Investigation of Quorum Quenching Property of a Novel *Bacillus velezensis* and its Application in Biofilm Control

by

Suryalekshmi V. A.
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Under the supervision of
Dr. Krishnakumar B.
Senior Principal Scientist

CSIR- National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram

Academy of Scientific and Innovative Research
AcSIR Headquarters, CSIR-HRDC campus
Sector 19, Kamla Nehru Nagar,
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(Thesis Supervisor)

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Date 11/7/2023

Thiruvananthapuram

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Suryalekshmi V. A.

To My Parents

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ABBREVIATIONS

3-O-C_{10}-HSL	N-(3-oxo Decanoyl)-L-HSL
3-O-C_{12}-HSL	N-(3-oxo Dodecanoyl)-L-HSL
3-O-C_4-HSL	N-(3-oxo Butanoyl)-L-HSL
3-O-C_6-HSL	N-(3-oxo Hexanoyl)-L-HSL
3-O-C_8-HSL	N-(3-oxo Octanoyl)-L-HSL
3-O-C_9-HSL	N-(3- oxo Nonanoyl)-L-HSL
3-OH-C_{10}-HSL	N-(3-hydroxy Decanoyl)-L-HSL
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3-OH-C_6-HSL	N-(3-hydroxy Hexanoyl)-L-HSL
3-OH-C_8-HSL	N-(3-hydroxy Octanoyl)-L-HSL
AHL	N-Acyl Homoserine Lactone
AIP	Auto-Inducer Peptide
AO	Acridine Orange
BHI	Brain heart Infusion
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
C_{10}-HSL	N-Decanoyl-L-HSL
C_{12}-HSL	N-Dodecanoyl-DL-HSL

C_{14}-HSL	N-(3-myristoyl)-DL-HSL
C_4-HSL	N-Butyryl-L-HSL
C_5-HSL	N-Pentanoyl-L-HSL
C_6-HSL	N-Hexanoyl-L-HSL
C_7-HSL	N-Heptanoyl-L- HSL
C_8-HSL	N-Octanoyl-L-HSL
CDD	Conserved Domains Database
CFL	Cell Free Lysate
CFS	Cell Free Supernatant
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal Laser scanning Microscopy
CRA	Congo Red Agar
DMSO	Dimethyl Sulfoxide
eDNA	Extracellular DNA
EPS	Extra Polymeric Substance
FI assay	Flask Incubation Assay
HHQ	4-hydroxy-2-heptyl quinoline
HPLC	High Performance Liquid Chromatography
Km	Micheal-Menten constant
LB media	Luria-Bertani media
LB plot	Lineweaver-Burk Plot
LB-EPS	Loosely Bound- Extra Polymeric Substance

LC-MS	Liquid Chromatography- Mass Spectrometry
MATH	Microbial Adhesion To Hydrocarbons
MATS	Microbial Adhesion To Silica
MBIC	Minimum Biofilm Inhibitory Concentration
MBL	Metallo β-lactamase
MBR	Membrane Bioreactor
MDR	Multi Drug Resistance
MIC	Minimum Inhibitory Concentration
MLL	Metallo-β-Lactamases Like Lactonases
NCBI	National Center for Biotechnology Information.
NGS	Next Generation Sequencing
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
OD	Optical Density
ORF	Open Reading Frame
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffer Saline
PI	Propidium Iodide
PIPES	Piperazine-N,N′-bis(2-Ethane Sulfonic acid)
PLL	Phosphotriesterase-Like Lactonases
PQS	2–heptyl-3,4-dihydroxy quinoline
QQ	Quorum Quenching

QS	Quorum sensing
QSI	Quorum Sensing Inhibitors
SAM	S-Adenosyl Methionine
SDS	Sodium Dodecyl Sulfate
SMP	Soluble Microbial Product
STP	Sewage Treatment Plant
SVI	Sludge Volume Index
TB-EPS	Tightly Bound- Extra Polymeric Substance
Vmax	Maximum velocity
β–MCP	β–Mercaptoethanol

Chapter 1

Introduction

The social intelligence of the unicellular bacterial kingdom was mostly unknown until the twenty-first century. Cooperation among bacteria was thought to be extremely improbable due to the presence of binary fission-mediated reproduction and self-non-self-recognition-led competition. However, a greater understanding of processes such as conjugation, symbiosis, production of antibiotics and other secondary metabolites, and niche adaptations has highlighted the need for a certain degree of communication among the participating prokaryotic cells (Whiteley et al., 2017). The unicellular existence of prokaryotic kingdom was challenged initially by studies on fruiting body formation in *Myxococcus xanthus* (McVittie et al., 1962) as well as streptomycin biosynthesis and aerial mycelium formation in *Streptomyces grisieus* (Hošt'álek, 1979). The possibility of a bacterial communication system was first mentioned in genetic competency studies of *Streptococcus pneumoniae* (Tomasz, 1965) and bioluminescence in marine bacteria (Nealson et al., 1970). The discovery of luminescence genes (*lux* genes) from the marine symbiotic bacteria *Vibrio fischeri* was considered as another milestone in this journey (Engebrecht et al., 1983). However, more details on this communication network was revealed only during 1990's due to the widespread use of DNA sequencing and comparative genomics studies (Whiteley et al., 2017). Progressing from these researches, bacterial communication is now considered as an essential process for their reproductive success which regulates a plethora of phenotypes.

Unicellular organisms favor diffusible signal mediated strategies that are similar to the endocrine systems in higher organisms rather than direct cell-cell contact mediated communication. Such signal molecules are broadly known as 'pheromones' and are released extracellular (Williams et al., 2007). The sub-class of pheromones which stimulates their own synthesis is referred as 'autoinducers' and they co-ordinate the social behavior of bacteria in a cell-density dependent manner. This phenomenon was discovered by Fuqua et al. (1994) and they introduced the term 'Quorum Sensing' (QS). Bacterial quorum sensing involves the local accumulation of autoinducer molecules to a certain threshold level beyond which specific set of genes are transcriptionally activated altering their behavior as a function of cell-density (Papenfort & Bassler, 2016). This process helps a bacterial population to behave as a multicellular organism. The production, secretion and reception of autoinducer molecules by the bioluminescent bacteria *Vibrio harveyi* and *Vibrio fischeri* which colonizes the light organ in the Hawaiian bobtail squid *Euprymna scolopus* is the first extensively studied QS mechanism (Fuqua et al., 1994). This led to an explosion in research regarding other bacterial QS networks and associated phenotypes.

1.1 Bacterial quorum sensing system

The fundamental mechanism of Quorum sensing involves the binding of an autoinducer molecule to its corresponding transcriptional regulator. Therefore, the basic components of a QS system is composed of an autoinducer synthesis system and a specific receptor associated signaling cascade network (Fig. 1.1) (Schaefer et al., 1996). To distinguish autoinducers from other extracellular metabolites, four criteria are put forward by Winzer et al., (2002) (i) The molecule should be produced during specific growth stages and under the influence of environmental changes, (ii) The molecule should accumulate extracellularly and recognize a specific receptor protein, (iii) the accumulation of the molecule beyond a certain threshold concentration should elicit transcriptional responses, and (iv) the cellular response should extend beyond physiological changes required to metabolize the molecule. Hence, any metabolite that accumulate and trigger the toxic stress response in bacteria on reaching a critical concentration cannot be considered as autoinducers. Gram negative and Gram positive bacteria employ different QS system to regulate their cellular responses, and also the autoinducer molecules vary intra- and inter specifically (Rutherford & Bassler, 2012). Therefore, the accurate comprehension and appropriate quorate response is a highly complex process in a multispecies population since the participants have to face a heterogeneous mixture of autoinducers (Whiteley et al., 2017).

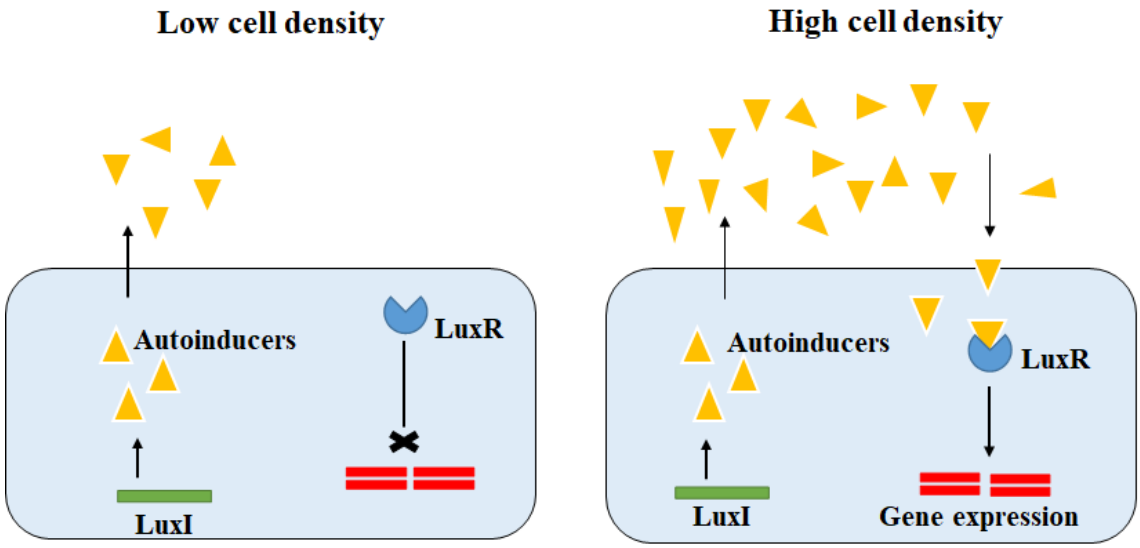

Fig. 1.1 Diagrammatic representation of the mechanism involved in the quorum sensing in Gram negative bacteria

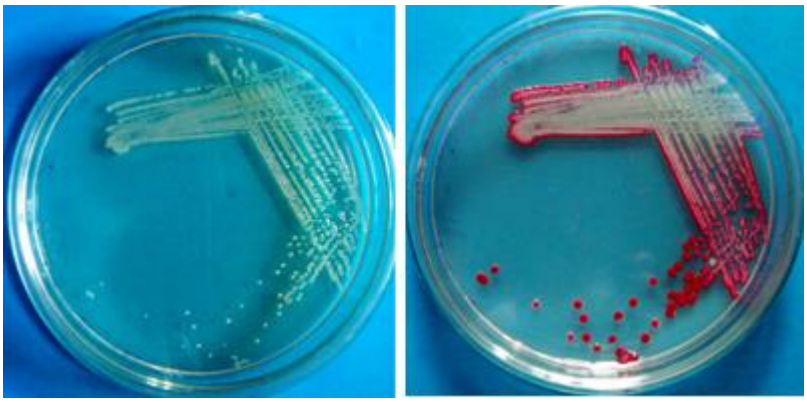

Fig. 1.2 Cell density dependent pigment (Prodigiosin) production by *Serratia marcescens* on solid agar medium (source CSIR-NIIST study).

In several known bacterial species, quorum sensing is pervasive. Pathogens such as *Vibrio, Pseudomonas, Yersinia, Serratia, Staphylococcus* and *Streptococcus* completely regulate their virulence mechanisms with the QS networks (Zhao et al., 2015). The pigment production in *Serratia* (Fig. 1.2) exotoxin production in *Bacillus* and *Enterococcus* genera, nitrogen fixation in *Rhizobium*, adaptation to extrophilic conditions in *Natronococcus occultus, Thermotoga maritima, Acidithiobacillus ferrooxidans* etc. are also coming under the quorate control (Koul et al., 2016).

1.2 Quorum sensing in Gram negative bacteria

The QS in Gram negative bacteria is characterized by four features. First, the autoinducer molecule involved are S-Adenosyl Methionine (SAM) derivatives (Wei et al., 2011). An exception to this is p-Coumaroyl homoserine lactone derived from p-coumarate in *Rhodopseudomonas palustris* (Schaefer et al., 2008). These molecules are highly permeable through the bacterial cell membrane. Second, the autoinducer binding receptors are located intracellularly *viz.*, either embedded in the inner cell membrane or within the cytoplasm (Houdt et al., 2007). Third, the QS networks trigger a cascade of responses involving multiple genes and finally (Four) a positive feed-back loop is promoted by the signal binding which results in the increased production of autoinducer molecules (Ng & Bassler, 2009).

The most widely distributed autoinducer in Gram negative bacteria are the N-Acyl Homoserine Lactones (AHLs) (Fig. 1.3), which are constituted by a homoserine lactone moiety and an acyl chain with an even number of carbons in the range C4 to C18 (Fig. 1.4). A hydroxyl or olefinic double bond modification is occasionally found at the C-3 position. The stability and specificity of an AHL molecule is highly dependent upon its chain length (Patel et al., 2016). Even though the molecules are believed to be freely diffusible through the membrane, studies have identified active efflux pumping systems involved in the translocation of AHL long chain. Hence it is possible that only short chain AHL molecules are passively transported across into the cell (Churchill & Chen, 2011). Major AHL signals produced in Gram negative bacteria and their biological functions are enlisted in Table 1.1.

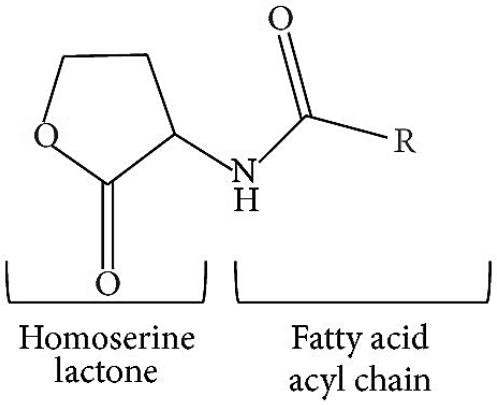

Fig. 1.3 The general structure of N-Acyl Homoserine Lactone (AHL)

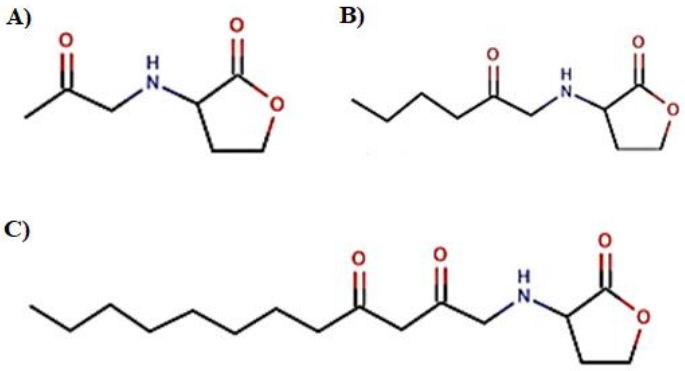

Fig. 1.4 Examples of AHL molecules with variable side chain length A) N-Butanoyl Homoserine Lactone (C_4-HSL) B) N-Hexanoyl Homoserine Lactone (C_6-HSL) C) N3-Oxododecanoyl Homoserine Lactone (3O-C_{12} HSL) (Acet et al., 2021)

Table 1.1 Major N-Acyl Homoserine Lactonase signals and their functions in Gram negative bacteria

Sl. No.	Organism	AHL signal	Regulatory genes	Major Phenotypes controlled	Reference
1	*Vibrio fischeri*	3-Oxo-C_6-HSL	LuxI/R	Bioluminescens	(Fuqua et al., 1994)
2	*Vibrio harveyi*	3-OH-C_4-HSL	LuxLM/N	Bioluminescens	(Defoirdt et al., 2010)
3	*Chromobacterium violaceum*	C_6-HSL	CvilI/R	Violacein production	(McClean et al., 1997)
4	*Serratia marcescens*	C_4-HSL	SmaI/R	Prodigiosin production, Motility, biofilm formation	(Van Houdt et al., 2007)
5	*Pseudomonas aeruginosa*	3-Oxo-C_{12}-HSL C_4-HSL	LasI/R, RhlI/R	Virulence factors, biofilm formation	(Steindler et al., 2009)
6	*Pseudomonas aeruofaciens*	C_6-HSL	PhzI/R	Phenazine antibiotics	(Venturi, 2006)
7	*Erwinia chrysanthemi*	3-Oxo-C_6-HSL C_6-HSL	ExpI/R	Pectate lyase production	(Hussain et al., 2008)
8	*Rhizobium leguminosarum*	C_6-HSL	RhlI/RhlR	Rhizosphere expressed proteins	(Bloemberg & Lugtenberg, 2001)
9	*Agrobacterium tumefaciens*	3-Oxo-C_8-HSL	TraI/R	Ti plasmid conjugation	(Zhang et al., 2002)
10	*Pseudomonas flourescens*	3-Oxo-C_{10}-HSL	LasI/R Rhl I/R	Mupirocin resistance, biofilm formation	(El-Sayed et al., 2001)
11	*Erwinia carotovora*	3-Oxo-C_6-HSL	ExpI/R	Exoenzyme production	(Welch et al., 2005)
12	*Rhodospirillum rubum*	3-OH-C_{14}-HSL	Lux I/R homologue	Plant pigmentation	(Mastroleo et al., 2013)
13	*Escherichia coli*	C_6-HSL	Lux I/R homologue	biofilm formation, multidrug resistance	(Wu & Luo, 2021)
14	*Acinetobacter baumanii*	C_6-HSL	AbaI/R	biofilm formation, carpospores liberation, pili assembly	(Sun et al., 2021)

Most of the Gram negative bacteria uses the autoinducer synthase enzyme belonging to the *lux I* family that transfers the fatty acyl side chains via acyl-acyl carrier proteins to the lactonized methionine moiety from SAM (Wei et al., 2011). Generally, *lux I type* proteins are highly specific for the acyl chain length, but multiple AHL producing types are also identified. Several *lux I* homologue protein are identified in gram negative bacteria. CviI in *Chromobacterim violaceum,* LasI and RhI in *Pseudomonas aeruginosa,* SwrI in *Serratia marcescens,* Ras I in *Ralstonia solanacearum,* AhyI in *Aeromonas hydrophila,* etc. are some of the major members in the family (Kiratisin et al., 2002; McClean et al., 1997; Morohoshi et al., 2007; Ponnusamy et al., 2009). These homologs are ~200 amino acid long, and bear conserved amino acids in their carboxy terminal half. Biosynthesis of AHLs are not exclusive to LuxI proteins. Non-*lux I* type autoinducers synthases such as AinS and LuxLM in *Vibrio harveyi,* HdtS in *Pseudomonas flourescens,* are also present along with this family (Papenfort & Bassler, 2016). The AHL molecules in Gram- negative bacteria are detected by the cytoplasmic transcription factor Lux R containing an amino terminal ligand binding domain and a carboxy terminal DNA binding domain. AHL reception stabilizes the lux R proteins by dimerizing them and subsequently promotes the transcription activation by DNA binding. The AHL-lux R complexes generally binds with the lux box regions in the upstream regions of the target genes (Houdt et al., 2007). These receptor proteins can exist as either solo class or paired transcription factors with the corresponding lux I homolog. Two component membrane bound histidine kinase receptor proteins such as LuxN, CqsS and LuxQ are also present in some Gram negative bacteria as the autoinducer acceptors (Montánchez & Kaberdin, 2020).

The characterization and quantification of AHL signals is facilitated by various biosensor assays. The purple blue pigment Violacein induction in *Chromobacterium violaceum* and its mini Tn5 mutant *Chromobacterium substugae* (non-pigment producing) are the most primitive and simplified AHL detection system used today (Harrison & Soby, 2020). Several reporter gene fusions *viz., gfp, lacz* or *lux* are also used for the process. The Lacz reporter based *Agrobacterioum tumefaciens* biosensor strain NTL4 is widely used for the detection of short and long chain AHLs (Zamani et al., 2014). However, high sensitive analytical methods such as Liquid Chromatography coupled with Mass spectrometry (LC-MS) and Nuclear Magnetic Resonance (NMR) are required for the confirmation of the chemical identity of the AHL signals (Zhao et al., 2021).

Gram negative bacteria employs QS mediated regulation in a variety of biological processes. The implication of QS in bacterial pathogenesis is a well explored area. Many

animal and plant pathogenic bacteria employ QS network to efficiently invade the hosts and establish virulence factor production. In Gram negative phyto-pathogens such as *Pseudomonas syringae, Ralstonia solanacearum, Agrobacterium tumefaciens* with *Ti plasmid, Erwinia amylovora, Pectobacterium carotovorum* and *Burkholderia glumae*, AHLs play critical role in pathogenesis of agricultural crops and results in yield loss (Gutiérrez-Pacheco et al., 2019). Animal pathogens also employ AHLs for their mobility, virulence factor production and localization in the host tissue(De Kievit & Iglewski, 2000). Other major biological processes involving QS mechanisms are sporulation, pigment production, antibiotic synthesis, fermentation and biofilm formation (Hooshangi & Bentley, 2008). Recent applications of QS in synthetic biology concentrates in combining the autoinducer signals and their associated machineries in developing genetic circuits for dynamic control of both Gram-negative and Gram-positive bacterial populations. This enables metabolic engineering control and regulation of physiological activities in Gram-negative bacterial population. The AHL based QS genetic circuits in Gram negative bacteria such as oscillators, toggle switches, and logic gates are gaining immense attention due to their potential in therapeutic applications (Ge et al., 2020).

1.3 Quorum sensing in Gram positive bacteria

While Gram negative bacteria employ low molecular weight hydrophobic signal molecules as their autoinducers, Gram positive bacteria generally relies on post-translationally modified oligopeptides *viz.,* Autoindicer Peptides (AIPs). They are produced as precursors within the cell and transported across the cell membrane using dedicated oligopeptide exporters which also help in their processing into mature signal peptides (Banerjee & Ray, 2017). Functional AIPs are 5-17 amino acids long and exists in either linear or cyclic form. Many of the AIPs contain lactone, thiolactone, lanthionines and isoprenyl groups as their post translational modification. Similar to the LuxI/R system in Gram negative bacteria, AIPs also facilitate specific intraspecific communication in a cell density dependent manner. These signals are detected using a two component membrane bound sensor kinases receptors (Bhatt, 2018). The ligand binding initiates auto-phosphorylation in a conserved histidine residue of the sensor kinases which in turn proceeds through a phosphorylation cascade downstream and ends in a transcriptional activator response regulator protein (McBrayer et al., 2020). Gram positive QS systems are more complex than Gram negative systems due to the multiplicity of the AIPs and the complex signalling cascades involved. The competence in *Streptococcus pneumaniae,* sporulation in *Bacillus subtilis,* virulence in *Staphylococcus aureus, Listeria* and *Enterococcus* are some of the widely studied QS Controlled phenotypes. Agr QS network in *S. aureus* is

considered as one of the extensively studied networks in Gram positive bacteria (Neelapu et al., 2018).

Apart from the above mentioned common signalling mechanisms in Gram negative and Gram positive bacteria, a third class of hybrid QS system was also reported such as in bacteria *V. harveyi* and *Salmonella typhimurium*. In this system, the bacteria adopts a mechanism which is a combination of mechanisms in Gram negative and positive bacteria where a low molecular weight signalling molecule acts as the autoinducer and they are detected with two component sensor kinase receptors (Rezzonico et al., 2012) . Numerous minor QS systems that are highly specific to certain organisms are also discovered (Sifri, 2008).

1.4 Quorum sensing in *P. aeruginosa*

Pseudomonas aeruginosa, is a ubiquitous and highly adaptive Gram negative bacterium, considered as a promising candidate for QS related studies. The QS network of this nosocomial opportunistic pathogen is well-established and widely researched, and it consists of two different types of systems that are controlled by AHL and alkyl quinolones (Fig. 1.5) (Lee & Zhang, 2015).

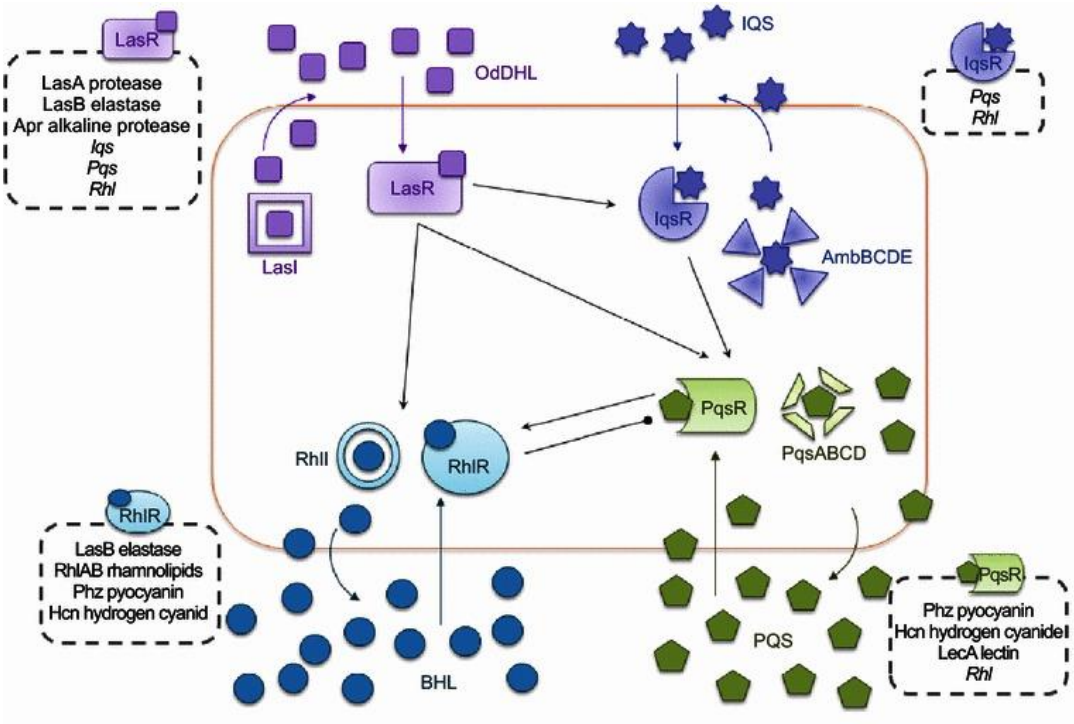

Fig. 1.5 QS network in *Pseudomonas aeruginosa* (Adapted from (Lee & Zhang, 2015)

The AHL controlled network is the major contributor to the signaling cascades in this organism regulating 11% of the total genome (Rutherford & Bassler, 2012). The system is again divided into two components *viz.*, Las and Rhl system. The Las system is composed of the signal synthase LasI and the signal receptor cum transcriptional activator LasR controlled by the N-3-oxododecanoyl homoserine lactone (3-O-C_{12}-HSL). This long chain substituted AHL is transported to the cell exterior with the help of an efflux pump. However, a small degree of transport also takes place via passive diffusion. The Rhl system is regulated by the short chain AHL, N-Butyryl homoserine lactone (C_4-HSL) which is produced by the signal synthase RhlI and detected by the transcriptonal activator RhlR. Since the C_4-HSL readily diffuses across the membrane, it does not require any active efflux (Kiratisin et al., 2002; Ventre et al., 2003). In addition to these systems, an alkyl quinolone based QS network *viz.* PQS system also reported in *P. aeruginosa* which regulates approximately 170 genes (Venturi, 2006). The cognate receptor PqsR in the PQS network is compatible with two ligands, 4-hydroxy-2-heptyl quinoline (HHQ) and 2–heptyl-3,4-dihydroxy quinoline (PQS). The PQS has less affinity towards the receptor and it is associated with membrane vesicles due to its relatively high hydrophobicity (Lee & Zhang, 2015).

The QS system in *P. aeruginosa* works in a hierarchical manner with Las system as the dominant component which dimerizes upon the 3-O-C_{12}-HSL, binding and imparts a positive feedback loop on the Rhl system and other genes under its regulation. A second positive feedback loop executes when Rhl binds with the C_4-HSL. The PQS system is not independent from the AHL dependent network (Glessner et al., 1999). The Las system imparts a positive regulation upon PQS by activating pqsR and pqsH while Rhl system negatively regulates the PQS synthesis. However, the PQS is found to enhance the production of C_4-HSL by activating RhlI. The interconnection between the QS networks is presented in Fig. 1.5. The evolutionary fitness of *P. aeruginosa* is highly related to the multiplicity of virulence factors and complex genetic regulatory network. Together these QS systems control a plethora of virulence factors in *P. aeruginosa* including motility, pyocyanin productions, resistance against oxidative stress, siderophores, exoenzyme production and biofilm formation (Lin *et al.,* 2019, Jimenez *et al*., 2012). The important virulence factor, regulatory genes and related functions in *P. aeruginosa* are summarized in Table 1.2.

Table 1.2 QS controlled virulence factors in *P. aeruginosa*

Sl. No.	Virulence factor	Gene	Function	Reference
1	Las A protease	las A	Host tissue protein degradation	(Kessler et al., 1997)
2	Alkaline protease	aprA	Protection from host defence	(Marzhoseyni et al., 2023)
3	Elastase B	las B	Host tissue protein degradation	(Galdino et al., 2019)
4	Catalase	katA	Protection from oxidative stress	(da Cruz Nizer et al., 2021)
5	Exotoxin	toxA	Host cell apoptosis initiation	(Wood et al., 2023)
6	Cyanide	hcnAB	Host cell toxicity	(Zdor, 2015)
7	Chitinase	chicC	Cell lysis	(Folders et al., 2001)
8	Type IV pili	pilC	Attachment to host cells, motility	(Wozniak & Keyser, 2004)
9	Pyocyanin	phz	Immune response suppression, cytotoxic	(Wierre-Gore, 2021)
10	Pyoverdine	pvd	Iron chelating agent, growth promoter	(Liao et al., 2022)
11	Rhamnolipid	rhlABC	Biofilm formation, immune evasion	(Williamson et al., 2008)
12	Flagellin	fliC	Bacterial adhesion, swarming motility	(Luo et al., 2015)
13	Alginate	algR, algB	Biofilm formation, immune evasion	(Morici et al., 2007)

1.5 Bacterial biofilms

Bacteria can survive in extreme and diverse ecological niches due to their remarkable adaptability. This property is partly dictated by their ability to form biofilms. Biofilms are considered as microbial survival structures that has transformed from a planktonic state to a surface attached sessile form (Vishwakarma, 2020). The first observation of biofilms dates back to 1684, when Antoine van Leeuwenhoek documented the microscopic image of dental

plaques. Unbeknown to Leeuwenhoek, it was the initial discovery of a biofilm. For a greater period of time, microbiological studies focused on the planktonic growth only. The concept that bacteria develop into community structures rather than planktonic form in nature outside the laboratory conditions was first emerged on observing microcolony formation on glass surfaces submerged in sea water (Kalgudi, 2018). The term 'Biofilm' was coined by Costerton (Costerton, 1995) describing *P. aeruginosa* cells entrapped in glycocalyx found in cystic fibrosis lungs. Later on, the ubiquitous existence of many bacterial biofilms was extensively studied and documented.

Biofilms are defined as sessile microbial communities comprised of cells attached to a substratum (surface), interface and to each other in a self-produced matrix. Biofilms are remarkably recalcitrant structures that can be formed on any biotic or abiotic substratum. They are several fold tolerant to stress factors and antimicrobial agents than their planktonic equivalents (Mah & O'Toole, 2001). The formation of biofilms is of greater concern in industrial, environmental and medical sectors (Vishwakarma, 2020). The practical challenges due to biofilms in industrial operations include increased fluid friction, biofouling in membrane bioreactors, corrosion and heat transfer obstructions in tanks and pipelines (Kalgudi, 2018). The spoilage caused by biofilm-mediated contamination, particularly in the food industry, results in a significant increase in operational and maintenance costs (Shen et al., 2021). It is beyond dispute that biofilms play a crucial role in the invasion and progression of bacterial infections. The bacterial cells are protected from immune system reactions by the biofilm's matrix components. For instance, *Vibrio cholera* biofilms shield the cells from antibiotics and the acidic pH of the gastrointestinal system (Yin et al., 2019). The resilience of biofilms aids in the more effective transmission of diseases from one person to another. Nosocomial infections are another major impact of biofilm formation. The possibility of developing opportunistic infections such as fasciitis and endocarditis are elevated as a result of biofilm (Driscoll et al., 2007). Contamination of sterile medical surfaces and equipment, such as catheters, prosthetic valves, contact lenses, and intrauterine devices, is another grave disadvantage associated with biofilms (Donlan, 2001). These hospital-associated infections are often caused by significant biofilm formers such *P. aeruginosa*, *Klebsiella pneumoniae, Staphylococcus aureus, Proteus mirabilis*, etc. (Assefa & Amare, 2022).

Contrary to the negative impacts of biofilms, they are highly beneficial and hence important in many other scenarios. The primary benefit is that biofilms are used in the bioremediation of harmful contaminants such azo dyes, heavy metals, and herbicides. Human

skin and gut microbiota also create biofilms, which have been shown to be highly advantageous (Mangwani et al., 2016). The development of biofilms is also discovered to be effective in a number of plant-microbial interactions (Rudrappa et al., 2008).

1.6 Stages of Biofilm development

The intricate process of biofilm development is heavily reliant on environmental cues including nutrient deprivation, oxidative stress, shear force, etc. Bacterial cells and extrapolymeric matrix are the two essential parts of a biofilm, regardless of surface where the biofilm is formed, and the bacterial species involved. Transcriptomic and proteomic studies have revealed that the participating cells in a biofilm are physiologically heterogeneous in nature (Tolker-Nielsen, 2015). The EPS matrix of the biofilms are also highly characterized using analytical techniques such as HPLC and microscopic examinations. A variety of polysaccharides, proteins, glycoproteins, glycolipids, and extracellular DNA (eDNA) are all present in EPS, albeit their exact composition varies from biofilm to biofilm. The compositional and physiological heterogeneity in biofilms greatly facilitate the adaptation to environmental conditions. This in turn creates other issues including the growth of persister cells and the emergence of antibiotic resistance (Flemming et al., 2016).

Despite the fact that biofilms are formed by different types of bacteria, they follow a general developmental process in a sequence like- (i) reversible and irreversible attachment to substratum, (ii) formation of microcolonies (iii) formation of macrocolonies and maturation and (iv) dispersion (Kalgudi, 2018) (Fig. 1.6) . Every biofilm development process begins with a reversible stage in which planktonic cells attach to the surface using a weak interaction force, such as Van der Waal's force. This step is preceded by a conditioning layer formation derived from the surrounding medium on the surface. Additional gene expression adjustments and the emergence of specialized structures like pili and adhesins gradually make the attachment process irreversible (Tolker-Nielsen, 2015).

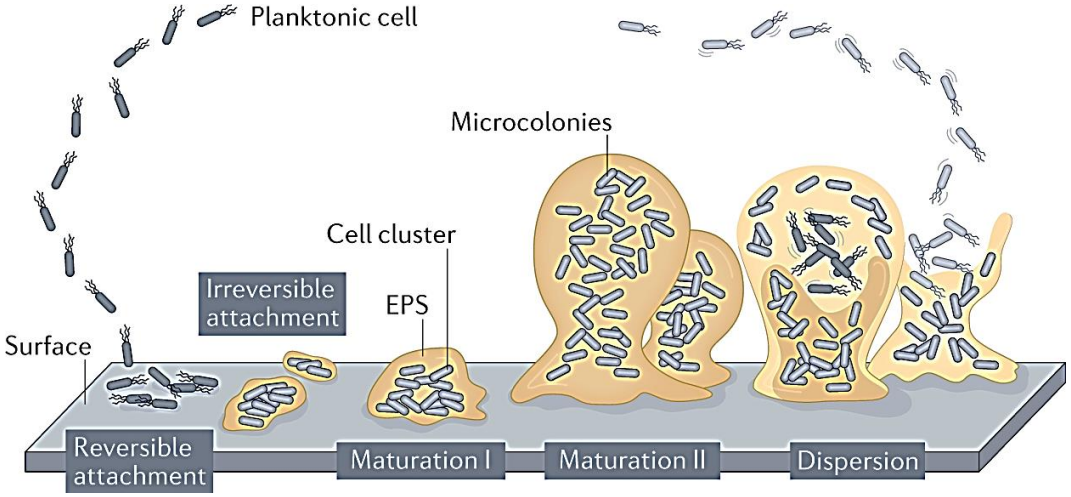

Fig. 1.6 Different stages of a biofilm formation (Adapted from Kalgudi, (2018))

A solid substratum (surface) facilitates the bacterial attachment in many ways. They can function as a medium for nutrient adsorbing, provide minimal shear and flow velocity over the liquid solid interface, physical properties such as surface roughness and hydrophobicity favors the attachment. Other factors affecting the cell adhesion includes chemical composition and ionic strength of culture medium and surface charges. The irreversible binding of the cells also depends upon the expression of certain protein such as diguanylate cyclases (SadC) and phosphodiesterases (BifA) in *P. aeruginosa.* These type of protein complexes are responsible for swarming motility and aids in attaching cells irreversibly by reducing the flagellar movement (Banerjee et al., 2021).

Once the attachment process is established, the EPS producing genes are upregulated and the cells are encapsulated in viscous matrix. In a biofilm, 50-90% of the total organic matter is constituted by EPS. The cells then start to divide rapidly and develop into mature microcolonies (Fig. 1.7). These steps are collectively known as maturation. Steep oxygen and nutrient gradients can be found in a fully developed bacterial biofilm. Nevertheless, biofilms can have channels and cavities that help the diffusion of nutrients and other components across the biofilm (Quan et al., 2022). This non-uniformity in resource supply is considered as a reason for the existence of various mutational and phenotypic variations within a biofilm. A certain degree of division of labor exists among cells within a mature biofilm which strongly determines its architecture. For example, the cells closer to the substratum (inside biofilm)

displays slower metabolic rates compared to the surface cells due to the restriction in oxygen supply. As a result, clustered cells with different organizations are frequently observed in biofilms (Eg: Flat, cylindrical and mushroom like structures in *P. aeruginosa* biofilm (Reichhardt & Parsek, 2019)

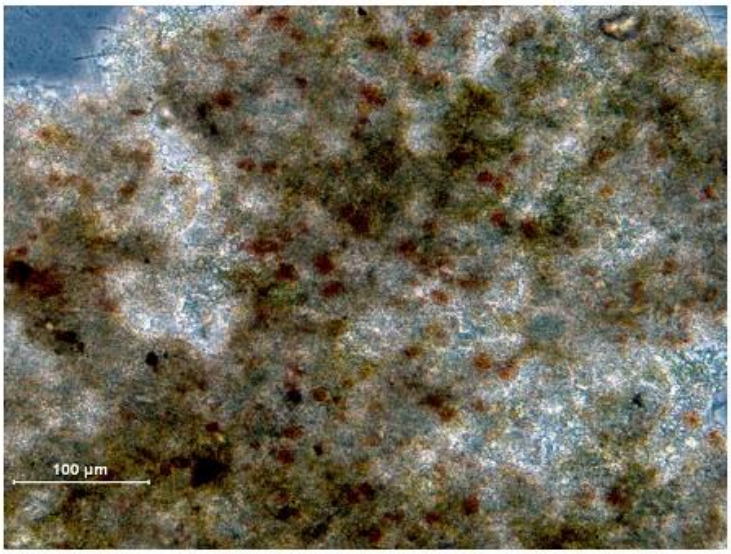

Fig. 1.7 Phase contrast microscopic image of Microcolonies (red spots) of *Serratia marscens* in a complex biofilm (Source CSIR-NIIST study).

The final step in microbial biofilm life cycle is the dispersal of cells into surrounding medium. The dispersed cells invade newer surfaces and propagate the biofilms to newer niches. This stage also requires environmental cues like nutrient availability and dispersing agents such as Nitric Oxide (NO), sodium ascorbate, D-amino acids and Iron. The gram negative autoinducer molecules AHLs also play a vital role in biofilm dispersion (Rumbaugh & Sauer, 2020).

1.7 Existing strategies for controlling biofilms

Considering the adverse effects of biofilms as elaborated in previous section, different approaches are adopted for managing biofilms. Prevention and eradication are the two broadly applied strategies to control biofilm associated biofouling. Usually, a combinatorial approach will be required for completely handling the biofilm formation (Hawas et al., 2022). In health sector, biofilms mainly cause persistent and recurring infections. Specific therapeutic regimens including antibiotic prophylaxis and sterilization of infected site and contact surfaces are followed. In food industry also similar strategies are adopted. Use of preservatives, sterilization of contact surfaces and physical removal are practiced here (Verderosa et al., 2019). In industrial settings much sophisticated techniques such as steam sterilization, ultrasonication

and membrane back washing etc. are frequently used (Alfa, 2019; Mattila-Sandholm & Wirtanen, 1992; Rather et al., 2021). Table 1.3 enlists the important conventional biofilm eradication methods employed in industrial and health sector. However, these practices have their own demerits. Most of these methods are highly labor and energy intensive, and hence more cost involving. Moreover, the antimicrobial resistance, which is of alarming importance now, has brought thought about extensive use of antimicrobial agents such as antibiotics and other preservatives for biofilm control purposes. Additionally, the use of high strength chemicals for biofouling control causes several ecological damages and habitat loss and a sustainable solution has become the need of the hour. In this scenario, inhibition of quorum sensing (quorum quenching) is emerging as an alternate strategy for biofilm control (Rather et al., 2021).

Table 1.3 Conventional and advanced biofilm control strategies

Sl.No.	Treatment method	Examples	Reference
1	Chemical treatment	NaOCl, Peracetic acid, NaOH, H_2O_2	(Kalgudi, 2018)
2	High hydrostatic pressure	H_2O	(Kulawik et al., 2022)
3	Photocatalysis	UV plus O_2, He, N_2 etc.	(Biswas et al., 2017)
4	Bacteriophages	P100	(Tian et al., 2021)
5	Membrane back washing	Air or water	(Cogan et al., 2016)
6	Surface modifications	Phosphorylcholine containing polymers (Medical equipment)	(Russell, 2000)
		Arsenic, mercuric oxide, tributyltin coatings (Marine equipment)	(Selim et al., 2017)
		Silver, furanone coatings (medical)	(Tran et al., 2014)
		Low surface free energy and microroughness	(Song et al., 2020; Tsibouklis et al., 1999)
		Silicon and floropolymer based non-stick coatings	(Tsibouklis et al., 2000)
		Functionalized coatings (nisin, lysozyme)	(Blackman et al., 2020; Roupie et al., 2021)
		'Theta' surface concept	(Vladkova, 2008)
7	Nanotechnology	Silver nanoparticles	(Markowska et al., 2013)

		Titanium nanoparticles	(Al-Shabib et al., 2020)
		Silicon and organic quaternary ammonium salts antimicrobial sprays	(Etim et al., 2018)
		Micro and nano emulsions	(Song et al., 2016)
8	Enzyme treatment	DNAse	
		Lysostaphin	
		a-amylase	
		Lyase	(Thallinger et al., 2013)
9	Biosurfactants	Lactobacilli biosurfactant	(Tahmourespour et al., 2011)
10	Bioelectric approach	pH modification, electrophoresis, hyperoxyanion generation	(Kim et al., 2015)
11	Ultrasonic treatment	High power streaming (eradicating preformed biofilms)	
		Low power streaming (Biofilm formation inhibition	(Sadekuzzaman et al., 2015)
		Piezoelectric elements	(Montoya et al., 2021)
12	Antibiofilm agents	Chitosan	(Carlson et al., 2008)
		Povidone iodine (PVP)	(Hosaka et al., 2012)
		Gallium	(Ma et al., 2013)
		Lactoferrin	(Ammons et al., 2011)

1.8 Quorum Quenching mediated biofilm control

Although quorum sensing (QS) aids in community-based behavior coordination, it is not necessary for bacterial survival or proliferation. Therefore, interference with quorum sensing may prevent the development of desired phenotypes like biofilms. Quorum quenching (QQ) is the process of preventing autoinducer-mediated quorum sensing. In a highly competitive microbial world, the development of antagonistic processes against QSis highly plausible. Since the process does not involve any cell death, the probability for developing resistance against QS disrupting agents are fairly low (García-Contreras et al., 2013). There are a number of QQ techniques that can be used to prevent quorum sensing, including: i) Inhibiting auto inducer signal synthesis by inhibiting synthase proteins (ii) Enzymatic degradation of autoinducer accumulation iii) Interference with signal receptors. Significant number of prokaryotes, as well as several eukaryotes, including some plants (example) used in traditional medicine, can produce QS-inhibiting substances (Choudhary & Schmidt-Dannert, 2010) Numerous chemicals that can interfere with the QS system have been found, and their processes have been uncovered. (Kalia, 2013). Several QS inhibitory strategy targeting AHL networks are reported due to the extensive researches in the field (Fetzner, 2015; A. Kim et al., 2014; Ponnusamy et al., 2009).

1.9 Quorum sensing inhibitors of natural origin

Numerous organic substances of plant and animal origins are known to prevent QS without hindering bacterial growth. Secondary metabolites produced by algae, fungus, bacteria, and higher plants have been discovered as compounds that can interfere with the quorum sensing mechanism of bacteria and prevent the formation of biofilms (Kalia, 2013). QSIs can be structurally similar (analogue) to the AHL molecules and thus blocks the LuxR receptors thereby disrupting the AHL detection. Halogenated furanones produced by the Australian red alga *Delisea pulchra,* which block AHLs-mediated gene expression by sabotaging AHL signal from its reporter protein, represent a promising class of natural QSI (Manefield et al., 1999). They are also known to suppress quorum sensing by boosting and destabilizing the turnover rate of LuxR, reducing its capacity to bind DNA and start transcription. Furanones are demonstrated to prevent the growth of biofilms by inhibiting AHL-mediated quorum sensing in several Gram-negative bacteria as *P. aeruginosa*, *V. harveyi*, and *E. coli* (Hentzer et al., 2002). There have been reports of flavonoids such kaempferol, naringenin, quercetin, and apigenein inhibiting AHL-mediated pathogenic characteristics in *P. aeruginosa PAO1, E. coli*

O157:H7, and *V. harveyi* (Nazzaro et al., 2013). It is interesting to note that several well-known natural substances found in food, such as eugenol, curcumin, and ajoene from garlic, can likewise block acyl-homoserine lactone receptors (Abraham, 2016). Given that fungi and quorum sensing bacteria have coexisted for millions of years, it is hypothesised that they can create QSI substances. Some of the examples are penicillic acid and patulin generated by *Penicillium radiciola* and *Penicillium coprobium*. They can target 45-60% of genes regulated by the LasI/R, RhlI/R systems in *P. aeruginosa*. Natural QSI therefore present a viable remedy for the multidrug resistance linked to conventional antibiotics or antimicrobial substances. These substances have primarily been used against pathogenic bacteria, but new research has shown that natural QSI can also be used to decrease membrane biofouling. However, further research is needed to determine more potential QSI present in natural sources and the mechanisms they use to counteract quorum sensing. For example, Vanillin was successfully used to mitigate *A. hydrophila* biofilms from five different surfaces by degrading both short and long chain AHLs (Ponnusamy et al., 2014). It has also been discovered that the Piper betle extract reduces membrane biofouling in ultrafiltration MBR treating textile wastewater (Siddiqui et al., 2013). The aforementioned examples imply that artificial membranes incorporating QSI substances are anticipated to be particularly beneficial in several industrial sectors for biofilm/biofouling control.

1.10 Enzymatic quorum quenching

Quorum quenching can also be achieved by enzymatic degradation of AHL signals. There are naturally occurring QQ bacteria and eukaryotes that are capable of producing AHL degrading enzymes such as lactonases, acylases and oxidoreductases. Lactonases catalyses the lactone ring hydrolysis of AHLs while acylases hydrolyse the amide bond between homoserine moiety and acyl chain. Oxidoreductases oxidize the C3 position of AHL promoting the subsequent degradation by amidohydrolase. AHL-degrading enzymes originate naturally from bacteria, archaea, and eukaryotes (Fetzner, 2015). Using quorum quenching enzymes, AHL signal molecules can be completely degraded or rendered inactive. These enzymes are produced by organisms either to compete with their co-existing QS bacteria or to regulate their own autoinducer levels. PvdQ and BlcC, the analogous enzymes produced by bacteria such as *Pseudomonas* and *Agrobacterium* can degrade their own AHL signals and are thought to be responsible for AHL recycling (Sikdar et al., 2020). The major classes of QQ enzymes are described below:

(i) AHL lactonases

The activity of AHL lactonase is through hydrolyzing the lactone ring in the AHL molecule. They may exhibit preferences for specific AHL subtypes and lactone substrates such δ, ε and γ lactones (Fig. 1.8) (Wang et al., 2004). AHL lactonases are found among four different super families *viz* Phosphotriesterase-like Lactonases (PLL), metallo-β-lactamases like lactonases (MLLs), α/β hydrolase fold lactonases and paraoxanases. The PLLs are found to be produced by bacteria and archaea, and prefer long chain AHLs as their substrate. They are metalloenzymes and contain many thermo-stable representatives with various biotechnological applications. Eg: SacPox, VmoLac and GkL. SsoPox from *Saccharolobus solfataricus* is a widely studied member of this class of enzyme (Ng et al., 2011). The MLLs are also produced by bacteria, archaea and eukaryotes and possess a HXHXDX motif. They are also metalloenzymes found in association with divalent metal ions like Zn^{2+} (Rajesh & Rai, 2014). MLLs are the most characterized enzymes in the AHL lactonase category. AiiA type homologs produced mostly in *Bacillus* genera are considered as the representative enzyme of this class. Compared to the other classes, MLLs have broad substrate specificity. α/β hydrolase fold lactonases are solely produced by bacteria and similar to the MLL class they bear the α/β hydrolase fold and exhibit broad substrate specificity but lacks HXHXDX motif (Lade et al., 2020). AidH from *Ochrobactrum* sp. is the well characterized member in the class. The paraoxanases are characterized with six bladed β-propeller fold and produced by mammals and bacteria. These enzymes have a broad range of substrate specificity which includes long chain AHLs, organophosphates and δ&γ lactones (Mei et al., 2010).

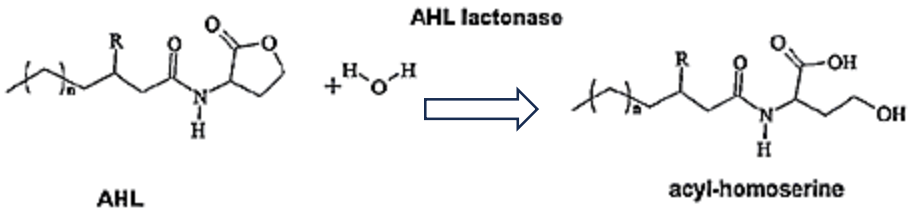

Fig. 1.8 The mechanism of AHL degradation by AHL lactonases (Czajkowski et al, 2009)

(ii) AHL acylases

The AHL acylases hydrolyse the amide bond between the lactone ring and acyl chain of AHLs (Fig. 1.9). Large hydrophobic binding pockets are visible in their active sites, which can hold the lengthy acyl chains of AHL substrates. *Ralstonia* sp. XJ12B and *P. aeruginosa*

PAO1 both produce a variety of AHLs degrading AHL-acylases. One of the few published examples of bacterial AHL acylases is AiiD from *Ralstonia* sp. XJ12B. AiiC from *Anabaena* sp. PCC7120, PvdQ and QuiP from *P. aeruginosa* PAO1 and AhlM from *Streptomyces* sp. It has been demonstrated that the AHL-acylase generated by *Streptomyces* sp. has the capacity to breakdown particular AHLs with 6 or more carbon in acyl chain (Maisuria & Nerurkar, 2015; Romero et al., 2008).

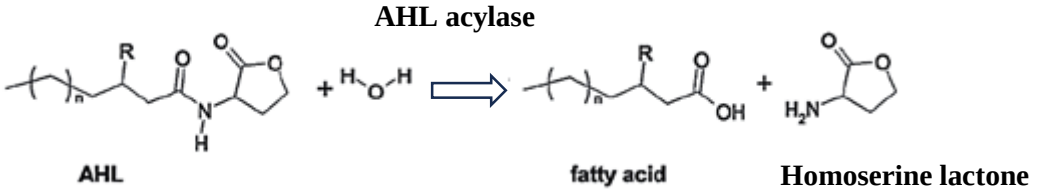

Fig. 1.9 mechanism of AHL degradation by AHL acylase (Czajkowski et al. 2009)

(iii) Oxidoreductases

Unlike lactonases and acylases, the oxidoreductases modify the AHL signals, but not degrade it. They can either reduce the C3 carboxyl group of AHLs, converting it into a hydroxyl group or oxidise the ω1, ω2, or ω3 carbons of the acyl chain (Fig. 1.10). *Acidobacterium* sp.'s BpiB09 reductase converts 3-oxoAHLs to 3-hydroxy-AHLs and can modify *P. aeruginosa's* QS response. P450BM3 is another major class of oxidoreductase produced by *Bacillus megaterium* CYP102A1which can oxidize a broad range of AHLs including 3-oxo-C_6-HSL, C_6-HSL, C_8-HSL, C_4-HSL, C_8-HSL, and C_{10}-HSL (Fetzner, 2015).

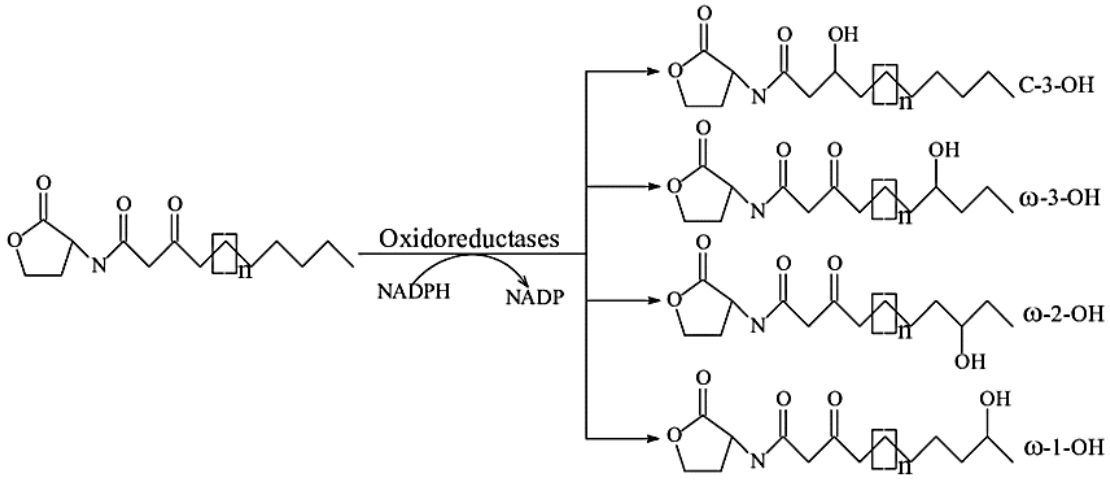

Fig. 1.10 The mechanism of AHL degradation by AHL oxidoreductases (Paul et al., 2009)

1.11 Application of quorum quenching in biofilm control

The application of AHL-acylase and AHL-lactonase, the two major quorum quenching enzymes can be applied for managing biofouling (Shah & Choo, 2020). According to studies, Gram-negative bacteria and their metabolites are primarily responsible for biofilm development. Quorum quenching mediated biofilm reduction is thought to be a feasible strategy because the AHL-driven quorum sensing system is connected to nearly all stages of biofilm formation, including initial surface attachment, bacterial growth, maturation, and removal of aged cells. Many studies have used the enzymatic quorum quenching approach to reduce membrane biofouling in membrane bioreactors (MBRs) for wastewater treatment (Fig. 1.11) (Yeon et al., 2009). For instance, *A. hydrophila* and *P. putida* biofilm formation on three different membrane surfaces were decreased by the pure AHL-degrading enzyme porcine kidney acylase I (Yeon et al., 2009). As a strategy to control biofouling, immobilizing QQ enzymes on magnetic entrapments, alginate capsules, silica beads and membrane surfaces were reported previously (Kaur & Yogalakshmi, 2022). Moreover, it has been proposed to use microorganisms that produce quorum quenching enzymes and aid in the breakdown of pollutants as an alternative to using purified enzymes in wastewater treatment (Maqbool et al., 2015; Oh et al., 2012).

