacteria differing in a range of antibiotic susceptibility are also the reason for antibiotic resistance (Kester and Fortune 2014). Antibiotic degrading enzymes may also be produced by these subpopulations (Hoiby, Bjarnsholt et al. 2010). Bacterial cell community within a biofilm also confer oxidative stress on membrane causing them to acquire an enhanced state of mutability (Boles and Singh 2008; Driffield, Miller et al. 2008; Conibear, Collins et al. 2009). So the biofilms are described as responsible for resistance against broad range of antibiotics (Campoccia, Montanaro et al. 2010; Hoiby, Bjarnsholt et al. 2010).

Metabolically aged populations of bacteria usually reside within biofilm (Kester and Fortune 2014). It's an important aspect to develop therapeutic control because antibiotics act differently e.g. beta lactams attack and degrade cell wall synthesis in actively growing *P. aeruginosa* (Tenover

2006; Hoiby, Bjarnsholt et al. 2010) and quinolone insert nicks in replicating DNA (Gradelski, Kolek et al. 2002). In vitro experiments have repeatedly established that slow growing or no growing cells are less susceptible to antibiotics (Finkel 2006). Cells in the stationary phase show enhanced survival and persistence when treated with antibiotics as compared to those in logarithmic phase (Jayaraman 2008). Key component of pathogenicity is biofilm formation. So many transcription factors collectively regulate its formation in an intricate pattern. They can reverse their substrate attachment with the help of flagella, pili and fimbriae but only when they are loosely attached. However their attachment becomes irreversible after the secretion of adhesion molecules and extracellular matrix. When a biofilm matures, production of extracellular matrix also increases which helps to maintain the biofilm in three dimensional structure (Sauer, Camper et al. 2002; Agladze, Jackson et al. 2003). Other virulent genes are also activated after successful establishment of biofilms through Quorum Sensing (Lebreton and Cossart 2017). Virulence and antibiotic resistance is spread among cells effectively within biofilms due to compact association of cells and the process is enhanced under stress conditions such as antimicrobial exposure (Deziel, Gopalan et al. 2005). Increased biofilm formation was seen in antibiotic resistant strains of *P. aeruginosa* (Drenkard and Ausubel 2002). Biofilms consisting of single bacterial species have been dreadful as in case of cystic fibrosis in patients of *P. aeruginosa* infections. However the identification of biofilms comprising of multiple bacterial species have worsen the situation even more as compared to infections caused by multiple bacterial species comprising of planktonic cells (Lee, Periasamy et al. 2014). In vivo studies showed longer healing time for wound and increased host immune response in host rabbit infected simultaneously by *S. aureus* and *P. aeruginosa* (Seth, Geringer et al. 2012; Pastar, Nusbaum et al. 2013).

2.4 Enzymatic Quenching of Quorum:-

In general, any strategy that can disrupt Quorum Sensing can be potentially adapted to quench quorum sensing and combat infections. In last some years, so many strategies have been identified to quench the quorum of bacteria. These include synthetic chemicals and halogenated furanone produced by *Delisea pulchra* that inhibit regulatory R protein (Hentzer and Givskov 2003), synthetic analogues of AHLs and AIPs that mimic the original quorum sensing signals (Lyon, Mayville et al. 2000; Smith, Bu et al. 2003), and the enzymes that degrade AHLs known as Quorum Quenching enzymes such as paraoxonases, AHL acylase and AHL lactonase . QQ enzymes are divided into two classes on the basis of their mode of AHL degradation. Class I includes AHL lactonase which inactivates AHL by hydrolysis of lactone ring and AHL acylase that cleaves the bond between fatty acids and homoserinelactone. This class also includes paraoxonases. These enzymes generally inactivate AHLs by breaking their structure. Whereas class II includes oxidoreductases which reduce carbonyl to hydroxyl (Dong, Xu et al. 2000; Leadbetter and Greenberg 2000).

2.4.1 AHL Lactonase:

AHL lactonase is a quorum quenching enzyme reported for AHL degradation through hydrolysis of lactone ring in its structure. The gene AiiA encoding AHL lactonase was first time reported in *Bacillus* sp. Strain 240B1. It was successfully cloned in *Escherichia coli* to study its effect on AHL substrate. When expressed in the transformed pathogen *E. carotovora* strain SCG1, it successfully decreased its pathogenicity for Chinese cabbage, cauliflower, tobacco etc. (Dong, Xu et al. 2000). Plants cloned with this gene showed enhanced resistance to soft rot (Dong, Wang et al. 2001). AHL degrading mechanisms vary in different organisms. *variovorax paradoxus* use AHLs as their carbon and nitrogen source. This confirms the presence of AHL degrading enzymes in this

bacteria. AHL lactonase contains 'His^{104}-X-His^{106}-X-Asp^{108}-His^{109}' motif that is similar to zinc binding motif of metalloenzymes (Dong, Xu et al. 2000).

Two families of lactonases have been discovered yet. AiiA lactonase is well studied and characterized and needs Zn^{2+} ions for effective activity (Kim, Choi et al. 2005; Riaz, Elmerich et al. 2008). Substitutions in AHL structure and length of side acyl chain does not affect this enzyme`s activity. QsdA is another type of lactonase found in *Rhodococcus erythropolis*. Both QsdA and AiiA are Zn^{2+} dependent (Uroz, Oger et al. 2008). Other type of lactonases have also been discovered such as AidH, AiiM and BpiB (Schipper, Hornung et al. 2009; Mei, Yan et al. 2010; Wang, Morohoshi et al. 2010). Zinc ion dependence is common in all of these lactonases and found in different bacterial genera such as *Klebsiella* (Park, Lee et al. 2003), *Pseudomonas* (Sio, Otten et al. 2006), *Arthrobacter* (Park, Lee et al. 2003), *Streptomyces* (Park, Kang et al. 2005) , *Rhodococcus* (Park, Hwang et al. 2006), *Agarobacterium* (Carlier, Uroz et al. 2003), *Bacillus* (Lee, Park et al. 2002).

2.4.2 AHL acylase:-

This QQ enzyme breaks the amide bond between homoserine part and acyl chain fatty acids releasing homoserine lactones in the process (Lin, Xu et al. 2003). This enzyme was first time discovered in *Variovorax paradoxus* strain VAI-C showing enhanced level of AHL degradation (Leadbetter and Greenberg 2000).

It has also been reported in a variety of bacterial species e.g. *Ralstonia* sp. XJ12B 10, QuiP and PvdQ genes in *P. aeruginosa* PAO1 (Sio, Otten et al. 2006), AhlM in *Streptomyces* sp. M664 (Park, Kang et al. 2005). Resemblance to Ntn Hydrolases is common in almost all AHL acylase enzymes. They show a specificity for substrate commonly depending on the length of acyl side chain e.g. both long chain and short chain AHLs are degraded by AiiD, AHLs shorter than eight

carbons are not degradable by PvdQ gene whereas AhlM can show degradation activity only for AHLs having shorter than eight carbon chain (Lin, Xu et al. 2003).

2.4.3 Oxidoreductases:

These enzymes bring changes in chemical structure of AHLs instead of degrading them. This cause the change in activation state of signaling molecule (Chan, Atkinson et al. 2011). These enzymes can oxidize AHLs at various points.

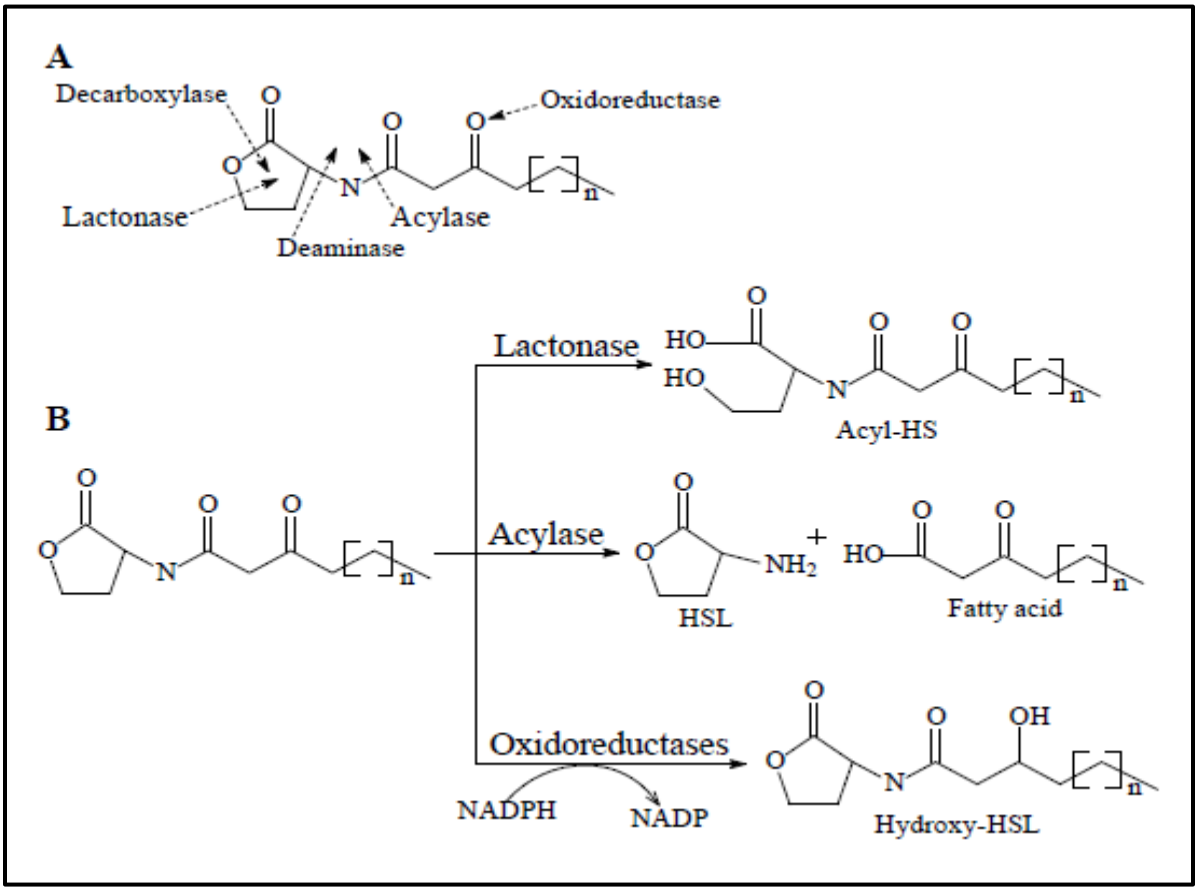


Figure 3. Possible sites for degradation on AHL structure (A). Possible mechanisms of degradation by QQ enzymes (B). Adopted from (Chen, Gao et al. 2013).

2.5 QQ Enzymatic Strategy to Control Diseases:-

Quorum quenching is a potential strategy to control infections than antibiotics as it imposes least risk of selection pressure on microbe. This approach has successfully decreased plant diseases

when used as therapeutic tool. In co-culture of quorum quenching bacteria along with QS pathogen, the QQ enzyme successfully diminished the QS associated phenotype. Similarly transgenic plants transformed with gene encoding QQ enzyme showed much decreased susceptibility to infection when encountered with QS pathogen.

QQ strategy was first time applied when *Erwinia carotovora* was transformed with AiiA gene. This diminished its decay phenotype in Chinese cabbage (Dong, Xu et al. 2000). Bacterial cells in biofilms are very much resistant to antibiotics and macrophages because of its adaptability to environment and proactive components. Dental plaque is an excellent example of biofilms.

Biofouling on membrane bioreactors (MBR) has greatly decreased the efficiency of this system for wastewater treatment. Biofilms grow in membrane bioreactor are difficult to remove causing economic losses. Experimental data suggests the effectiveness in controlling the QS mediated biofilms in MBR without compromising the performance of membrane. a magnetic carrier containing AHL acylase was applies to MBR and it significantly inhibited the biofouling (Yeon, Lee et al. 2009). Nanofilteration membranes are also incorporated with AHL acylase to interfere with QS biocake formation in MBR and significant biofouling reduction was seen (Xiong and Liu 2010).

A large number of pathogens have been found to use AHLs as their signaling molecules to develop virulence. So QQ is promising strategy for control of such infections. AiiA is required by rhizosphere bacteria for colonization and its defective strain is unable to grow in plant root. This suggests the importance of lactonase to make bacteria competitive enough to survive on plant root (Gao, Chen et al. 2007).

So QQ enzymes are highly anti-QS and bacteria expressing them can successfully prevent infections and can use signaling molecules of neighboring bacteria as their source of energy. QQ

enzymes also eliminate infections without posing risk of antibiotic resistance. They can be effective antibacterial therapy to combat dreadful infections by QS bacteria.

Research Objectives:-

1. Isolation and Identification of Quorum Quenching Bacteria
2. Confirmation of their Quorum Quenching activity through AHL degradation assay
3. To analyze Quorum Quenching effect of current bacterial isolates on *P. aeruginosa* biofilm
4. Identification and confirmation of genes responsible for production of Quorum Quenching enzymes

Chapter 3

MATERIALS AND METHODS

3.1 Bacterial Isolation and Identification:

Bacterial consortia was isolated from sludge samples collected from a full-scale membrane bioreactor situated at H-12 Campus of National University of Science and Technology Islamabad. A previously used established method was used for isolation of bacterial species able to survive on AHLs as the sole carbon source (Christian et al., 2011).

3.1.1 Sample Collection and Isolation of Bacteria:

Sludge samples were collected from a full-scale membrane bioreactor from three points: biotank, membrane tank and sludge wastage point. The samples collected in glass bottles were mixed and prepared within an hour for subsequent bacterial isolation. In laboratory, the following steps were performed for sample preparation:

- Proper mixing of the sample collected from three points of membrane bioreactor.
- A 5 ml of sample mixed with 50 ml of 0.5% normal saline solution.
- Mixing and two-minute vortex with half minute sonication.
- Centrifugation at 3000 rpm for a minute and removal of debris.
- Collection of supernatant and another cycle of centrifugation for five minutes at 4500 rpm.
- Re-suspension of pellets in 15 ml of saline solution (0.9%).
- The suspension formed is used for further bacterial isolation.

A total 200 milliliter minimal media was prepared for enrichment process of bacterial isolation. The composition per liter is provided as annexure- A. The preparation steps include:

- Compounds (annexure A) were dissolved in 190 ml distilled water and before adding trace elements, the solution was acidified to 5.5 by adding 1M HCL.
- Distilled water was used to fill the volume to 200 ml.
- After final addition of trace elements, media was filtered through 0.45 mM syringe filters.
- Finally, the media was autoclaved for sterilization.

