# Detection of Quorum Quenching Enzymes Producing Genes in Membrane Bioreactor

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# Certificate

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**To my Teachers**

**and**

**Fatherly Brothers**

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Shabila Perveen

# Contents

# List of Tables

# List of Figures

# List of Abbreviations

| | | | |
|---|---|---|---|
| **%** | Percentage | **DI** | Deionized |
| **+ve** | Positive | **DNA** | Deoxyribonucleic acid |
| **°C** | Degrees Celsius | **DOM** | Dissolved organic matter |
| **µl** | Microliter | **ED** | Electrodialysis |
| **µm** | Micromolar | **EPS** | Extracellular polymeric substances |
| **100x** | 100 times | **$Fe(OH)_3$** | Iron(III)-hydroxide |
| **A/O-MBR** | Anoxic/oxic membrane bioreactor | **FISH** | Florescence in-situ hybridization |
| **AHLs** | Acyl-homoserine lactones | **F:M** | Food-to-microorganism |
| **A136** | *Agrobacterium tumefaciens* | **g/gVSS** | Gram per gram volatile suspended solids |
| **API** | Analytical profile index | **$H_2O_2$** | Hydrogen peroxide |
| **AIP** | Autoinducer peptide | **HCl** | Hydrochloric acid |
| **BH4** | *Rhodococcus* BH4 | **HRT** | Hydraulic retention time |
| **BLAST** | Basic Local Alignment Search Tool | **kDa** | Kilodalton |
| **BLASTN** | Basic Local Alignment Search Tool Nucleotide | **L** | Litre |

| | | | |
|---|---|---|---|
| **bp** | Base pair | **Lab scale** | Laboratory scale |
| **C-3** | Carbon at third position | **LB** | Luria-Bertani |
| **$CaCl_2$** | Calcium chloride | **M** | Molar |
| **$CaCO_3$** | Calcium carbonate | **PCR** | Polymerase chain reaction |
| **CEBS** | Cell entrapping beads | **PES** | Polyethersulfone |
| **CTA** | Cellulose Triacetate | **pH** | Potential of hydrogen |
| **CV026** | *Chromobacterium violaceum* | **MEGA** | Molecular Evolutionary Genetic Analysis |
| **COD** | Chemical oxygen demand | **MBR** | Membrane bioreactor |
| **Conc. $H_2SO_4$** | Concentrated sulphuric acid | **DGGE** | Denaturing gradient gel electrophoresis |
| **Min** | Minutes | **PS** | Polysaccharide |
| **MLSS** | Mixed liquor suspended solids | **PN** | Protein |
| **ml** | Millilitre | **Psf** | Polysulfone |
| **mm** | Millimetre | **PVDF** | Polyvinylidene fluoride |
| **3O$C_6$HSL** | N- (3-oxo-hexanoyl)-L-homoserine lactone | **QQ HC** | Quorum quenching hollow cylinder |
| **3O$C_{12}$HSL** | N-(3-oxo-dodecanoyl)-L-homoserine lactone | **QQ** | Quorum quenching |
| **NF** | Nanofiltration | **QS** | Quorum sensing |

| | | | |
|---|---|---|---|
| **NCBI** | National Center for Biotechnology Information | **RO** | Reverse osmosis |
| **NG** | No growth | **rpm** | Revolutions per minute |
| **$NH_3$-N** | Ammonia | **SBR** | Sequencing batch reactor |
| **NMP** | N-Methyl-2-pyrrolidone | **$SiO_2$** | Silicon dioxide |
| **$NO_2$-N** | Nitrite-nitrogen | **SRT** | Sludge retention time |
| **NUST** | National University of Science and Technology | **SMP** | Soluble microbial products |
| **$OD_{600}$** | Optical density at a wavelength of 600 nm | **sp.** | Species |
| **PAC-MBR** | Powdered activated carbon-Membrane Bioreactor | **TMP** | Trans Membrane pressure |
| **-ve** | Negative | **TOC** | Total organic carbon |
| **VSS** | Volatile suspended solids | **UF** | Ultrafiltration |
| **w/v** | Weight/volume | **x-gal** | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |
| **MF** | Microfiltration | **$Zn^{2+}$** | Zinc ion |

# Abstract

Biological techniques to control the membrane biofouling issue in membrane bioreactors (MBR) for wastewater treatments are emerging since over a decade. One such an approach is the use of quorum quenching (QQ) by bacterial cells to interrupt the interspecies quorum sensing process, the later responsible for aiding in biofilm formation leading to biofouling. Therefore, to make membrane bioreactor more efficient for use, lowering the persistence biofouling is important and QQ has the potential to lower biofouling through degradation of the acyl-homoserine lactones (AHLs) involved in biofilm formation.

The study attempted to isolate indigenous quorum quenching bacterial species from MBR treating real wastewater and to perform biosensor based biochemical analysis. The QQ genes in the selected species were detected by polymerase chain reaction. Furthermore, impact analysis of the QQ activity on extracellular polymeric substances (EPS) production in sequencing batch reactors (SBRs) treating wastewater inoculated with entrapped isolates in alginate beads was conducted.

Majority of the bacterial isolates belonged to the genus *Bacillus* and *Enterobacter* based on 16srRNA sequencing. Biosensors analysis revealed prominent QQ activity in *Pseudomonas* and *Bacillus* species. The reported QQ genes: two acylase producing PvdQ and QuiP genes in *Pseudomonas* sp. QSP01, and lactonase producing AiiA gene in *Bacillus* sp. QSP03 and *Bacillus* sp. QSP10 were detected during gel documentation. These three-species entrapped in alginate beads inoculated individually and in consortia in SBRs indicated lower EPS production in comparison to non-inoculated bioreactor, all containing seeding sludge from full scale MBR treating real wastewater.

It may be concluded from the study that the QQ bacterial profile of full scale MBR is different from that of pilot or laboratory scale MBR. The detection of AHL acylases and AHL lactonases

producing genes in the species verified their AHL degrading capacity and gives a deeper insight into the mechanism involved in AHL degradation in MBR. Moreover, lowered EPS level was observed in SBRs having QQ entrapped alginate beads. Therefore, the QQ based biofouling control technique has the potential to lower biofouling in MBR.

# 1. Introduction

## 1.1 Background

Industrialization and increasing world population are causing increased water depletion and decreased natural clean water resource availability, adding to the demand for reuse. Efforts are made to preserve available water resources by more stringent effluent standards (Visvanathan, 2000). Adding to this situation is the climate change impacts on fresh water resources and Pakistan is predicted to be a water scarce country in the decades ahead.

Among the wastewater treatment technologies, MBRs of many types has emerged in the recent decades as an important biological wastewater treatment innovation. A micro or ultra-filtration membrane is used in MBR to separate the treated water from the suspended solids (Cicek, 2003). The advantages include higher biodegradation efficiency, lesser sludge production and smaller footprint (Drews, 2010; Fan et al., 2006; Howell et al., 2003). The market of MBR technology is also growing faster than advance technologies of other wastewater treatment processes and membrane technologies. It is assumed that the markets of MBR will double every seven years (Stephenson et al., 2007).

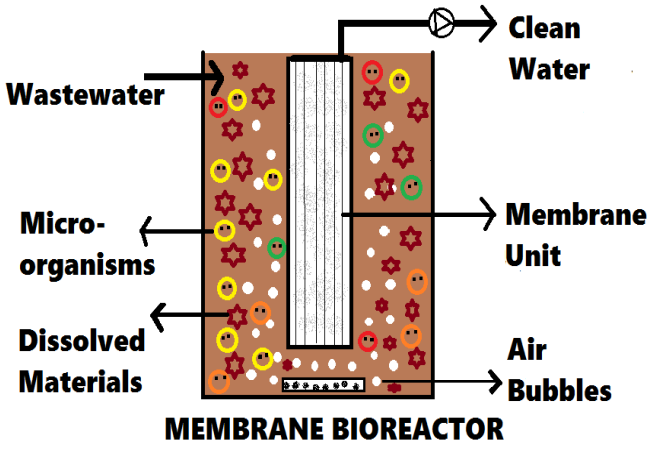



**Figure 1.1**: The interaction of bioreactor and membrane in a submerged MBR.

In MBRs the bioreactor and membrane stages interact with regards to both biological reactions and membrane filtration (Drews & Kraume, 2005; Fane, 2005), it depends on the state of biomass especially on the interactions of microbiology-membrane (Drews, 2010). Activated sludge is the core of biological treatment system. It contains diverse microorganisms including bacteria, protozoa, fungi, viruses, algae and metazoan. Bacteria is dominant, constituting almost 95% of the microbial population (Jenkins et al., 2003). Therefore, the information on composition and diversity of microbial community of biological wastewater treatment systems is important for better operation and efficiency of the system unlike the traditional focus majorly on process parameters optimization (Wan et al., 2011).

One of the major problems that adds to the cost, minimizes membrane lifespan, reduces efficiency and ultimately impedes the widespread use of MBR is membrane fouling, the biofouling being the most persistent one. In recent years, biological methods are being developed to control biofouling. The biological interventions require further screening of bacterial communities and an understanding of their role in the whole treatment process. Furthermore, ultimately the microbial community is responsible for membrane fouling (Zhun et al., 2012).

Quorum sensing (QS) has acquired consideration in the past decade due to its role in membrane biofouling. This term is used for the environmental sensing system in which the bacterial species monitor their own population density through signal molecules called autoinducers, including AHLs of varying chain length, saturation and substitution at the third carbon in its chemical structure (Anbazhagan et al., 2012). Therefore, QS is termed a regulatory mechanism by which specific genes (LuxI homologue) involved in biofilm formation in bacteria is activated by binding to a cytoplasmatic LuxR homologue (Camilli & Bassler, 2006).

AHL based QS in gram negative has three points of target: the signal generating point, the signal molecules and the signal receptor (Zhang et al., 2016). The QQ enzymes degrade signal molecules into by-produces. These by-products are unable to induce QS and are most probably utilized as sources of carbon, nitrogen or energy (Amara et al., 2010).

Previously, different approaches including QS inhibitor molecules (Brackman et al., 2009; Galloway et al., 2011) and QS signal degrading enzymes (Dong et al., 2000; Ogez and Uroz, 2010) have been used to degrade QS signal molecules. Later, bacterial species producing these degrading enzymes have been used (Kim et al., 2012; Maqbool et al., 2015; Waheed et al., 2017). The three-major QQ enzymes reported to control membrane biofouling are AHL acylases, AHL lactonases and AHL oxidoreductase (Uroz et al., 2005; Christiaen et al., 2011).

What makes QQ based biofouling control technique more attractive is its nominal or no negative impact on treatment efficiency and no production of by-products that pose threat to health or the environment. Therefore, the continuous search for novel QQ strain capable of quenching QS signal molecule remains important. To date relatively few bacterial strains have been characterized as QQ species and the most suitable strain for application in real MBR is under investigation (Cheong et al., 2013). Further study on QQ strategy to control biofouling is required in bringing the technique to full- scale application in MBR treating real wastewater.

## 1.2 Membrane biofouling

The compounds excreted by microorganisms involved in biofouling are of major concern in MBR. These compounds are the slimy and sticky substances, for example extracellular polymeric substances (EPS) or soluble microbial products (SMP) that bound to the flocs or remain free in suspension. Both these compounds consist of polysaccharides, proteins, lipids, nucleic acid while the first two components are major contributors to fouling (Drew, 2010).

While after the role of EPS, SMP and other excretions by microorganisms was proved, the focus has been expanded to more biological processes involved in membrane fouling.

Traditionally correlation of mixed liquor suspended solids (MLSS) concentration with fouling remained majorly under focus. The biomass retained in the MBR during sludge retention time (SRT) undergoes endogenous decay (autolysis) releasing dissolved organic matter (DOM) eventually contributing to membrane biofouling (Miura et al., 2007). Optimization of operating conditions, such as the hydraulic retention time (HRT), sludge retention time (SRT), MLSS and aeration rate are important for decreased DOM that will result in reduced biofouling and better effluent quality (Miura et al., 2007). The regular physical and chemical cleaning involved are not energy efficient and effective (Xiong & Liu, 2010)

Upon the relationship found between QS and biofilm formation, an anti-fouling strategy called QQ is being studied in the past few years for its potential application in MBR. Reduced biofouling by AHL-based QQ has been demonstrated in various laboratory and pilot scale studies on MBR. Still with various control strategies available, the membrane biofouling remains undesirable in the application of membrane filtration for wastewater treatment. During the filtration, the membrane is clogged with deposits of inorganic, organic and microorganism (Lewandowski & Beyenal, 2005). Hardest to deal with is the microbial biofilm (Zhang et al., 2016). This area therefore remains a main area and concern in research and development in wastewater by MBR.

## 1.3 Problem statement

Despite extensive research on the cause of membrane fouling, the problem persists. The solutions are either temporary or have various short-comings. The high biological activities in the bioreactor are central to the function of MBR and at the same time, the role of microbiological activity in biofouling cannot be ignored. Studies on this path are in infancy

due to required specialized skills, resources, time and equipment to carry out extensive studies. Having insight into the biological processes are key to finding a better solution to membrane biofouling.

Although previous studies indicated that AHL degradation by quorum quenching species delay biofilm formation in MBR, still the detailed mechanism behind such an activity is limited. Moreover, a limited number of signal molecules are targeted and major studies are based on using a single species of *Rhodococcus* sp. BH4.

The bacterial profile of sludge or cake on filtration surface is more complex and signal molecules/ autoinducers of various sizes are involved in quorum sensing. The singularity cannot be fully generalized for such a complex web of interactions. Further studies are lacking to better understand quorum quenching mechanism within this complex environment.

Microbial diversity in MBR varies from place to place, time to time and across different conditions. The screening of QS and QQ species of the MBR under study becomes viable for planning inhibition measures. Therefore, the MBR located at H-12 National University of Science and Technology (NUST) Islamabad Pakistan is screened for QQ species instead of relying on previous studies on laboratory and pilot scale MBR at the site or elsewhere. This anti-biofouling method holds great promises for the future. It has negligible side effects as compared to chemical and physical cleaning methods.

## 1.4 Research objectives of study

The study aimed at screening activated sludge from MBR treating real wastewater for QQ bacterial species. This biological pathway explores a natural solution within the MBR for membrane biofouling. It is fundamental to detect the genes responsible for disruption of QS to authenticate the role of QQ strains. Molecular analysis included PCR polymerase chain reaction (PCR) along with biosensor based biochemical analysis. Furthermore, for analysing

the long term and stable application in MBR, selected QQ species entrapped in beads were introduced for analysis for their impact on EPS level in mixed liquor.

In summary, the main objectives of the study were;

i. Isolating QQ bacterial species from full scale MBR treating real wastewater

ii. Identifying the genes responsible for QQ mechanism

iii. Studying the QQ impact on EPS level in SBRs

# 2. Literature Review

## 2.1 Wastewater treatment options

To allow the human and industrial effluent's safe disposal, avoiding danger to human health and natural environment, wastewater treatment is required. Irrigation is considered a disposal and utilization of wastewater but it requires some degree of treatment due to its impact on the performance and operation of aquaculture system (Clark et al., 1989). Therefore, there is a pressing need to innovate and improve wastewater treatment techniques for safe wastewater disposal and reuse.

The treatment options for sewage grouped per processes and function are; preliminary, primary, secondary and tertiary. Preliminary involves simple processes like screening and grit removal. This removes solid pollution. The primary treatment, usually a plain sedimentation involves settlement of solid materials which decreases the pollution load. Secondary usually involves the biological process to remove common pollutants and tertiary removes specific pollutants.

The challenges for implementation of treatment options in low and middle-income communities are high energy requirements, operational and maintenance cost including sludge disposal, and such options deals with environmental protection only, the effluent can still contain pathogenic materials with potential human health hazards. Some of the secondary treatment options available are summarized in the table below, adapted from Crites et al. (2014).

**Table 2.1**: Secondary treatment options for wastewater.

| Treatment process | Description | Treatment process | Description |
|---|---|---|---|
| Activated sludge process | Involves biological treatment that requires aeration. | Aerated lagoons | Resembles water-stabilization ponds but aeration is provided. |
| Land treatment | Under controlled conditions, sewage is supplied to the soil. | Oxidation ditch | Aerated channels of oval shape |
| Reed beds/wet lands | Flow of sewage through an area of reeds | Rotating biological contractor | Surface areas provided for bacterial growth by vertical plates |
| Trickling filters | Loose bed of stones provides surface for bacterial growth. Sewage passes down this media. | Upflow anaerobic sludge blanket | Anaerobic process, pollutants absorbed on blanket of bacteria |
| Waste-stabilization ponds | Ponds of large surface area | | |

Activated sludge based biological wastewater treatment process are the most elaborated man-made microbial systems aimed at water cleaning (Liu & Jansson, 2010).

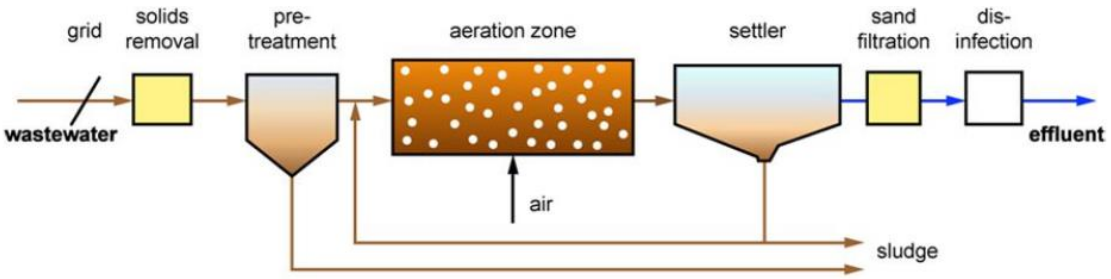



**Figure 2.1**: Conventional activated sludge system (Drews & Kraume, 2005).

## 2.2 Membrane bioreactor

The improvement of conventional activated sludge process with the application of membrane bioreactor (MBR) is a promising technology in wastewater treatment processes (Stephenson et al., 2007) where for separation of particulate material from water, activated sludge process and

membrane technology are employed without requirement of secondary classifier (Chang et al., 2011).

MBR has a high biodegradation capacity and efficiency that results into high quality permeate and lower sludge production. Effluent standards are expected to became stringent and the cost associated with MBR is falling, making it reliable for treatment of industrial and municipal wastewater (Judd, 2008).

One major reason for not utilizing MBR widely is membrane fouling especially the biofouling that causes declined permeate flux and additional requirement for cleaning and foul membrane replacement adding to treatment cost, and shortening the membrane life (Ahmed et al., 2007; Ahmed et al., 2008; Fan et al., 2006).

MBRs are typically operated at low food-to-microorganism ratio with high sludge retention time (SRT). With limited energy availability, microorganisms prefer available energy sources as opposed to biomass growth (Low & Chase, 1999; Muller, Stouthamer et al., 1995).

### 2.2.1 Membrane filtration

Microfilters or nanofilters are employed during wastewater treatment by MBR. The membrane surface receives the water to be treated. After the feed water passes through the membrane surface, the product named permeate joins surface water sources for reuse. The rejects are named concentrate or retentate.

### 2.2.2 Membrane types

Separation processes in water and wastewater treatment prefer membranes over traditional water technology based upon its good performance and process economics. Membranes are made of plastic, ceramic or metals. Few polymeric materials, widely used in making membranes are celluloses, polysulphones (including charged polysulphones), polyamides,

polyacrylonitrile, polyvinylidene difluoride, polyethylsulphone, polyethylene and polypropylene due to their desirable chemical and physical resistance (Radjenović et al., 2008).