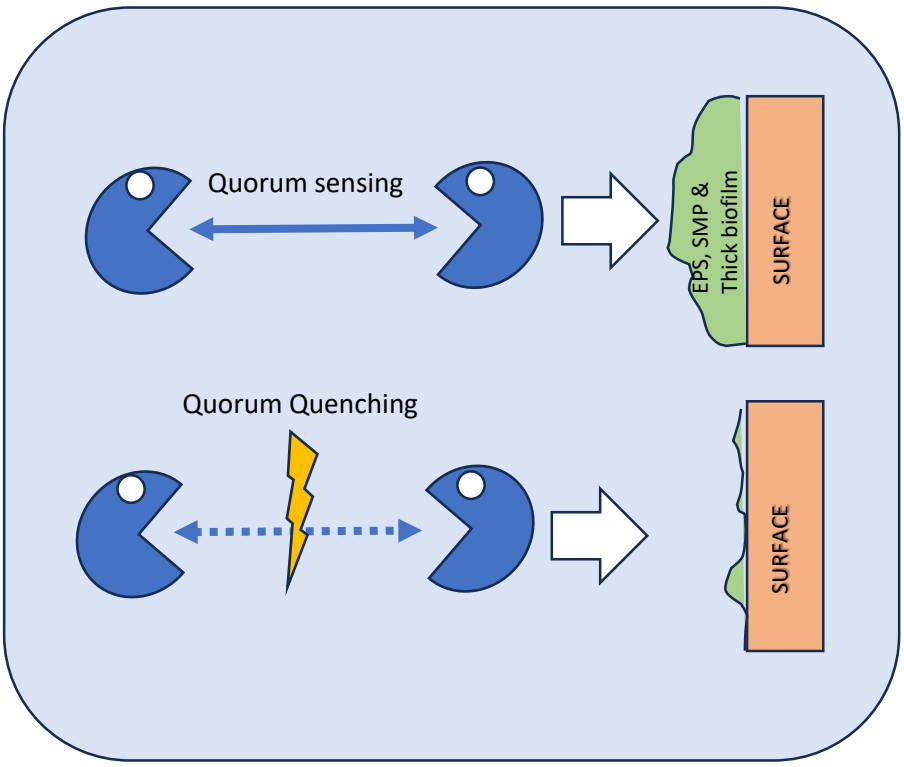

Fig. 1.11 Diagrammatic representation of Quorum Quenching mediated biofilm control

A major milestone in the study of quorum sensing and quorum quenching has reached with the identification of quorum quenching mechanisms in many bacterial species. It has also been claimed that certain bacterial families with a history of producing quorum quenching enzymes could be applied as commercially viable membrane bioreactor anti-biofouling tools. In this view, major quorum quenching bacteria and associated technologies used in biofilm mitigation techniques are enlisted in Table 1.4.

Table 1.4 Application of quorum quenching enzymes in biofilm control on various surfaces

Sl. No.	Application	Organism	QQ enzyme	Reference
1	Membrane Bioreactors	Porcine Kidney	Acylase I	(Yeon et al., 2009)
2.	Membrane filters	*Rhodococcus* sp. BH4	AHL lactonase	(Oh et al., 2012)
		Recombinant *E. coli*		(Maqbool et al., 2015)
2	Non-alumina membrane	*S. solfataricus*	SsoPox (Lactonase)	Ng et al., 2011
3	Functionalized catheter	*Aspergillus melleus*	Acylase	(Ivanova et al., 2015)
4	Topical treatment	*Bacillus sp.*ZA12	Lactonase	(Gupta et al., 2015)
5	Aerosolization	*S. solfataricus*	SsoPox (Lactonase)	(Hraiech et al., 2014)
6	Activated carbon QQ beads	*Rhodococcus* sp. BH4	AHL lactonase	(Yu et al., 2022)
7	Alginate biofouling beads	*Comamonas* sp.	Acylase	(Xu et al., 2020)
8	Alginate biofouling beads	*Pantoaea stewartii*	Lactonase	(S. Lee et al., 2021)
9	Alginate polysulfone activated carbon	Bacterial consortium (*Rhodococcus* sp. BH4	Enzyme cocktail	(Xiao et al., 2018)
		Enterococcus cloaca, Microbacterium sp. QQ1, *Pseudomonas* sp. QQ3, *E. coli* lux1)		
10	Dental chair unit coating	*Bacillus velezensis*	YtnP Lactonase	Liu et al., 2020

1.12 Patents on enzyme mediated biofilm control

Numerous patents cover QQ based approaches for controlling biofilm/biofouling for practical applications. Methods using QQ bacteria as well as enzymes have been disclosed in these patents. The outcomes of a thorough patent search using popular patent databases and search engines such as Google patent, Patent Lens, US PTO, Espacenet, etc. are shown in Table 1.5.

Table 1.5 List of patents disclosed for enzymatic quorum quenching mediated biofilm control

Sl. No.	Technology	Inventors	Assignee	Patent No.	Published year
1	Proteins and methods for disrupting bacterial communication	Bergonzi, *et al*	University of Minnesota	US20220154156A1	2022
2	Multi-target quorum sensing quenching enzyme preparation as well as preparation method and application thereof	Zhao Jing *et al.*	Dalian Minzu University	CN115478064A·	2022
3	MBR membrane pollution control method based on microbial population induction quenching	Junsong *et al.*	Uzhou Shengsiphon Environmental Protection Science & Tech Limited Company;	CN114455697A	2022
4	*Delftia* sp for regulating quorum sensing quenching and separation method and application thereof	Liao *et al.*	Shenzhen University	CN112940971A	2021
5	Enzyme-based membrane with antifouling activity for water treatment and method for manufacturing thereof	Jung-Bae *et al.*	Korea University Industry-University Cooperation Foundation	KR102276596B1	2021
6	Novel quorum sensing quenching bacterium, and culture and screening method and application thereof	Xiaochi *et al.*	Harbin Institute of Technology Shenzhen	CN113215069A	2021
7	Method for controlling membrane pollution by using quorum sensing quenching immobilized strains	Wenzhao *et al*	Peking University Shenzhen Graduate School	CN107158957B	2020

8	The composition and preparation method of quorum quenching media of core-shell structure for anti-biofouling strategy	Kwangho Choo *et al.*	Kyungpook National University Industry-University Cooperation Foundation	KR102093432B1	2020
9	A kind of MBR control membrane fouling method based on micropopulation induction quenching	Huarong Du *et al.*	Guangzhou University	CN110028152A	2019
10	Extraction method of microbial lysate producing enzyme and reduction apparatus of biofouling	Seok-tae *et al.*	Korea Advanced Institute of Science And Technology	KR20190064213A	2019
11	Method for preparing nanometer material/quorum quenching enzyme hydrogel Abstract	Zhenya *et al.*	Xi'an University of Architecture and Technology	CN108949740A	2018
12	HEMM-1 Enterococcus durans HEMM-1 with quorum quenching activity and uses thereof	Hee-Dueng *et al.*	Korea University Research and Business Foundation	KR101845663B1·	2018
13	Quorum quenching enzyme ZD03-aiiA gene, protein encoded thereby and cloning method thereof	Ding Xian *et al.*	South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences	CN109022378A·	2018
14	The biological beads controlled for biofouling in membrane bioreactor	Zhou Yan *et al*	Nanyang Technological University	CN110023251A	2017

15	Preventing and reducing biofilm formation and planktonic proliferation	McHatton *et al.*	Novozymes Biologicals Inc	US9809477B2	2017
16	Quorum sensing quenching bacteria preparation method, MBR membrane pollution prevention and control method, and MBR membrane pollution prevention and control apparatus	Wenzhao *et al.*	Forigin Environmental Protection Technology (Shenzhen) Co., Ltd.	CN106399140A	2017
17	Quorum sensing quenching enzyme and bacteriocin combined anticorrosion and fresh-keeping method	Li Pinglan *et al.*	China Agricultural University	CN107183146A·	2017
18	Quorum quenching *Bacillus amyloliquefaciens* LBA6 and method of using thereof	Hee-deung *et al.*	Korea University Research & Business Foundation	KR20160011058A	2016
19	Immobilization column for biofilm formation-inhibiting microorganisms and membrane water treatment apparatus using the same	Jeong-Hak *et al.*	Seoul National University Industry-University Cooperation Foundation	KR101585169B1	2016
20	RO1S-5 RO1S-5 strain having capability of inhibiting quorum sensing and use thereof	Young-suk	Myongji University Industry-University Cooperation Foundation	KR101598425B1	2016
21	Inhibition of biofilm formation with genetically engineered bacteriophages	Ruoting Pei	University of Texas System	US20150237870A1	2015
22	A method for quorum quenching	Gopalan Suresh *et al*	Council of Scientific and Industrial Research	WO2014174531A1	2014

23	Fluidizable carrier with biofilm formation-inhibiting microorganisms immobilized therein and membrane water treatment apparatus using the same	Chung-Hak *et al.*	SNU R&DB foundation	KR101270906B1	2013
24	Use of bacteria of the genus *Tenacibaculum* for quorum quenching	Ostero Catal *et al.*	University of Santiago Compostela	EP2356991A1	2011
25	Use of a novel alpha-proteobacteria for quorum quenching	Otero Casal Ana Maria	University Of Santiago De Compostela	WO2011154585A1	2011
26	Magnetic carrier comprising enzyme for inhibiting biofilm formation immobilized thereon, and membrane bioreactor system and method for inhibiting biofilm formation using the same	Chung-Hak *et al.*	SNU R & DB foundation	US2009159533A1	2009

1.13 Aim and objectives

AHL-mediated quorum sensing system in Gram-negative bacteria has a critical role in the development of biofilms. The implementation of quorum quenching as an alternate strategy for preventing membrane biofouling has been suggested as a sustainable solution in this scenario. Recently, attempts to minimise biofouling in MBRs and other biofilm forming scenarios using microorganisms with the ability to produce AHL-degrading or modifying enzymes have proven successful. These facts strongly suggest that biofouling could be controlled by quorum quenching bacteria and the underlying mechanism involved. In this scenario, our major objectives of this research work include:

1. Isolation and characterization of quorum quenching bacteria from environmental samples.
2. Establishing the quorum quenching impact of the isolate on *Pseudomonas aeruginosa* quorum sensing network with special reference to the biofilm induced antibiotic resistance.
3. Elucidating the mechanism involved in the quorum quenching by the isolate
4. Investigation on the antibiofilm property of the isolate against gram negative bacteria on various surfaces.

Chapter 2

Isolation and Characterization of Quorum Quenching Bacteria from Environmental Samples

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2.1Introduction

Bacteria develop quorum sensing (QS) disrupting strategies in a polymicrobial environment as one of the competition mechanisms. The disruption of quorum sensing can be caused by signal degradation, AHL reception interference or AHL production inhibition. Among these, the signal degradation strategy, generally considered quorum quenching (QQ), is an effective adaptive method by bacterial cells to survive and maintain a niche. From an evolutionary point of view, cells develop the QQ mechanisms to utilize the autoinducer signals as resources or regulators. Prokaryotes are known to produce compounds or enzymes capable of attaining quorum quenching. Compared to the currently used antimicrobial compounds, quorum quenching agents may impart less selection pressure and lower the risk of developing antibiotic resistance because they do not include bactericidal or bacteriostatic activity. Hence, isolating naturally occurring microorganisms with QQ property is an emerging approach in QS inhibition studies. It has a huge application potential in areas like biofilm mitigation, antiviral agents, biocontrol agents, as well as synergistic interactions with antibiotics and probiotics for better efficacy.

The two major approaches to isolate bacteria exhibiting QQ properties are direct isolation and enrichment culture. Direct isolation from environmental samples and the ensuing confirmatory studies are labour- and resource-intensive. Whereas, in enrichment culture technique, the growth of QQ bacteria is promoted, or they are subjected to targeted evolution. Hence, this chapter describes the isolation and characterization of quorum-quenching bacteria from environmental samples through enrichment culture method. Activated sludge, a biocenosis of abundant microorganisms, was selected as the initial inoculum for the enrichment culture, and the Gram-negative quorum sensing system is mainly targeted here.

2.1 Materials and Methods

2.2.1 Bacterial Strains and culture conditions

The bacterial strains used in this study were as follows: the biomarker strains *Chromobacterium violaceum* CV026 (ATCC 31532) reclassified as *C. substugae* (Fig. 2.1a), the bio-reporter strains *Chromobacterium violaceum*. NIIST (GenBank No. FJ 982784) (Fig. 2.1b), *Serratia marcescens* NIIST (Genbank No. JQ807993) (Fig. 2.1c), and *Staphylococcus aureus* (MTCC 902). Except for *C.violaceum*, all bacterial strains were grown in Luria Bertani (LB) Miller medium (pH-7.0) and kept at a temperature of 37°C. The bacterial cultures were sub-cultured up to an optical density (OD) of 0.5 at 600 nm for all experimental procedures.

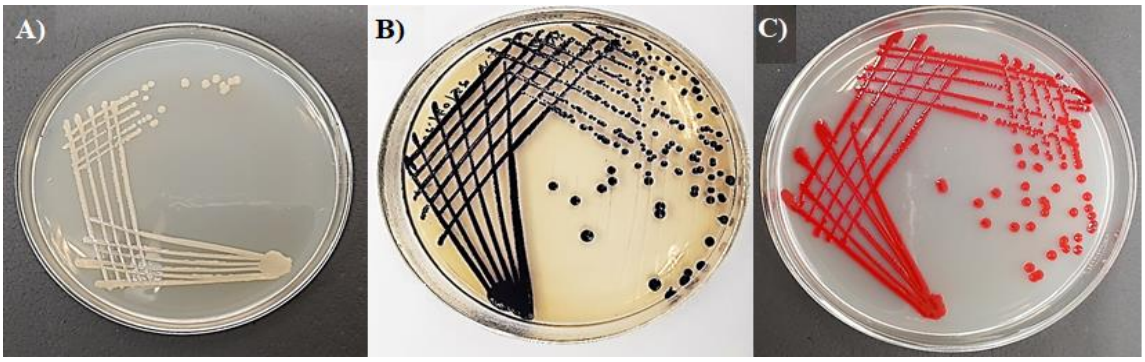


Fig. 2.1 Bio-sensors/reporters used in the study **a)** *Chromobacterium substugae* **b)** *Chromobacterium violaceum* NIIST **c)** *Serratia marcescens* NIIST.

2.2.2 Enrichment culture for isolating Quorum Quenching (QQ) bacteria

The start-up inoculum for the study was activated sludge collected from the Sewage Water Treatment Plant (STP), Muttathara, Thiruvananthapuram, Kerala, India. The sample was prepared as described (Christiaen, 2011) with slight modifications. Briefly, activated sludge was suspended in 0.9% saline and subjected to three alternative cycles of vortexing (30 s) and sonication (30 S, 20 KHz). The suspension was centrifuged at 1000 g for 5 min. at 25 °C. The pellet obtained was resuspended in 5 ml saline and inoculated in M9 minimal medium (Table 2.1) containing 1 mM N-Hexanoyl-L-Homoserine Lactone (C_6-HSL) (Sigma Aldrich, MA, USA) as the sole carbon source. The medium, inoculum and carbon source were provided in a 5:4:1 ratio throughout the enrichment cycle. The cultures were incubated at 30 °C with shaking for five days and re-inoculated to a fresh medium. The process was repeated for up to 7 cycles. From the enrichment culture, samples were spread-plated on M9 agar plates supplemented with

C_6-HSL. Plates were incubated overnight at room temperature. Single and discrete colonies were sub-cultured.

Table 2.1 Composition of M9 minimal media used for enrichment culture

Ingredient	Concentration/L
Macro-nutrients	
Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g
Micronutrients	
$MgSO_4$	1 mM
$CaCl_2$	100 µl
$(NH_4)_6Mo_7O_{24}.4H_2O$	3×10^{-9} M
H_3BO_3	4×10^{-7} M
$CoCl_2.6H_2O$	3×10^{-8} M
$CuSO_4.5H_2O$	1×10^{-8} M
$MnCl_2.4H_2O$	8×10^{-8} M
$ZnSO_4.7H_2O$	1×10^{-8} M
$FeSO_4$	1×10^{-6} M

2.2.3 Preparation of Cell-Free Supernatant (CFS) and Cell Free Lysate (CFL)

The pure bacteria cultures were inoculated into the Nutrient Broth and incubated at 30 °C and 200 rpm for 48 hrs. After incubation, the cultures were centrifuged at 5800 g for 10 minutes, and the supernatant was collected, filtered through a 0.2 µm syringe filter and the resulting CFS was stored at -20 °C. The CFS was freeze-dried using a lyophilizer (VirTis Genesis, USA). Both CFS as liquid and the lyophilized form is used in different experiments.

For CFL preparation, pellets were resuspended in Pottassium phosphate buffer (100 mM, pH-7) and lysed with sonication. The suspension was centrifuged, and the supernatant was collected, filtered, and kept at -20 °C (Rajesh *et al*., 2014).

2.2.4 Primary screening for quorum quenching activity of the isolates (Flask Incubation assay)

The isolates obtained from the enrichment culture were screened for their QQ activity using flask incubation assay(Nithya et al., 2010) with the *C. violaeum* NIIST. This pigment-producing strain was inoculated into Nutrient Broth medium and treated with 10% (v/v) Cell-Free Supernatant (CFS) or Cell Free Lysate (CFL) of the above isolates and incubated at 30 °C for 18 hr. in a rotary shaker with 150 rpm. The medium was buffered with PIPES (1M, pH-6.7) to prevent AHL's alkaline hydrolysis. The cultures were visually examined for colour reduction and isolates producing pigment reduction were selected for further experiments.

2.2.5 Extraction and quantification of violacein and prodigiosin

The selected isolates from the primary screening were subjected to Flask Incubation (FI) assay with *C. subtsugae* and *S. marcescens* as aforementioned, with varying concentrations of CFS (1-10% v/v), and monitored for the violacein and prodigiosin production respectively. The *C. subtsugae* cultures were supplemented with 10 μM C_6-HSL (Sigma Aldrich, USA). One ml of culture was collected and centrifuged at 5800 g (25 °C, 2 min.). The supernatants were discarded and Pellet was added with 1 ml of DMSO and 1ml Methanol for *Chromobacterium* (Choo *et al*., 2006) and *Serratia* (Gulani *et al.*, 2012), respectively. The solution was vortexed vigorously for solubilising the pigments and centrifuged at 8000 g (25°C, 5 min.). The absorbance of the supernatant was taken at 545 nm and 499 nm for violacein and prodigiosin, respectively. The pellet obtained was resuspended in 0.01M PBS and checked for the viable cell count in Plate count agar.

2.2.6 Swarming and swimming motility assay

Semisoft nutrient agar medium of agar concentration 0.3% and 0.8 % (wt/v) were used to determine the effect of CFS on *Serratia*'s swimming and swarming motility, respectively. Samples from the FI assay were stabbed onto the plates for the swimming motility test, and 1 μl of the sample was inoculated onto the surface of the agar plate for the swarming motility test (Morohoshi et al., 2007). The growth pattern of the plates was monitored after 24 hr. incubation at 37 °C.

2.2.7 Salt aggregation assay

The biosurfactant production by *S. marcescens* was analysed using a salt aggregation assay (Coria-Jimenez et al., 1998). The FI assay was briefly carried out with CFS treatment (1-25 % v/v). The culture suspension from each treatment was mixed with varying concentrations of ammonium sulphate precipitating agent, vortexed for 30 s and allowed to settle for 30 min. The minimal salt concentration necessary for cell aggregation was recorded.

2.2.8 Soft Agar Overlay assay

The direct interaction of the selected isolate and the *C. substugae* strain was analysed using the soft agar overlay method (Chu *et al.*, 2011). The isolate was streaked onto a nutrient agar plate in a short stretch of line and incubated overnight at 32 °C. Five ml soft nutrient agar with 0.5% (wt/v) agar was prepared, autoclaved and cooled to 50 °C in a water bath and inoculated with 5µl overnight *C. violaceum* NIIST culture. The preparation was poured over the previously streaked nutrient agar plate. The soft agar was allowed to solidify and incubated overnight at 32 °C and inspected for depigmentation.

2.2.9 Estimation of External AHL degradation by the isolate

The AHL degradation activity of isolate CFS was analysed using *C. substugae.* Briefly, 1 mM C_6-HSL was incubated with CFS for 3 hrs at 30 °C. The medium was buffered with PIPES to prevent alkaline hydrolysis of the AHL. Samples were taken every 30 minutes. The residual AHL from the mixture was extracted with acidified ethyl acetate. 100 µl of an overnight culture of *C. violaceum* CV026 (OD_{600}-0.1) was spread plated on nutrient agar plates (2% agar). Wells of 2 mm diameter was made on the agar surface, and 50 µl of ethyl acetate extract was added to the well. C_6-HSL incubated in PIPES buffer alone was used as the positive control. The plates **were** incubated at 30 °C for 24 hrs and were checked for a purple-halo around the wells, and the diameter was noted.

2.2.10 Lipase assay

The anti-QS effect of isolating CFS on the Gram-positive bacteria was tested using lipase assay in S. *aureus* (Vijayakumar et al., 2020). The bacterial culture was grown in BHI broth in the presence (5 % and 10 % v/v) and without CFS for 24 hr. at 37 °C. The culture was centrifuged at 3000g for 10 min, and the supernatant was collected. Lipase substrate buffer (0.3

% p-nitrophenyl palmitate in isopropanol and 50 mM Na_2PO_4 buffer with 0.2 % sodium deoxycholate in the ratio 1:9) was incubated in the dark with the *S. aureus* culture supernatants at room temperature for 1 hr. the activity was terminated after that with 1M Na_2CO_3. The suspension was centrifuged at 10,000 g for 10 min. The absorbance of the supernatant was measured at 410 nm.

2.2.11 Antibacterial activity test

The bacteriocidal activity of CFS on different bacteria was assessed using the Kirby-Bauer disc diffusion method recommended by CLSI (AWS Bauer *et al.,* 1966). The bacteria used in the study are listed in Table 2.2.

Table 2.2 Bacteria used in the Kirby-Bauer disc diffusion assay

Bacteria used	MTCC number
Bacillus cereus	MTCC 1305
Mycobacterium smegmatis	MTCC 993
Staphylococcus aureus	MTCC 902
Escherichia coli	MTCC 2622
Klebsiella pneumoniae	MTCC 109
Proteus mirabilis	MTCC 425
Pseudomonas aeruginosa	MTCC 2642
Salmonella typhi	MTCC 3216

2.2.12 Genetic Identification of the QQ isolate

The whole genomic DNA of the isolate was purified using Nucleospin DNA isolation kit (Machery-nagel, Germany) and the 16S rRNA gene was amplified with the universal primers 27F (5′GAGTTTGATCATGGCTCAG3′) and 1492R (5′ GGTTACCTTGTTACGA CTT3′) in MyCycler Thermal cycler system (BIORAD, USA). The purified PCR product was sequenced using Sanger's method. The isolate was identified by homology analysis of the 16SrRNA gene sequence by the NCBI BLAST tool, and the sequence was deposited in NCBI GenBank. The phylogenetic analysis of the isolate was conducted in MEGA X.

2.3 Results and Discussion

2.3.1 Isolation of quorum quenching bacteria by enrichment culture.

The proliferation of naturally occurring quorum quenching bacteria in activated sludge was promoted through an enrichment culture approach, providing C_6-HSL as the sole carbon and energy source. After seven cycles of enrichment, where each cycle consisted of five day-long incubation, the suspension was spread on M9-C_6-HSL (100 µM) agar plates (Fig. 2.2). Based on the colony morphology, nine discrete bacterial colonies formed were isolated from the agar plates. All the isolates were viable on Nutrient Agar (NA) plates. Stock cultures were maintained in minimal media containing C_6-HSL.

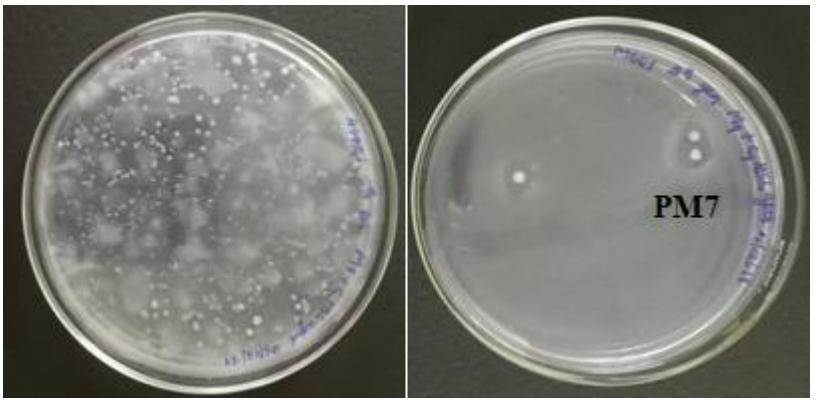


Fig. 2.2 M9-C_6-HSL agar plates showing mixed bacterial cultures from the final enrichment cycle

The Cell-Free Supernatants (CFS) and Cell-Free Lysate (CFL) from all the isolates were screened for quorum quenching activity using C_6-HSL as a substrate for *C. violaceum* CV026. It is a mini Tn5 mutant that lacks CVil-encoded AHL synthase and thus can only produce violacein in response to externally supplied cognate short-chain AHL signal molecules (Harrison *et al.*, 2020). In the case of CFS treatment, only one among the nine isolates, namely PM7, showed significant ($p<0.05$, One way ANOVA) violacein reduction (67.4%) compared to the untreated control. To confirm the QQ activity, 100 µL from each flask was spread-plated on Nutrient Agar plates and incubated at 30 °C for 24 hr. The bacterial count showed no significant difference ($P<0.05$) in the number (as colony-forming units) compared with the control untreated sample. These results substantiate the anti-quorum sensing activity of the CFS of the PM7 isolate as opposed to its bacterial growth inhibition. In contrast, there was no discernible quorum quenching activity in any of the isolates with the CFL treatment, which is indicated by the absence of pigment reduction. (Fig. 2.3). Hence, PM7, which exhibits

extracellular anti-QS activity, was selected for subsequent investigations. The isolate produces white, opaque, medium-sized colonies with a rough, matted surface and irregular edge on Nutrient Agar in 24hr (Fig. 2.4).

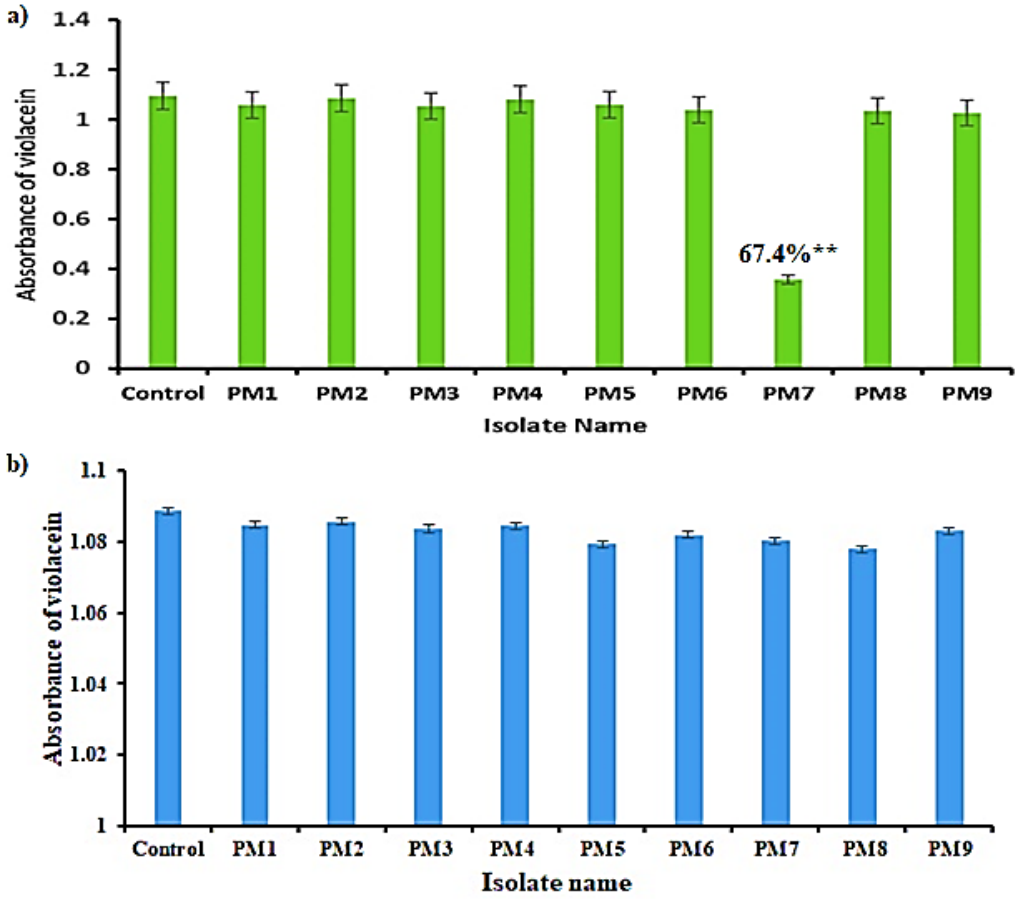


Fig. 2.3 Extracellular and intracellular QQ activity of the isolates obtained from enrichment culture regarding the absorbance of violacein. The data represents QQ activity of a) Cell Free Supernatants b) Cell Free Lysates. (**p<0.05, One-way ANOVA)

Fig. 2.4 Typical colonies of the Isolate PM7 on Nutrient Agar

2.3.2 Quorum quenching effect of PM7 CFS on Gram-negative bioreporter strains

The quorum sensing inhibitory effect of PM7 cell-free supernatant was tested on Gram-negative bioreporter strains such as *C. violaceum* and *S. marcescens*. As observed in the flask incubation assay, a significant ($p<0.0001$, linear regression analysis) dose-dependent decrease in pigment (violacein) production was observed in *C. violaceum* (Fig. 2.5a) and *S. marcescens* (prodigiosin) (Fig. 2.5b) with an increase in CFS concentration (1-10% v/v). At 10% (v/v) CFS treatment, violacein and prodigiosin production decreased to 73% and 67.2 %, respectively. However, the viable cell count of treated and untreated samples showed no significant difference. *C. violaceum* and *S. marcescens* overexpress the pigments violacein and prodigiosin in response to cognate short-chain AHLs like C_4-HSl and C_6-HSL. Violacein production in *C. violaceum* is regulated by the LuxI/LuxR homologues Cvil I/Cvil R (Mclean *et al*., 1997). Whereas, SmaI/SmaR QS system regulates the PIG gene cluster comprising of 15 genes responsible for the prodigiosin production in *S. marcescens* (Coulthurst et al., 2006). Treatment with CFS significantly decreased pigment production in these bioreporter strains without causing apparent cell death, indicating that the PM7 has extracellular QQ activity.

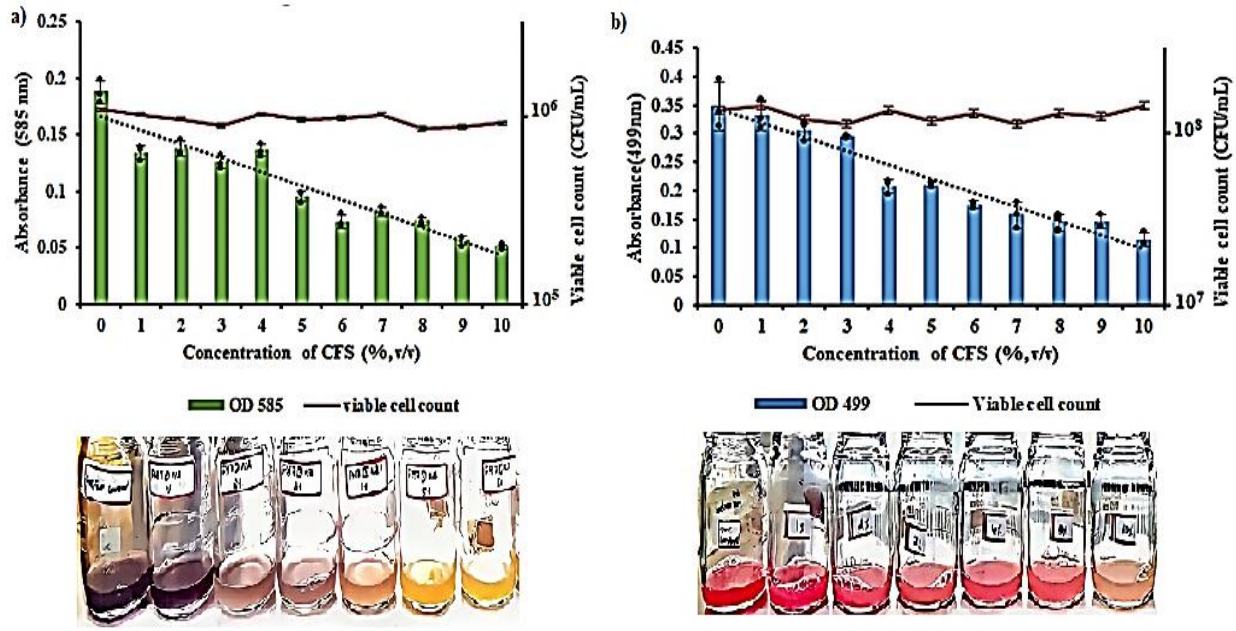


Fig. 2.5 Effect of PM7 CFS on the pigment production and cell viability Data represents a) Absorbance of violacein (OD 585) and viable cell count (CFU/mL) in *C. violaceum*. (R^2-0.9, $p<0.01$ linear regression analysis) b) Absorbance of prodigiosin (OD 499) and viable cell count of *S. marcescens* (R^2-0.94, $p<0.01$ linear regression analysis). Experiments are conducted in triplicates, and values are expressed as mean ± *standard error.*

Besides the prodigiosin production, their QS network also regulates *Serratia*'s swarming and swimming motility. Serrawetin W2, the biosurfactant produced by *Serratia* in response to the short-chain AHL system, is responsible for lowering the surface tension of the substratum and helping in their motility (Morohoshi *et al*., 2007). The treatment with CFS visibly reduced the swarming and swimming motilities of *S. maracescens* NIIST (Fig. 2.6). Even though the diameter of motilities was decreased, the prodigiosin production on agar plates showed no significant reduction. However, Morohoshi *et al.* (2007) have already reported a disparity in the concentration of AHLs required for prodigiosin production and the swarming motility of *Serratia* sp., which accounts for this observation.

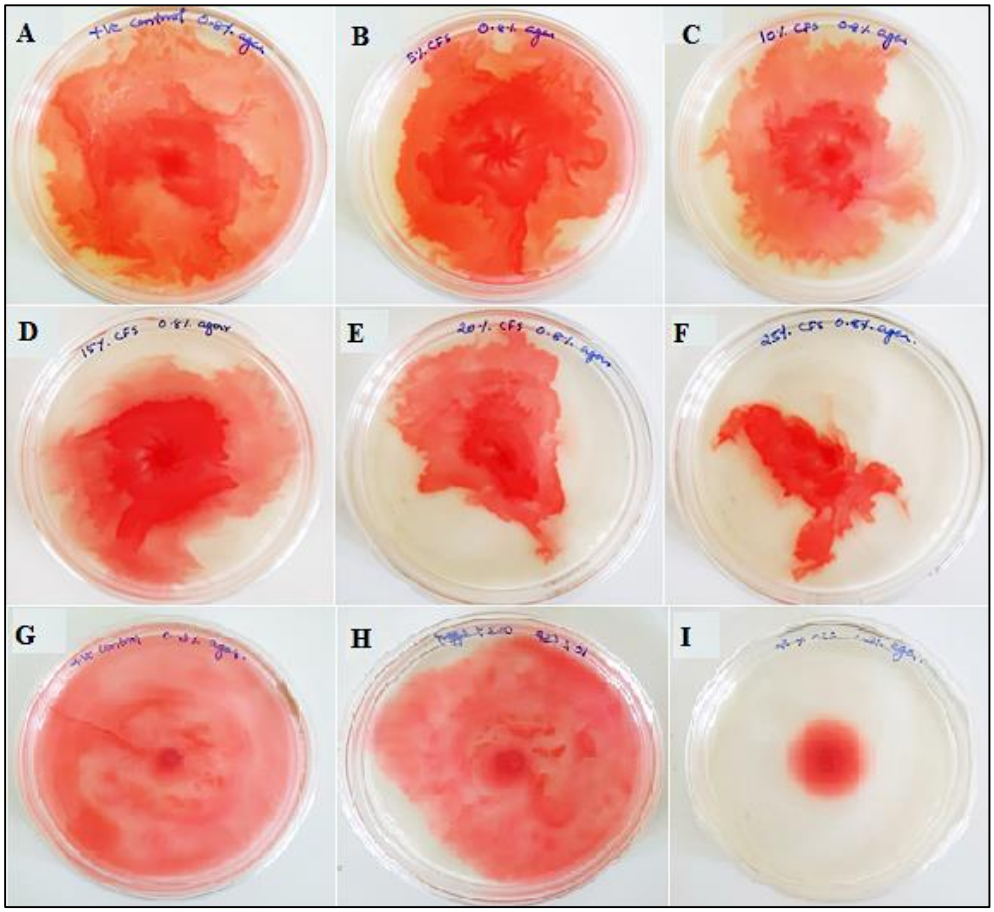


Fig. 2.6 Effect of CFS on the motility of *S. marcescens*. Variations in swarming motility is shown in A-F. CFS addition A) 0%, B) 5%, C) 10%, D) 15% E) 20% F) 25%. Variations in swimming motility is shown in G-I. CFS addition G) 0%, H) 10% I) 25%

A salt aggregation assay was conducted to substantiate the variation in the motility of *S. marcescens* with the CFS addition. Ammonium sulphate addition promotes cell precipitation, a sign of the hydrophobicity imparted on cells by the biosurfactants. The lower the salt concentration necessary to combine the cells, the more hydrophobic the cell surface.

As evident from Fig. 2.7, the ammonium sulphate concentration required to precipitate *S. marcescens* cells increases with the CFS concentration. The minimum concentration of $(NH_4)_2SO_4$ required to aggregate 25% (v/v) CFS treated cultures increases to 2M while the control sample precipitates at 0.625M.

S / C	10M (+VE)	4M	3M	2M	1M	0.5M	0.25 M	0.125 M	0.625 M	0.031 M	0.015 M	0M (-VE)
0%	■	■	■	■	■	■	■	■	■			
1%	■	■	■	■	■	■	■	■	■			
5%	■	■	■	■	■	■	■	■				
10%	■	■	■	■	■	■	■					
15%	■	■	■	■	■							
20%	■	■	■	■								
25%	■	■	■	■								

■ - Precipitation C- Concentration of ammonium sulfate S-CFS concentration

Fig. 2.7 Effect of PM7 CFS on the hydrophobicity of *S. marcescens*. a) Chart showing the trend in ammonium sulphate precipitation at different CFS concentration b) *S. marcescens* culture suspensions treated with different concentration of CFS and 0.5M $(NH_4)_2SO_4$. Cell aggregation decreases with the increase in CFs concentration.

The soft-agar overlay assay with *C. violaceum* also yielded concordant results. This sensitive plate-based assay showed a colourless zone in *C. violaceum* around PM7 growth (Fig. 2.8). The AHL degradation property of PM7 CFS was analysed using the biosensor strain *C. substugae*. Following incubation with 10% (v/v) CFS, the decreasing diameter of violacein zones generated in *C. substugae* culture plates demonstrated a time-dependent decline in the C_6-HSL concentration. Three hours of incubation resulted in the complete deactivation of 1 mM C6-HSL (Fig. 2.9).

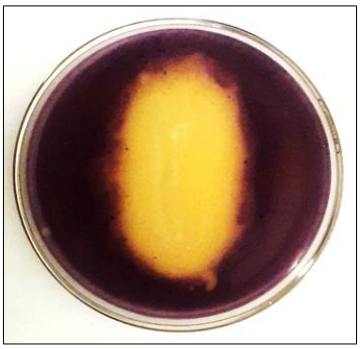


Fig. 2.8 Agar over lay assay showing zone of depigmentation around the growth of isolate PM7

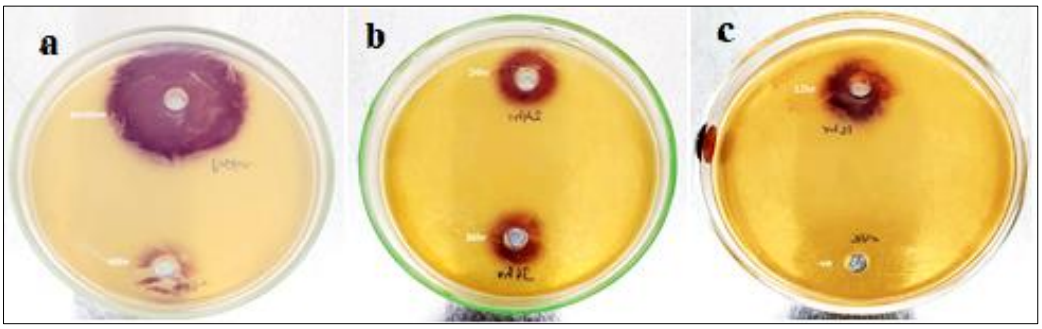


Fig. 2.9 Time dependent AHL degradation by CFS. A) top- positive control, bottom- 2hr. B) top- 30 min. bottom-1.30 hr. c) top-1 hr. bottom-3 hr

2.3.3 Quorum quenching effect of PM7 CFS on Gram-positive bacteria

The effect of PM7 cell free supernatant on the QS-controlled phenotypes of Gram-positive bacteria was also tested. The study was conducted with *S. aureus*, which is a frequent human commensal and opportunistic pathogen which co-ordinate several properties such as biofilm formation, exo-enzyme production, and toxin expression with the help of an Accessory gene regulator (Agr)-QS system. A post-translationally modified thiolactone-containing peptide molecule act as their autoinducer signal (Vijayakumar *et al.* 2020). The extracellular lipase production, which is a QS-controlled phenotype in *S. aureus*, was found to be unaffected by the PM7 CFS treatment (Fig. 2.10). There was no significant difference ($p<0.05$, unpaired t-test) in the production of lipase even at a 10% (v/v) CFS concentration. The inference that the isolate PM7's anti-QS activity is limited to Gram-negative bacteria alone can be drawn from this observation.

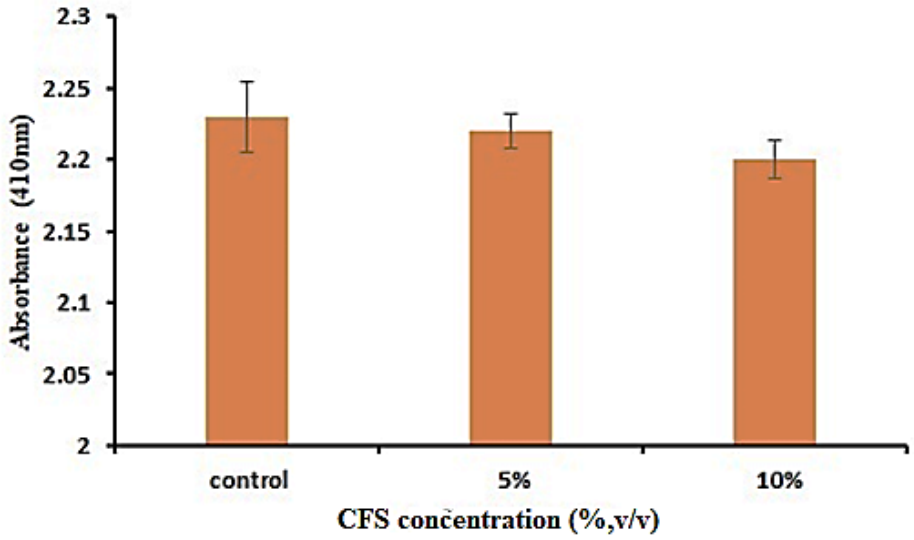


Fig. 2.10 Effect of PM7 CFS on the extracellular lipase production by *S. aureus*. Experiments were conducted in triplicates and values are expressed as mean ± *standard error.*

2.3.4 Antibacterial potential of PM7 cell free supernatant

The antibacterial potential of the PM7 CFS was evaluated against seven bacterial strains using the classic Kirby-Bauer disc diffusion assay. Among the test organisms, 3 were Gram-positive, and four were Gram-negative. As apparent from Fig. 2.11, none of the test organisms exhibits any inhibition zones due to the CFS treatment.

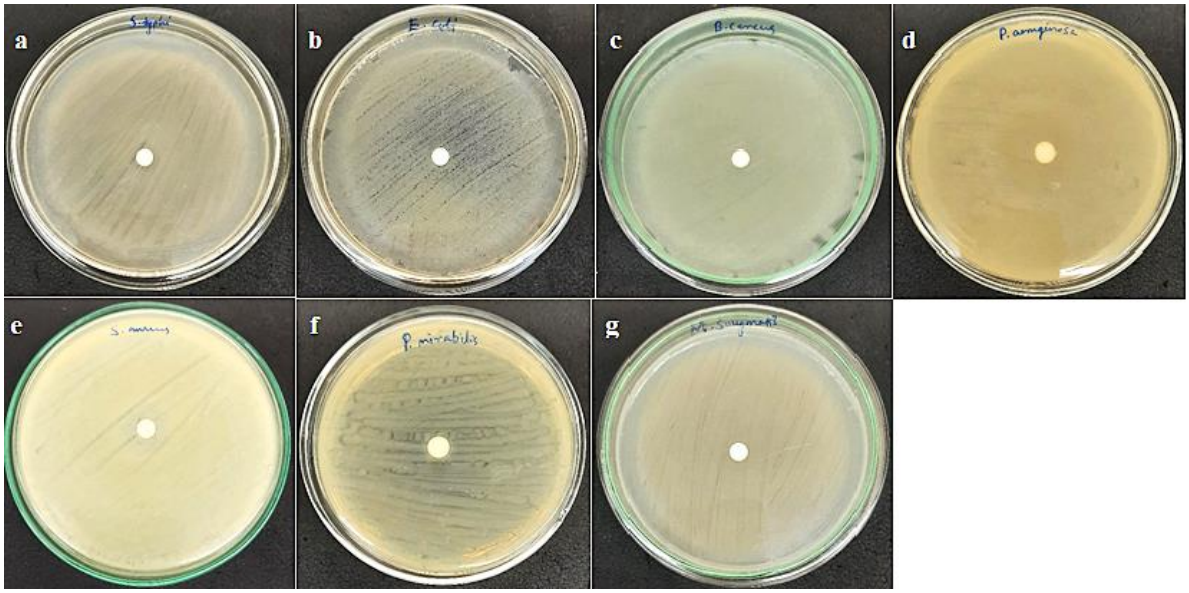


Fig. 2.11 Assessment antibacterial potential of PM7 CFS. Muller Hinton agar plates showing disc diffusion assay with CFS against a) *S. typhi*, b) *E. coli*, c) *B. cereus*, d) *P. aeruginosa*, e) *S. aureus*, f) *P. mirabilis* and g) *M. smegmatis.*

Several previous reports have demonstrated that QS does not influence bacterial growth and fitness *in vivo* and *in vitro* and there is a low risk of resistance development (Anandan & Vittal, 2019; Packiavathy et al., 2021; Utari et al., 2018). However, Defoirdt et al., (2010) put forwards the importance of deriving conclusions about selective pressure posed by QS inhibition using competition experiments in *in vivo* models between QS mutants and wild types.

2.3.5 Identification of the QQ isolate

The isolate PM7 was identified as *Bacillus velezensis* PM7 by 16S rRNA sequencing and subsequent BLAST similarity searches, with 96.17% similarity to previously reported bacteria of the same species. The strain's genetic relatedness to the *Bacillus subtilis* group, including *Bacillus atropheus* and *Bacillus amyloliquifaciens,* was revealed by the phylogenetic tree constructed by using the closest BLAST hits to assess the clustering of the isolate with other species of the *Bacillus* genus (Fig. 2.12). The isolate was identified as a new strain, and the 16S rRNA sequence was deposited in GenBank under the accession number MZ234152.

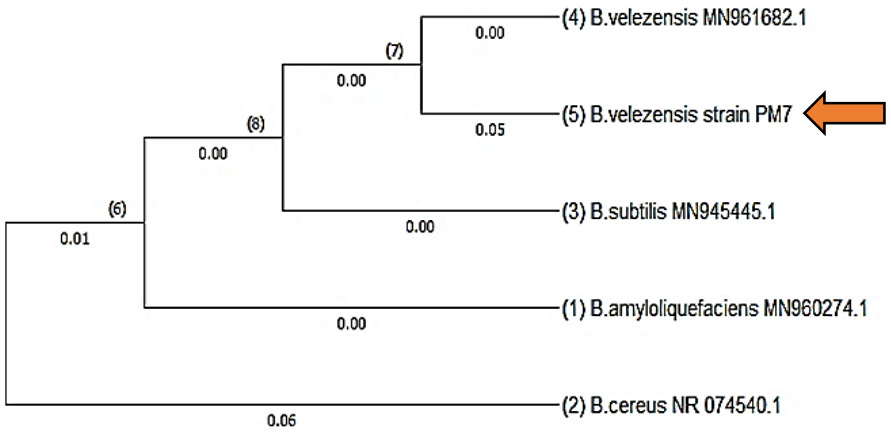


Fig. 2.12 Phylogram (Neighbour Joining Tree) shows the position of PM7 among with closely related strains. The phylogenetic tree was constructed in MEGA X V. 10.2.6.

In previous studies, soil samples (Christiaen et al., 2011) and activated sludge (Kim et al., 2014; Gu et al., 2018) were used as initial inoculums, and AHLs were provided as the only carbon and nitrogen sources in the enrichment cultures. In these studies, *Bacillus, Pseudomonas, Arthrobacter, Delftia, Aeromonas, Rhodococcus*, and *Micrococcus* genera were most commonly observed QQ isolates. However, only *Staphylococcus*, *Micrococcus*, and *Pseudomonas* were shown to have extracellular QQ activity (Kim et al., 2014). Hence, the present study reports for the first time an extracellular quorum quenching strain in *Bacillus* genus.

2.4 Chapter Summary

The first part of this research study was to isolate bacteria exhibiting significant quorum quenching property. Enrichment culture method using C_6-HSL as the sole carbon source in a nutrient/mineral salt medium, followed by screening methods was adopted for the isolation of potential strains. A novel strain of *Bacillus velezensis* (PM7) isolated from the enrichment culture exhibited significant extracellular quorum quenching activity against Gram-negative bacteria. It is the first report of extracellular QQ activity in *Bacillus* genus. On treatment with the cell-free supernatant (CFS) of PM7, a dose-dependent decrease in pigment (Violacein and Prodigiosin) production due to QQ was observed in two bio-reporter strains such as *C. violaceum* and *S. marcescens*. Moreover, a significant reduction in QS-controlled phenotypes such as motility and cell surface hydrophobicity of *S. marcescens* were also observed, reconfirming the extracellular QQ activity of PM7. The isolate did not have any QQ activities against Gram-positive bacteria. Moreover, no significant bacteriocidal activity was observed against a spectrum of bacteria (both Gram negative and positive) with the isolate's CFS,that strongly supports its use to control QS associated properties without exerting any selection pressure.

Chapter 3

Quorum Quenching effect of *B. velezensis PM7* on the Quorum Sensing Network and Antibiotic Susceptibility of *P. aeruginosa*

3.1 Introduction

Pseudomonas aeruginosa, is a ubiquitous and highly adaptive Gram-negative bacteria, which is considered as a model organism for QS-related studies. The QS network of this nosocomial opportunistic pathogen is thoroughly studied and well-established. It consisting of two systems that are controlled by N-Acyl Homoserine Lactone (AHL) and Alkyl quinolones (Schaber et al., 2004). The AHL-controlled network is the major contributor to the signaling cascades, which is divided into two components *viz.,* Las and Rhl system controlled by the N-3-oxododecanoyl homoserine lactone (3-O-C_{12}-HSL) and N-Butyryl homoserine lactone (C_4-HSL) respectively. Together these systems control a plethora of virulence factors in *P. aeruginosa* including motility, pyocyanin production, resistance against oxidative stress, production of siderophores/ exoenzyme and biofilm formation (Dekimpe & Deziel, 2009). *P. aeruginosa* can form biofilms on contact lenses, artificial valves, and other prosthetics in addition to being linked to conditions such as cystic fibrosis, bacteremia, and endocarditis (Di Onofrio et al., 2019; Gürtler et al., 2019; Strateva & Mitov, 2011).

Considering this organism's high degree of adaptability to the environment, intricate genetic regulatory networks, and ability to form biofilms, it develops resistance to a wide range of antibiotics (Botelho et al., 2019). Therefore, interfering the QS system of *P. aeruginosa* is recognized as an effective anti-virulence treatment method since it applies less selection pressure on the organism. Additionally, it reduces biofilms' growth, which increases bacteria's susceptibility to diffusion-controlled antibiotics. Hence, this chapter evaluates the impact of *B. velezensis* PM7 CFS the in vitro virulence factor production and antibiotic sensitivity of *P. aeruginosa*.

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

The impact of *B. velezensis* PM7 on the QS network was tested using the standard strains *P. aeruginosa* ATCC 27853 and MTCC 2642 (Kindly provided from the Department of Biotechnology, University of Kerala, Thiruvananthapuram, Kerala, India and Agro Processing and Technology Division, CSIR-NIIST, Thiruvananthapuram, Kerala, India respectively). Ten *P. aeruginosa* strains were isolated from the activated sludge collected from the sewage treatment plant (STP) in Thiruvananthapuram, Kerala, India, and these isolates were used for the antibiotic sensitivity assays. Cetrimide agar (Himedia) was used to identify the isolates as *Pseudomonas*, and Kovac's oxidase test and Kligler iron agar were used for the confirmation. (Kiran et al., 2011). All the isolated *P. aeruginosa* strains were grown in Luria Bertani (LB) - Miller medium at 37 °C with shaking (200 rpm). The conventional Kirby-Bauer disc diffusion assay was used to screen the multi-drug resistant properties of these strains. (Hudzicki, 2012). *Staphylococcus aureus* MTCC 902 was used to assess the staphylolytic activity.

3.2.2 Effect of PM7 on the Growth of *P. aeruginosa*

Overnight cultures of *P. aeruginosa* (OD_{600}-0.4) were inoculated in 50 mL of LB broth containing 10 mg/mL of lyophilized CFS (as described in Section 2.2.3) to study the impact of CFS on bacterial growth. The flasks were incubated at 30 °C under 180 rpm. The growth curves were constructed by acquiring a viable cell count on plate count agar at 1-hr intervals up to 21 hrs. Untreated *P. aeruginosa* cultures were taken as controls.

3.2.3 Effect of PM7 CFS on the virulence factors of *P. aeruginosa*

To evaluate the effects of *B. velezensis* PM7 on its virulence factors, *P. aeruginosa* ATCC 27853 was incubated overnight (15 hrs.) in nutrient broth medium and treated with different concentrations (1–10%, v/v) of CFS. Subsequently, the culture suspension was centrifuged at 11000 g for 10 minutes at 4 °C, and the supernatant was filtered through 0.2 µm cellulose acetate filter paper. This filtrate and the culture suspension were employed in the subsequent assays, along with the untreated *P. aeruginosa* cultures as the positive control. All the experiments were carried out in triplicates. The effect of *B. velezensis* PM7 was tested on the following virulence factors:

a) Staphylolytic activity

The Las A staphylolytic activity of *P. aeruginosa* culture supernatants was assessed according to Kessler et al., (1997) with slight modifications. *S. aureus* MTCC 901 overnight culture suspension in LB medium was boiled for 10 minutes and then centrifuged at 11000 g (25 °C) for 10 minutes. The cell pellet was resuspended in 10 mM Na_2PO_4 (pH – 4.5) and adjusted to an OD_{600} of 0.5. The 900 µL suspension of *S. aureus* was mixed with a 100 µL aliquot of *P. aeruginosa* supernatant, and the mixture was then incubated at 25 °C for 30 minutes under static conditions. The OD_{600} of the reaction mixture was determined, and percentage inhibition was calculated.

b) Motility tests

The swimming and swarming motility of *P. aeruginosa* was assessed according to (Blus-Kadosh et al., 2013) with slight modifications. Briefly, semisoft M9 agar medium supplemented with 0.5% casamino acid and 50µM $FeCl_3$ was prepared. The agar concentrations were selected as 0.3% and 0.5% for assessing swimming and swarming motility, respectively. CFS was added to the medium at three concentrations (1, 5 and 10 mg/mL). The overnight *P. aeruginosa* culture was spot-inoculated (2.5 µL) in the centre of each plate and incubated for 24 hours at 37 °C, and the bacterial migration was monitored. Positive controls comprised plates without CFS additives.

c) Cell surface hydrophobicity

The effect of CFS on the cell surface hydrophobicity of *P. aeruginosa* was estimated using MATH (Microbial Adhesion To Hydrocarbons) assay (Viswapriya *et al*., 2016) and MATS (Microbial Adhesion To Silica) assay (Déziel et al., 2001). The overnight grown CFS treated and untreated *P. aeruginosa* culture suspensions were adjusted to an OD_{600} of 0.5 and mixed with equal volumes of hydrophobic substrates (Toluene, Xylene and Decane) for MATH assay and with 900 mg fine granular silica for MATS, assay. The mixtures were vortexed for 2 minutes and kept undisturbed at room temperature to allow the phase separation. The aqueous layer was collected to examine the cell density at 600 nm. The adherence is calculated with the formula cell surface hydrophobicity (%) = [1-(OD_{600} after vortexing/OD_{600} before vortexing)] x100.

d) Pyocyanin production

The extraction and quantification of pyocyanin were carried out as previously described (El-Mowafy et al., 2014). Briefly, *P. aeruginosa* cells were inoculated in Kings A media (peptone 20 g/L, K_2SO_4 -10g/L & $MgCl_2$ - 1.4 g/L) with and without CFS treatment and incubated for 24 hr. at 37 °C and 200 rpm shaking. The *P. aeruginosa* culture suspensions were centrifuged at 11000g for 10 min. at 4 °C. Five mL aliquot of the supernatant was extracted with 3 mL of chloroform and left to separate. The organic layer was then re-extracted with 0.2 N HCl. The absorbance of the resulting pink solution was measured at 520 nm, and the pyocyanin concentration was calculated using the formula: pyocyanin (µg/mL) = (OD_{520} x 17.072).

e) Pyochelin production

The standard strain of *P. aeruginosa* (MTCC 2642) was employed to determine the effect of CFS on the synthesis of pyochelin. The 0.5 N HCl, nitrite molybdate reagent, and 1 N NaOH were mixed with the treated and untreated bacterial culture supernatants. The final volume of the reaction mixture was made up to 5 mL with distilled water, and absorbance was taken at 510 nm (Harjai et al., 2014).

f) Exopolysaccharide production

P. aeruginosa culture treated with lyophilized CFS at a varying concentration (1–10 mg mL^{-1}) were prepared. As previously explained by Packiavathy et al., (2014), the EPS generated in the samples was precipitated using the ethanol precipitation method and estimated employing the phenol-sulfuric acid method. As previously detailed by Khalil et al., (2019), the visualisation of the EPS reduction was carried out in a separate experiment using slime staining with safranin. Concentrations of CFS were 0.25, 0.5, and 1 mg mL^{-1}.