AHL standards were supplemented during the enrichment process for isolation of quorum quenching bacterial species as the sole source of Carbon and Nitrogen. AHL standard used were;

- N- (3-oxo-hexanoyl)-L-homoserine lactone ($3OC_6HSL$) standard of 0.5 mg/ml
- N-(3-oxo-dodecanoyl)-L-homoserine lactone ($3OC_{12}HSL$) standard of 0.5 mg/ml
- N-(3-Hydroxybutanoyl)-L-homoserine lactone
- N-(3-Oxodecanoyl)-L-homoserine lactone

3.1.2 Enrichment method for bacterial isolation

Experiment was set up in 1.5 ml centrifuge tubes. In all the reactions, a mixture of all AHLs was provided as sole carbon and nitrogen source. Blank containing distilled water was included. The enrichment process was concluded in the following manner:

- 750 µl of the inoculums prepared from the sample was added to the six micro-centrifuge tubes
- 700 µl of the minimal media was added in each tube including blank containing 750 µl distilled water

- 10 µl of mixture AHLs was added to the set of micro tubes containing the sample suspension and minimal media
- All tubes were incubated at 37°C for three days during the first cycle
- 100 µl of the culture was transferred to 1 ml fresh minimal media with addition of AHLs in the same quantities
- The samples were incubated for three more days at the same temperature
- The cycle was repeated again in a similar fashion. After third enrichment cycle, 100 µl of the aliquot was spread on nutrient media.

3.1.3 Colony purification:

On the spread plates, limited number of colonies appeared that had survived on AHLs as sole carbon source. Each colony was picked and streaked on separate agar plates. The plates were incubated for 48 h at room temperature. Based on morphological difference, colonies were further separated and streaked again until same colonies were achieved on separate nutrient agar plates. The subcultures were streaked multiple times until pure cultures were achieved.

Table 1. Composition of minimal media prepared for bacterial isolation from sludge of membrane bioreactor.

Compounds	**Quantity per Liter (grams)**
Sodium chloride	1
Potassium chloride	0.5
Magnesium chloride	0.4
Calcium chloride	0.1
Sodium sulphate	0.15
Potassium dihydrogen phosphate	2
Disodium phosphate	2.25
Trace elements per Liter (milligrams)	
Ferric chloride	1
Manganese(II) chloride	100
Zinc chloride	46

3.1.4 Preparation of Cryostocks:

Pure cultures obtained were preserved by making their cryostocks. A 2 ml of overnight liquid cultures were taken in centrifuge tubes and centrifuged at 5000 rpm for 5 min. Supernatant was discarded and again 1 ml of culture was added and centrifuged. The step was repeated until thick pellet was obtained. Then pellet was re-suspended in 1 ml of LB media and mixed with 0.5 ml of glycerol and transferred to cryovials labelled with strain name and date. These cryovials were kept in liquid nitrogen for 5-10 min and then transferred to -20°C and -80°C for long term storage.

3.2 Biochemical Analysis:-

Following tests were performed for biochemical analysis of bacteria.

3.2.1 Gram Staining:

A fresh colony of each bacterial strain was picked and heat fixed on glass slide by passing it over flame. Fixed smear was then flooded with crystal violet solution and allowed to remain for 1 min. Crystal violet was rinsed with distilled water. Slides were then flooded with iodine solution and allowed to remain for 1 min. Iodine solution was rinsed with distilled water. Slides were then flooded with decolorizer (mostly alcohol) for 1-5 seconds. Decolorizer was rinsed with distilled water. Slides were flooded with safranin and allowed to remain for 1 min. Slide were then dried on a tissue paper and placed in upright position.

Slides were examined under compound microscope and retained color of stain was observed. Bacterial morphology was observed too.

3.2.2 Catalase Test:

Four to five drops of 3% hydrogen peroxide were added in a test tube. A well isolated 18-24 hour colony of test microorganism was collected by sterile loop and placed in a test tube. Test tube was placed against a dark background and immediate bubble formation was checked.

3.2.3 Oxidase Test:

The analysis was carried out by filter paper test method. A small piece of filter paper was soaked in 1% Kovac`s oxidase reagent and let dry. A well isolated colony was picked from 18-24 hour culture and rubbed onto treated filter paper. Change in color of treated filter paper was observed.

3.2.4 Growth on EMB agar:

EMB agar plates were prepared to test the growth of bacterial isolates on it. 18.75 g of EMB agar was dissolved in distilled water and autoclaved. Plates were prepared and each bacterial isolate was streaked on it. Plates were incubated for 24 hours and color change was observed.

3.2.5 Growth on MacConkey agar:

MacConkey agar plates were prepared to check the growth of bacterial isolates on MacConkey agar. A 25 g of MacConkey agar was dissolved in 500 ml of distilled water and autoclaved. Plates were prepared and each bacterial isolate was streaked and colony color was observed after incubation of 24 h.

3.3 AHL Degradation Assays:-

AHL degradation tests were performed by agar overlay method adopting the protocol described by (Chen, Chen et al. 2009; Lade, Paul et al. 2014) with some modifications and disk diffusion method.

3.3.1 Agar Overlay Method:

Genetically engineered microorganisms, *chromobacterium violecium* CV026 and *Agarobacterium tumefaciens* A136 were used to make indicator agar plates to confirm quorum quenching.

- CV026 was grown in LB with 20 µg of Kanamycin for overnight.
- 50 µl from overnight culture of CV026 was added to 5 ml molten LB agar.
- It was poured on the surface of pre-warmed LB agar plates.
- The overlay plate was left for solidification in sterile conditions.
- About 6 mm holes were made in overlay plate and 50 µl of each bacterial isolate was poured in them along-with 5 µM of C_6-HSL.
- A well was left in which no AHL degrading agent was added. Only C_6-HSL was added in it.
- Plates were incubated for 48 h at 28° C. Color in surrounding areas of well was observed.

CV026 is for the confirmation of short chain AHLs. To confirm the degradation of long chain AHLs by bacterial isolates, indicator plates of A136 were prepare.

- A136 was grown in LB with 50 µg/ml of Spectinomycin and 4.5 µg/ml of Tetracycline for overnight.
- A 50 µl from overnight culture of A136 was added to 5 ml of molten LB agar supplemented with 80 µl of X-gal.
- It was poured on the surface of pre-warmed LB agar plates and left for solidification in sterile conditions
- About 6 mm holes were made in overlay plate and 50 µl of each bacterial isolate was poured in them along-with 5 µM of C_{10}-HSL.

- A well was left in which no AHL degrading agent was added. Only C_{10}-HSL was added in it.
- Plates were incubated for 48 hours at 28°C. Color in surrounding areas of well was observed.

3.3.2 Disk Diffusion Method:

AHL degradation assay was performed by Disk Diffusion method as described by (Chan, Atkinson et al. 2011; Chong, Koh et al. 2012)

CV026 was grown in LB with 20 µg of Kenamycin for overnight.

- A 50 µl from overnight culture of CV026 was added to 5 ml molten LB agar.
- It was poured on the surface of pre-warmed LB agar plates.
- The overlay plate was left for solidification in sterile conditions.
- Sterile 0.45 µM filter paper was dipped in 10 mg/ml of C_6-HSL.
- The soaked filter paper was put over the respective indicator plates.
- Fresh colonies from 24 h growth on plates of QQ bacterial isolates were loaded on filter paper.
- All the plates were incubated for 48 hours at 28° C.
- In colored background, colony color was noted and compared.
- Process was repeated to check long chain AHL degradation by A136.
- A136 was grown in LB with 50 µg/ml of Spectinomycin and 4.5 µg/ml of Tetracycline for overnight.
- A 50 µl from overnight culture of A136 was added to 5 ml of molten LB agar supplemented with 80 µl of X-gal.

- It was poured on the surface of pre-warmed LB agar plates and left for solidification in sterile conditions.
- After solidification of agar, a sterile membrane filter disk was dipped in 10 mg/ml of C_{10}-HSL and placed over the surface of agar.
- Fresh colonies from 24 hour culture of each bacterial isolate were loaded on membrane filter disk.
- Plates were incubated for 48 hours at 28°C. After incubation color of plates was checked.

3.4 AHL Degradation assay by Microtiter Plate Method:-

Microtiter plate assay was carried out to quantify the amount of AHL degradation by quorum quenching bacteria. Cell free lysates of QQ bacterial isolates were prepared for this assay (Christiaen, Brackman et al. 2011).

3.4.1 Cell Free Lysate Preparation:

Isolated bacterial strains were incubated in LB for 48 h at 30°C in shaker incubator. The culture was centrifuged at 12,000 rpm for 10 min. The cell pellets were collected and re-suspended in 100 mM potassium phosphate buffer with pH set at 7.0. This suspension was sonicated and centrifuged. Supernatant obtained was passed through 0.45 µM syringe filter. Filtrate obtained by this method is “cell free lysate " and stored at -20°C until use.

3.4.2 Quantification of AHL Degradation by Microtiter Plate Assay:

AHL degradation was quantified by previously described method (Zhu, He et al. 2011).

- CV026 was incubated for 18 h in LB in shaker incubator.

- A 50 µl of this culture was inoculated to fresh LB medium supplemented with 2 µl of 0.5 mg/ml of C_4-HSL and 100 µl cell free lysate of bacterial isolates in 100 ml conical flasks. Incubation was carried out at 30°C at 120 rpm for 24 hours in shaker incubator.
- A 1 ml culture from each flask was then poured in microcentrifuge tubes and 1 ml of dimethyl sulphoxide was added in it. It was centrifuged at 8000 rpm for 5 min to solubilize violacein and for cell removal.
- A 200 µl of violacein containing supernatant was added to wells of 96-well microtiter plate. Harvested bacterial cells were resuspended in 1 ml of sterile water and 200 µl was transferred to wells of microplate.
- Absorbance was read in microplate reader at 550 nm wavelength for violacein quantification and at 630 nm wavelength to evaluate cell growth. The results were compared with assay controls which contained CV026 supplemented with C_4-HSL but without cell free lysates.

3.5 Quorum Quenching Effect On *P. aeruginosa* Biofilms:-

- Static Microtiter Plate assay was performed to check the effect of quorum quenching activity of bacterial isolates on *P. aeruginosa* biofilms (Cady, McKean et al. 2012).
- *P. aeruginosa* was cultured in Tryptic Soy Broth in flask for 18 hours at 37° C with shaking at 150 rpm.
- This culture was inoculated in fresh TSB media and O.D was adjusted to 0.5 at 600 nm with the help of spectrophotometer.
- A 200 µl reaction containing 20 µl of *P. aeruginosa* culture, 20 µl of cell free lysate of each QQ bacterial isolate and fresh media was prepared in different wells.

- The assay control was maintained without cell free lysate treatment.
- The plate was incubated at 37°C for 24 hours without agitation.
- After the respective incubation time, planktonic cell growth was read at 630 nm.
- Bacterial culture was then discarded and plate was washed twice with 0.85% saline to remove loosely attached cells of bacteria. Plate was air dried for 30 min.
- A 200 µl of methanol was added per well to fix the tightly bound cells. Fixative was then discarded after 15 min and plate was air dried.
- Tightly bound cells were stained with 200 µl of 0.1 % crystal violet per well for 10 min.
- After 10 min stain was discarded and excess stain was removed by washing each well thrice with saline. Plates were air dried.
- For the quantification of tightly bound cells, 200 µl of 33% glacial acetic acid was added in each well as crystal violet solvent.
- Plate was read at 550 nm to detect the biofilms formed.
- To confirm the effect of quorum quenching on biofilm formation by cell free lysates, *P. aeruginosa* was grown in Tryptic Soy broth for 18 h at 37°C with shaking at 150 rpm. This culture was inoculated in fresh TSB and O.D was set 0.5 at 600 nm.
- A 200 µl reaction containing 20 µl of P. aeruginosa culture, 20 µl of cell free lysate of each QQ bacterial isolate, 10 µl of 10 µM C_4-HSL and fresh media was prepared in different wells.
- Control wells contained only media, *P. aeruginosa* cells without autoinducers, *P. aeruginosa* cells with autoinducers but without cell-free lysate, cells with cell-free lysate without auto-inducers.
- Plate was incubated at 37°C for 24 hours.

- After incubation plate was read at 620 nm for planktonic cell growth measurement and was washed and stained in the same way and read at 550 nm for biofilms formed.

3.6 Statistical Analysis:-

Experiments were repeated twice. Statistical significance of data was verified by ANOVA. Quorum quenching effect on biofilm formation and AHL degradation quantification data were inspected by one way ANOVA followed by Tukeys test. Graphpad Prism 7 was used to perform these statistical analysis.

3.7 Antibiotic Susceptibility Assay:-

Antibiotic susceptibility of bacterial isolates was checked using antibiotic disks of Ampicillin-Sulbactam, Chloramphenicol, Cefepime, Gentamicin, Ceftazidime, and Cefazolin by following method.

- Saline (0.9%) was prepared and 5 ml poured in sterile test tube.
- Fresh bacterial culture of each bacterial isolate was picked with sterile loop and suspended in respective test tube containing saline. Culture enough to match the turbidity of 0.5 McFarland standard was added.
- 200 µl of this suspension was swabbed on Muller Hinton agar plates.
- Antibiotic disks were placed on plates and then incubated at 37°C for 24 h.
- Zone of inhibition around disk was measured in mm.
- Isolates were classified as susceptible, Intermediate and resistant according to CLSI (clinical and laboratory standard institute) standards for antimicrobial susceptibility testing.

3.8 Genotypic Characterization:-

Following steps were performed for genotypic identification of isolated strains.

3.8.1 16S rDNA Sequencing:

Pure colonies of isolated strains were sent to Macrogen for 16S rDNA sequencing. BLAST was run on all the sequences and closest BLAST hits for each sequence were determined by NCBI. Species and Genus of isolates were determined on the basis of their alignment to already published sequences in NCBI database. These sequences were deposited to GenBank database and accession number for each sequence was obtained.

3.8.2 Phylogenetic Analysis:

16S rDNA sequences of all isolated strains were subjected to BLAST and 5 closest BLAST hits were selected for each strain. MEGA7 was used for the generation of phylogenetic tree. All the sequences were obtained in the FASTA format file. All the sequences were aligned by using CLUSTAL W and T-coffee. Unrooted phylogenetic tree from neighbor joining method showing evolutionary relationship was constructed with bootstrap value of 1000.

3.9 Identification of Quorum Quenching Genes:-

Molecular basis of AHL degradation activity in bacterial isolates was confirmed by PCR identification of genes that encode AHL lactonase and AHL acylase enzymes well reported for quorum quenching.

3.9.1 DNA Extraction:

DNA was extracted by using Invitrogen Purelink bacterial DNA extraction kit. Overnight bacterial cultures were used to pellet down the cells through centrifugation. Pellets were resuspended in 180

µl of genomic digestion buffer. 20 µl of proteinase K was added to lyse the cells. Incubation was carried out at different temperatures for different bacterial strains from 55-75°C from 30 min to 3 h until lysis was complete. 20 µl of RNAase A was added to lysate and incubated at room temperature for 2 min. 200 µl of genomic lysis/binding buffer was added to obtain a homogenous solution. 200 µl of 96-100% ethanol was added to lysate and it was then mixed well to obtain a homogenous solution. Lysate was transferred to spin column and centrifuged at 10,000×g for 1 min. Collection tube was discarded and column was transferred to a new collection tube. 500 µl of wash buffer I was added to column and centrifuged at 10,000×g for 1 min. Column was placed in new collection tube and 500 µl of wash buffer II was added and centrifuged for three minutes. Column was then placed in 1.5 ml microcentrifuge tube and 50 µl of elution buffer was added to it. It was incubated at room temperature for 1 min and centrifuged at maximum speed for 1 min. Tube contained the purified genomic DNA. Extracted DNA was stored at -20°C until use.