**Table 2.2**: Membrane types that are used in water and wastewater treatment technologies.

| **Membrane Type** | **Separation Range** |
|---|---|
| Microfiltration (MF) | 100-1000 nm |
| Ultrafiltration (UF) | 5-100 nm |
| Nanofiltration (NF) | 1 to 5 nm |
| Reverse Osmosis (RO) | 0.1 to 1 nm |
| Electrodialysis (ED) | <0.1 nm |

MBR has emerged over the past decade as an effective secondary treatment technology usually apply membranes in the range of those of Microfiltration and Ultrafiltration (Radjenović, et al., 2008). The membranes that are hydrophobic in nature make them more prone to fouling upon the interaction with foulants. Commercially available membranes go through certain modifications for achievement of hydrophilic surface through chemical oxidation, organic chemical reaction, grafting and plasma treatment (Chang & Lee, 1998; Choi et al., 2002; Radjenović et al., 2008).

Application of membrane in treatment of water and wastewater are usually pressure-driven. NF and RO are high-pressure membranes characterized by smaller pore size for separation process of small molecules and ions. MF and UF are low-pressure membranes, typically employed to remove larger particles (Vanysacker et al., 2014).

### 2.2.3 Membrane bioreactor configuration

Membrane separation involves pressure-driven filtration (in side-stream MBRs), or vacuum-driven membranes. The later operates in dead-end mode and is directly immersed into the bioreactor.

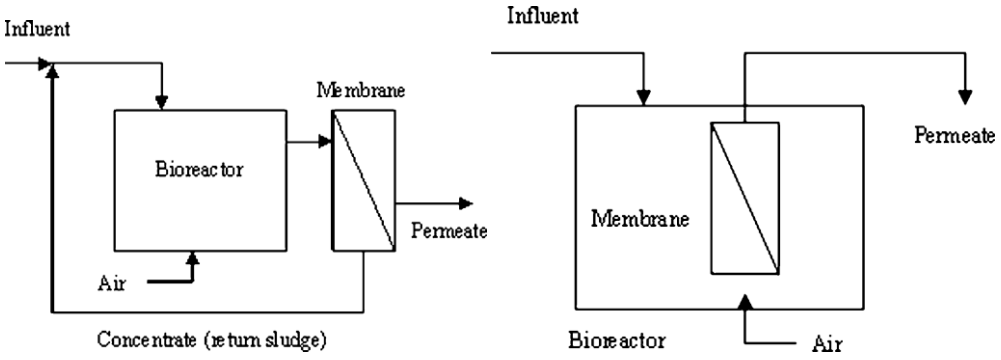



**Figure 2.2**: a) External pressure-driven membrane filtration in side-stream MBR and, b) Internal vacuum-driven membrane filtration in submerged MBR (Radjenovic et al., 2008).

The side-stream configuration requires pumping the wastewater through the membrane module, after which it is returned to the bioreactor.

The vacuum-driven membrane filtration configuration for wastewater treatment is most commonly used as it consumes lower energy for filtration. Membrane fouling is prevented in both configurations by the shear over the membrane surface provided by pumping in side-stream and by aeration in immersed processes. Configurations of MBR have either planar or cylindrical geometry.

In practice, five membrane configurations are used: Hollow fiber, spiral-wound, plate-and-frame (flat sheet), pleated filter cartridge and tubular. The first three types are widely used in MBR while plated filter cartridge and tubular module are not used widely (Radjenović et al., 2008).

## 2.3 Biological profile of membrane bioreactor

Structure and dynamics of microbial communities in MBR helps understand the biological wastewater treatment system (Muñoz et al., 2009). Moreover, knowledge of the microbial community ecology involved in biofouling, the influence of plant operation mechanisms on the structure and dynamics are significant for MBR technology optimization through anti-

fouling strategies (Herzberg et al., 2010; Huang et al., 2008; Miura et al., 2007). The microbial diversity of activated sludge is high. It constitutes prokaryotes, eukaryotes and viruses while bacteria (prokaryotes) dominate the microbial population, playing main role in the degradation process (Radjenović et al., 2008).

High bacterial diversity and adaptability to environmental changes results in more stable and better effluent quality (Miura et al., 2007). The dominant bacterial communities in raw sewage do not appear in the activated sludge. This indicates that the dominant bacterial community of raw sewage may not have any important role to play during treatment of wastewater (Liu et al, 2007).

The bacterial dynamics in MBR system have been studied by quinine profiling as well. Results indicated UQ-8 (ubiquinone) which is the derivative of subclass β-*Proteobacteria* as the dominant quinine species of the microbial community structure (Ahmed, et al., 2007). Filamentous *Chloroflexi* along with α, β, γ- *Proteobacteria* were found in both hybrid MBR and Conventional MBR. A class taxonomical scale of bacterial clones acquired from four bench scale MBRs also indicated β-*Proteobacteria* (majorly of taxonomic orders *Rhodocyclales* and *Burkholderiales*) as the dominant species (58%), followed by *Sphingobacteria* (22%) and *Flavobacteria* (8%) (Falk et al., 2009).

A study found the minimum number of bacterial species in a full scale MBR as estimated by nonparametric estimators to be 300. The clone distribution in the library indicated that β-*Proteobacteria* is 27%, Bacteroidetes 25% and 14% α- *Proteobacteria* along with other phylogenetic groups making up 34% of the bacterial species (γ- *Proteobacteria, Actinobacteria, Acidobacteria, Chorobi and Firmicutes.* Majority of the ammonia oxidizing bacteria were affiliated with *Nitrosomonas oligotropha* (Wan, et al., 2011). β-*Proteobacteria* was also found to be predominant in activated sludge of two sewage treatment plants (Liu, et al., 2007).

Another study also indicated that identified sequences in a pilot scale MBR mostly belonged to α, β, γ- Proteobacteria (majorly including classes of *Caulobacteraceae*, *Sphingomonadaceae*, *Comamonadaceae* and *Xanthomonadaceae*) along with representatives of *Deltaproteobacteria*, *Actinobacteria* and *Gemmatimonadetes* (Muñoz, et al., 2009). While α- *Proteobacteria* was dominant among the TGGE bands acquired from an MBR (Calderón et al., 2012).

Previous studies have suggested that a large and more dynamic metacommunity exists for a less specific functional group and feed complexity negatively correlates with the size of metacommunity (Curtis & Sloan, 2005; McGuinness et al., 2006). While the strength of ammonia level in wastewater show prominent impacts on AOB community structure (Kuo et al., 2006; Lydmark et al., 2007).

Altogether, studies indicate *Proteobacteria* as the dominant bacterial group. There are different results for dominance of *β - Proteobacteria* or *α- Proteobacteria* (Calderón, et al., 2012). This could be due to biases introduced by culture dependent methods (Eschenhagen et al., 2003; Xia et al., 2010).

In another study on a pilot scale submerged MBR, predominant phyla at all temperatures were *Proteobacteria, Nitrospira* and *Bacteroidetes*. Due to strong influence of temperature on the bacterial community however, *α- Proteobacteria* and few filamentous bacteria (*Actinobacteria, Haliscomenobacteria and Thiothrix*) were dominant at low temperature while *Zoogloea* appeared at higher temperature (Ma et al., 2013). These studies confirm the occurrence of changes in bacterial community dynamics across differing physical conditions, like temperature.

## 2.4 Understanding membrane biofouling

Fouling is the membrane's external and internal surface coverage by deposits during MBR operation. The term fouling is generally used for all phenomena that cause permeability loss that adds to the overall cost due to elevated energy demands. Due to fouling the productivity or permeate yield is decreased, inefficient chemical cleaning may reduce modules' lifespan requiring replacement, and the aeration requirement increases (Drews, 2010). Hence fouling is the main drawback of MBR, making it costly.

Few of the major causes of membrane fouling are: Macromolecular and colloidal matter adsorption; biofilms formation; inorganic matter precipitation and membrane aging (Radjenović et al., 2008). Biofouling includes the microbial cell and microbial metabolites accumulation on membrane causing membrane fouling. These metabolites, either in bound form (EPS) or soluble form (SMP), produced by microorganisms and cells are considered the drivers of biofouling in MBR. The microbial metabolites are majorly constituted by proteins and polysaccharides (Gao et al., 2013; Laspidou & Rittmann, 2002; Le-Clech et al., 2006; Meng et al., 2005; Rosenberger et al., 2006).

Understanding the mechanisms and identifying the responsible substances can help control fouling. Factors that are commonly quoted to affect fouling include the membrane, sludge characteristics and operation (Chang et al., 2002; Clech et al., 2006). Hence it includes the biological, membrane operation and design parameters altogether (Judd, 2008). Major contributors to fouling are assumed to be polysaccharides (PS) and proteins (PN). Thus the EPS or SMP concentration relies on measuring the PN and PS (Drews, 2010). This would reflect microbial proliferation and their activities, making microbial community central during membrane fouling process (Yeon et al., 2009).

Study on the impact of microbial ecology in MBR on membrane biofouling (Ahmed et al., 2007) and on its treatment efficiency is neglected. Studies have indicated pre-treatment of wastewater as an important measure for controlled membrane fouling in MBR in case of low MLSS concentration (Miura et al., 2007) while positive impact on membrane fouling due to excessive mixed liquor viscosity has been observed (Itonaga & Watanabe, 2004). Membrane biofouling also depends on the composition of substrate (Ahmed et al., 2008).

### 2.4.1 Fouling components in MBR

There are various fouling components in MBR. They can be categorized into three groups: organic fouling, inorganic fouling and biological fouling (Meng et al., 2009).

**Table 2.3**: Fouling categories in MBR.

| **Fouling category** | **Fouling components** |
|---|---|
| Organic fouling | polysaccharides and proteins |
| Inorganic fouling | $CaCO_3$, $SiO_2$ and $Fe(OH)_3$ |
| Biological fouling | Bacterial cells |

Source: Vanysacker et al. (2014)

Bacterial cells attach to wet surfaces, they multiple and capsulate themselves in a slimy matrix produced naturally by themselves in the form of extracellular polymeric substances (EPSs) called biofilm. More than 99 percent of bacteria in the natural world are said to be residing in biofilms (Costerton et al., 1987).

### 2.4.2 Mitigation strategies for membrane-fouling in MBR

In consideration of the economic and ecological issues associated with membrane fouling, many strategies to cater the fouling issue have emerged that are biological, chemical, mechanical and/or hydrodynamic in nature. Biofouling control is applied at bubbling, suction

of permeate, during backflushing, dosing additive and other means. The additives used have associated adverse impact along with elevated energy demand and decreased productivity (Drews, 2010).

Aeration in aerobic MBRs is employed to provide the microbial biomass dissolved oxygen and help keep solids in suspension, also to minimize membrane fouling (Calderón et al., 2011; Germain & Stephenson, 2005).

Another measure is the addition of flux enhancers. Adding cationic polymer to the mixed liquor of MBR enhances the filterability (Yoon et al., 2005). Addition of nanomaterials, Fullerence $C_{60}$ inhibits the respiratory activity and attachment of the bacterial species *Escherichia coli* (Chae et al., 2009).

Mitigating membrane biofouling in MBR by powdered activated carbon (PAC) has also been studied (Khan et al., 2012). It provides large surface area, support medium and habitat for bacterial activities at low temperature (Ma et al., 2012; Seo et al., 2002).

Along with traditional methods, design parameters also determine fouling while new strategies are emerging like inhibition of quorum sensing, an anti-fouling strategy (Lee et al., 2007). Quorum sensing is the communication among bacteria by signal molecules such as AHL and others contribute to biofilm formation (Drews, 2010).

AHL activity is found to be low at early stages of filtration in MBR and mature when fouling is fully developed. Addition of acylase enzyme to disrupt these signal molecules (autoinducers) show controlled fouling (Yeon et al., 2008). Magnetic enzyme carries to overcome the limitation of using this technique (Yeon, et al., 2009) can be retained and recovered by magnetic capture along with other benefits of high stability and delayed fouling (Drews, 2010).

## 2.5 Factors affecting biological processes in MBR

The bacterial community structure in MBR is more impacted by the quality (turbidity, TOC, DOC, TP and pH) of influent than by the MLSS concentration (Miura, et al., 2007). The main environmental factors influencing microbial growth are pH value and temperature. Temperature governs the rate of treatment and impact the composition of bacterial population. Optimal performance in MBR is achieved at temperature range of 15 to 25 °C and pH range of 7.2-8.5 (Radjenović et al., 2008). Studies suggest that the role of solid retention time (SRT) and biomass is important in determination of the microbial diversity (bacterial and AOB community) (Cook et al., 2006; Franklin et al., 2001).

The operational aspects including longer SRT, shorter HRT and shear forces also impact the microbial community in the activated sludge of MBR (Luxmy et al., 2000). The impact due to strength of feed and invasion by exotic species through influent are considered less important in case of MBR as it is operated at lower F: M ratio, and high biomass concentration with limited sludge wasting by membrane rejection (Wan et al., 2011).

Volatile suspended solid (VSS) concentration influence the spread of bacterial population in the sludge of a pilot scale MBR operated for domestic wastewater treatment (Muñoz et al., 2009) and the effect was more profound for VSS concentration exceeding 8000 mg/L (Munoz et al., 2007). Bacterial diversity in MBR is strongly influenced by the volume of the bioreactor (van der Gast et al., 2006). While the performance of the bioreactor (effluent quality) was found to be unaffected by changes in bacterial dynamics during the study period due to bacterial species redundancy and common function of diverse bacterial community (Muñoz et al., 2009).

But the source of diversity developing a specific community must be considered in order to link community structure and function (Curtis & Sloan, 2004). Moreover, rate of sludge

mineralization is impacted by bacterial diversity and is an important factor to be considered for its disposal or usage as organic fertilizer (Muñoz et al., 2009; Spinosa, 1998).

Operating an MBR with high viscosity impacts the energy requirements for pumping, air scour of the membrane and aeration for the microorganisms. Although there have been extensive studies on MBR processes, no systematic study encompassing all interactions have been made so far (Drews, 2010).

## 2.6 Bacterial communication system and inhibition

Bacteria have their own way of communication. Quorum sensing, discovered in 1970's was defined as a mechanism that coordinates phenotypic expressions at the population level, like bioluminescence (Nealson, 1977). Quorum sensing (QS) is a cell-to-cell communication mechanism; microbial cells use this mechanism to assess their local densities or gradients of diffusion resulting in control of gene expression (Shao & Bassler, 2012). The mechanism is driven by signal molecules production, secretion and sensing. When the signal molecules are accumulated to a threshold concentration, a change in gene expression is triggered in the population (Waters & Bassler, 2005).

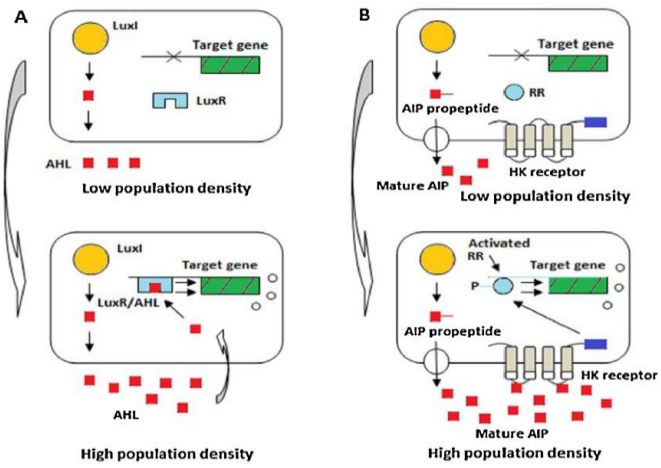



**Figure 2.3**: Quorum sensing in bacteria. A) AHL based QS in gram-negative bacteria. B) Autoinducer peptide based QS in gram-positive bacteria. (Thiel et al., 2009).

At a low population density or high diffusion rate, acylhomoserine lactone (AHL) are at low concentration and the LuxR (gets activated at high cell density) receptor is degraded. The receptor is activated when the AHL concentration reaches a specific concentration by forming AHL/LuxR complex. Studies show AHL based quorum sensing in about 10% *proteobacteria* classified as gram-negative. The system in gram-positive QS bacterial species acts in an analogous fashion, the signal involves autoinducer peptide (AIP).

The mechanism involves production of AIP precursors, post-transcription modification and secretion through specific transporters. At maturity, AIP concentration increases and they bind to transmembrane histidine kinases which get activated and in turn activate the downstream response regulator. This process of activated regulators initiates specific genes transcription (Siddiqui et al., 2015).

To control the phenotypic expressions such as biofilm formation, virulence, motility, luminescence, competence that are regulated by QS, anti-QS techniques are being explored. Controlling QS is considered a better option to avoid these phenotypic expressions as loss of QS activities are found to pose no threat to the cell activities (Siddiqui et al., 2015).

### 2.6.1 QS controlling strategies

Different mechanisms have been studied for the inhibition of QS activities, including;

- QS signal production control (Chen et al., 2011). This strategy involves techniques to disrupt the signal molecules production. For instance, the *LuxI* genes in gram-negative bacterial species produces AHLs. The target in this mechanism is therefore the *LuxI* genes. It aims at complete disruption of signal molecules production.
- QS signal (AHL) degradation (Sio et al., 2006). The targets in this strategy are the signal molecules. The production is not stopped while after production the density is controlled by degradation of signal molecules. Majorly this technique is in practice.

- QS signal activity (AHL cognate receptor protein or AHL synthase) control (Parveen & Cornell, 2011). This method involves the control at expression site, that is the signal molecule interaction with genes that are expressed is targeted. In case of AHL based QS, the *LuxR*-AHL complex triggers the biofilm formation, hence this the technique avoids the complex from forming.
- QS signal mimicking by synthetic compounds as signal molecule's analogues (Chen et al.,2011). This is a rare technique in which such compounds are introduced in the system, which have more affinity towards the group of genes otherwise expressed by signal molecules attachment.

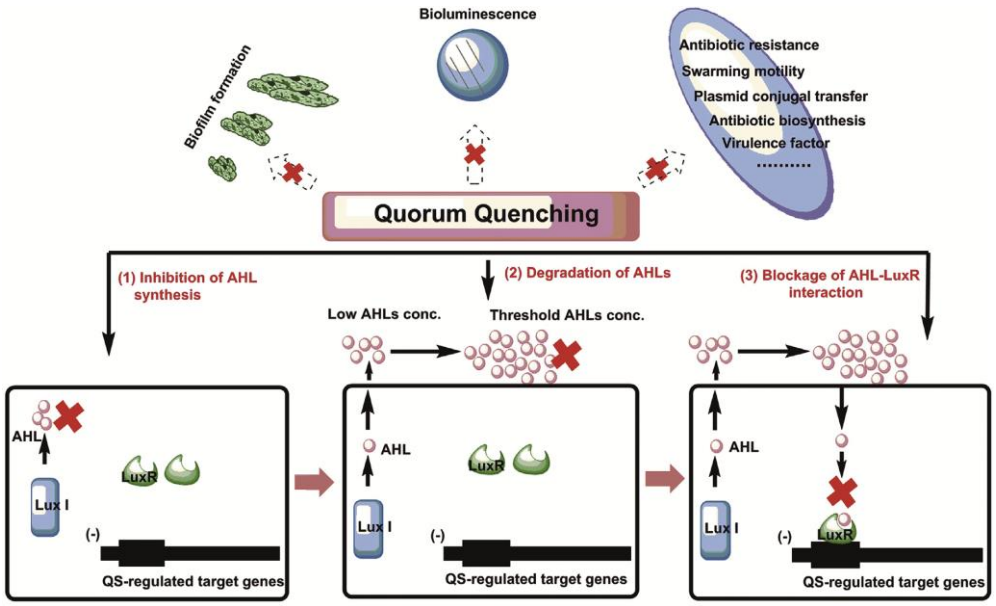



**Figure 2.4**: Few AHL based QS control strategies: 1) AHL synthesis blockage, 2) Signal receptor protein interference, 3) Inactivation of AHLs (Lade et al., 2014).