The effect of *B. velezensis* PM7 on the exopolysaccharide production is visualized using Congo Red Agar (CRA) plate assay. The CRA plates (Brain Heart Infusion (BHI) agar 37 g/L, sucrose- 5% wt. /V, Aqueous Congo red dye 0.8 g/L) were prepared according to (Rajkumari et al., 2017). The test plates were amended with varying concentrations (1, 5, 7 and 10 mg/mL) of CFS and overnight grown *P. aeruginosa* culture (adjusted to OD_{600} – 0.5) was swabbed on CRA plates. The plates were incubated at 37 °C for 48-72 hr. and observed for the development of black colonies. Untreated plates were used as the positive control.

g) Rhamnolipid production

The rhamnolipid quantification was carried out as previously reported (Pearson *et al*., 1997) with slight modification. Briefly, *P. aeruginosa* cultures, both untreated and treated, were cultured for up to 96 hr. and centrifuged at 11000 g for 10 minutes at 4 °C. The supernatants were extracted with diethyl ether and filtered through 0.2 µM filters. The pooled extracts were re-extracted with 20 mM HCl and the organic phase was dried off. The remaining mass was dissolved in water estimated with the orcinol method with rhamnose as the standard molecule. One mg of rhamnose corresponds to 2.5 mg of rhamnolipid.

h) Alginate production

The extraction and quantification of alginate from *P. aeruginosa* cultures were carried out as previously described with slight modifications (Franklin & Ohman, 1996). Briefly, 48 hr. old *P. aeruginosa* cultures with and without CFS treatment were mixed with 0.9% saline to reduce viscosity and centrifuged at 12000g for 30 minutes. Alginate was precipitated from the supernatant using 2% cetyl pyridinium chloride and dissolved in 1M NaCl. One cycle of re-precipitation of the alginate pellet with cold isopropanol and dissolution in 0.9% NaCl was also carried out. The alginate mixture was quantified by carbazole-borate reagent, and absorbance was measured at 530 nm. D- mannuronic acid was used as the standard molecule for the assay (Heidari et al., 2017).

3.2.4 Effect of *B. velezensis* PM7 CFS on the sensitivity of *P. aeruginosa* to oxidative stress

To examine the sensitivity to oxidative stress, the *P. aeruginosa* (ATCC 27853) culture suspensions were diluted to an OD_{600} of 0.3 and swabbed on LB agar plates (1.5% wt/v). Sterile paper discs (Himedia, India) saturated with 30% H_2O_2 were aseptically placed on the agar surface, and the plates were incubated at 37 °C for 24 hr. To determine the resistance to H_2O_2 thereafter, the zone of inhibition around the paper discs was measured (Sethupathy et al., 2016).

3.2.5 Effect of *B. velezensis* PM7 CFS on the Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Inhibitory Concentration (MBIC) values of *P. aeruginosa*

Gentamycin and Chloramphenicol (Himedia, India) were used to study CFS's effect on the antibiotic sensitivity of *P. aeruginosa.* Broth-microdilution method recommended by Clinical and Laboratory Standard Institute (CLSI) was used for the detection of inhibitory concentration breakpoint values (Clinical and Laboratory Standards Institute, 2012). For the

MIC detection, wells were observed for turbidity and viability was determined using resazurin (0.4 mg/mL) staining after 12 hr. The minimum biofilm inhibitory concentrations (MBIC) were identified according to (Kordbacheh et al., 2017). The biofilm formation and viability were observed using crystal violet and resazurin staining respectively. Test wells were treated with 10% (v/v) CFS. MBIC values were defined as the minimum concentration of antibiotics required to inhibit biofilm formation completely.

3.2.6 Assessing the efflux pump assay for MexAB-OprM activity of *P. aeruginosa.*

Overnight cultures of *P. aeruginosa* ATCC 27853 in LB broth were adjusted to an OD_{600} of 0.4 and centrifuged at 1700 g for 5 minutes at 25 °C. The cell pellets were collected and re-suspended in PBS. The suspension was provided with 500 µg/mL Ethidium Bromide (EtBr) as the efflux pump substrate and test samples were supplemented with 10% (v/v) CFS. The control and test samples were incubated at 37 °C for 1hr. under static conditions and centrifuged at 3000 rpm for 5 min. at 4°C. The supernatant was discarded, and the pellet was re-suspended in ice-cold PBS. The cells were immediately transferred to 96 well plates containing 0.4% (w/v) glucose as the energy source and 10% (v/v) CFS. The efflux pump activity was measured in terms of EtBr fluorescence (λ_{ex} – 540nm, λ_{em} – 590 nm) taken at an interval of 1 min. for 60 min. using Biotek Synergy 4 hybrid multi-mode microplate reader at 37°C. Phenyalanine-arginine β-naphthylamide (Paβn) – a known efflux pump inhibitor- is used as the positive control (Kalgudi, 2018)

3.2.7 Effect of PM7 CFS on persister cell formation in *P. aeruginosa*

The fate of high dose antibiotic-induced persister cell formation in *P. aeruginosa* isolates/strains upon CFS treatment was tested using 10 isolates/strains upon Gentamycin exposure. Overnight culture of each isolate/strain is adjusted to an OD_{600} of 0.2 and inoculated to fresh LB broth in 96 well plates. The test samples are treated with 10% (v/v) CFS. The cultures are incubated statically at 37 °C for 24 hr. The planktonic cells are gently aspirated, and unbound cells are washed off with PBS. The biofilm-embedded cells are incubated with gentamycin at a concentration of 5X MIC of respective isolate/strain for 6 hr. Thereafter, the biofilm is rinsed and re-suspended in 0.9% saline solution. The suspension is serially diluted, and viable cell count was determined by plating on an LB agar (Patel et al., 2022)

3.3 Results and Discussion

3.3.1 Effect of *B. velezensis* PM7 on the QS dependent virulence factors in *P.aeruginosa*

The absence of a bacteriocidal or bacteriostatic action is the primary advantage of quorum quenching system. The growth of *P. aeruginosa* in the presence and absence of PM7 CFS was closely monitored, and the results are presented in Figure 3.1. As it is evident from the results, the CFS treated *P. aeruginosa* showed a characteristic sigmoid growth curve similar to untreated cells, with no significant difference in the specific growth rates (Table 3.1).

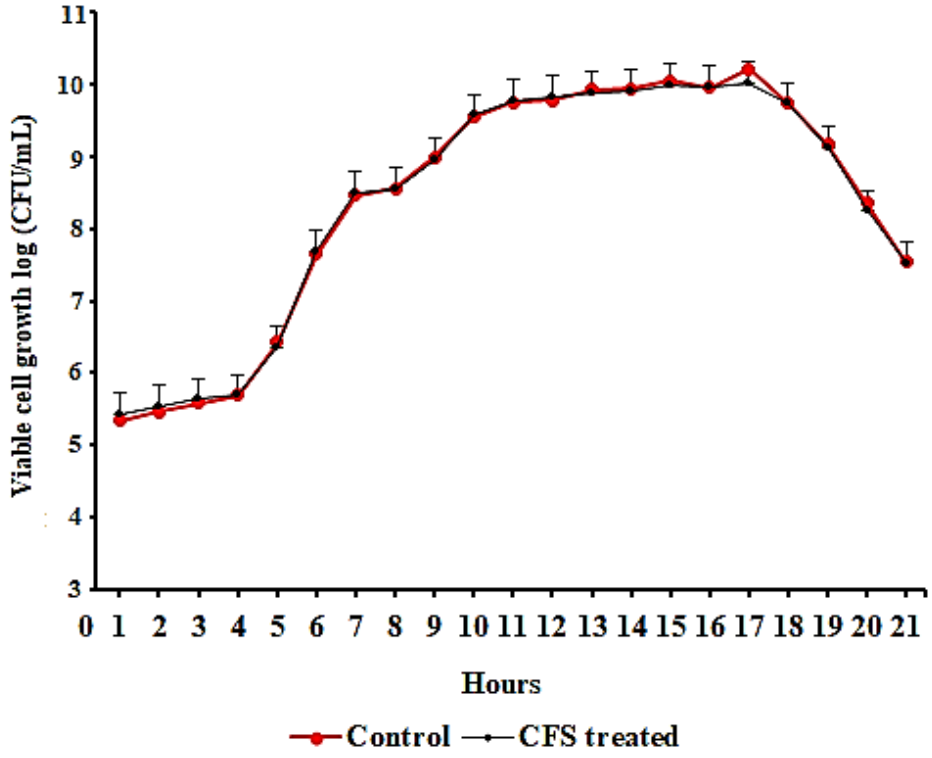


Fig. 3.1 Growth curve of untreated control (red) and CFS (10 mg/mL) treated (black) *P. aeruginosa* cultures. ($p<0.01$, unpaired t-test)

Table 3.1: Comparison between growth characteristics of untreated and CFS treated *P. aeruginosa*

Parameter	Untreated	Treated
Cell count	6hr-8.1×10^6	6hr-6.2×10^6
(CFU/mL)	7hr-9.3×10^8	7hr-8.8×10^8
Specific growth rate, μ (hr^{-1})	4.74	4.95

The arsenal of virulence factors that *P. aeruginosa* produces is crucial to their capacity to enter and persist in a wide range of environments and host tissues. They gain various evolutionary benefits as a result, which makes them a powerful pathogen (Venturi, 2006). Since it is known that *B. velezensis* PM7 has QQ activity against *P. aeruginosa*, this work investigates the impact of this activity on the virulence variables.

The LasI-LasR R QS system mediated virulent phenotype such as staphylolytic activity, resistance against oxidative stress and motility were found to be significantly decreasing in a concentration dependent manner with the CFS treatment. At a concentration of 10% (v/v), the Las A staphylolytic activity decreased up to 49.45% as compared to the untreated control (Fig. 3.2). The phenotype is mediated by a zinc metalloprotease enzyme which is involved in the pathogenesis by degrading the elastin in host tissues. The endopeptidase is also capable of breaking the pentaglycine cross links in cell wall of *S. aureus* like bacteria (Kong et al., 2005).

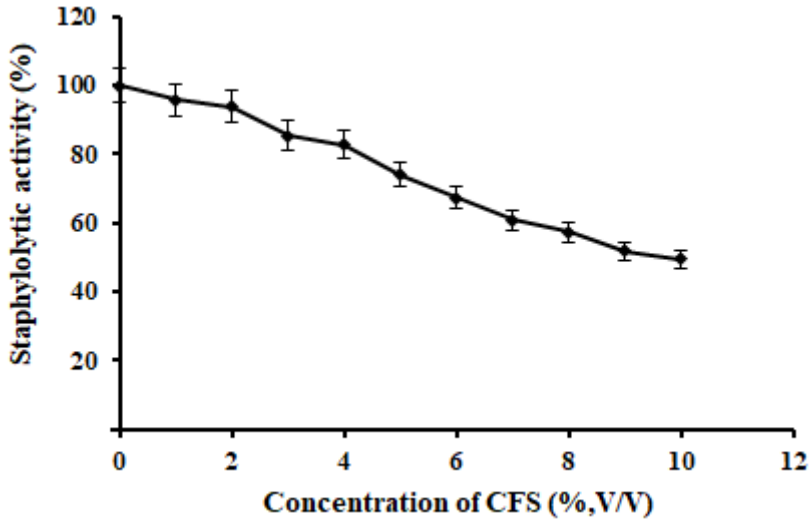


Fig. 3.2 Quantification of the changes in Staphylolytic activity (R^2- 0.96) of *P. aeruginosa* with the PM7 CFS treatment (10%, v/v). Data are expressed as Mean ± SE, **p<0.05, linear regression analysis.

The resistance towards oxidizing agents such as H_2O_2, due to the activities of Super Oxide Dismutase and Catalase enzymes (Sethupathy et al., 2016), also decreased upon CFS treatment (Fig. 3.3 A). A 4.14-fold increase in zone of inhibition zone was observed upon 10% (v/v) CFS treatment (Fig. 3.3 B).

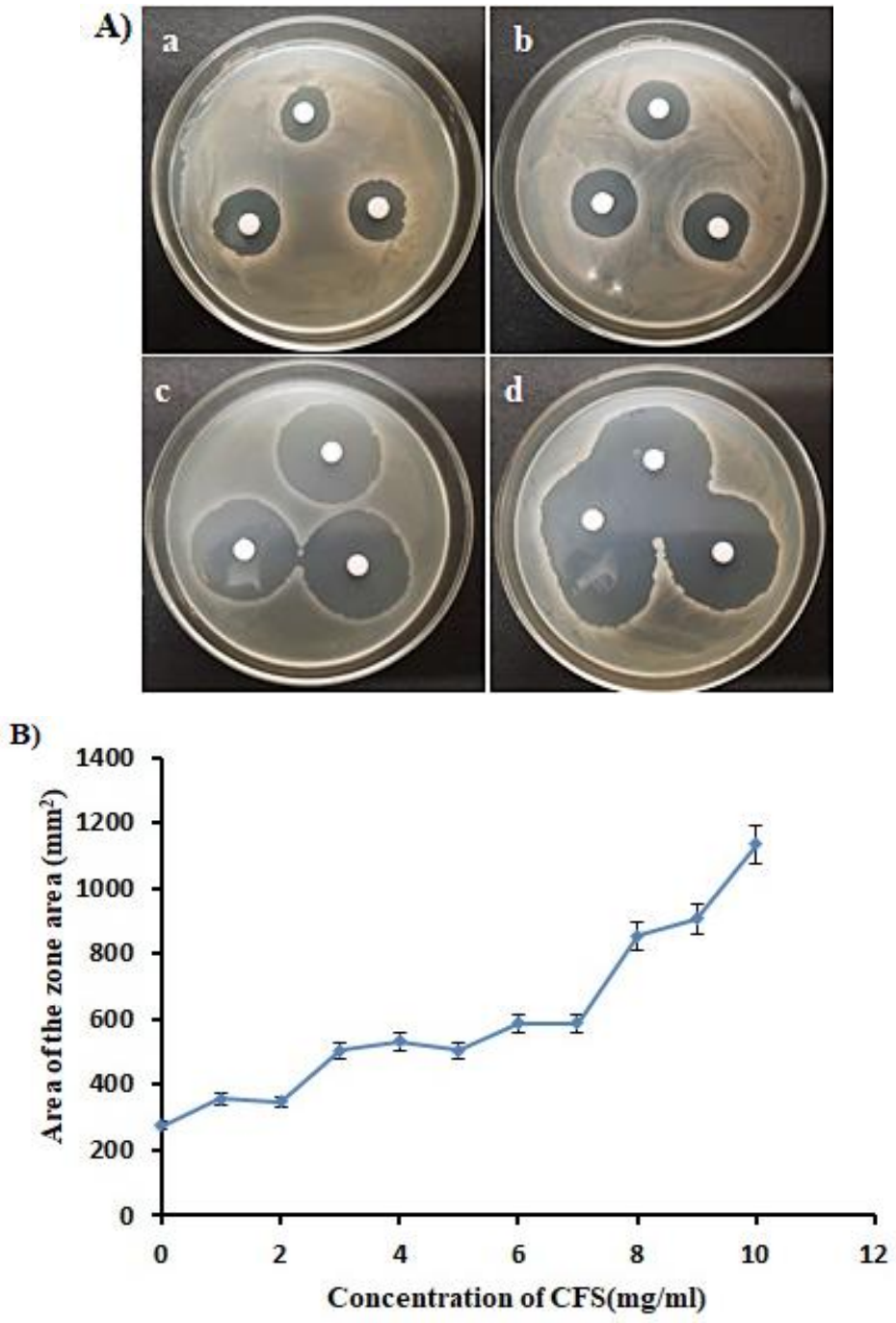


Fig. 3.3 A) Nutrient agar plates showing the increase in bacterial growth inhibition zones around H_2O_2 discs with higher CFS treatment a-control b-1mg/mL CFS c-5 mg/mL, d-10 mg/mL. B) Quantification of clear zone formation (R^2- 0.89, **$p<0.05$, linear regression analysis).

Similarly, the swarming and swimming motility of the *P. aeruginosa* cells were also severely hampered with the CFS treatment as evident from the Fig. 3.4.

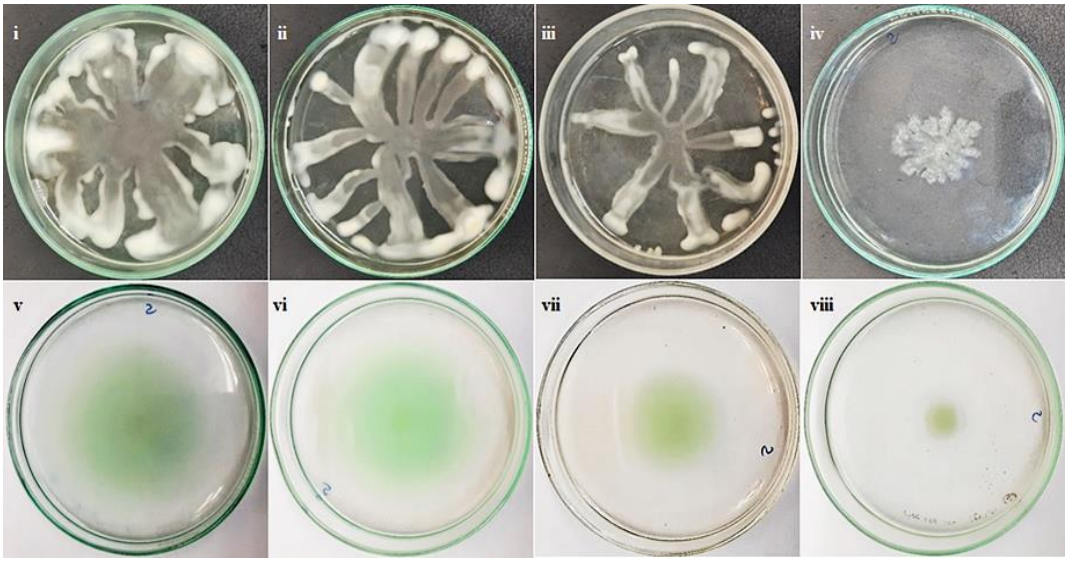


Fig. 3.4 Effect of CFS on the motility of *P. aeruginosa*. Swarming motility variations are shown in (i-iv) and swimming motility in (v-viii). (i,v)- Control, CFS treatment concentrations- (ii, vi) - 1mg/mL, (iii, vii)-5mg/mL, (iv, viii)-10 mg/mL.

The impact of CFS on the cell surface hydrophobicity was assessed using MATH and MATS assays. For all the tested hydrocarbons the adherence of the bacteria was significantly declined. Decane (C10) had a reduction of 35.2%, Xylene (C8) of 42.8%, and Toluene (C7) of 52.5% (Fig. 3.5).

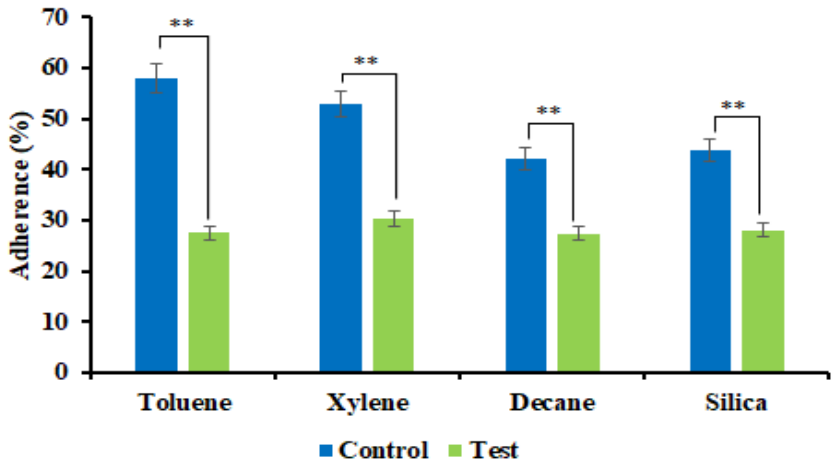


Fig. 3.5 Quantification of Cell Surface Hydrophobicity by MATH/MATS assays. Data represents the difference in percentage adherence of *P. aeruginosa* cells to hydrocarbons/silica due to CFS (10% v/v) treatment. (**p<0.05, unpaired t-test).

Additionally, the decrease in hydrophobicity is negatively correlated with the number of carbon atoms. However, this conclusion cannot be drawn definitively with just the three solvents. Similar to the hydrocarbons, the adherence of *P. aeruginosa* towards silica was also reduced upon CFS treatment by 36.3%.

Other prominent virulent factors in *P. aeruginosa* include pyocyanin, pyochelin, rhamnolipid and alginate production. The CFS treatment significantly affected the production of all of them in a dose-dependent manner. Pyocyanin, a blue-green pigment, was diminished by 63.94% (Fig 3.6). The tricyclic phenazine compound pyocyanin can release reactive oxygen intermediates, thereby altering host immune responses. It is essential in establishing chronic *Pseudomonas* infections (Strateva & Mitov, 2011). The glycolipid type biosurfactant Rhamnolipid and the linear exopolysaccharide of mannuronic acid, Alginate, are two major components of *P. aeruginosa* biofilms. The extracellular QQ activity of *B. velezensis* PM7 is also negatively affecting the production of these two components significantly. A decline up to 73. 7% and 40.6% are observed in the case of Rhamnolipid and Alginate respectively on CFS treatment (Fig. 3.6). Since these two compounds are inevitable in the biofilm composition and architecture, the inhibitory effect of CFS on them contributes prominently in the antibiofilm property of *B. velezensis* PM7.

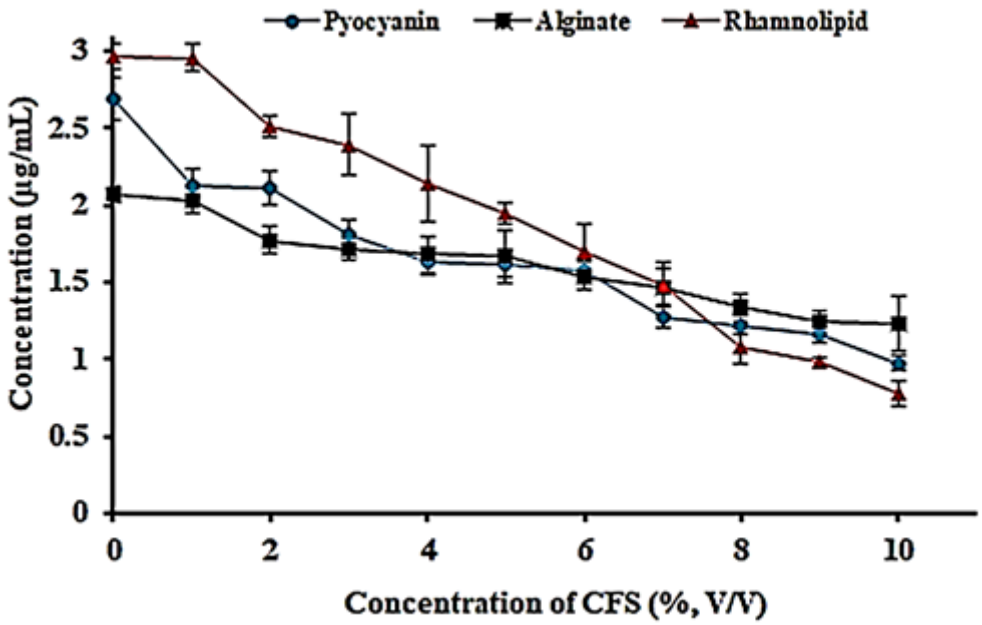


Fig. 3.6 Effect of *B. velezensis* PM7 CFS treatment on the QS controlled virulence factors of *P. aeruginosa* compared to untreaed control. Quantification of Pyocyanin (R^2- 0.93), Rhamnolipid (R^2- 0.96) and Alginate (R^2- 0.9). Data is expressed as Mean ± SE, **p<0.05, linear regression analysis.

The pyochelin, an iron-chelating siderophore capable of causing oxidative damage and inflammations (Harjai et al., 2014) had an acute decrease of 93.3% (Fig. 3.7).

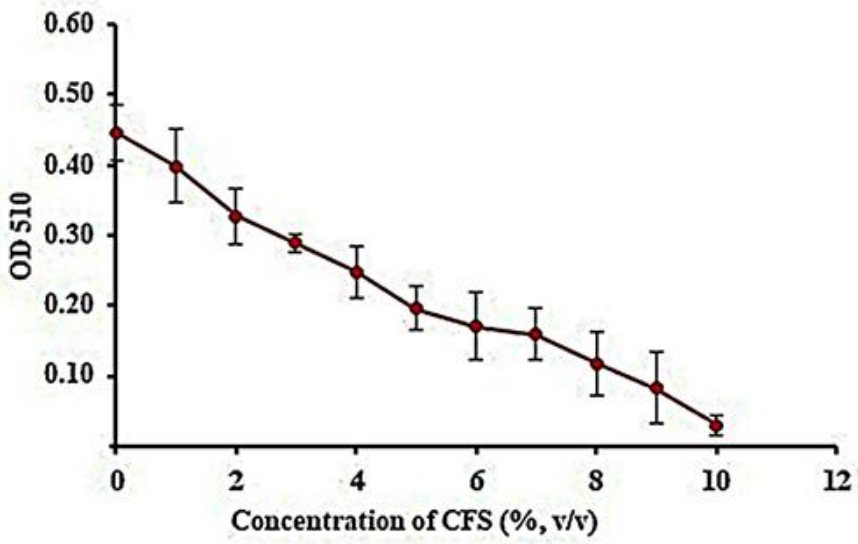


Fig. 3.7 Quantification of the changes in pyochelin production (Absorbance at 510 nm) (R^2-0.96) of *P. aeruginosa* with the PM7 CFS treatment (10%, v/v). Data are expressed as Mean ± SE, **p<0.05, linear regression analysis.

The quantitative EPS inhibition assay in *P. aeruginosa* yielded a 97.9% (p= 0.0014) reduction upon treatment with 10 mg mL^{-1} PM7 CFS (Fig. 3.8 A). Concordant results were also obtained from the slime staining procedure (Fig. 3.8 B) where significant production in the EPS production can be observed. These results are substantiated in the Congo red agar plates also. The non-biofilm-forming colonies primarily form as the concentration of CFS rises, while the black-colored biofilm-forming colonies are limited in the control plates. QS-controlled EPS production is regarded as a significant step in biofilm architecture development in *P. aeruginosa* (Yang et al., 2011), which is regulated by two extensively studied QS networks, namely, Las I – Las R and Rh1 I- Rh1R systems (Sakuragi & Kolter, 2007).

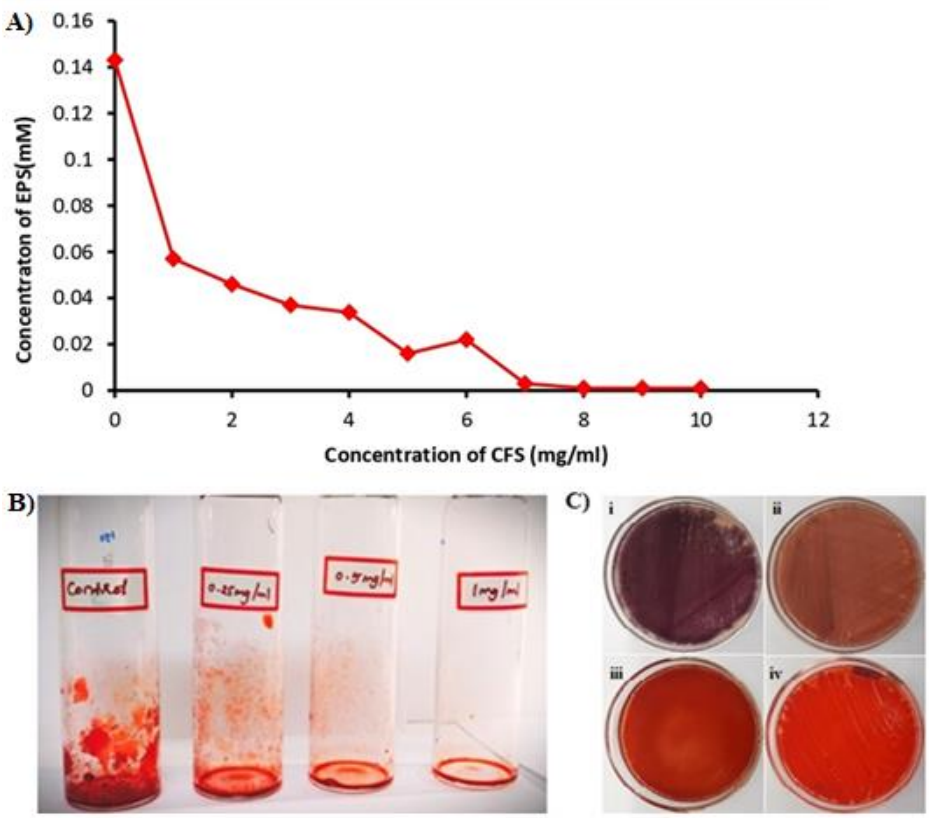


Fig. 3.8 A) Quantitative analysis of the EPS inhibition in *P. aeruginosa* cells treated with CFS ($p<0.01$, unpaired t-test). B) Decline in *P. aeruginosa EPS* production visible in slime staining procedure. C) Congo red agar plates showing inhibition in acidic extra polymeric substance production of *P. aeruginosa* by CFS treatment i-control, CFS treatment ii-5mg/mL, iii-7 mg/mL, iv-10 mg/mL

3.3.2 Antibiotic susceptibility of *P. aeruginosa* biofilm embedded cells is enhanced by CFS treatment

Multidrug resistant (MDR) environmental isolates of *P. aeruginosa* were screened via Kirby Bauer disc diffusion assay. The standard strains used in the study (ATCC 27853 and MTCC 2642) were also found to be MDRs. The antibiogram of 8 environmental isolates (SRL1 – SRL4 and HRK1- HRK4) and the standard strains are provided with Appendix.I. The minimum inhibitory concentrations for both planktonic and biofilm embedded cells of all the isolates used in the study was determined by broth dilution method for the antibiotics Gentamycin and Ciprofloxacin. The MIC values for Gentamycin was found to be in the range of 2-10 µg/mL with the least and highest values for SRL1 and MTCC strain respectively. However, the MBIC values for all tested bacteria were higher than their MIC values which varied between of 8-19 µg/mL. As illustrated in Fig. 3.9, the isolate SRL3 exhibited the highest increase in inhibitory concentration, and SRL2, the least.

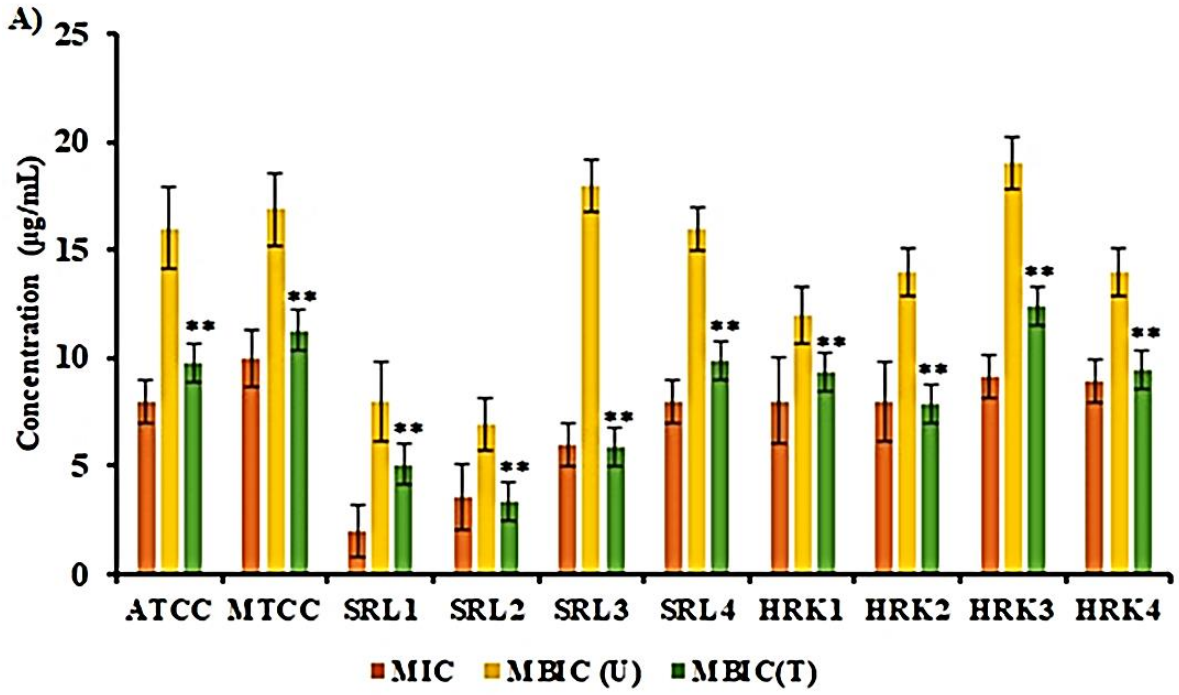


Fig. 3.9 Effect of *B. velezensis* PM7 Cell Free supernatant on the antibiotic susceptibility of *P. aeruginosa* towards Gentamicin. MIC- minimum inhibitory concentrations for the planktonic cells, MBIC – MIC of biofilm embedded cells U- untreated T- CFS treated ** $p<0.05$, one-way ANOVA.

The MBIC values were greater than the corresponding MIC values for ciprofloxacin as well in all the tested bacteria. Here, the highest and lowest increments were for the isolates SRL2 and HRK 4 respectively (Fig. 3.10). The biofilm forming capacity of the isolates/strains were also tested and classified into 4 categories (Table 3.2) according to Stepanović et al., (2007). Higher increases in MBIC values for Gentamycin were seen in *P. aeruginosa* isolates that belong to the category of strongly adherent biofilm formers. On the contrary, no such association can be observed with Ciprofloxacin.

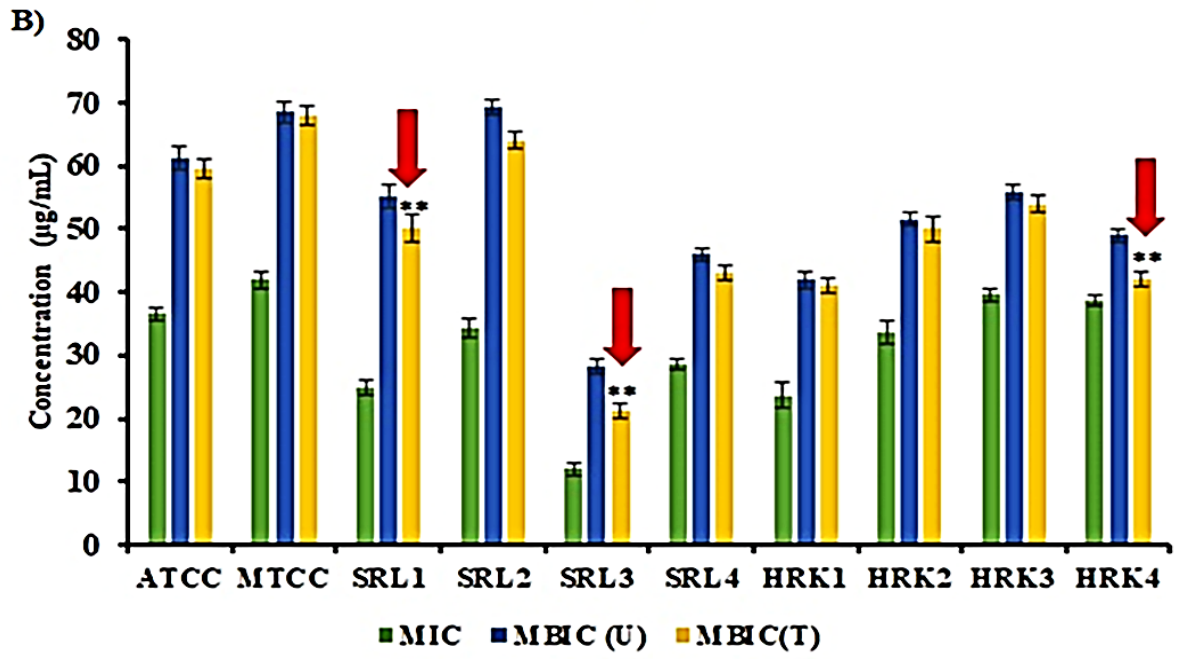


Fig. 3.10 Effect of *B. velezensis* PM7 Cell Free supernatant on the antibiotic susceptibility of *P. aeruginosa* to Ciprofloxacin. MIC- minimum inhibitory concentrations for the planktonic cells, MBIC – MIC of biofilm embedded cells U- untreated T- CFS treated ** $p<0.05$, one-way ANOVA.

Table 3.2: Biofilm forming capacity of the Multi Drug Resistant (MDR) *P. aeruginosa* strains/isolates.

Strain/isolate	Biofilm category
ATCC	+++
MTCC	++
SRL1	+++
SRL2	+
SRL3	+++
SRL4	+++
HRK1	++
HRK2	++
HRK3	+++
HRK4	++

0-Non-biofilm formers, + - weakly adherent, ++- moderately adherent, +++ - strongly adherent

Significant decline in the MBIC values of Gentamycin is observed upon the CFS treatment in all the tested *P. aeruginosa* isolates with the most being exhibited by the isolates SRL3 (67.2%) and SRL3 (50.7%). The percentage reduction was least for HRK1 (21.7%). But, the activity of ciprofloxacin was not found to be considerably influenced by the CFS treatment. Significant reduction in MBIC values was observed for only three isolates in the case of Ciprofloxacin *viz.,* SRL3 (25.4), SRL1 (9.1%) and HRK4 (14.3%). This disparity in antibiotic susceptibility enhancement can be due to the differences in the chemical composition of these antibiotics. Gentamicin is a positively charged aminoglycoside antibiotic that can bind to extracellular polymeric substances in bacterial biofilms such as alginate. Due to this electrostatic interaction, the penetration of the antibiotics like Gentamicin and Tobramycin towards the biofilm-embedded cells is substantially lowered (Gordon et al., 1988). This slow diffusion is a major reason for the emergence of resistance against them. As a neutral antibiotic coming under the flouroquinolone class, Ciprofloxacin readily diffuses through the biofilms (Tseng et al., 2013).

The QQ activity of PM7 CFS can considerably decrease the total biofilm formation in *P. aeruginosa*. The biofilm matrix components like alginate and rhamnolipids are also declined due to CFS treatment which hinders the sequestration of gentamycin-like antibiotics. Sequestering antibiotics to the biofilm matrix can develop more resistance mechanisms in the bacteria by giving sufficient exposure and time (Bagge et al., 2004). Even though, ciprofloxacin diffusion is not affected by the biofilm matrix components, a slight enhancement in susceptibility can be observed due to CFS treatment in at least 3 isolates. Ciofu & Tolker-Nielsen, (2019) have reported that *P. aeruginosa* biofilm components and architecture can alter the physiology of participating cells and modify the antibiotic susceptibility towards Ciprofloxacin-like antibiotics.

MexAB-OprM is a critical efflux pump in *P. aeruginosa* that is responsible for imparting resistance towards a wide range of antibiotics such as quinolones, macrolides, tetracyclins and β-lactam antibiotics (Verchère et al., 2015). A real-time fluorescence monitoring assay using Ethidium Bromide as the substrate revealed that the QQ activity of CFS has no impact on the efflux pump activity (Fig. 3.11). A decline in fluorescence emission indicates the pump to be actively transporting EtBr from cytoplasm to outer space. But the relative fluorescence of the CFS-treated and untreated cells kept uniformly decreasing with time. Whereas, the known MexAB-OprM inhibitor PaβN treated cells maintained the fluorescence over the 60 minutes.

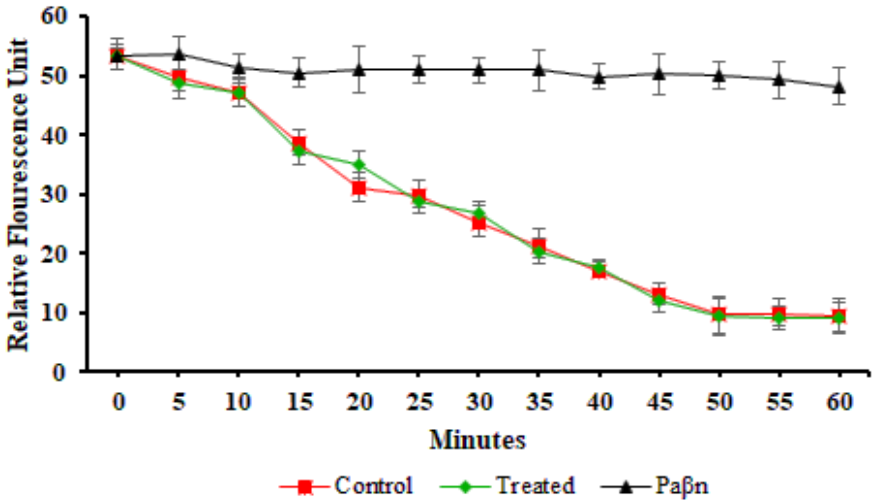


Fig. 3.11 MexAB-OprM efflux pump activity of *P. aeruginosa* at 10% (v/v) CFS treatment compared against the positive control Paβn and untreated negative control.

The effect of *B. velezensis* PM7 CFS on the *P. aeruginosa* persister cells under high-dose antibiotic exposure was also assessed (Fig. 3.12). 5X MIC Gentamicin is the stress condition for eight environmental isolates and two standard strains. SRL 4, HRK 2 and HRK 3 were not exhibiting any persister cell formation under the given condition. A significant reduction in the persister cell formation was observed in all the remaining isolates/strains upon CFS treatment (10%, v/v); for SRL3 and HRK4, a 100% reduction was obtained. The least decline was obtained for the MTCC strain (37.5%). Due to their slow metabolism and lack of growth, persister cells have the potential to evade host immune response and antibiotic treatments. They are also responsible for the recurrent character of the biofilms (Patel et al., 2022). Thus, it can be demonstrated that the CFS treatment can be very effective in properly eradicating biofilms.

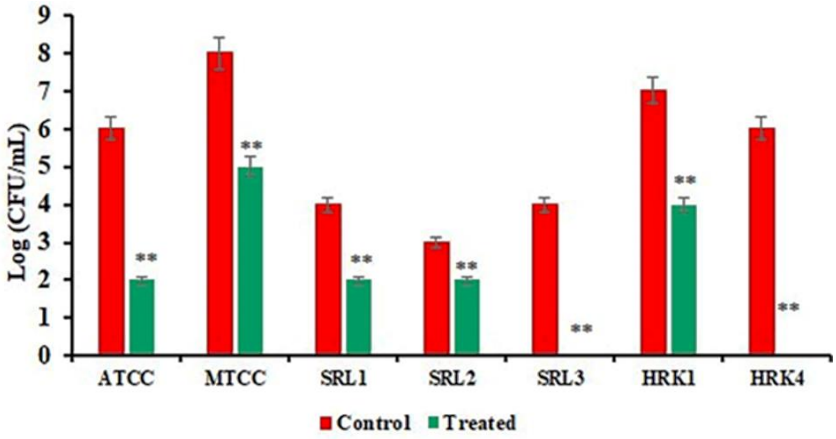


Fig. 3.12 Effect of CFS on the persister cell formation in *P. aeruginosa* under high dose antibiotic (5X MIC Gentamicin) exposure. **p<0.05, unpaired t-test.

3.4 Chapter summary

The model Gram negative bacterium *P. aeruginosa* was studied in detail to evaluate the extracellular QQ activity of the isolated strain *B. velezensis* PM7. It was found that the growth of *P. aeruginosa* was unaffected by the Cell-Free Supernatant of *B. velezensis* PM7. However, the QS-controlled phenotypes such as virulence factor production, exoenzyme secretions and biofilm formation were significantly reduced by the CFS treatment. The impact of the isolate PM7 on the antibiotic susceptibility of selected *P. aeruginosa* isolates/strains was also examined. In all the tested Multi-Drug Resistant (MDR) *Pseudomonas* isolates/strains, the inhibitory concentration for biofilm-embedded cells was higher than planktonic cells. However, the elevated resistance to cationic aminoglycoside antibiotic (Gentamicin) declined after treatment with MP7 CFS. But, significant reduction in antibiotic resistance for Ciprofloxacin was observed only for 3 isolates. The disparity in resistance between these two antibiotics can be attributed to their mode of action. Gentamicin is hindered mainly by the diffusion inhibition, while the efflux pumps mainly resist Ciprofloxacin. It was also found that the MexAB-OprM efflux pump activity of *P. aeruginosa* was unaffected by the treatment with PM7 CFS. The QQ activity of PM7 also impaired the persister cell formation in *P. aeruginosa* upon exposure to high dose of antibiotic. The observations of all these studies clearly demonstrated the extracellular QQ activity of the present isolate *B. velezensis* PM7. Further, it proposes the synergistic use of QQ agents along with conventional antibiotics for tackling biofilms without avoiding the chances of antibiotic resistance development.

Chapter 4

Identifying the Mechanism of Quorum Quenching in *Bacillus velezensis* PM7

4.1 Introduction

The process of disrupting the quorum sensing network can be attained by either quorum sensing inhibitors (QSIs) or Quorum Quenching (QQ) agents (Anju et al., 2021). Many natural and synthetic QSIs are identified as structural analogues to AHLs that can block the receptors by outcompeting AHLs (Kalia, 2013). On the other hand, QQ agents degrade the autoinducer molecules produced by their target organisms. The enzyme-based degradation of signal molecules is one of the extensively studied mechanisms. Some naturally occurring QQ bacteria and eukaryotes can produce AHL-degrading enzymes such as lactonases, acylases and oxidoreductases (Fetzner, 2015). Lactonases catalyses the hydrolysis of lactone ring in AHLs, while acylases hydrolyse the amide bond between homoserine moiety and the acyl chain. Oxidoreductases oxidize the C3 position of AHL, promoting the subsequent degradation by amidohydrolase (Koul & Kalia, 2016). Due to these multiple possibilities, it is imperative to determine a specific QS disruption mechanism, which will help to identify the target bacterial population and to enhance the efficacy of treatment. Therefore, this chapter, cover the results of multiple bioassays and analysis performed with *B. velezensis* PM7 CFS to establish the mechanism involved in its QQ activity. The isolate was screened for the presence of the aiiA homologue gene proposing the presence of a secretary AHL lactonase enzyme. Also, *in silico* 3D structure prediction and molecular docking analysis were carried out to find the substrate affinity of the $AiiA_{PM7}$ enzyme towards AHLs.

4. 2. Materials and methods

4.2.1 Bacterial strains and growth conditions

The reporter assays in this chapter were carried out using the mini Tn5 mutant biomarker strain *Chromobacterium substugae.* It was cultured and maintained at 30 °C in LB broth/agar supplemented with 20 µg/mL Kanamycin. The medium was supplemented with 1 mM C_6-HSL for inducing violacein production. The *B. velezensis* PM7 culture was prepared in Nutrient HiVeg^{TM} broth (Himedia) at 30 °C and 200 rpm shaking. The strain's Cell-Free Supernatant (CFS) was prepared as mentioned in section 2.3.2. The bacterial cultures were incubated up to an optical density (OD) of 0.5 at 600 nm for all experimental procedures.

4.2.2 Effect of heat, solvent and proteinase K on the QQ activity of *B. velezensis* PM7

As a preliminary method to identify the mechanism of quorum quenching by *B. velezensis* PM7, the CFS was incubated at various temperatures such as 30 °C, 50 °C, 80 °C and 90 °C, each for 10 min. One mL CFS was treated with 1 mg/mL proteinase K for 15 minutes for assessing the proteinaceous nature of the QQ activity of the CFS. For assessing the effect of solvents, equal volumes of *B. velezensis* PM7 CFS was mixed with organic solvents and vortexed thoroughly. The solvents used were Hexane, Benzene, Chloroform, Ethyl acetate and Ethanol (in the increasing order of polarity). The mixtures were left overnight for phase separation, and the organic layers were collected, evaporated and resuspended in distilled water or Dimethyl Sulfoxide. The quorum quenching activity of heat, proteinase K and solvent-treated CFS was tested using FI assay using *C. substugae* as aforementioned. Untreated CFS applied to the *C. substugae* culture and *C. substugae* alone were used as positive and negative controls, respectively (Packiavathy et al., 2021a)

4.2.3 Ring closure assay to assess the quorum quenching mechanism

The mechanism of quorum quenching by *B. velezensis* PM7 was analyzed previously through the ring closure assay, as reported by Packiavathy *et al.* (2021) with slight modification. Briefly, 5 mM C_6-HSL was treated with 500 µL *B. velezensis* PM7. The pH of the reaction mixture was brought down to 2 by adding HCl and kept at 4°C for 48 hr. After the incubation, the HCl was evaporated, and the residue was resuspended in LB broth. The violacein induction property of the resultant mixture was tested using *C. substugae*. Unacidified C_6-HSL CFS mixture served as the positive control, while *C. substugae* culture supplemented with C_6-HSL as the negative control.

4.2.4 Colorimetric detection of AHL

The quantification of AHL molecules was carried out using Hydroxylamine-$FeCl_3$ method (Taghadosi et al., 2015). Briefly, *B. velezensis* PM7 CFS was subjected to the FI assay with *C. substugae* at concentrations ranging from 1-10% (v/v). The cultures from each flask were centrifuged at 11,000 g for 10 minutes, and the pellets were discarded. The supernatant was extracted using acidified ethyl acetate for three times, and the organic phases were pooled together. 40 µL of the extract was mixed with 50 µL of a 1:1 mixture of hydroxyl amine (2 M): NaOH (3.5 M) and vortexed. Subsequently, 50 µL of 1:1 mixture of ferric chloride (10% in 4 M HCl) and 95% ethanol was added to the solution and kept for 10 minutes. The OD of the samples was measured at 520 nm. Untreated *C. substugae* cultures were used as the control.

4.2.5 LC-MS Analysis

To reconfirm the lactonolysis activity of the CFS of the isolate PM7, degradation of 3-oxo-C_8-HSL was qualitatively analyzed using LC-MS. Briefly, the CFS was incubated with the 3-oxo-C_8-HSL in phosphate-buffered saline for 30 min. Before and after treatment, the samples were extracted three times with acidified (glacial acetic acid) ethyl acetate. The organic phase was collected and dried in rotavapor at 30 °C, and the residue was resuspended in 1 mL acetonitrile. 10 µL of the sample was injected into UHPLC LC-30A in conjunction with Triple Quadrupole Mass spectrometer LC-MS-8045 with GISS-C18 column (Shimadzu, Japan). The gradient elution method used a mixture of deionized water (30% with 0.1% formic acid) and methanol (70%) for 10 minutes and at a column temperature of 30 °C. The compound detection was carried out by ESI/MS in the positive mode with interface temperature and interface voltage set to 400 °C and 4 kV, respectively. The detector voltage was set at 1.8 kV, and nebulizing gas flow of 3 L/min was maintained. The samples were analyzed in triplicates (Patel et al., 2016).

4.2.6 Amplification of aiiA homologous gene

The presence of an AHL lactonase encoded by an aiiA homologous gene in the *B. velezensis* PM7 was analysed through gene amplification. The whole genomic DNA was extracted using Nucleospin DNA isolation kit (Machery-Nagel, Germany) and aiiA homologous gene was amplified using the primers aiiA F2 (5'-CGG AAT TCA TGA CAG TAA AGA AGC TTT A-3') and aiiA R2 (5'-CGC TCG AGT ATA TAT TCA GGG AAC ACT T-3') and PCR conditions were maintained as reported earlier (Rajesh & Rai, 2014) (Initial denaturation 95 °C - 3 min, denaturation- 95 °C - 30 s, Annealing- 50 °C - 1 min.,

Extension – 72 °C - 2 min., Final extension - 72°C - 10 min., Cycle no. 35). The resultant PCR product was purified and the sequencing of the amplicon was carried out through Sanger's method. The consensus sequence of the gene was generated using BioEdit version 7.2. The gene sequence was compared and identified using BLASTn search against the non-redundant database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and deposited in the NCBI GenBank database. The conserved domains in the gene sequence were identified using the CDD database (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

4.2.7 Protein sequence analysis and tertiary structure prediction

The Open Reading Frame (ORF) of the aiiA$_{PM7}$ homologue was deduced using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) and translated to protein sequences. The protein sequence similarity search was conducted using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), and theoretical computation of various physicochemical parameters such as molecular weight, isoelectric point, aliphatic index etc. was carried out using the ProtParam tool by ExPasy (https://web.expasy.org/protparam/). The phylogenetic analysis of the protein sequence was conducted with MEGA X. V. 10. 2. 6. The putative tertiary structure of AiiA$_{PM7}$ was predicted using Swiss-Model workspace (https://swissmodel.expasy.org/) (Shastry et al., 2019).

4.2.8 Docking studies

The docking process between the predicted AiiA$_{PM7}$ protein structure and N-Acyl Homoserine Lactone (AHL) molecules was carried out using Biovia Discovery Studio 2021. The process was evaluated by LibDock program. The binding modes of all 19 AHL molecules were obtained from PubChem compound database. The 2D structure of the compounds was drawn by Marvin sketch v.5.3 in LibDock. Both the putative protein structure and substrates were prepared for the molecular interaction studies using Discovery Studio v. 21. All the parameters for the study were kept default. CHARMM 36 force field was applied to minimize the structures. Docking was performed with the defined binding sites. The free energy of the binding was calculated using the empirical scoring function LibDock score. The nature of bonding and bond length of the docked ligands were also determined. The docked complexes and interactions were visualized by Discovery Studio visualizer (Younus et al., 2021).

4.2.9 AHL degrading Bioassay

The degradation of AHL molecules by the lactonase activity of CFS was tested as previously described with slight modifications (Anandan & Vittal, 2019). Briefly, selected

AHLs (C_4-HSL, C_6-HSL, 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL, and 3-OH-C_{12}-HSL) were individually incubated under static conditions with CFS at 30 °C for 2hr. The pH of the reaction mixture was maintained with PIPES buffer to avoid the alkaline hydrolysis of AHL molecules. After the incubation, AHLs were extracted using ethyl acetate acidified with 0.02% (v/v) glacial acetic acid. The organic fraction was evaporated, and the residue was dissolved in acetonitrile. AHLs in the PIPES buffer alone were used as the positive control. Overnight grown *C. violaceum* CV026 culture was adjusted to an OD_{600} of 0.5. One mL of the culture was mixed with 24 mL of 1.5% molten LB agar (50 °C) and poured into the plates. The long chain AHLs (3-oxo-C_{10}-HSL and 3-OH-C_{12}-HSL), the molten agar was supplemented with 1mM C_6-HSL in DMSO. Wells of 6 mm diameter were punched aseptically into the agar and filled with 50 µL AHL samples. The plates were incubated at 30 °C for 24 hr. and observed for purple-colored and depigmented zones for the short and long-chain AHLs.

4.3 Results and Discussion

4.3.1 Effect of heat, solvent and Proteinase K on the QQ activity of *B. velezensis* PM7 CFS

The *C. subtsugae* cultures supplemented with exogenous AHL signal was used in the flask incubation assay to examine the effect of heat treatment on the QQ activity of *B. velezensis* PM7 CFS. A decline in violacein production inhibition was observed at higher temperatures (Fig 4.1). At 50 °C, the recovery of violacein production was 13.4% compared to the control, and it increased to 98.9% at 80 °C. Additionally, Proteinase K treatment at 1 mg/mL inactivated the QQ property of the CFS resulting in a violacein retrieval of 99.06% (Fig. 4.1). Similar observations are reported in previous studies where enzyme-mediated quorum quenching was confirmed as the mechanism of quorum quenching with subsequent analyses (Koch et al., 2014; Packiavathy et al., 2021b; Shastry et al., 2019).