3.9.2 Primer Designing:

Primers for amplification of quorum quenching genes were designed for each bacterial isolate from the quorum quenching gene sequences of closest BLAST hits of 16S rDNA of bacterial isolates. Quorum quenching gene sequences of closest BLAST hits were obtained from NCBI and their conserved sequences were determined. Primers were then designed both manually and using NCBI primer designing tool. Primers for two main categories of quorum quenching genes (Lactonase and Acylase) were designed and their properties were determined by using OLIGOANALYZER tool.

Table 2. Primers for amplification of QQ genes are listed below in table.

Name	Sequence 5` to3`	% GC content	T_m
AiiA-F	GATGGCCTGGAGAATGAC	55.6	59.9
AiiA-R	GCGTGTAGGGTATGAGCC	61.1	62.2
PQnhm-F	GTTCTGCACGAAGTCCCTG	57.9	62.3
PQnhm-R	GCTGTTGGGTTCGATGATG	52.6	60.2
QPnhm-F	GTCGGCCAGGTAATAGAGC	57.9	62.3
QPnhm-R	GCTACCGTCCGGAATACTG	57.9	62.3
Pvd-F	AAGACGTTCCATCGCCTTGT	50	60.4
Pvd-R	CCCTGTAGGAGCGGGTTTAC	60	64.5

3.9.3 PCR Reaction:

Phusion polymerase master mix was used for amplification via Pvd-F and Pvd-R primers. 50 µl reaction was prepared as follows;

Table 3. PCR reaction protocol for Pvd gene amplification

REAGENT	Volume in µl
Phusion Master Mix	25 µl
Forward Primer	1.5 µl
Reverse Primer	1.5 µl
DNA	2 µl
Nuclease Free Water	20 µl

3.9.4 PCR Conditions:

PCR was performed in following cyclic conditions.

Table 4. Cyclic condition for amplification of Pvd gene.

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98°C	30s	1
Denaturation **Annealing:** **AiiA** **PvdQ** **QuiP** **Pvd** **Extension**	98°C 59.9°C 62.3°C 62.3°C 62°C 72 °C	10s 30s 30s 30s 30s 90s	35 x
Final Extension	72°C	10min	1
Hold	4°	-----	----

Table 5. PCR reaction protocol for amplification of AiiA, PvdQ and QuiP genes.

Genes	Reaction mixture
PvdQ (*Pseudomonas* sp.) and AiiA gene (*Bacillus ceureus*)	12.5 µl of 2X master mix, 1 µl DNA, 2 µl of each forward and reverse primers with 32.5 µl nuclease free water
For QuiP (*Pseudomonas* sp.) and AiiA gene (*Bacillus subtilis*)	12.5 µl 2X master mix, 0.5 µl DNA, 2 µl of each forward and reverse primers, 1 µl $MgCl_2$ with 32 µl nuclease free water

Table 6. Cyclic conditions for amplification of AiiA, PvdQ and QuiP genes.

Gene	Cycling program for PCR reaction
AiiA gene	30 seconds of initial denaturation (1 cycle) at 95°C, 30 sec. of denaturation at 95°C (30 cycles), 60 sec. of annealing (30 cycles) at 59.9°C, 60 sec. of extend at 62.2°C (30 cycles) and five minutes of post-extension at 62.2°C (1 cycle)
PvdQ gene	30 seconds of initial denaturation (1 cycle) at 95°C, 30 sec. of denaturation at 95°C (30 cycles), 60 sec. of annealing (30 cycles) at 62.3°C, 60 sec. of extend at 60.2°C (30 cycles) and five minutes of post-extension at 60.2°C (1 cycle)
QuiP gene	30 seconds of initial denaturation (1 cycle) at 95°C, 30 sec. of denaturation at 95°C (30 cycles), 60 sec. of annealing (30 cycles) at 62.3°C, 60 sec. of extend at 62.3°C (30 cycles) and five minutes of post-extension at 62.3°C (1 cycle)

3.9.5 Gel Electrophoresis:

Amplified PCR products were separated on 1% agarose gel in 1X TAE buffer. 0.5 g of agarose gel is added on 50 ml of 1X TAE buffer. It was heated until clear solution was obtained of mixed agarose and buffer. 3 µl of ethidium bromide was then added in it. 1 Kb DNA ladder was used as marker. Wells were loaded with sample mixture containing 3 µl of loading dye along with 4 µl of PCR product. Conditions were set at Voltage 80V, Current 500A and Time 40 min. Gel was viewed under UV light using UV Transilluminator.

3.9.6 PCR Product Purification:

Thermo scientific GeneJET PCR purification Kit was used for the purification of PCR product. For the purification of PCR product binding buffer was added in 1:1 volume. This solution was then added to the GeneJET purification column and centrifuged for 30-60s. Flow through was discarded. 700 µl of wash buffer was added to the purification column and centrifuged. Residual wash buffer was removed by more centrifugation. 50 µl of elution buffer was added, centrifuged and eluted DNA was stored at -20 in micro-centrifuge tubes.

3.10 Gene Sequencing:-

Purified PCR products were sent to Macrogen Korea for sequencing.

3.10.1 Sequence Analysis:

Consensus sequence of each sequenced gene was determined using BioEdit tool and subjected to Blast analysis. ExPASy tool was used to determine open reading frames and annotated sequences were submitted to GENBANK and accession numbers were obtained. Conserved domains in sequenced amplicons were determined by Conserved Domain Database (CDD) available from NCBI. Amino acid sequence of sequenced gene amplicons was retrieved by ExPASy tool available online and Phylogenetic tree showing the evolutionary relationship of sequenced amplicons with other published data of quorum quenching genes was built using MEGA 7.

Chapter 4

RESULTS

4.1 Bacterial strain culturing:-

Samples were collected from a full-scale membrane bioreactor situated at H-12 Campus of National University of Science and Technology Islamabad. Experiment was set up for enrichment process of bacterial isolation. AHLs were provided as sole source of Carbon and Nitrogen. Inoculum prepared from sample was added to minimal media. AHLs were also added to micro centrifuge tubes containing minimal media and inoculum. Tubes were incubated at 37°C for three days during the first cycle. A hundred microliters of the culture was transferred to 1 ml fresh minimal media with addition of AHLs in the same quantities. The samples were incubated for three more days at the same temperature. The cycle was repeated again in a similar fashion. After third enrichment cycle, 100 µl of aliquot from each centrifuge tube was spread on nutrient media plates. A combination of different colonies was observed after incubation of plates at 37°C for 24 h. Limited number of colonies appeared that have survived on AHLs as the sole carbon source. This method of culturing inoculum in minimal media with AHLs the only source of carbon narrows down the bacterial screening and QQ positive strains are isolated by enrichment culture method.

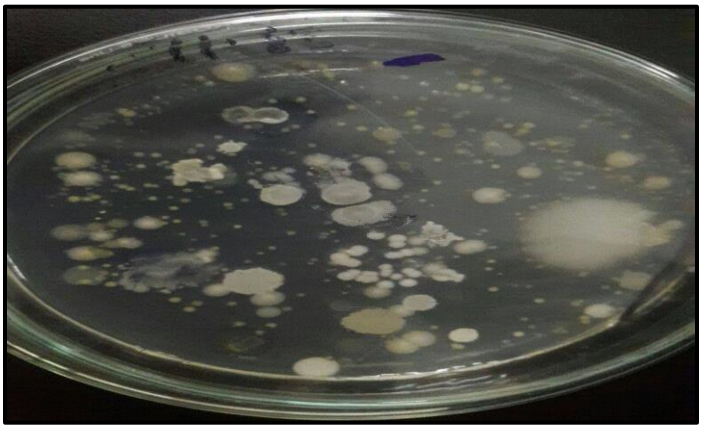


Fig 4. Colonies of the mixed bacterial consortia obtained after culturing the sludge sample on nutrient agar plate

4.1.1 Colony Purification:

Each colony was picked and streaked on separate agar plates. The plates were incubated for 48 h at room temperature. Based on morphological difference, colonies were further separated and streaked on new plate until same colonies were achieved on separate plates. The subcultures were streaked multiple times until pure cultures were achieved. A total of 12 isolates were obtained and codes were assigned to each of them. Out of 12 isolates, 4 isolates showed significant growth on minimal media using AHLs as carbon and nitrogen source which were selected for further studies.

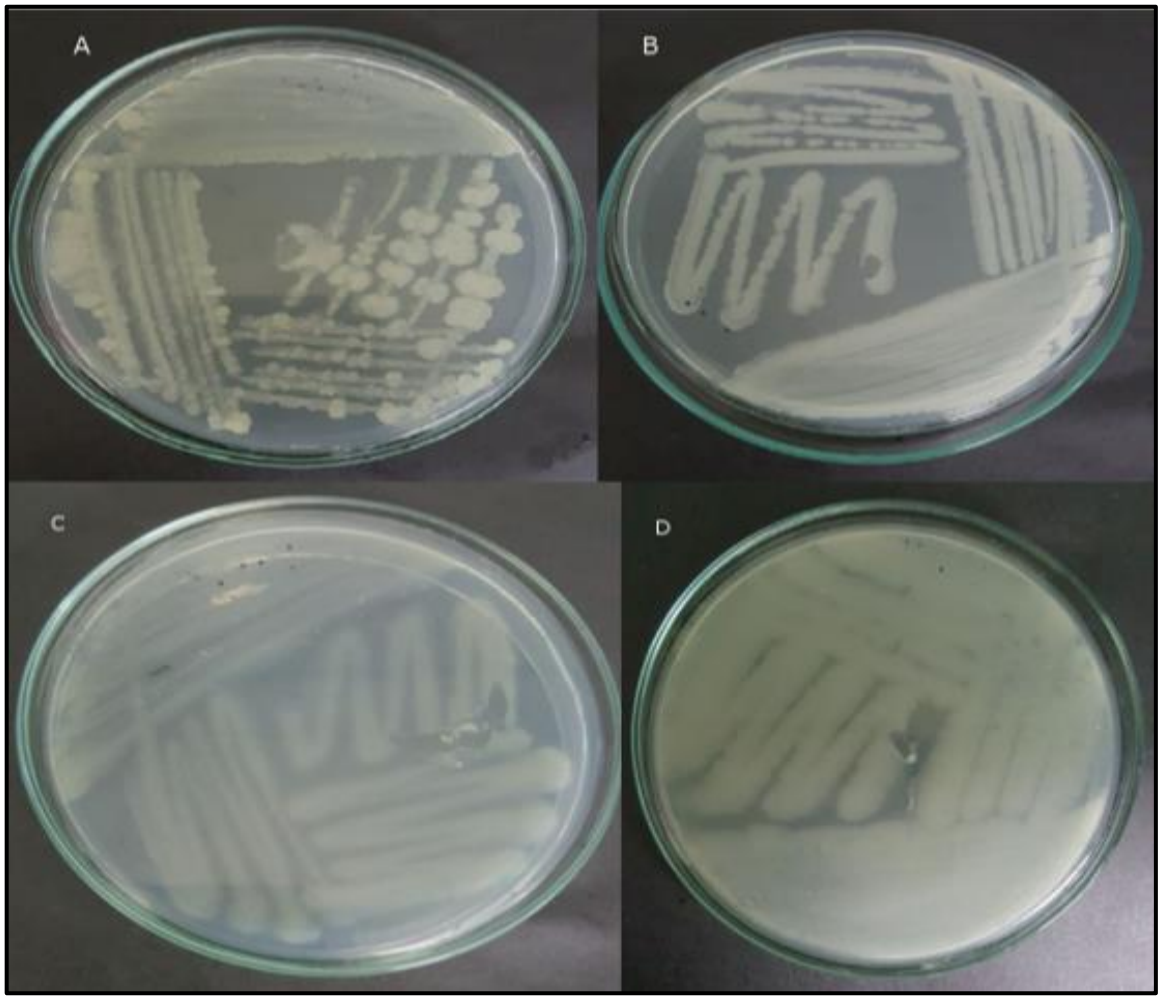


Figure 5. Purified colonies of bacterial strains isolated from mix consortia obtained from sludge. Codes assigned to each of these isolated bacterial strains shown are; QSP10: Flat, Sticky and irregular colonies with lobate margins (A). QSP03: White, pasty and round shaped colonies (B). QSP01: White colored, round shaped and sticky colonies (C). QQ3: White colored, raised and convex shaped, highly dense growth on nutrient agar plate (D).

4.2 Biochemical Analysis:-

Following tests were performed for biochemical analysis of isolated bacterial colonies.

4.2.1 Gram Staining:

Gram staining was performed for confirmation of selected bacterial strains. Bacterial morphology showed current isolates to be rod shaped. Among them QSP03 and QSP10 were gram positive whereas QSP01 and QQ3 were gram negative. QSP03 and QSP10 cells appeared in chain or cluster arrangement whereas QSP01 and QQ3 were diploid cells.

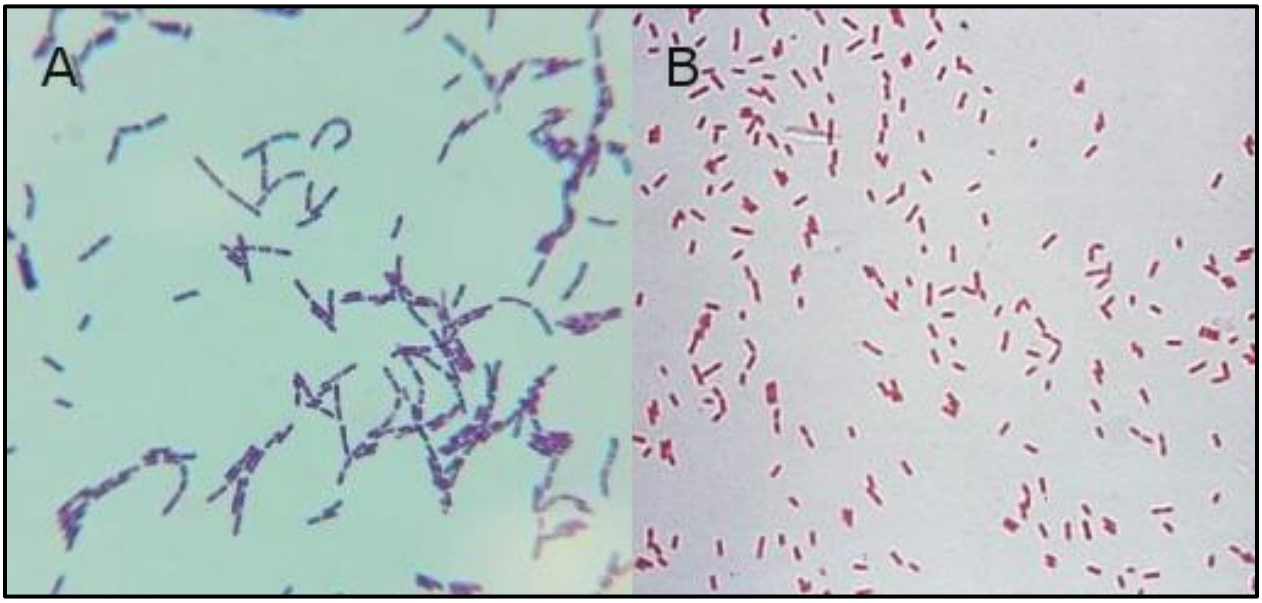


Figure 6. Different morphologies of bacterial isolates as observed by Gram staining.