## 2.7 QQ bacterial species and enzymes

During the past decade, attempts are made to isolate and identify quorum quenching bacterial species in MBR for biofouling control. Kim et al. (2014) indicated that QQ enzymes are produced by *Afipia* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Micrococcus* sp., *Microbacterium*

sp. and *Rhodococcus* sp. strains. Majorly *Rhodococcus* sp. strains are being utilized around the world in similar studies owing to their ability to degrade various kind of AHL chains.

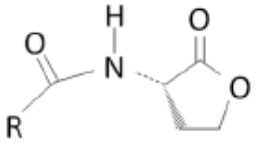
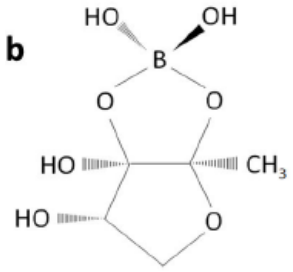
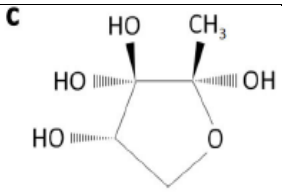
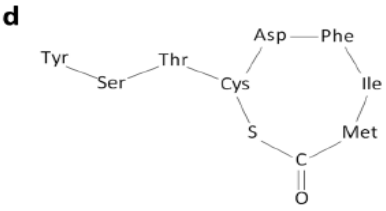
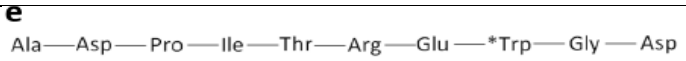
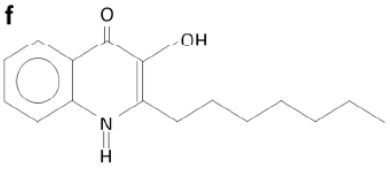
QQ enzymes were discovered in a wide range of bacteria and were classified into three major types according to their enzymatic mechanisms: AHL lactonase (lactone hydrolysis), AHL acylase (amidohydrolysis) and AHL oxidase and reductase (oxidoreduction). The metal centres at the active sites of these enzymes are considerably diverse (Lee et al., 2014).

**Table 2.4**: Representative QQ enzymes, degrading mechanism and host.

| QQ Enzymes | Properties/degradation mechanism | Few Producer Bacteria |
|---|---|---|
| **Lactonases** | ▪ Conserved $Zn^{2+}$ binding domain HXHXDH<br>▪ *Bacillus* sp. molecular weight is 33.6 kDa with *N*-glycosylation<br>▪ Inactivate AHLs by hydrolyzing the ester bond of the lactone ring of the molecules yielding a homoserine<br>▪ AHL-lactonase exhibits excellent thermal stability at temperatures below 37 °C<br>▪ AHL-lactonase encoded by the *aiiA* gene is an acidic protein with its isoelectric point at 4.7 pH<br>▪ AHL-lactonase enzyme activity is pH-dependent with the optimal pH at 8 | -*Bacillus* sp.<br>-*Arthrobacter* sp.<br>-*Agrobacterium tumefaciens*<br>-*Rhodococcus* sp.<br>- *Streptomyces* sp.<br>-*Klebsiella* sp.<br>-*Ralstonia* sp. |
| **Acylases** | ▪ Cleave the amide bond of AHLs into free homoserine lactone and a fatty acid | -*Streptomyces* sp.<br>-P*seudomonas* sp.<br>-*Ralstonia* sp.<br>-*Comamonas* sp.<br>-*Shewanella* sp.<br>-*Streptomyces* sp. |
| **Oxidoreductase** | ▪ Targets the acyl side chain by oxidative or reducing activities | -*Rh. erythropolis* |

The common acyl-groups of AHLs identified so far vary from 4 to 18 carbons in length; they may be saturated or unsaturated, and with or without a C-3 substitution (usually hydroxy- or oxo).

**Table 2.5:** Some identified autoinducers and their chemical structures.

| Autoinducer | Chemical structure |
|---|---|
| a. Base AHL molecule |  |
| b. AI-2 in *Vibrio harveyi* |  |
| c. AI-2 in *Salmonella enterica* |  |
| d. AIP in *Staphylococcus aureus* |  |
| e. AIP in *B. subtilis* |  |
| f. *Pseudomonas aeruginosa* quinolone |  |

Camilli & Bassler (2006); Zhang et al. (1993)

The mechanism involved in the degradation of AHL is illustrated by the diagrams adapted from Chen et al. (2013).

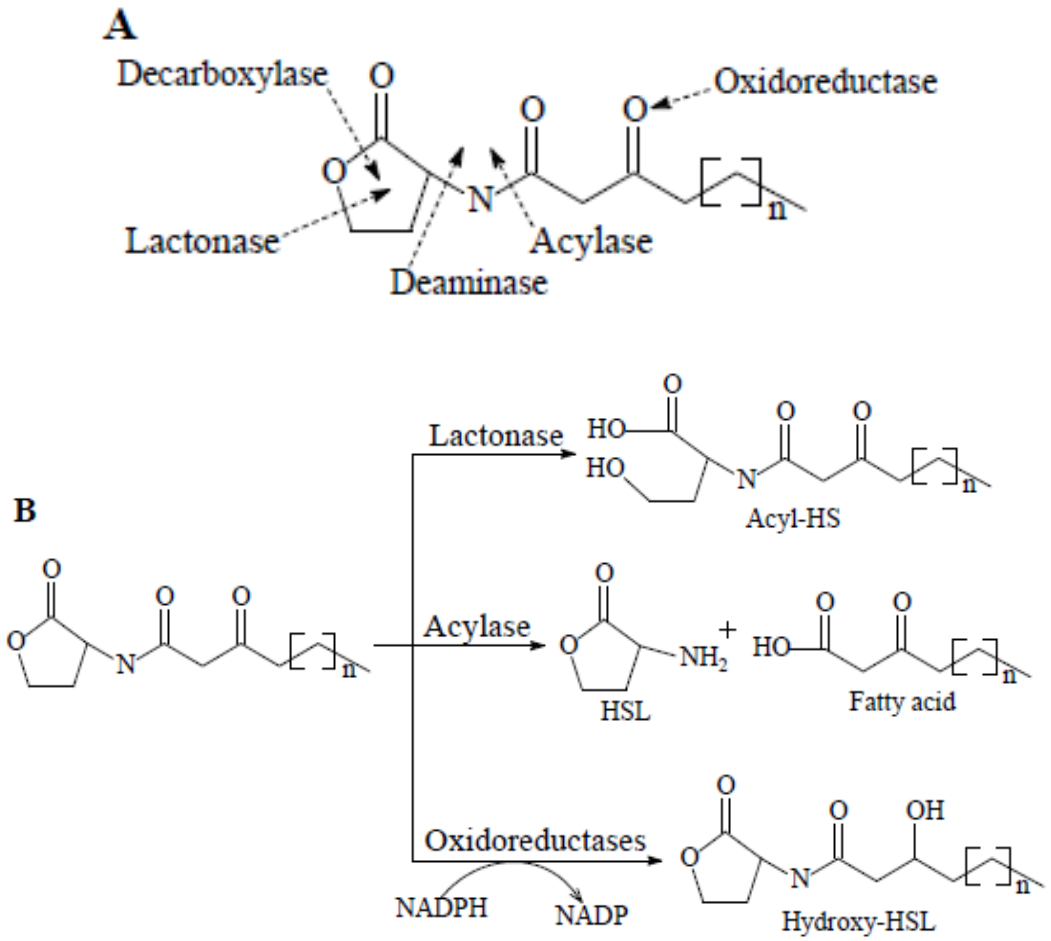



**Figure 2.5**: (A) Possible linkage degraded by QQ enzymes in quorum sensing molecule N-acyl homoserine lactone (B) Degradation mechanism of QQ enzymes. (Chen et al., 2013).

## 2.8 Bacterial immobilizing materials and techniques

To utilize QQ bacteria in MBRs for long-term sustainable operations, one important strategy is cell immobilization to restrict the cells and prevent gradual decrease of QQ bacteria population. Few materials in literature are:

- Cellulose Triacetate (CTA) Polymeric Gel Matrix
- Alginate
- Chitin
- K-Carrageenan
- Polyacrylamide

Initially, AHL degrading enzymes were introduced by immobilizing the enzymes in nanofiltration membrane (Kim et al., 2014) and magnetic carriers (Yeon et al., 2009). Later to overcome the associated cost and enzyme loss through enzyme degradation, bacterial cells producing these enzymes are being introduced in MBR.

Such attempts have resulted into cell entrapped micro-vessels (Jahangir et al., 2012; Oh et al., 2012), beads (Kim et al., 2014; Maqbool et al., 2015; Weerasekara et al., 2016), macrocapsules coated with polymer (Lee, et al., 2014), hollow cylinders (Lee et al., 2016) and sheets (Nahm et al., 2017). Other attempts include hybridizing quorum quenching with other anti-fouling methods. A couple of examples include QQ with backpulse method and relaxation (Weerasekara et al., 2014) and QQ with chlorination technique (Weerasekara, et al., 2016).

### 2.8.1 Methods of entrapping QQ bacteria

The table summarizes the techniques involved in entrapping QQ bacteria for application in MBR.

**Table 2.6:** Few QQ bacteria entrapping methodologies.

| Media | Preparation | Reference |
|---|---|---|
| QQ sheets | - QQ bacteria pallet resuspended in DI water mixed with polyvinyle alcohol and sodium alginate (10:1) solution<br>- Mixture with the aid of micrometer film applicator, casted over a glass board<br>- Cross-linking by boric acid and $CaCl_2$ solution (7:4) and 0.5 M sulphate solution<br>- Finally cut into 10mm × 10mm for application in MBR | Nahm et al., 2017 |
| QQ hollow cylinder (QQ HC) | - Bacterial aliquot contains 7mg of BH4 per dry weight of QQ-HC<br>- Polyvinyl alcohol-alginate matrix mixed with bacterial suspension<br>- Solution extruded through nozzle into $CaCl_2$ and boric acid solution for cross-linking | Lee et al., 2016 |
| Coated QQ beads | - 2% sodium alginate solution mixed with 2mg per gram of dry weight of solution<br>- Dripped into 4% $CaCl_2$ solution | Kim et al., 2015 |

| Media | Preparation | Reference |
|---|---|---|
| | - Phase inversion technique for coating with PVDF, PES, Psf | |
| QQ beads | - Polyvinyl alcohol- alginate matrix mixed with BH4 suspension (7mg per dry weight of alginate solution)<br>- Dripped by peristaltic pump into $CaCl_2$ and boric acid solution | Kim, et al., 2014 |
| Cell entrapping beads (CEBs) | - 4% (w/v) sodium alginate and BH4 suspension<br>- Suspension dripped into 3% $CaCl_2$ with a flow rate of 1.6mL/min. | Kim et al., 2013 |

## 2.9 Common bacterial structure analysis techniques

Molecular biological tools employed for the study of the microbial communities in bioreactors and other engineered systems has made it possible to link diversity and dynamics to process stability. Previously cultivation-based studies have been conducted for analysing microbial diversity but it gave a partial picture (Briones & Raskin, 2003). Advent of molecular tools improved the scenario, from which PCR is widely used for study of microbial community structure because of its simplicity and sensitivity.

Moreover, DNA fingerprinting methods can be used to analyse PCR products (Hill et al., 2000; Ogram, 2000). Furthermore, the PCR products sequenced through cloning or fingerprinting electrophoresis gels helps identify population and characterize the structure of community. The biases in amplification products of PCR can be overcome by using specially designed primers and sensitive optical detection (Lim et al., 2001; Stults et al., 2001; Wintzingerode et al., 1997).

The commonly used microbial biological tools include florescence in-situ hybridization (FISH), polymerase chain reaction coupled with either denaturing gradient gel electrophoresis or temperature gradient gel electrophoresis (PCR-DGGE/TGGE). These methods have yielded information on microbial diversity and structure in natural and engineered habitats including

microbial species that were previously not known due to limitations of cultural-based approaches (Lorenzo et al., 2006; Luxmy et al., 2000; Muñoz et al., 2009).

Polymerase chain reaction- Denaturing gradient gel electrophoresis (PCR-DGGE) method enables simultaneous analysis of many samples while preferential DNA amplification and efficiency of DNA extraction may affect its accuracy (LaPara et al., 2002).

Florescence in-situ hybridization (FISH) method has been used in combination with PCR-DGGE method to overcome the limitations and to quantify bacterial structure (Miura, et al., 2007). FISH in MBRs is limited due to low percentage of detectable fluorescent cells (Rosenberger et al., 2000).

DGGE fingerprinting technique has been used to examine the structure of bacterial populations in varying environmental conditions. For detailed molecular examination of diversity and composition, clone library analysis of phylogenetic and functional markers have been employed (Wan et al., 2011).

Both DGGE and TGGE are tools for monitoring variations in structure of bacterial community and taxonomic identification of dominant community members (Miura et al., 2007; Muñoz et al., 2009) but the limitation includes comigration of fragments with common electrophoretic behaviour (LaPara et al., 2006).

Quinone profiling had also been used to detect bacterial diversity in MBR system. Quinone is represented in mole fraction of each quinone type, which is considered to be specific for microbial communities (Ahmed et al., 2007; Ahmed et al., 2008). The variations in enzyme activities are also used as an indicator for the evaluation of mixed species community's physiology in the sludge (Munoz et al., 2007).

## 2.10 Relevance of bench studies for full scale membrane bioreactor

The interpretation of bench scale or lab-scale results to full- scale plant operation requires the understanding of severe differences in typical conditions of operation. The time scale, feed parameters and temperature fluctuations are the important aspects to compare. Moreover, in lab scale studies, synthetic wastewater is used that may give non-representative microbial communities (Drews, 2010).

Another aspect is the size of the facility. The soluble microbial products (SMP) concentrations have been found elevated in lab and pilot plant in comparison to full-scale plants (Drews, 2010; Drews & Kraume, 2005; Judd, 2008), the reason for which has not been found yet while difference in energy inputs is given as a possible reason for it (Drews, 2010). These different aspects indicate that the sludge and fouling process might be different at different scales. A study found that fouling mechanism cannot be interpreted with current information available from lab-scale results (Lyko et al., 2008).

While in many cases bench scale trials are the only way to analyse relationships between certain variables because in a complex network, at least few variables can be fixed or kept constant. Moreover, to avoid the interactions of ambient conditions in full-scale plant, lab-scale studies remain the only option to independently analyse influences on biological kinetics in MBR (Drews et al., 2007). Therefore bench scale studies are important for the fundamental researches on MBR but the restrictions in applicability in full-scale plant shall be considered (Kraume et al., 2009).

# 3. Methodology

## 3.1 Bacterial isolation and identification

Bacterial isolation was carried out from sludge samples collected from a full-scale MBR situated at H-12 Campus of National University of Science and Technology Islamabad Pakistan. Method previously used for isolation of bacterial species prepared in minimal media with AHLs as the sole carbon source were used, adopted from Christiaen et al. (2011) with slight modifications.

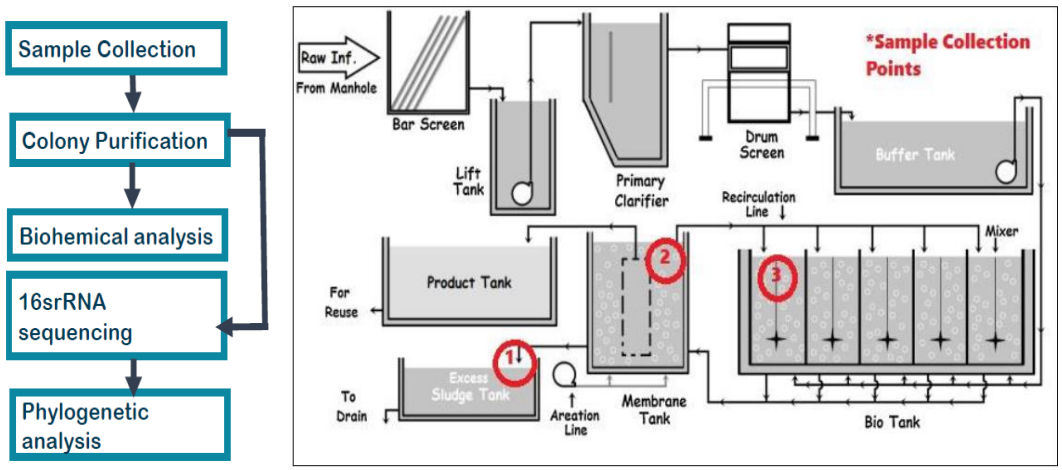



**Figure 3.1:** Schematic diagram of initial stage methodologies and sample collection points (Khan et al ., 2017).

### 3.1.1 Sample collection and preparation

Sludge samples were collected from a full-scale membrane bioreactor from three points: biotank, membrane tank and sludge tank. The samples collected in glass bottles were mixed and prepared within an hour for subsequent bacterial isolation. In laboratory, the following steps were followed, adopted from Christiaen et al. (2011) for sample preparation:

- Proper mixing of samples collected from three points of MBR

- 05 mL of the sample mixed with 50 mL of 0.9 percent saline solution
- Mixing and two minutes vortexing followed by two min sonication
- Centrifugation at 3000rpm for a minute and removal of debris
- Collection of the supernatant and another cycle of centrifugation for five minutes at 4500rpm
- Resuspension of pallets in 15 mL saline solution (0.9%)
- Suspension formed is used for further bacterial isolation.

### 3.1.2 Minimal media preparation and AHL mixtures

The minimal media (MM) protocol followed was adopted from Cheong et al. (2011). In total 200 mL MM was prepared for enrichment process of bacterial isolation. The composition per litre is provided as annexure- A. The preparation steps include:

- Compounds (Annexure- A) were mixed in 190 mL distilled water and the solution was acidified to pH 5.5 with 1M HCl
- Trace elements were added and filtered through 0.45 µL
- Media was autoclaved for sterilization
- AHLs mixture was supplemented as sole carbon source during enrichment process for isolation of QQ bacterial species.

### 3.1.3 Bacterial isolation

In a set containing three replicates, mixture of all AHLs was provided as sole carbon source. In this set, blank containing distilled water was included. The enrichment process was concluded in the following manner, adopted from Christiaen et al. (2011):

- 750 µL of the inoculums from the prepared sample was added to the six micro-centrifuge tubes
- 700 µL of the minimal media were added in each tube including blank containing 750 µL distilled water
- 10 µL of mixture AHLs (0.5mg/L) was added to the second 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 were transferred to 1 µL fresh minimal media with addition of AHLs in the same quantities
- Samples were incubated for three more days at the same temperature
- Cycle was repeated in a similar fashion
- After third enrichment cycle, 100 µL of the aliquot were spread on LB, TSA and nutrient media.