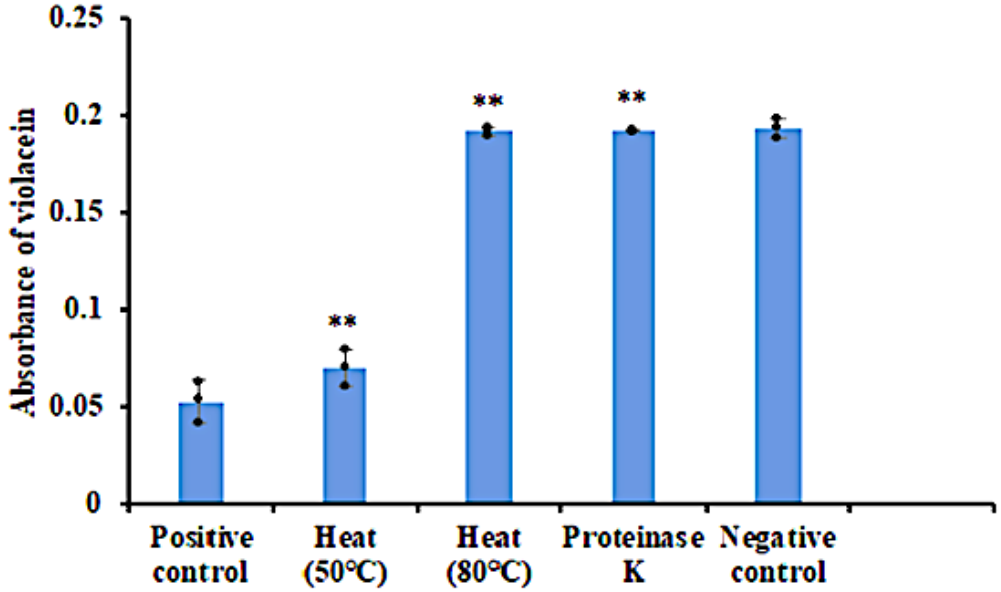


Fig. 4.1 Effect of heat treatment (50 °C and 80 °C) and proteinase K treatment (1 mg/mL) in the quorum quenching activity of *B. velezensis* PM7 on *C. substugae*. ** Significant reduction of QQ activity at $p<0.05$ (unpaired t-test) concerning the positive control

Extraction of the CFS with organic solvents also abolished the extracellular QQ activity of the bacterium (Table 4.1). The loss of activity at higher temperatures, solvent extraction and after proteinase K treatment directly indicates the enzymatic nature of the QQ activity of CFS released by PM7.

Table 4.1 Effect of organic solvents on the QQ activity of *B. velezensis* PM7

	Solvents	Presence of QQ activity
Non-polar ⇩ Polar	Hexane	X
	Benzene	X
	chloroform	X
	Ethyl acetate	X
	Ethanol	X

4.3.2 Identification of lactonase activity in the cell free supernatant (CFS)

Both enzymatic and non-enzymatic QQ activity against gram-negative bacteria were reported previously. Hassan *et al.* (2016) reported an extracellular non-enzymatic QQ activity in *Streptomyces coelicoflavus* isolated from soil. The QQ molecule identified from the culture supernatant of the organism was 1H-pyrrole-2-carboxylic acid which suppressed the expression of QS genes in *P. aeruginosa*. Whereas, the major extracellular enzymatic QQ activities reported in bacteria includes the AHL lactonases produced by *Psychrobacter* sp. (Packiavathy et al., 2021a) and *Muricauda olearia* (Tang et al., 2015). Both strains were isolated from marine sediments and were found interfere with the QS network of *C. violaceum* and *P. aeruginosa.*

The mechanism of AHL degradation by *B. velezensis* PM7 was studied by the acidification assay with *C. subtsugae*. After acidification, it was observed that the CFS pre-treated AHL has significantly recovered as evidenced by the induction of violacein, its retrieval was ~97.91% (Fig. 4.2). The AHL recovery after acidification suggests the enzyme as an extracellular Acyl Homoserine Lactonase. AHL lactonases hydrolyze the lactone ring targeting the ester bond resulting in the formation of acyl homoserine which can be recyclized to functional AHLs upon protonation in lower pH (Tang et al., 2015; Yates et al., 2002).

The AHL degradation activity of CFS by PM7 was analysed using the Hydroxylamine – $FeCl_3$ method on *C. substugae* flask incubation assay. The quantification of spent AHLs from the assay revealed a gradual decline upon the treatment with different levels of CFS (Fig. 4.3). However, the reduction reaches a plateau phase after the 4% (v/v) CFS treatment due to the assay's lesser sensitivity.

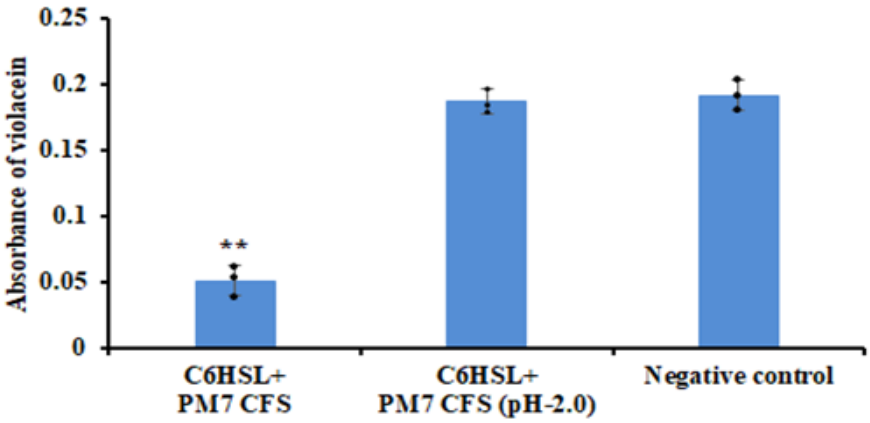


Fig. 4.2 Retrieval of the quorum sensing activity of the extracted C_6-HSL from the PM7 CFS treated *C. violaceum* CV026 upon acidification to pH 2.0. ** Significant violacein reduction at $p<0.05$ (Unpaired t-test) concerning the negative control.

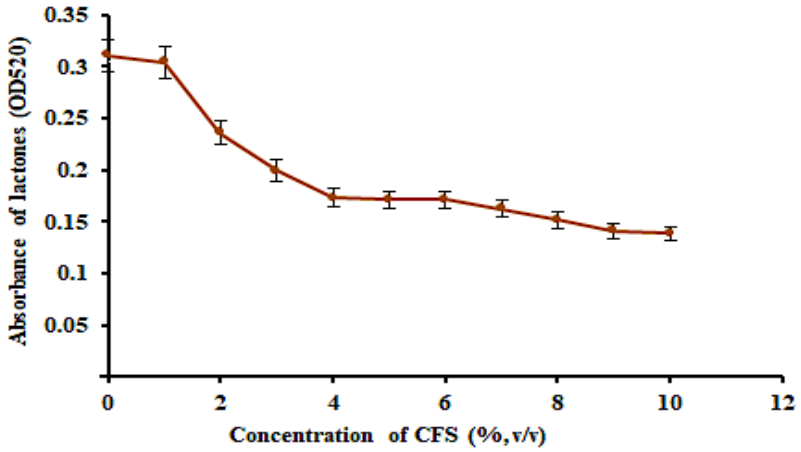


Fig. 4.3 Quantification of AHLs from the Flask Incubation assay with *C. substugae* by Hydroxylamine – $FeCl_3$ method.

To reconfirm the mechanism of QQ exhibited by PM7, 3-oxo-C_8-HSL was digested by the CFS and the reaction products were examined by LC-MS. The *m/z* value of standard 3-oxo-C_8-HSL is 242 (Fig. 4.4A), which can also be found in the *B. velezensis* PM7 CFS treated samples at time t=0 (Fig. 4.4B). However, the prominent 242 *m/z* peak was absent in the treated cultures after 30 minutes of incubation indicating the AHL degradation property of the CFS (Fig. 4.4C). Instead, the sample contained an *m/z* peak at 261, corresponding to the ring-opened product 3-oxo-C_8-HS.H (M-H ion). These results are comparable with a previous report where the hydrolysed AHL's molecular mass increased due to the addition of a water molecule to the ester bond (Patel et al., 2016). The formation of similar LC-MS chromatograms for various AHL molecules by extracellular (Tang et al., 2015) and intracellular (Anandan & Vittal, 2019) lactonases has already been reported.

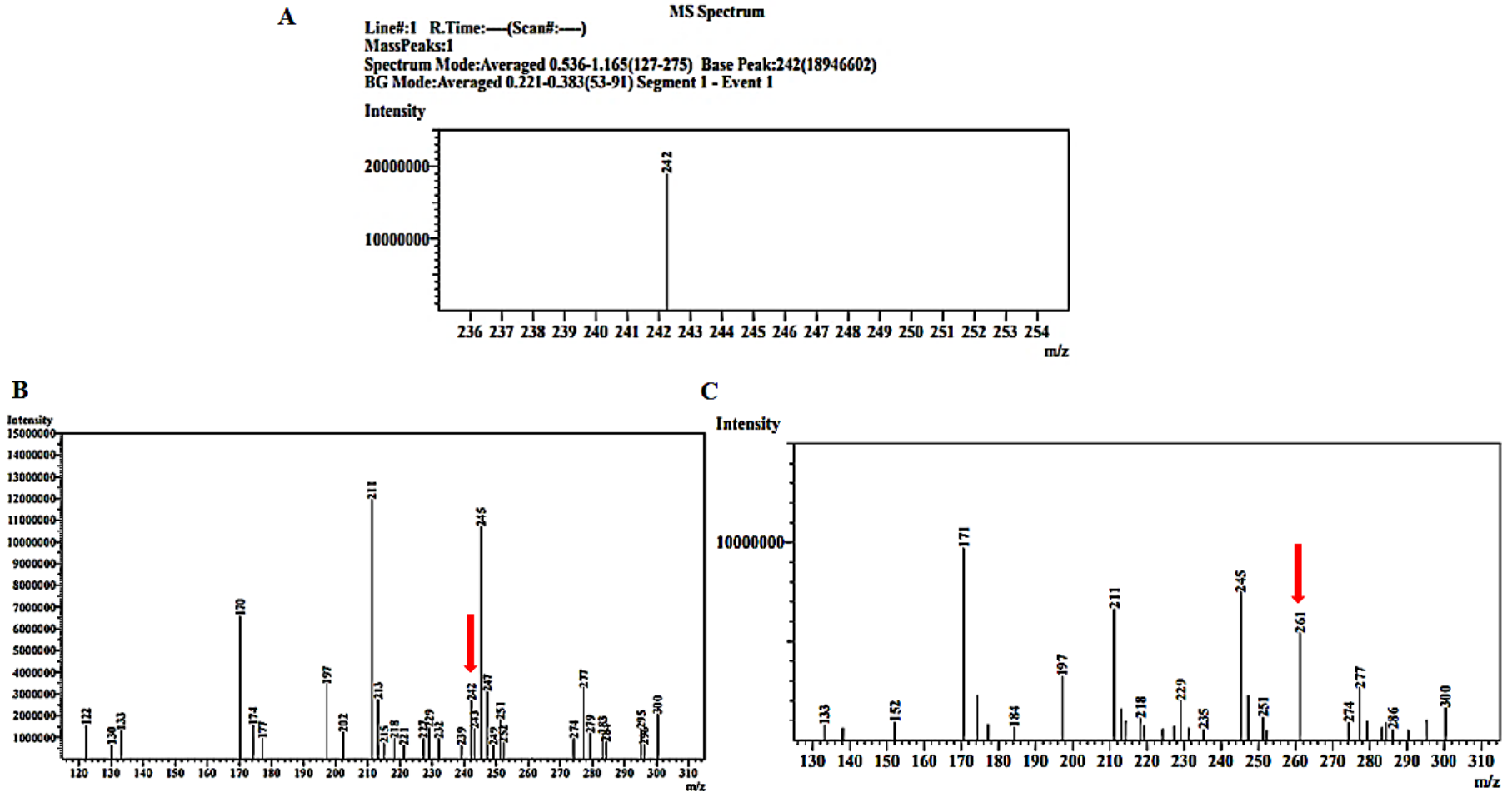


Fig. 4.4 LC-MS analysis of the degradation product of CFS with 3-oxo-C_8-HSL. A) Spectra of 3-oxo-C_8-HSL showing 242 *m/z* peak. B) 3-oxo-C_8-HSL incubated with CFS at time t=0 C) Fragmentation spectra of 3-oxo-C_8-HSL showing 261 *m/z* peak (M-H) alone, indicating complete degradation of the test molecule.

4.3.3 PCR amplification of N-acyl Homoserine Lactonase gene

The primer pair aiiAF2 and aiiaR2 was used to confirm the presence of an aiiA homologue responsible for the production of AHL lactonase in the *Bacillus* genus (Fig. 4.5). The 753 bp long sequence revealed 99.60 % identity to the *B. thuringiensis* AHL Lactonase aiiA-B22 gene. The AHL lactonase domain, which is an αββα Metallo β-lactamase (MBL) fold was identified from the gene sequence via conserved domain analysis using the CDD database (Fig. 4.6).

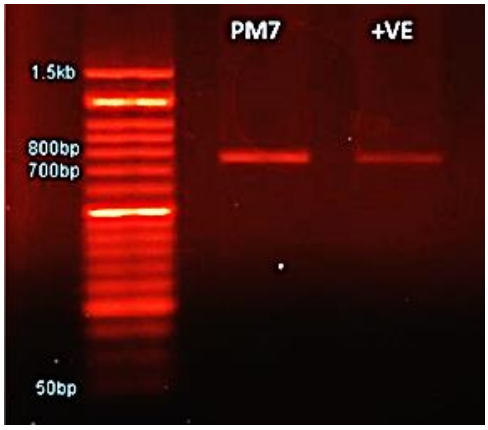


Fig 4.5. Amplified aiiA gene (753 bp) from *B.velezensis* PM7. *B. cereus* is used as the positive control

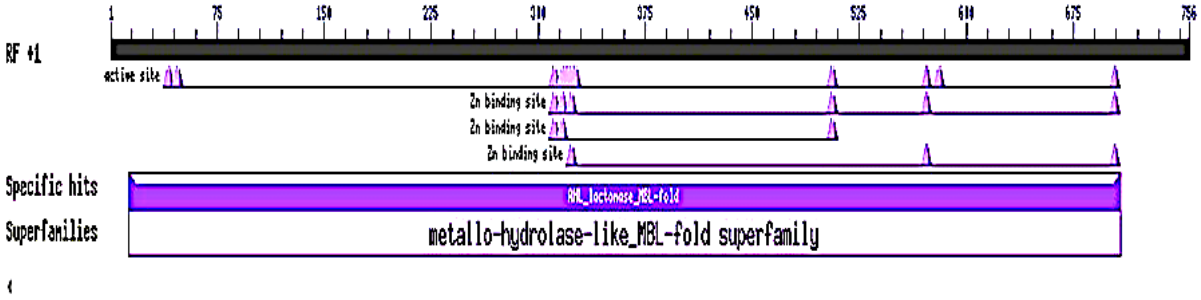


Fig. 4.6 Conserved domain analysis of the aiiA$_{PM7}$ gene sequence

4.3.4 Sequence analysis and putative structure prediction of AiiA$_{PM7}$ from *B. velezensis* PM7

This result suggests that the gene product belongs to the Metallo-hydrolase-like MBL-fold superfamily that includes enzymes catalyzing redox and hydrolysis reactions. The aiiA lactonase cluster is widely spread in the *Bacillus* genus (Anandan & Vittal, 2019; Bergonzi *et al*., 2016; Liu *et al.*, 2008; Vinoj *et al*., 2014). AHL degrading lactonase from *B. velezensis* was

previously reported by Sun *et al*., (2021), the heterologous expression of which was applied to maintain dental chair unit hygiene.

The aiiA$_{PM7}$ gene sequence was used to deduce an open reading frame in the +1 strand spanning from 1 to 753 bp (Fig. 4.7). The translated protein sequence from the ORF consisted of 250 amino acids. Protein BLAST analysis of the sequence revealed 95.6 % identity to N-Acyl Homoserine Lactonase of *B. thuringiensis* (GenBank Acc. A3FJ64.2). Various physical, chemical parameters and amino acid composition of the predicted protein were also computed using the ProtParam tool of ExPasy (Table 4.2). The amino acid composition analysis of the enzyme revealed a higher proportion of Glutamate (10.8 %) and Leucine (10.4 %). In contrast, Tryptophan has the lowest percentage (0.4 %) (Appendix II).

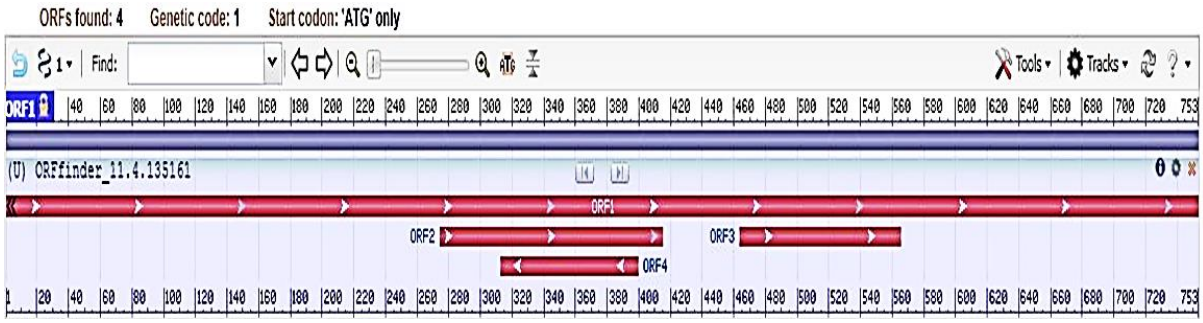


Fig. 4.7 Open Reading Frames (ORF) in the aiiA$_{PM7}$ gene sequence

Table 4.2 Theoretical physical/chemical parameters of the predicted protein AiiA$_{PM7}$

Physical/chemical parameter	**Theoretical value**
No. of amino acids	250
Molecular weight (Da)	27945.81
Isoelectric point (pI)	4.72
Ext. coefficient	22140
Estimated half-life (hr.)	>10
Instability index	48.46
Aliphatic index	94.32
Grand average of hydropathicity (GRAVY)	-0.129

The amino acid sequence of the proposed protein was compared with the other known N-Acyl Homoserine Lactonase of metallo-β-lactamase superfamily by multiple sequence alignment to identify the conserved sequences or motifs. The catalytically important amino acids Tyrosin 194, Aspartate 191, and the dinuclear Zinc binding motif HXHXDX, which are essential for the AHL lactonase activity, have been identified in the $AiiA_{PM7}$ sequence (Fig 4.8). A neighbor joining phylogenetic tree was also generated based on the evolutionary similarities between these representative enzymes, and it revealed that $AiiA_{PM7}$ and AiiA from *Bacillus cereus* shared the highest degree of similarity. Other significant AHL lactonase enzyme classes were identified to be rooted to $AiiA_{PM7}$, with the AidC class being the most evolutionary distant clad (Fig. 4.9).

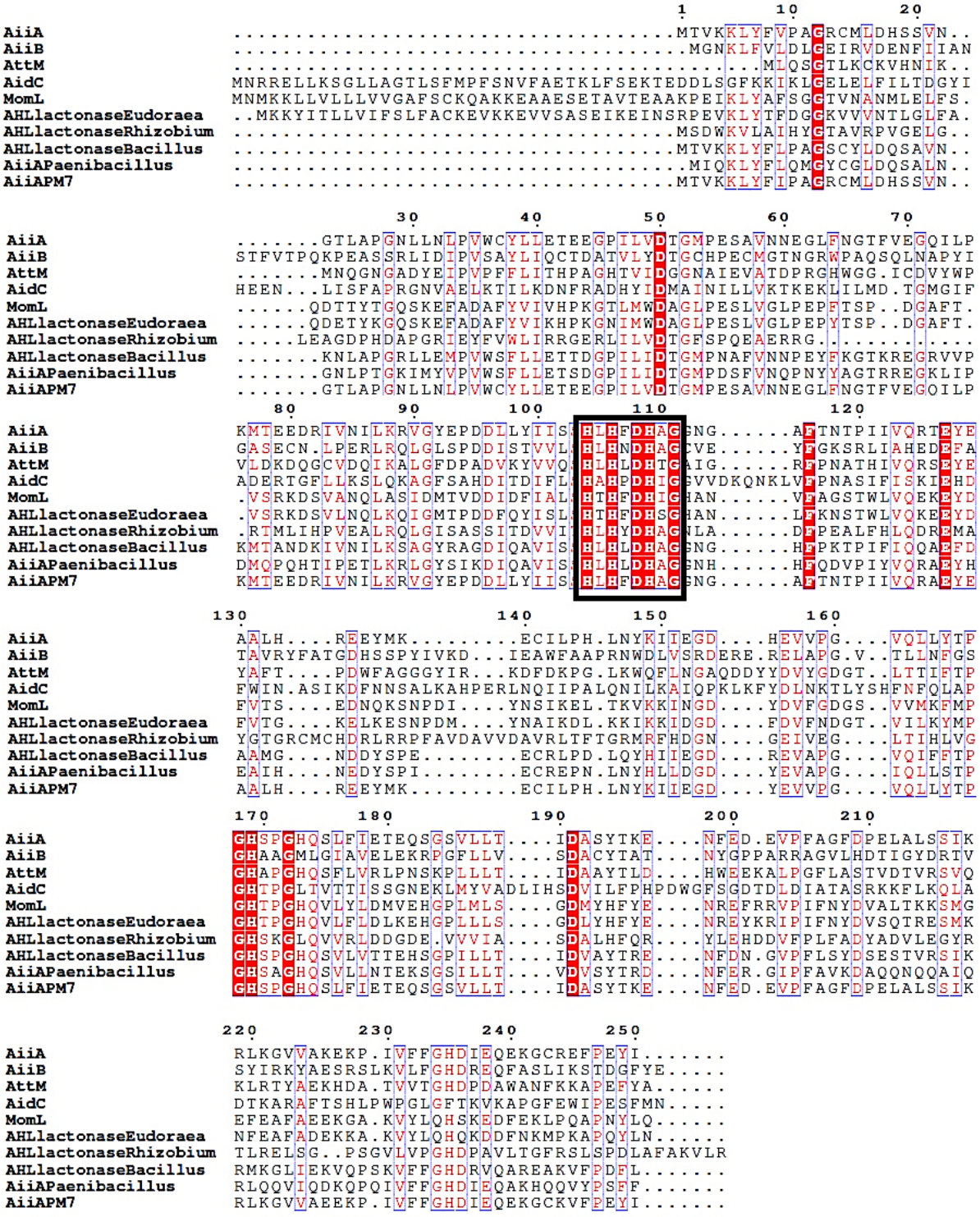


Fig. 4.8 Multiple Sequence alignment of AiiA$_{PM7}$ amino acid sequence with other known AHL lactonases. The metallo β lactamase motic HXHXDX is boxed. Alignment candidates used are 1. *Bacillus cereus* AiiA (Genbank ID: UAT16591.1) 2. AiiB *Agrobacterium tumefaciens* (WP_172691130.1) 3. AttM Agrobacterium fabrum (AAD43990.1) 4. AidC *Chryseobacterium* sp. (BAP32158.1) 5. MomL *Muricauda olearia* (AIY30473.1) 6. AHL lactonase family protein *Eudoraea adriatica* (WP_019670967.1) 7. AHL Lactonase family protein *Rhizobium acidisoli* (QAS81050.1) 8. AHL lactonase family protein *Bacillus atropheus* (ARW07811.1) 9. AHL lactonase *Paenibacillus nuruki* (ODP27172.1)

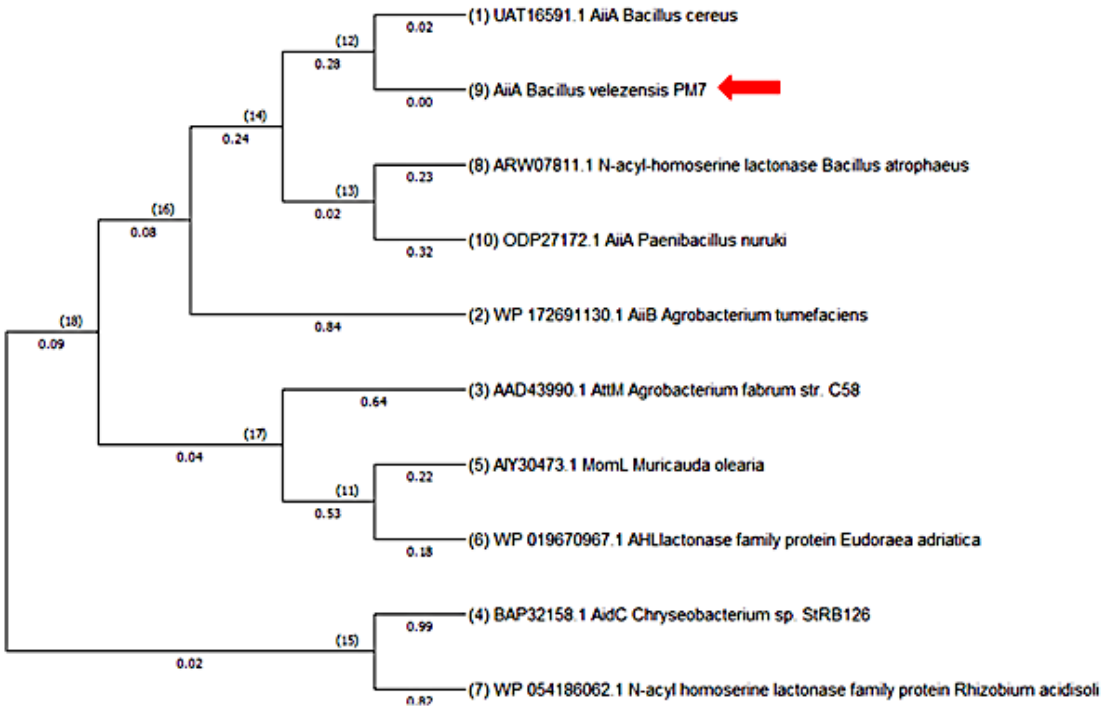


Fig. 4.9 Neighbour-joining phylogenetic tree showing the evolutionary relationship between the AiiA$_{PM7}$ and other major known AHL lactonases. The evolutionary distance was computed using Poisson correction and are in the units of the number of amino acid substitutions per base.

The putative three-dimensional structure of AiiA$_{PM7}$ was constructed using homology modelling by SWISS-MODEL server with the N-Acyl Homoserine Lactone hydrolase of *B. thuringiensis* (2a7m.1.A) as the template. The projected model has a QMEAN4 score of 0.861 and features two potential locations for Zn^{2+} ion binding, both of which are essential for the appropriate alignment of the substrate with the enzyme active site and charge stabilization (Fig. 4.10). The modelled structure was validated and refined using Molprobity server (Fig. 4.11).

The aiiA homologs reported in the earlier studies were exclusively showing intracellular expression (Anandan & Vittal, 2019; Rajesh & Rai, 2014; Shankar et al., 2014). However, the extracellular expression of AHL lactonases, as observed in this study, was reported only in marine bacteria (Packiavathy et al., 2021a; Tang et al., 2015). Also, the previously reported secretory AHL lactonase is encoded with an N-terminal signal peptide for its outer cellular transport (Tang et al., 2015). However, the AiiA$_{PM7}$ sequence lacked such signal peptides. Hence, further analyses are needed to confirm the extracellular localization of the AiiA$_{PM7}$.

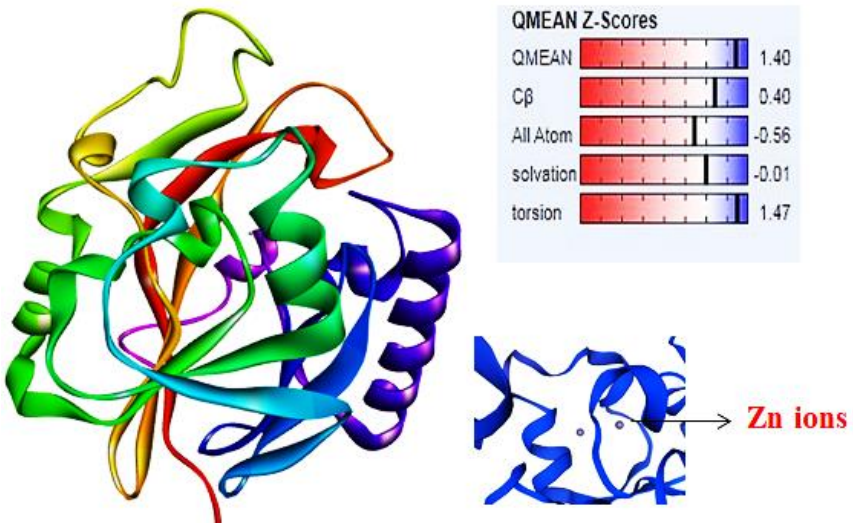


Fig. 4.10 Putative three-dimensional structure of $AiiA_{PM7}$

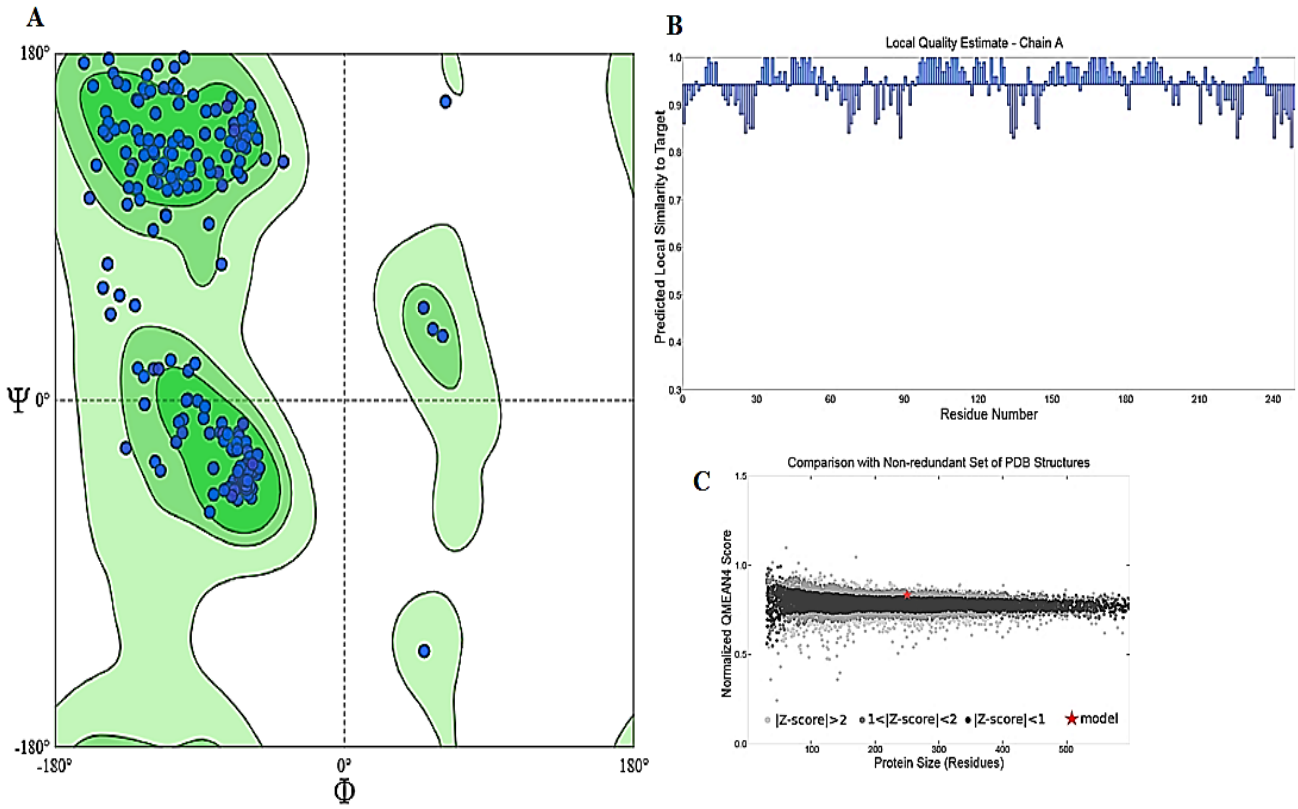


Fig. 4.11 Structure validation of the putative $AiiA_{PM7}$ A) Ramachandran plot B) Local quality estimate C) Comparison with non-redundant set of PDB structures.

The general extracellular secretion mechanism in Gram-positive bacteria includes Sec and Tat transport pathway which is regulated by the N terminal signal peptides present in the protein to be transported (Beckwith, 2013; Stanley et al., 2000). Given the existence of these two systems, around 25 % of released proteins lack identified export signals (Briaud & Carroll, 2020). *Bacillus subtilis*, a model organism for the Gram-positive category, is reported to produce extracellular vesicles for the purpose (Brown et al., 2014). Since *B. velezensis* belongs

to the *B. subtilis* group, it is conceivable that vesicular transport could be responsible for the extracellular localization of the lactonase enzyme.

4.3.5 *In silico* analysis of AiiA$_{PM7}$ binding affinity towards AHL molecules

The catalytic interaction of the predicted AiiA$_{PM7}$ protein with 19 different AHL molecules was analyzed through molecular docking. Both unsubstituted and substituted (3-Oxo and 3-Hydroxy) AHLs with varying N-acyl side chain lengths were selected. A total of 2128 poses were generated for the ligands in the docked complex. With the lactone ring positioned at an adequate distance, enabling efficient hydrolysis, all tested ligands interacted well with the predicted protein's catalytic site. This demonstrates the enzyme's broad substrate specificity. However, the affinity of the enzyme/LibDock score towards the AHL increases with the N-acyl side chain length, with some exceptions due to the substitutions (Table 4.3). For example, the N-(3-hydroxy Dodecanoyl)-L-HSL (3-OH-C_{12}-HSL) is having higher LibDock score (122.83) than N-(3-hydroxy Decanoyl)-L-HSL (3-OH-C_{10}-HSL). Weak bonds such as hydrophobic interactions caused by varying substrate orientations in the enzyme active site account for the differences in binding affinity towards specific AHLs (Liu et al., 2008).

With the general trend being this, substitutions in the acyl chain play an important role in rendering the affinity. For AHLs with the same number of carbon atoms in the side chain, the greatest LibDock score was for 3-hydroxy substituted, followed by 3-Oxo substituted and unsubstituted AHLs. In the case of C_{12}-HSLs, 3-OH-C_{12}-HSL has the highest affinity (LibDock score- 122.83) towards the enzyme with eight hydrophobic interactions, three hydrogen bonds and two metal (Zn^{2+}) acceptor interactions to the AiiA$_{PM7}$ active site. Regarding 3-O-C_{12}-HSL (116.88), the number of interactions decreases to 6 hydrophobic interactions, two hydrogen bonds and one metal acceptor interaction. For the unsubstituted C_{12}-HSL (101.88), only hydrophobic interaction (11) and Hydrogen bond (1) are forming in the enzyme-substrate complex (Fig 4.12)

N-acyl homoserine lactonases have been known to have broad substrate preferences. The AiiA lactonase homologues AiiA$_{KMMI17}$ (Anandan & Vittal, 2019), AhlX (P. Liu et al., 2019) and AiiA$_{B546}$ (Chen et al., 2013) exhibited catalytic affinity towards a wide range of AHL molecules. According to Aslanli & Efremenko, (2020), the strength of the interaction between the AHLs and the surface of enzymes increases with an increase in the acyl side chain length. Also, the presence of substitutions elevates the affinity towards the active site.

Table 4.3 Binding affinity of the putative $AiiA_{PM7}$ protein towards different AHL molecules

Sl. No.	AHL	Formula	LibDock Score
1	N-(3-hydroxy Dodecanoyl)-L-HSL	3-OH-C_{12}-HSL	122.832
2	N-(3-oxo Dodecanoyl)-L-HSL	3-O-C_{12}-HSL	116.88
3	N-(3-hydroxy Decanoyl)-L-HSL	3-OH-C_{10}-HSL	109.233
4	N-Dodecanoyl-DL-HSL	C_{12}-HSL	106.474
5	N-(3-oxo Decanoyl)-L-HSL	3-O-C_{10}-HSL	104.735
6	N-(3-myristoyl)-DL-HSL	C_{14}-HSL	102.329
7	N-(3- oxo Nonanoyl)-L-HSL	3-O-C_9-HSL	101.88
8	N-(3-hydroxy Octanoyl)-L-HSL	3-OH-C_8-HSL	101.732
9	N-Decanoyl-L-HSL	C_{10}-HSL	101.061
10	N-(3-oxo Octanoyl)-L-HSL	3-O-C_8-HSL	98.6415
11	N-Octanoyl-L-HSL	C_8-HSL	95.1216
12	N-(3-hydroxy Hexanoyl)-L-HSL	3-OH-C_6-HSL	92.9243
13	N-(3-oxo Hexanoyl)-L-HSL	3-O-C_6-HSL	92.4479
14	N-Heptanoyl-L- HSL	C_7-HSL	90.8091
15	N-Hexanoyl-L-HSL	C_6-HSL	86.5227
16	N-(3-hydroxy Butanoyl)-L-HSL	3-OH-C_4-HSL	85.6587
17	N-(3-oxo Butanoyl)-L-HSL	3-O-C_4-HSL	84.4456
18	N-Pentanoyl-L-HSL	C_5-HSL	84.2557
19	N-Butyryl-L-HSL	C_4-HSL	81.0254

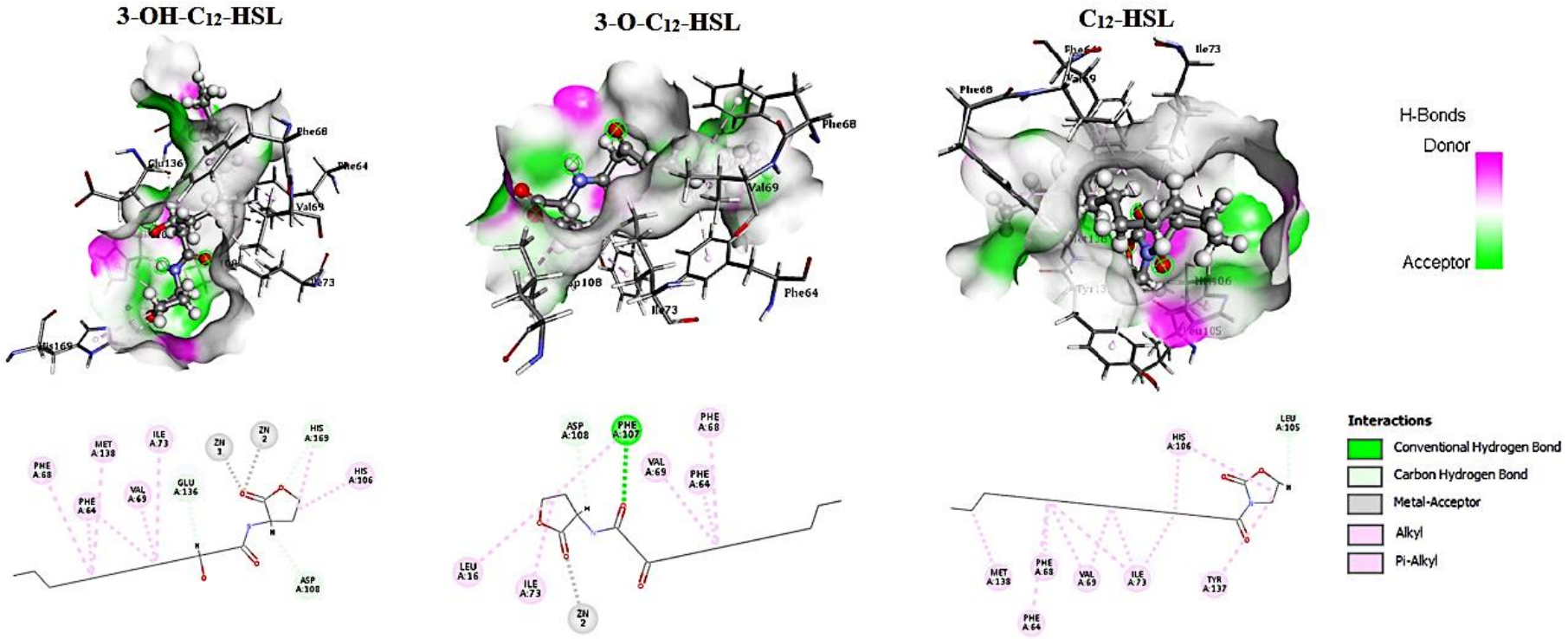


Fig. 4.12 Molecular docking analysis of AHL signals with different substitutions and an equal number of carbon atoms (C12) in the acyl side chain. The images represent product-bound forms of putative $AiiA_{PM7}$ active sites with 3-OH-C_{12}HSL (A), 3-O-C_{12}HSL (B) and C_{12}HSL (C) and 2D representations of the AHL molecules with their interactions with the active site amino acids

4.3.6 *In vitro* analysis of AHL degradation

The lactonolysis activity of *B. velezensis* PM7 CFS towards 5 different AHL molecules *viz.*, 3-OH-C_{12}-HSL, 3-oxo-C_{10}-HSL, 3-oxo-C_8-HSL, C_6-HSL and C_4-HSL were tested using the biosensor strain *C. violaceum* CV026. The concentration of all the AHL molecules was significantly reduced with CFS treatment. However, a complete degradation was observed in the case of 3-OH-C_{12}-HSL only, indicated by the absence of depigmented zone in the assay plates. The lowest degradation was obtained for C4-HSL (6.81%). The degradation of AHLs by CFS increased with the length of the acyl chain (Fig. 4.13 & Fig. 4.14). This observation is consistent with the molecular docking data, which suggest the extracellular presence of AHL lactonase in *B. velezensis* PM7.

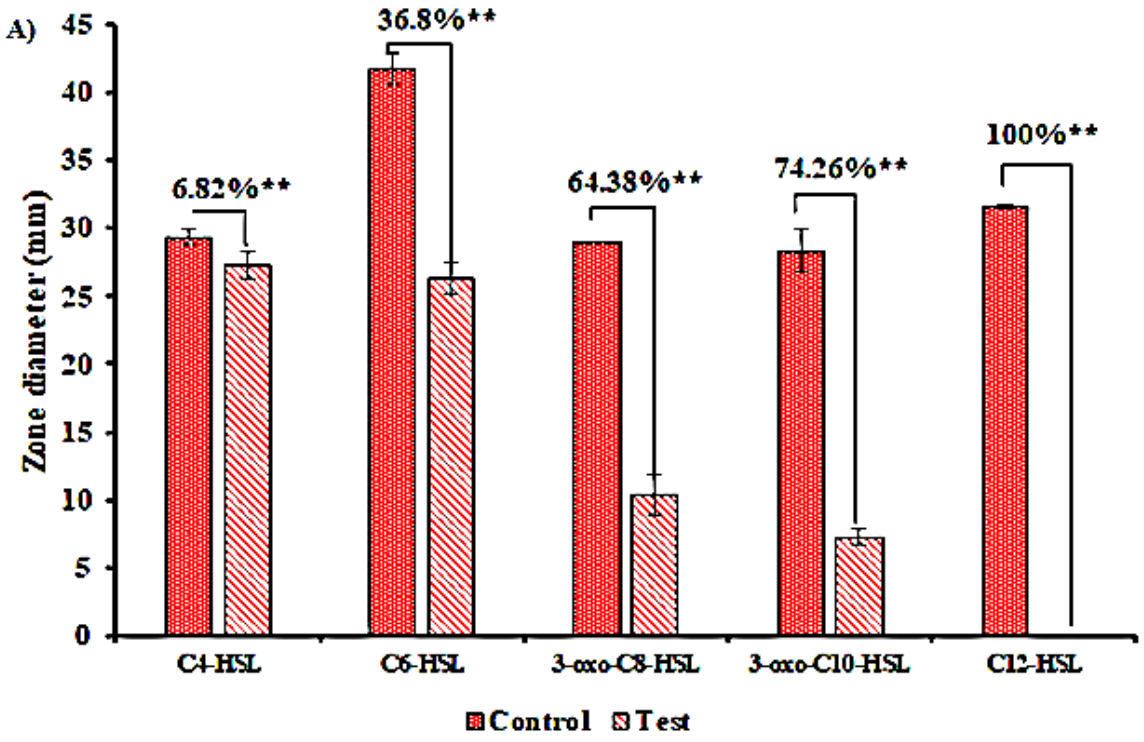


Fig. 4.13 *In vitro* analysis of the AHL degradation by *B. velezensis* PM7. A) Data represents the affinity of *B. velezensis* PM7 towards different AHL molecules measured in terms of reduction in zone diameter as compared to the untreated control. **p<0.05, unpaired t-test.

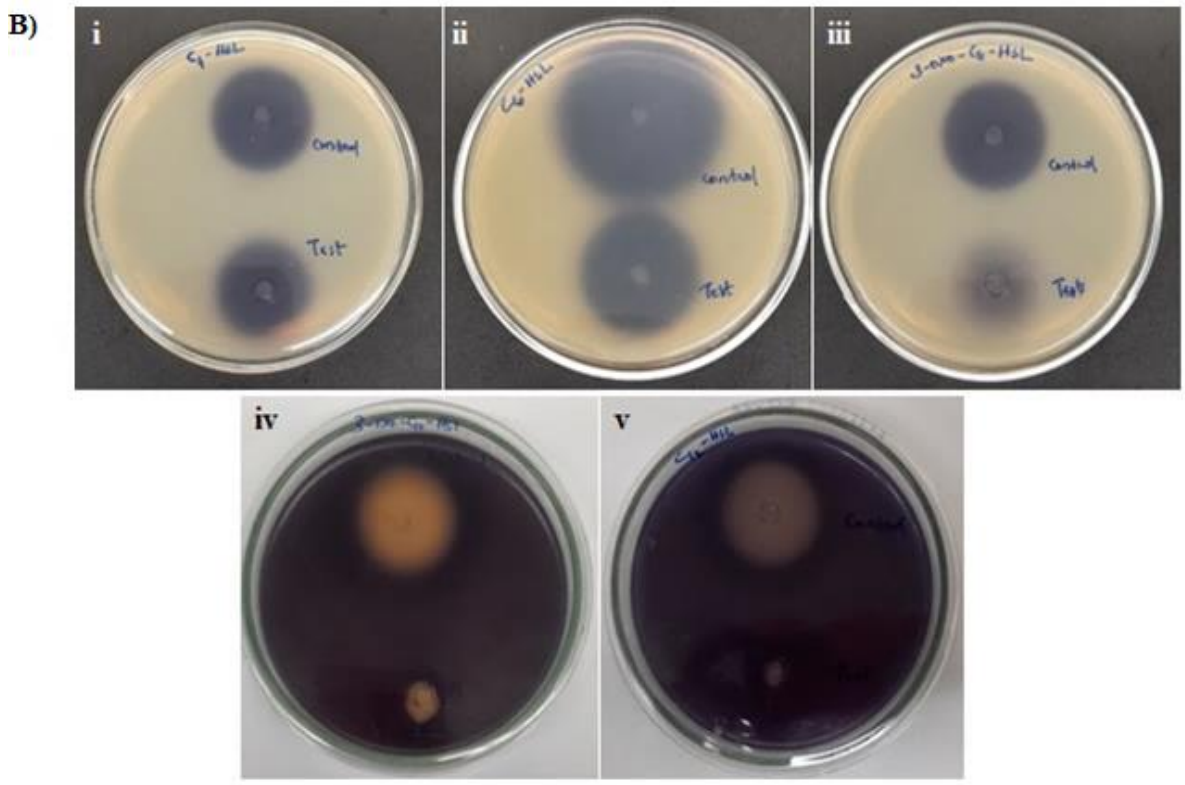


Fig. 4.14 *Chrombacterium violaceum* CV026 plates showing pigmentation/depigmentation zones in response to untreated (upper zone) and CFS-treated (lower zone) AHLs. Purple zones in culture plates indicate the presence of short chain AHLs i- C_4-HSL, ii-C_6-HSL, iii-3-Oxo-C_8-HSL and depigmented zones in plates supplemented with C_6-HSL indicate long chain AHLs iv- 3-Oxo-C_{10}-HSL and v- C_{12}-HSL.

4.4 Chapter Summary

The QS disruption can be achieved by inactivating autoinducer synthases/receptors with the quorum sensing inhibitors (QSI) or degrading autoinducers with quorum quenching (QQ) enzymes. The most widely studied class of QQ enzymes is AHL lactonases which hydrolyze the lactone ring, forming N-acyl homoserine. The mechanism of extracellular QQ in *B. velezensis* PM7 was elucidated in this chapter. Heat, solvents and proteinase K treatment abolished the QQ activity of the CFS indicating the proteinaceous nature of the underlying mechanism. Analytical studies of the AHL degradation using LC-MS and bio-reporters revealed the mechanism of QQ as lactone ring hydrolysis. The observations suggested the presence of an extracellular AHL lactonase enzyme in the CFS. Subsequently, a homolog of N-acyl homoserine lactonase ($aiiA_{PM7}$) was amplified from *B. velezensis* PM7 genome. The genomics and proteomics analysis of the homolog affirmed its similarity to *B. thuringiensis* AHL lactonase (aiiA-B22) gene/protein. The putative three-dimensional structure of the protein was formulated using homology modelling from the protein sequence data. The modelled structure was docked against 19 AHL molecules, revealing a broad-spectrum affinity of the enzyme towards all the ligands. The affinity was found decreasing with the decrease in the number of C atoms in the side chain, which was confirmed using the biosensor *C. substugae.* Due to the extracellular expression and wide spectrum substrate specificity, $AiiA_{PM7}$ could be used as a promising candidate for QQ applications.

Chapter 5

Application of *Bacillus velezensis* PM7 in Gram-negative Bacterial Biofilm Control

5.1 Introduction

Quorum quenching is considered as a vital process with ecological and evolutionary importance. The co-existence, competition and adaptation of microbial communities in their respective environment are highly influenced by the QS-QQ interactions. The transformation of bacteria from their planktonic form to the sessile substrate-attached biofilm form is a remarkable adaptation mechanism (Skariyachan et al., 2018). The biofilm formation occurs ubiquitously in almost all biotic and abiotic surfaces and this complex process is governed by quorum sensing regulatory networks. The composition and architecture of biofilms vary drastically among different species and substrata to which the bacteria attach (Kalgudi, 2018). Besides these, environmental factors like nutrient gradient, supply of oxygen, shear stress, temperature, and pH also contribute significantly. Due to a high degree of tolerance towards the stress factors, complete removal of biofilms from surfaces is hardly possible (El-Mowafy et al., 2014). Therefore, biofouling has become a significant concern in the industrial (Vishwakarma, 2020), and environmental and health sectors (Pinto et al., 2021). In this scenario, applying quorum quenching agents has emerged as a promising strategy in biofilm mitigation (Oh & Lee, 2018; Syafiuddin et al., 2021; Utari et al., 2018). The lack of bactericidal and bacteriostatic activity imparts less selection pressure in counteracting organisms, and thus the possibility of resistance development is low (Fetzner, 2015). Hence, this chapter explores the application of *B. velezensis* PM7 in biofilm formation and dispersion regulation on medically and industrially relevant surfaces. The catalytic efficiency of the bacterium's extracellular lactonase activity and the physical and chemical parameters affecting the stability and activity of extracellular lactonase are also analyzed here.

5.2 Materials and Methods

5.2.1 Estimating the catalytic activity of extracellular lactonase

The catalytic activity of the extracellular lactonase was assessed as reported earlier with slight modification (Sakr et al., 2013). Briefly, 500 µL crude CFS was incubated with AHLs at 30 °C with shaking at 150 rpm. C_6-HSL (10 µM) and 3-OH-C_{12}-HSL (30 µM) were the selected AHLs for the study. Samples were taken at the time intervals of 30 minutes up to 4 hr. The reaction was terminated by adding 2% SDS. The residual amount of AHLs was analyzed using AHL biodegradation assay with *C. substugae* as elaborated in section 5.2.9. C_6-HSL and 3-OH-C_{12}-HSL were quantified by measuring the violacein-pigmented and depigmented zone formed, respectively and comparing them to the standard curve showing the diameter of the zone as a function of different AHL concentrations. One unit of AHL lactonase activity was defined as the amount of enzyme hydrolysed 1nM of corresponding AHL.

5.2.2 Determination of kinetic parameters

The crude CFS was incubated with different concentrations of C_6-HSL and 3-OH-C_{12}-HSL for 90 minutes at 30 °C with shaking at 150 rpm. The reaction was terminated by adding 2% SDS. As previously mentioned, the residual AHL concentration was measured by AHL biodegradation assay. The initial velocity of the AHL degradation reactions in each tube was calculated using the formula, Rate, $V_0 = -d[A]/dt$; A- AHL concentration, t – time in minutes. The data was plotted into a Lineweaver-Burk plot, and the kinetic parameters, Km and Vmax, were calculated from the graphs (Wang et al., 2004).

5.2.3 Effect of temperature on the enzyme stability and catalytic activity.

For measuring the thermal stability of lactonase, 1 mL crude CFS (containing 146 units of enzyme concerning C_6-HSL) was incubated at different temperatures (10 – 90 °C) for 60 minutes. After the incubation, the CFS was used for flask incubation assay (10% v/v) with *C. substugae* supplemented with 10 µM C_6-HSL. The violacein produced in each flask was extracted in DMSO and absorbance at 585 nm was noted, and percentage inhibition by CFS was calculated by comparing to the untreated control.

The effect of temperature on the catalytic activity of the lactonase was analyzed by incubating the CFS along with 10 µM C_6-HSL at different temperatures for 60 minutes. The changes in AHL degradation property of the CFS after the incubation was detected using AHL

biodegradation assay. The percentage activity was calculated by comparing to the untreated control.

5.2.4 Effect of pH on the enzyme stability and catalytic activity.

The assay for analyzing the effect of pH on the storage stability was carried out as previously described, with slight modifications (Sakr et al., 2013). Briefly, 1 mL of the crude CFS was brought to the desired pH using suitable buffers. The selected buffers are as follows: Citrate-phosphate buffer (pH range 3.0-6.0), Phosphate buffer (pH 6.0-8.0), Tris-HCl buffer (pH 8.0-9.0) and Glycine- NaOH buffer (9.0-10.0). The tubes were incubated at 30 °C for 60 minutes, and the reaction was terminated by neutralizing to pH-7 using NaOH or HCl appropriately. The treated CFS' were subjected to flask incubation assay as mentioned above, and percentage activity was calculated by comparing to the untreated control.

For measuring the influence of pH on the catalytic activity, the crude CFS was modified to different pHs using buffers and mixed with 10 µM C_6-HSL. The mixture was kept at 30 °C for 60 minutes, and the reaction was terminated by adding 2% SDS. The residual amount of C_6-HSL was estimated with AHL biodegradation assay and percentage activity was calculated.

5.2.5 Effect of metal ions, oxidizing agents, surfactants and inhibitor compounds on the stability of extracellular lactonase

The effect of various classes of chemicals on the catalytic activity of CFS was analyzed by similar experiments. The compounds used, and their test concentrations are given in Table 6.1. One mL crude CFS (146 enzyme units) was treated with these agents for 60 minutes at 30 °C and incubated with 10 µM C_6-HSL for 90 minutes. The reaction was stopped by adding 2% SDS. The residual AHL concentration was measured using the AHL biodegradation assay.

Table 5.1 Treatment agents and concentration range used to assess CFS catalytic in their presence.

Treatment agent	Concentrations	
	Higher	**lower**
Metal ions		
1. $FeSO_4$	1 mM	10 mM
2. $MgSO_4$	1 mM	10 mM
3. $CaSO_4$	1 mM	10 mM
4. $ZnSO_4$	1 mM	2 mM*
5. $CuSO_4$	1 mM	2 mM*
Oxidizing agents		
1. H_2O_2	0.1%	1%
2. NaOCl	0.1%	1%
3. Na_2BO_3	0.1%	1%
Surfactants		
1. Triton-X-100	0.1%	1%
2. Tween- 40	0.1%	1%
3. Tween -80	0.1%	1%
4. SDS	0.1%	1%
Inhibitors		
1. EDTA	2 mM	5 mM
2. Urea	2 mM	5 mM
3. PMSF	2 mM	5 mM
4. β -mercaptaethanol	2 mM	5 mM

5.2.6 Effect of CFS on biofilm formation

The effect of *B. velezensis* PM7 CFS on the biofilm formation was tested using static microtiter plate assay by Anandan & Vittal, (2019) with modifications. The test bacteria (*C. violaceum* NIIST, *S. marcescens* NIIST and *P. aeruginosa* ATCC 27853) were grown in LB broth (Himedia, India) for 12 hrs. at 30 °C under shaking incubation at 180 rpm. The initial bacterial density (OD 600) was set to 0.01. Freshly inoculated culture (190 µl) along with filter sterilized (0.22 µm) 100 µl CFS (14.6 enzyme units for 10% v/v) were dispensed into a 96-well microtiter plate. Untreated cultures were used as the positive control. The plate was incubated at 30 °C statically for 24 hrs. The planktonic cells were decanted, and respective cell density was read at 600 nm. Biofilms formed were stained with 100 µl of crystal violet [0.1%

(w/v) in water] and incubated for 15 min at room temperature and then washed thoroughly with sterile distilled water. The residual crystal violet was dissolved in 95% ethanol and the absorbance was measured at 550 nm.