4.2.2 Catalase Test:

This test confirms the presence or absence of catalase production in microorganisms. Bacteria living in oxygenated environment usually have catalase enzyme to protect themselves from toxic oxygen metabolites such as Hydrogen peroxide. Hydrogen peroxide effect is neutralized by enzyme catalase. Enzyme Catalase is responsible for degradation of hydrogen peroxide in water

and oxygen. All isolates were catalase positive. Bubbles were produced when colonies were treated with 3% Hydrogen Peroxide

4.2.3 Oxidase Test:

Oxidase test confirms the presence or absence of Cytochrome oxidase in bacteria. This test involves the use of an artificial compound tetra-methyl- *p*-phenylenediamine dihydrochloride. When this artificial compound is oxidized by Cytochrome C, its color is changed to dark blue or purple indicating the presence of enzyme Cytochrome oxidase. A well isolated colony was picked from 18-24 h culture and rubbed onto filter paper treated with Kovac`s oxidase reagent (1%tetra-methyl- *p*-phenylenediamine dihydrochloride, in water). All strains were positive for oxidase test except QSP03 as color change to dark blue was observed for all strains except QSP03.

4.2.4 EMB Agar Growth:

EMB (eosin methylene blue) is a selective media for gram negative bacteria. It's toxic for gram positive. It also distinguishes lactose fermenting bacteria from non-fermenting among gram negative bacteria. Pink to dark purple colonies on this purple colored media due to change in pH induced by lactose fermenting bacteria upon fermentation of lactose. QSP03 and QSP10 did not grow on EMB agar plates. QSP01 and QQ3 showed growth but no color change in colonies was observed which show that they are Gram negative but do not ferment lactose.

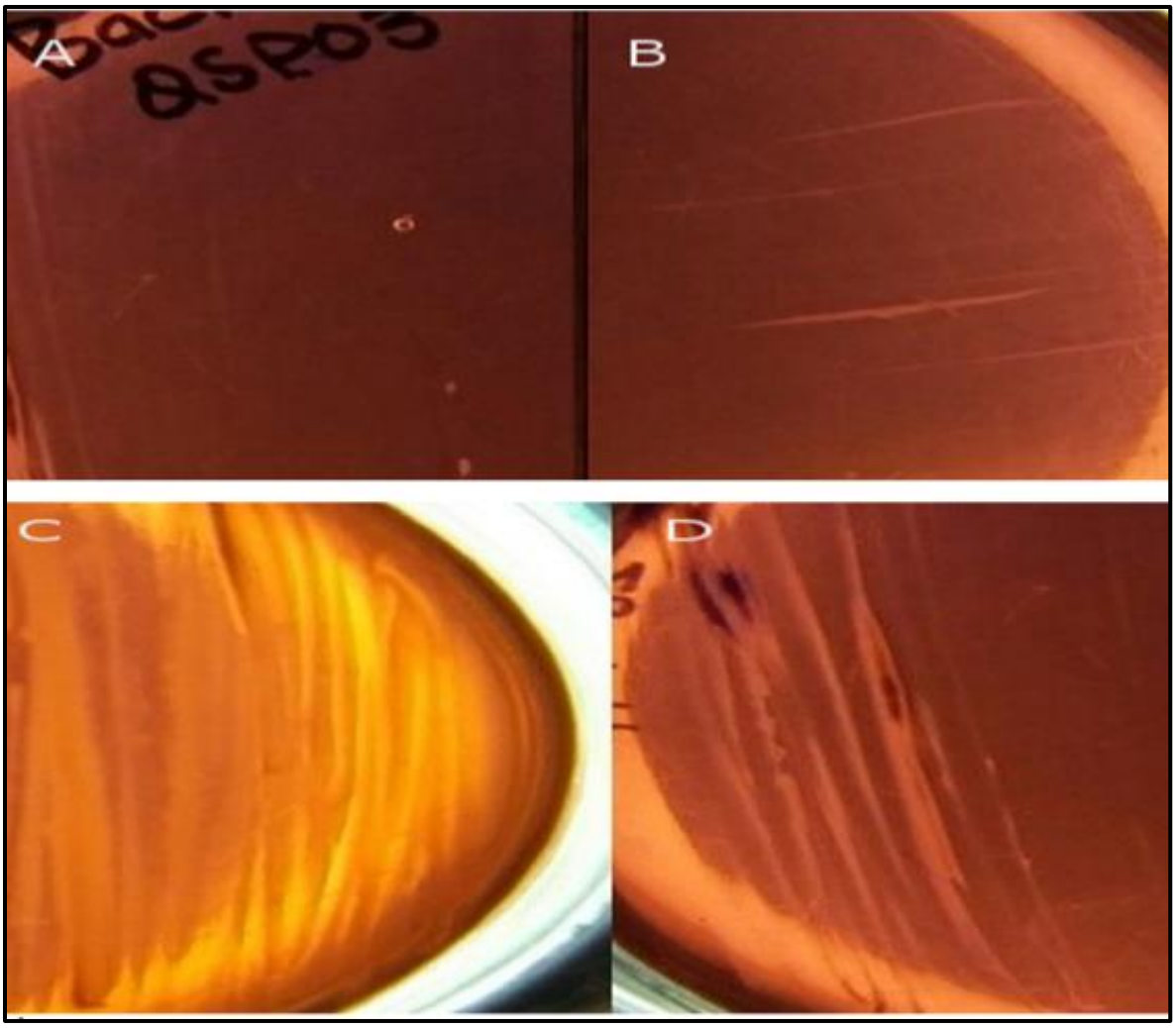


Figure 7. Growth analysis of selected bacterial strains on EMB agar. QSP03 and QSP10 did not show any growth (A and B). Whereas colorless colonies of QSP01 and QQ3 were observed (C and D).

4.2.5 MacConkey Agar Test:

This is also a selective media for Gram negative bacteria and it differentiates between lactose fermenting strains and non-lactose fermenting strains. Lactose fermenting bacteria changes pH to acidic by fermenting lactose and produce pink colored colonies because of lower pH. All the selected strains were streaked on MacConkey agar plates alongwith *E. coli* as positive control for lactose fermentation. QSP03 and QSP10 did not show growth at all which shows them to be Gram

positive. QSP01 and QQ3 showed growth but no lactose fermentation activity. *E. coli* produced pink colored colonies which indicate lactose fermentation.

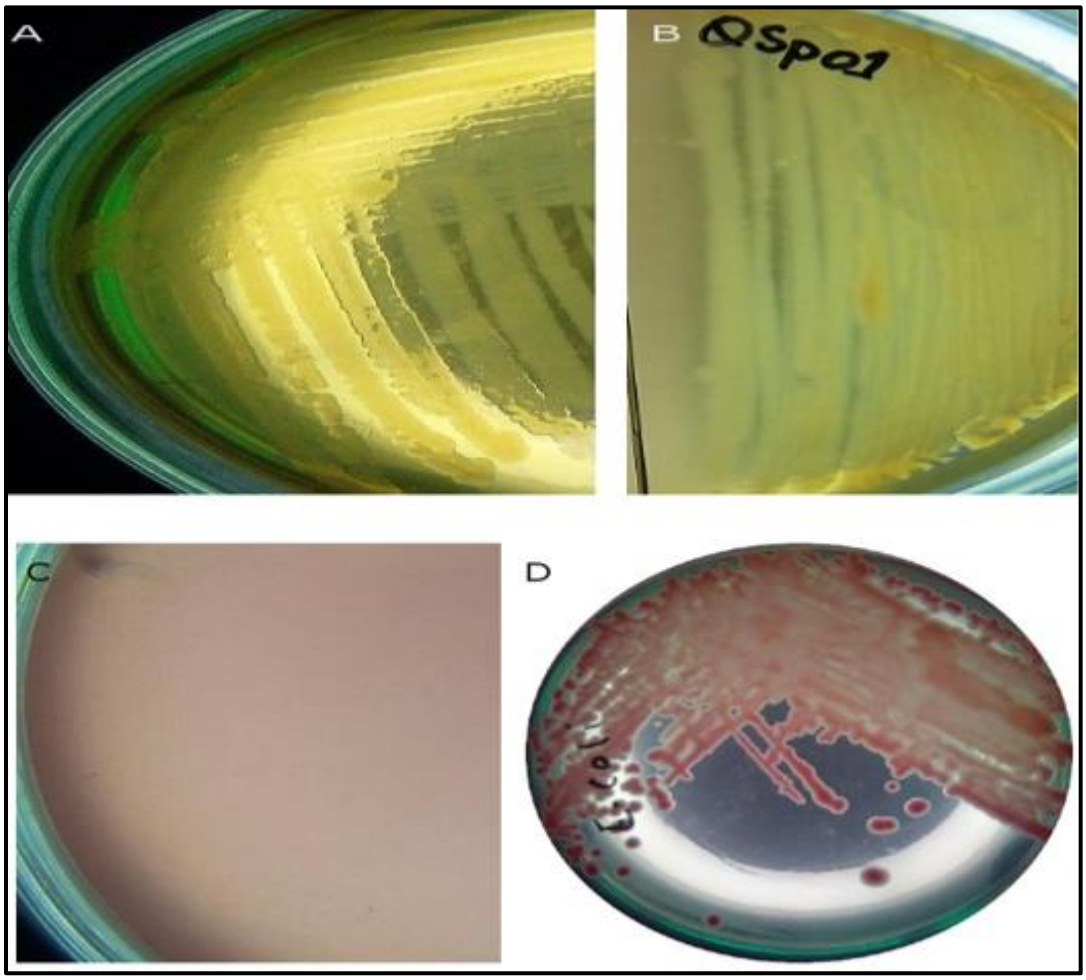


Figure 8. Growth and Lactose fermentation analysis of isolated bacterial strains on MacConkey agar. Brown colonies of QSP01 and QQ3 were observed (A and B). QSP03 and QSP10 did not show growth (C). E. *coli* (positive contol) produced pink colored colonies (D).

Table 7. Biochemical Characterization Summary

Isolates	Gram Staining	Morphology	Catalase Test	Oxidase Test	EMB agar Test	McConkey agar Test
QSP01	-ve	Rods	+ve	+ve	No lactose fermentation	Brown colonies, no lactose fermentation
QSP03	+ve	Rods	+ve	-ve	No Growth	No Growth
QSP10	+ve	Rods	+ve	+ve	No Growth	No Growth
QQ3	-ve	Rods	+ve	+ve	No lactose fermentation	Brown colonies, no lactose fermentation

4.3 AHL Degradation Assays to Confirm the Quorum Quenching Activity in Bacterial Isolates:-

Chromobacterium violecium is a gram negative bacteria mostly present in water and soil. *C. violecium* naturally produces an antibacterial, water insoluble purple-green pigment called violecein. This pigment is produced bacause of quorum sensing including AHL signalling molecule. CV026 is mutant strain of *C. violecium* derived by mini TN 5 transposon mutagenesis

lacking the ability to produce AHLs and thus intiating quorum sensing and so bioluminiscence. But when it gets AHL from exogenous source, it restores its pigment production capability. So CV026 is commonly used in studies involving quorum sensing and detection of AHLs. CV026 strain is resistant to kenamycine. It detects short chain AHLs.

A. tumefaciens derived A136 is another genetically engineered bacterium which express β-galactosidase gene in response to AHLs. This expression can be qualitatively measured by X-gal or its variants such as Y-gal serving as substrate for β-galactosidase activity and results in blue or pink color accordingly when gene expressed. Strain A136 is resistant to spectinomycine and tetracycline. It detect long chain AHLs.

Both CV026 and A136 strains were used in this study to confirm quorum quenching potential in our isolates.

4.3.1 Agar Overlay Assay:

Agar plates harboring CV026 and A136 strains for detection of short chain and long chain AHLs respectively were prepared. Holes of 6 mm were made and 50 µl test strains were added alongwith 5 µM of C_6-HSL in different wells of CV026 indicating agar plate for short chain AHL degradation test. In the control well, no AHL degrading agent was added. It contained only C_6-HSL. Plates were incubated for 48 h at 28°C. Color in surrounding areas of well was observed.

Color around the well containing only C_6-HSL was changed from light yellow to violet. It confirms the restoration of quorum sensing in CV026 in the surrounding areas of well because of exogenous source of AHL i.e. C_6-HSL in well. Color around the wells containing bacterial Isolates did not change much despite the presence of C_6-HSL. This confirms these bacterial isolates to be quorum quenching. They degraded the provided AHL.

The process was repeated on A136 indicating agar plate for long chain AHL detection. C_{10}-HSL was added as exogenous long chain AHL source. Plates were incubated for 48 h at 28°C.

Color in surrounding areas of well was observed. Color surrounding the well containing only C_{10} HSL was changed from light yellow to blue-green. C_{10}-HSL here worked as signaling molecule and induced the expression of β -galactosidase gene in A136. When the enzyme acted upon its substrate X-gal, it produced greenish color. Color around the wells containing Bacterial isolates did not change despite the presence of C_{10}-HSL. It confirms the bacterial isolates to be quorum quenching and capable of degrading long chain AHL too. Experiment was repeated twice and average of AHL degradation zones was taken.