### 3.1.4 Colony purification and biochemical analysis

After 24 hours of incubation, on the LB, TSA and nutrient media plates with spread cultures, limited number of colonies appeared that had survived on AHLs as sole carbon source. Colonies that were morphologically different were picked and streaked on separate agar media plates. The plates were incubated for 48 hours at room temperature.

Based on morphological difference, colonies were further separated and restreaked until same colonies were achieved on separate media plates. The subcultures were streaked multiple times until pure cultures were achieved. These were subjected to the following analysis:

- Colony morphology- Each pure culture's colony was observed on three agar media plates: nutrient agar, LB agar and TSA agar for form (circular, irregular, filamentous,

rhizoid, curled); size (punctiform or diameters in mm); surface (shinny, smooth, veined, rough, dull, wrinkled, glistening); texture (dry, moist, mucoid, brittle, viscous, butyrous); color (opaque, cloudy, translucent, iridescent); elevation (flat, raised, umbonate, crateriform, convex, pulvinate); margin (entire, undulate, lobate, curled, filiform); smell (pungent, non-pungent) and growth (thick, thin).

- Bacterial cells morphology- Gram test was carried out and the observations of bacterial cells were made under light microscope (100x). Bacterial cell shape (cocci, bacilli, spiral, vibrio) and arrangement (single, diploid, triplets, chain, cluster, random) were studied.
- Catalase test- Slide method was used for catalase test. Colony picked by a heat sterilized loop was transferred to a clean glass slide containing a drop of 03 percent $H_2O_2$. Bubble formation was observed in few seconds in catalase positive strains.
- Oxidase test- Swab method was adopted for oxidase test. Colony picked by a female swab was dipped in oxidase reagent. The change in colony's colour was observed after few seconds for oxidase positive and negative strains.
- Differential media (MacConkey and EMB agar)- Colonies were further streaked on MacConkey and EMB media agar plates. Colonies grown on the media plates were noted as gram negative bacteria. Colour of the colonies were observed as it gives information on lactone fermenting and non-lactone fermenting ability of bacterial species.

### 3.1.5 16SrRNA sequencing for identification

Glycerol stocks of pure colonies were prepared for identification, storage and further experimental analysis. For the preparation of stocks, 0.5 millilitre LB broth in micro-centrifuge

tubes was autoclaved for 20 minutes at temperature 121°C. The tubes were brought to room temperature in laminar flowhood to acquire sterile environment for stock preparation.

The micro-centrifuge tubes were given unique identification codes and inoculated with pure colonies. After incubation for 24 hours at 37°C, 0.4 µL of 50 percent glycerol (prepared with distilled water and heat sterilized) was added to the micro-centrifuge tubes containing the cultures. After proper mixing, the stocks were stored at -20°C. The glycerol stocks were sent for identification by 16SrRNA sequencing (Macrogen, Korea). All raw sequence result files were received in .ab1 data file format.

### 3.1.6 Sequence processing and phylogeny analysis

Majorly the acquired sequence data was processed with multiple software available for sequence processing, alignment and making consensus sequences. Closest related sequences were obtained from the BLASTN function in NCBI. The data files were subjected to the following processing with the aid of various software, before submission to NCBI to acquire accession numbers:

- BioEdit sequence alignment editor version 7.2.5- all the .ab1 result files were compared against the result graphs for mismatches between graph peaks and nucleotide base reported in file. The editor was used for correction of mismatches in both forward and reverse sequence data for each strain.
- The forward and reverse sequence data of each strain were opened in a single window in BioEdit software again. After reverse complementing the sequences, a single consensus sequence was acquired under the function: Alignment- Create Consensus Sequence

- MEGA 7 – the masking function was used to mask out noises in the file. The benefit of the function is the visual representation of graphs that enables manual masking. Phylogenetic trees were constructed on MEGA 7 after conversion to data format to .meg. Neighbour-joining method was selected for the analysis.
- NCBI BLASTN- The consensus sequence acquired from forward and reverse sequence data were examined in BLASTN in NCBI database. Sequences showing 99-100 percent similarities and lower exponential (e) values were acquired
- NCBI GenBank- The processed consensus sequence of each strain was summed up in a single file following the submission direction given by BankIt tool. Accession numbers were acquired after few weeks for each strain.

### 3.1.7 Analytical profile index (API) kit

An attempt was made to identify and study the properties of seven non-sequenced species by API 20E text kit (Biomeurix, Canada). The kit is employed for enteric and non-fastidious bacterial species identification. The 20 capsules in the kit has media for specific biochemical characterization. For the performance of the test, the guidelines provided with the kit were followed, briefly:

- Autoclaved saline suspension (0.85%) was used for preparation of fresh colony suspensions
- Suspension in capsules of the API kit with guidelines for additional reagents where required was incubated for 24 hours at 37˚C
- Additionally, the oxidase test results were consulted in which 1% N,N- dimethyl-p-phenylenediamine dihydrochloride reagent had been used
- Colours produced in the capsules were noted and studied against the colour code for result interpretation available for API 20E

- Code of seven digits acquired was used on the online platform of Biomeurix, Canada. Bacterial species showing highly similar codes based on the biochemical characterization were acquired and reported

## 3.2 Biosensors for QQ and QS activity analysis

Three biosensor specie stocks were acquired from a PhD scholar Dr. Hira Waheed, National University of Science and Technology Islamabad, Pakistan. The biosensors stocks were used to study the QS and QQ activity of all isolated strains. The biosensors used in the present study are,

- *Chromobacterium violaceum* CV026- *C. violaceum* via Cvil/R AHL quorum sensing system regulates violacein production. It responds to C6-AHL, a short-chain AHL. CV026 is constructed as a violacein and AHL- negative double miniTn5 mutant of ATCC 31532. Exposure to external short chain AHL produces visual purple pigment (McClean et al., 1997).
- *Agrobacterium tumefaciens* A136- the strain is constructed by eliminating the TraI/R QS system. It has two plasmids, pCF218 that produces the TraR response regulator. The second plasmid is pMV26, containing the traI promoter joined to the luxCDABE operon (Watson et al, 1975). The strain is sensitive to long-chain AHLs (C8-HSL, 3-O-C8-HSL, C10-HSL, C12-HSL, 3-O-C12-HSL and C14-HSL). In the presence of x-gal (β-galactosidase), blue pigment can be observed visually upon its interaction with exogenous AHL.
- *Pseudomonas aeruginosa* QSIS2- The strain contains pLasB-SacB1 that encodes killing induced by exogenous AHLs. It is a *Pseudomanas aeruginosa* lasI rhll double mutant strain (Rasmussen et al., 2005).

### 3.2.1 QS activities

The methodology as described by Lade et al. (2014) is adopted for the screening of QS activity among the gram-negative isolated strains. The isolated pure cultures were screened by well-diffusion assay and parallel streak methods by using CV026 and A136 biosensor strains. Indicator plates were formed by,

- LB agar plates were prepared and incubated for 24 hours at 37°C for sterility test
- Flask containing LB agar cooled to 50°C was supplemented with 20µg/ml kanamycin and 10µl/ml CV026 liquid culture. After proper mixing, it was poured as thin layer above the previously prepared LB plates as top layer.
- Another flask of LB agar cooled to 50°C was supplemented with 50µg/ml spectinomycin and 4.5 µg/ml tetracycline. It was inoculated with A136 biosensor liquid culture (10µl/ml). X-gal in concentration of 80µg/ml was added. Same procedure was followed for top layer pouring
- Double layered plates were left in a sterile condition for solidification
- Sterilized micropipettes of 5mm diameter were used to make wells in the solidified indicator plates
- Each well was filled with 50 microliter test strain liquid culture
- Wells were filled in duplicate with positive (known QS species) and negative control (sterile LB broth)
- Plates were incubated upright up at 28°C for 48 hours or more where required
- Observation of colony produced around the well were made for colour changes
- Results were noted as diameter of color produced around colonies in well

Further verification was done by streaking CV026 parallel to the test strains on LB agar plates with appropriate antibiotics. LB agar plates supplemented with x-gal was solidified before

streaking A136 parallel to the test colonies. Positive and negative controls were included for comparison and result interpretation.

Colour changes were observed to verify quorum sensing activity of each strain producing short or long chain AHLs.

### 3.2.2 QQ activities

In all the biosensor based screening analysis of QQ activity by methods introduced by Lade et al., 2014, non-inoculated sterile broth with Milli-Q water was used as a negative control. Positive control was either a known QQ species of *Rhodococcus* sp. BH4 or AHL supplemented media or both as required.

#### a) CV026 well-diffusion assay

The well-diffusion assay for QQ activity study had an additional ingredient. A short chain AHL was supplemented to the indicator plates. For the preparation,

- 50µl CV026 was added to 5ml LB agar with supplemented 5µM C6HSL
- 20µg/ml kanamycin was added in the molten LB agar It was poured on the surface of prewarmed LB agar plate
- Overlay plate was left for solidification in a sterile condition
- 6mm holes were made with clean pipette tips
- Wells were filled with test strains of 50µl
- Negative control was LB broth itself
- Plates were incubated for three days at 28°C
- White colony in the background of coloured indicator plate was observed and noted in terms of diameter in millimetres

## b) A136 well-diffusion assay

- 50µl A136 was added to 5ml LB agar with supplemented 5µM C10HSL
- 50µg/ml spectinomycin and 4.5 µg/ml tetracycline and 80µg/ml x-gal were added.
- It was poured on the surface of prewarmed LB agar plate, solidified and wells made with pipette tips to contain sample cultures (50µl)
- After incubation at 28°C for three days, inhibition zone around colonies formed were observed and noted down

## c) QS1S2 assay

QQ activities were studied both in bacterial consortia and pure cultures. This biosensor strain is a *lasI rhII* double-mutant harbouring pLasB-SacBI (received from Dr. Hira Waheed, IESE NUST). It encodes an AHL-induced killing system. For QQ activity analysis in consortia,

- ABT minimal media was prepared by autoclaving solution A and solution B. Glucose, casamino acid, gentamicin and thiamine were added in the solution (Annexure- B)
- *Pseudomonas aeroginosa* QSIS2 was cultured in the ABT minimal medium at 37°C for 24 hours
- Spent medium from sample preparation step of all three cycles were autoclaved for sterilization and filtered
- Media were diluted by the ratio 1:100
- 01 ml of each diluted medium was added to 12-well microtiter plates and a replicate was added in microvials
- 03ml QSIS2 biosensor culture was added in the samples
- Minimal media with AHL was added as control in both experiment set

For the study of QQ activity in pure colonies that has been previously isolated from the sample,

- Liquid culture of all pure colonies was made in LB broth upon incubation at 37°C

- 800nM of each C4HSL and 3OC12HSL were added in the vials containing 3 ml of each test cultures
- Vials were incubated for 24 hours at 37°C
- Samples were sterilized and filtered through microfilters of pore size 0.45µm
- 01 ml of the sterilized samples were transferred to 12-wells MTP and new vials
- 03 ml QSIS2 biosensor was added in each
- Non-inoculated LB broth mixed with distil water and another non-inoculated LB broth with 800nM of both AHLs were used as reference in both sets on experiment

Employing the *P.aerogenosa* QSIS2 strain, QQ activity by the same method as used was performed to study the QQ activity in supernatant, cell pallets and whole cell of QQ pure colonies. The LB culture of each strain were subjected to,

- Centrifugation at 4,000 rpm for five minutes at room temperature
- Supernatant was collected in a separate vial
- Whole cell was resuspended in 01ml LB broth
- Biosensor culture of 03ml was added in 01ml of each sample
- Same procedure was followed as above to elucidate the QQ activity by QSIS2 biosensor

For heat treatment, the 24-hour cell culture in LB broth were sterilized and for the next 24 hours, sterile supernatant of cultures was incubated with supplemented AHLs. The assumption was, in case of extracellular QQ activity due to heat stable molecules, QSIS2 assay will indicate QQ activity in the samples.

In past studies, the calculation of remaining AHL level had been made by using the reverse relationship between QSIS2 growth and remaining AHL in percentage. The equation used by Waheed et al. (2017) is,

$$Remaining\ AHL\ level\ (\%) = \{(Final\ OD_{600} - Initial\ OD_{600}) / Initial\ OD_{600}\} \times 100$$

While in the present study the calculation is made by considering the QSIS2 growth in non-inoculated sterile broth to be maximum (0% AHL), considered the negative control having no AHL induced death of the biosensor species. The AHL supplemented broth growth is considered minimum (100% AHL remaining). The decrease in QSIS2 growth was evaluated as:

$$Decrease\ in\ QSIS2\ growth\ (\%) = \{(OD_{600}\ of\ negative\ control - OD_{600}\ of\ sample) / OD_{600}\ of\ negative\ control\} \times 100$$

The remaining AHL level is calculated from the decrease in growth equation in comparison to positive (100% AHL remaining) and negative control (0%AHL).

### 3.2.3 Bio agar assay

An attempt was made to further understand the location and predict the enzymes involved in QQ extracellular activities by method described by Cheong et al. (2013)

- CV026 and A136 indicator plates were prepared by mixing 24 hours' biosensor cultures in LB agar
- Sterile 0.45µm filter papers were dipped in 100mg/L of C6HSL in case of CV026 and 100mg/L C10HSL in case of A136 indicator plates
- Soaked filter papers were put over the respective indicator plates prepared with short and long chain AHL
- QQ active pure colonies were loaded on the filter with reference species *Rhodococcus* BH4
- Plates were incubated for 48hours at 28°C

- In coloured background, inhibition zones and their diameter were observed and recorded

## 3.3 QQ gene identification by PCR

DNA extraction kit was used for DNA extraction. Genomic DNA Mini Kit (PureLink, USA) was used for DNA extraction. The protocol provided with the kit was followed, briefly:

- Lysates preparation for gram-negative bacteria: Cell pallets were made by centrifugation. After resuspension in 180µl digestion buffer, 20µl Proteinase K was added before proper mixing/vortexing.
- The suspension was incubated at 55°C with frequent vortexing for 45 minutes. 20µl RNase A was added to the lysate, mixed and kept for two minutes at room temperature.200µl lysis/binding buffer and 96-100% ethanol each were added and vortexed for 5 seconds. A homogenous solution was formed.
- Lysates preparation for gram-positive bacteria: Lysozyme digestion buffer prepared was added in 200µl/sample. Fresh Lysozyme was added to the concentration of 20 mg/ml. pallets were resuspended in 180 Lysozyme digestion buffers. After mixing and incubation at 37°C for half hour, proteinase K in concentration 20µl was added. 200µl lysis/binding buffer was added, mixed and incubated at 55°C for another half hour. 200µl 96-100% ethanol was added and mixed to acquire a homogenous solution.
- Binding DNA: In a spin column fixed in collection tube, lysate of about 640µl was added and centrifuged at 10,000 rpm for a minute at room temperature. The spin column was put into a clean collection tube and the previous collection tube discarded in wastebin.
- Washing DNA: 500µl wash buffer was added to the column and centrifuged at room temperature at 10,000 for a minute. Collection tubes were discarded and spin column

kept in clean collection tubes. 500µl wash buffer 2 was added to the column. Both buffers had been prepared with ethanol. After centrifugation for three minutes at 14,000rpm, collection tubes were again discarded.

- Eluting DNA: The columns were placed in microcentrifuge tubes. 50µlelution buffer was added to the column and incubated for a minute at room temperature before centrifugation or a minute at 14,000 rpm.
- Storing DNA: The purified DNA was stored at -20°C for subsequent use.

### 3.3.1 Primer design

Primers specific to the identified species and QQ enzyme producing genes were designed manually and by software primer3 plus. Results from both were verified by primer blast service of NCBI and *In-silico* PCR amplification service. The selected primers were purchased from a third party dealing in primer synthesis (Operon, EU).

#### a) Conditions

The primer design condition followed were,

- Primer size: 18-22 bp
- Melting temperature (Tm): 52-62°C
- Difference between Tm of both primers: 2°C
- GC content: 50-60%
- No runs and repeats
- No secondary structure formation probability

Oligocalc software was used for evaluating whether the primer design conditions were fulfilled by the three set designed primers.

**Table 3.1**: List of designed primers.

| **Oligo name** | **Sequence (5' to 3')** |
|---|---|
| PvdQ- For<br>PvdQ- Rev | GTTCTGCACGAAGTCCCTG<br>GCTGTTGGGTTCGATGATG |
| AiiA- For<br>AiiA-Rev | GATGGCCTGGAGAATGAC<br>GCGTGTAGGGTATGAGCC |
| QuiP-For<br>QuiP-Rev | GTCGGCCAGGTAATAGAGC<br>GCTACCGTCCGGAATACTG |

## b) Steps

- NCBI Blast of partial sequence to acquire full genome sequence showing 98-99% similar identity
- Uniprot service to retrieve sequences of identified QQ genes specific to the species under consideration for the present study
- NCBI Blast of the full genome sequence and the gene sequence to retrieve the conserved area showing maximum (99%) similarities
- PrimerBlast, Pick Primer and Primer3plus for primer options service provided by NCBI
- Examination of each results for conditions fulfilment for primer design
- Manually highlighting regions for forward and reverse templates. Reverse complementing the later template
- Primerblast and Oligocalc of all manually designed primers to check its condition, repeat as many times as possible to acquire primers fulfilling all conditions
- *In-silico* PCR amplification to examine the probable results

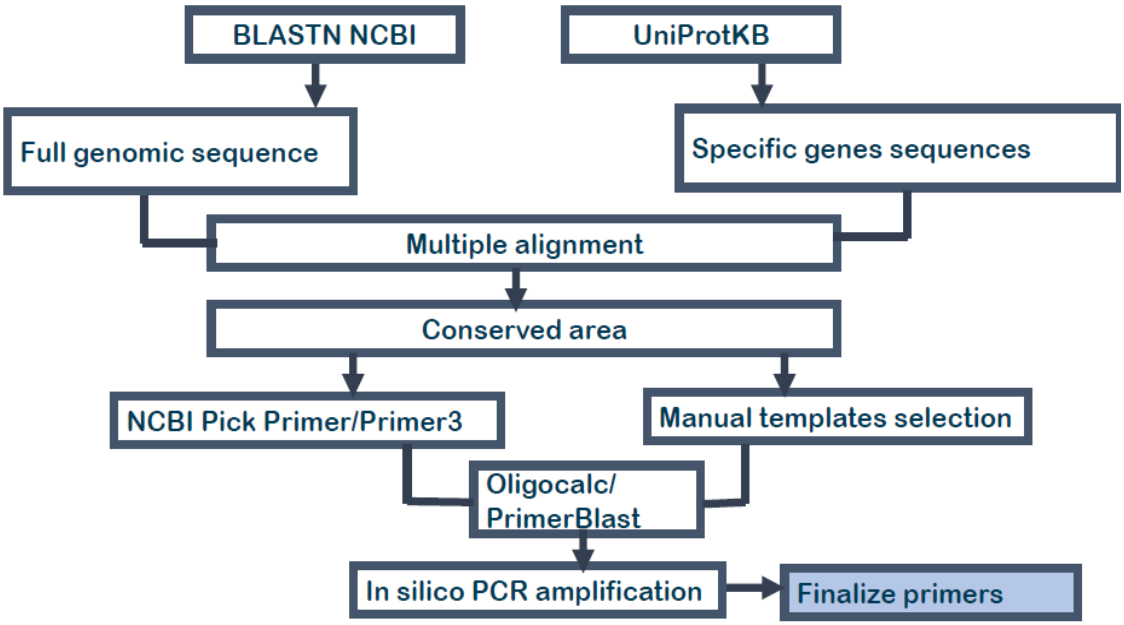



**Figure 3.2**: QQ specific genes primer designing steps.

### 3.3.2 PCR amplification

After repeated PCR reactions by varying concentrations of reagents in reaction mixture during the optimization process, the optimum concentrations for the selected strains were finalized. Step up and step down in temperature settings were made in trials. Clear bands of target gene size on gel were resulted from contents tabulated below.