5.2.7 Effect of CFS on biofilm maturation

The effect of PM7 CFS on the biofilm maturation was also tested using a static microtitre plate assay (Djokic et al., 2022). *P. aeruginosa* was grown in LB broth (Himedia, India) for 12 hrs. at 30°C under 180 rpm. The initial bacterial density (OD 600) was set to 0.01, and 190 µl of the bacterial culture was dispensed into the wells of 96 well plates that were incubated at 37°C for 48 hours. After incubation, CFS was added to the wells in a concentration ranging from 1% – 10% (14.6 enzyme units for 10% v/v) in all the wells except for the positive control. The total volume of the well was made up to 200 µl using sterile LB broth. The plate was incubated at 37 °C for 18 – 24 hours. The planktonic cells from the wells were decanted after the incubation, and the non-adherent cells were washed out using sterile PBS. Subsequently, the biofilm was fixed using 0.2% sodium acetate treatment for 15 minutes. Biofilm was quantified using the crystal violet method, as described earlier.

5.2.8 Confocal Laser Scanning Microscopy (CLSM)

A non-invasive procedure was adopted for visualizing the biofilm architecture via confocal laser scanning microscopy. *P. aeruginosa* biofilms with and without CFS treatment for 48 hr. were prepared on glass coverslips. To assess the impact of CFS on biofilm maturation, 24 hr. old biofilms formed on coverslips surfaces were placed into fresh medium containing 10% (v/v) CFS and incubated for 24 hrs. The biofilms were later aseptically retrieved , fixed and stained with acridine orange according to a method adapted from Lynch et al., (2002). Briefly, the coverslips were stained with 100 µL of 0.1% acridine orange and incubated for 2 minutes. The excess stain was removed by washing with sterile PBS buffer; for imaging using CLSM, a Zeiss LSM 980 (Airyscan 2) (Zeiss, Germany) with an argon/ krypton laser was used. The CLSM was run in conjunction with Zen Blue image analysis software. The excitation wavelength used was 488 nm. Samples were scanned in the z-direction to give an overall thickness, generating a series of x-y images.

5.2.9 Anti-biofilm Effect of *B. velezensis* PM7 on different surfaces

The anti-biofilm effect of *B. velezensis* PM7 CFS was tested on different material surfaces such as stainless steel, borosilicate glass and urinary catheters. The test bacteria used

for the analysis were *P. aeruginosa*, *S. marcescens* and *C. violaceum.* The bacteria were grown in LB broth for 12 hr at 30 °C under shaking incubation under 180 rpm. The initial bacterial density (OD 600) was set to 0.01 and inoculated into fresh LB broth supplemented with PM7 CFS at a concentration of 10% (v/v) (146 enzyme units). The testing materials, such as steel coupon, glass slides and catheter fragments, were immersed in the bacterial culture and incubated at 37 °C for 72 hrs. under static conditions. Bacterial cultures with the test material and devoid of PM7 CFS and un-inoculated LB broth with the test material were used as the positive and negative controls, respectively.

a) Crystal Violet Assay

Quantification of biofilms on the tested materials was carried out using a crystal violet assay (Viszwapriya et al., 2016). The tested materials were removed from the culture and washed with sterile PBS to remove non-adherent cells. The materials were then soaked in 0.1% (w/v) crystal violet solution for 45 minutes, after which they were allowed to dry at room temperature. The adherent crystal violet was dissolved in 20% acetic acid, and OD was measured at 595 nm.

b) Extraction of EPS from the tested surfaces

The Extra Polymeric Substance (EPS) from the biofilm formed on the different tested materials was extracted using formaldehyde-NaOH as reported with slight modifications (Pan et al., 2010). Briefly, after the incubation for biofilm formation, the test material was aseptically retrieved from the bacterial culture and suspended in sterile PBS. The total biofilm formed on the surfaces was transferred to the PBS by vigorous vortexing for 2 minutes and subsequent sonication (1 min). The cycle was repeated up to 5 times. To this biofilm-infused PBS solution 6% (v/v) formaldehyde was added and incubated at 4°C for 60 minutes. Subsequently, 40% (v/v) 1M NaOH was added and again incubated at 4°C for 3hr. This biofilm dispersion was centrifuged at 11000g, and the supernatant was filtered through 0.2 µm filter paper to remove cell debris.

c) Exopolysaccharide Quantification

The exopolysaccharide from the biofilm was estimated using phenol–sulphuric acid method (Oliveira et al., 1999). 50 µL of 80% phenol and 5 mL of 95% sulphuric acid were added to the extracted EPS suspension. The suspensions were incubated at 25 °C for 30 minutes and then incubated at room temperature for 4 hr. The absorbance of the reaction mixture was

taken at 480 nm. Sterile PBS treated with a phenol-sulphuric acid mixture was used as the blank. The concentration of exopolysaccharide was calculated using the standard curve prepared using glucose as the substrate.

d) Total Protein Quantification

To the extracted EPS suspension (1 mL), add 4.5 ml reagent I (2% Na_2CO_3 in 0.1N NaOH + 1% sodium potassium tartrate + 0.5% $CuSO_4.5H_2O$) and incubated for 10 minutes. To this solution, 0.5 ml reagent II (1:1 Folin reagent and water) was added and incubated for 30 minutes. The absorbance of the resultant solution was taken at 660 nm. Sterile PBS treated with Lowry's reagents was used as the blank. The concentration of the protein was estimated from the standard curve prepared by using Bovine Serum Albumin (BSA) as the substrate (Felz et al., 2019)

e) eDNA Quantification

The biofilm's extracellular DNA (eDNA) concentration was quantified according to Allesen-Holm et al. (2006) with modification. As previously mentioned, the biofilm-formed tested materials were aseptically transferred to sterile PBS and subjected to alternative cycles of sonication and vortexing. 200 µL of the suspension was used for measuring the OD600. The remaining suspension was then added with 0.05 mM Propidium Iodide and incubated at 28 °C for 30 minutes. The fluorescence of the PI-bound eDNA was measured at excitation/emission 535/610 nm. The data was normalized by dividing it with the OD 600 values.

f) Cell Viability Analysis

The fraction of viable cells on the tested material surface was calculated using plate count and fluorescent microscopy-assisted direct cell counting (Winding et al., 1994). For the same, biofilm formed on the surfaces was completely dispersed into sterile PBS in aseptic conditions. The suspension was then spot-plated on plate count agar from a dilution of 10^{-1} to 10^{-7} for measuring the viable cell count of the biofilm formed. For measuring the total cell count, the biofilm suspension was added with Acridine Orange (0.1 mg/mL) solution and incubated at room temperature in the dark for 10 minutes. The samples were then filtered through a black polycarbonate filter (DMF no. 111156; pore size: 0.2 µm.). The filter was rinsed with 50 mL of sterile distilled water and dried in a hot air oven at 50°C. The preparation was then hydrated with a drop of buffered glycerine and covered with a coverslip. The filters were examined under an epiflourescent microscope (excitation filter 460/526 nm) with an oil

immersion objective (100X). Highly and poorly fluorescent cells in 30 fields were counted, and the result was expressed as cell/mL. The proportion of viable cells was calculated by dividing the viable plate count by total cell count.

5.2.10 Effect of *B. velezensis* PM7 CFS on biofilms formed under dynamic conditions

To assess the effect of *B. velezensis* PM7 CFS in the biofilm formation under dynamic flow, a continuous flow system was designed. (Fig. 5.1) Two 500 ml screw cap bottles (Schott Duran), each with 150 ml of LB broth containing sodium citrate (2 g/L) and 3 ml of *P. aeruginosa* inoculum adjusted to 0.5 McFarland standard units, were used for this study. One was maintained as a test, and the other as control. To the test bottle cell-free supernatant was added (10% v/v, 2190 enzyme units). Whereas the control bottle was maintained without the CFS treatment. Folley's urinary catheter (medical grade silicon) was aseptically inserted into the culture bottles. With the help of a peristaltic pump, the bacterial suspension with the medium from the bottle was re-circulated through the catheter tube by maintaining a flow rate of 20 mL/min. The liquid recirculation was continued for 15 days; during that period, the medium was replenished every 48 hr. After developing a prominent biofilm within, the tubes were removed from the pump, and the biofilm formed within the tube was quantified and quantified differentially. The total exopolysaccharide formed and protein formed was estimated as per the methods described above (section 6.2.9.3, 6.2.9.4). The biofilm formed was separated into soluble microbial products (SMP), loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) according to standard procedures, and the components of the same were differentially estimated (Xing et al., 2020).

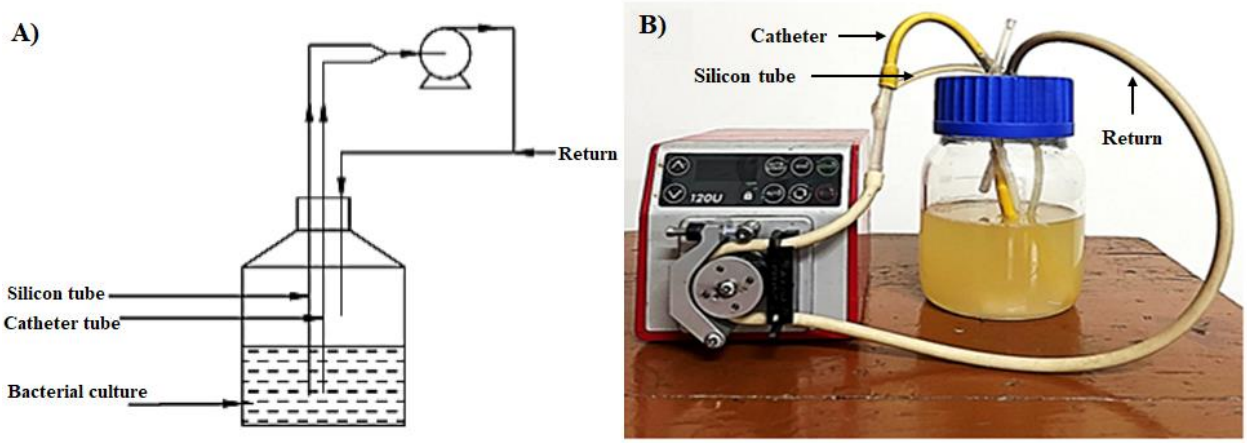


Fig. 5.1 Effect of PM7 CFS on biofilms formed by *P. aeruginosa* under dynamic conditions **A)** Diagrammatic representation and **B)** Experimental set-up of the biofilm formation

5.2.11 Effect of *B. velezensis* PM7 CFS on biofilm dispersal

The effect of PM7 CFS on the biofilm dispersion was analysed according to (Johansson et al., 2008) with modifications. Briefly, the test materials for biofilm formation *viz.*, sterile stainless-steel coupons, borosilicate glass slides and urinary catheter fragments, were incubated in nutrient broth inoculated with *P. aeruginosa* for 48 hrs. at 37 °C. The spent medium from the culture was aspirated, and the test materials with preformed biofilms were gently washed with 0.5 % nutrient broth. To this material, 0.5% nutrient broth containing 10% (v/v) CFS (292 enzyme units) was added and incubated at 37 °C for 2 hrs under shaking at 60 rpm. After the incubation, the media was aseptically collected and briefly vortexed. Samples were taken from the suspension and after appropriate serial dilution, viable cell count was estimated on plate count agar. Tested materials incubated in uninoculated nutrient broth were taken as the control. The experiments were carried out in triplicates and the values were expressed with standard deviation.

5.2.12 Metagenomic analysis for identifying the effect of PM7 CFS on the bacterial community structure

The extracellular quorum quenching effect *B. velezensis* PM7's on the bacterial community structure in activated sludge was analysed. Freshly collected activated sludge from the SWTP, Muttathara, Thiruvananthapuram, Kerala, was used for this study. It was seeded into synthetic wastewater at 0.1 g/mL level. To examine the effect of CFS, the suspension was treated with 10% (v/v) crude CFS (730 enzyme unit) and incubated at 32°C for seven days under shaking at 150 rpm. Meanwhile, the wastewater with activated sludge alone was maintained as the positive control. After incubation, the total DNA was extracted from the suspension and subjected to Next Generation Sequencing (NGS). From this, 25 ng of DNA was used to amplify 16S rRNA hypervariable region V3-V4. The reaction mixture used was KAPA HiFi HotStart Ready Mix and 100 nm final concentration of modified 341F and 785R primers. The amplicons were purified using Ampure beads to remove unused primers. Additional eight cycles of PCR were performed using Illumina barcoded adapters to prepare the sequencing libraries. The sequence data were generated using Illumina MiSeq. The data was checked for quality parameters and sequencing adapter contamination.Adapter sequences used:

P7 adapter read1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

P5 adapter read2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT.

Data Analysis

The reads were trimmed (20 bp) from the 5’ end to remove the degenerate primers and processed to remove adapter sequences and low-quality bases using Trimgalore. The QC passed reads were imported into Mothur, and the pairs were aligned to form contigs. The contigs were screened for errors, and only those between 300 bp and 532 bp were retained. Any contig with ambiguous base calls was rejected. The high-quality contigs were checked for identical sequences, and duplicates were merged. The contigs were aligned to a known database for 16s rRNA for eliminating non-specific amplification. After this process, the gaps and the overhang at the ends from the contigs were removed and processed for chimera removal. UCHIME algorithm was used to flag contigs with chimeric regions. A known reference of all the chimeric sequences was used to identify and remove possible chimeric sequences. The filtered contigs were processed and classified into taxonomical outlines based on the GREENGENES v.13.8-99 database. The contigs were then clustered into OTUs (Operational Taxonomic Units). After the classification, OTU abundance was estimated.

5.3 Results and Discussion

5.3.1 Catalytic activity and kinetic parameters of the crude extracellular lactonase in *B. velezensis* PM7

The crude lactonase's catalytic activity and kinetic parameters from *B. velezensis* PM7 CFS were analyzed here concerning two AHL molecules *viz.*, C_6-HSL and 3-OH-C_{12}-HSL. The initial rate of the reaction was 64.6 nM/min. for C_6-HSL after 30 minutes, which declined to 6.44 nM/min after 4 hr. incubation. In the case of 3-OH-C_{12}-HSL, the initial velocity was 226.21 nM/min. and dropped to 31.28 nM/min. after 5 hr. From the straight-line segment of the residual substrate concentration against time graph (Fig. 5.2), it was calculated that 500 µL of the crude CFS contains approximately 146.05 units/mL of lactonase enzyme concerning C_6-HSL. Whereas in terms of 3-OH-C_{12}-HSL, the enzyme concentration estimated was approximately 296 units/mL.

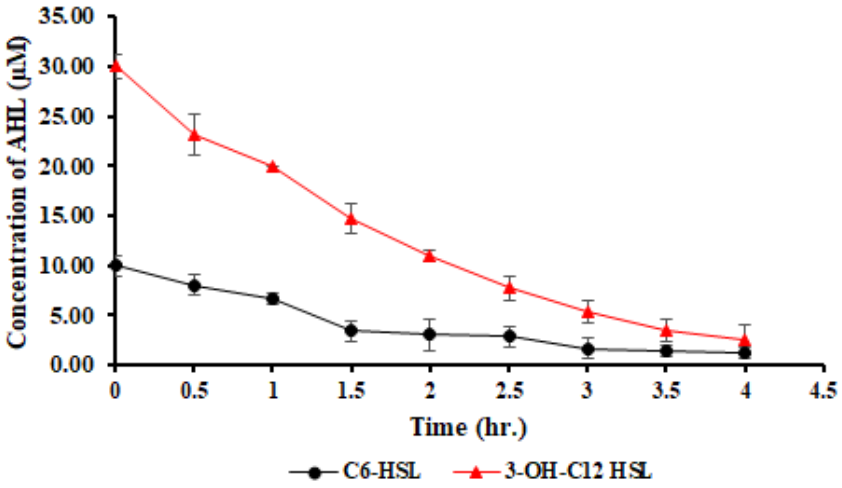


Fig. 5.2 Estimation of the extracellular lactonase catalytic activity of *B. velezensis* PM7 using C_6-HSL and 3-OH-C_{12}-HSL as substrate

The kinetic parameters *viz.*, maximum velocity Vmax and Michaels-Menten constant Km, of the crude extracellular lactonase were estimated with the help of the Lineweaver-Burk (LB) plot for both the AHL molecules (Fig. 5.3). The Vmax for C_6-HSLwas found to be 391.7 nM/min. while Km was 43.2 µM. whereas for 3-OH-C_{12}-HSL, the parameters were 522.4 nM/ min and 37.98 µM, respectively. The lower Km value of the crude lactonase for 3-OH-C_{12}-HSL points towards the higher catalytic affinity of the enzyme active site towards this substrate than C_6-HSL. The substrate preference of AHL lactonases towards long chain AHL were also reported with the help of kinetic data (Tang et al., 2015; Wang et al., 2004). In similar studies,

(Sakr et al., 2013) obtained a Vmax of 460.5 nM/min and Km of 77.13 nM with respect to C_6-HSL for the crude AHL lactonase enzyme obtained from *Bacillus weihenstephanensis.* However, enzyme purification is crucial in enhancing the apparent kinetic parameters. For example, the purified AHL lactonase homologue AiiO-AIO6 reportedly has a Km value of 0.415 mM and Vmax of 16.9 mM/min (Xia et al., 2020).

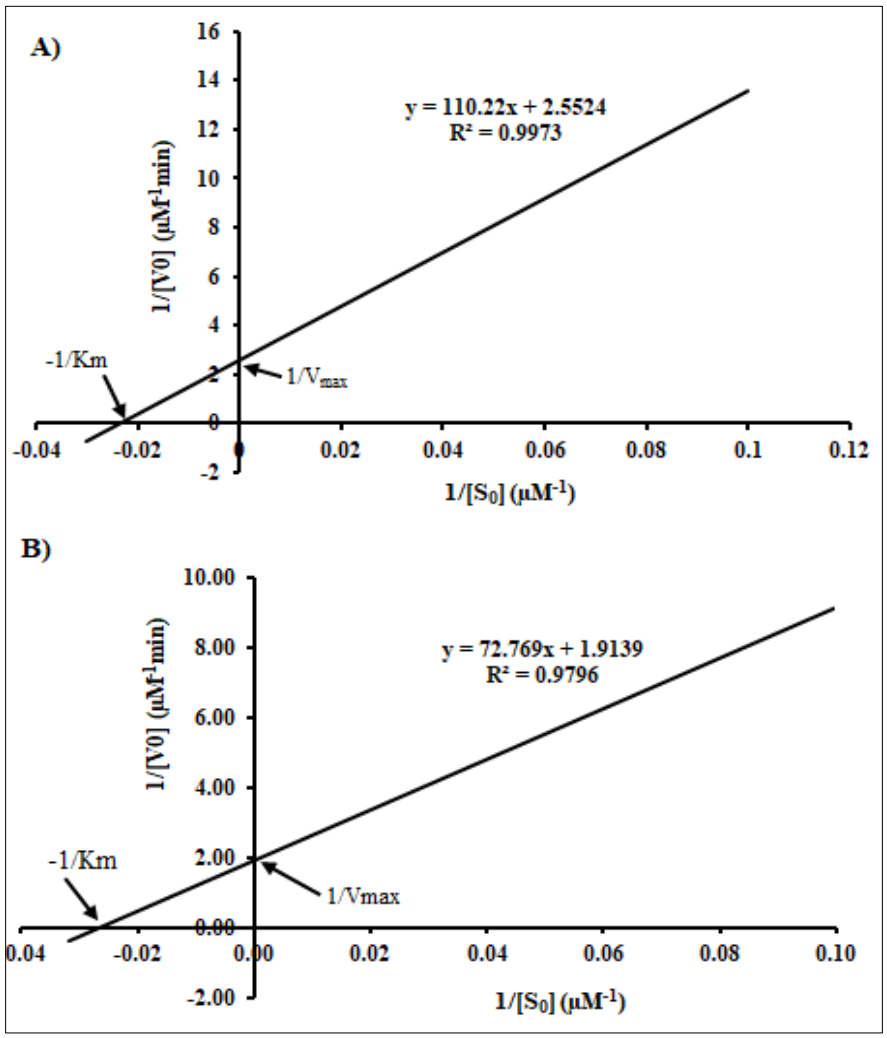


Fig. 5.3 Lineweaver-Burk plot of the crude CFS lactonase activity concerning C_6-HSL(A) and 3-OH-C_{12}-HSL (B)

5.3.2 Effect of temperature on the stability and catalysis of *B. velezensis* PM7 CFS

The crude CFS demonstrated remarkable stability over a wide range of temperatures (Fig. 5.4). From 10-50 °C storage temperature, the CFS retained activity in the 95-100 % range. The enzyme activity declined to 83.2% at 60 °C and completely lost above 90 °C treatment. However, the maximum catalytic efficiency of the crude extract was observed only in the range of 30-50 °C. An increase or decrease of 10 °C resulted in a decline of approximately 85% of the enzyme activity. Similar to the enzyme stability, the catalysis also abolished above 90 °C.

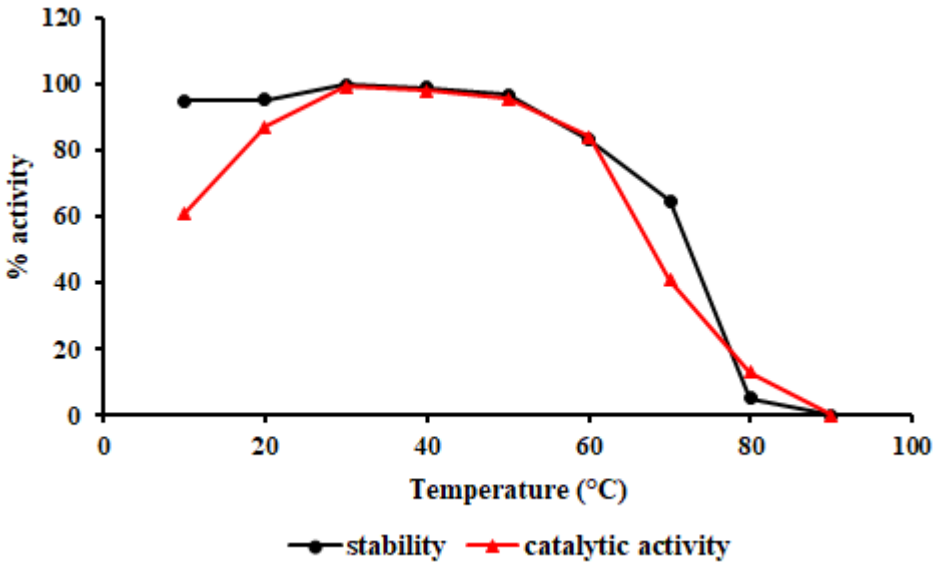


Fig. 5.4 Effect of temperature on the stability and catalytic activity of the crude CFS

Acyl homoserine lactonases demonstrate a wide range of temperature stability. Some of the major AHL lactonase homologs and their optimum temperature ranges are listed in Table 5.2. The PM7 CFS lactonase activity is also comparable with the given examples.

5.3.3 Effect of pH on the stability and catalysis of *B. velezensis* PM7 CFS

As shown in Fig. 5.5, the CFS of *B.velezensis* PM7 was stable in the pH range of 6-8. The optimum pH for storage was observed at pH 7, where 99.76% of its activity was retained. The crude CFS was also found stable under comparatively basic pHs. The optimum pH range for the lactonase catalytic activity was observed in the range of 6-8, where more than 96% activity was retained. However, unlike the stability, a rise in pH beyond eight sharply declined the enzyme activity. The loss of enzyme activity can be attributed to the alkaline hydrolysis property of AHL molecules. The acidic pHs are also not in favor of lactonase activity. Exposure to pH 5 resulted in a decline in activity up to 68.4%.

The optimum pH range of most of the AHL lactonases are reported in the pH range 6-8 (Table. 5.2). A slight variation was reported in the case of the thermophile derived VmoLac and SSPOX from *Sulfobulus* with an optimum pH in the range of 4-7. The stability of the PM7 extracellular lactonase enzyme in a wide pH range can be considered as an added advantage for many applications.

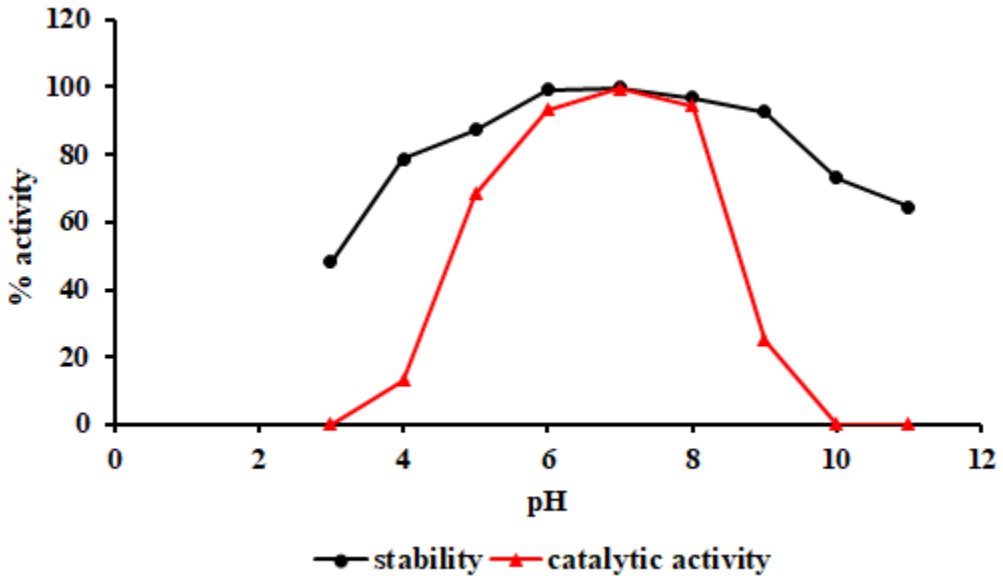


Fig. 5.5 Effect of pH on the stability and catalytic activity of the crude CFS

Table 5.2 Optimum temperature and pH ranges of some AHL lactonase homologs

Enzyme	Source	Temperature range	pH range	Reference
AiiA	*Bacillus licheniformis* DAHB1	30-50 °C	6-8	(Vinoj et al., 2014)
AiiA	*Bacillus Weihenstephansis* P65	28-50 °C	6-8	(Sakr et al., 2013)
AiiA	*Bacillus thuringiensis*KMCL07	18-45 °C	5-7	(Anandan & Vittal, 2019)
AiiA	*Bacillus* sp. A196	10-40 °C	5-7	(Beaz-Hidalgo & Figueras, 2013)
PPH	*Mycobacterium Tuberculosis*	30-50 °C	6-8	(Afriat et al., 2006)
VmoLac	*Vulcanisaeta mountnosvika*	>100 °C	4-7	(Hiblot et al., 2015)
SSOPOX	*Sulfobulus sulfotaricus*	10-100 °C	4-6	(Suzumoto et al., 2020)
AidP	*Antartica planococcus*	<32 °C	4-8	(See-Too et al., 2018)
AiiK	*Kurthia huakui* LAM0618	< 45 °C	6-8	(Dong et al., 2018)

5.3.4 Effect of metal ions, oxidizing agents, surfactants and inhibitor compounds on the stability of extracellular lactonase.

The applied lower concentration (1 mM) of the divalent metal ions Mg^{2+}, Cu^{2}, and Zn^{2+} and both higher and lower concentrations of Ca^{2+} imparted no significant impact on the stability of crude CFS lactonase from the PM7. However, a slight decline in lactonase activity was observed on incubation with higher concentration of Mg^{2+}, Cu^{2}, and Zn^{2+}. The crude lactonase activity was significantly reduced upon Fe2+ treatment at either of its applied concentrations (Fig. 5.6A).

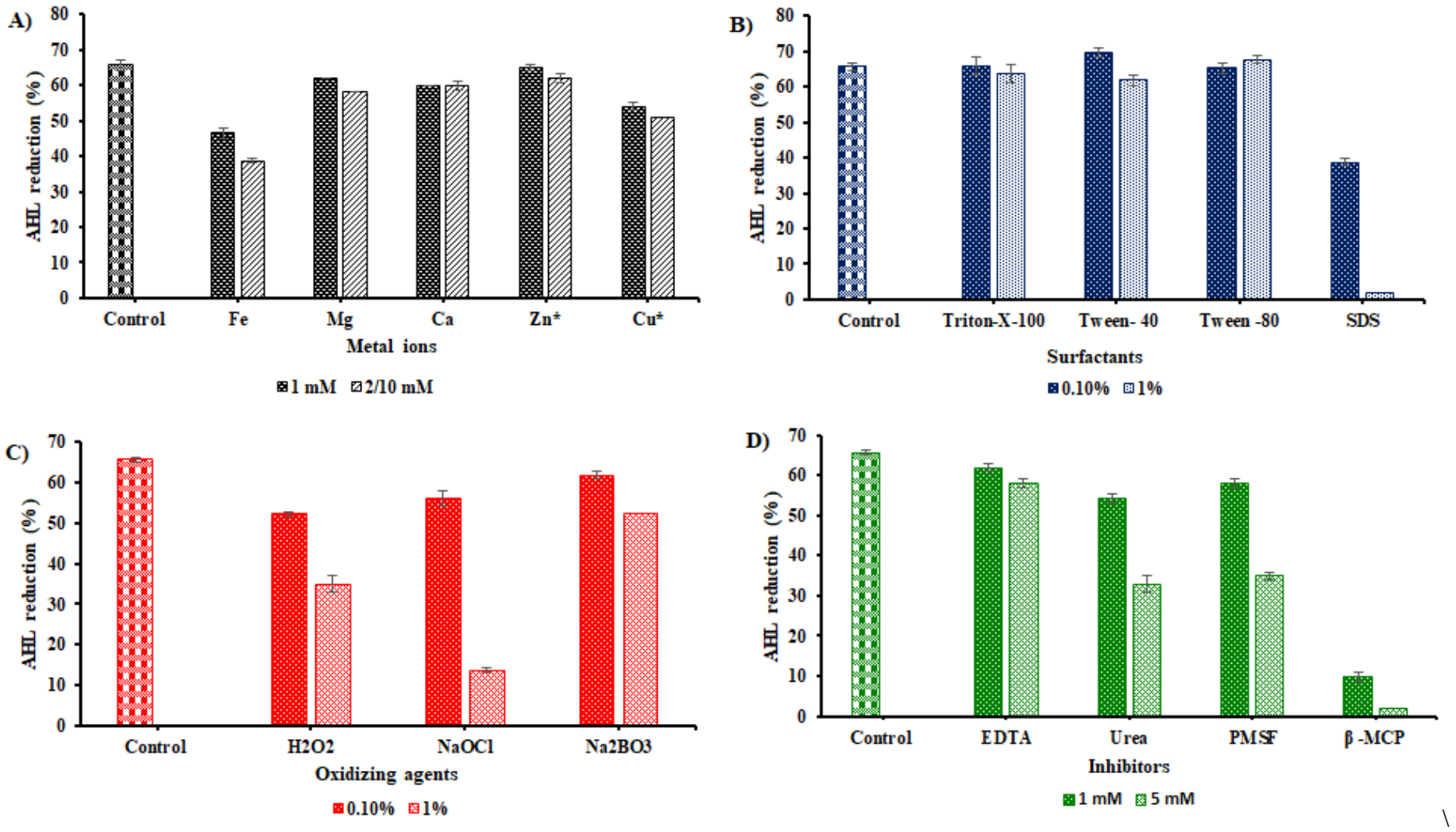


Fig. 5.6 Effect of A) Metal ions B) Surfactants C) Oxidizing agents and D) Inhibitors on the stability of crude CFS

The surfactants Triton X-100, Tween-80 and Tween-40 had no significant impact on the stability of crude CFS. At 1% concentration Tween-40 declined the quorum quenching activity by 3.8%. In contrast, Tween–80 at its higher concentration (1%) was found to be slightly enhancing the lactonase activity. The SDS produced a 41.09% and 96.08% decline in the lactonase activity upon treatment, with 0.1% and 1%, respectively, the most significant decline among the tested surfactants (Fig. 5.6B). However, all the tested oxidizing agents had significant effect on the stability of crude CFS lactonase (Fig. 5.6C). The least decline was observed upon the Na_2BO_3 treatment. Only a 20.39% reduction was imparted by 1% Na_2BO_3 treatment. The highest decline was produced by 1% NaOCl treatment (79.29%). The metal chelating agent EDTA had little influence on the extracellular lactonase activity of *B. velezensis* PM7 (Fig. 5.6D). However, urea and PMSF have significant inhibitory effect on the enzyme stability, especially at the higher concentration (5 mM). β-Mercaptoethanol, the protein denaturing agent, had the most drastic effect on the enzyme stability. A decline of 96.57% was produced by the treatment with 5 mM β-MCP. Apparently, 1 mM of β -MCP was sufficient to provide an 85.08% decrease in the extracellular lactonase activity of CFS.

5.3.5 Effect of *B. velezensis* PM7 CFS on Biofilm formation and maturation of Gram-Negative bacteria.

Biofilm control is one of the main applications of bacterial quorum quenching. In this study, the effect of *B. velezensis* PM7's extracellular quorum quenching property on biofilm formation and maturation of three Gram-negative bacteria were analyzed. A decline in biofilm formation was observed with the treatment of PM7 cell-free supernatant in *C. violaceum, P. aeruginosa* and *S. marcescens* (Fig.5.7A). A concentration-dependent biofilm inhibitory activity ($p<0.001$, linear regression analysis) was observed via the classic crystal violet assay. However, the percentage of antibiofilm activity varied among the bacteria. At a CFS concentration of 10 mg/mL, 58.15%, 65.8% and 70.34% were inhibited for *P. aeruginosa*, *C. violaceum* and *S. marcescens* respectively. However, the effect of CFS on biofilm maturation was not apparent, as seen in the biofilm formation inhibition (Fig. 5.7B). Only a 16.4% reduction was observed in pre-formed biofilms of *P. aeruginosa* after CFS application. *C. violaceum* and *S. marcescens* displayed only 19.8% and 21.05% reduction in total pre-formed biofilm content. This performance discrepancy may be due to the interference of biofilm components like EPS with the penetration or diffusion of QQ agents. In a recent study, Packiavathy *et al.* (2021) reported that a marine isolate *Psychrobacter* sp. at 20% (v/v) CFS concentration inhibited biofilm formation by 89%, 71%, 58% and 60% against *P. aeruginosa,*

S. marcescens, *Vibrio parahaemolyticus* and *Vibrio vulnificus* respectively. The strain *M. olearia* Th120 showed 20% inhibition in *P. aeruginosa* PAO1 biofilm development (Christiaen et al., 2011; Tang et al., 2015). The test organisms used in this study releases different types and concentrations of AHLs to regulate their QS network. Short-chain AHLs like C_6-HSL and C_4-HSL serve as the autoinducer molecules for *C. violaceum* and *S. marcescens*, respectively. While *P. aeruginosa* generates a wide range of AHL molecules to control their two complex QS systems, the LasI-LasR and Rh1I-Rh1R networks. The difference in the auto-inducer and QS network may account for the variation in the effectiveness of biofilm inhibition by CFS.

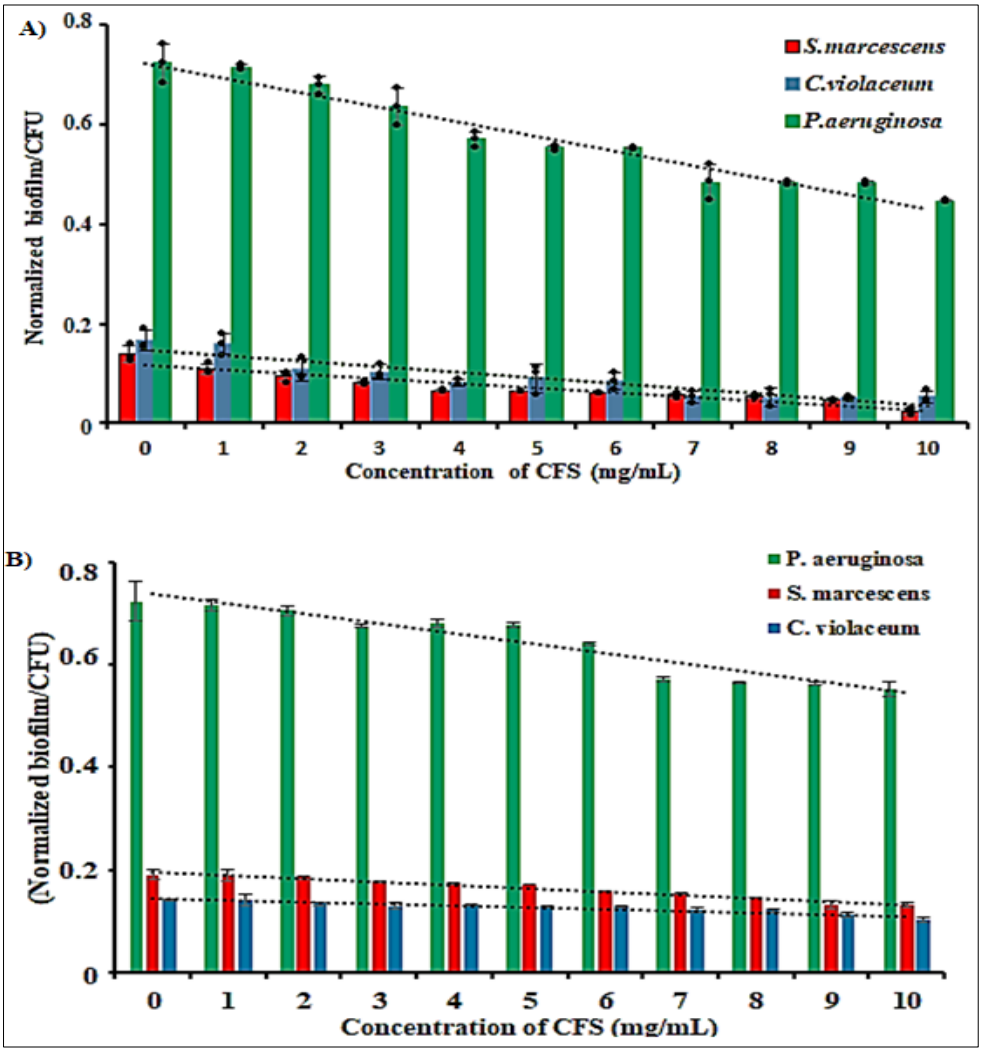


Fig. 5.7 Effect of *B. velezensis* PM7 CFS on A) biofilm formation and B) biofilm maturation in three Gram negative bacteria *viz.*, *C. violaceum*, *S. marcescens* and *P. aeruginosa* from Microtiter plate-based crystal violet assay. Mean values of three independent experiments are represented, and standard errors (SE) are shown along with the line of regression for normalized biofilm formation concerning the CFS concentration for each bacterium.

5.3.6 Confocal Laser Scanning Microscopy analysis

Confocal laser scanning microscopy was used to qualitatively assess the impact of *B. velezensis* PM7 on the biofilm formation and maturation in *P. aeruginosa.* Consistent with the result of Microtiter plate assay, the CFS treatment had less effect on pre-formed biofilms (Fig. 5.8 B). However, the biofilm formation was significantly declined with the lactonase activity (Fig. 5.8 C & D). These observations are also consistent with the microtiter plate assay. The three-dimensional projection of the CLSM also revealed the biofilm architecture disruption (Fig. 5.8 E, F & G).

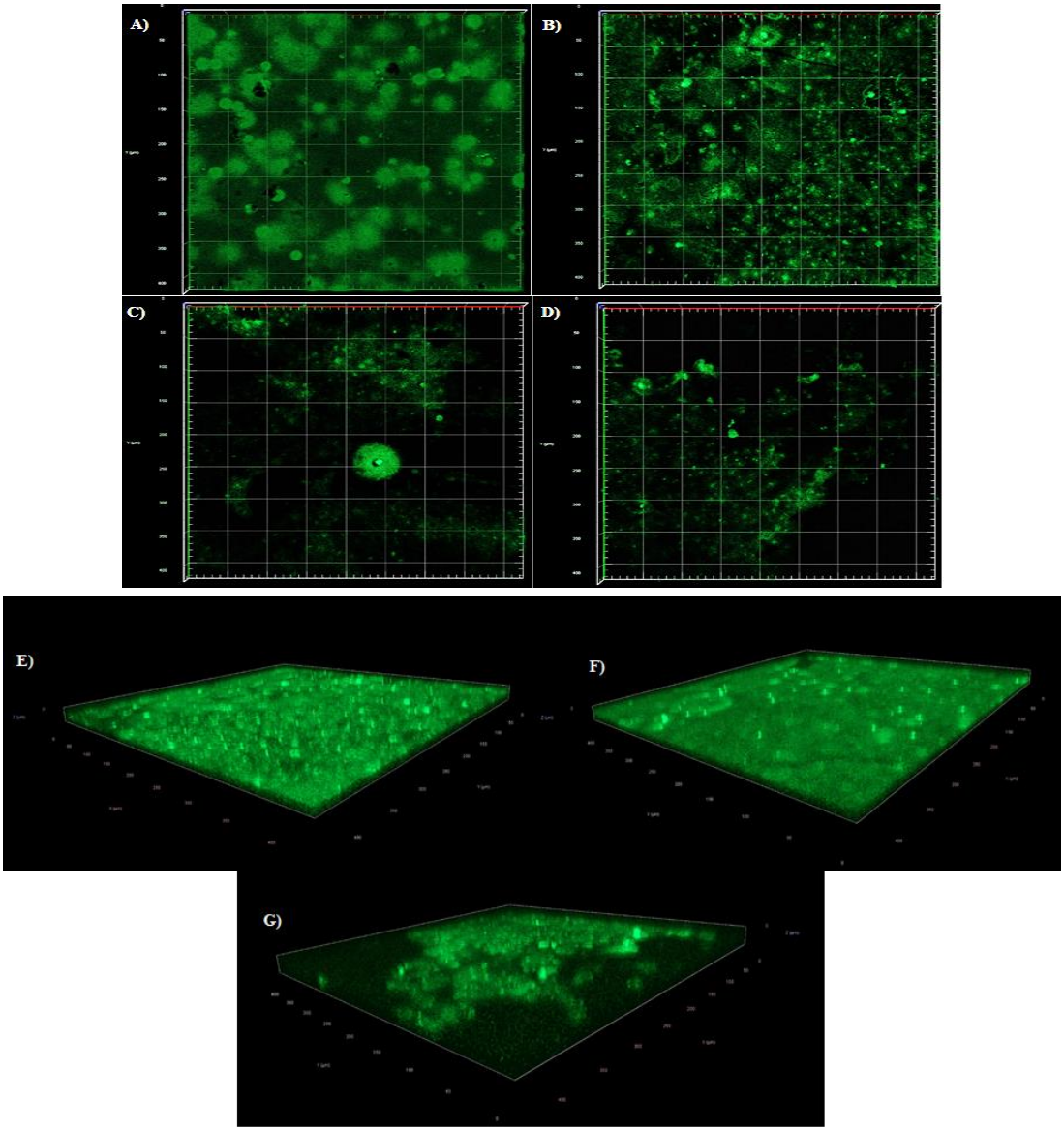


Fig. 5.8 Confocal laser scanning micrographs of *P. aeruginosa in-vitro* biofilms on glass coverslips. 3-dimensional z projection of A) control (48 hr. old untreated biofilm) B) 24 hr. preformed biofilm treated with CFS after 24 hr. incubation C&D) 48 hr. old biofilms treated with CFS. 3-dimensional y projection of E) control F) preformed biofilm treated with CFS G) 48 hr. old biofilm treated with CFS

5.3.7 Effect of *B. velezensis* PM7 on the Biofilm Formation on different surfaces

The effect of cell free supernatant of *B. velezensis* PM7 in biofilm formation on different material surfaces, under static conditions was assessed. Incubation with the tested concentration of 10% (v/v) (292 enzyme units) of the CFS resulted in a considerable decline of biofilm formation by all the tested bacteria in stainless steel, borosilicate glass and catheter. Microscopic examination of the glass surface also revealed considerable variation in the microscopic structure of biofilms formed under CFS treatment (Fig. 5.9)

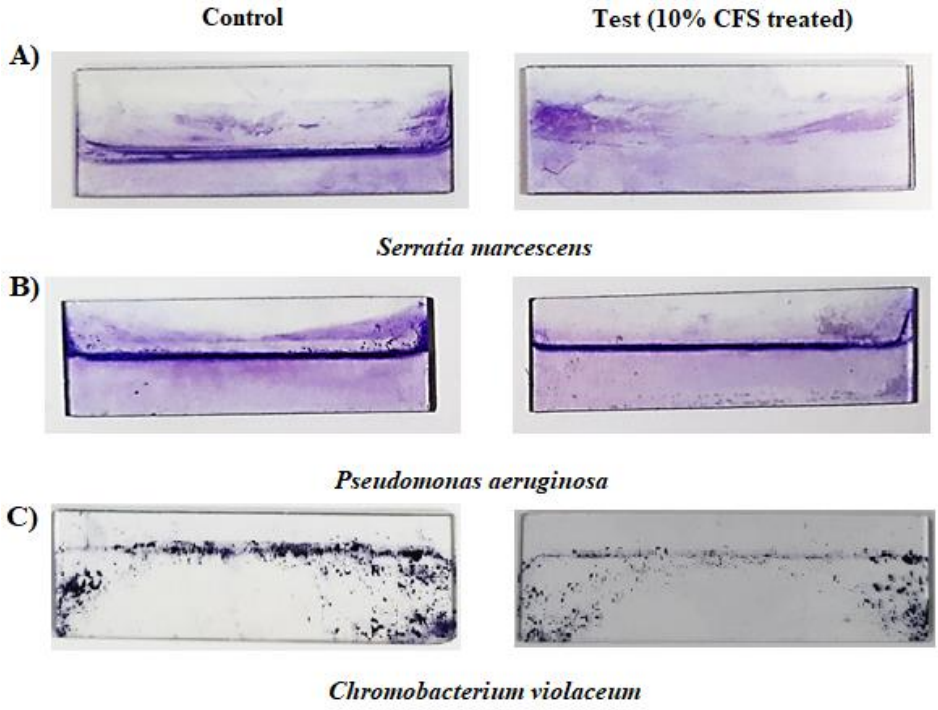


Fig. 5.9 Effect of CFS in the biofilm formation on borosilicate glass by A) *S. marcescens* B) *P. aeruginosa* visualized by crystal violet staining and C) *C. violaceum* (without staining)

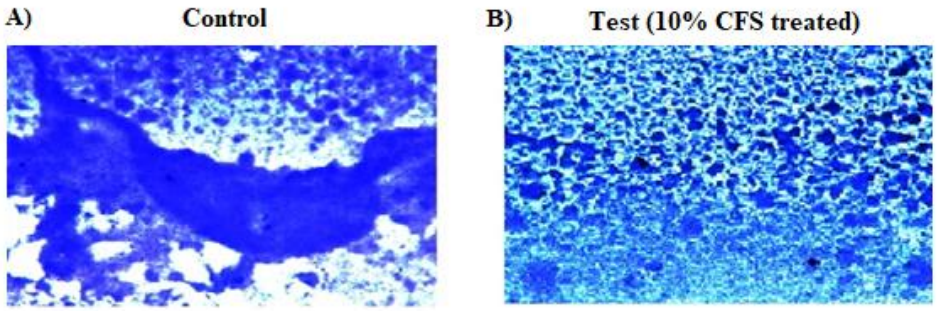


Fig. 5.10 Bright field microscopic image (Magnification 20x) of biofilm formed by *P. aeruginosa* on borosilicate glass A) Control B) Test. Image captured using Leica DM 2500 epiflourescent microscope (Leica microsystems, Germany)

The quantification of the biofilm formed was carried out using crystal violet method which revealed a significant ($p<0.05$, unpaired t-test) reduction of 57.64%, 68.36% and 80.01% for *P. aeruginosa*, *C. violaceum* and *S. marcescens* respectively (Fig. 5.11).

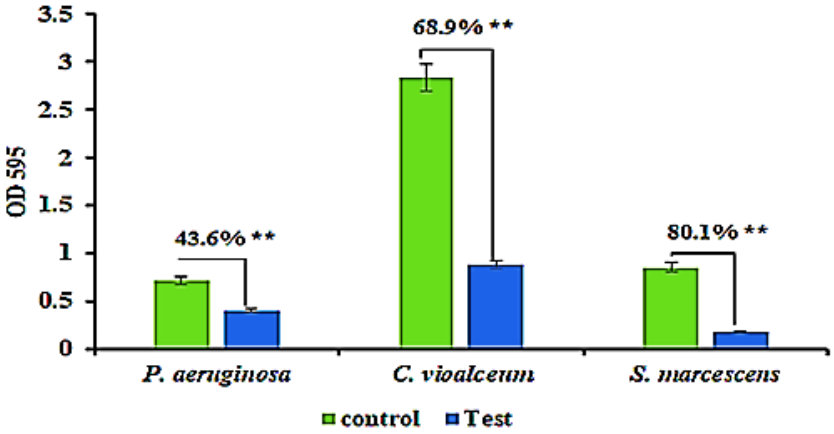


Fig. 5.11 Biofilm formed (as OD value) on borosilicate glass. Data represents mean values of three different experiments and standard errors are represented. **p<0.05, unpaired t-test

Biofilms are communities of microorganisms embedded in extracellular polymeric substance (EPS) matrix. The EPS of the biofilm consists of a mixture of polysaccharides, proteins and extracellular DNA that function as cell-to-cell as well as cell-to-substratum adherent. The EPS assists both as a structural scaffold and protection barrier.

The viable cell fraction of the biofilms formed on the glass surfaces were analysed with the help of conventional plate-based colony counting and flourescent microscopic evaluation. No discernible impact was seen on the percentage of viable cells on the control and test surfaces (Fig. 5.12A). Quorum quenching lacks any bacteriocidal activity, thus, there cannot be an increase in the number of dead cells, which accounts for this observation. However, the extracellular polysaccharide concentration was found to be declining up to 73.93% for *P. aeruginosa* 36.34% for *C. violaceum* and 64.36% for *S. marcescens* (Fig. 5.12B). Similarly, other major components in the biofilm *viz.*, total protein and extracellular DNA were also significantly declined upon CFS treatment. The highest total protein concentration reduction was observed in *P. aeruginosa* (56.58%) while *S. marcescens* and *Chromobacterium* had comparable declines (Fig. 5.12C). *S. marcescens* exhibited the greatest loss (75.8%), while *P. aeruginosa* had the least decline (38.6%) in extracellular DNA, which is crucial for the biofilm's architecture (Fig. 5.12D).

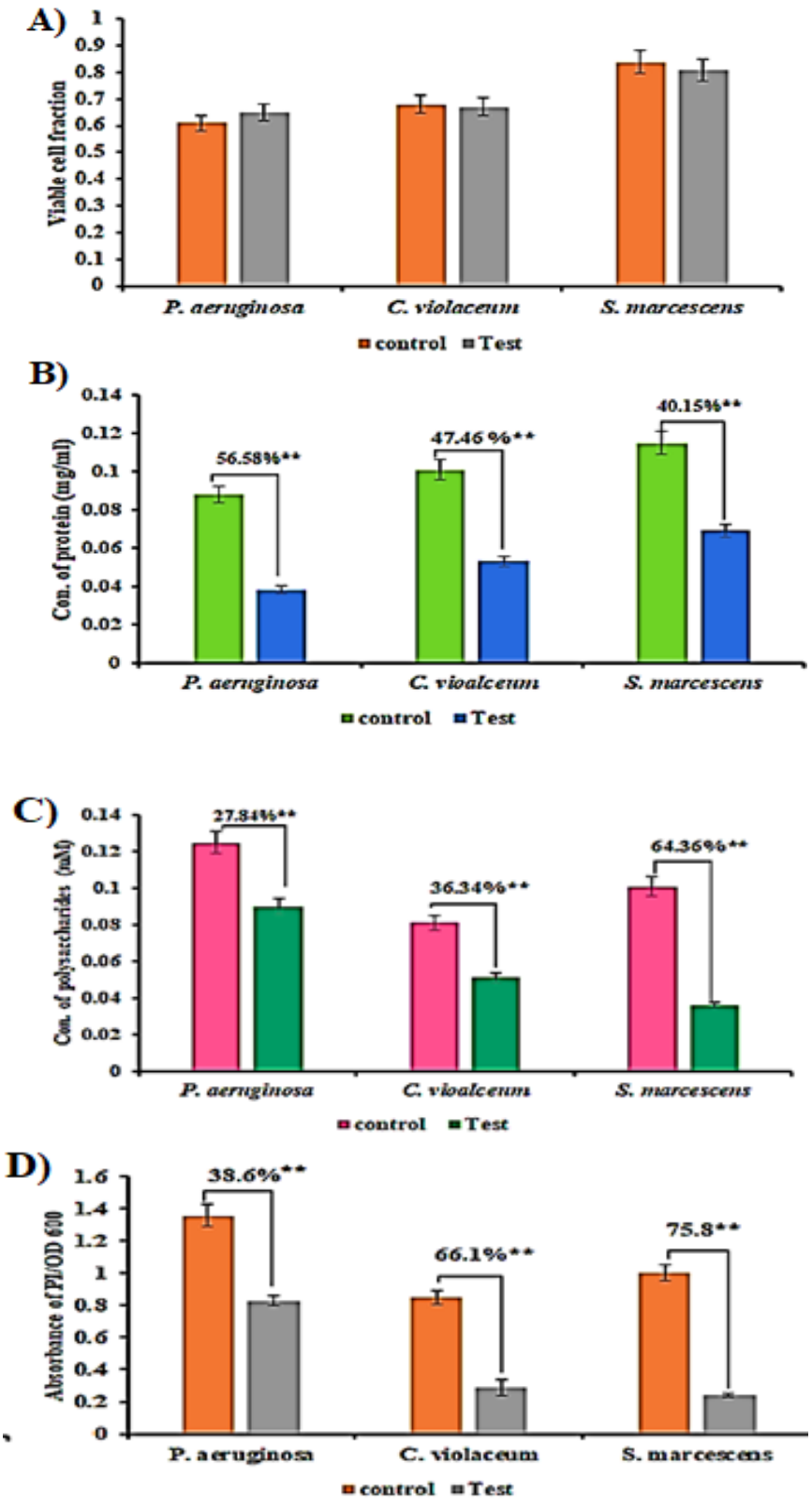


Fig. 5.12 Interference of *B. velezensis* PM7 CFS on the biofilm composition on borosilicate glass. A) Viable cell fraction, Concentration of B) Total protein C) Total Polysaccharide and D) eDNA. Data represents mean values of three different experiments and standard errors are represented. **p<0.05, unpaired t-test.

The extent of biofilm formation in stainless steel (SS) surfaces was different from borosilicate glass. Most notably, significantly higher biofilm formation was observed in the steel surface as compared to the borosilicate glass. However, upon CFS treatment, a significant declined in biofilm formation was observed in all the tested Gram-negative bacterial strains (Fig. 5.13 A,B,C). Unlike the glass surface, the highest reduction reported is for *P. aeruginosa* (85.5%) and the lowest for *C. violaceum* (55.34%) (Fig. 5.13D).