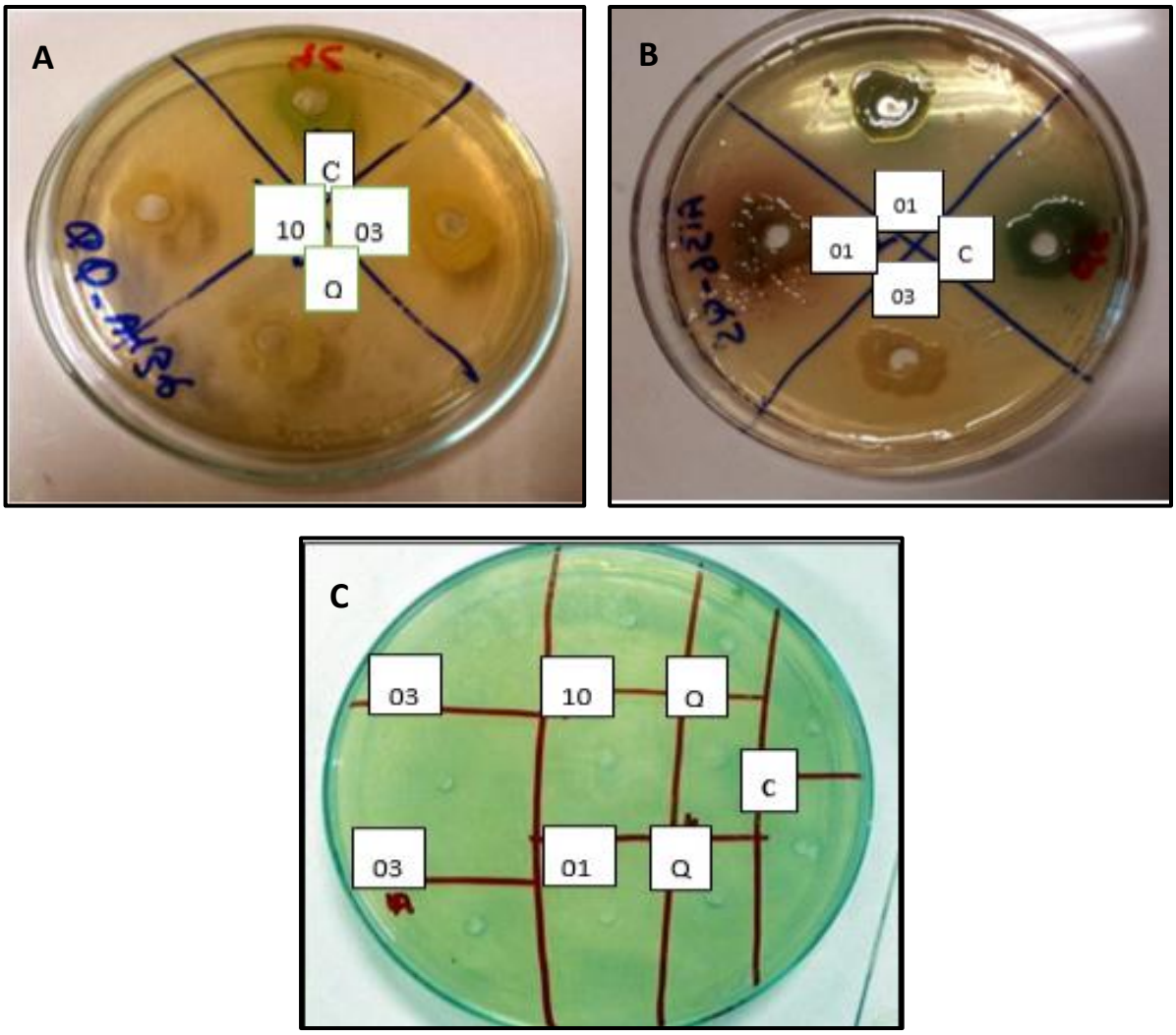


Figure 9. AHL degradation analysis on indicating agar plates of CV026 and A136 strains. Blue-Green color was observed around control well of plate harboring A136 strain whereas well 10, 03, 01 and Q containing culture of bacterial isolates QSP10, QSP03 and QQ3 alongwith AHLs retained same light yellow color or produced very light color (A). Agar plate of CV026 strain also showed violet color around control well. Whereas other wells 03, 10, Q and 01 containing culture of bacterial isolates alongwith AHLs showed very fade color or no color change (B and C).

4.3.2 Disk Diffusion Assay:

CV026 and A136 indicating agar plates for detection of short chain and long chain AHLs respectively were prepared as described in methodology section 3.2.

A sterile membrane filter disk was dipped in 10 mg/ml of C_6 -HSL and placed over the surface of agar in CV026 plate. Fresh colonies from 24 h culture plates of each bacterial isolate were loaded on membrane filter disk. Plates were incubated for 48 h at 28° C. after incubation color of colonies was checked. Inhibition of violacein production occurred because of AHL degradation at site where colonies of bacterial isolates were loaded. Colorless spots were seen at those points.

Similarly a sterile membrane filter disk was dipped in 10 mg/ml of C_{10}-HSL and placed over the surface of A136 plate. Fresh colonies from 24 h culture of each bacterial isolate were loaded on membrane filter disk. Plates were incubated for 48 h at 28°C. After incubation color of plates was checked. Absence of color change was observed in the form of colorless spots in case of QSP03 and QSP10 and very fade color in case of QSP01 and QQ3. It confirms the long chain AHL degradation by these quorum quenching bacterial isolates.

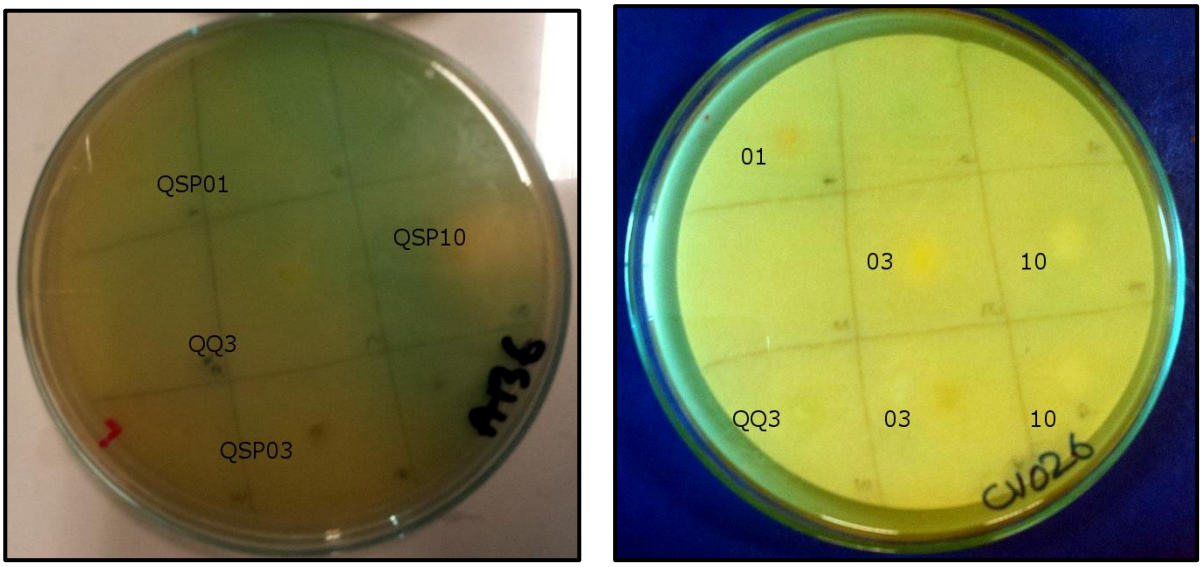


Figure 10. Quorum quenching by bacterial isolates in the form of colorless spots can be seen in colored background in both A136 and CV026 plates (A and B respectively).

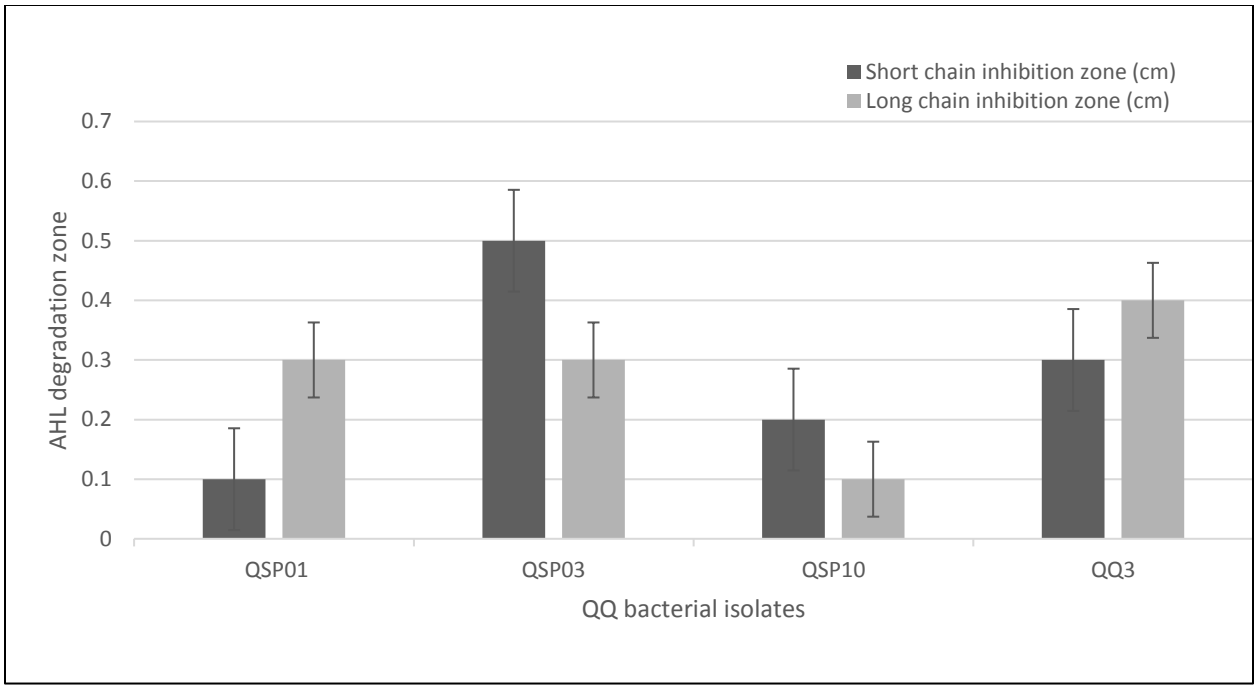


Figure 11. Zone of inhibition obtained after AHL degradation. Bars are indicating AHL degradation appeared in the form of colorless zones around wells containing bacterial isolates. X-axis shows bacterial isolates and Y-axis represents AHL degradation zones by current isolates in centimeters.

4.4 AHL Degradation Assay by Microtiter Plate Method:-

Microtiter plate assay was carried out to quantify the amount of AHL degradation by quorum quenching bacteria. Cell free lysate of each bacterial isolate were prepared. CV026 was incubated in LB alongwith C_4-HSL as exogenous AHL supply and cell free lysate of bacterial isolates in different flasks. Control reaction contained only CV026 and C_4-HSL. Incubation was carried out at 30°C at 120 rpm for 24 h in shaker incubator. 1 ml culture from each flask was taken into microcentrifuge tubes and 1 ml of dimethyl sulphoxide was added, centrifuged at 8000 rpm for 5 min to solubilize violacein and for cell removal. 200 µl of violacein containing supernatant was added to wells of 96-well microplate. Harvested bacterial cells were re suspended in 1 ml of sterile water and 200 µl was transferred to wells of microplate. Absorbance was read in microplate reader at 550 nm wavelength for violacein quantification and at 630 nm wavelength to evaluate cell growth. The results were compared with assay control which contained only CV026 supplemented

with C_4-HSL but without cell free lysates treatment. Presence of AHL in media initiate quorum sensing in CV026 and so violacein production. Amount of violacein produced by CV026 is inversely related to AHL degradation by quorum quenching bacterial isolates. Each bacterial isolate inhibited violacein production to different extents. 60% violacein inhibition was seen for QSP03 and QSP10 whereas 50% violacein inhibition was observed for QSP01 and QQ3 ($p > 0.0001$).

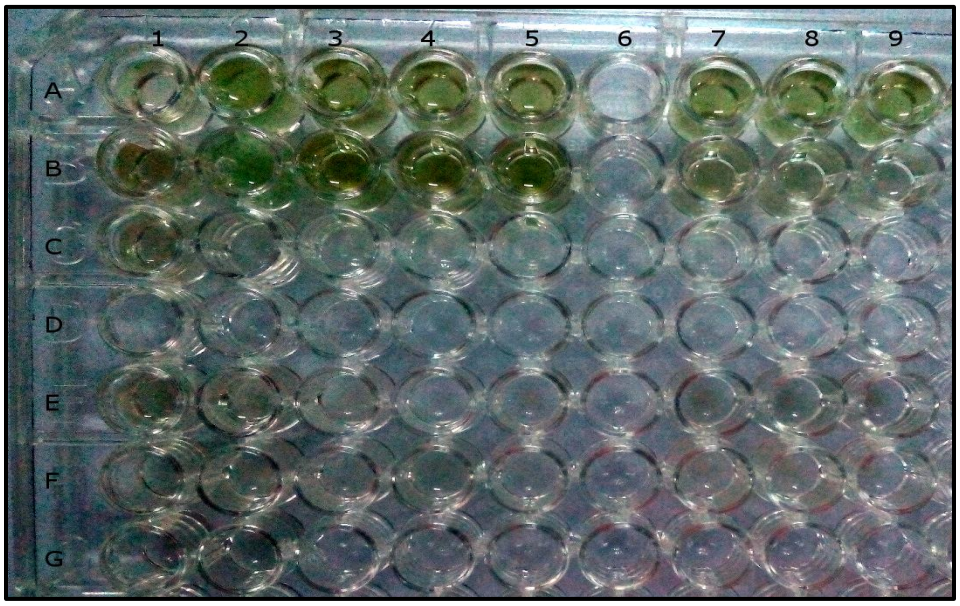


Figure 12 A. AHL degradation was quantified by microtiter plate method. Intensity of violacein production was measured to analyze the AHL degradation by current bacterial isolates. A2 and B2 contain CV026 cultures with exogenous AHL source. A3-A5 contain CV026 treated with QSP01, A7-A9 treated with QQ3, B3-B5 treated with QSP10 and B7-B9 treated with QSP03. Column D-F contains cells for growth measurement.

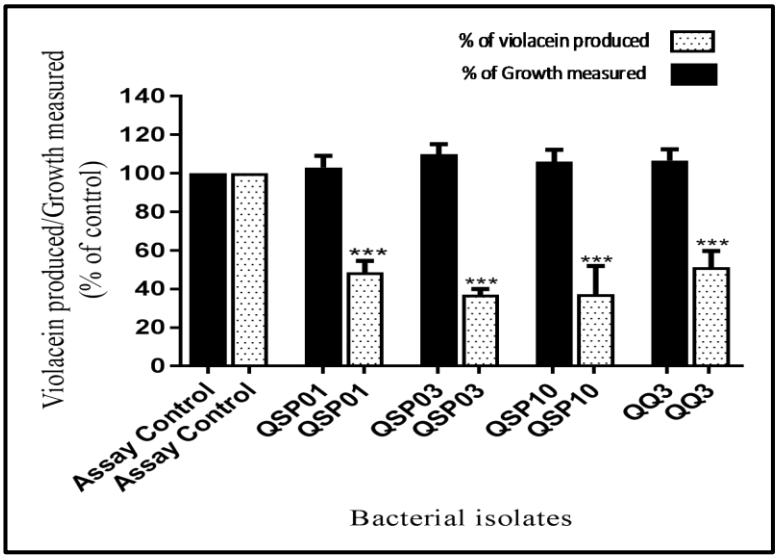


Figure 12 B. Violacein estimation by AHL degradation assay. X-axis represents CV026 culture treated with QSP01, QSP03, QSP10 and QQ3 isolates and supplemented with C_4-HSL. Assay control involves CV026 culture supplemented with C_4-HSL with no AHL degrading agent treatment. Y-axis shows the intensity of violacein produced and growth measured in treated and untreated cultures.