**Table 3.2**: PCR reaction mixture contents.

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

These reaction mixtures of total volume 50uL were amplified in a thermocycler machine. The programs set for the reactions are;

**Table 3.3**: Cycling programs for performed PCR reactions.

| Gene | Cycling program for PCR reaction |
|---|---|
| AiiA gene | 30 seconds of pre-duration (1 cycle) at 95°C, 30 sec. of duration at 95°C (30 cycles), 60 sec. of aneal (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 pre-duration (1 cycle) at 95°C, 30 sec. of duration at 95°C (30 cycles), 60 sec. of aneal (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 pre-duration (1 cycle) at 95°C, 30 sec. of duration at 95°C (30 cycles), 60 sec. of aneal (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) |

## 3.4 Bacterial immobilization

Beads were prepared as per methods used by Kim et al. (2010) and Kim et al. (2014) with few modifications. For the first batch of SBR, coated and uncoated beads were made by the following methods:

**Table 3.4**: QQ entrapping media preparation.

| QQ bead | Preparation |
|---|---|
| Coated beads | Cell pallets and measurement of their concentration were performed as above for uncoated beads. Bacterial suspension (120 mg) was mixed with sodium alginate (2%) and polyvinyl matrix (2%) and intruded into a 4% $CaCl_2$ solution. After several hours, the beads were washed with distil water.<br>Polysulfone pallets were dissolved in N-Methyl-2-pyrrolidone (NMP) by stirring at 80°C for 24 hours to acquire 10% Psf solution and PVDF.<br>The beads were coated by PVDF and Psf by the inversion technique. After 30sec in psf solution, the beads remained in distilled water bath for 3 hours. The coated beads were washed twice with distil water and stored at 4°C. |

## 3.5 SBR

Sequencing batch study was carried out to study the impact of beads on EPS level. The batch included six reactors of 850 ml working volume. Sludge was received from a full scale MBR and acclimatized in laboratory by synthetic feed provision and aeration. An effective volume of 5% beads were inoculated in the reactor.

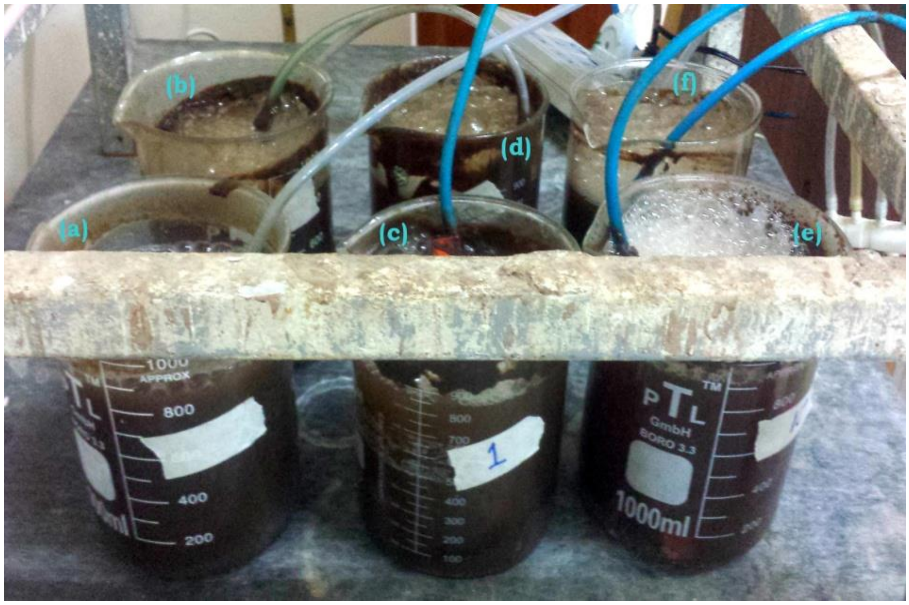



**Figure 3.3**: Six batch reactors inoculated with: a) Non-inoculated, b) Vacant beads, c) *Pseudomonas*-QSP1 entrapped beads, d) *B. ceureus*-QSP3 entrapped beads, e) *B. subtilis*-QSP10 entrapped beads, f) Mixed beads of all three QQ strains.

The composition of feed and other conditions is shown in the table below.

**Table 3.5**: Working conditions and wastewater composition of sequencing batch study.

| | |
|---|---|
| Working conditions | 10 hours of aeration<br>Half hour settling time/ half hour decanting<br>Half hour idle |
| Wastewater composition (g/L) | Glucose (1), $NH_4Cl$ (0.382), $K_2H_2PO4$ (0.0477), $CaCl_2$ (0.00973), $MgSO_4.H_2O$ (0.00973), $FeCl_3$ (0.001), $NaHCO_3$ (0.180) |

## 3.6 EPS analysis

Soluble and bound EPS were determined based on protein and carbohydrate analysis of sludge sample collected from the reactors. The steps involved in EPS analysis are shown in the table.

**Table 3.6**: Method of EPS extraction and determination.

| | | |
|---|---|---|
| EPS extraction | 50 ml sample centrifugation (4000 rpm, 4°C) yielded suspension containing soluble EPS<br><br>Resuspension of pellet in EPS buffer to previous volume, shaking (300 rpm, 1 hour) and centrifugation (4000 rpm, 4°C, 20min) yielded loosely bound EPS in the suspension<br><br>Resuspension of pallet in EPS buffer again with addition of 70g/gVSS Dowex cations exchange resins (Sigma-aldrich) shaking (2 hours) and centrifugation (4000 rpm, 4°C, 20min) yielded tightly bound EPS in the suspension | Frølund et al., 1996 |
| Lowry method for protein analysis | 2:1 for soluble EPS sample and 1:1 dilution of bound EPS was prepared<br><br>With the addition of prepared reagents (Folin-Ciocalteu phenol reagent and others), the samples were kept for 20min in dark condition<br><br>Absorbance was taken at 750 nm<br><br>Protein was determined in each sample by using standard curve/ straight line equation | Lowry et al., 1951 |
| Dubois method for total polysaccharide analysis | 1:1 dilution of all samples with distil water were prepared (1ml each)<br><br>1 ml of 5% phenol solution was added<br><br>5 ml conc. $H_2SO_4$ was rapidly added<br><br>After waiting for samples to cool down and proper mixing, absorbance 490 nm was taken<br><br>Straight line equation was used for carbohydrate determination | DuBois et al., 1956 |

The results for protein and carbohydrates were reported in mg/L for soluble and mg/gMLSS bound EPS.

## 3.7 Statistical analysis

Two software employed to analyse and represent results are Microsoft excel and SPSS 16.0.

# 4. RESULTS

## 4.1 Bacterial species in MBR

After BLASTN in NCBI, the highest similar sequence alignment acquired helped in identification of the strains. The results were deposited in Genbank NCBI and accession numbers were assigned to them.

**Table 4.1:** 16srRNA sequence alignment result in NCBI and the sole carbon sources provided.

| Isolates | Carbon source | Closest 16srRNA identity | Accession numbers |
|---|---|---|---|
| *Pseudomonas aeruginosa* QSP01 | AHL mixture | *Pseudomonas aeruginosa* VSS6/ 99% | KY576793 |
| *Corynebacterium striatum* QSP02 | AHL mixture | *Corynebacterium striatum* 1954BRRJ/ 94% | KY576794 |
| *Bacillus cereus* QSP03 | AHL mixture | *Bacillus cereus* PSMRRAAGRI15/ 99% | KY576795 |
| *Enterobacter cloacae* QSP04 | 3OC6HSL and 3OC12HSL | *Enterobacter cloacae* B3/ 100% | KY576796 |
| *Kocuria flava* QSP05 | 3OC6HSL and 3OC12HSL | *Kocuria flava* HO-9041/99% | KY576797 |
| *Lysinibacillus sp.* QSP06 | AHL mixture | *Lysinibacillus sp.* BAB-4376/99% | KY576798 |
| *Enterobacter sp.* QSP08 | AHL mixture | *Enterobacter cloacae* APSAC 03/99% | KY576799 |
| *Brucella suis* QSP09 | AHL mixture | *Brucella suis* AAg01/ 99% | KY576800 |
| *Bacillus subtilis* QSP10 | AHL mixture | *Bacillus subtilis* LG4 | KY576801 |

The phylogenetic analysis shows that some species are in abundance while majority are related closely. The Figure 4.1 shows relationship among bacterial species isolated in the present study and few others reported in literature based on evolutionary relationship among taxa. Strains with blue diamond in the phylogenetic tree are the ones presently isolated. It is a mixture of quorum sensing and quorum quenching bacterial species.

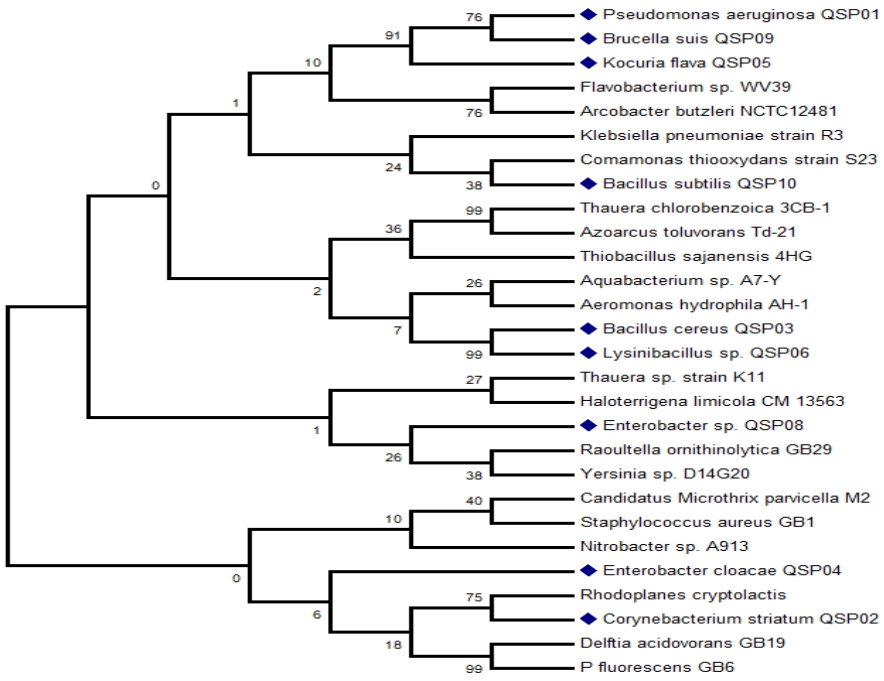



**Figure 4.1**: Evolutionary relationships of taxa (by Neighbor-Joining method) among the bacterial isolates from membrane bioreactor at NUST, Islamabad and those reported in literature (MEGA 7).

### 4.1.1. Comparison of QQ isolates from pilot and full-scale MBR

The species isolated by enrichment technique surviving on AHLs as sole carbon source are assumed to be able to degrade and use up AHLs as energy source.

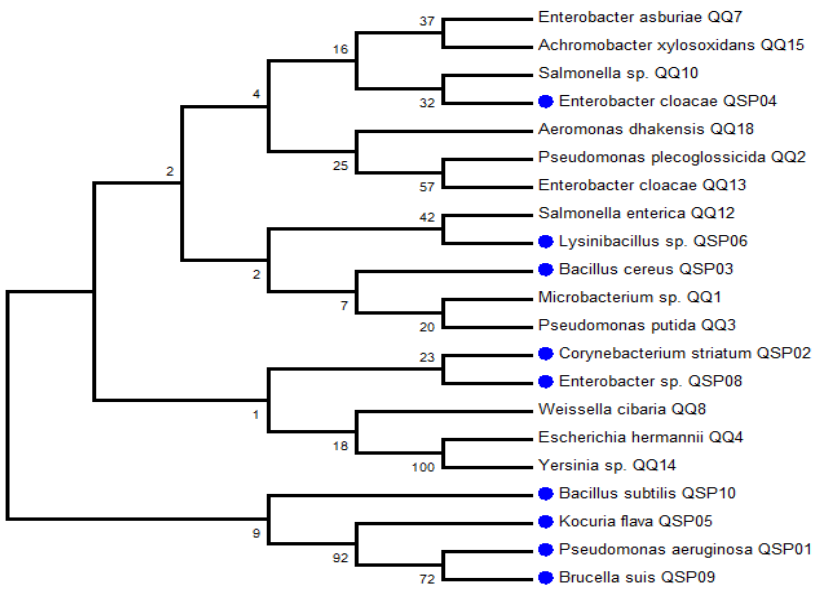



**Figure 4.2**: Evolutionary relationships of taxa (by Neighbor-Joining method) among quorum quenching bacterial isolates from pilot and full-scale membrane bioreactor at NUST, Islamabad (MEGA 7).

The isolates of full scale membrane bioreactor are shown with blue nodes. The abundant species is *Pseudomonas* sp. that occurs in both reactors. *Bacillus* sp. is a major constituent in full scale MBR. Strains without nodes were isolated previously by the similar method from a semi pilot scale MBR system of 35 L effective volume seeded with sludge from the full scale MBR (Waheed et al., 2017). The present isolation is made from the full scale MBR itself. The difference in QQ species distribution is reflected by the evolutionary relationship. The difference may have occurred due to shift from full to pilot scale MBR. Bacterial profile is sensitive to even minor changes in the environment. The wastewater composition and physical conditions also determine the bacterial diversity in MBR.

Furthermore, the species from full scale MBR are closely related to each other and majority have distant evolutionary relationship with QQ strains isolated from a semi-pilot scale MBR.

### 4.1.2 Phenotypic characteristics of isolates

Colony morphology assists in acquisition of pure cultures and is also an indicator of diversity, as different species tend to form colonies differing in size, texture, form, boundary, opacity and other characteristics. Moreover, the colonies may not be same on agar plates of differing types. The agars employed in the present study were MM media, nutrient agar, LB agar and TSA.

Secondly, the bacterial morphology was studied under light microscope by gram-staining method gave an insight into the cell structure (gram-positive and gram-negative), shape and arrangement of bacterial cells. Further characteristics were studied by biochemical analysis of the pure colonies.

**Table 4.2**: Phenotypic characteristics of isolated bacterial species from membrane bioreactor.

| **Isolates** | **Gram test** | **Bacterial morphology** | **Catalase test** | **Oxidase test** | **EMB agar** | **McConkey agar** |
|---|---|---|---|---|---|---|
| *P.aeruginosa* QSP01 | -ve | Bacilli, diploid | +ve | +ve | Lactonase -ve | Lactonase -ve |
| *Corynebacterium striatum* QSP02 | +ve | Cocci, diploid | +ve | -ve | NG | NG |
| *Bacillus cereus* QSP03 | +ve | Bacilli, chain | +ve | +ve | NG | NG |
| *Enterobacter cloacae* QSP04 | -ve | Bacilli, diploid | +ve | -ve | Lactonase +ve | Lactonase +ve |
| *Kocuria flava* QSP05 | +ve | Cocci, no special arrangement | +ve | -ve | NG | NG |
| *Lysinibacillus sp*. QSP06 | +ve | Cocci, single | +ve | +ve | NG | NG |
| *Enterobacter sp.* QSP08 | -ve | Bacilli, diploid | +ve | +ve | NG | NG |
| *Brucella suis* QSP09 | -ve | Cocci, diploid | +ve | +ve | Lactonase -ve | Lactonase - ve |
| *Bacillus subtilis* QSP10 | +ve | Bacilli, clusters | +ve | +ve | Lactonase -ve | Lactonase - ve |

*NG=No growth

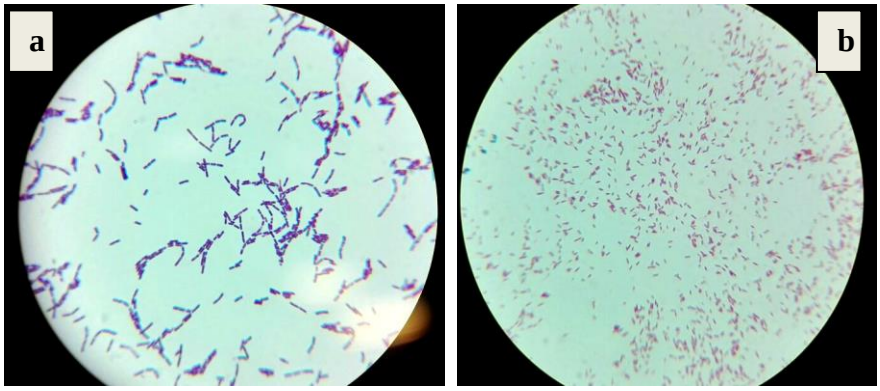



**Figure 4.3**: Colony morphology under 100× light microscope. (a) *Bacillus sp.* QSP03 (b) *Brucella suis* QSP09.

## 4.2 Bacterial isolates identified by API 20E kit

Apart from the selected isolates identified through 16srRNA sequencing, four out of seven other pure colonies were identified through biochemical testings aided by API 20E kit to have a better idea of bacterial species abundance in membrane bioreactor.

**Table 4.3:** Isolated bacteria from sludge of membrane bioreactor able to survive on AHL as sole carbon source with the API 20E result.

| **Isolates** | **Carbon source** | **Closest API 20E identity** | **Bacterial Morphology** | **Gram test** |
|---|---|---|---|---|
| QSP-A | AHL mixture | - | Bacilli, chain | Positive |
| QSP-B | AHL mixture | *Klebsiella pneumoniae* | Bacilli, random | Negative |
| QSP-C | AHL mixture | *Enterobacter cloacae* | Cocci, chain | Negative |
| QSP-D | AHL mixture | *Salmonella sp.* | Bacilli, diploid | Negative |
| QSP-E | AHL mixture | - | Bacilli, diploid | Positive |
| QSP-F | AHL mixture | - | Cocci, diploid | Positive |
| QSP-G | AHL mixture | *Aeromonas hydrophila* | Vibrio, chain | Negative |

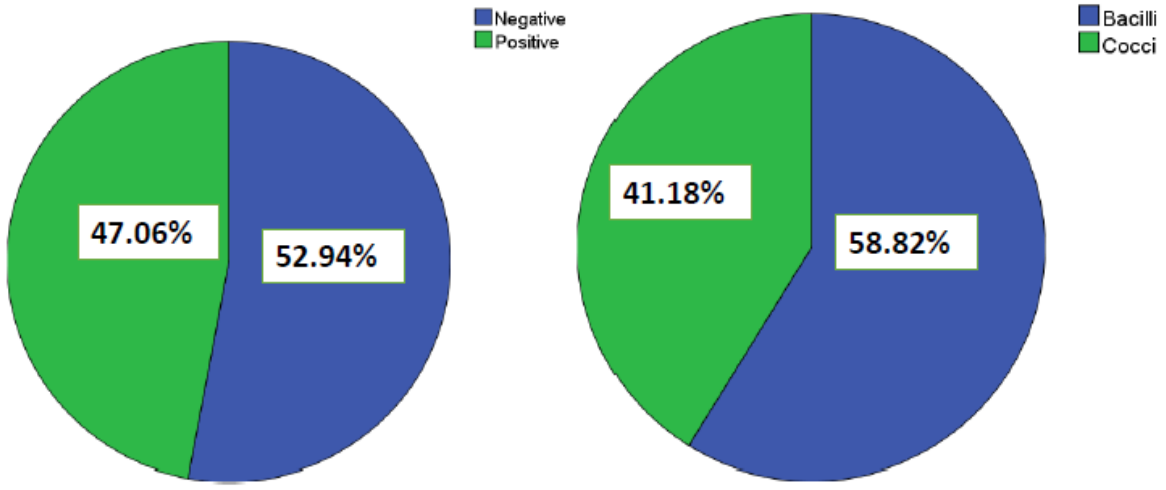



**Figure 4.4**: (a) Percent of gram-positive and gram-negative bacteria in membrane bioreactor. (b) Percent abundance of bacilli and cocci bacterial species isolated from membrane bioreactor.