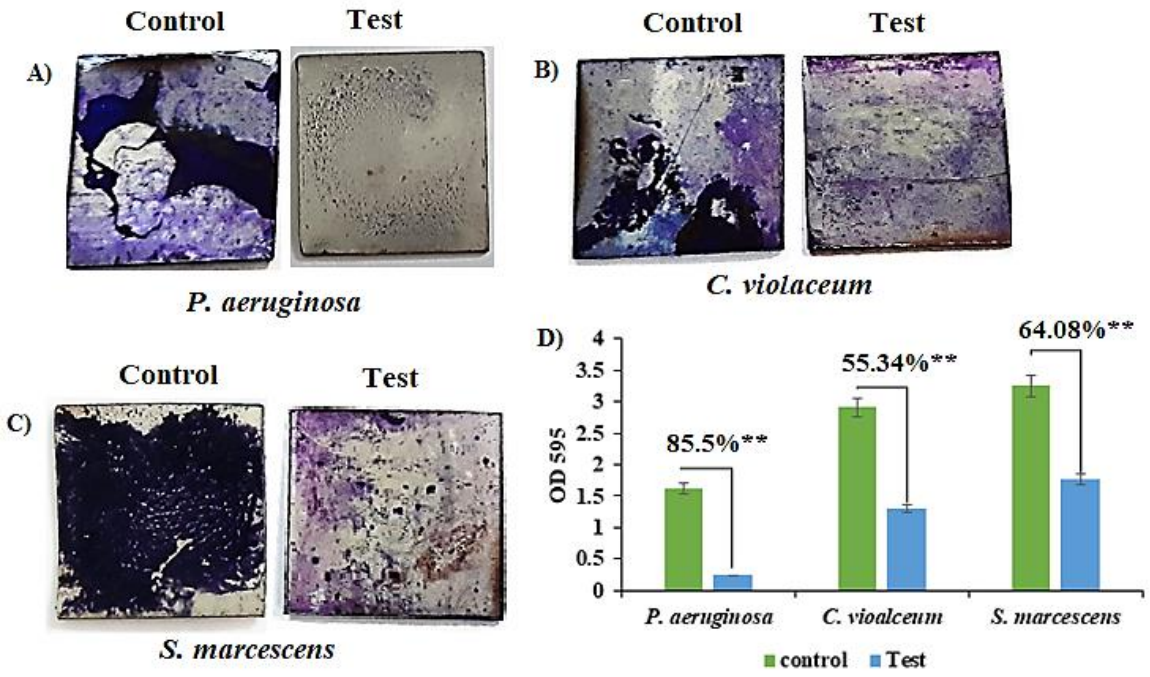


Fig. 5.13 Effect of CFS in biofilm formation on stainless steel by A) *S. marcescens* B) *P. aeruginosa* and C) *C. violaceum* visualized by crystal violet staining. D) Quantification of total biofilm formed. Data represents mean values of three different experiments, and standard errors are represented. **p<0.05, unpaired t-test

The differential component analysis of the biofilm formed on SS also yielded similar results as observed with borosilicate glass. No statistically significant difference was observed in viable cell fraction between the control and treated samples in all the tested bacteria. The extracellular polysaccharide concentration was found to be declining up to 72.55 % for *P. aeruginosa*, 49. 39 % for *C. violaceum* and 65.60 % for *S. marcescens* on SS surfaces upon the PM7 CFS treatment at a concentration of 10 % (v/v). With the PM7 CFS treatment, the concentration of the protein was found to be declining. The highest reduction was exhibited by *Pseudomonas* biofilm (63.7 %). For the eDNA content also, *Pseudomonas* displayed the greatest reduction. However, *Chromobacterium was* found to have the least decline in all the parameters irrespective of the tested material (Fig. 5.14).

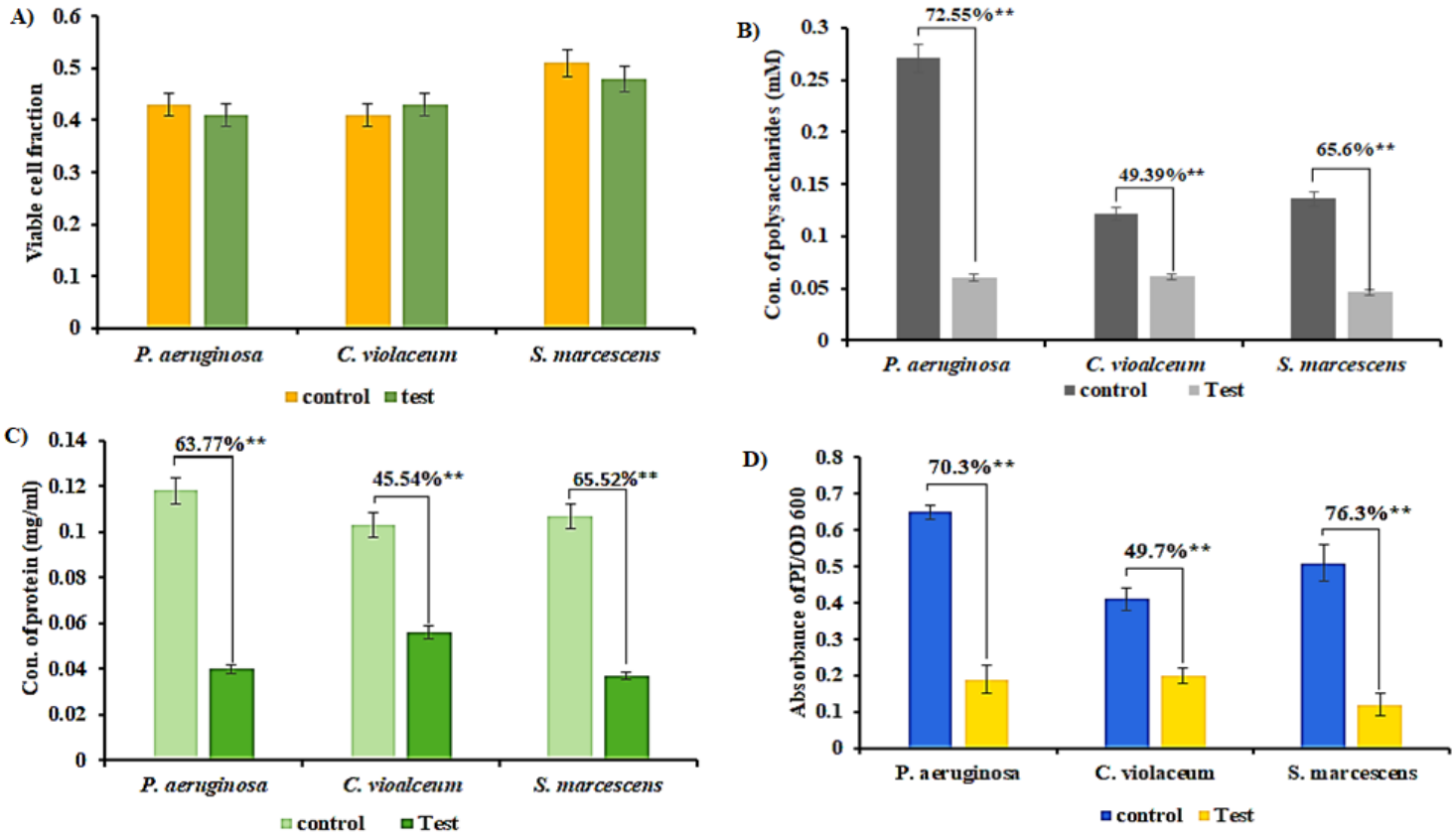


Fig. 5.14 Interference of *B. velezensis* PM7 CFS on the biofilm composition on stainless steel. A) Viable cell fraction, Concentration of B) Total Polysaccharide, C) Total protein and D) eDNA. Data represent mean values of three different experiments, and standard errors are represented. **p<0.05, unpaired t-test

The effect of PM7 CFS on the biofilm formation in Foley's urinary catheter was assessed using the strain *Pseudomonas aeruginosa* MTCC 2642. Similar to the other tested surfaces, the extracellular lactonase significantly decreased the biofilm formation within urinary catheters under static conditions (Fig. 5.15A). Based on the crystal violet assay, the total biofilm production in the catheter was reduced by 48.2% (Fig. 5.15B) without producing any significant variation in viable cell fraction between the control and test (Fig. 5.15C). However, a considerable decline up to 42.9%, 37.5% and 34.9% was observed in the EPS components polysaccharides, total protein and eDNA, respectively, with CFS treatment (Fig. 5.15 D, E, F).

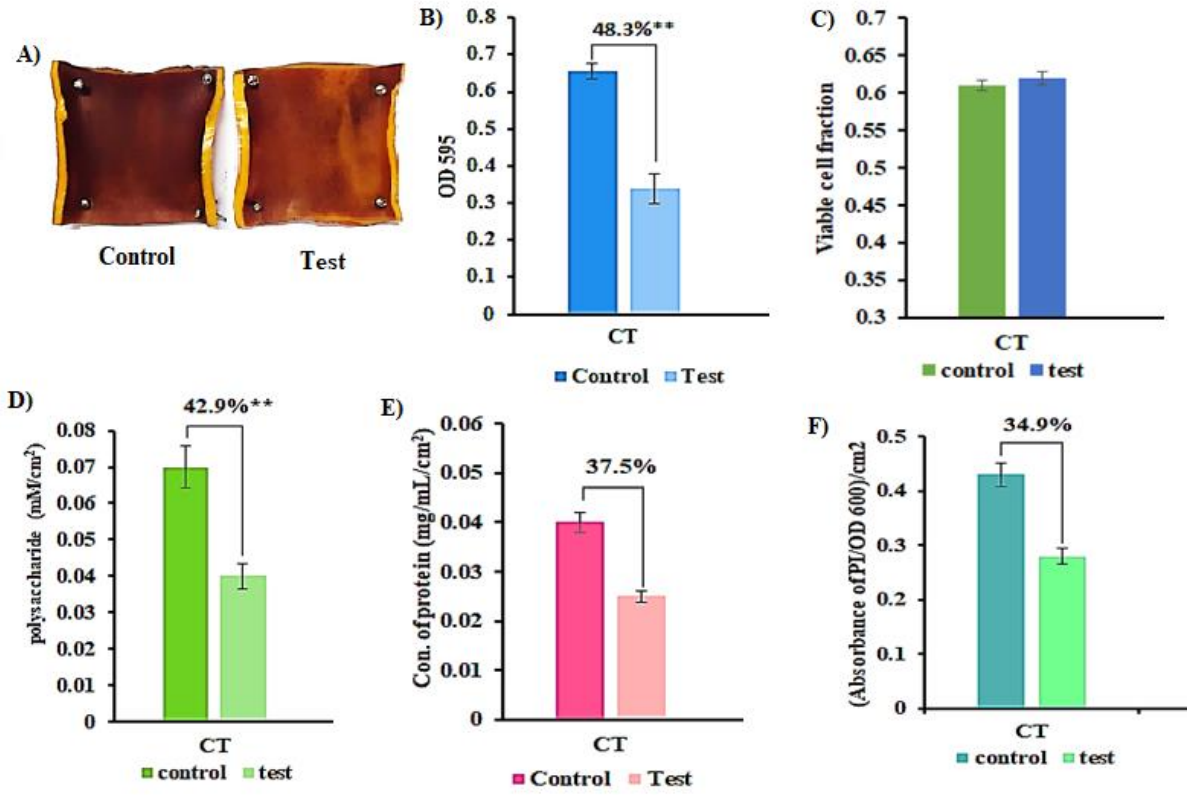


Fig. 5.15 A) Effect of CFS in forming a biofilm on Foley's urinary catheter by *P. aeruginosa.* B) Quantification of total biofilm formed. Interference on the biofilm composition on stainless steel. A) Viable cell fraction, Concentration of B) Total Polysaccharide C) Total protein and D) eDNA. Data represent mean values of three different experiments, and standard errors are represented. **p<0.05, unpaired t-test

The effect of the extracellular lactonase activity of *B. velezensis* PM7 on biofilm formed under flow (dynamic condition) was also analyzed. A reduced biofilm formation was observed in the test unit compared to the control unit. Compared to the static biofilms formed in the previous experiments, the biofilms' thickness was higher. Thus, the components of the biofilm could be differentiated into soluble microbial products (SMP), loosely bound EPS and tightly bound EPS. A significant reduction ($p < 0.05$, unpaired t-test) of exopolysaccharide and protein concentration was observed in all the biofilm components (Fig. 5.16). The highest percentage reduction is observed for the soluble microbial products for both total polysaccharide and total protein content. Conversely, the least reduction was found in tightly bound EPS components.

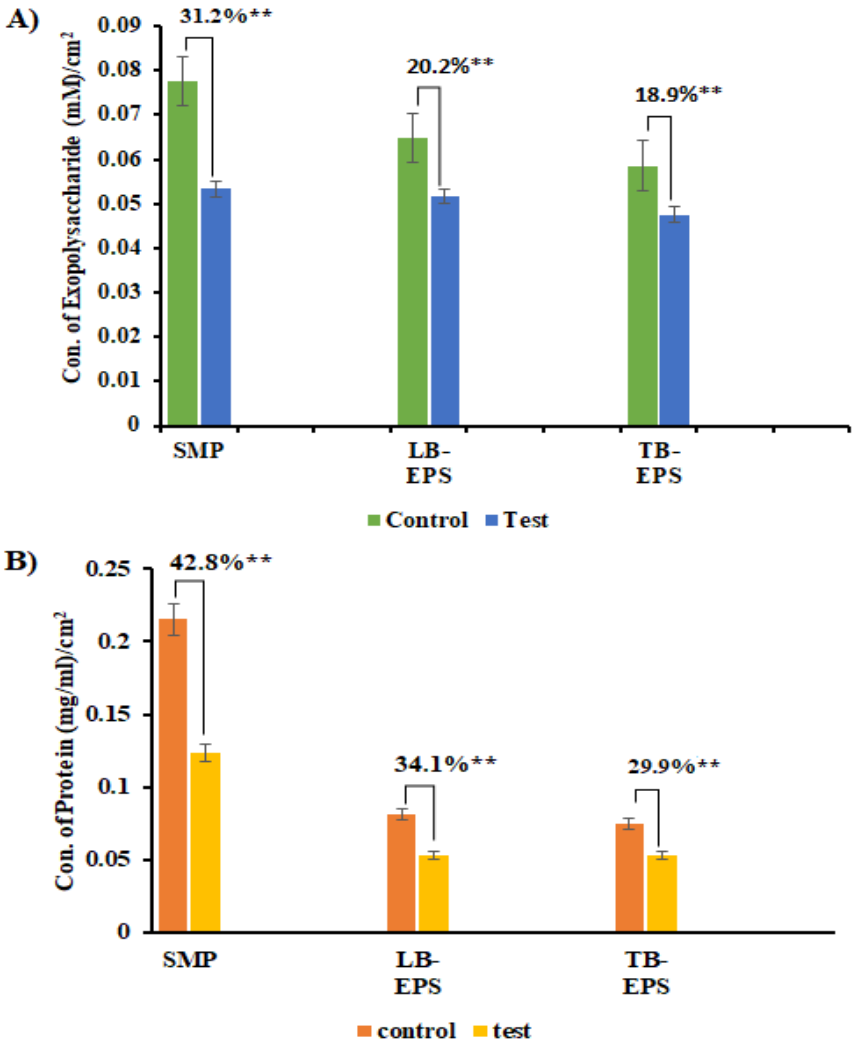


Fig.5.16 Effect of PM7 CFS on the concentrations of the A) exopolysaccharide and B) protein content in the biofilm components (SMP- Soluble Microbial products, LB-EPS- loosely Bound EPS, TB-EPS – Tightly Bound EPS) formed under dynamic conditions by *P. aeruginosa* on Foley's urinary catheter. Data represents mean values of three different experiments and standard errors are represented. **p<0.05, unpaired t-test.

The use of quorum quenching enzymes in regulating biofilm formation in industrially and medically relevant surfaces is an emerging field of research. In a previous work by (dos Reis Ponce et al., 2012), an aiiA transconjugant *Enterococcus cloacae* that synthesise lactonase was demonstrated to reduce the adherent cell numbers in the stainless steel surface. The AHL acylase homologue PF-1240 also exhibited remarkable antibiofilm activity against the food spoilage bacterium *Hafnia alvei* on stainless steel surfaces (Shen et al., 2021). Several earlier reports have shown that lactonase regulates biofilm growth on borosilicate glass surfaces also (Dong et al., 2018; Kim et al., 2014; Packiavathy et al., 2021b). Guendouze et al., (2017) demonstrated the AHL lactonase enzyme *Sso*Pox-W263I as a promising candidate for functionalized catheters and anti-virulence dressings due to their remarkable antibiofilm property. In another study, Ivanova et al., (2015) exploit the synergism between quorum quenching and matrix-degrading enzymes to control the biofilm formation in urinary catheters. In this scenario, the $AiiA_{PM7}$ also can be considered as a potential surface-modifying enzyme for biofouling control.

5.3.8 Effect of *B. velezensis* PM7 on the biofilm dispersal

Dispersion is considered the final step in the lifecycle of a biofilm, and hence it plays a crucial role in controlling biofouling. AHL molecules are also reported to initiate the dispersion of cells from matured biofilms (Krzy\.zek & Gościniak, 2018). Therefore, the effect of *B. velezensis* PM7 extracellular lactonase on the biofilm dispersion on different surfaces was analysed using *P. aeruginosa*. A significant reduction ($p<0.05$, unpaired t-test) was observed in all the tested surfaces (Fig. 5.17). The highest percentage reduction was observed for stainless steel, whereas catheters exhibited the lowest.

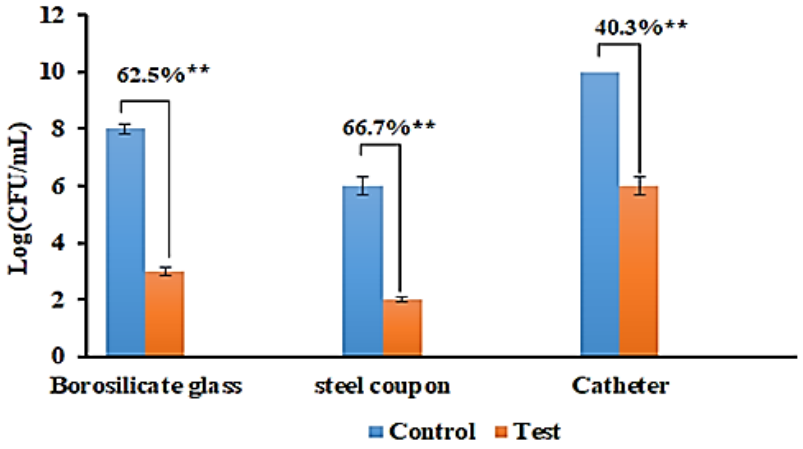


Fig. 5.17 Effect of PM7 CFS on the biofilm dispersion on different surfaces. Data represents mean values of three different experiments and standard errors. **p<0.05, unpaired t-test.

Dispersion involves biofilm matrix modification and subsequence reduction in bacterial adhesion. It is a complex process involving combinatorial effects of several factors such as c-di-GMP levels, Nitric oxide (NO) concentration, auto-inducers, nutrient supply, carbon substrate, etc. (Skariyachan et al., 2018). Since lactonases promote the degradation of biofilm matrix components they can also promote biofilm dispersion (Zhang et al., 2023). Therefore, the decrease in biofilm dispersal by PM7 CFS treatment can also be due to the overall inhibition in biofilm formation.

5.3.9 Effect of *B. velezensis* PM7 on bacterial community structure in activated sludge.

Microbial flocs in activated sludge comprise a dense microbial consortium in a matrix of EPS and adsorbed wastewater particles. They behave as biofilms without any surface association. Previous reports have elucidated the role of quorum sensing and AHLs in forming and regulating activated sludge flocs, aerobic granules, and associated microbial compositions and activities (Li et al., 2023). Hence, the influence of PM7 CFS on the bacterial community structure of activated sludge was briefly addressed here. The sludge volume index (SVI), which is a settle ability parameter of activated sludge, was found to be comparable in the case of both control and test samples. For the control samples, the observed SVI was 55.06 ± 0.86 mL/g; for the control, the values changed only up to 58. 13 ± 0.77 mL/g. The microscopic evaluation revealed differences in floc sizes between the control and test. The average floc size of the untreated sludge was slightly higher than the CFS-treated sludge (Appendix III).

To investigate the microbial community composition and diversity, bacterial community profiling using 16S rRNA V3-V4 metagenomic sequencing was carried out before and after the addition (10% v/v) of PM7 CFS. The various alpha diversity indices were also comparable for the control and test samples, as indicated in Table 5.3. In Fig. 5.18, heat maps depict the top operational taxonomic units (OTUs) classified under phylum, genus and species levels with the relative abundance above a specific cut-off value in the control and test-activated sludge. As evident from the image, the microbial community structure of the activated sludge with the CFS treatment is significantly different from the untreated control at all levels. The OTUs with the highest relative abundance at the phylum level indicated the dominance of Proteobacteria and Chloroflexi in both samples.

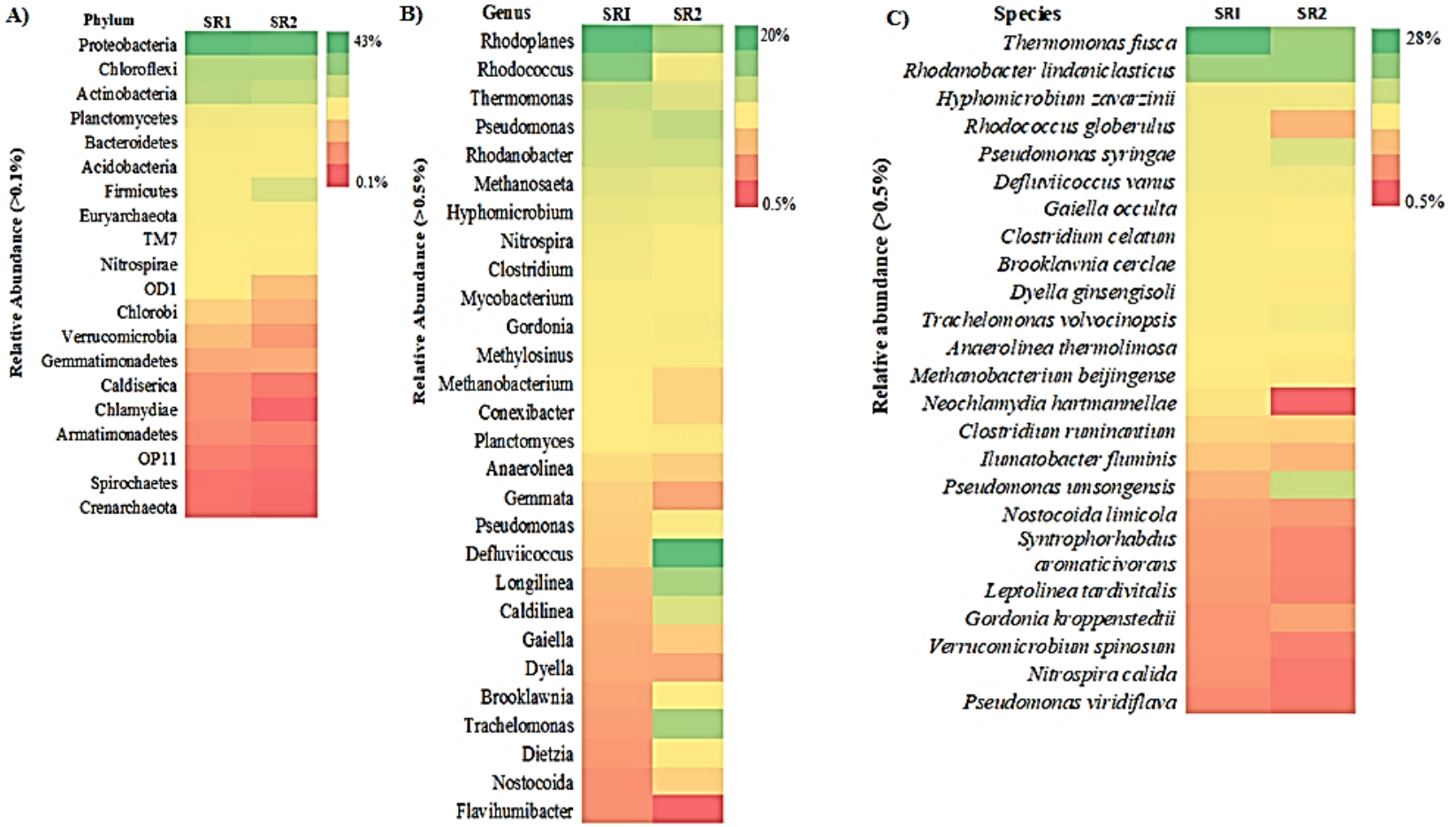


Fig. 5.18 Heatmaps showing relative abundance of bacterial community present in activated sludge in A) Phylum B) Genus and C) species levels. SR1- Control SR2- Test (treated with 10% v/v CFS)

Table 5.3 Alpha diversity indices of the control and CFS treated activated sludge

Index	Control	Test
Chao1	1119.845	1115.2
se.chao1	44.11986	44.42416
ACE	1110.589	1082.475
se.ACE	17.10163	16.45878
Shannon	4.290914	4.198921
Simpson	0.96637	0.962276
InvSimpson	29.7357	26.50799
Fisher	121.4804	118.5781

However, the abundance of the phylum Firmicutes increased upon the CFS treatment. In the genus level, the dominance shifted from *Rhodoplanes* and *Rhodococcus* in control to *Defluvicoccus* and *Loginilea* in the CFS treated samples. In the species level also, significant differences can be observed. The proportion of bacteria like *Pseudomonas syringae, Defluvicoccus vanus*, *Pseudomonas umsongensis*, *Ilumatobacter fluvinus* etc were increased on CFS treatment. At the same time, the species *Thermononas fusca*, *Rhodococcus globerulus*, *Neochlamydia haetmanillae* and *Pseudomonas virdiflava* were declined. The proportion of archeae was 2% for both untreated and treated activated sludge (Appendix IV).

Significant divergence in microbial community composition in a bioreactor-activated sludge due to the treatment with an indigenous quorum quenching *Bacillus cereus* strain is reported by Xu et al., (2019). The immobilised cells dramatically altered the community structure at class, order, phylum, family and genus taxonomic levels. The enriched genus in the study included, *Delftia*, *Bacillus*, *Acinetobacter*, *Aeromonas* and *Pseudomonas.* Quorum sensing signals play a critical role in activated sludge's structural and functional aspects. A shared system of QS signal synthesis and degradation (quorum quenching) is reported to co-exist among the microbial community in activated sludge, which regulate the floccular or granular assembly (Tan et al., 2015). In this scenario, Li et al., (2017) demonstrated the augmentation of bioreactors with AHL producers or degraders, facilitating the desired up/down-regulation of quorum sensing level and sludge granulation in a bioreactor. The *B.*

velezensis PM7 slightly altered the microbial floc structure at the CFS concentration of 10% (v/v). The decrease in floc size can lead to the possibility of granulation initiation.

Meanwhile, decreased floc size can lead to poor sludge settling, followed by sludge washout in treatment systems. Thus, further study on the impacts of PM7 CFS via various approaches, such as different concentrations or immobilisation, can help apply this extracellular QQ system in several sectors, such as wastewater treatment and membrane bioreactor fouling control. Also, a detailed analysis of the microbial community impairments caused by the CFS treatment would contribute to deciphering the mechanism of biofouling control in complex environments.

5.4 Chapter Summary

The formation, maturation and dispersion of biofilms differ among surfaces and inhabiting bacterial species. This study revealed the catalytic activity and parameters of the extracellular lactonase activity concerning the two AHL molecules *viz.,* C_6-HSL and 3-OH-C_{12}-HSL were comparable to the previously reported AHL lactonases. The enzymatic activity was found stable at 30-50 °C and pH range 4-7. Other major chemical factors affecting the enzyme stability included the metal ion $Fe^{2+,}$ surfactants like SDS, oxidizing agents like Hydrogen Peroxide and Sodium hypochlorite and inhibitors like Urea, PMSF and β-mercaptoethanol. The classic microtiter plate assay revealed the CFS to be less efficient in controlling biofilm maturation, while the biofilm formation was significantly declined with the treatment in Gram-negative bacteria. Significant reduction in static biofilm formation was observed on borosilicate glass, stainless steel and Foley's catheter upon the CFS treatment. The reduction was also evident in the differential composition of the respective biofilms. Under the dynamic conditions also, the CFS treatment significantly decreased the *P. aeruginosa* biofilm formation on the catheter surface. Besides, the biofilm dispersion by all the tested bacteria was found to be disrupted by CFS treatment on all the treated materials. A change in microbial community composition and microbial floc size in activated sludge samples was also observed upon the treatment with 10% (v/v) CFS. The findings of this study suggests that *B. velezensis* PM7 is a promising antibiofilm agent for different industrial, medical and environmental applications.

Chapter 6

Summary and Conclusion

Quorum sensing (QS), which is mediated by small, diffusible autoinducer molecules like acyl homoserine lactones (AHL), is a prevalent method for Gram-negative bacteria to regulate their gene expression in a cell density dependent manner. The phenotypes such as virulence, conjugation, antibiotic production and biofilm formation are intricately regulated by this network. Particularly, the development of biofilms poses a major threat to healthcare, many industrial, agriculture and food processing sector. Due to the extreme recalcitrance of biofilms, the complete removal is almost impossible.

Diverse traditional and advanced techniques are practised in different industrial sectors to control biofilm. However, majority of these methods are energy intensive, not environmentally benign and not commercially viable. Therefore, as a sustainable alternative, Quorum sensing disruption (quorum quenching) mediated biofilm control is an emerging area of high significance. The entire research study covered in this thesis deals with the isolation and characterization of novel quorum sensing disrupting bacteria from environmental samples and evaluation of their biofilm mitigation properties under various circumstances.

In the first part of this study, enrichment culture method, using N- Hexanoyl Homoserine Lactone as the sole carbon source in minimum media, was used to foster the growth of quorum quenching bacteria. Among different isolates obtained, significant extracellular quorum quenching activity was observed in a Gram-negative bacterium, which was identified through polyphasic approach as a novel strain of *Bacillus velezensis* (PM7). It is the first description of extracellular QQ activity in the *Bacillus* genus. When exposed to the cell free supernatant (CFS) of PM7, the bio-reporter microorganisms *Chromobacterium violaceum* and *Serratia marcescens* showed a dose-dependent reduction in corresponding pigment (Violacein and Prodigiosin) synthesis. Additionally, a notable decline in QS-controlled phenotypes of *S. marcescens* was seen, including motility and cell surface hydrophobicity. The isolate, however, does not possess anti-QS activity against Gram-positive bacteria. The CFS also lacked any antibacterial activity against several tested bacteria including Gram positive and Gram negative.

Due to the well-researched autoinducer network made up of two HSL regulated systems, the Las network and Rhl network, the ubiquitous bacterium *P. aeruginosa* is regarded as an ideal Gram-negative species for QS related studies. Therefore, in the second part of this study, the impact of *B. velezensis* PM7 on various QS controlled phenotypes of *P. aeruginosa* was assessed. The CFS of *B. velezensis* PM7 had no effect on the growth of *P. aeruginosa*.

However, the CFS treatment substantially declined many of the QS controlled phenotypes such as virulence factor production, exoenzyme secretion and biofilm formation. It was also evaluated whether *B. velezensis* PM7 affected the antibiotic susceptibility of *P. aeruginosa* that was chosen for the testing. The inhibitory concentration for biofilm embedded cells was found to be higher than for planktonic cells in all the tested multidrug resistant isolates/strains. The treatment with PM7 CFS showed different response to the antibiotics Gentamycin and Ciprofloxacin in the multidrug resistant *P. aeruginosa* tested. The disparity in removal of resistance between these two antibiotics can be attributed to their mode of action. Gentamycin is hindered mainly by the diffusion inhibition while ciprofloxacin is mainly resisted by the efflux pumps. Meanwhile, the QQ activity prevented the production of persister cells after receiving an antibiotic in high dose. These results indicate, in order to combat the emergence of antibiotic resistance, the synergistic use of QQ compounds together with traditional antibiotics could be a better option.

The QS disruption can be performed by either inhibiting autoinducer synthases/ receptors with quorum sensing inhibitors (QSI) or destroying autoinducers with quorum quenching (QQ) enzymes. The AHL lactonases, which hydrolyze the lactone ring to produce N-acyl homoserine, are the class of QQ enzymes that have received the most attention. Hence, the mechanism of QQ by *B. velezensis* PM7 was also studied. The response of QQ activity of CFS under high temperature, solvents, and proteinase K treatment suggests proteinaceous nature of the active compound responsible for the QQ. Moreover, the mechanism of QQ was identified as lactone ring hydrolysis through LC-MS analysis of AHL degradation products, and using bio-reporters. These findings indicated the presence of an extracellular AHL lactonase enzyme in the CFS.

As next part of this study, the N-acyl homoserine lactonase homolog ($aiiA_{PM7}$) of *B. velezensis* PM7 was amplified using specific primers. Through homology modelling from the protein sequence data, a prospective three-dimensional structure of the protein was generated. The modeled structure was docked against 19 AHL molecules, demonstrating a wide range of ligand preferences for the lactonase in this study. The biosensor *C. substugae* based reporter assay confirmed that the affinity declined as the number of C atoms in the side chain decreased. $AiiA_{PM7}$ is a viable candidate for QQ applications because of its wide spectrum substrate specificity and extracellular expression.

The antibiofilm property of *B. velezensis* PM7 was analyzed in the last part of this study. For this purpose, the catalytic activities and parameters of the CFS were calculated with respect to C_6-HSL and 3-OH-C_{12}-HSL and were found to be comparable to already reported AHL lactonases. The enzymatic activity was found stable in the temperature range 30 to 50 °C and pH range 4 to 7. The influence of other major chemicals and inhibitors such as metal ions, oxidizing agents, surfactants and inhibitors on the enzyme stability was also examined. The *B. velezensis* PM7 CFS demonstrated a significant decline in biofilm formation by three tested Gram-negative bacteria However, the biofilm maturation inhibition property was comparatively low in all scenarios. The reduction in biofilm formation was also evident on different tested surfaces such as borosilicate glass, stainless steel and urinary catheters under static and dynamic conditions. The CFS treatment also substantially reduced dispersion, which is the final phase of a biofilm lifecycle, which would be very beneficial in preventing the spread of biofilms. The metagenomic analysis of the activated sludge treated with CFS revealed the involvement of its QQ activity in altering the microbial composition and community structure.

In conclusion, Quorum quenching is proposed as a sustainable alternate strategy for controlling quorum sensing mediated manifestations like biofilm formation, pathogenicity and antimicrobial resistance. The empirical data generated and subsequent in-silico analysis in this study explicitly demonstrated the potential of extracellular (cell free supernatant) lactonase mediated quorum quenching activity of the novel strain *B. velezensis* PM7 in this study. The traditional biofouling management techniques with inherent limitations can be avoided through the more sustainable approach as proposed in this study. More importantly, the threat of antimicrobial resistance development as a result of the continued and extensive application of biocidal chemicals, including for biofilm control can also be avoided through the QQ approach as covered in this thesis. The unique strain of *B. velezensis* PM7 described in this study, with its lactonase secretory characteristic and broad-spectrum action against various Gram-negative bacteria, would be helpful in this scenario for preventing biofouling or other undesired QS-related properties. In-depth research is further required to study the extracellular QQ activity on bacterial population dynamics and its implications on biofilm in natural environments.

References

Abraham, W.-R. (2016). Going beyond the Control of Quorum-Sensing to Combat Biofilm Infections. *Antibiotics*, *5*(1). https://doi.org/10.3390/antibiotics5010003

Acet, Ö., Erdönmez, D., Acet, B. Ö., & Odabaşı, M. (2021). N-acyl homoserine lactone molecules assisted quorum sensing: effects consequences and monitoring of bacteria talking in real life. *Archives of Microbiology*, *203*(7), 3739–3749. https://doi.org/10.1007/s00203-021-02381-9

Afriat, L., Roodveldt, C., Manco, G., & Tawfik, D. S. (2006). The latent promiscuity of newly identified microbial lactonases is linked to a recently diverged phosphotriesterase. *Biochemistry*, *45*(46), 13677–13686.

Alfa, M. J. (2019). Biofilms on instruments and environmental surfaces: Do they interfere with instrument reprocessing and surface disinfection? Review of the literature. *American Journal of Infection Control*, *47*, A39–A45.

Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., Molin, S., Givskov, M., & Tolker-Nielsen, T. (2006). A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. *Molecular Microbiology*, *59*(4), 1114–1128.

Al-Shabib, N. A., Husain, F. M., Qais, F. A., Ahmad, N., Khan, A., Alyousef, A. A., Arshad, M., Noor, S., Khan, J. M., Alam, P., & others. (2020). Phyto-mediated synthesis of porous titanium dioxide nanoparticles from Withania somnifera root extract: broad-spectrum attenuation of biofilm and cytotoxic properties against HepG2 cell lines. *Frontiers in Microbiology*, *11*, 1680.

Ammons, M. C. B., Ward, L. S., & James, G. A. (2011). Anti-biofilm efficacy of a lactoferrin/xylitol wound hydrogel used in combination with silver wound dressings. *International Wound Journal*, *8*(3), 268–273.

Anandan, K., & Vittal, R. R. (2019). Quorum quenching activity of AiiA lactonase KMMI17 from endophytic Bacillus thuringiensis KMCL07 on AHL-mediated pathogenic phenotype in Pseudomonas aeruginosa. *Microbial Pathogenesis*, *132*, 230–242.

Anju, V. T., Dyavaiah, M., & Siddhardha, B. (2021). Quorum Sensing as a Therapeutic Target in the Treatment of Chronic Wound Infections. In P. Kumar & V. Kothari (Eds.),

Wound Healing Research: Current Trends and Future Directions (pp. 643–659). Springer Singapore. https://doi.org/10.1007/978-981-16-2677-7_20

Aslanli, A. G., & Efremenko, E. N. (2020). In silico determination of substrate spectrum of lactonases, hydrolyzing various N-acyl homoserine lactones. *IOP Conference Series: Materials Science and Engineering*, *848*(1). https://doi.org/10.1088/1757-899X/848/1/012006

Assefa, M., & Amare, A. (2022). Biofilm-Associated multi-drug resistance in hospital-acquired infections: a review. *Infection and Drug Resistance*, 5061–5068.

Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E. P., & Høiby, N. (2004). Pseudomonas aeruginosa Biofilms Exposed to Imipenem Exhibit Changes in Global Gene Expression and β-Lactamase and Alginate Production. *Antimicrobial Agents and Chemotherapy*, *48*(4), 1175–1187. https://doi.org/10.1128/AAC.48.4.1175-1187.2004

Banerjee, G., & Ray, A. K. (2017). Quorum-sensing network-associated gene regulation in Gram-positive bacteria. *Acta Microbiologica et Immunologica Hungarica*, *64*(4), 439–453.

Banerjee, P., Sahoo, P. K., Adhikary, A., Ruhal, R., Jain, D., & others. (2021). Molecular and structural facets of c-di-GMP signalling associated with biofilm formation in Pseudomonas aeruginosa. *Molecular Aspects of Medicine*, *81*, 101001.

Beaz-Hidalgo, R., & Figueras, M. J. (2013). A eromonas spp. whole genomes and virulence factors implicated in fish disease. *Journal of Fish Diseases*, *36*(4), 371–388.

Beckwith, J. (2013). The Sec-dependent pathway. *Research in Microbiology*, *164*(6), 497–504.

Bergonzi, C., Schwab, M., & Elias, M. (2016). The quorum-quenching lactonase from Geobacillus caldoxylosilyticus: purification, characterization, crystallization and crystallographic analysis. *Acta Crystallographica Section F: Structural Biology Communications*, *72*(9), 681–686.

Bhatt, V. S. (2018). Quorum sensing mechanisms in gram positive bacteria. *Implication of Quorum Sensing System in Biofilm Formation and Virulence*, 297–311.

Biswas, A., Salunke, G., Khandelwal, P., Das, R., & Poddar, P. (2017). Surface disordered

rutile TiO 2--graphene quantum dot hybrids: a new multifunctional material with superior photocatalytic and biofilm eradication properties. *New Journal of Chemistry*, *41*(7), 2642–2657.

Blackman, L. D., Fros, M. K., Welch, N. G., Gengenbach, T. R., Qu, Y., Pasic, P., Gunatillake, P. A., Thissen, H., Cass, P., & Locock, K. E. S. (2020). Dual Action Antimicrobial Surfaces: Alternating Photopatterns Maintain Contact-Killing Properties with Reduced Biofilm Formation. *Macromolecular Materials and Engineering*, *305*(10), 2000371.

Bloemberg, G. V, & Lugtenberg, B. J. J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Current Opinion in Plant Biology*, *4*(4), 343–350.

Blus-Kadosh, I., Zilka, A., Yerushalmi, G., & Banin, E. (2013). The Effect of pstS and phoB on Quorum Sensing and Swarming Motility in Pseudomonas aeruginosa. *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0074444

Botelho, J., Grosso, F., & Peixe, L. (2019). Antibiotic resistance in Pseudomonas aeruginosa – Mechanisms, epidemiology and evolution. *Drug Resistance Updates*, *44*(April), 100640. https://doi.org/10.1016/j.drup.2019.07.002

Briaud, P., & Carroll, R. K. (2020). Extracellular Vesicle Biogenesis and Functions in Gram-Positive Bacteria. *Infection and Immunity*, *88*(12). https://doi.org/10.1128/IAI.00433-20

Brown, L., Kessler, A., Cabezas-Sanchez, P., Luque-Garcia, J. L., & Casadevall, A. (2014). Extracellular vesicles produced by the Gram-positive bacterium Bacillus subtilis are disrupted by the lipopeptide surfactin. *Molecular Microbiology*, *93*(1), 183–198. https://doi.org/10.1111/mmi.12650

Carlson, R. P., Taffs, R., Davison, W. M., & Stewart, P. S. (2008). Anti-biofilm properties of chitosan-coated surfaces. *Journal of Biomaterials Science, Polymer Edition*, *19*(8), 1035–1046.

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R., & Guo, J. (2013). Biocontrol of tomato wilt disease by B acillus subtilis isolates from natural environments depends on conserved genes mediating biofilm formation. *Environmental Microbiology*, *15*(3), 848–864.

Choudhary, S., & Schmidt-Dannert, C. (2010). Applications of quorum sensing in

biotechnology. *Applied Microbiology and Biotechnology*, *86*(5), 1267–1279. https://doi.org/10.1007/s00253-010-2521-7

Christiaen, S. E. A., Brackman, G., Nelis, H. J., & Coenye, T. (2011). Isolation and identi fi cation of quorum quenching bacteria from environmental samples. *Journal of Microbiological Methods*, *87*(2), 213–219. https://doi.org/10.1016/j.mimet.2011.08.002

Churchill, M. E. A., & Chen, L. (2011). Structural basis of acyl-homoserine lactone-dependent signaling. *Chemical Reviews*, *111*(1), 68–85. https://doi.org/10.1021/cr1000817

Ciofu, O., & Tolker-Nielsen, T. (2019). Tolerance and resistance of pseudomonas aeruginosabiofilms to antimicrobial agents-how P. aeruginosaCan escape antibiotics. *Frontiers in Microbiology*, *10*(MAY). https://doi.org/10.3389/fmicb.2019.00913

Clinical and Laboratory Standards Institute. (2012). *Performance standards for antimicrobial disk susceptibility tests: Approved standard - Eleventh edition* (Vol. 32, Issue 1). https://doi.org/M02-A11

Cogan, N. G., Li, J., Badireddy, A. R., & Chellam, S. (2016). Optimal backwashing in dead-end bacterial microfiltration with irreversible attachment mediated by extracellular polymeric substances production. *Journal of Membrane Science*, *520*, 337–344.

Coria-Jimenez, R., Romero-Olvera, J., & Celis-Cruz, C. (1998). Superficial hydrophobicity in Serratia marcescens strains of clinical origin. *Research in Microbiology*, *149*(1), 27–29.

Costerton, J. W. (1995). Overview of microbial biofilms. *Journal of Industrial Microbiology and Biotechnology*, *15*(3), 137–140.

Coulthurst, S. J., Williamson, N. R., Harris, A. K. P., Spring, D. R., & Salmond, G. P. C. (2006). Metabolic and regulatory engineering of Serratia marcescens: mimicking phage-mediated horizontal acquisition of antibiotic biosynthesis and quorum-sensing capacities. *Microbiology*, *152*(7), 1899–1911.

Czajkowski, R., & Jafra, S. (2009). Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules. *Acta Biochimica Polonica*, *56*, 1–16. https://doi.org/10.18388/abp.2009_2512

da Cruz Nizer, W. S., Inkovskiy, V., Versey, Z., Strempel, N., Cassol, E., & Overhage, J.

(2021). Oxidative stress response in Pseudomonas aeruginosa. *Pathogens*, *10*(9), 1187.

De Kievit, T. R., & Iglewski, B. H. (2000). Bacterial quorum sensing in pathogenic relationships. *Infection and Immunity*, *68*(9), 4839–4849.

Defoirdt, T., Boon, N., & Bossier, P. (2010). Can bacteria evolve resistance to quorum sensing disruption? *PLoS Pathogens*, *6*(7), 1–6. https://doi.org/10.1371/journal.ppat.1000989

Dekimpe, V., & Deziel, E. (2009). Revisiting the quorum-sensing hierarchy in Pseudomonas aeruginosa: the transcriptional regulator RhlR regulates LasR-specific factors. *Microbiology*, *155*(3), 712–723.

Déziel, E., Comeau, Y., & Villemur, R. (2001). Initiation of biofilm formation by Pseudomonas aeruginosa 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *Journal of Bacteriology*, *183*(4), 1195–1204. https://doi.org/10.1128/JB.183.4.1195-1204.2001

Di Onofrio, V., Gesuele, R., Maione, A., Liguori, G., Liguori, R., Guida, M., Nigro, R., & Galdiero, E. (2019). Prevention of pseudomonas aeruginosa biofilm formation on soft contact lenses by allium sativum fermented extract (BGE) and cannabinol oil extract (CBD). *Antibiotics*, *8*(4), 1–12. https://doi.org/10.3390/antibiotics8040258

Djokic, L., Stankovic, N., Galic, I., Moric, I., Radakovic, N., Šegan, S., Pavic, A., & Senerovic, L. (2022). Novel Quorum Quenching YtnP Lactonase From Bacillus paralicheniformis Reduces Pseudomonas aeruginosa Virulence and Increases Antibiotic Efficacy in vivo. *Frontiers in Microbiology*, *13*(June), 1–17. https://doi.org/10.3389/fmicb.2022.906312

Dong, W., Zhu, J., Guo, X., Kong, D., Zhang, Q., Zhou, Y., Liu, X., Zhao, S., & Ruan, Z. (2018). Characterization of AiiK, an AHL lactonase, from Kurthia huakui LAM0618T and its application in quorum quenching on Pseudomonas aeruginosa PAO1. *Scientific Reports*, *8*(1), 1–11. https://doi.org/10.1038/s41598-018-24507-8

Donlan, R. M. (2001). Biofilms and device-associated infections. *Emerging Infectious Diseases*, *7*(2), 277.

dos Reis Ponce, A., Martins, M. L., de Araujo, E. F., Mantovani, H. C., & Vanetti, M. C. D.

(2012). AiiA quorum-sensing quenching controls proteolytic activity and biofilm formation by Enterobacter cloacae. *Current Microbiology*, *65*, 758–763.

Driscoll, J. A., Brody, S. L., & Kollef, M. H. (2007). The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections. *Drugs*, *67*(3), 351–368. https://doi.org/10.2165/00003495-200767030-00003

El-Mowafy, S. A., Shaaban, M. I., & Abd El Galil, K. H. (2014). Sodium ascorbate as a quorum sensing inhibitor of Pseudomonas aeruginosa. *Journal of Applied Microbiology*, *117*(5), 1388–1399. https://doi.org/10.1111/jam.12631

El-Sayed, A. K., Hothersall, J., & Thomas, C. M. (2001). Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in Pseudomonas fluorescens NCIMB 10586. *Microbiology*, *147*(8), 2127–2139.

Engebrecht, J., Nealson, K., & Silverman, M. (1983). Bacterial bioluminescence: isolation and genetic analysis of functions from Vibrio fischeri. *Cell*, *32*(3), 773–781.

Etim, I.-I. N., Wei, J., Dong, J., Xu, D., Chen, N., Wei, X., Su, M., & Ke, W. (2018). Mitigation of the corrosion-causing Desulfovibrio desulfuricans biofilm using an organic silicon quaternary ammonium salt in alkaline media simulated concrete pore solutions. *Biofouling*, *34*(10), 1121–1137.

Felz, S., Vermeulen, P., van Loosdrecht, M. C. M., & Lin, Y. M. (2019). Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS). *Water Research*, *157*, 201–208.

Fetzner, S. (2015). Quorum quenching enzymes. *Journal of Biotechnology*, *201*, 2–14. https://doi.org/10.1016/j.jbiotec.2014.09.001

Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9), 563–575.

Folders, J., Algra, J., Roelofs, M. S., van Loon, L. C., Tommassen, J., & Bitter, W. (2001). Characterization of Pseudomonas aeruginosa chitinase, a gradually secreted protein. *Journal of Bacteriology*, *183*(24), 7044–7052.

Franklin, M. J., & Ohman, D. E. (1996). Identification of algI and algI in the Pseudomonas aeruginosa alginate biosynthetic gene cluster which are required for alginate O

acetylation. *Journal of Bacteriology*, *178*(8), 2186–2195. https://doi.org/10.1128/jb.178.8.2186-2195.1996

Fuqua, W. C., Winans, S. C., & Greenberg, E. P. (1994). Quorum sensing in bacteria: The LuxR-LuxI family of cell density- responsive transcriptional regulators. *Journal of Bacteriology*, *176*(2), 269–275. https://doi.org/10.1128/jb.176.2.269-275.1994

Galdino, A. C. M., de Oliveira, M. P., Ramalho, T. C., de Castro, A. A., Branquinha, M. H., & Santos, A. L. S. (2019). Anti-virulence strategy against the multidrug-resistant bacterial pathogen Pseudomonas aeruginosa: pseudolysin (elastase B) as a potential druggable target. *Current Protein and Peptide Science*, *20*(5), 471–487.

García-Contreras, R., Maeda, T., & Wood, T. K. (2013). Resistance to quorum-quenching compounds. *Applied and Environmental Microbiology*, *79*(22), 6840–6846.

Ge, C., Sheng, H., Chen, X., Shen, X., Sun, X., Yan, Y., Wang, J., & Yuan, Q. (2020). Quorum sensing system used as a tool in metabolic engineering. *Biotechnology Journal*, *15*(6), 1900360.

Glessner, A., Smith, R. S., Iglewski, B. H., & Robinson, J. B. (1999). Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of twitching motility. *Journal of Bacteriology*, *181*(5), 1623–1629. https://doi.org/10.1128/jb.181.5.1623-1629.1999

Gordon, C. A., Hodges, N. A., & Marriott, C. (1988). Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived pseudomonas aeruginosa. *Journal of Antimicrobial Chemotherapy*, *22*(5), 667–674. https://doi.org/10.1093/jac/22.5.667

Guendouze, A., Plener, L., Bzdrenga, J., Jacquet, P., Rémy, B., Elias, M., Lavigne, J.-P., Daudé, D., & Chabrière, E. (2017). Effect of quorum quenching lactonase in clinical isolates of Pseudomonas aeruginosa and comparison with quorum sensing inhibitors. *Frontiers in Microbiology*, *8*, 227.

Gupta, P., Chhibber, S., & Harjai, K. (2015). Efficacy of purified lactonase and ciprofloxacin in preventing systemic spread of Pseudomonas aeruginosa in murine burn wound model. *Burns*, *41*(1), 153–162. https://doi.org/https://doi.org/10.1016/j.burns.2014.06.009

Gürtler, N., Osthoff, M., Rueter, F., Wüthrich, D., Zimmerli, L., Egli, A., & Bassetti, S. (2019). Prosthetic valve endocarditis caused by Pseudomonas aeruginosa with variable

antibacterial resistance profiles: A diagnostic challenge. *BMC Infectious Diseases*, *19*(1), 4–9. https://doi.org/10.1186/s12879-019-4164-3

Gutiérrez-Pacheco, M. M., Bernal-Mercado, A. T., Vázquez-Armenta, F. J., Mart ínez-Tellez, M. A., González-Aguilar, G. A., Lizardi-Mendoza, J., Madera-Santana, T. J., Nazzaro, F., & Ayala-Zavala, J. F. (2019). Quorum sensing interruption as a tool to control virulence of plant pathogenic bacteria. *Physiological and Molecular Plant Pathology*, *106*, 281–291. https://doi.org/https://doi.org/10.1016/j.pmpp.2019.04.002

Harjai, K., Gupta, R. K., & Sehgal, H. (2014). Attenuation of quorum sensing controlled virulence of Pseudomonas aeruginosa by cranberry. *Indian Journal of Medical Research*, *139*(MAR), 446–453.

Harrison, A. M., & Soby, S. D. (2020). Reclassification of Chromobacterium violaceum ATCC 31532 and its quorum biosensor mutant CV026 to Chromobacterium subtsugae. *Amb Express*, *10*(1), 1–7.

Hassan, R., Shaaban, M. I., Abdel Bar, F. M., El-Mahdy, A. M., & Shokralla, S. (2016). Quorum sensing inhibiting activity of Streptomyces coelicoflavus isolated from soil. *Frontiers in Microbiology*, *7*, 659.

Hawas, S., Verderosa, A. D., & Totsika, M. (2022). Combination therapies for biofilm inhibition and eradication: a comparative review of laboratory and preclinical studies. *Frontiers in Cellular and Infection Microbiology*, *12*, 850030.

Heidari, A., Noshiranzadeh, N., Haghi, F., & Bikas, R. (2017). Inhibition of quorum sensing related virulence factors of Pseudomonas aeruginosa by pyridoxal lactohydrazone. *Microbial Pathogenesis*, *112*, 103–110. https://doi.org/10.1016/j.micpath.2017.09.043

Hentzer, M., Riedel, K., Rasmussen, T. B., Heydorn, A., Andersen, J. B., Parsek, M. R., Rice, S. A., Eberl, L., Molin, S., Høiby, N., & others. (2002). Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound. *Microbiology*, *148*(1), 87–102.

Hiblot, J., Bzdrenga, J., Champion, C., Chabriere, E., & Elias, M. (2015). Crystal structure of VmoLac, a tentative quorum quenching lactonase from the extremophilic crenarchaeon Vulcanisaeta moutnovskia. *Scientific Reports*, *5*(1), 8372. https://doi.org/10.1038/srep08372

Hooshangi, S., & Bentley, W. E. (2008). From unicellular properties to multicellular behavior: bacteria quorum sensing circuitry and applications. *Current Opinion in Biotechnology*, *19*(6), 550–555.

Hosaka, Y., Saito, A., Maeda, R., Fukaya, C., Morikawa, S., Makino, A., Ishihara, K., & Nakagawa, T. (2012). Antibacterial activity of povidone--iodine against an artificial biofilm of Porphyromonas gingivalis and Fusobacterium nucleatum. *Archives of Oral Biology*, *57*(4), 364–368.

Hošt'álek, Z. (1979). Regulation of gene expression in secondary biosynthesis. In *Regulation of secondary product and plant hormone metabolism* (pp. 111–120). Elsevier.

Houdt, R. Van, Moons, P., Aertsen, A., Jansen, A., Vanoirbeek, K., Daykin, M., Williams, P., & Michiels, C. W. (2007). *Characterization of a luxI / luxR -type quorum sensing system and N -acyl-homoserine lactone-dependent regulation of exo-enzyme and antibacterial component production in Serratia plymuthica RVH1*. *158*, 150–158. https://doi.org/10.1016/j.resmic.2006.11.008

Hraiech, S., Hiblot, J., Lafleur, J., Lepidi, H., Papazian, L., Rolain, J. M., Raoult, D., Elias, M., Silby, M. W., Bzdrenga, J., Bregeon, F., & Chabriere, E. (2014). Inhaled lactonase reduces pseudomonas aeruginosa quorum sensing and mortality in rat pneumonia. *PLoS ONE*, *9*(10), 1–8. https://doi.org/10.1371/journal.pone.0107125

Hudzicki, J. (2012). Kirby-Bauer Disk Diffusion Susceptibility Test Protocol Author Information. *American Society For Microbiology, December 2009*, 1–13. https://www.asm.org/Protocols/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Pro

Hussain, M. B. B. M., Zhang, H.-B., Xu, J.-L., Liu, Q., Jiang, Z., & Zhang, L.-H. (2008). The acyl-homoserine lactone-type quorum-sensing system modulates cell motility and virulence of Erwinia chrysanthemi pv. zeae. *Journal of Bacteriology*, *190*(3), 1045–1053.

Ivanova, K., Fernandes, M. M., Francesko, A., Mendoza, E., Guezguez, J., Burnet, M., & Tzanov, T. (2015). Quorum-quenching and matrix-degrading enzymes in multilayer coatings synergistically prevent bacterial biofilm formation on urinary catheters. *ACS Applied Materials & Interfaces*, *7*(49), 27066–27077.

Ivanova, K., Fernandes, M. M., Mendoza, E., & Tzanov, T. (2015). Enzyme multilayer

coatings inhibit Pseudomonas aeruginosa biofilm formation on urinary catheters. *Applied Microbiology and Biotechnology*, *99*(10), 4373–4385. https://doi.org/10.1007/s00253-015-6378-7

Johansson, E. M. V, Crusz, S. A., Kolomiets, E., Buts, L., Kadam, R. U., Cacciarini, M., Bartels, K.-M., Diggle, S. P., Camara, M., Williams, P., & others. (2008). Inhibition and dispersion of Pseudomonas aeruginosa biofilms by glycopeptide dendrimers targeting the fucose-specific lectin LecB. *Chemistry & Biology*, *15*(12), 1249–1257.

Kalgudi, R. (2018). *WestminsterResearch Quorum quenching : a study of the inhibition of pseudomonas aeruginosa quorum sensing-controlled biofilm formation and virulence*.