4.5 Quorum Quenching Effect on *P. aeruginosa* Biofilms:-

P. aeruginosa Biofilms were subjected to treatment with cell free lysate of QQ bacteria to study their effect on biofilms by static microtiter plate method. *P. aeruginosa* was cultured and O.D was adjusted to 0.5 at 600 nm. A 200 µl reaction containing 20 µl of *P. aeruginosa* culture, 20 µl of cell free lysate of each QQ bacterial isolate and fresh media was prepared in different wells. The assay control was maintained without cell free lysate treatment. The plate was incubated at 37°C for 24 h without agitation. Planktonic cell growth was read at 630 nm. Bacterial culture was discarded and plate was washed with saline, 200 µl Methanol per well was added to fix the cells, discarded after 15 min and plate air dried. Tightly bound cells were stained with 0.1% crystal violet and plate was read at 550 nm to detect biofilms formed. Significant biofilm inhibition effect was observed for each bacterial isolate treatment. About 50% biofilm inhibition was observed by QQ3 and QSP01, almost 60% by QSP10 and QSP03 bacterial isolates ($p = 0.0007$). Growth inhibition

was not seen with cell free lysate treatment of any bacterial isolate. To confirm the role of quorum sensing in biofilm formation and quorum quenching effect of cell free lysate on it, reactions were prepared with addition of 10 μM of C_4-HSL. The results showed significant regeneration of biofilms because of exogenous AHL supply in QSP03 and QQ3 lysate treated cells. It confirms that biofilm inhibition in QQ bacterial lysate treated cells is due to AHL degradation effect.

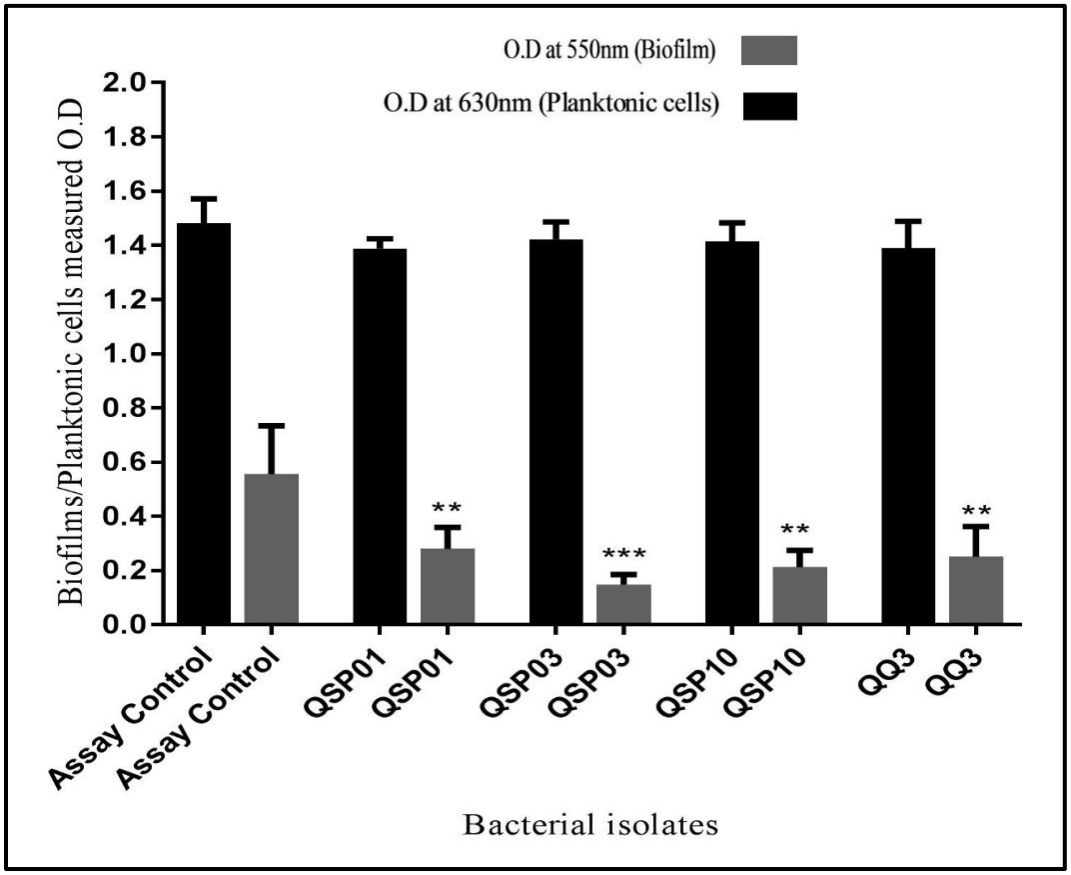


Figure 13. Biofilm degradation assay. Quorum quenching effect of current bacterial isolates on *P. aeruginosa* biofilm was analyzed by static microtiter plate method. X-axis represents *P. aeruginosa* cells treated with QSP01, QSP03, QSP10 and QQ3 bacterial isolates. Assay control involves *P. aeruginosa* cell growth in Tryptic Soy Broth without any quorum quenching agent. Y-axis represent the planktonic cell growth and adherent cells (Biofilm) persistence in both treated and untreated cultures.

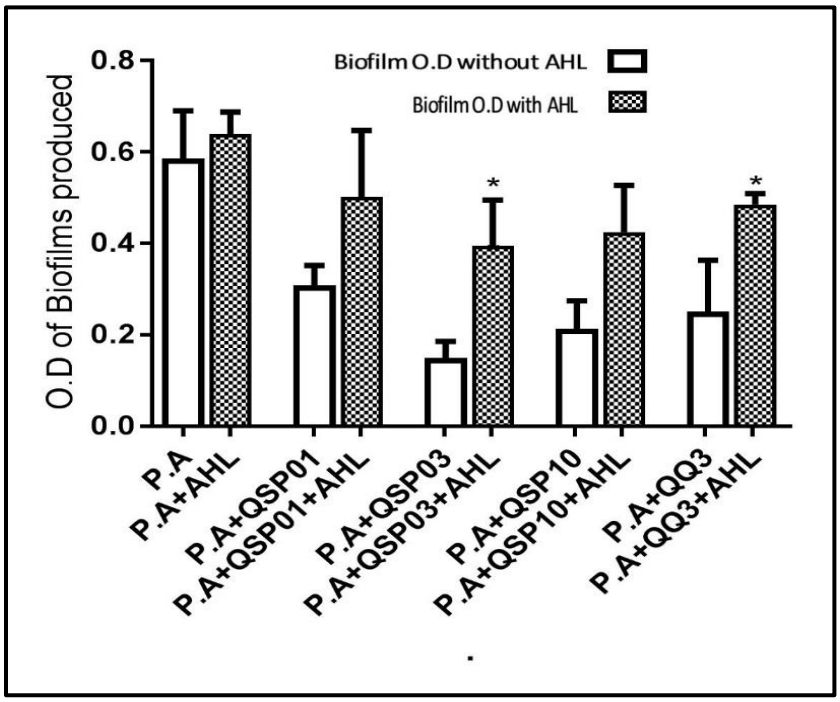


Figure 14. Analysis of role of Quorum sensing in biofilm formation. Biofilm formation in *P. aeruginosa* with and without exogenous supply of AHL and alongwith cell free lysate treatment and without its treatment is shown. X-axis represents the biofilm formation in *P. aeruginosa* with and without treatment of bacterial isolates and C_4-HSL. Y-axis represents the O.D of biofilms produced.

4.6 Antibiotic Susceptibility Test:-

Antimicrobial resistance in microorganisms is often related to characteristices like virulence, infections and pathogenecity. So antibiotic susceptibility test was performed to check wether the isolated bacteria are antibiotic resistant or not. Antibiotic susceptibility of bacterial isolates was checked using antibiotic disks of Ampicillin/sulbactam, Cefepime, Gentamicin, Ceftazidime, Cefazolin, and Chloramphenicol by disk diffusion method. Fresh bacterial culture of each isolate was suspended in 0.9% saline solution and turbidity was matched with 0.5 McFarland standard. 200 µl suspension of each isolate was spread on Muller Hinton agar plates and antibiotic disks were placed on surface of media. Zones of inhibition around antibiotic disks were measured after incubation at 37° C for 18 hours. Isolates were classified as susceptible, Intermediate and resistant

according to CLSI (clinical and laboratory standard institute) standards for antimicrobial susceptibility testing.

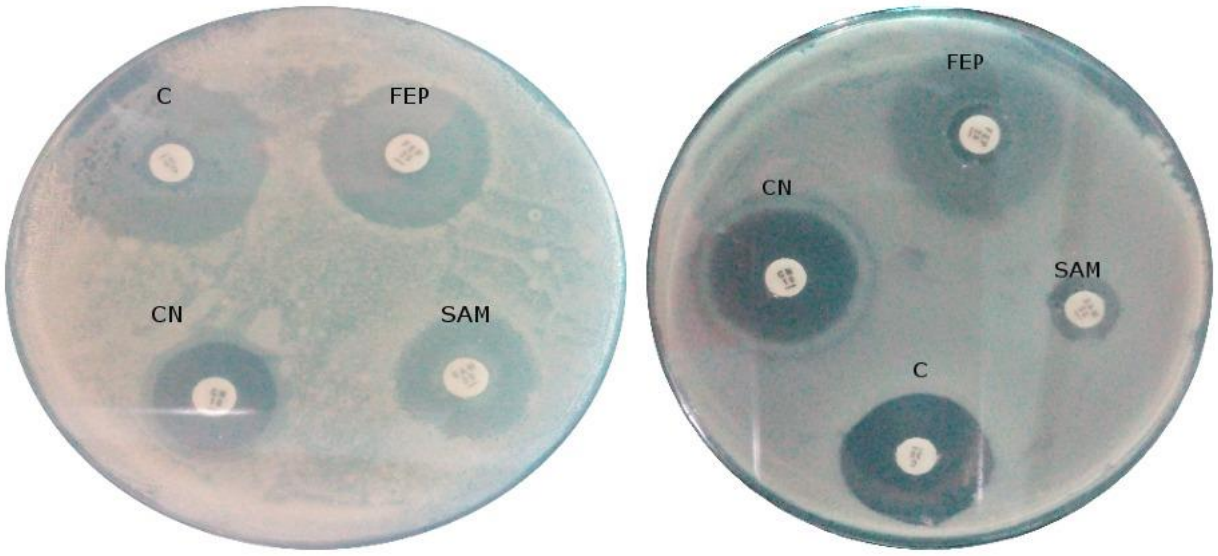
Table 8. Antibiotic susceptibility of isolated bacterial strains.

Isolate	Ampicillin-Sulbactam	Cefepime	Gentamycin	Cefazolin	Ceftazidime	Chloramphenicol
QSP01	S	S	S	S	S	S
QSP03	S	S	S	S	S	S
QSP10	S	I	S	S	S	S
QQ3	S	S	S	R	R	S

S: Susceptible

I: Intermediate

R: Resistant



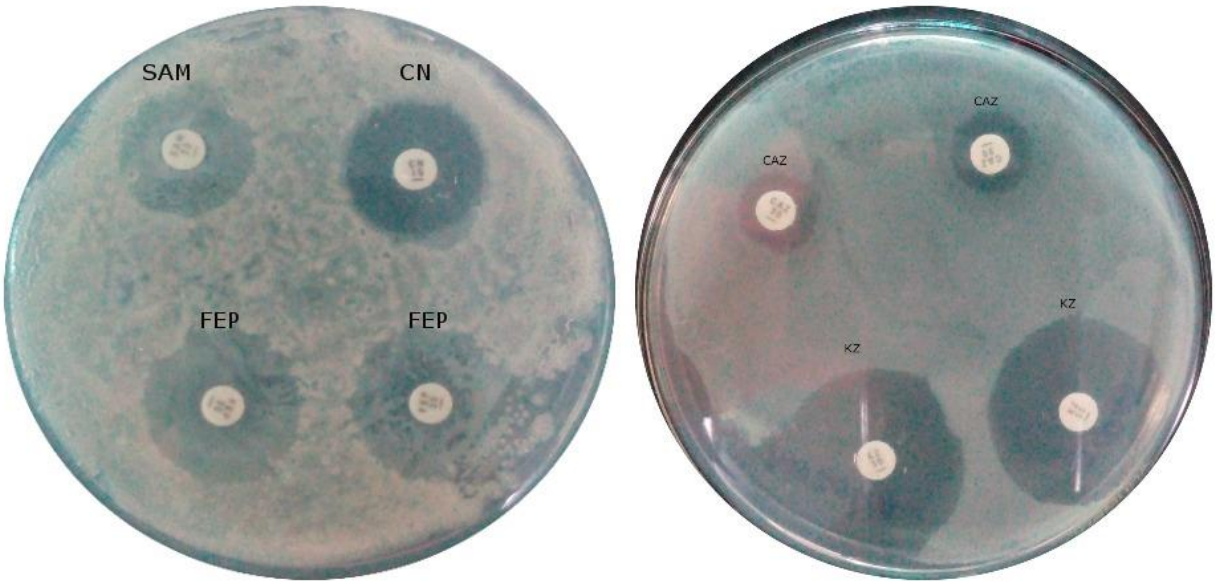


Figure 15. Zone inhibition assay. Different diameters of inhibition zones were observed for all bacterial isolates against antibiotic disks of Ampicillin/sulbactam (SAM), Cefepime (FEP), Gentamicin (CN), Ceftazidime (CAZ), Cefazolin (KZ), and Chloramphenicol (C).

4.7 Genotypic Characterization:-

Pure colonies of isolated strains were sent to Macrogen for sequencing of 16S rDNA. BLAST was run on all the sequences and closest BLAST hits for each sequence were determined by NCBI. Species and genus of isolates were determined on the basis of their alignment to already published sequences in NCBI database. These sequences were deposited To GenBank database and accession number for each sequence was obtained. On the basis of 16S rDNA sequencing QSP01, QSP03, QSP10 and QQ3 were identified as *Pseudomonas aeruginosa* strain QSP01, *Bacillus cereus* strain QSP03, *Bacillus subtilis* strain QSP10 and *Pseudomonas putida* strain QQ3.

Table 9. NCBI accession numbers of isolated bacterial strains and closest BLAST hit.