Christiaen et al. (2011) found that 75 % of the isolates from different environmental samples were gram- negative. Gram-negative and rod-shaped bacteria were also abundant in lab scale MBR (Waheed et al., 2017). Although gram-negative and rod shaped QQ bacteria were abundant in the present study but by a small percentage. This indicates that gram-positive bacterial species cannot be ignored for their potential role in QS and QQ. The difference of ratio in pilot/lab scale MBRs and full scale MBR may be due to the difference in physical conditions and influent characteristics. Furthermore, the abundance is evaluated based on only seventeen strains. The low isolate number may introduce biasness in the study.

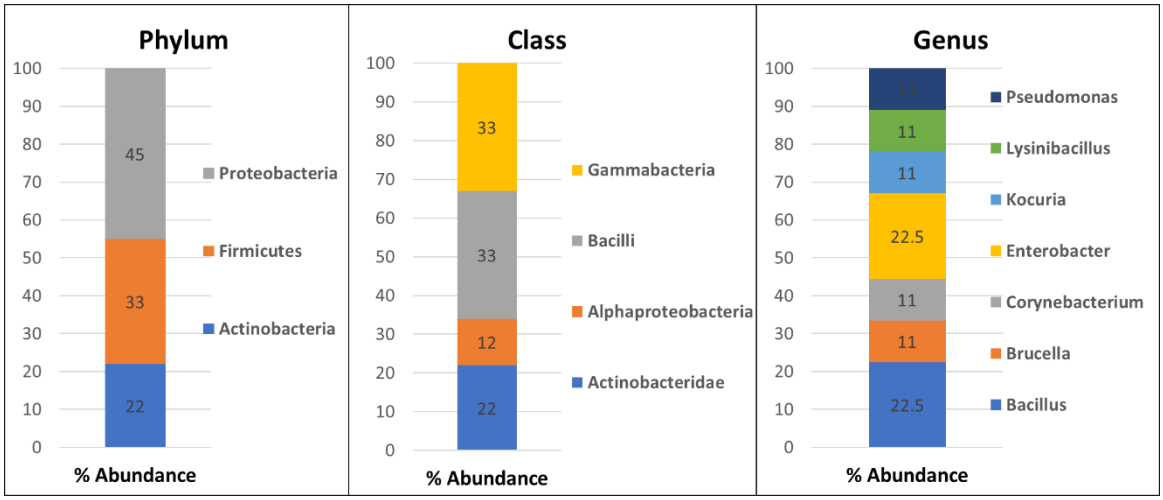
## 4.3Bacterial abundance in MBR

The QQ strains isolated from various environmental samples by Christiaen et al., (2011) mostly belonged to the genera *Pseudomonas* while it appeared the second abundant genera among strains isolated from laboratory scale MBR, *Serratia* sp appeared abundantly and *Enterobacter* sp was the third abundant species (Waheed et al., 2015). In a study on a pilot scale submerged

MBR, Ma et al. (2013) found the predominant phyla at all temperatures were *Proteobacteria*, *Nitrospira* and *Bacteroidetes*. Studies conducted by Calderon et al. (2012) indicated *Proteobacteria* as the dominant bacterial group.

Another study indicated that identified sequences in a pilot scale MBR mostly belonged to α, β, γ- *Proteobacteria* (Muñoz et al., 2009). While most studies indicate α- and β- *Proteobacteria* as the most abundant among *Proteobacteria*. The difference in finding could be due to biases introduced by culture dependent methods (Eschenhagen et al., 2003; Xia et al., 2010). Various other factors may have contributed to the biases including wastewater characteristics, operating conditions, sludge characteristics, influent and feed composition among others.

The distribution frequency of the 09 identified strains at phylum, class and genus level indicated Proteobacteria as the most abundant phylum and Gammaproteobacteria as the abundant class. Bacillus and Enterobacter appeared the abundant Genus. All strains belonged to different species. The species belonging to these genera can be explored for their ability to quench AHLs on priority basis.




**Figures 4.5**: Percentage distribution of QQ species in MBR.

*Pseudomonas* sp are found in diverse environment in abundance due to their high adaptability. It plays role in denitrification process in MBR (Khan et al., 2013). *Enterobacter* sp. contribute

in biofilm development in MBR (Lim et al., 2012). *Bacillus cereus* genera was abundant among QQ strains isolated from MBR (Steven et al., 2011).

## 4.4 AHLs based QS profile of isolated bacteria

Gram-negative bacterial species play a key role in AHL production, moreover gram-negative bacteria are reported to dominate in membrane bioreactor (Yeon et al., 2009; Waheed et al., 2017). Previous studies have shown that some bacteria may be involved simultaneously in QS and QQ (Chong et al., 2012). The activities were screened by using biosensor species for long chain and short chain AHLs detection. The biosensors produce blue or violet colour when exposed to exogeneous AHLs. The screening helps in final selection of QQ strains for application in SBRs for QQ activity study. In the biosensor based screening for AHL production and degradation capacity, distilled water and sterile LB broth were added as negative controls.

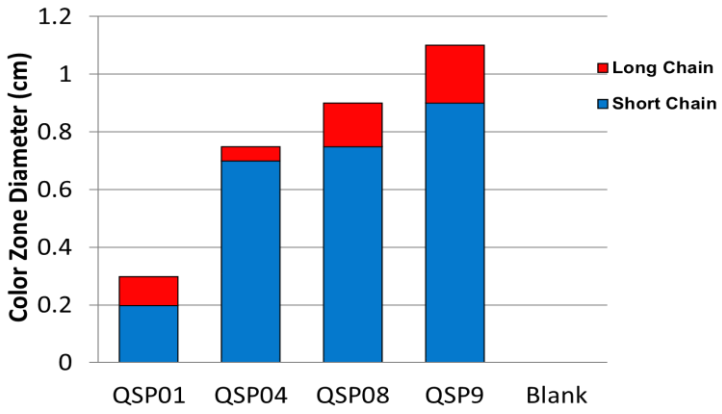
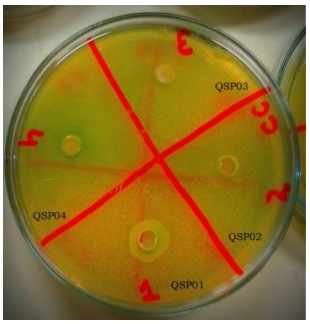
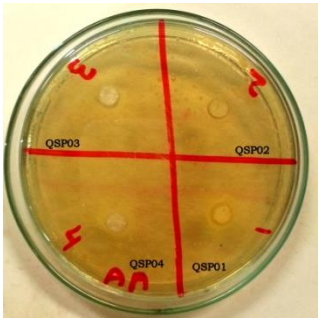
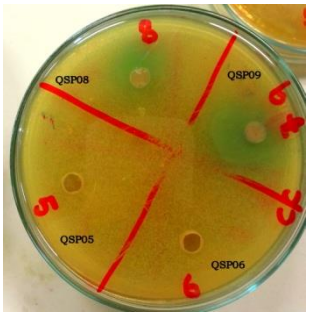
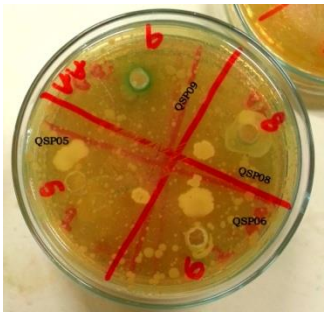
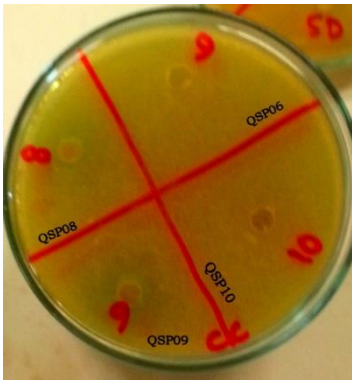
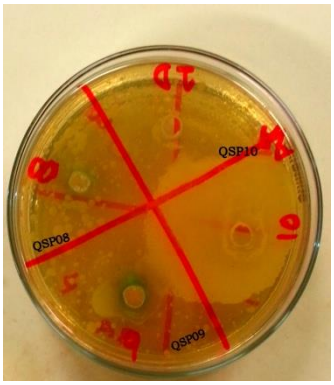


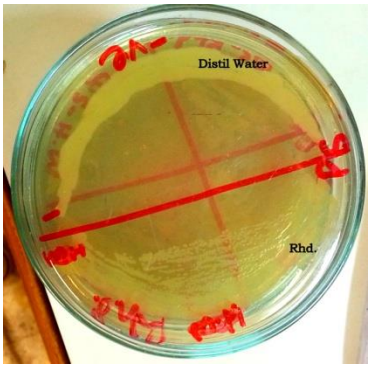

**Figure 4.6**: QS in the gram-negative bacterial species for short and long chain AHLs.

High AHL production was observed by *Pseudomonas kilonensis* and *Psychrobacter* sp in case of pilot scale MBR. All QS species screened belonged to *Proteobacteria* (Waheed et al., 2015). *P. aeruginosa* PAO1is reported to produce BHL and is positive for QS production (Zhang et al., 2016).

**Table 4.4**: AHL based QS activities detected by parallel streak method and well-diffusion assay.

| S. no. | Strains | Parallel streak | | Pictorial representation of well-diffusion assay | |
|---|---|---|---|---|---|
| | | Short-chain AHLs | Long-chain AHLs | Short-chain AHLs | Long-chain AHLs |
| 1 | *Pseudomonas aeruginosa* QSP01 | - | + |   |   |
| 2 | *Enterobacter cloacae* QSP04 | +++ | ++ | | |
| 3 | *Enterobacter* sp. QSP08 | + | ++ |   |   |
| 4 | *Brucella suis* QSP09 | + | + |   |   |

* - for QS negative, + for positive, ++ for higher QS activity



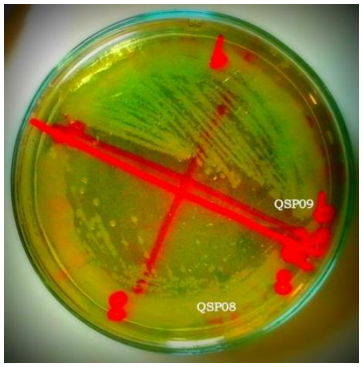




**Figure 4.7**: (a) Parallel streak of biosensor species in the outer layer with negative controls (*Rhd*. BH4 and water) on the inner layer. (b) QS in strain QSP08 and QSP09 detected by parallel streak method.

## 4.4 QQ profile of isolated bacteria

The intensity of the pigment produced or inhibited on a CV026 indicator plate corresponds to the QS and QQ ability respectively (Zhang et al., 2016). Moreover, isolated QQ strains from laboratory scale MBR degraded AHLs with C12 more than those with shorter acyl chain side length including C6 and C8-HSL indicating different QQ preferences of QQ strains (Cheong et al., 2013). Enzymatic degradation of AHL is reported in many bacterial species. The most common are the *Bacillus* and *Pseudomonas* strains reported to degrade AHLs by lactonases and acylases (Morohoshi et al., 2009).

*B. cereus* ATCC927 is known for the enzymatic degradation of AHLs upon CV026 screening with heat treatment (Christiaen et al., 2011). Zang et al. (2016) attributed the decreased OHL observed by *Pseudomonas* sp to low molecular weight compounds other than the known QQ enzymes (Zang et al., 2016). Byers et al. (2002) studied *P. aeruginosa* and found its ability to accumulate the AHLs secreted by the same bacteria during log-phase and its degradation in the stationary phase. *B.cereus* was employed as positive control in screening QQ isolates by CV026 biosensor (Zhang et al., 2016).

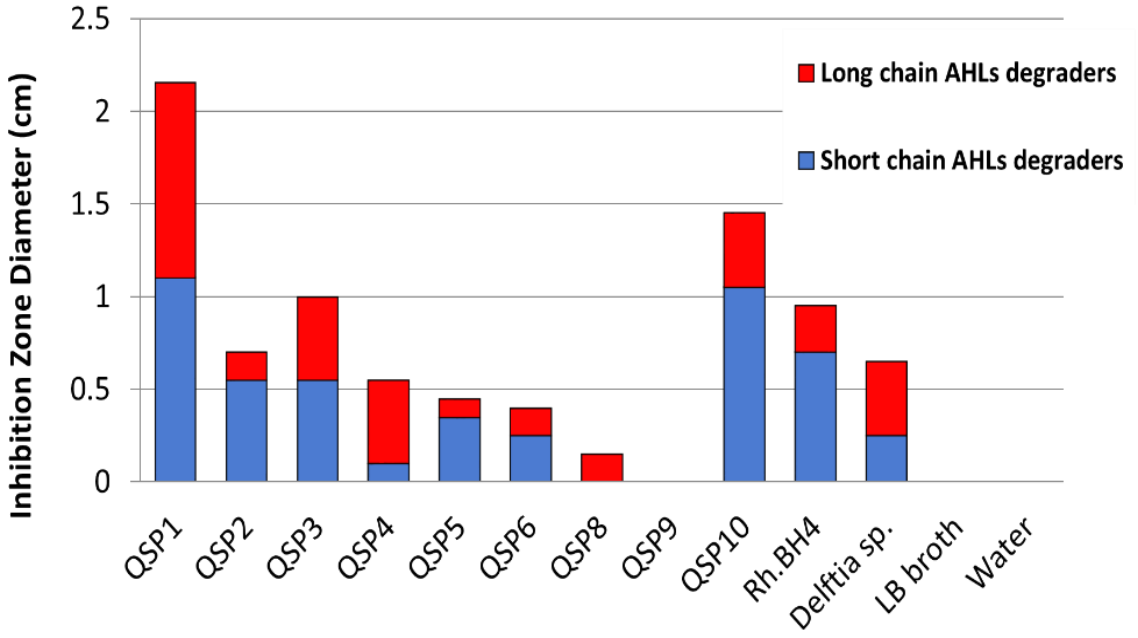



**Figure 4.8**: QQ activity in the isolated bacterial species for short and long chain AHLs.

In the present study, on the well diffusion assay that was supplemented by short chain C6HSL and long-chain C10HSL, *Pseudomonas* sp. and *Bacillus* sp. were more able to degrade them as indicated by the diameter of inhibition zones in the graphs, on coloured background of the indicator biosensor agar plates. Their ability to degrade C6HSL and C10HSL exceeds that of reference bacterial species namely *Delftia* sp. (Waheed et al., 2015) and *Rhoducoccus* BH4 sp. (Kim et al., 2012).

## 4.5 QQ enzyme location prediction via bio agar assay

Filter papers dipped in standard AHLs solution and overlaid over indicator agar plate aids in the prediction of QQ enzyme activities in the isolate (Cheong et al., 2013). The inhibition zone is either in an outer ring or inner spherical form. Cheong et al. (2013) also indicated that those bacteria releasing endoenzymes produce inhibition zone without outer ring while exoenzymes produce ring around the inhibition zone on the bio agar assay. From the known QQ enzymes, lactonase is endoenzyme while acylase is exoenzyme.

The filter (0.45µm) was loaded with QQ strain colonies as per the method introduced by Cheong et al. (2013). White ring appeared around the colony of *P.aerogenosa* QSP01 strain. This indicates that the QQ enzyme produced by this strain is secreted out of the cell (Cheong et al., 2012). The ring around the colony was not observed around reference species *Rhodococcus* sp. BH4 in the present as well as previous study by Cheong et al. (2012). Moreover, no ring appeared around QSP03 and QSP10, both strains belonging to *Bacillus* genus as per 16srRNA partial sequence result. This indicates that the QQ enzymes produced by these species is kept inside the cell.