Kalia, V. C. (2013). Quorum sensing inhibitors: An overview. *Biotechnology Advances*, *31*(2), 224–245. https://doi.org/10.1016/j.biotechadv.2012.10.004

Kaur, J., & Yogalakshmi, K. N. (2022). Degradation of n-hexanoyl homoserine lactone with quorum quenching bacteria immobilised magnetic nanocomposite beads. *Environmental Technology*, *43*(6), 885–892. https://doi.org/10.1080/09593330.2020.1811389

Kessler, E., Safrin, M., Abrams, W. R., Rosenbloom, J., & Ohman, D. E. (1997). Inhibitors and specificity of Pseudomonas aeruginosa LasA. *Journal of Biological Chemistry*, *272*(15), 9884–9889. https://doi.org/10.1074/jbc.272.15.9884

Khalil, A. L., Alawi, S. R., & others. (2019). ISOLATION, ANTIBIOTIC SENSITIVITY AND QUALITATIVE DETERMINATION OF SLIME LAYER FORMATION TEST FOR P. AERUGINOSA ISOLATED FROM WOUND AND URINARY TRACT INFECTION PATIENT. *Biochemical and Cellular Archives*, *19*(1), 1509–1514.

Kim, A., Park, S., Lee, C., Lee, C., & Lee, J. (2014). *Quorum Quenching Bacteria Isolated from the Sludge of a Wastewater Treatment Plant and Their Application for Controlling Biofilm Formation*. *24*(11), 1574–1582.

Kim, Y. W., Subramanian, S., Gerasopoulos, K., Ben-Yoav, H., Wu, H.-C., Quan, D., Carter, K., Meyer, M. T., Bentley, W. E., & Ghodssi, R. (2015). Effect of electrical energy on the efficacy of biofilm treatment using the bioelectric effect. *Npj Biofilms and Microbiomes*, *1*(1), 1–8.

Kiran, S., Sharma, P., Harjai, K., & Capalash, N. (2011). Enzymatic quorum quenching increases antibiotic susceptibility of multidrug resistant Pseudomonas aeruginosa.

Iranian Journal of Microbiology, *3*(1), 1–12.

Kiratisin, P., Tucker, K. D., & Passador, L. (2002). LasR, a transcriptional activator of Pseudomonas aeruginosa virulence genes, functions as a multimer. *Journal of Bacteriology*, *184*(17), 4912–4919. https://doi.org/10.1128/JB.184.17.4912-4919.2002

Koch, G., Nadal-Jimenez, P., Cool, R. H., & Quax, W. J. (2014). Deinococcus radiodurans can interfere with quorum sensing by producing an AHL-acylase and an AHL-lactonase. *FEMS Microbiology Letters*, *356*(1), 62–70.

Kong, K. F., Jayawardena, S. R., Indulkar, S. D., Del Puerto, A., Koh, C. L., Høiby, N., & Mathee, K. (2005). Pseudomonas aeruginosa AmpR is a global transcriptional factor that regulates expression of AmpC and PoxB β-lactamases, proteases, quorum sensing, and other virulence factors. *Antimicrobial Agents and Chemotherapy*, *49*(11), 4567–4575. https://doi.org/10.1128/AAC.49.11.4567-4575.2005

Kordbacheh, H., Eftekhar, F., & Ebrahimi, S. N. (2017). Anti-quorum sensing activity of Pistacia atlantica against Pseudomonas aeruginosa PAO1 and identification of its bioactive compounds. *Microbial Pathogenesis*, *110*, 390–398. https://doi.org/10.1016/j.micpath.2017.07.018

Koul, S., & Kalia, V. C. (2016). Multiplicity of Quorum Quenching Enzymes : A Potential Mechanism to Limit Quorum Sensing Bacterial Population. *Indian Journal of Microbiology*. https://doi.org/10.1007/s12088-016-0633-1

Koul, S., Prakash, J., Mishra, A., & Kalia, V. C. (2016). Potential Emergence of Multi-quorum Sensing Inhibitor Resistant (MQSIR) Bacteria. *Indian Journal of Microbiology*, *56*(1), 1–18. https://doi.org/10.1007/s12088-015-0558-0

Krzy\.zek, P., & Gościniak, G. zyn. (2018). A proposed role for diffusible signal factors in the biofilm formation and morphological transformation of Helicobacter pylori. *The Turkish Journal of Gastroenterology*, *29*(1), 7.

Kulawik, P., Rathod, N. B., Ozogul, Y., Ozogul, F., & Zhang, W. (2022). Recent developments in the use of cold plasma, high hydrostatic pressure, and pulsed electric fields on microorganisms and viruses in seafood. *Critical Reviews in Food Science and Nutrition*, 1–15.

Lee, J., & Zhang, L. (2015). The hierarchy quorum sensing network in Pseudomonas

aeruginosa. *Protein and Cell*, *6*(1), 26–41. https://doi.org/10.1007/s13238-014-0100-x

Lee, S., Xu, H., Rice, S. A., Chong, T. H., & Oh, H.-S. (2021). Development of a quorum quenching-column to control biofouling in reverse osmosis water treatment processes. *Journal of Industrial and Engineering Chemistry*, *94*, 188–194. https://doi.org/https://doi.org/10.1016/j.jiec.2020.10.037

Li, Y.-S., Li, B.-B., Tian, T., & Yu, H.-Q. (2023). Quorum sensing unveils the sludge floccule-assisted stabilization of aerobic granules in granule-dominated sequencing batch reactors. *Biotechnology and Bioengineering*, *120*(2), 444–455.

Li, Y.-S., Pan, X.-R., Cao, J.-S., Song, X.-N., Fang, F., Tong, Z.-H., Li, W.-W., & Yu, H.-Q. (2017). Augmentation of acyl homoserine lactones-producing and-quenching bacterium into activated sludge for its granulation. *Water Research*, *125*, 309–317.

Liao, C., Huang, X., Wang, Q., Yao, D., & Lu, W. (2022). Virulence factors of Pseudomonas aeruginosa and antivirulence strategies to combat its drug resistance. *Frontiers in Cellular and Infection Microbiology*, *12*, 926758.

Liu, D., Momb, J., Thomas, P. W., Moulin, A., Petsko, G. A., Fast, W., & Ringe, D. (2008). Mechanism of the quorum-quenching lactonase (AiiA) from Bacillus thuringiensis. 1. Product-bound structures. *Biochemistry*, *47*(29), 7706–7714.

Liu, J., Sun, X., Ma, Y., Zhang, J., Xu, C., & Zhou, S. (2020). Quorum quenching mediated bacteria interruption as a probable strategy for drinking water treatment against bacterial pollution. *International Journal of Environmental Research and Public Health*, *17*(24), 1–13. https://doi.org/10.3390/ijerph17249539

Liu, P., Chen, Y., Shao, Z., Chen, J., Wu, J., Guo, Q., Shi, J., Wang, H., & Chu, X. (2019). AhlX, an N-acylhomoserine lactonase with unique properties. *Marine Drugs*, *17*(7), 387.

Luo, Y., Zhao, K., Baker, A. E., Kuchma, S. L., Coggan, K. A., Wolfgang, M. C., Wong, G. C. L., & O'Toole, G. A. (2015). A hierarchical cascade of second messengers regulates Pseudomonas aeruginosa surface behaviors. *MBio*, *6*(1), 10–1128.

Lynch, M. J., Swift, S., Kirke, D. F., Keevil, C. W., Dodd, C. E. R., & Williams, P. (2002). The regulation of biofilm development by quorum sensing in Aeromonas hydrophila. *Environmental Microbiology*, *4*(1), 18–28.

Ma, H., Darmawan, E. T., Zhang, M., Zhang, L., & Bryers, J. D. (2013). Development of a poly (ether urethane) system for the controlled release of two novel anti-biofilm agents based on gallium or zinc and its efficacy to prevent bacterial biofilm formation. *Journal of Controlled Release*, *172*(3), 1035–1044.

Mah, T.-F. C., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, *9*(1), 34–39.

Maisuria, V. B., & Nerurkar, A. S. (2015). Interference of quorum sensing by Delftia sp. VM4 depends on the activity of a novel n-acylhomoserine lactone-acylase. *PLoS ONE*, *10*(9), 1–15. https://doi.org/10.1371/journal.pone.0138034

Manefield, M., de Nys, R., Naresh, K., Roger, R., Givskov, M., Peter, S., & Kjelleberg, S. (1999). Evidence that halogenated furanones from Delisea pulchra inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology*, *145*(2), 283–291.

Mangwani, N., Kumari, S., & Das, S. (2016). Bacterial biofilms and quorum sensing: fidelity in bioremediation technology. *Biotechnology and Genetic Engineering Reviews*, *32*(1-2), 43–73.

Maqbool, T., Khan, S. J., Waheed, H., Lee, C.-H., Hashmi, I., & Iqbal, H. (2015). Membrane biofouling retardation and improved sludge characteristics using quorum quenching bacteria in submerged membrane bioreactor. *Journal of Membrane Science*, *483*, 75–83. https://doi.org/https://doi.org/10.1016/j.memsci.2015.02.011

Markowska, K., Grudniak, A., & Wolska, K. (2013). Silver nanoparticles as an alternative strategy against bacterial biofilms. *Acta Biochimica Polonica*, *60*(4), 523–530.

Marzhoseyni, Z., Mousavi, M. J., Saffari, M., & Ghotloo, S. (2023). Immune escape strategies of Pseudomonas aeruginosa to establish chronic infection. *Cytokine*, *163*, 156135.

Mastroleo, F., Van Houdt, R., Atkinson, S., Mergeay, M., Hendrickx, L., Wattiez, R., & Leys, N. (2013). Modelled microgravity cultivation modulates N-acylhomoserine lactone production in Rhodospirillum rubrum S1H independently of cell density. *Microbiology*, *159*(Pt_12), 2456–2466.

Mattila-Sandholm, T., & Wirtanen, G. (1992). Biofilm formation in the industry: a review.

Food Reviews International, *8*(4), 573–603.

McBrayer, D. N., Cameron, C. D., & Tal-Gan, Y. (2020). Development and utilization of peptide-based quorum sensing modulators in Gram-positive bacteria. *Organic & Biomolecular Chemistry*, *18*(37), 7273–7290.

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., & others. (1997). Quorum sensing and Chromobacterium violaceum: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology*, *143*(12), 3703–3711.

McVittie, A., Messik, F., & Zahler, S. A. (1962). Developmental biology of Myxococcus. *Journal of Bacteriology*, *84*(3), 546–551.

Montánchez, I., & Kaberdin, V. R. (2020). Vibrio harveyi: A brief survey of general characteristics and recent epidemiological traits associated with climate change. *Marine Environmental Research*, *154*, 104850.

Montoya, C., Jain, A., Londoño, J. J., Correa, S., Lelkes, P. I., Melo, M. A., & Orrego, S. (2021). Multifunctional dental composite with piezoelectric nanofillers for combined antibacterial and mineralization effects. *ACS Applied Materials & Interfaces*, *13*(37), 43868–43879.

Morici, L. A., Carterson, A. J., Wagner, V. E., Frisk, A., Schurr, J. R., zu Bentrup, K. H., Hassett, D. J., Iglewski, B. H., Sauer, K., & Schurr, M. J. (2007). Pseudomonas aeruginosa AlgR represses the Rhl quorum-sensing system in a biofilm-specific manner. *Journal of Bacteriology*, *189*(21), 7752–7764.

Morohoshi, T., Shiono, T., Takidouchi, K., Kato, M., Kato, N., Kato, J., & Ikeda, T. (2007). Inhibition of quorum sensing in Serratia marcescens AS-1 by synthetic analogs of N-acylhomoserine lactone. *Applied and Environmental Microbiology*, *73*(20), 6339–6344.

Nazzaro, F., Fratianni, F., & Coppola, R. (2013). Quorum Sensing and Phytochemicals. *International Journal of Molecular Sciences*, *14*(6), 12607–12619. https://doi.org/10.3390/ijms140612607

Nealson, K. H., Platt, T., & Hastings, J. W. (1970). Cellular control of the synthesis and activity of the bacterial luminescent system. *Journal of Bacteriology*, *104*(1), 313–322.

Neelapu, N. R. R., Dutta, T., & Challa, S. (2018). Quorum sensing and its role in

Agrobacterium mediated gene transfer. *Implication of Quorum Sensing System in Biofilm Formation and Virulence*, 259–275.

Ng, F. S. W., Wright, D. M., & Seah, S. Y. K. (2011). Characterization of a phosphotriesterase-like lactonase from Sulfolobus solfataricus and its immobilization for disruption of quorum sensing. *Applied and Environmental Microbiology*, *77*(4), 1181–1186. https://doi.org/10.1128/AEM.01642-10

Ng, W.-L., & Bassler, B. L. (2009). Bacterial quorum-sensing network architectures. *Annual Review of Genetics*, *43*, 197–222.

Nithya, C., Aravindraja, C., & Pandian, S. K. (2010). Bacillus pumilus of Palk Bay origin inhibits quorum-sensing-mediated virulence factors in Gram-negative bacteria. *Research in Microbiology*, *161*(4), 293–304.

Oh, H.-S., & Lee, C.-H. (2018). Origin and evolution of quorum quenching technology for biofouling control in MBRs for wastewater treatment. *Journal of Membrane Science*, *554*, 331–345.

Oh, H.-S., Yeon, K.-M., Yang, C.-S., Kim, S.-R., Lee, C.-H., Park, S. Y., Han, J. Y., & Lee, J.-K. (2012). Control of Membrane Biofouling in MBR for Wastewater Treatment by Quorum Quenching Bacteria Encapsulated in Microporous Membrane. *Environmental Science & Technology*, *46*(9), 4877–4884. https://doi.org/10.1021/es204312u

Oliveira, R., Marques, F., & Azeredo, J. (1999). Purification of polysaccharides from a biofilm matrix by selective precipitation of proteins. *Biotechnology Techniques*, *13*, 391–393.

Packiavathy, I. A. S. V., Kannappan, A., Thiyagarajan, S., Srinivasan, R., Jeyapragash, D., Paul, J. B. J., Velmurugan, P., & Ravi, A. V. (2021a). AHL-Lactonase Producing Psychrobacter sp. From Palk Bay Sediment Mitigates Quorum Sensing-Mediated Virulence Production in Gram Negative Bacterial Pathogens. *Frontiers in Microbiology*, *12*(April). https://doi.org/10.3389/fmicb.2021.634593

Packiavathy, I. A. S. V., Kannappan, A., Thiyagarajan, S., Srinivasan, R., Jeyapragash, D., Paul, J. B. J., Velmurugan, P., & Ravi, A. V. (2021b). AHL-Lactonase producing Psychrobacter sp. from Palk Bay sediment mitigates quorum sensing-mediated virulence production in Gram negative bacterial pathogens. *Frontiers in Microbiology*, *12*, 748.

Packiavathy, I. A. S. V., Priya, S., Pandian, S. K., & Ravi, A. V. (2014). Inhibition of biofilm development of uropathogens by curcumin--an anti-quorum sensing agent from Curcuma longa. *Food Chemistry*, *148*, 453–460.

Pan, X., Liu, J., Zhang, D., Chen, X., Li, L., Song, W., & Yang, J. (2010). A comparison of five extraction methods for extracellular polymeric substances (EPS) from biofilm by using threedimensional excitation-emission matrix (3DEEM) fluorescence spectroscopy. *Water Sa*, *36*(1).

Papenfort, K., & Bassler, B. L. (2016). Quorum sensing signal-response systems in Gram-negative bacteria. *Nature Reviews Microbiology*, *14*(9), 576–588. https://doi.org/10.1038/nrmicro.2016.89

Patel, H., Buchad, H., & Gajjar, D. (2022). Pseudomonas aeruginosa persister cell formation upon antibiotic exposure in planktonic and biofilm state. *Scientific Reports*, *12*(1), 1–12. https://doi.org/10.1038/s41598-022-20323-3

Patel, N. M., Moore, J. D., Blackwell, H. E., & Amador-Noguez, D. (2016). Identification of unanticipated and novel N-acyl L-homoserine lactones (AHLs) using a sensitive non-targeted LC-MS/MS method. *PloS One*, *11*(10), e0163469.

Paul, D., Kim, Y. S., Ponnusamy, K., & Kweon, J. H. (2009). Application of Quorum Quenching to Inhibit Biofilm Formation. *Environmental Engineering Science*. https://doi.org/10.1089/ees.2008.0392

Pinto, H., Simões, M., & Borges, A. (2021). Prevalence and impact of biofilms on bloodstream and urinary tract infections: A systematic review and meta-analysis. *Antibiotics*, *10*(7), 825.

Ponnusamy, K., Paul, D., & Kweon, J. H. (2009). Inhibition of Quorum Sensing Mechanism and Aeromonas hydrophila Biofilm Formation by Vanillin. *Environmental Engineering Science*. https://doi.org/10.1089=ees.2008.0415

Quan, K., Hou, J., Zhang, Z., Ren, Y., Peterson, B. W., Flemming, H.-C., Mayer, C., Busscher, H. J., & van der Mei, H. C. (2022). Water in bacterial biofilms: pores and channels, storage and transport functions. *Critical Reviews in Microbiology*, *48*(3), 283–302.

Rajesh, P. S., & Rai, V. R. (2014a). Molecular identification of aiiA homologous gene from

endophytic Enterobacter species and in silico analysis of putative tertiary structure of AHL-lactonase. *Biochemical and Biophysical Research Communications*, *443*(1), 290–295. https://doi.org/10.1016/j.bbrc.2013.11.101

Rajesh, P. S., & Rai, V. R. (2014b). Molecular identification of aiiA homologous gene from endophytic Enterobacter species and in silico analysis of putative tertiary structure of AHL-lactonase. *Biochemical and Biophysical Research Communications*, *443*(1), 290–295.

Rajkumari, J., Busi, S., Vasu, A. C., & Reddy, P. (2017). Facile green synthesis of baicalein fabricated gold nanoparticles and their antibiofilm activity against Pseudomonas aeruginosa PAO1. *Microbial Pathogenesis*, *107*, 261–269. https://doi.org/10.1016/j.micpath.2017.03.044

Rather, M. A., Gupta, K., & Mandal, M. (2021). Microbial biofilm: formation, architecture, antibiotic resistance, and control strategies. *Brazilian Journal of Microbiology*, 1–18.

Reichhardt, C., & Parsek, M. R. (2019). Confocal laser scanning microscopy for analysis of Pseudomonas aeruginosa biofilm architecture and matrix localization. *Frontiers in Microbiology*, *10*, 677.

Rezzonico, F., Smits, T. H. M., & Duffy, B. (2012). Detection of AI-2 receptors in genomes of Enterobacteriaceae suggests a role of type-2 quorum sensing in closed ecosystems. *Sensors*, *12*(5), 6645–6665.

Romero, M., Diggle, S. P., Heeb, S., Cámara, M., & Otero, A. (2008). Quorum quenching activity in Anabaena sp. PCC 7120: identification of AiiC, a novel AHL-acylase. *FEMS Microbiology Letters*, *280*(1), 73–80. https://doi.org/10.1111/j.1574-6968.2007.01046.x

Roupie, C., Labat, B., Morin-Grognet, S., Echalard, A., Ladam, G., & Thébault, P. (2021). Dual-functional antibacterial and osteogenic nisin-based layer-by-layer coatings. *Materials Science and Engineering: C*, *131*, 112479.

Rudrappa, T., Biedrzycki, M. L., & Bais, H. P. (2008). Causes and consequences of plant-associated biofilms. *FEMS Microbiology Ecology*, *64*(2), 153–166.

Rumbaugh, K. P., & Sauer, K. (2020). Biofilm dispersion. *Nature Reviews Microbiology*, *18*(10), 571–586.

RUSSELL, J. C. (2000). Bacteria, biofilms, and devices: the possible protective role of

phosphorylcholine materials. *Journal of Endourology*, *14*(1), 39–42.

Rutherford, S. T., & Bassler, B. L. (2012). Bacterial quorum sensing: Its role in virulence and possibilities for its control. *Cold Spring Harbor Perspectives in Medicine*, *2*(11), 1–26. https://doi.org/10.1101/cshperspect.a012427

Sadekuzzaman, M., Yang, S., Mizan, M. F. R., & Ha, S. D. (2015). Current and recent advanced strategies for combating biofilms. *Comprehensive Reviews in Food Science and Food Safety*, *14*(4), 491–509.

Sakr, M. M. ohamme., Aboshanab, K. M. ohame. A., Aboulwafa, M. M. abrou., & Hassouna, N. A. bde. H. (2013). Characterization and complete sequence of lactonase enzyme from Bacillus weihenstephanensis isolate P65 with potential activity against acyl homoserine lactone signal molecules. *BioMed Research International*, *2013*, 192589. https://doi.org/10.1155/2013/192589

Sakuragi, Y., & Kolter, R. (2007). Quorum-sensing regulation of the biofilm matrix genes (pel) of Pseudomonas aeruginosa. *Journal of Bacteriology*, *189*(14), 5383–5386.

Schaber, J. A., Carty, N. L., McDonald, N. A., Graham, E. D., Cheluvappa, R., Griswold, J. A., & Hamood, A. N. (2004). Analysis of quorum sensing-deficient clinical isolates of Pseudomonas aeruginosa. *Journal of Medical Microbiology*, *53*(9), 841–853.

Schaefer, A. L., Greenberg, E. P., Oliver, C. M., Oda, Y., Huang, J. J., Bittan-Banin, G., Peres, C. M., Schmidt, S., Juhaszova, K., Sufrin, J. R., & others. (2008). A new class of homoserine lactone quorum-sensing signals. *Nature*, *454*(7204), 595–599.

Schaefer, A. L., Val, D. L., Hanzelka, B. L., Cronan, J. E., & Greenberg, E. P. (1996). Generation of cell-to-cell signals in quorum sensing: Acyl homoserine lactone synthase activity of a purified Vibrio fischeri LuxI protein. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(18), 9505–9509. https://doi.org/10.1073/pnas.93.18.9505

See-Too, W. S., Convey, P., Pearce, D. A., & Chan, K. G. (2018). Characterization of a novel N-acylhomoserine lactonase, AidP, from Antarctic Planococcus sp. *Microbial Cell Factories*, *17*(1), 1–14. https://doi.org/10.1186/s12934-018-1024-6

Selim, M. S., Shenashen, M. A., El-Safty, S. A., Higazy, S. A., Selim, M. M., Isago, H., & Elmarakbi, A. (2017). Recent progress in marine foul-release polymeric nanocomposite

coatings. *Progress in Materials Science*, *87*, 1–32.

Sethupathy, S., Prasath, K. G., Ananthi, S., Mahalingam, S., Balan, S. Y., & Pandian, S. K. (2016). Proteomic analysis reveals modulation of iron homeostasis and oxidative stress response in Pseudomonas aeruginosa PAO1 by curcumin inhibiting quorum sensing regulated virulence factors and biofilm production. *Journal of Proteomics*, *145*, 112–126. https://doi.org/10.1016/j.jprot.2016.04.019

Shah, S. S. A., & Choo, K.-H. (2020). Isolation and characterization of novel indigenous facultative quorum quenching bacterial strains for ambidextrous biofouling control. *Bioresource Technology*, *308*, 123269. https://doi.org/https://doi.org/10.1016/j.biortech.2020.123269

Shankar, P., Kushwah, J., Bhushan, A., Joshi, J., & Mukherjee, T. (2014). *Diversity and Polymorphism in AHL-Lactonase Gene (aiiA) of Bacillus Diversity and Polymorphism in AHL-Lactonase Gene (aiiA) of Bacillus*. *May*. https://doi.org/10.4014/jmb.1105.05056

Shastry, R. P., Rekha, P. D., & Rai, V. R. (2019). Biofilm inhibitory activity of metallo-protein AHL-lactonase from cell-free lysate of endophytic Enterobacter species isolated from Coscinium fenestratum Gaertn. *Biocatalysis and Agricultural Biotechnology*, *18*. https://doi.org/10.1016/j.bcab.2019.01.047

Shen, Y., Cui, F., Wang, D., Li, T., & Li, J. (2021). Quorum Quenching Enzyme (PF-1240) Capable to Degrade AHLs as a Candidate for Inhibiting Quorum Sensing in Food Spoilage Bacterium Hafnia alvei. *Foods*, *10*(11), 2700.

Sifri, C. D. (2008). Quorum sensing: bacteria talk sense. *Clinical Infectious Diseases*, *47*(8), 1070–1076.

Skariyachan, S., Sridhar, V. S., Packirisamy, S., Kumargowda, S. T., & Challapilli, S. B. (2018). Recent perspectives on the molecular basis of biofilm formation by Pseudomonas aeruginosa and approaches for treatment and biofilm dispersal. *Folia Microbiologica*, *63*, 413–432.

Song, B., Zhang, E., Han, X., Zhu, H., Shi, Y., & Cao, Z. (2020). Engineering and application perspectives on designing an antimicrobial surface. *ACS Applied Materials & Interfaces*, *12*(19), 21330–21341.

Song, Z., Sun, H., Yang, Y., Jing, H., Yang, L., Tong, Y., Wei, C., Wang, Z., Zou, Q., & Zeng, H. (2016). Enhanced efficacy and anti-biofilm activity of novel nanoemulsions against skin burn wound multi-drug resistant MRSA infections. *Nanomedicine: Nanotechnology, Biology and Medicine*, *12*(6), 1543–1555.

Stanley, N. R., Palmer, T., & Berks, B. C. (2000). The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in Escherichia coli. *Journal of Biological Chemistry*, *275*(16), 11591–11596.

Steindler, L., Bertani, I., De Sordi, L., Schwager, S., Eberl, L., & Venturi, V. (2009). LasI/R and RhlI/R quorum sensing in a strain of Pseudomonas aeruginosa beneficial to plants. *Applied and Environmental Microbiology*, *75*(15), 5131–5140.

Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. Di, Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates. *Apmis*, *115*(8), 891–899.

Strateva, T., & Mitov, I. (2011). Contribution of an arsenal of virulence factors to pathogenesis of Pseudomonas aeruginosa infections. *Annals of Microbiology*, *61*(4), 717–732. https://doi.org/10.1007/s13213-011-0273-y

Sun, X., Hill, P., Liu, J., Qian, J., Ma, Y., & Zhou, S. (2021). Marine-Source Quorum Quenching Enzyme YtnP to Improve Hygiene Quality in Dental Units. *Marine Drugs*, *19*(4), 225.

Sun, X., Ni, Z., Tang, J., Ding, Y., Wang, X., & Li, F. (2021). The abaI/abaR quorum sensing system effects on pathogenicity in Acinetobacter baumannii. *Frontiers in Microbiology*, *12*, 679241.

Suzumoto, Y., Dym, O., Roviello, G. N., Worek, F., Sussman, J. L., & Manco, G. (2020). Structural and functional characterization of new ssopox variant points to the dimer interface as a driver for the increase in promiscuous paraoxonase activity. *International Journal of Molecular Sciences*, *21*(5), 1–26. https://doi.org/10.3390/ijms21051683

Syafiuddin, A., Boopathy, R., & Mehmood, M. A. (2021). Recent advances on bacterial quorum quenching as an effective strategy to control biofouling in membrane bioreactors. *Bioresource Technology Reports*, *15*, 100745. https://doi.org/https://doi.org/10.1016/j.biteb.2021.100745

Taghadosi, R., Shakibaie, M. R., & Masoumi, S. (2015). Biochemical detection of N-Acyl homoserine lactone from biofilm-forming uropathogenic Escherichia coli isolated from urinary tract infection samples. *Reports of Biochemistry & Molecular Biology*, *3*(2), 56–61. http://www.ncbi.nlm.nih.gov/pubmed/26989738

Tahmourespour, A., Salehi, R., & Kermanshahi, R. K. (2011). Lactobacillus acidophilus-derived biosurfactant effect on gtfB and gtfC expression level in Streptococcus mutans biofilm cells. *Brazilian Journal of Microbiology*, *42*, 330–339.

Tan, C. H., Koh, K. S., Xie, C., Zhang, J., Tan, X. H., Lee, G. P., Zhou, Y., Ng, W. J., Rice, S. A., & Kjelleberg, S. (2015). Community quorum sensing signalling and quenching: microbial granular biofilm assembly. *Npj Biofilms and Microbiomes*, *1*(1), 1–9.

Tang, K., Su, Y., Brackman, G., Cui, F., Zhang, Y., Shi, X., Coenye, T., & Zhang, X.-H. (2015). MomL, a novel marine-derived N-acyl homoserine lactonase from Muricauda olearia. *Applied and Environmental Microbiology*, *81*(2), 774–782.

Thallinger, B., Prasetyo, E. N., Nyanhongo, G. S., & Guebitz, G. M. (2013). Antimicrobial enzymes: an emerging strategy to fight microbes and microbial biofilms. *Biotechnology Journal*, *8*(1), 97–109.

Tian, F., Li, J., Nazir, A., & Tong, Y. (2021). Bacteriophage--a promising alternative measure for bacterial biofilm control. *Infection and Drug Resistance*, 205–217.

Tolker-Nielsen, T. (2015). Biofilm development. *Microbial Biofilms*, 51–66.

Tomasz, A. (1965). Control of the competent state in Pneumococcus by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria. *Nature*, *208*(5006), 155–159.

Tran, P. L., Hamood, A. N., & Reid, T. W. (2014). Antimicrobial coatings to prevent biofilm formation on medical devices. *Antibiofilm Agents: From Diagnosis to Treatment and Prevention*, 175–204.

Tseng, B. S., Zhang, W., Harrison, J. J., Quach, T. P., Song, J. L., Penterman, J., Singh, P. K., Chopp, D. L., Packman, A. I., & Parsek, M. R. (2013). The extracellular matrix protects Pseudomonas aeruginosa biofilms by limiting the penetration of tobramycin. *Environmental Microbiology*, *15*(10), 2865–2878. https://doi.org/10.1111/1462-2920.12155

Tsibouklis, J., Stone, M., Thorpe, A. A., Graham, P., Nevell, T. G., & Ewen, R. J. (2000). Inhibiting bacterial adhesion onto surfaces: the non-stick coating approach. *International Journal of Adhesion and Adhesives*, *20*(2), 91–96.

Tsibouklis, J., Stone, M., Thorpe, A. A., Graham, P., Peters, V., Heerlien, R., Smith, J. R., Green, K. L., & Nevell, T. G. (1999). Preventing bacterial adhesion onto surfaces: the low-surface-energy approach. *Biomaterials*, *20*(13), 1229–1235.

Utari, P. D., Setroikromo, R., Melgert, B. N., & Quax, W. J. (2018). PvdQ quorum quenching acylase attenuates Pseudomonas aeruginosa virulence in a mouse model of pulmonary infection. *Frontiers in Cellular and Infection Microbiology*, *8*, 119.

Van Houdt, R., Givskov, M., & Michiels, C. W. (2007). Quorum sensing in Serratia. *FEMS Microbiology Reviews*, *31*(4), 407–424.

Ventre, I., Ledgham, F., Prima, V., Lazdunski, A., Foglino, M., & Sturgis, J. N. (2003). Dimerization of the quorum sensing regulator RhlR: Development of a method using EGFP fluorescence anisotropy. *Molecular Microbiology*, *48*(1), 187–198. https://doi.org/10.1046/j.1365-2958.2003.03422.x

Venturi, V. (2006). Regulation of quorum sensing in Pseudomonas. *FEMS Microbiology Reviews*, *30*(2), 274–291.

Verchère, A., Dezi, M., Adrien, V., Broutin, I., & Picard, M. (2015). In vitro transport activity of the fully assembled MexAB-OprM efflux pump from Pseudomonas aeruginosa. *Nature Communications*, *6*, 1–6. https://doi.org/10.1038/ncomms7890

Verderosa, A. D., Totsika, M., & Fairfull-Smith, K. E. (2019). Bacterial biofilm eradication agents: a current review. *Frontiers in Chemistry*, *7*, 824.

Vijayakumar, K., Bharathidasan, V., Manigandan, V., & Jeyapragash, D. (2020). Quebrachitol inhibits biofilm formation and virulence production against methicillin-resistant Staphylococcus aureus. *Microbial Pathogenesis*, *149*, 104286.

Vinoj, G., Vaseeharan, B., Thomas, S., Spiers, A. J., & Shanthi, S. (2014). Quorum-quenching activity of the AHL-lactonase from Bacillus licheniformis DAHB1 inhibits Vibrio biofilm formation in vitro and reduces shrimp intestinal colonisation and mortality. *Marine Biotechnology*, *16*(6), 707–715.

Vishwakarma, V. (2020). Impact of environmental biofilms: Industrial components and its

remediation. *Journal of Basic Microbiology*, *60*(3), 198–206.

Viszwapriya, D., Prithika, U., Deebika, S., Balamurugan, K., & Pandian, S. K. (2016). In vitro and in vivo antibiofilm potential of 2,4-Di-tert-butylphenol from seaweed surface associated bacterium Bacillus subtilis against group A streptococcus. *Microbiological Research*, *191*, 19–31. https://doi.org/10.1016/j.micres.2016.05.010

Vladkova, T. (2008). *Surface modification approach to control biofouling*. Springer.

Wang, L. H., Weng, L. X., Dong, Y. H., & Zhang, L. H. (2004). Specificity and Enzyme Kinetics of the Quorum-quenching N-Acyl Homoserine Lactone Lactonase (AHL-lactonase). *Journal of Biological Chemistry*, *279*(14), 13645–13651. https://doi.org/10.1074/jbc.M311194200

Wei, Y., Perez, L. J., Ng, W.-L., Semmelhack, M. F., & Bassler, B. L. (2011). Mechanism of Vibrio cholerae autoinducer-1 biosynthesis. *ACS Chemical Biology*, *6*(4), 356–365.

Welch, M., Dutton, J. M., Glansdorp, F. G., Thomas, G. L., Smith, D. S., Coulthurst, S. J., Barnard, A. M. L., Salmond, G. P. C., & Spring, D. R. (2005). Structure--activity relationships of Erwinia carotovora quorum sensing signaling molecules. *Bioorganic & Medicinal Chemistry Letters*, *15*(19), 4235–4238.

Whiteley, M., Diggle, S. P., & Greenberg, E. P. (2017). Progress in and promise of bacterial quorum sensing research. *Nature*, *551*(7680), 313–320. https://doi.org/10.1038/nature24624

Wierre-Gore, N. A. E. (2021). *Phenotypic adaptation of Pseudomonas aeruginosa to the Cystic Fibrosis lung environment*.

Williams, P., Winzer, K., Chan, W. C., & Cámara, M. (2007). Look who's talking: Communication and quorum sensing in the bacterial world. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *362*(1483), 1119–1134. https://doi.org/10.1098/rstb.2007.2039

Williamson, N. R., Fineran, P. C., Ogawa, W., Woodley, L. R., & Salmond, G. P. C. (2008). Integrated regulation involving quorum sensing, a two-component system, a GGDEF/EAL domain protein and a post-transcriptional regulator controls swarming and RhlA-dependent surfactant biosynthesis in Serratia. *Environmental Microbiology*, *10*(5), 1202–1217.

Winding, A., Binnerup, S. J., & Sørensen, J. (1994). Viability of indigenous soil bacteria assayed by respiratory activity and growth. *Applied and Environmental Microbiology*, *60*(8), 2869–2875.

Winzer, K., Hardie, K. R., & Williams, P. (2002). Bacterial cell-to-cell communication: sorry, can't talk now—gone to lunch! *Current Opinion in Microbiology*, *5*(2), 216–222.

Wood, S. J., Goldufsky, J. W., Seu, M. Y., Dorafshar, A. H., & Shafikhani, S. H. (2023). Pseudomonas aeruginosa Cytotoxins: Mechanisms of Cytotoxicity and Impact on Inflammatory Responses. *Cells*, *12*(1), 195.

Wozniak, D. J., & Keyser, R. (2004). Effects of subinhibitory concentrations of macrolide antibiotics on Pseudomonas aeruginosa. *Chest*, *125*(2), 62S – 69S.

Wu, L., & Luo, Y. (2021). Bacterial quorum-sensing systems and their role in intestinal bacteria-host crosstalk. *Frontiers in Microbiology*, *12*, 611413.

Xia, R., Yang, Y., Pan, X., Gao, C., Yao, Y., Liu, X., Teame, T., Zhang, F., Hu, J., Ran, C., & others. (2020). Improving the production of AHL lactonase AiiO-AIO6 from Ochrobactrum sp. M231 in intracellular protease-deficient Bacillus subtilis. *AMB Express*, *10*(1), 1–9.

Xiao, Y., Waheed, H., Xiao, K., Hashmi, I., & Zhou, Y. (2018). In tandem effects of activated carbon and quorum quenching on fouling control and simultaneous removal of pharmaceutical compounds in membrane bioreactors. *Chemical Engineering Journal*, *341*, 610–617. https://doi.org/https://doi.org/10.1016/j.cej.2018.02.073

Xing, X., Li, T., Bi, Z., Qi, P., Li, Z., Wang, H., Lyu, L., Gao, Y., & Hu, C. (2020). Efficient removal of disinfection by-products precursors and inhibition of bacterial detachment by strong interaction of EPS with coconut shell activated carbon in ozone/biofiltration. *Journal of Hazardous Materials*, *392*, 122077.

Xu, B., Albert Ng, T. C., Huang, S., Shi, X., & Ng, H. Y. (2020). Feasibility of isolated novel facultative quorum quenching consortiums for fouling control in an AnMBR. *Water Research*, *169*, 115251. https://doi.org/https://doi.org/10.1016/j.watres.2019.115251

Xu, F., Zhao, C., Lee, C. H., Wang, W., & Xu, Q. (2019). Anti-biofouling performance of an immobilized indigenous quorum quenching bacterium bacillus cereus hg10 and its influence on the microbial community in a bioreactor. *International Journal of*

Environmental Research and Public Health, *16*(19), 3777.

Yang, L., Hu, Y., Liu, Y., Zhang, J., Ulstrup, J., & Molin, S. (2011). Distinct roles of extracellular polymeric substances in Pseudomonas aeruginosa biofilm development. *Environmental Microbiology*, *13*(7), 1705–1717.

Yates, E. A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S. R., Sockett, R. E., Goldner, M., Dessaux, Y., Cámara, M., Smith, H., & others. (2002). N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of Yersinia pseudotuberculosis and Pseudomonas aeruginosa. *Infection and Immunity*, *70*(10), 5635–5646.

Yeon, K.-M., Cheong, W.-S., Oh, H.-S., Lee, W.-N., Hwang, B.-K., Lee, C.-H., Beyenal, H., & Lewandowski, Z. (2009). Quorum Sensing: A New Biofouling Control Paradigm in a Membrane Bioreactor for Advanced Wastewater Treatment. *Environmental Science & Technology*, *43*(2), 380–385. https://doi.org/10.1021/es8019275

Yin, W., Wang, Y., Liu, L., & He, J. (2019). Biofilms: the microbial "protective clothing" in extreme environments. *International Journal of Molecular Sciences*, *20*(14), 3423.

Younus, S., Vinod Chandra, S. S., & Nair, A. S. S. (2021). Docking and dynamic simulation study of Crizotinib and Temozolomide drug with Glioblastoma and NSCLC target to identify better efficacy of the drug. *Future Journal of Pharmaceutical Sciences*, *7*(1). https://doi.org/10.1186/s43094-021-00323-2

Yu, H., Du, C., Qu, F., He, J., & Rong, H. (2022). Efficient biostimulants for bacterial quorum quenching to control fouling in MBR. *Chemosphere*, *286*, 131689. https://doi.org/https://doi.org/10.1016/j.chemosphere.2021.131689

Zamani, M., Behboudi, K., & Ahmadzadeh, M. (2014). *Biocontrol Science and Technology Quorum quenching by Bacillus cereus U92 : a double-edged sword in biological control of plant diseases*. *December*. https://doi.org/10.1080/09583157.2013.787046

Zdor, R. E. (2015). Bacterial cyanogenesis: impact on biotic interactions. *Journal of Applied Microbiology*, *118*(2), 267–274.

Zhang, H.-B., Wang, L.-H., & Zhang, L.-H. (2002). Genetic control of quorum-sensing signal turnover in Agrobacterium tumefaciens. *Proceedings of the National Academy of Sciences*, *99*(7), 4638–4643.

Zhang, Y., Wei, W., Wen, H., Cheng, Z., Mi, Z., Zhang, J., Liu, X., & Fan, X. (2023). Targeting Multidrug-Recalcitrant Pseudomonas aeruginosa Biofilms: Combined-Enzyme Treatment Enhances Antibiotic Efficacy. *Antimicrobial Agents and Chemotherapy*, e01358–22.

Zhao, J., Ai, G., Yang, S., Zhang, X., & Zhang, G. (2021). Detection, structural elucidation, and biological effects of diverse N-acyl-homoserine lactone signaling molecules in the plant-promoting endophytic bacterium Rhizobium oryzihabitans M15. *Journal of Agricultural and Food Chemistry*, *69*(33), 9693–9705.

Zhao, J., Chen, M., Quan, C. S., & Fan, S. D. (2015). Mechanisms of quorum sensing and strategies for quorum sensing disruption in aquaculture pathogens. *Journal of Fish Diseases*, *38*(9), 771–786.

Appendix I

Antibiogram of the Multi Drug Resistant (MDR) *P. aeruginosa* strains/isolates.

Isolate	AMK	TOB	CEF	AMC	AMP	COT	IPM	ERY	PIP	CAZ
ATCC	R	S	R	R	R	R	S	R	R	R
MTCC	R	S	R	R	R	R	S	R	R	S
SRL1	R	S	S	R	R	R	S	R	R	R
SRL2	S	S	S	R	R	R	S	R	R	R
SRL3	S	S	R	R	R	R	S	R	S	S
SRL4	S	S	R	R	R	R	S	R	R	S
HRK1	S	S	R	R	R	R	S	R	R	R
HRK2	R	S	R	R	R	R	S	R	R	R
HRK3	S	S	R	R	R	R	S	R	R	R
HRK4	S	S	R	R	R	R	S	R	R	R

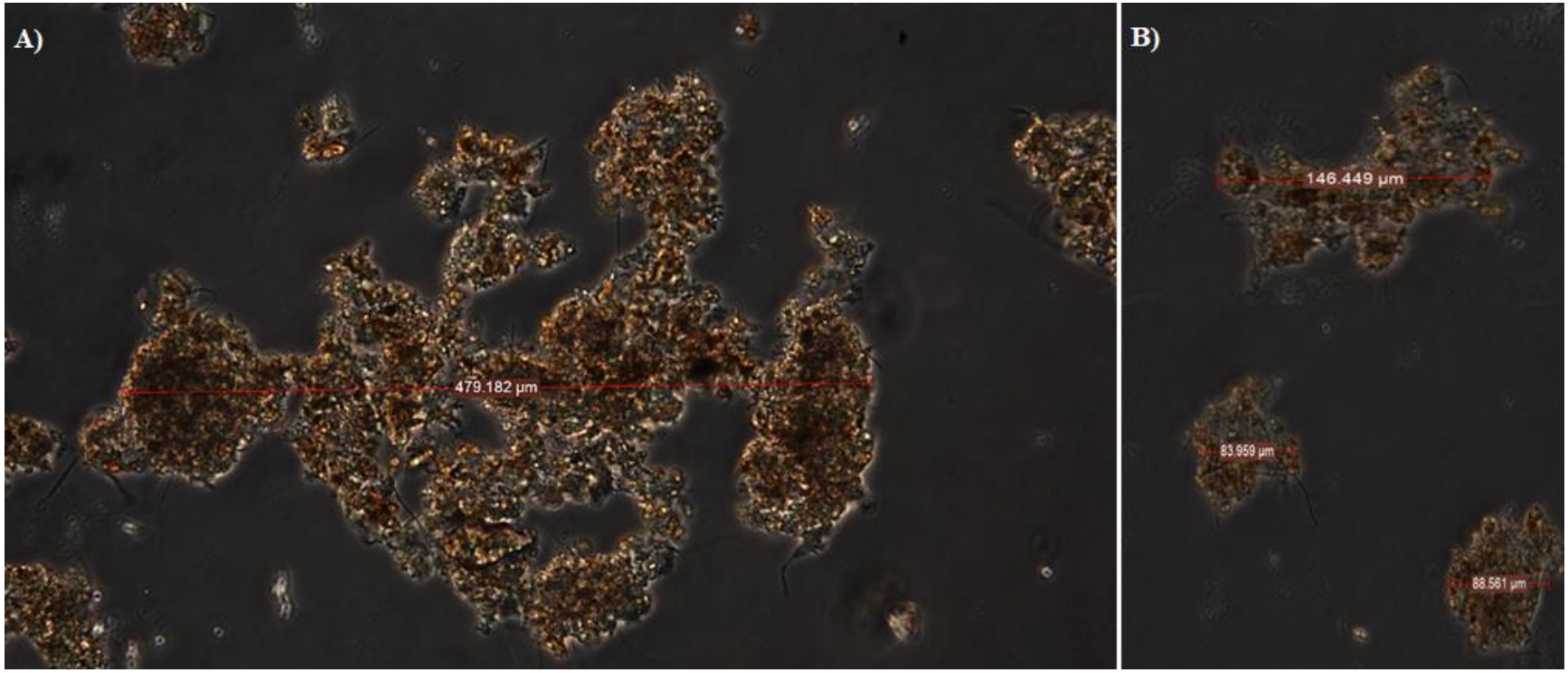
AMK-Amikacin, **TOB**- Tobramycin, **CEF**- Cefepime, **AMC**- Amoxicillin+Clavulanic acid, **AMP**- Ampicillin, **COT**- Co-trimoxazole, **IPM**- Imipenem, **ERY**- Erythrimycin, **PIP**- Piperacillin, **CAZ**- Ceftazidime **R**-Resistant **S-** Susceptible

Appendix II

Amino acid composition:		
Ala (A)	12	4.8%
Arg (R)	6	2.4%
Asn (N)	11	4.4%
Asp (D)	11	4.4%
Cys (C)	4	1.6%
Gln (Q)	6	2.4%
Glu (E)	27	10.8%
Gly (G)	21	8.4%
His (H)	9	3.6%
Ile (I)	18	7.2%
Leu (L)	26	10.4%
Lys (K)	12	4.8%
Met (M)	5	2.0%
Phe (F)	12	4.8%
Pro (P)	16	6.4%
Ser (S)	12	4.8%
Thr (T)	12	4.8%
Trp (W)	1	0.4%
Tyr (Y)	11	4.4%
Val (V)	18	7.2%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%

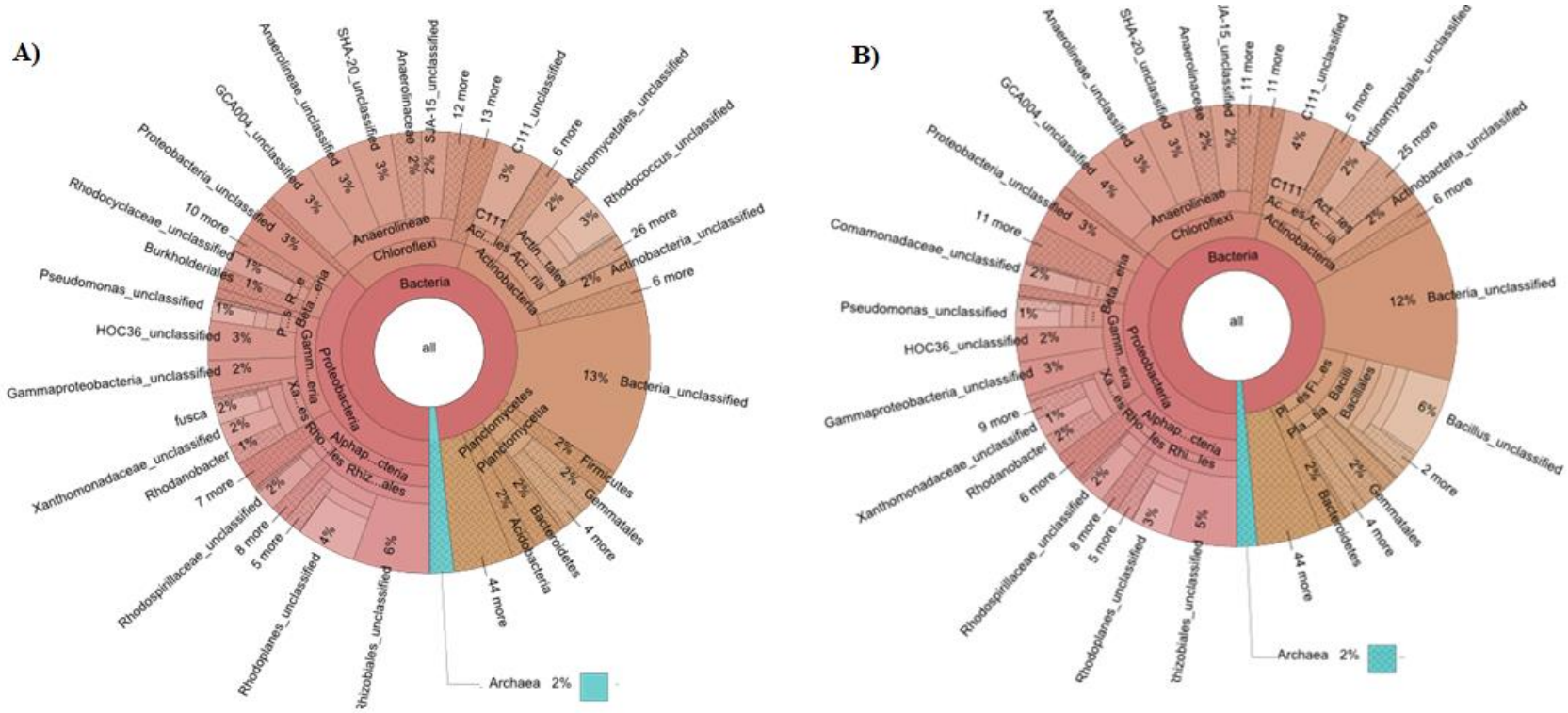
Amino acid composition of $AiiA_{PM7}$ (From ProtParam analysis)

Appendix III



Phase contrast image of microbial flocs (20X) found in A) untreated activated sludge B) 10% v/v CFS treated activated sludge

Appendix IV



Relative abundance of bacterial and archae community found in A) untreated activated sludge B) 10% (v/v) CFS treated activated sludge.

ABSTRACT

Name of the Student: Suryalekshmi V.A **Registration No. :10BB16A39018**

Faculty of Study: Biological Sciences **Year of Submission: 2023**

CSIR Lab : NIIST **Name of the supervisor: Dr. Krishnakumar B.**

Title of the thesis: Investigation of quorum quenching property of a novel *Bacillus velezensis* and its application in biofilm control.

Quorum sensing (QS) is a prevalent method for Gram-negative bacteria to regulate many of their gene expression in a cell density dependent manner. Important phenotypic traits such as virulence, secondary metabolites including antibiotic production and biofilm formation are intricately regulated through QS. Among these, biofilms due to its adverse impacts have high practical significance in healthcare, many industrial, agriculture and food processing sector. The conventional methods for biofilm control have inherent limitations. But, QS disruption (quorum quenching, QQ) mediated biofilm control is emerging as an alternate strategy with many advantages. The present study mainly focus on bacterial QQ. In this study, a novel strain of *Bacillus velezensis* (PM7) was isolated from environmental samples through enrichment culture, and its biofilm mitigation properties were evaluated under various conditions. The cell free supernatant (CFS) of PM7, showed dose-dependent reduction of QS controlled phenotypes in two bio-reporters, *Chromobacterium violaceum* and *Serratia marcescens*. This is the first report of extracellular QQ activity in *Bacillus*. The CFS treatment substantially declined many of the QS controlled phenotypes and resistance towards diffusion controlled antibiotics such as Gentamycin in *P. aeruginosa*, the model organism for QS related studies in Gram negative bacterium. The mechanism of extracellular QQ by *B. velezensis* PM7 was studied in detail, and demonstrated as lactonase mediated AHL degradation. Molecular docking studies of the putative lactonase from the bacterium also revealed a broad substrate specificity of the enzyme towards AHLs. The PM7 CFS exhibited a significant decline in biofilm formation by three Gram-negative bacteria on different surfaces such as stainless steel, borosilicate glass and urinary catheters. Hence, the unique strain of *B. velezensis* PM7 described in this study, with its lactonase secretory characteristic and broad-spectrum action against various Gram-negative bacteria, would be helpful in this scenario for preventing biofouling or other undesired QS-related properties. In-depth research is also required into the impact of extracellular QQ activity on bacterial population dynamics and its implications for biofilm in natural environments.

List of publication emanating from the thesis work

1. **Suryalekshmi Vijaya Ayyappan**, Krishnakumar Bhaskaran, Extracellular lactonase-mediated quorum quenching by a novel *Bacillus velezensis*, *FEMS Microbiology Letters*, Volume 369, Issue 1, 2022, fnac095, https://doi.org/10.1093/femsle/fnac095
2. **Suryalekshmi V.A**., Haritha. K., Athira A., Rinsiya Rahim A., Krishnakumar B., The extracellular lactonase activity of *Bacillus velezensis* PM7 impairs the quorum sensing controlled phenotypes and antibiotic resistance in *P. aeruginosa*. (Manuscript submitted).
3. **Suryalekshmi V.A.,** Rinsiya Rahim A., Krishnakumar B., Broad spectrum quorum quenching activity of Bacillus velezensis regulates biofilm formation against Gram negative bacteria. (Manuscript under preparation)

Conference presentation

1. **Suryalekshmi V. A.,** Ayana B. and Krishnakumar B., "Development of a microbial system for controlling quorum sensing mediated biofilm control", 33rd Kerala Science Congress on 25th - 30th January 2021. **(Best Poster Award)**
2. **Suryalekshmi V. A.** and Krishnakumar B., "Quorum quenching activity of an isolate Bacillus sp. And its application in Biofilm control", International Conference on Biotechnology for Resource Efficiency, Energy, Environment, Chemicals and Health, on 1st – 4th December 2021 at CSIR-Indian Institute of Petroleum, Dehradun, India
3. **Suryalekshmi V. A.** and Krishnakumar B., "Quorum Quenching Activity of Lactonase from an Isolated Bacillus sp."National webinar on " Advances in Industrial Biotechnology", on 3rd – 4th February 2022 at Dept. of Biotechnology, University of Kerala, Kariavattom, Trivandrum, Kerala. **(Best Poster Award)**
4. **Suryalekshmi V. A.** and Krishnakumar B., "Quorum quenching activity of a novel strain of bacillus velezensis and its application in biofilm control", 34th Kerala Science Congress on 10th - 12th February 2022.

   Suryalekshmi V. A., Haritha K. and Akhina M.K., "Effect of chlorination in the residual pathogenic bacterial removal and antibiotic resistance pattern in a full scale Waste Water Treatment Plant" , 12th International Conference on Sustainable Waste management & Circular Economy and IPLA Global Forum, on 30th November- 03rd December 2022 at Sri Venkateswara University, Tirupati, Andrapradesh, India.

GenBank Submissions

- MH234575
- MH234574
- MH234573
- KY689157
- KY689153
- KY689148
- KY689156
- KY689154
- MZ234152

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Extracellular lactonase-mediated quorum quenching by a novel *Bacillus velezensis*

Suryalekshmi Vijaya Ayyappan [1,2] and Krishnakumar Bhaskaran [1,2,*]

[1]Environmental Technology Division, CSIR-NIIST, Industrial Estate PO Pappanamcode, Thiruvananthapuram 695019, India
[2]Academy for Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India
*Corresponding author: Environmental Technology Division, CSIR NIIST, Industrial Estate, P. O., Thiruvananthapuram - 695019, India. Tel: 9447386706; E-mail: krishna@niist.res.in
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Abstract

Many Gram-negative bacteria coordinate their gene expression via quorum sensing (QS) mediated by small diffusible autoinducer molecules such as Acyl Homoserine Lactones (AHL). The degradation of AHLs or quorum quenching (QQ) imparts less selection pressure on the target organisms leading to a possible alternative to antimicrobial agents and traditional biofilm control methods. Here, a novel strain of *Bacillus velezensis*, strain PM7, exhibiting extracellular QQ activity against Gram-negative bacteria has been isolated. Analytical studies of the AHL degradation using LC-MS as well as bioreporters revealed the mechanism of QQ as homoserine lactone ring hydrolysis. Using molecular techniques, the presence of an aiiA homologues gene, specific for acyl homoserine lactonase enzyme, was confirmed in the bacterium. A biofilm inhibitory activity in the range of 36.9%–77.4% was achieved by the PM7 against three different Gram-negative bacteria. Moreover, a significant reduction ($P < .05$) in the QS-controlled traits such as violacein production in *Chromobacterium violaceum* (73%), swarming motility and prodigiosin production (67.2%) in *Serratia marcescens*, and exopolysaccharide production (97.9%) in *Pseudomonas aeruginosa*, was also observed. The extracellular and broad-spectrum lactonase of this novel strain can be beneficial for its use in QQ applications.