Isolate	Identification	Closest 16S rDNA	Accession Number
QSP01	*Pseudomonas aeruginosa* strain QSP01	*Pseudomonas aeruginosa* NBRC 12689/ 99%	KY576793
QSP03	*Bacillus cereus* strain QSP03	*Bacillus cereus* CCM2010/ 99%	KY576795
QSP10	*Bacillus subtilis* strain QSP10	*Bacillus subtilis* 168/ 99%	KY576801
QQ3	*Pseudomonas putida* strain QQ3	*Pseudomonas putida* KT2440/ 99%	KR058848

4.7.1 Phylogenetic Tree:

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 24 nucleotide sequences. Codon

positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 43 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

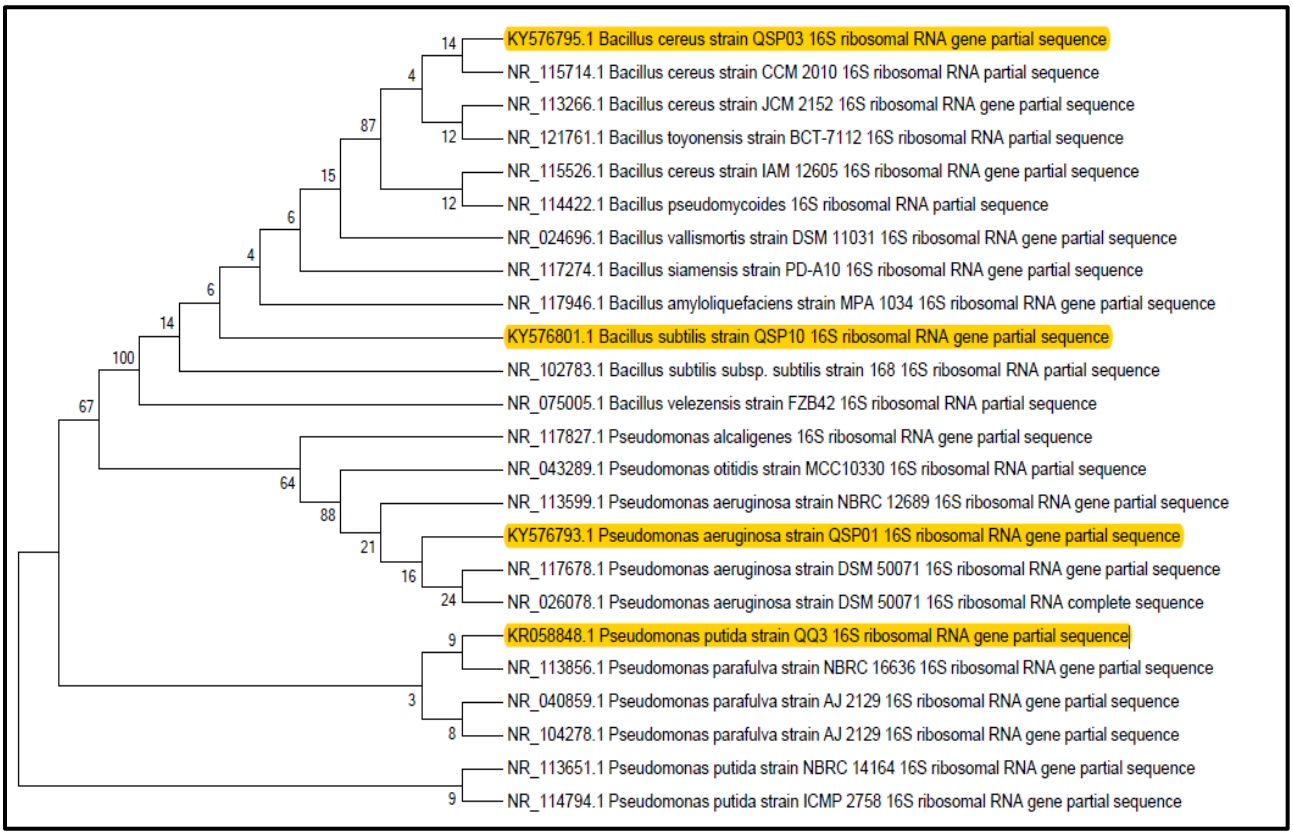


Figure 16. Phylogenetic analysis of isolated bacterial strains on the basis of 16S rDNA using MEGA 7. Phylogenetic tree is showing the evolutionary relationship of isolates (highlighted ones) with their closest BLAST hits and among themselves. Unrooted phylogenetic tree from neighbor joining method showing evolutionary relationship was constructed with bootstrap value of 1000.

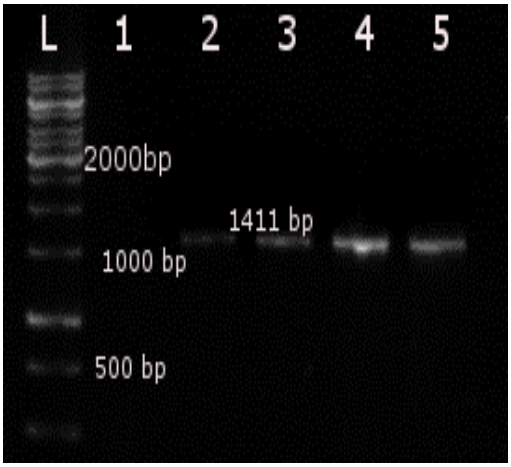
4.7.2 Identification of Genes Resposible for Quorum Quenching in Isolated Bacteria:

DNA was extracted by using Invitrogen Purelink bacterial DNA extraction kit. Primers for amplification of quorum quenching genes were designed for each bacterial isolate from the quorum quenching gene sequences of closest BLAST hits of 16S RNA of bacterial isolates. Our strains were identified as *Pseudomonas aeruginosa* QSP01, *Pseudomonas putida* QQ3, *Bacillus cereus* QSP03 and *Bacillus subtilis* QSP10. Literature confirms the presence of AHL lactonase in gram

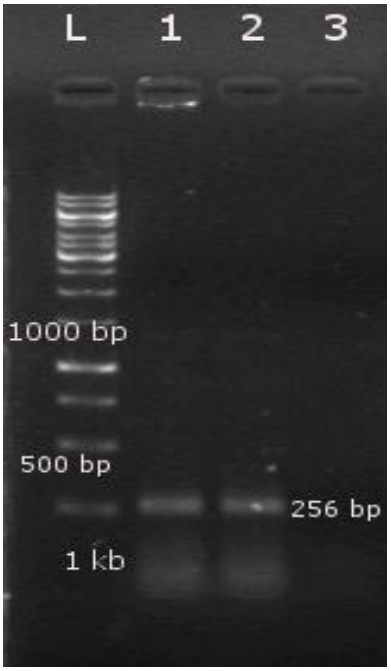
positive bacteria and AHL acylase in Gram negative bacteria. Both are the well reported quorum quenching enzymes. Primers for each enzyme gene were designed accordingly. 1411 bp and 2kb PvdQ gene fragment encoding AHL acylase was amplified in pseudomonas aeruginosa QSP01 and pseudomonas putida QQ3 respectively, 572 bp band of QuiP gene also encoding a homologue of AHL acylase was amplified in both *Pseudomonas aeruginosa* QSP01 and 257 bp fragment of gene AiiA encoding AHL lactonase in bacillus cereus and bacillus subtilis were amplified and bands were visualized on gel.

A.

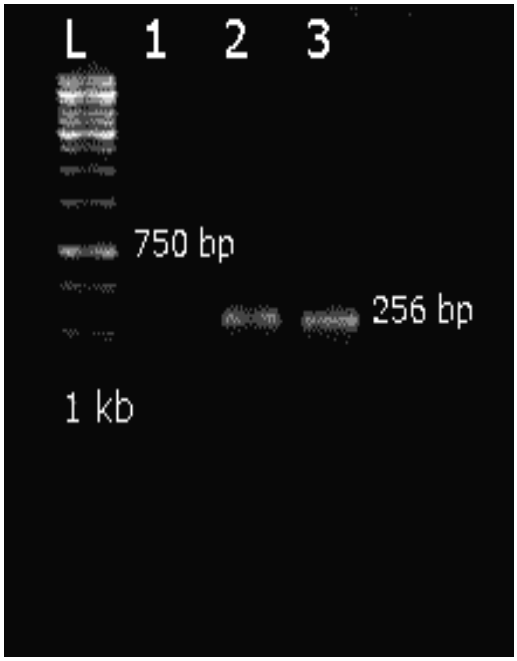
B.



C.



D.



E.

Figure 17. Gene identification through PCR. A gene amplicon of 576 bp and 1411 bp amplified in *P. aeruginosa* (A and B), 256 bp amplified in *B. cereus* and *B. subtilis* (C and D) and 2000 bp in *P. putida* QQ3 (E)

4.8 Gene Sequencing:-

All PCR products were sent to Macrogen Korea after purification. Consensus sequence of all the sequenced amplicons were determined using BioEdit and subjected to BLAST analysis to compare them with already published data in NCBI. Consensus sequences of *Pseudomonas aeruginosa* QSP01 and *Bacillus cereus* QSP03 showed high similarity to coding sequences of already published PvdQ, QuiP and AiiA gene. Open reading frame of these sequences were obtained from ExPASy tool and annotated sequences were submitted to GENBANK database and accession numbers were obtained. Whereas sequences from other gene products of Bacillus subtilis QSP10 and Pseudomonas putida QQ3 had unreliable sequence result of forward and reverse primer in their chromatogram.

Table 10. Identified genes with their GenBank accession numbers and reported functions.

Isolated Bacteria	**Gene Identified**	**Enzyme encoded**	**Reported function**	**GenBank Accession number**
***Bacillus cereus* QSP03**	AiiA	AHL lactonase	AHL degradation by opening the homoserine lactone ring in AHL structure	MG213739
***Pseudomonas aeruginosa* QSP01**	PvdQ	AHL acylase	AHL degradation by cleaving the amide bond between acyl chain and homoserine lactone ring	MG356411
***Pseudomonas aeruginosa* QSP01**	QuiP	AHL acylase	AHL degradation by cleaving the amide bond between acyl chain and homoserine lactone ring.	MG356412

4.8.1 Gene Sequence Analysis:

Amino acid sequences were retrieved by ExPASy and subjected to BLAST analysis to compare with already published data of protein in NCBI. AHL lactonase sequence of our isolate *Bacillus cereus* QSP03 got perfectly aligned with AHL lactonase sequences of *Bacillus cereus* present in online NCBI database. Analysis of conserved domains in our AHL lactonase sequence using CDD (conserved domain database) showed two conserved domains belonging to Metallo- β -lactamase and metal dependent hydrolase. CDD analysis showed the sequenced amplicon of AHL lactonase belong to the Metallo-Hydrolase-like_MBL-fold superfamily of proteins.

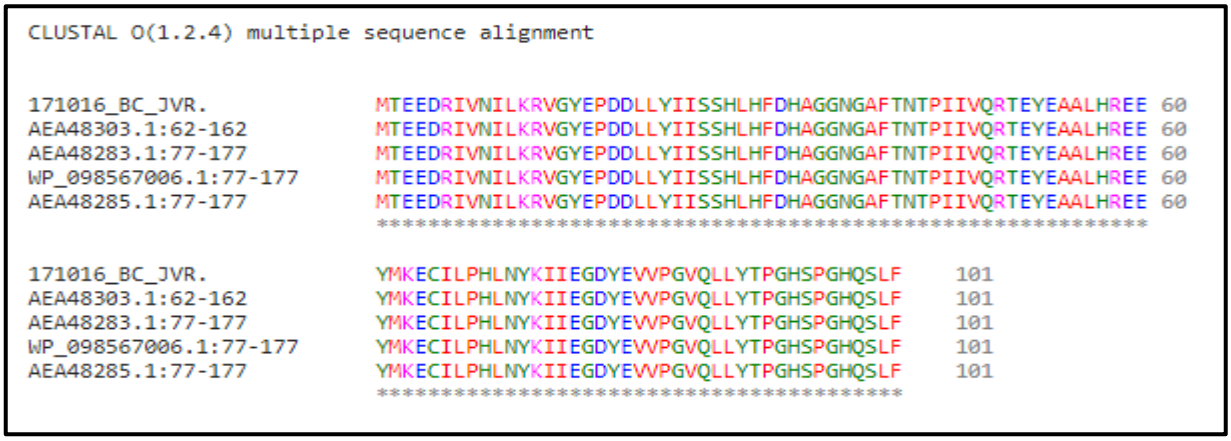


Figure 18. AHL lactonase partial *B. cereus*: ACCESSION AEA48303, AHL lactonase partial *B. cereus*: ACCESSION AEA48283, AHL lactonase family protein *B. cereus*: ACCESSION WP_098567006, AHL lactonase partial *B. cereus:* ACCESSION AEA48285 partial CDS is perfectly matched with partial CDS of our isolate *B. cereus* QSP03; 171016_BC_JVR.

Sequenced amplicons of PvdQ and QuiP both encoding AHL acylase also showed high similarity with AHL acylase sequence of *P. aeruginosa* published in NCBI. CDD analysis showed that both belong to Ntn-hydrolase superfamily and penicil-amidase superfamily of proteins.

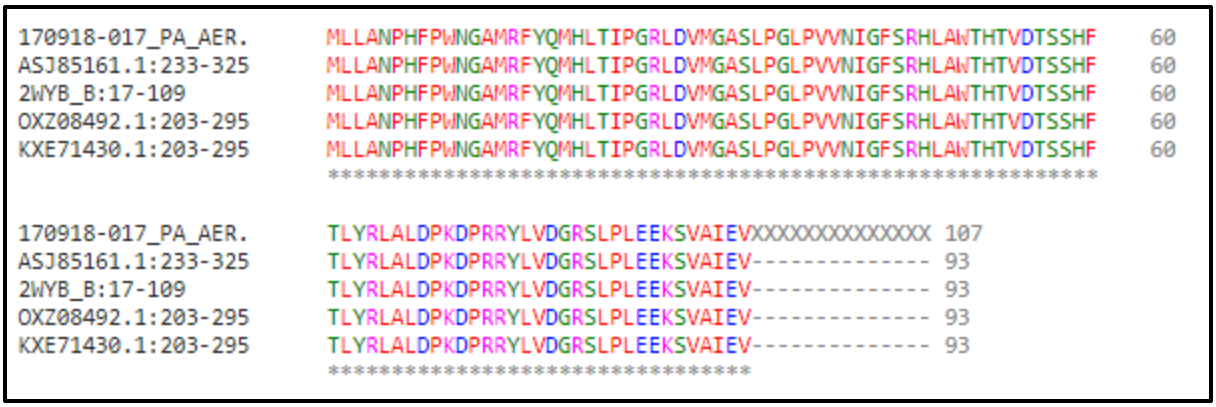


Figure 19. *P. aeruginosa*: ACCESSION ASJ85161, *P. aeruginosa* PAO1: ACCESSION 2WYB_B, AHL acylase subunit beta *P. aeruginosa*: ACCESSION OXZ08492, *P. aeruginosa*: ACCESSION KXE71430. Showing high similarity to PvdQ gene of our isolate *P. aeruginosa* QSP01; 170918-017_PA_AER.