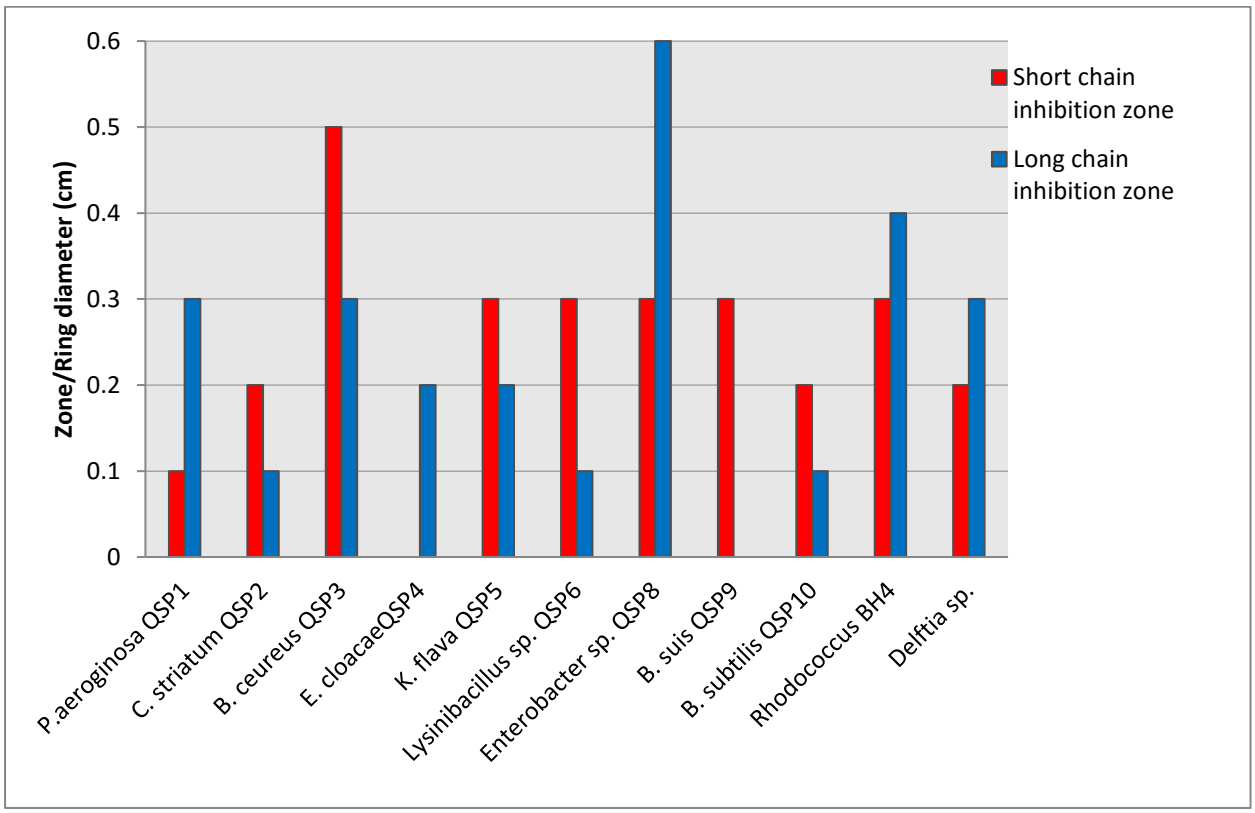
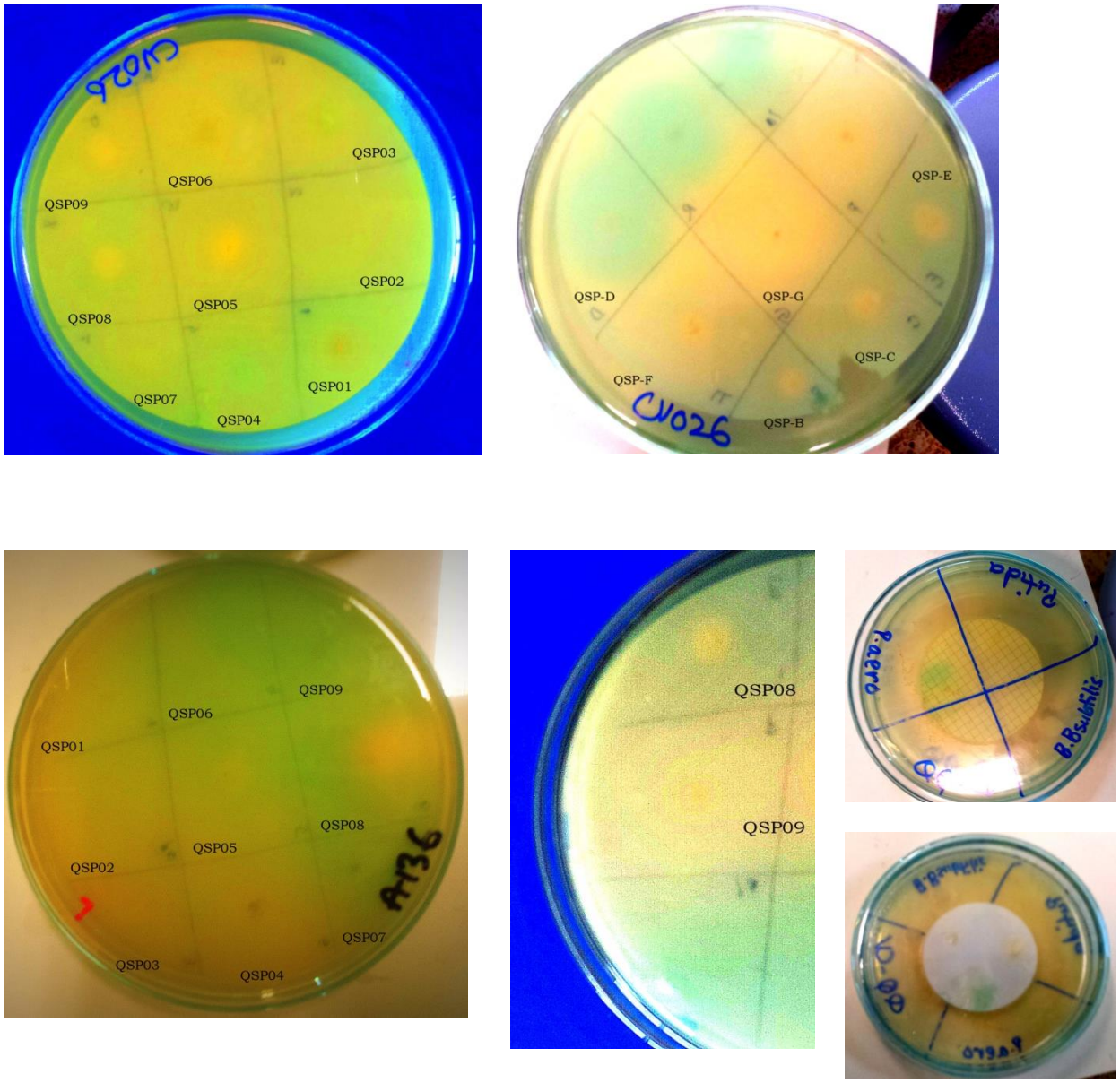



**Figure 4.9**: Zone/ring diameters formed on filter overlaid the indicator plate containing CV026 and A136 strains.




**Figures 4.10**: (a) Inhibition zone on indicator plate of CV026 for strains QSP01 to QSP10. (b) Inhibition zone on indicator plate of CV026 for other strains. (c) Inhibition zone on indicator plate of A136 for strains QSP01 to QSP10. (d) Inhibition zone on indicator plate of CV026 for strains QSP03, QSP09 and QSP10. Inhibition zone on indicator plate of A136 for *P. putida*, QSP10 and a negative control species *P. aeruginosa*.

**Table 4.5**: Predicted nature of QQ enzymatic activity by the isolates.

| S.no. | Isolates | Probable QQ enzyme location |
|---|---|---|
| 1 | *P.aeruginosa QSP01* | Exoenzyme |
| 2 | *Corynebacterium striatum* QSP02 | Not predicted |
| 3 | *Bacillus cereus* QSP03 | Endoenzyme |
| 4 | *Enterobacter cloacae* QSP04 | Exoenzyme |
| 5 | *Kocuria flava* QSP05 | Exoenzyme |
| 6 | *Lysinibacillus sp*. QSP06 | Exoenzyme |
| 7 | *Enterobacter sp.* QSP08 | Exoenzyme |
| 8 | *Brucella suis* QSP09 | Endoenzyme |
| 9 | *Bacillus subtilis* QSP10 | Endoenzyme |

## 4.6 QQ activity by QSIS2 biosensor

The previous results upon use of biosensor strains CV026 and A136 indicated the prevalence of QS and QQ activity among majority isolated species. For the final selection of QQ strain for application in membrane bioreactor, another biosensor QSIS2 was used with supplemented short- and long-chain AHLs to study the degradation by the isolated bacterial strains.

### 4.6.1 QQ activity in consortia

The quorum quenching in the minimal media after each cycle is determined from the growth decrease of QSIS2 induced by AHLs, measured as $OD_{600}$, an indirect indication of AHL level in the media. It is anticipated that with each enrichment cycle, the AHL levels would decrease and the species able to utilize AHL as sole carbon, nitrogen or energy source would survive through all cycles (Christiaen et al., 2011).

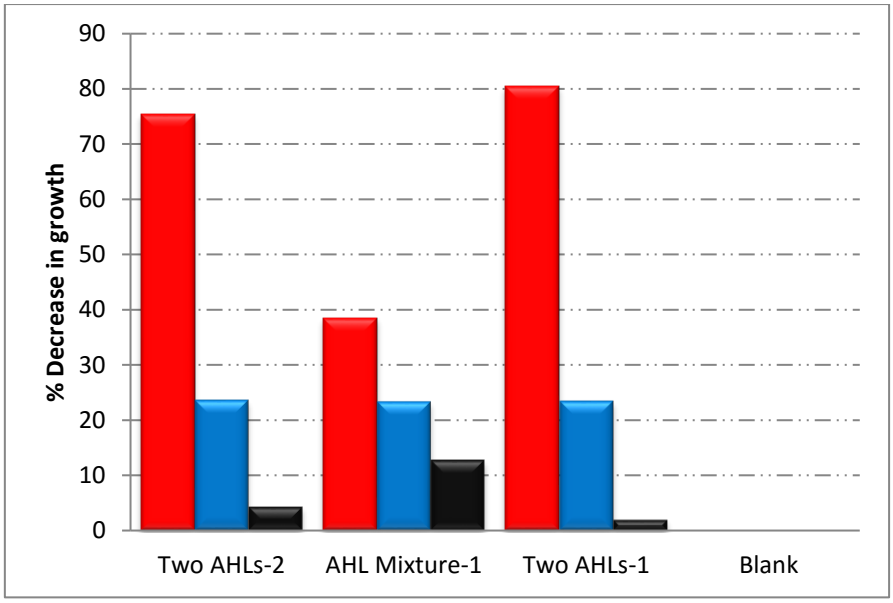



**Figure 4.11**: Decrease in growth of indicator species expressed as percent decrease in growth relative to blank (100%growth) during three cycles.

The degradation of another two AHLs: long chain 3OC12HSL and short chain C4HSL in the spent media from cycle 1, 2 and 3 during sample preparation gives an insight into the joint QQ activity by all unknown species in the sample. We assume, the QQ activity is high in 3rd cycle because till 3rd cycle only QQ bacteria remains. The 1st cycle may have mixture of QS and QQ species along others.

### 4.6.2 QQ activity in pure colonies

Each isolate evaluated for QQ activity in the QSIS2 assay is studied by the growth difference in blank and in 24 hours incubated isolates with AHLs. Christiaen et al. (2011) found high QQ activity among taxa *Pseudomonas* sp., *Arthrobacter* sp., and *Delftia* spp screened by QSIS2 assay.

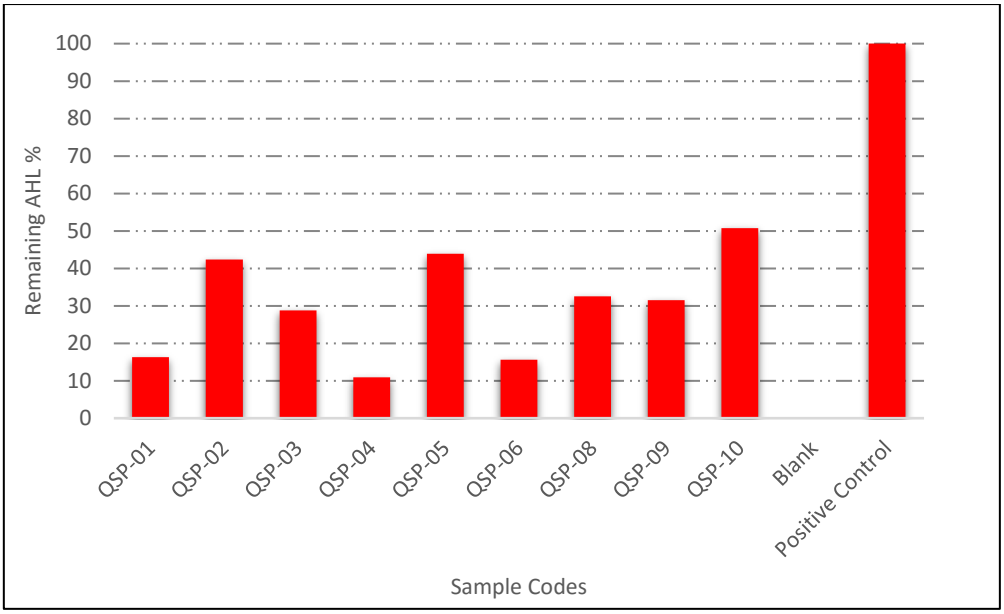



**Figure 4.12**: Percent remaining AHL level relative to positive and negative control taken as 100% and 0% AHL remaining respectively.

As the positive control is supplemented with known amount of short and long chain AHLs, the % remaining AHLs in the liquid cultures is evaluated by % decrease in growth relative to positive and negative control. *Pseudomonas* sp. QSP01, *Enterobacteria* sp QSP04 and QSP06 strain degraded more than half of the AHLs quantity supplemented. *Bacillus* sp QSP10 degraded half that is 800 nM of the total supplemented AHLs of 1600 nM in 24 hours.

### 4.6.3 QQ activity after heat treatment

Past studies have shown that it is possible that some bacteria produce heat-stable molecules of low weight that are capable of degrading QS signal molecules (Christiaen et al., 2011). Mandrich et al., (2010) reported thermal resistance and thermophilicity in microbial lactonases family of thermophilic archaea. Cheong et al., (2013) cultured bacteria for 24 hours without supplemented AHLs and digested them at 121˚C for 20 mins. After which the digested media was cooled and supplemented with AHLs and incubated for another round of 24.

Moreover, Christiaen et al. (2011) observed that QQ activity was retained after heat treatment mainly in genera *Arthrobacter*, *Pseudomonas* and *Delftia*. Furthermore, Zhang et al. (2016)

observed heat-stable degradation of AHLs isolated from RO membrane for application in MBR in *Bacillus* species and another strain of *Delftia* sp.

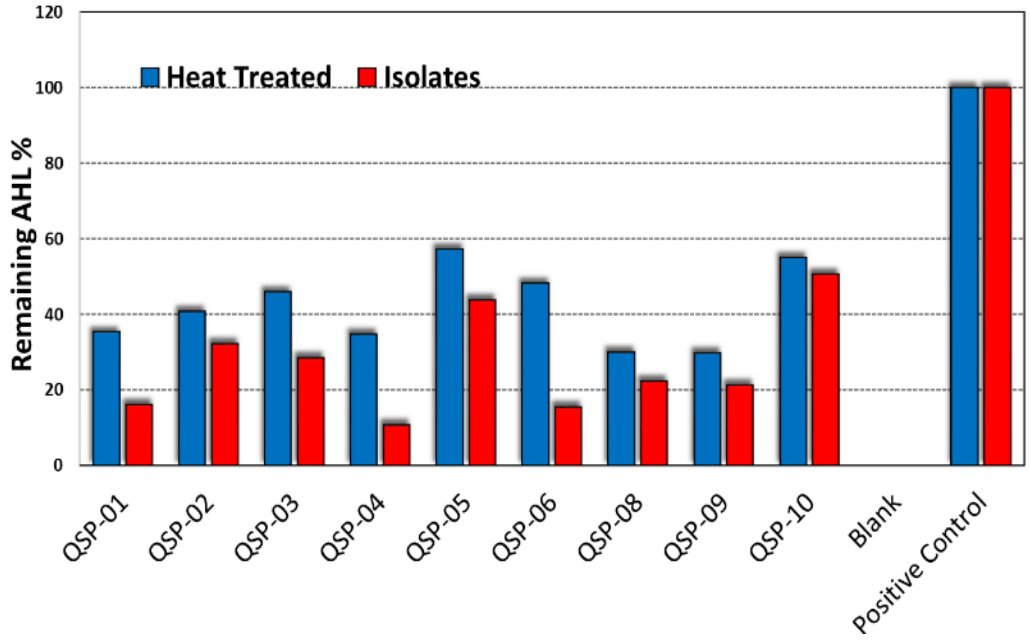



**Figure 4.13**: QQ activity studied by QSIS2 assay in heat treated verses without heat treatment of bacterial isolates.

In the present study, all the bacterial strains indicated heat-stable enzymes production while in all cases the enzyme activity decreased after heat-treatment. The AHLs degrading enzymes may have lost some of their degrading capacity due to heat denaturation during autoclaving and sterilization. The highest heat-stability was indicated by *Corynebacterium* sp. QSP02 and *Bacillus* sp. QSP10, followed by *Enterobacteria* sp. QSP08.

## 4.7 QQ specific genes

PCR primers designed for detection of acylase and lactonase producing genes were used to verify the presence of predicted gene contributing to QQ enzyme production.

**Table 4.6**: Primers designed and used for specific genes identification.

| Oligo name | Sequence (5' to 3') | Length | Target gene |
|---|---|---|---|
| AiiA-For: | GATGGCCTGGAGAATGAC | 18 | AiiA encoding lactonase |
| AiiA Rev: | GCGTGTAGGGTATGAGCC | 18 | AiiA encoding lactonase |
| PQnhm- For: | GTTCTGCACGAAGTCCCTG | 19 | PvdQ encoding acylase |
| PQnhm- Rev: | GCTGTTGGGTTCGATGATG | 19 | PvdQ encoding acylase |
| QPnhm-For: | GTCGGCCAGGTAATAGAGC | 19 | QuiP encoding acylase |
| QPnhm-Rev: | GCTACCGTCCGGAATACTG | 19 | QuiP encoding acylase |

### 4.7.1 QQ genes in *Pseudomonas aeruginosa* QSP01

Many species of *Pseudomonas* genus in literature are shown to produce QQ enzymes (Sio et al., 2006; Chong et al., 2012; Cheong et al., 2013). Cheong et al. (2013) further reported that QQ strain belonging to *Pseudomonas* species isolated from lab scale MBR was responsible for QQ enzyme named acylase, based on bio agar assay screening and they termed it extracellular QQ activity. This helped in final selection of strains for genes screening through PCR.

Primers designed for the detection of two genes in *Pseudomonas aeroginosa* QSP01 were PvdQ and QuiP genes. The ladder used for quality and product size analysis on gel is of 1kb. It is reported that one of the specific gene named PvdQ is responsible for AHL acylase production in Pseudomonas species. If this gene is present in our present strain identified by 16srRNA, it is an indication of its QQ ability. AHL acylase is of size 1411bp (Bokhove et al, 2010). This product was detected upon amplification with gene specific primers for PvdQ gene. Shown on gel, the band can be clearly viewed in Figure 4.14.

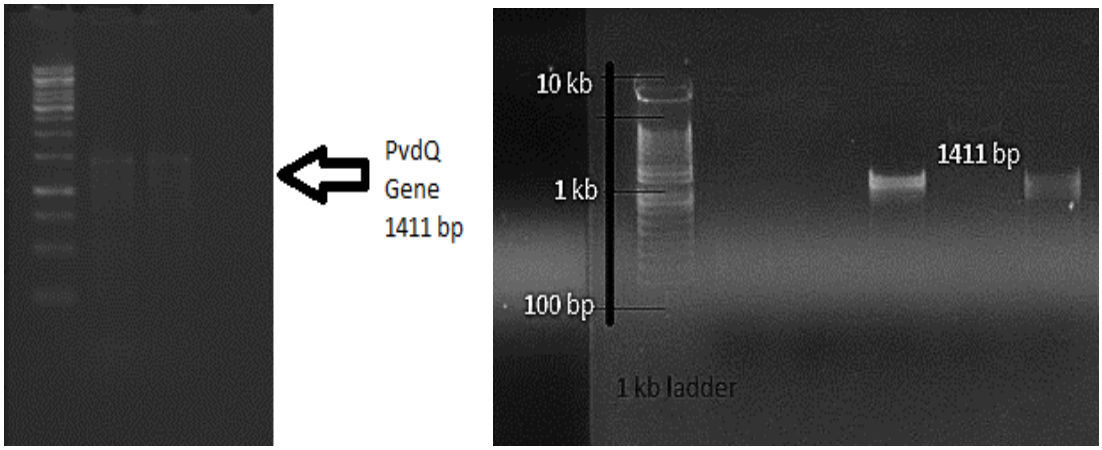



**Figure 4.14**: PvdQ gene responsible for QQ enzyme production detected in *P. aeruginosa* QSP01.

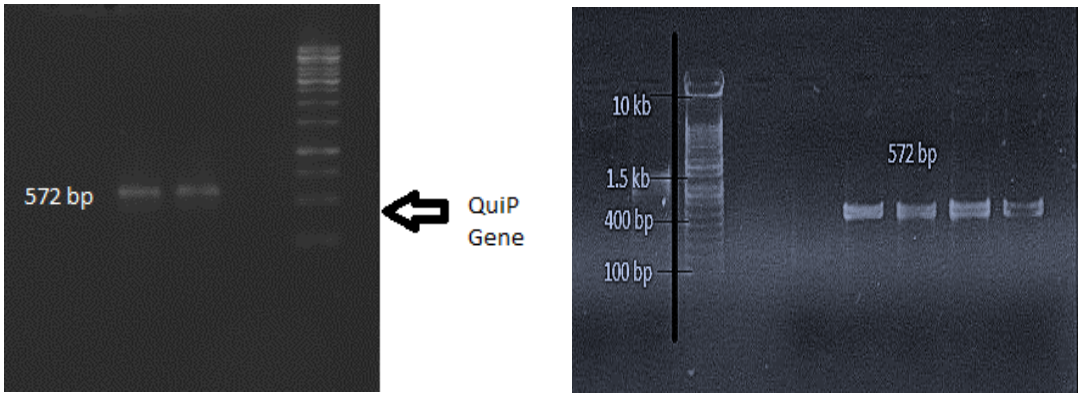



**Figure 4.15**: QuiP gene responsible for QQ enzyme production detected in *P. aeruginosa* QSP01.

Another gene QuiP encoding acylase, the second reported gene in *Pseudomonas* sp is shown at 572 bp (Huang et al., 2006). Therefore, both acylase encoding genes in QSP01 strain verifies its ability in AHLs degradation.