Keywords: quorum sensing, AHL lactonases, *Bacillus velezensis*, quorum quenching, biofouling control

Introduction

Quorum Sensing (QS) is one of the mechanism used by several bacterial species to monitor their local cell density and act cooperatively facilitating population dependent adaptive behavior. Diffusible signals (autoinducers) that vary intraspecifically and interspecifically mediate QS in a concentration dependent manner (Fuqua et al. 1994). The predominant autoinducer for QS in Gram-negative bacteria are N-Acyl Homoserine Lactones (AHL), which bind to their corresponding receptor proteins to initiate the expression of QS-controlled genes. QS regulates a plethora of biological traits in bacteria including bioluminescence (Fuqua et al. 1994), virulence (Dekimpe and Deziel 2009), motility (Glessner et al. 1999, Rice et al. 2005), DNA transfer (Mhedbi-Hajri et al. 2016), and biofilm formation (Hawver et al. 2016).

Biofilms are highly complex structures that predominate in diverse ecosystems. The formation of biofilms is beneficial in sectors, such as environmental bioremediation (Russel et al. 2021), waste water treatment (Hou et al. 2022, Khan et al. 2022), and bioleaching (Castro et al. 2017). However, studies indicate that biofilms have adverse effects on productivity in many industrial (Vishwakarma 2020) and health care (Pinto et al. 2021) sectors. While, the effects of biofilms on agriculture sector have been found to be both positive (Chen et al. 2013, Ansari and Ahmad 2018) and negative (Rather et al. 2021).The extracellular polymeric substances (EPS) encapsulation in biofilms elevates resistance to antibiotics and other conventional biocides as compared to their planktonic equivalent (Shih and Huang 2002). Disruption of the QS network, called Quorum Quenching (QQ), has been demonstrated as a substitute for antimicrobials in several fields including bacterial infections in plants (Singh et al. 2021, Verma et al. 2021) and animals (Khan et al. 2015, Utari et al. 2018, Anju et al. 2021), membrane biofouling in water treatment plants (Oh and Lee 2018, Shah et al. 2021, Syafiuddin et al. 2021), aquaculture (Defoirdt et al. 2006, Zhao et al. 2015, Raissa et al. 2020), and biocorrosion of oil wells and shipping vessels (Ejileugha et al. 2021).

Enzymatic and nonenzymatic degradation of AHL molecules has been put forward as an effective strategy of QQ in Gram-negative bacteria (Hassan et al. 2016, Chbib 2020, Packiavathy et al. 2021). AHL degrading enzymes such as lactonases (Torres et al. 2017, Anandan and Vittal 2019), acylases (Chan 2013, Koch et al. 2014), and oxidoreductases (Uroz et al. 2005) have been previously reported. The extensively explored of these are lactonases, which are divided into three protein superfamilies: *viz.* paraoxonases (PONs; Bar-Rogovsky et al. 2013), phosphotriesterases like lactonase (PLL; Hiblot et al. 2015), and metallo-lactamases like lactonases (MLLS; Thomas et al. 2005). Autoinducer inactivator A (AiiA) from *Bacillus thuringiensis* is a well-characterized lactonase from the MLL superfamily (Kim et al. 2005). Several homologues of AiiA have been identified in a wide range of bacteria, predominantly in *Bacillus* genera that has been shown as promising QQ agents with remarkable antibiofilm property (Tang et al. 2015, See-Too et al. 2018, Liu et al. 2019). Lactonases may possess a broad range of substrate specificity irrespective of the acyl chain length due to their ability to target the lactone ring moiety. Gram-negative bacteria such as *Agrobacterium*, *Pseudomonas*, and *Klebsiella* and Gram-positive bacteria such as *Bacillus*, *Rhodococ-*

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cus, *Streptomyces*, and *Arthrobacter* are reported to have lactonase-based QQ activity (Khan and Farrand 2009, Oh and Lee 2018, Anandan and Vittal 2019).

In the present study, a novel strain of *Bacillus velezensis* was isolated that can disrupt the QS networks of Gram-negative bacteria by degrading AHL molecules. Unlike the QQ *Bacillus* strains reported previously, the AHL degradation by the present isolate was extracellular. The bacterium was found to possess an aiiA homologous gene, and it exhibited antibiofilm activity against three Gram-negative bacteria, indicating a broad range of potential targets.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study included the wild type *Chromobacterium violaceum* NIIST (MTCC 5522), the bioreporter strains *C. violaceum* CV026, reclassified as *Chromobacterium subtsugae* (ATCC 31532), which is a mini Tn5 mutant, *Serratia marcescens* NIIST5 (MTCC 5821), *Pseudomonas aeruginosa* (ATCC 27853), and *Bacillus cereus* (MTCC 1305). All the test bacteria except *C. violaceum* was subcultured in Luria Bertani (LB)–Miller medium at 37°C. For *C. violaceum*, the culture temperature was maintained as 30°C.

Enrichment culture for isolating QQ bacteria

The activated sludge collected from the sewage treatment plant (STP), Muttathara, Thiruvananthapuram, Kerala was used as the inoculum for the enrichment culture. The sample was prepared as previously described (Christiaen et al. 2011). Briefly, 1 g of activated sludge was suspended in 10 ml of saline (0.9% NaCl) and subjected to three cycles of 30 s vortexing and 30 s sonication in a bath sonicator (Elmasonic, Germany) at 20 kHz. To settle down larger particles, the resultant suspension was centrifuged at 1000 *g* for 5 min at 25°C. The final suspension was inoculated in M9 minimal medium containing *N*-hexanoyl-L-homoserine lactone (C_6-HSL; Sigma Aldrich, USA), as the sole carbon and energy source, at a final concentration of 1 mM. The medium, inoculum and AHL were provided in 5:4:1 ratio, respectively. After 5 days of shaking incubation at 30°C, the cultures were reinoculated into a fresh medium. The process was repeated up to seven cycles. From the enrichment culture, samples were spread on M9 agar plates supplemented with C_6-HSL. Plates were incubated overnight at 30°C and discrete colonies were subcultured as axenic culture.

Preparation of cell-free supernatant

The pure cultures of bacteria isolated from the enrichment culture were inoculated separately into 100 ml of nutrient broth. The culture was incubated at 30°C for 48 h in an incubator shaker at 200 rpm. After incubation, the cultures were centrifuged at 5800 *g* for 10 min (25°C) and the supernatant was collected. It was filtered through 0.2 μm cellulose acetate syringe filters (Millipore, USA) to prepare the cell-free supernatant (CFS) and stored at −20°C. The CFS was freeze-dried using a lyophilizer (VirTis Genesis, USA). Both CFS as liquid and the lyophilized form is used in different experiments.

Primary screening for QQ activity of the isolates

The isolates obtained from the enrichment culture were screened for their QQ activity by Flask Incubation (FI) assay according to Zhu et al. (2011). Briefly, *C. violaceum* NIIST was inoculated into nutrient broth medium containing the CFS of the above isolates in varying concentrations (1%–10% v/v) and incubated at 30°C for 18 h at 150 rpm in an incubator shaker. For preventing the alkaline hydrolysis of AHL, the medium was buffered with PIPES (1 M, pH 6.7). After incubation, the cultures were visually examined for any pigment reduction. Untreated cultures and uninoculated nutrient broth were used as positive and negative controls, respectively. Cultures with significant pigment reduction were selected for further studies.

Assessment of QQ activity of the isolate PM7 on Gram-negative bacteria

Based on the results of primary screening, one of the isolates, PM7, was selected for detailed assessments of its QQ activity through phenotypic traits. The isolate was subjected to FI assay with *C. subtsugae* and *S. marcescens* as described in primary screening, and monitored for the violacein and prodigiosin production, respectively. The *C. subtsugae* cultures were supplemented with 10 μM C_6-HSL (Sigma Aldrich). Quantification of the pigment production from the assays was carried out using solvent extraction. Briefly, 1 ml of the culture suspension was centrifuged at 5800 *g* (2 min, 25°C) and the supernatant was discarded. The cell pellet was mixed with 1 ml of DMSO or methanol for *Chromobacterium* and *Serratia*, respectively, and pigments were extracted. The absorbance of the samples was taken at 545 nm and 499 nm for violacein and prodigiosin, respectively. The viable cell count of the cultures was taken using plate count agar (Himedia, India).

The effect of CFS on the motility of *S. marcescens* was tested according to Morohoshi et al. (2007). Briefly, semisoft nutrient agar medium of agar concentrations 0.3% and 0.8% (w/v) were prepared for the swimming and swarming motility, respectively. For the swimming motility test, samples from the FI assay were inoculated by stabbing the plates using inoculation needles, while 1 μl of the sample was inoculated on the surface of the agar plate for assessing swarming motility. The plates were incubated for 24 h at 37°C and observed for motility.

The variations in exopolysaccharide (EPS) production by *P. aeruginosa* upon CFS treatment was monitored using an EPS inhibition assay. Overnight cultures of *P. aeruginosa* treated with the lyophilized CFS in different concentrations (1–10 mg/ml) were prepared. The EPS produced in the samples was precipitated using the ethanol precipitation method and estimated by the phenol–sulfuric acid method as previously described by Packiavathy et al. (2014). In a separate experiment, the visualization of the EPS reduction was done via slime staining with safranin as previously described by Khalil and Alawi (2019). CFS concentrations were 0.25, 0.5, and 1 mg/ml.

To analyze the effect of CFS on bacterial growth, overnight cultures from *P. aeruginosa* (0.4 OD at 600 nm) were inoculated in 50 ml of LB broth containing lyophilized CFS at 10 mg/ml. The flasks were incubated at 30°C under 180 rpm. Samples were taken at 1 h intervals up to 21 h, and growth curves were prepared by taking viable cell count on plate count agar. Untreated *P. aeruginosa* cultures were taken as controls.

The CFS was incubated at temperatures ranging from 30 to 90°C for 15 min for heat sensitivity assay. A volume of 1 ml CFS was treated with 1 mg/ml proteinase K (Sigma Aldrich) for 15 min for assessing the proteinaceous nature of the QQ activity of the CFS. The QQ activity of heat and proteinase K-treated CFS was tested using FI assay using *C. subtsugae* as aforementioned. Untreated CFS applied to culture and *C. subtsugae* culture alone were used as positive and negative controls, respectively. The reversal

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of QQ activity of the CFS was analyzed using an acidification test as described by Packiavathy et al. (2021).

Identification of the isolated QQ bacterium

The 16S rRNA gene of the isolated strain was amplified using the universal 16S rRNA primers 27F and 1492R. The PCR products were sequenced using Sanger's method. The isolate was identified by homology analysis of the 16S rRNA gene sequence by the NCBI BLAST tool and the sequence was deposited in the NCBI GenBank. The phylogenetic analysis of the sequence was carried out using MEGA X (Kumar et al. 2018)

Amplification of aiiA homologous gene

The presence of an AHL lactonase encoded by an aiiA homologous gene in the QQ isolate was analyzed using gene amplification. The whole genomic DNA was isolated using Nucleospin DNA isolation kit (Machery-Nagel, Germany) and amplified using the primers aiiA F2 (5′-CGG AAT TCA TGA CAG TAA AGA AGC TTT A-3′) and aiiA R2 (5′-CGC TCG AGT ATA TAT TCA GGG AAC ACT T-3′) and PCR conditions were maintained as reported earlier (Rajesh and Rai 2014). The gene sequencing was carried out using Sanger's method. The consensus sequence was generated using BioEdit version 7.2.

LC-MS analysis

To reconfirm the lactonolysis activity of the CFS of the QQ isolate, degradation of 3-oxo-C_8-HSL was qualitatively analyzed using LC-MS. Briefly, the CFS was incubated with the 3-oxo-C_8-HSL in phosphate buffered saline for 30 min. The samples before and after treatment were extracted three times with ethyl acetate acidified with glacial acetic acid. The organic phase was collected and dried in rotavapor at 30°C and the residue was resuspended in 1 ml acetonitrile. A volume of 10 μl of the sample was injected into UHPLC LC-30A in conjunction with Triple Quadrupole Mass spectrometer LC-MS-8045 with GISS-C18 column (Shimadzu, Japan). The gradient elution method used a mixture of deionized water (30% with 0.1% formic acid) and methanol (70%) both for 10 min and a column temperature of 30°C. The compound detection was carried out by ESI/MS in the positive mode with interface temperature and interface voltage set to 400°C and 4 kV, respectively. The detector voltage was set at 1.8 kV and nebulizing gas flow of 3 l/min was maintained. The samples were analyzed in triplicates.

Microtiter plate assay for biofilm prevention analysis

The effect on biofilm formation by the CFS of *B. velezensis* PM7 was tested using a static microtiter plate assay with modifications as reported by Anandan and Vittal (2019). *Serratia marcescens* and *P. aeruginosa* were grown in LB–Miller broth (Himedia) at 37°C, while *C. violaceum* at 30°C in an incubator shaker at 180 rpm. The initial bacterial density (OD 600) for the microtitre plate assay was set to 0.01 using LB broth and dispensed to a 96-well microtitre plate. The CFS was added to the wells in varying concentration (1–10 mg/ml). Untreated cultures were used as the positive control. The plate was incubated at 30°C for 24 h statically. The OD of the planktonic population.

Statistical analysis

Data analysis was carried out using Graphpad Prism (version 9.4.1). All experiments were conducted in triplicates. Unpaired *t*-tests and linear regression analyses were adopted for the statistical significance analyses.

Results and discussion

Isolation and characterization of QQ bacteria

The growth of QQ bacteria was promoted via an enrichment culture strategy using C_6-HSL as the sole carbon and energy source**.** Based on colony morphology, nine different bacteria were isolated from the enrichment culture on M9-C_6-HSL agar plates. When the CFS from each isolate was separately screened for the presence of QQ activity using *C. violaceum* NIIST, one of the isolates, strain PM7, showed the highest inhibition (67.4%; Figure S1, Supporting Information) in violacein production compared with the control (untreated sample).

Through 16S rRNA sequencing, and subsequent BLAST similarity searches, the isolate PM7 was identified as *B. velezensis* PM7 with 96.17% similarity to previously identified bacteria of the same species. The phylogenetic tree constructed by taking the closest BLAST hits to test the clustering of the isolate with other species of the *Bacillus* genera showed the strain's genetic similarity to *Bacillus subtilis* group (Figure S2, Supporting Information). The 16S rRNA sequence was deposited in the GenBank under the accession number MZ234152. In previously reported studies, QQ bacteria were isolated from enrichment cultures using soil samples (Christiaen et al. 2011) and activated sludge (Kim et al. 2014, Gu et al. 2018) as initial inoculums and AHLs as sole carbon and nitrogen sources. The most frequently encountered QQ isolates in these studies include *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Delftia*, *Aeromonas*, *Rhodococcus*, and *Micrococcus* genera. However, QQ activity of culture supernatants was only reported from *Staphylococcus*, *Micrococcus*, and *Pseudomonas* (Kim et al. 2014).

The QQ effect of *B. velezensis* PM7 on Gram-negative bacteria

The extracellular QQ activity of *B. velezensis* PM7 on different Gram-negative bacteria was demonstrated through its effect on QS-controlled phenotypic traits such as pigment production, motility, and EPS formation without affecting the normal growth of the respective bacteria. In the FI assay, *C. subtsugae* (Fig. 1A) and *S. marcescens* (Fig. 1B) exhibited a dose-dependent significant decrease in pigment production with an increase in PM7 CFS concentration from 1% to 10% (v/v). Violacein production was inhibited up to 73% ($P < .0001$ linear regression analysis) in the presence of CFS at 10% (v/v), whereas prodigiosin production declined up to 67.2% ($P < .0001$, linear regression analysis). *Chromobacterium violaceum* and *S. marcescens* are Gram-negative bacteria that produce the pigments violacein and prodigiosin, respectively in response to cognate short-chain AHLs like C_4-HSL and C_6-HSL. The LuxI/LuxR homolog, CviI I/CviI R circuit, regulates the synthesis of violacein in *C. violaceum* (McLean et al. 2004). Whereas, the PIG gene cluster in *S. marcescens* that produces prodigiosin is controlled by SmaI/SmaR QS system (Van Houdt et al. 2007). The significant reduction in pigment production in these bioreporter strains upon treatment with CFS without causing any cell death indicates a potential extracellular QQ activity of the *B. velezensis* PM7.

Besides prodigiosin production, *S. marcescens*' QS network also regulates motility (Houdt et al. 2007). As observed on agar plates, the CFS treatment reduced the swarming and swimming motilities of *S. marcescens* (Fig. 2). Even though the diameter of spread was decreased, the prodigiosin production on agar plates showed no significant reduction. Contrary to our findings, Morohoshi et al. (2007) reported a disparity in the concentration of AHLs required for prodigiosin production and swarming motility of *Serratia* sp. They have demonstrated that the concentration of AHL necessary

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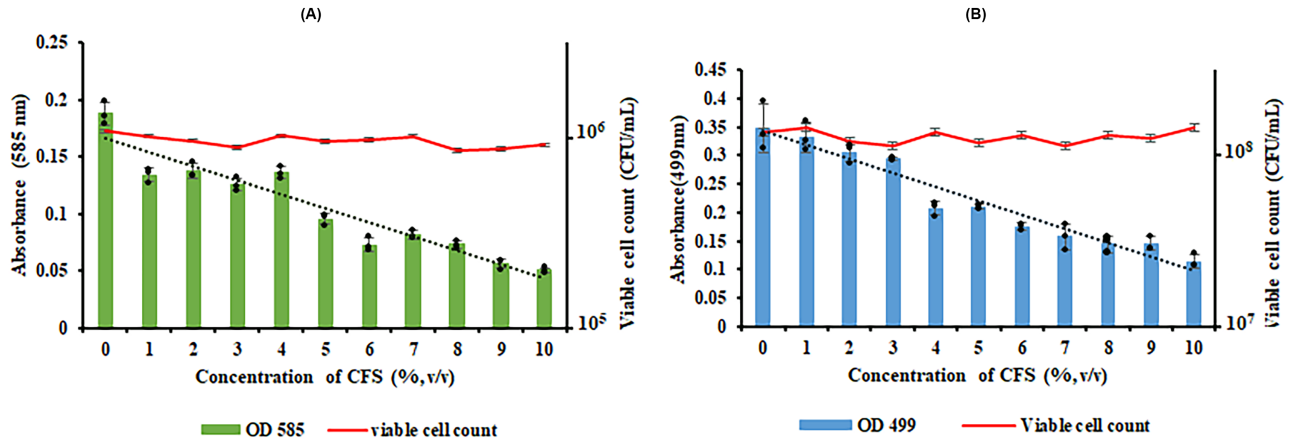

Figure 1. Effect of *B. velezensis* PM7 CFS on the pigment production and cell density of *C. violaceum* and *S. marcescens* from FI assay. Data represents (A) absorbance of violacein at 585 nm and viable cell count (CFU/ml) in *C. violaceum* [R^2: 0.9 (Sy.x: 0.015), slope: −0.012 (SE-0.014), y intercept: 0.166 (SE-0.008), linear regression analysis]. (B) Absorbance of prodigiosin at 499 nm and viable cell count (CFU/ml) in *S. marcescens* [R^2: 0.94 (Sy.x- 0.02), slope- −0.024 (SE- 0.001), y intercept: 0.344 (SE- 0.011), linear regression analysis]. Mean values of three independent experiments are represented and standard errors (SE) are shown along with the line of regression for pigment production with respect to the CFS concentration.

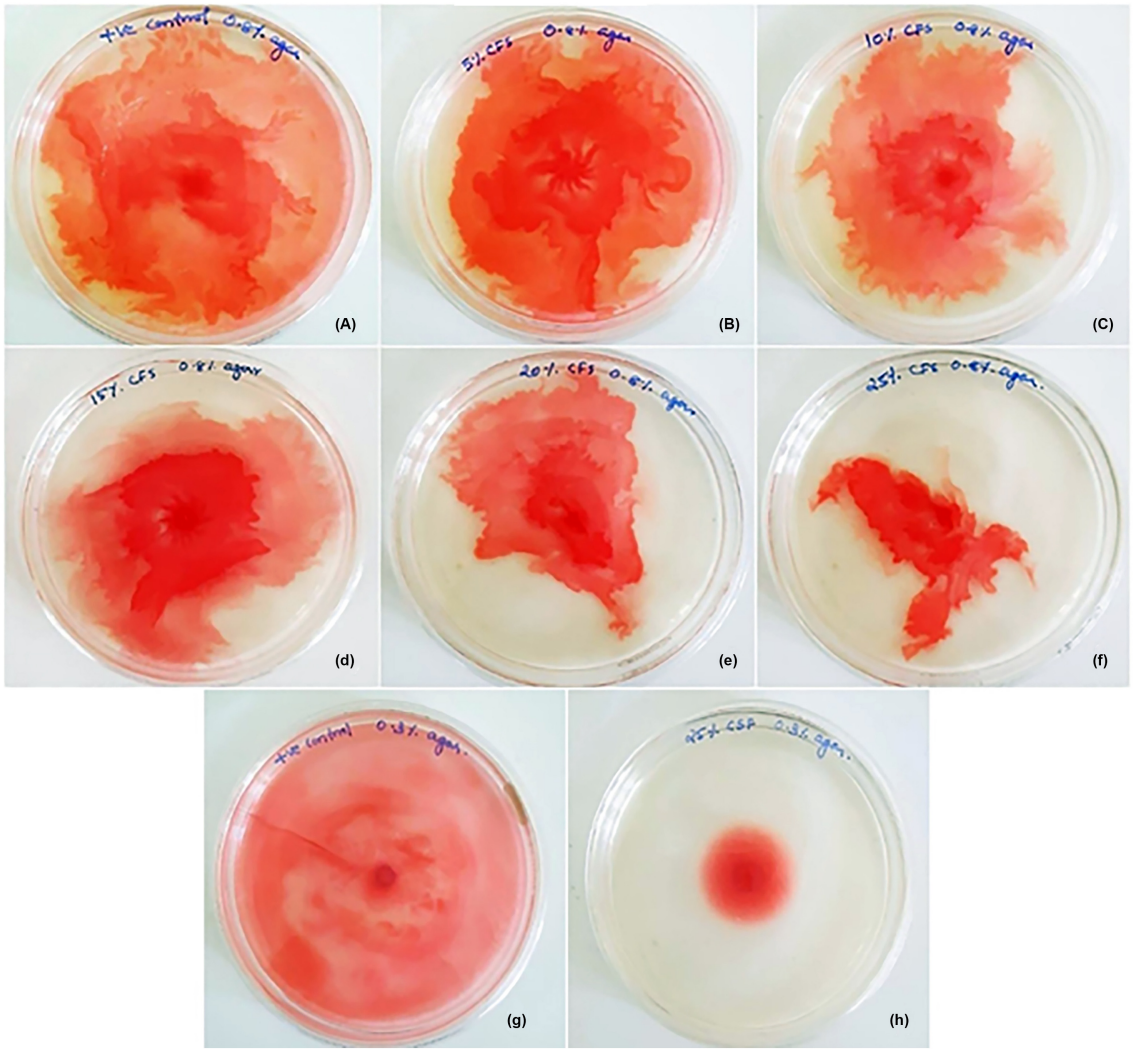

Figure 2. Effect of *B. velezensis* PM7 CFS on the motility of *S. marcescens*. Variations in swarming motility are shown in (a)–(f). CFS addition (A) 0%, (B) 5%, (C) 10%, (D) 15%, (E) 20%, and (F) 25%. Variations in swimming motility are shown in (g)–(h), CFS addition (g) 0%, and (h) 25%.

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to restore swarming motility is only 50% responsible for inducing prodigiosin production to its maximum.

The results of growth studies with PM7 at 10 mg/ml showed that *B. velezensis* PM7 CFS has no significant impact ($P > .05$, unpaired *t*-test) on *P. aeruginosa* growth (Fig. 3A). However, the quantitative EPS inhibition assay in *P. aeruginosa* yielded a 97.9% ($P = .0014$) reduction upon treatment with 10 mg/ml PM7 CFS (Fig. 3B). Concordant results were also obtained from the slime staining procedure (Fig. 3C). QS-controlled EPS production is regarded as a significant step in biofilm architecture development in *P. aeruginosa* (Yang et al. 2011), which is regulated by two extensively studied QS networks namely, Las I—Las R and Rh1 I- Rh1R systems (Sakuragi and Kolter 2007). The extracellular QQ activity of PM7 remarkably decreased the EPS production. Antimicrobial resistance development in biofilms is due to multiple factors such as presence of multidrug efflux pumps (Whiteley et al. 2001) and persister cells (Broun et al. 2000). Additionally, restricted penetration of antimicrobial agents is a significant contributing factor (Shigeta et al. 1997). Therefore, *B. velezensis* PM7's inhibition of EPS production may enhance the susceptibility of biofilms toward other mitigation agents.

Chromobacterium subtsugae was used in the FI assay to examine the effect of heat treatment on QQ activity. A decline in violacein production inhibition was observed at higher temperatures (Fig 4A). At 50°C, the recovery of violacein production was 13.4% compared to the control, and it increased to 98.9% at 80°C. Additionally, proteinase K treatment at 1 mg/ml inactivated the QQ property of the CFS resulting in a violacein retrieval of 99.06% (Fig. 4A). The loss of activity at higher temperature and after proteinase K treatment is a direct indication of the enzymatic nature of the QQ activity of PM7 CFS.

To study the mechanism of AHL degradation by *B. velezensis* PM7, an acidification assay with *C. subtsugae* was carried out. After acidification, it was observed that the CFS-pretreated AHL has significantly recovered as evidenced by the induction of violacein (Fig. 4B). Violacein retrieval was estimated to be 97.91%. The AHL recovery after acidification suggests the enzyme to be an extracellular acyl homoserine lactonase. AHL lactonases hydrolyze the lactone ring targeting the ester bond resulting in the formation of acyl homoserine, which can be recyclized to functional AHLs upon protonation in lower pH (Yates et al. 2002, Tang et al. 2015).

Both enzymatic and nonenzymatic QQ activity against Gram-negative bacteria were reported previously. Hassan et al. (2016) reported an extracellular nonenzymatic QQ activity in *Streptomyces coelicoflavus* isolated from soil. The QQ molecule identified from the culture supernatant of the organism was 1H- pyrrole- 2- carboxylic acid, which suppressed the expression of QS genes in *P. aeruginosa*. Whereas, the major extracellular enzymatic QQ activities reported in bacteria includes the AHL lactonases produced by *Psychrobacter* sp (Packiavathy et al. 2021) and *Muricauda olearia* (Tang et al. 2015). Both strains were isolated from marine sediments and were found to be interfering with the QS network of *C. violaceum* and *P. aeruginosa*.

Biofilm inhibition potential of strain PM7 against Gram-negative bacteria

The effect of CFS on biofilm formation by the Gram-negative bacteria *C. violaceum*, *S. marcescens*, and *P. aeruginosa* was assessed using crystal violet assay. In all the tested bacteria, a concentration dependent biofilm inhibitory activity ($P < .001$, linear regression analysis) was observed in all the tested bacteria (Fig. 5). However, the strength of the effect varied. For *P. aeruginosa*, *C. violaceum*, and *S. marcescens*, an inhibition of 36.9%, 62.17%, and 77.4% was observed at a CFS concentration of 10 mg/ml. In a recent study, Packiavathy et al. (2021) have reported that a marine isolate *Psychrobacter* sp. at 20% (v/v) CFS concentration inhibited biofilm formation by 89%, 71%, 58%, and 60% against *P. aeruginosa*, *S. marcescens*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, respectively. Whereas, the strain *M. olearia* Th120 showed 20% inhibition in *P. aeruginosa* PAO1 biofilm development (Christiaen et al. 2011, Tang et al. 2015). The test organisms used in the experiment release different types and concentrations of AHLs to regulate their QS network. Short chain AHLs like C_6-HSL and C_4-HSL serve as the autoinducer molecules for *C. violaceum* and *S. marcescens*, respectively. While *P. aeruginosa* generates a wide range of AHL molecules to control their two complex QS systems, the LasI-LasR and Rh1I-Rh1R networks. The difference in the autoinducer and QS network may account for the variation in the effectiveness of biofilm inhibition by CFS.

LC-MS analysis of AHL degradation

To reconfirm the mechanism of QQ exhibited by PM7, 3-oxo-C_8-HSL was digested by the CFS and the reaction products were examined by LC-MS. The m/z value of standard 3-oxo-C_8-HSL is 242 (Fig. 6A). However, the prominent 242 m/z peak was absent in the treated cultures after 30 min of incubation indicating the AHL degradation property of the CFS (Fig. 6B). Instead, the sample contained an m/z peak at 261, which corresponds to the ring-opened product 3-oxo-C_8-HS.H (M-H ion). These results are comparable with a previous report where an increase in molecular mass of the hydrolyzed AHL due to the addition of a water molecule to the ester bond (Patel et al. 2016). The formation of similar LC-MS chromatogram for various AHL molecules by extracellular (Tang et al. 2015) and intracellular (Anandan and Vittal 2019) lactonases has already been reported.

PCR amplification of N-acyl homoseriene lactonase gene

The primer pair aiiAF2 and aiiaR2 was used to confirm the presence of an aiiA homologue responsible for the production of AHL lactonase in the *Bacillus* genus (Figure S3, Supporting Information). The 753-bp long sequence revealed 99.60% identity to the *B. thuringiensis* AHL lactonase aiiA-B22 gene. The AHL lactonase domain, which is an $\alpha\beta\beta\alpha$ metallo β-lactamase (MBL) fold was identified from the gene sequence via conserved domain analysis using the CDD database (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). This result suggests that the gene product belongs to the metallo-hydrolase-like MBL-fold superfamily that includes enzymes catalyzing redox as well as hydrolysis reactions.The aiiA cluster of lactonases is widely spread in the *Bacillus* genus (Liu et al. 2008, Vinoj et al. 2014, Bergonzi et al. 2016, Anandan and Vittal 2019). AHL degrading lactonases from *B. velezensis* were previously reported by Sun et al. (2021), the heterologous expression of which was applied to maintain dental chair unit hygiene.

The aiiA homologs reported in the earlier studies were exclusively showing intracellular expression (Rajesh and Rai 2014, Shankar et al. 2014, Anandan and Vittal 2019). However, the extracellular expression of AHL lactonases as observed in this study was reported previously only in marine bacteria (Tang et al. 2015, Packiavathy et al. 2021). Also, the reported secretory AHL lactonase is encoded with an N-terminal signal peptide for the outer cellular transport (Tang et al. 2015). However, the $AiiA_{PM7}$ sequence lacked such signal peptides. Therefore, primarily based on these findings, the $aiiA_{PM7}$ gene cannot be definitively attributed to the observed extracellular lactonase activity in *B. velezensis* PM7.

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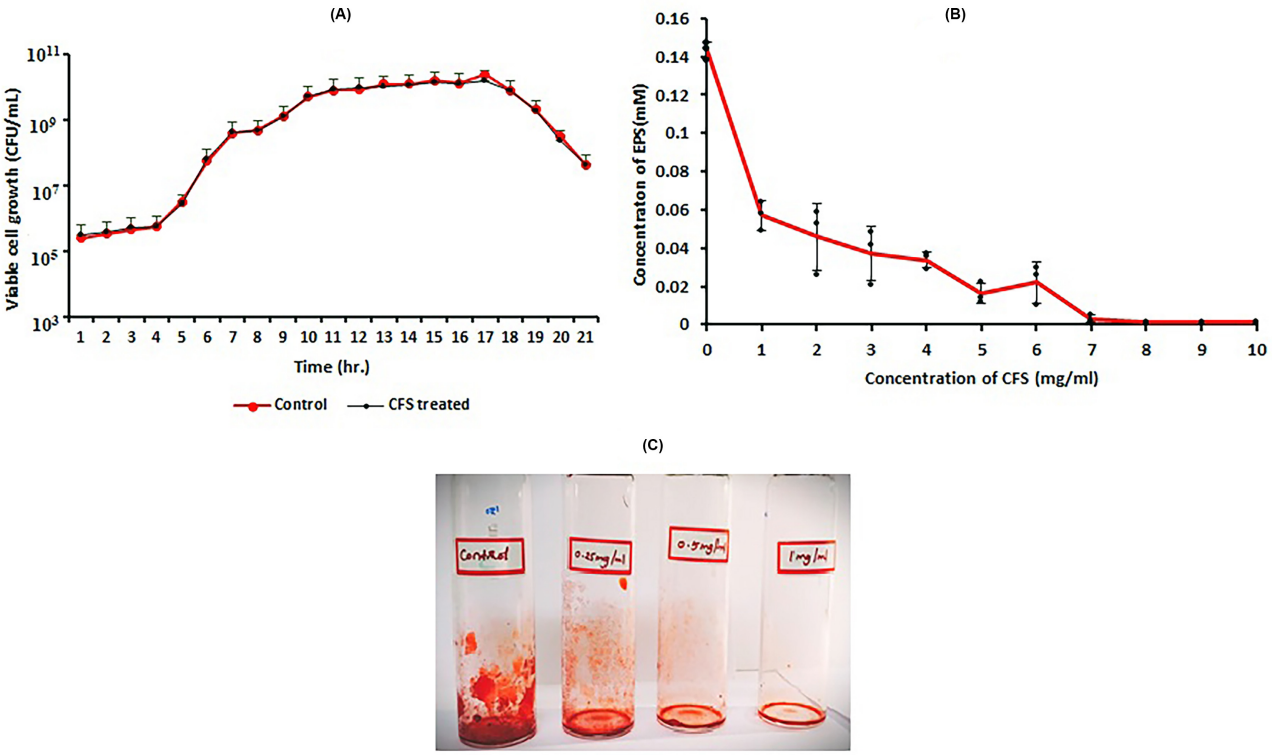

Figure 3. Effect of *B. velezensis* PM7 CFS on growth and EPS production of *P. aeruginosa*. (A) Growth curve of untreated control (red) and CFS (10 mg/ml)-treated (black) *P. aeruginosa* cultures ($P < .01$, unpaired *t*-test). (B) Quantitative analysis of the EPS inhibition in *P. aeruginosa* cells treated with CFS ($P < .01$, unpaired *t*-test). Mean values of three independent experiments are represented and standard errors are shown in both (A) and (B). (C) Decline in *P. aeruginosa* EPS production visible in slime staining procedure.

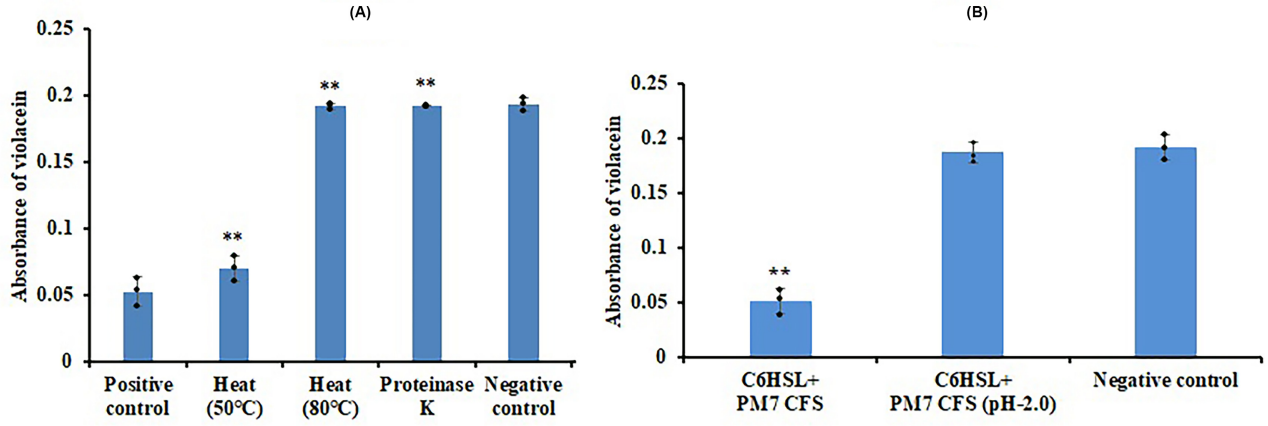

Figure 4. (A) Effect of heat treatment (50 and 80°C) and proteinase K treatment (1 mg/ml) in the QQ activity of *B. velezensis* PM7 on *C. violaceum* CV026. Activity is measured as absorbance of violacein produced (OD 585). Untreated CV026 culture supplemented with C_6-HSL and PM7 CFS-treated culture at 30°C is taken as positive and negative controls respectively. ** Significant reduction of QQ activity at $P < .05$ with respect to the positive control. (B) Retrieval of the QS activity of the extracted C_6-HSL from the PM7 CFS-treated *C. violaceum* CV026 upon acidification to pH 2.0. Activity is measured as absorbance of violacein produced (OD 585). Untreated CV026 culture supplemented with C_6-HSL is taken as the negative control** Significant violacein reduction at $P < .05$ with respect to the negative control.

The general extracellular secretion mechanism in Gram-positive bacteria includes Sec and Tat transport pathway, which is regulated by the N-terminal signal peptides present in the protein to be transported (Stanley et al. 2000, Beckwith 2013). Given the existence of these two systems, around 25% of released proteins lack identified export signals (Briaud and Carroll 2020). *Bacillus subtilis*, a model organism for Gram-positive category, is reported producing extracellular vesicles for the purpose (Brown et al. 2014). Since *B. velezensis* belongs to the *B. subtilis* group, it is conceivable that vesicular transport could be responsible for the extracellular localization of the lactonase enzyme.

Traditional biofouling control methods such as membrane backwashing, intermittent permeation, and the use of biocidal chemicals and detergents usually yield less satisfactory results. Additionally, the threat of antimicrobial resistance emergence due to continuous use of biocidal agents also prevails. The QQ en-

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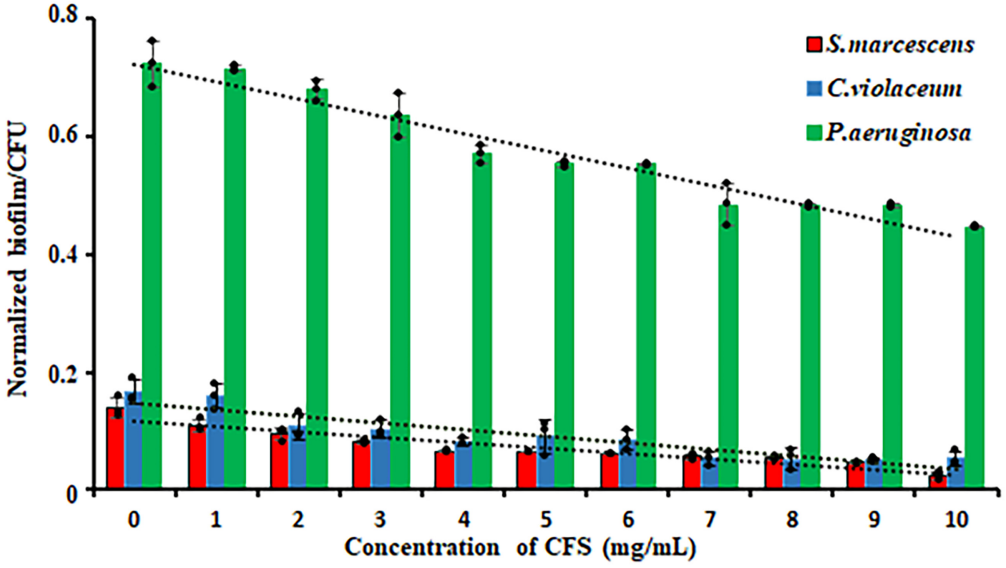

Figure 5. Antibiofilm activity of *B. velezensis* PM7 on three Gram-negative bacteria *viz. P. aeruginosa* [R^2: 0.95 (Sy.x −0.05), slope: −0.073 (SE- 0.005), y intercept- 0.76 (SE- 0.03)], *C. violaceum* [R^2: 0.86 (Sy.x −0.04), slope: −0.02 (SE- 0.003), y intercept: 0.18 (SE- 0.02)], and *S. marcescens* [R^2: 0.89 (Sy.x −0.02), slope: −0.02 (SE- 0.002), y intercept: 0.16 (SE- 0.03)] from crystal violet assay. The data is normalized using viable cell count (CFU/ml). Mean values of three independent experiments are represented and standard errors (SE) are shown along with the line of regression for normalized biofilm formation with respect to the CFS concentration for each bacteria.

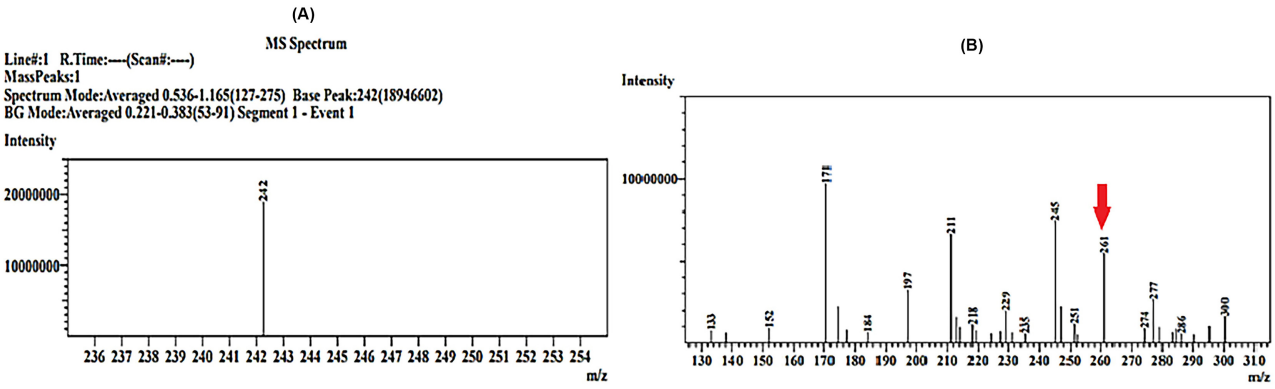

Figure 6. LC-MS analysis of the degradation product of CFS with 3-oxo-C_8-HSL. (A) Spectra of 3-oxo-C_8-HSL showing 242 m/z peak. (B) Fragmentation spectra of 3-oxo-C_8-HSL showing 261 m/z peak (M)–(H) alone indicating complete degradation of the test molecule.

zymes are, therefore, envisaged as an alternative mechanism. In this scenario, the novel strain of *B. velezensis* reported in this study with its lactonase secretory property, and broad-spectrum activity against different Gram-negative bacteria would be advantageous for controlling biofouling or other undesirable QS-related traits. Moreover, the effect of extracellular QQ activity on bacterial population dynamics and its implication on biofilm in natural environments would be an unexplored area that needs in-depth study.

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Supplementary data

Supplementary data are available at FEMSLE online.

Conflicts of interest. None declared.

References

Anandan K, Vittal RR. Quorum quenching activity of AiiA lactonase KMMI17 from endophytic *Bacillus thuringiensis* KMCL07 on AHL-mediated pathogenic phenotype in *Pseudomonas aeruginosa*. *Microb Pathog* 2019;**132**:230–42.

Anju VT, Dyavaiah M, Siddhardha B. Quorum sensing as a therapeutic target in the treatment of chronic wound infections. In: Kumar P, Kothari V (eds), *Wound Healing Research: Current Trends and Future Directions*. Singapore: Springer, 2021,643–59.

Ansari FA, Ahmad I. Biofilm development, plant growth promoting traits and rhizosphere colonization by *Pseudomonas entomophila* FAP1: a promising PGPR. *Adv Microbiol* 2018;**8**:235.

Bar-Rogovsky H, Hugenmatter A, Tawfik DS. The evolutionary origins of detoxifying enzymes: the mammalian serum paraoxonases (PONs) relate to bacterial homoserine lactonases. *J Biol Chem* 2013;**288**:23914–27.

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Beckwith J. The Sec-dependent pathway. *Res Microbiol* 2013;**164**:497–504.

Bergonzi C, Schwab M, Elias M. The quorum-quenching lactonase from *Geobacillus caldoxylosilyticus*: purification, characterization, crystallization and crystallographic analysis. *Acta Crystallogr Sect F Struct Biol Commun* 2016;**72**:681–6.

Briaud P, Carroll RK. Extracellular vesicle biogenesis and functions in Gram-positive bacteria. *Infect Immun* 2020;**88**:e00433–20.

Brooun A, Liu S, Lewis K. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 2000;**44**:640–6.

Brown L, Kessler A, Cabezas-Sanchez P *et al.* Extracellular vesicles produced by the Gram-positive bacterium *Bacillus subtilis* are disrupted by the lipopeptide surfactin. *Mol Microbiol* 2014;**93**:183–98.

Castro L, Blázquez ML, González F *et al.* Anaerobic bioleaching of jarosites by *Shewanella putrefaciens*, influence of chelators and biofilm formation. *Hydrometallurgy* 2017;**168**:56–63.

Chan K-G. Expression of *Klebsiella* sp. lactonase ahlK gene is growth-phase, cell-population density and N-acylhomoserine lactone independent. *Front Life Sci* 2013;**7**:132–9.

Chbib C. Impact of the structure-activity relationship of AHL analogues on quorum sensing in Gram-negative bacteria. *Bioorg Med Chem* 2020;**28**:115282.

Chen Y, Yan F, Chai Y *et al.* Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. *Environ Microbiol* 2013;**15**:848–64.

Christiaen SEA, Brackman G, Nelis HJ *et al.* Isolation and identification of quorum quenching bacteria from environmental samples. *J Microbiol Methods* 2011;**87**:213–9.

Defoirdt T, Crab R, Wood TK *et al.* Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* isolates. *Appl Environ Microbiol* 2006;**72**:6419–23.

Dekimpe V, Deziel E. Revisiting the quorum-sensing hierarchy in *Pseudomonas aeruginosa*: the transcriptional regulator RhlR regulates LasR-specific factors. *Microbiology* 2009;**155**:712–23.

Ejileugha C, Ezealisiji KM, Ezejiofor AN *et al.* Microbiologically influenced corrosion: uncovering mechanisms and discovering inhibitor—metal and metal oxide nanoparticles as promising biocorrosion inhibitors. *J Bio Tribo Corrosion* 2021;**7**:1–21.

Fuqua WC, Winans SC, Greenberg EP. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* 1994;**176**:269–75.

Glessner A, Smith RS, Iglewski BH *et al.* Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of twitching motility. *J Bacteriol* 1999;**181**:1623–9.

Gu Y, Huang J, Zeng G *et al.* Quorum quenching activity of indigenous quorum quenching bacteria and its potential application in mitigation of membrane biofouling. *J Chem Technol Biotechnol* 2018;**93**:1394–400.

Hassan R, Shaaban MI, Abdel Bar FM *et al.* Quorum sensing inhibiting activity of *Streptomyces coelicoflavus* isolated from soil. *Front Microbiol* 2016;**7**:659.

Hawver LA, Jung SA, Ng W-L. Specificity and complexity in bacterial quorum-sensing systems. *FEMS Microbiol Rev* 2016;**40**:738–52.

Hiblot J, Bzdrenga J, Champion C *et al.* Crystal structure of VmoLac, a tentative quorum quenching lactonase from the extremophilic crenarchaeon *Vulcanisaeta moutnovskia*. *Sci Rep* 2015;**5**:8372.

Hou F, Zhang T, Peng Y *et al.* Partial anammox achieved in full scale biofilm process for typical domestic wastewater treatment. *Front Environ Sci Eng* 2022;**16**:1–7.

Van Houdt R, Givskov M, Michiels CW. Quorum sensing in *Serrati a*. *FEMS Microbiol Rev* 2007;**31**:407–24.

Houdt R Van, Moons P, Aertsen A *et al.* Characterization of a luxI/luxR -type quorum sensing system and N -acyl-homoserine lactone-dependent regulation of exo-enzyme and antibacterial component production in Serratia plymuthica RVH1. *Res Microbiol* 2007;**158**:150–8.

Khalil AL, Alawi SR. Isolation, antibiotic sensitivity and qualitative determination of slime layer formation test for P . *aeruginosa* isolated from wound and urinary tract infection patient. *Biochem Cell Arch* 2019;**19**:1509–14.

Khan BA, Yeh AJ, Cheung GY *et al.* Investigational therapies targeting quorum-sensing for the treatment of *Staphylococcus aureus* infections. *Expert Opin Investig Drugs* 2015;**24**:689–704.

Khan NA, Khan AH, Ahmed S *et al.* Efficient removal of ibuprofen and ofloxacin pharmaceuticals using biofilm reactors for hospital wastewater treatment. *Chemosphere* 2022;**298**:134243.

Khan SR, Farrand SK. The BlcC (AttM) lactonase of *Agrobacterium tumefaciens* does not quench the quorum-sensing system that regulates Ti plasmid conjugative transfer. *J Bacteriol* 2009;**191**:1320–9.

Kim A, Park S, Lee C *et al.* Quorum quenching bacteria isolated from the sludge of a wastewater treatment plant and their application for controlling biofilm formation. 2014;**24**:1574–82.

Kim MH, Choi W-C, Kang HO *et al.* The molecular structure and catalytic mechanism of a quorum-quenching N-acyl-L-homoserine lactone hydrolase. *Proc Natl Acad Sci* 2005;**102**:17606–11.

Koch G, Nadal-Jimenez P, Cool RH *et al.* *Deinococcus radiodurans* can interfere with quorum sensing by producing an AHL-acylase and an AHL-lactonase. *FEMS Microbiol Lett* 2014;**356**:62–70.

Kumar S, Stecher G, Li M *et al.* MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 2018;**35**:1547.

Liu D, Momb J, Thomas PW *et al.* Mechanism of the quorum-quenching lactonase (AiiA) from *Bacillus thuringiensis*. 1. Product-bound structures. *Biochemistry* 2008;**47**:7706–14.

Liu P, Chen Y, Shao Z *et al.* AhlX, an N-acylhomoserine lactonase with unique properties. *Mar Drugs* 2019;**17**:387.

McLean RJC, Pierson LS , Fuqua C. A simple screening protocol for the identification of quorum signal antagonists. *J Microbiol Methods* 2004;**58**:351–60.

Mhedbi-Hajri N, Yahiaoui N, Mondy S *et al.* Transcriptome analysis revealed that a quorum sensing system regulates the transfer of the pAt megaplasmid in *Agrobacterium tumefaciens*. *BMC Genomics* 2016;**17**:1–14.

Morohoshi T, Shiono T, Takidouchi K *et al.* Inhibition of quorum sensing in *Serratia marcescens* AS-1 by synthetic analogs of N-acylhomoserine lactone. *Appl Environ Microbiol* 2007;**73**:6339–44.

Oh H-S, Lee C-H. Origin and evolution of quorum quenching technology for biofouling control in MBRs for wastewater treatment. *J Membr Sci* 2018;**554**:331–45.

Packiavathy IASV, Kannappan A, Thiyagarajan S *et al.* AHL-lactonase producing *Psychrobacter* sp. from Palk Bay sediment mitigates quorum sensing-mediated virulence production in Gram-negative bacterial pathogens. *Front Microbiol* 2021;**12**:748.

Packiavathy IASV, Priya S, Pandian SK *et al.* Inhibition of biofilm development of uropathogens by curcumin–an anti-quorum sensing agent from *Curcuma longa*. *Food Chem* 2014;**148**:453–60.

Patel NM, Moore JD, Blackwell HE *et al.* Identification of unanticipated and novel N-acyl L-homoserine lactones (AHLs) using a sensitive non-targeted LC-MS/MS method. *PLoS ONE* 2016;**11**:e0163469.

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Pinto H, Simões M, Borges A. Prevalence and impact of biofilms on bloodstream and urinary tract infections: a systematic review and meta-analysis. *Antibiotics* 2021;**10**:825.

Raissa G, Waturangi DE, Wahjuningrum D. Screening of antibiofilm and anti-quorum sensing activty of *Actinomycetes* isolates extracts against aquaculture pathogenic bacteria. *BMC Microbiol* 2020;**20**:1–10.

Rajesh PS, Rai VR. Molecular identification of aiiA homologous gene from endophytic *Enterobacter* species and in silico analysis of putative tertiary structure of AHL-lactonase. *Biochem Biophys Res Commun* 2014;**443**:290–5.

Rather MA, Gupta K, Mandal M. Impact of microbial biofilm on crop productivity and agricultural sustainability. In: *Microbes in Land Use Change Management*. Amsterdam: Elsevier, 2021, 451–69.

Rice SA, Koh KS, Queck SY *et al.* Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *J Bacteriol* 2005;**187**:3477–85.

Russel JG, Thulasiraman V, Nair RR *et al.* A novel bio-physical approach for perchlorate contaminated well water treatment. *Environ Adv* 2021;**4**:100058.

Sakuragi Y, Kolter R. Quorum-sensing regulation of the biofilm matrix genes (pel) of *Pseudomonas aeruginosa*. *J Bacteriol* 2007;**189**:5383–6.

See-Too WS, Convey P, Pearce DA *et al.* Characterization of a novel N-acylhomoserine lactonase, AidP, from Antarctic *Planococcus* sp. *Microb Cell Fact* 2018;**17**:1–14.

Shah SSA, De Simone L, Bruno G *et al.* Quorum quenching, biological characteristics, and microbial community dynamics as key factors for combating fouling of membrane bioreactors. *Npj Clean Water* 2021;**4**:19.

Shankar P, Kushwah J, Bhushan A *et al.* Diversity and polymorphism in AHL-lactonase gene (aiiA) of *Bacillus* diversity and polymorphism in AHL-lactonase gene (aiiA) of *Bacillu s*. *J Microbiol Biotechnol* 2014;**21**:1001–11. doi:10.4014/jmb.1105.05056.

Shigeta M, Tanaka G, Komatsuzawa H *et al.* Permeation of antimicrobial agents through *Pseudomonas aeruginosa* biofilms: a simple method. *Chemotherapy* 1997;**43**:340–5.

Shih P-C, Huang C-T. Effects of quorum-sensing deficiency on *Pseudomonas aeruginosa* biofilm formation and antibiotic resistance. *J Antimicrob Chemother* 2002;**49**:309–14.

Singh AA, Singh AK, Nerurkar A. Disrupting the quorum sensing mediated virulence in soft rot causing *Pectobacterium carotovorum* by marine sponge associated *Bacillus* sp. OA10. *World J Microbiol Biotechnol* 2021;**37**:1–11.

Stanley NR, Palmer T, Berks BC. The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in *Escherichia coli*. *J Biol Chem* 2000;**275**:11591–6.

Sun X, Hill P, Liu J *et al.* Marine-source quorum quenching enzyme YtnP to improve hygiene quality in dental units. *Mar Drugs* 2021;**19**:225.

Syafiuddin A, Boopathy R, Mehmood MA. Recent advances on bacterial quorum quenching as an effective strategy to control biofouling in membrane bioreactors. *Bioresour Technol Reports* 2021;**15**:100745.

Tang K, Su Y, Brackman G *et al.* MomL, a novel marine-derived N-acyl homoserine lactonase from *Muricauda olearia*. *Appl Environ Microbiol* 2015;**81**:774–82.

Thomas PW, Stone EM, Costello AL *et al.* The quorum-quenching lactonase from *Bacillus thuringiensis* is a metalloprotein. *Biochemistry* 2005;**44**:7559–69.

Torres M, Uroz S, Salto R *et al.* HqiA, a novel quorum-quenching enzyme which expands the AHL lactonase family. *Sci Rep* 2017;**7**:1–15.

Uroz S, Chhabra SR, Camara M *et al.* N-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities. *Microbiology* 2005;**151**:3313–22.

Utari PD, Setroikromo R, Melgert BN *et al.* PvdQ quorum quenching acylase attenuates *Pseudomonas aeruginosa* virulence in a mouse model of pulmonary infection. *Front Cell Infect Microbiol* 2018;**8**:119.

Verma R, Das A, Sarmah DK *et al.* A review article: anti-quorum sensing agents as a potential replacement for antibiotics in Phytobacteriology. *Phar Innov J* 2021;**10**:121–5.

Vinoj G, Vaseeharan B, Thomas S *et al.* Quorum-quenching activity of the AHL-lactonase from *Bacillus licheniformis* DAHB1 inhibits *Vibrio* biofilm formation in vitro and reduces shrimp intestinal colonisation and mortality. *Mar Biotechnol* 2014;**16**:707–15.

Vishwakarma V. Impact of environmental biofilms: industrial components and its remediation. *J Basic Microbiol* 2020;**60**:198–206.

Whiteley M, Bangera MG, Bumgarner RE *et al.* Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 2001;**413**:860–4.

Yang L, Hu Y, Liu Y *et al.* Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development. *Environ Microbiol* 2011;**13**:1705–17.

Yates EA, Philipp B, Buckley C *et al.* N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect Immun* 2002;**70**:5635–46.

Zhao J, Chen M, Quan CS *et al.* Mechanisms of quorum sensing and strategies for quorum sensing disruption in aquaculture pathogens. *J Fish Dis* 2015;**38**:771–86.

Zhu H, He C-C, Chu Q-H. Inhibition of quorum sensing in *Chromobacterium violaceum* by pigments extracted from *Auricularia auricular*. *Lett Appl Microbiol* 2011;**52**:269–74.

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