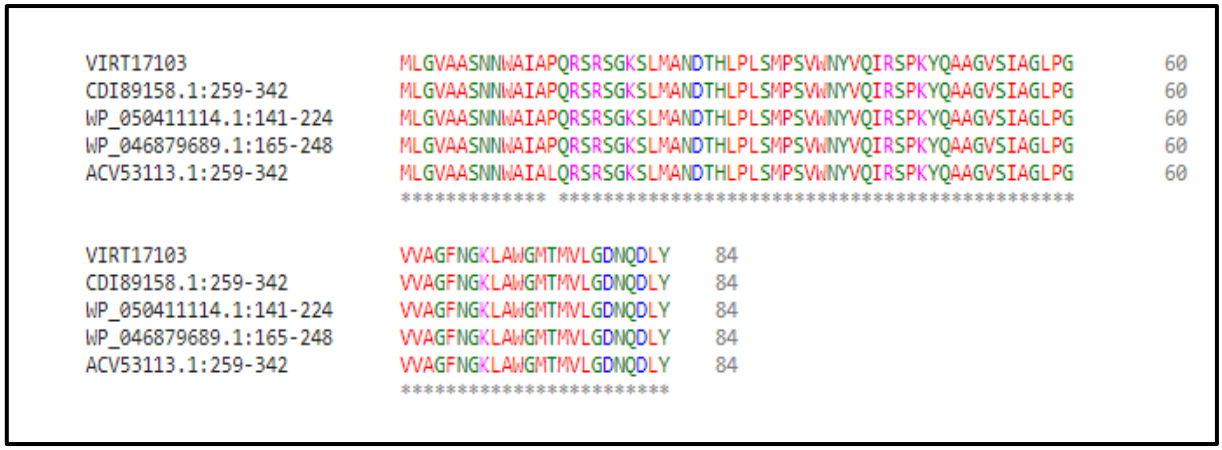


Figure 20. QuiP *P. aeruginosa*: ACCESSION CDI89158, AHL acylase subunit beta (partial) *P. aeruginosa*: ACCESSION WP_050411114, AHL acylase subunit beta *P. aeruginosa*: ACCESSION WP_046879689, Quip partial *P. aeruginosa*: ACCESSION ACV53113 show high similarity with partial AHL acylase subunit beta of our bacterial isolate *P. aeruginosa* QSP01; VIRT17103.

4.8.2 Evolutionary Relationship on The Basis of AHL lactonase and AHL acylase Sequence:

All these sequences were aligned with their closest BLAST hits and their phylogenetic relationship was inferred using MEGA 7. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 3.67548262 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap

test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 15 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 62 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

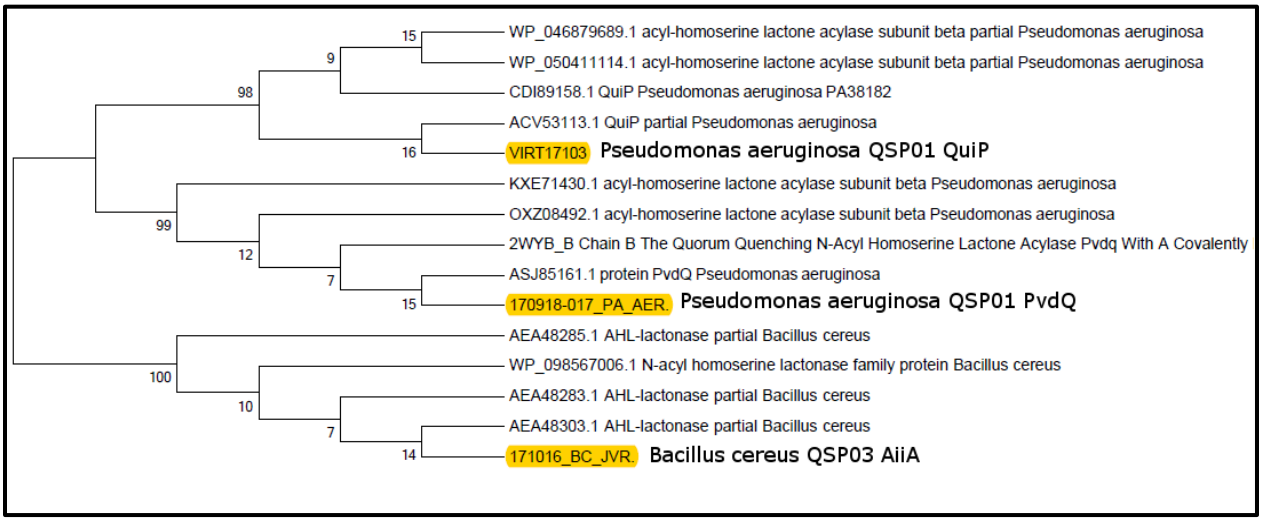


Figure 21. Unrooted phylogenetic tree derived from neighbor joining showing the evolutionary relationship of AHL lactonase; 171016, AHL acylase PvdQ; 170918-017_PA_AER and AHL acylase QuiP; VIRT17103 among themselves and with their closest BLAST hits.

Chapter 5

DISCUSSION

Many Quorum Quenching and Quorum Sensing bacteria have been reported in terrestrial as well as aquatic environment. In the present work we have isolated, identified and characterized quorum quenching bacteria from sludge of membrane bioreactor and confirmed their quorum quenching ability through different AHL degradation assays. Moreover genes encoding AHL lactonase and AHL acylase responsible for quorum quenching activity were also identified in bacterial isolates.

Bacteria are evolving continuously against antibiotics which has decreased the efficiency of this treatment against them (Beceiro, Tomas et al. 2013). Main purpose of the study was to find out a novel strategy to combat the increasing challenges posed by multi-drug resistant bacteria in healthcare sector.

The protocol for selective isolation of quorum quenching bacteria was optimized. Isolated Bacterial strains which showed significant growth on selective media with AHLs as sole source of Carbon and Nitrogen were selected for further studies. Isolation of quorum quenching bacteria involves cultivation of sample on minimal media containing AHL as sole source of Carbon and Nitrogen (Dong, Xu et al. 2000; Christiaen, Brackman et al. 2011).

These strains also showed AHL degradation capability when tested with biosensor strains CV026 and A136 which respond to exogenous AHLs by pigment production. Isolated bacterial strains significantly inhibited the pigment production by degrading AHLs provided from exogenous source (McClean, Winson et al. 1997).

To quantitate the AHL degradation by our isolated bacterial strains, AHL degradation assay by microtiter plate method was performed, using a biosensor strain CV026 which responds to exogenous AHLs by producing violacein. Current QQ bacterial strains were positive for AHL degradation to different extents as measured by intensity of violacein produced (Cady, McKean et al. 2012; Rajesh and Ravishankar Rai 2014).

P. aeruginosa is an opportunistic human pathogen and involves biofilm associated complicated infections and multidrug resistance. Its quorum sensing mechanism has been extensively characterized in all Gram negative bacteria (Rasamiravaka, Vandeputte et al. 2015). A complete quorum sensing mechanism is required in *P. aeruginosa* to be virulent and any disruption in this mechanism can lead to its decreased pathogenicity (Sio, Otten et al. 2006). *P. aeruginosa* mutants which lack proper quorum sensing mechanism show deficiency in biofilm formation (Shih and Huang 2002) . Quorum quenching enzymes AHL lactonase and AHL acylase have the ability to suppress biofilm formation in pseudomonas sp. (Dong, Gusti et al. 2002).

Inhibitory effect of cell free lysate of current bacterial isolates was tested against *P. aeruginosa* biofilms. Current bacterial isolates showed significant inhibition of biofilm formation in *P. aeruginosa* (Rajesh and Ravishankar Rai 2014). To confirm the fact that biofilm inhibition ability of our bacterial isolates is due to activity of QQ enzymes in their cell free lysates, exogenous C_4-HSL was added along with cell free lysates of bacterial isolates. Its addition caused regeneration of biofilms in treated cells more significantly in QSP03 and QQ3 strains. So this demonstrates the role of Alkyl Homoserine Lactones (AHLs) and quorum sensing in *P. aeruginosa* biofilm formation. Exogenous supply of AHLs restores the virulence factors such as biofilm formation (Favre-Bonte, Kohler et al. 2003).

C_4-HSL is a quorum sensing molecule and plays a significant role in biofilm formation of *P. aeruginosa* (Favre-Bonte, Kohler et al. 2003). Planktonic cells are not affected in this study which shows that cell free lysate only target quorum sensing by AHL degradation instead of whole biomass (Rajesh and Ravishankar Rai 2014).

Most of the human pathogens commonly including Gram negative bacteria maintain their threshold cell population by producing AHLs as their major signaling molecule and thus exhibiting the process of quorum sensing. Defects in AHL producing mechanism or inability to produce AHL causes the lack of pathogenicity in some plant pathogens (Rashid, Morohoshi et al. 2011). Hence, AHL degradation could be a potential strategy to suppress the pathogenicity and decrease in the severity of disease.

Isolated quorum quenching bacteria were tested for their antibiotic susceptibility as the antibiotic resistance is linked with pathogenic potential. Some reports also suggest the link between higher virulence and higher resistance in microorganisms (Beceiro, Tomas et al. 2013). All the isolates were susceptible to Ampicillin-Sulbactam, Chloramphenicol, Cefepime, Gentamicin, Ceftazidime, and Cefazolin and were generally regarded as non-pathogens on the basis of their susceptibility.

16S rDNA sequencing revealed the phylogenetic relationship of isolated bacterial strains with *Pseudomonas* and *Bacillus* species (Chen, Gao et al. 2013).

AHL lactonase and AHL acylase genes were amplified for identification of enzymes responsible for AHL degradation activity in isolated bacterial strains. Three gene including AiiA encoding AHL lactonase in *B. cereus* QSP03 and PvdQ and QuiP encoding AHL acylase in *P. aeruginosa* QSP01 were identified (Dong, Gusti et al. 2002; Sio, Otten et al. 2006). Amino acid sequence of AiiA amplicon was determined by online ExPASy tool. The AiiA amino acid sequence was significantly identical to AHL lactonase sequences of *Bacillus cereus* in NCBI database. Metallo-

β lactamase and metal dependent hydrolase were identified as two conserved domains in ExPASy retrieved AHL lactonase amino acid sequence of bacterial isolate *B. cereus* QSP03 through CDD analysis. According to a reported work (Dong, Gusti et al. 2002) AHL lactonase in *B. cereus* contained two conserved region which were Zinc containing glyoxylase and Metallo-Beta-lactamase II. These two conserved regions contain several glutamate and histidine residues required for binding of zinc and optimal enzyme activity (Crowder, Maiti et al. 1997). So the presence of these two domains i.e. Metallo- β -lactamase and metal dependent hydrolase (which could be zinc binding region in our enzyme), in our AHL lactonase sequence shows that these AHL degrading enzymes also have conserved zinc binding motif which is important for catalytic activity of enzyme. Moreover CDD analysis also showed the relatedness of AHL lactonase amino acid sequence of *B. cereus* QSP03 to Metallo-Hydrolase-like_MBL-fold superfamily. This superfamily of proteins specifically involve hydrolytic enzymes including AHL lactonase, beta-lactamases, and persulfide dioxygenase performing the number of biological functions (Marchler-Bauer, Bo et al. 2017).

Retrieved amino acid sequence of AHL acylase for both PvdQ and QuiP also showed high similarity to already published sequences of the AHL acylase encoding genes in NCBI. CDD analysis showed both belong to Ntn-hydrolase superfamily and penicil-amidase superfamily of proteins. PvdQ encoding quorum quenching enzyme AHL acylase is an Ntn-hydrolase (Bokhove, Nadal Jimenez et al. 2010) and well reported for its quorum quenching activity (Papaioannou, Wahjudi et al. 2009). Quip, an Ntn-hydrolase is also present in P. *aeruginosa* genome and exhibit same specificity for substrate as PvdQ (Huang, Petersen et al. 2006).

Chapter 6

CONCLUSION

In present study bacteria were isolated from sludge sample of membrane bioreactor, identified to species level and characterized for their AHL degradation characteristics. They can be referred to as "Quorum Quenching" bacteria. Genes responsible for quorum quenching activity are also identified. These genes encode for enzymes AHL lactonase and AHL acylase which are responsible for AHL degradation effect in these bacterial isolates. Among all isolated bacterial strains tested, *B. cereus* QSP03 showed highly significant results for AHL degradation assays. It also showed a significant quorum quenching effect on *P. aeruginosa* biofilm. The basic objective of this study was to search a novel strategy to inhibit quorum sensing mediated infections. The factors involved in quorum sensing can serve as a potential target to achieve the purpose. AHLs are one of the main signaling molecules in quorum sensing and can serve as the main target of quorum quenching.

A very recent review suggest the complex relationship between virulence and antibiotic resistance. Study of infections in animal models have suggested a direct link between virulence and antibiotic resistance (Roux, Danilchanka et al. 2015). Our bacterial isolates were checked for antibiotic resistance too to check if there is a possibility of them being pathogens. Almost all were susceptible to most of tested antibiotics.

- This study has shown that quorum quenching bacteria do not affect the planktonic cell growth of bacteria. This does not cause harsh environment for bacteria which usually occurs in case of treatment with antibiotics leading to mutations and resistance in bacteria. The quorum quenching strategy basically interrupts intercellular communication of QS

bacteria leading to suppression of quorum sensing regulated virulence factors i.e. biofilm formation (Rajesh and Ravishankar Rai 2014). Antibiotic treatment basically involves killing of pathogen by interfering with important housekeeping functions such as DNA, RNA and protein synthesis thus forcing a selection pressure and leading to antibiotic resistance (Dong, Wang et al. 2007). Antibiotic resistance is not a problem only in healthcare sector, in fact membrane biofouling in wastewater treatment plant is also due to microbes forming biofilms and all the available treatments fail to inhibit the formation of natural biofilms (Oh, Kim et al. 2013). So, biofilm degradation capability of QQ bacteria could not only be helpful in clinical application but they can be more effective in elimination of biofilm-based clogging of the filters

Chapter 7

FUTURE PROSPECTS

Biofilm is one of the main virulence related phenotypes regulated by quorum sensing in pathogens. In this study, we isolated bacteria producing anti-quorum sensing agents and found molecular basis of their production. It also proved AHLs as the potential target for inhibition of biofilm formation in *P. aeruginosa*. QQ strategy could be applied to treat infections. Biofouling in membrane bioreactor in wastewater treatment plants can also be dealt with successfully.

The ability of our bacterial isolates to quench the quorum sensing and identification of AHL lactonase and AHL acylase which are two main enzymes involved in AHL degradation provides a new source of these enzymes. Degradation of *P. aeruginosa* biofilms *in vitro* by these bacterial isolates could be feasible in the clinical situations too.

We can further characterize the expression of genes identified by cloning it into suitable vector and check its expression level. We can then enhance its expression by cloning it with a strong promotor and can further scale up the production of these novel anti-quorum sensing products.

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