### 4.7.2 QQ gene in *Bacillus cereus* QSP03

Lactonase producing gene in *Bacillus* species is responsible for the quorum quenching activity of the bacterial species. Lactonase encoding gene AiiA is reported in few *Bacillus* species. In

the present study, *Bacillus cereus* QSP03 screened for presence of AiiA gene is found of 257 bp (Reimmann et al., 2002) visualized on gel in Figure 4.16.

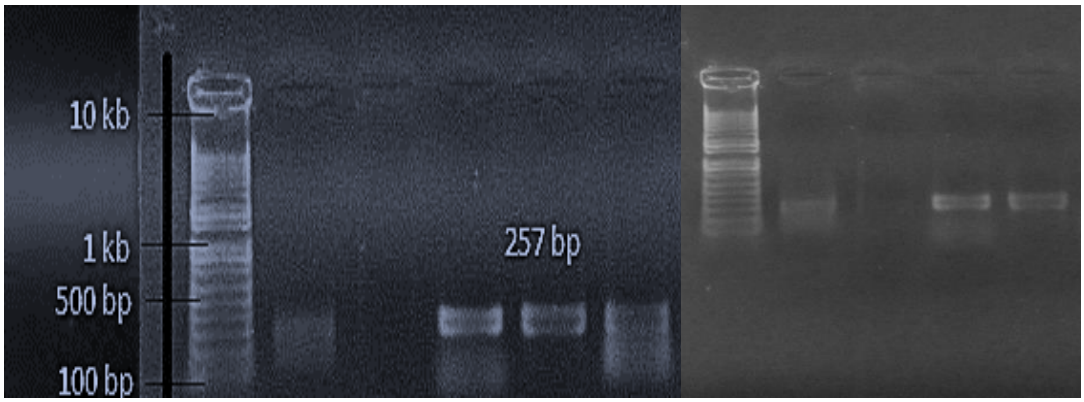



**Figure 4.16**: AiiA gene responsible for QQ enzyme production detected in *Bacillus cereus* QSP03.

### 4.7.3 QQ gene in *Bacillus subtilis* QSP10

The third species, *Bacillus subtilis* QSP10 amplified with AiiA gene specific for AHL lactonase production in Bacillus species presence is verified in QSP10 strain. Therefore, the enzymes produced by these three genes are responsible for the QQ activity imparted to them. AiiA gene is found of 257 bp (Reimmann et al., 2002).

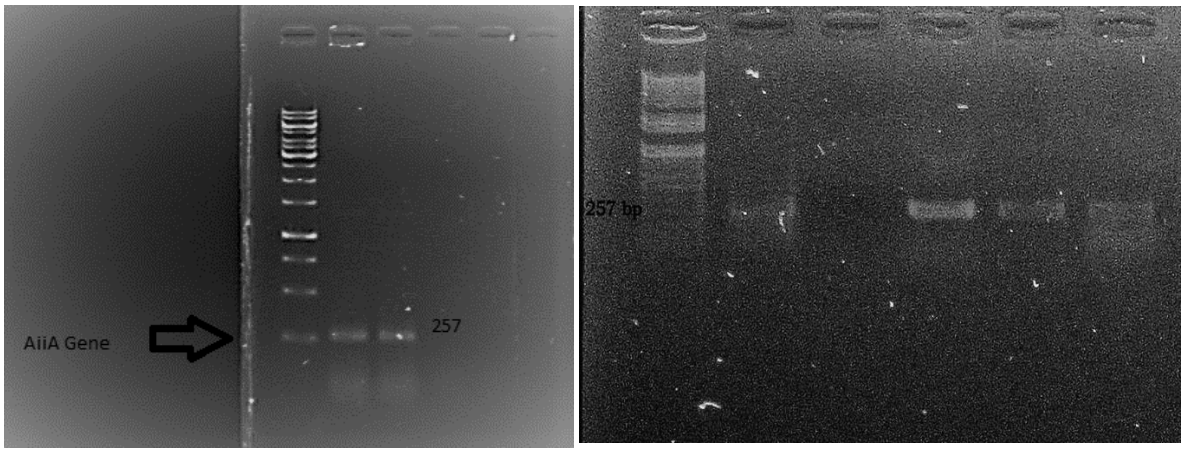



**Figure 4.17**: AiiA gene responsible for QQ enzyme production detected in *Bacillus subtilis* QSP10.

## 4.8 Beads entrapping bacterial cells

Bead entrapping bacterial species instead of QQ enzymes had been termed a reliable option for application in MBR (Kim et al., 2012). All previous studies showed lower EPS level in lab scale MBRs upon application of QQ strains in different media including beads (Kim et al., 2012; Maqbool et al, 2015), microcapsules (Kim et al, 2012) and micro vessels (Cheong et al., 2013). Delayed membrane biofouling was reported in all studies but unfortunately total inhibition of microbial floc deposition on membrane during filtration by convection could not be achieved by this method. From the many reasons given, one reason was the QQ species were not capable of degrading all signal molecules. Therefore, the eventual TMP rise-up could delayed (Zhang et al., 2016).

Different combinations of beads constituents were prepared to bring modification in bacterial cell entrapping media. Vacant beads were also made along with beads containing bacterial cells. Apart from modifications in entrapping material, the QQ species and combination of QQ species for consortia was also modified.

**Table 4.7**: Cell entrapping beads composition and physical condition at the start and end of inoculation.

| **S. no.** | **Composition** | **Initial physical condition** | **Final physical condition** |
|---|---|---|---|
| 1 | Alginate: 2%<br>Dipping solution: 4% $CaCl_2$ and 1% boric acid solution<br>Polymer: 1% Polyvinyl alcohol | Intact<br>  | Few broken beads<br>  |

| | | | |
|---|---|---|---|
| 2 | Alginate: 2%<br>Dipping solution: 4% $CaCl_2$<br>Polymer: 2% Polyvinyl alcohol<br>Coating material: Polysulfone | Intact<br>  | Intact<br>  |
| 3 | Alginate: 2%<br>Dipping solution: 4% $CaCl_2$<br>Polymer: 2% Polyvinyl alcohol<br>Coating material: PVDF | Intact<br>  | Alginate leaked<br>  |

After two weeks inoculation of coated and uncoated beads enlisted in table 4.7, it was observed that Psf coated beads are more stable in SBR. Cell entrapping beads face an issue of stability due to deterioration during continuous mixing in MBR. Maqbool et al. (2015) also observed reduction in beads size witnessed after 45 days' operation of a lab scale MBR which required reintroduction of fresh beads.

## 4.9 Impact of QQ beads inoculation on EPS in SBR

The role of EPS level in membrane biofouling is well documented and established fact. The role of QS in EPS production has recently acquired attention and it is observed that targeting QS system lower EPS level leading to delayed biofouling. The overall impact of coated beads containing selected QQ bacterial strains in comparison to non-inoculated SBR and vacant coated bead inoculated SBRs indicated lower soluble and total bound EPS. Waheed et al. (2017) also showed that soluble and bound EPS in mixed liquor and in membrane cake were lower in QQ MBR.

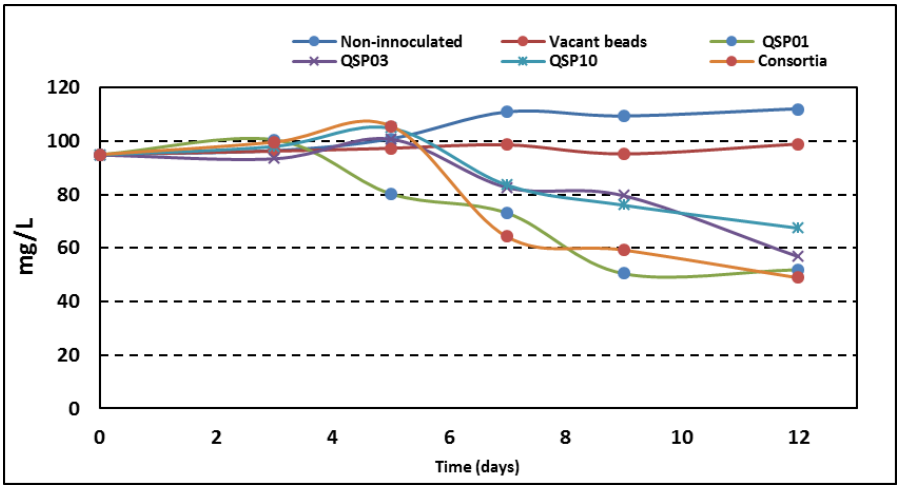



**Figure 4.18**: Impact of coated beads inoculation on soluble EPS during 12 days of batch study.

SBRs with vacant beads and the one with no beads had higher soluble EPS at the end of 12 days. The increase in soluble EPS initially in QQ SBRs may have been due to dissociation of bound EPS because of the physical impact of beads.

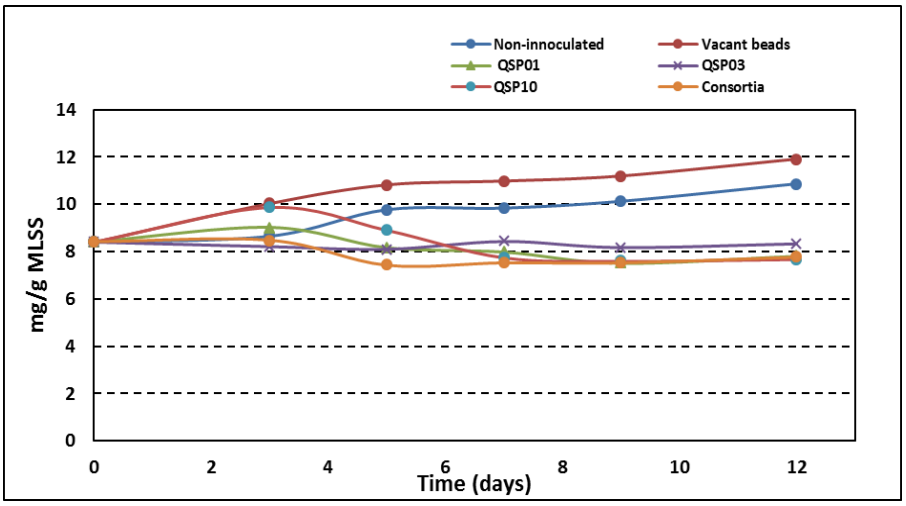



**Figure 4.19**: Impact of coated beads inoculation on LB EPS during 12 days of batch study.

Similarly, the LB EPS level decreased insignificantly as observed in Figure 4.19. The lower decrease level may have been due to the short duration of batch study, that is twelve days. While comparative to non-inoculated SBR and vacant bead SBR, all QQ inoculated SBRs

indicated lower LB EPS levels. QSP03 strain and consortia immobilized beads containing SBRs performed well.

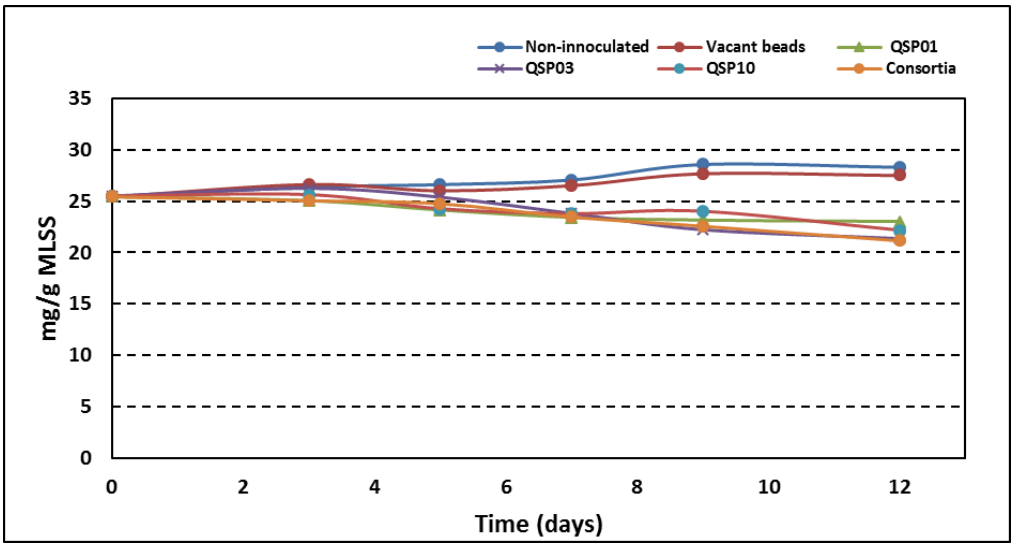



**Figure 4.20**: Impact of coated beads inoculation on TB EPS during 12 days of batch study.

The tightly bound EPS level in all SBRs performed almost same as in case of LB EPS level as shown in Figure 4.20.

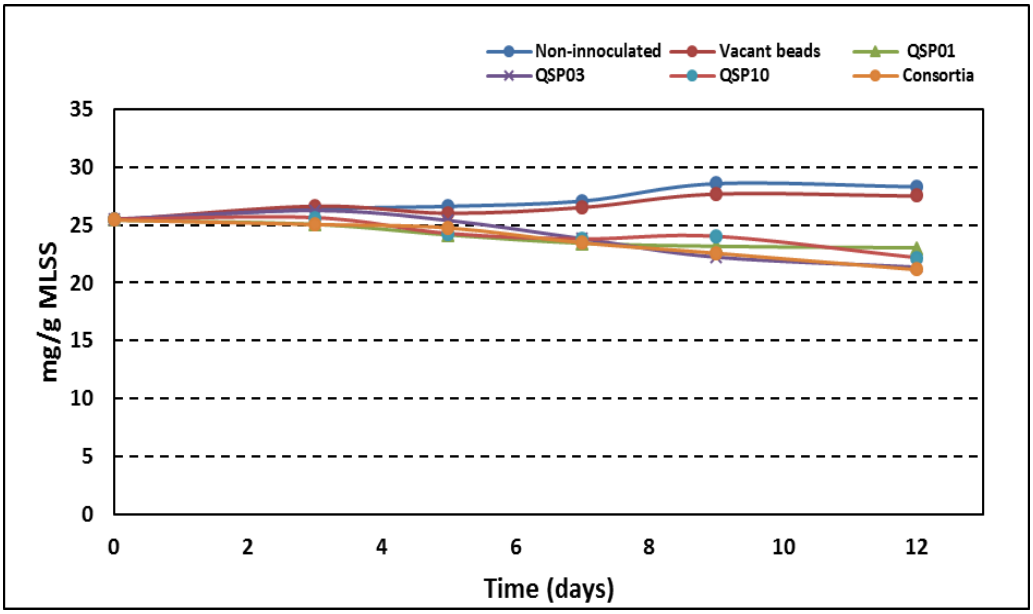



**Figure 4.21**: Impact of coated beads inoculation on total bound EPS during 12 days of batch study.

Therefore, we may attribute the lower soluble and total bound EPS level in QQ beads inoculated SBRs to the QQ bacterial strains. Total bound EPS appeared lower in MBR inoculated with polymer coated beads containing consortium of three QQ indigenous species as indicated in Figures 4.18 and Figure 4.21. Waheed et al. (2017) also indicating lower potential membrane biofouling in the bioreactors containing consortia instead of single species. Furthermore, the study also showed that soluble and LB EPS have major contribution towards biofouling in MBR. Due to the decreasing trend in EPS level in QQ SBRs, it may be assumed from the results shown in Figure 4.19 to Figure 4.21 that the biofouling of membrane would be delayed due to QQ activity by QQ bacterial species.

# 5. Conclusions and recommendations

Bacterial species able to survive on AHLs as sole carbon sources are considered the species with the ability to interrupt the quorum sensing process. The isolated bacterial species by providing AHL as sole carbon sources showed QQ activity of varying degree. Species have different tendencies to quench long and short chain AHLs as indicated by biochemical analysis employing biosensor species.

Detection of acylase and lactonase producing genes in three selected species by gene specific primers further verifies the ability of the indigenous species to quench quorum sensing molecules. The lower EPS in samples collected from SBR inoculated with beads containing quorum quenching bacterial species indicates lower membrane biofouling potential in such a bioreactor. This was due to the previous findings on role of QS in EPS production that ultimately causes membrane biofouling.

## 5.1 Conclusions

The conclusions drawn from the study are;

i. *Enterobacters* and *Bacillus* genus are abundant among the isolated QQ species. The gram-negative and rod-shaped bacteria dominates by small percentage
ii. Lactonase and acylase producing genes are detected in *Pseudomonas* sp QSP01, *Bacillus* sp QSP03 and QSP10 verifying their ability to quench QS
iii. Biosensor based screening indicated majority strains with dual role. *Brucella suis* QSP09 and *Kocuria flava* QSP05 showed high QS and QQ activity
iv. Mixed species beads inoculation in SBRs reduced total EPS in comparison to non-inoculated SBR.

## 5.2 Recommendations

For further study, few of the recommendations are;

i. Evaluate the kinetics of quorum quenching enzymes from isolates of membrane bioreactor
ii. Screening the AIP based quorum sensing activity of the isolated species
iii. Analysing the quorum quenching enzyme specificity and preference for all AH

# APPENDICES

## Annexure- A

Composition of minimal media prepared for bacterial isolation from sludge sample.

| **Compounds** | **Quantity per Litre (g)** |
|---|---|
| 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 Litre** | **(mg)** |
| Ferric chloride | 1 |
| Manganese(II) chloride | 100 |
| Zinc chloride | 46 |

## Annexure- B

ABT Media Protocol: growth media for *P.aerogenosa* QSIS2 strain

| **Solution A** | |
|---|---|
| Distilled water | 100 ml |
| $(NH_4)_2SO_4$ | 1 g |
| $Na_2HPO_4$ | 3 g |

| | |
|---|---|
| $KH_2PO_4$ | 1.5 g |
| $Na_2SO_4$ | 5.5 mg |
| NaCl | 1.5 g |
| **Solution B** | |
| Distilled water | 395 ml |
| $MgCl_2$ | 1 M |
| $CaCl_2$ | 0.1 M |
| $FeCl_3.7H_2O$ | 0.003 M |
| **Solution A+B= AB medium** | |
| **For ABT Medium** | |
| Thiamine | 1.25 mg |
| Glucose | 2.5 g |
| Casamino acid | 2.5 g |
| Gentamicin | 40,000 mg |

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