

**Mechanisms of the inhibition of biofilm formation
by *Sphingomonas* spp.**

A thesis

*Submitted in fulfillment of the requirements for the award of the
degree of*

**DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY**

**By
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**Under the supervision of
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February 2018**

CERTIFICATE

Certified that the thesis "**Mechanisms of the inhibition of biofilm formation by *Sphingomonas spp.***" which is submitted by **Ms. Parul**, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is a record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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DECLARATION

I hereby declare that the work presented in the thesis entitled “**Mechanisms of the inhibition of biofilm formation by *Sphingomonas* spp.**” in the fulfillment of the requirement for the award of the Degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is an authentic record of my own work during the period July 2012 to December 2017, under the supervision of **Dr. Moushumi Ghosh**, Professor, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala. The material embodied in this thesis has not been submitted in parts or full in any other university or institute for the award of any degree in India or Abroad.

Place: Patiala

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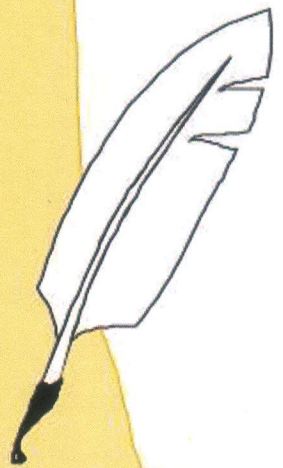
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Dedicated To

My

Parents



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LIST OF ABBREVIATIONS

4-NPO	4-nitropyridine-N-oxide
ACP	Acyl carrier protein
AHL	N-acyl homoserine lactones
AI-2	Autoinducer 2
AIP	Autoinducing peptide
Al	Aluminium
APHA	American Public Health Association
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
ATR	Attenuated Total Internal Reflection
BATH	Bacterial adhesion to hydrocarbon
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BP	<i>Klebsiella</i> biopolymer
CCD	Central Composite design
Cu	Copper
CPS	Capsular polysaccharides
CV	Crystal Violet
DAPI	4',6-diamidino-2-phenylindole
DBHB	Dibromohemibastadin-1
DMS	Dimethyl Sulphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dntp	deoxyribonucleotide triphosphate
DOC	Dissolved Organic Carbon
DQ	Degree of Quaternization
DWDS	Drinking Water Distribution System
EDTA	Ethylenediamine-tetra acetic acid
EDX	Energy Dispersive X-ray Spectroscopy
EPS	Extracellular Polymeric Substances

FOS	Fibre Optical Sensors
FTIR	Fourier Transformation Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LB	Luria-Bertani
LDH	lactate dehydrogenase
MgCl ₂	Magnesium chloride
MIC	Microbiological Influences Corrosion
MTCC	Microbial Type Culture Collection and Gene Bank
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCO	Molecular weight cut-off
NCBI	National Center for Biotechnology Information
NMB	N-methylbiopolymer
NMR	Nuclear magnetic resonance
O.D.	Optical Density
ONPG	ortho-Nitrophenyl- β -galactoside
PAS	Photo Acoustic Spectroscopy
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PE	Polyethylene
PP	Polypropylene
Ppm	Parts per million
PVC	Polyvinyl chloride
QB	Quaternized Biopolymer
QCM	Quartz Crystal Microbalance
QS	Quorum Sensing
QSI	Quorum Sensing Inhibitors
RLU	Relative Light Units
RO	Reverse Osmosis
Rpm	Revolutions per minute
RSM	Response Surface Methodology

SAM	S-adenosylmethionine
SA-PAE	Salicylic acid- poly (anhydride ester) polymers
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscopy
SRB	Sulphate-Reducing Bacteria
SSG	<i>Sphingomonas</i> selective growth
TAE	Tris-acetate-EDTA
TGA	Thermogravimetric Analysis
TLC	Thin Layer Chromatography
UV	Ultra Violet
WHO	World Health Organization

LIST OF SYMBOLS

%	Percentage
mg/ L	Milligram per litre
°C	Degree celcius
Psi	Pound per square inch
g	Gram
g/L	Gram per litre
µg /mL	Micrograms per millilitre
min	Minutes
mM	Millimolar
µM	Micromolar
mL	Millilitre
µL	Microlitre
h	Hours
v/v	Volume by volume
w/v	Weight by volume
µm	Micrometer
mm	Millimeter
nm	Nanometer
mg /mL	Milligram per millilitre
mg /L	Milligram per litre
m	Meter
cm	Centimeter
L/ min	Litre per minute
ng	Nanogram
CFU/mL	Colony forming units per millilitre
mg	Milligram
mmole	Millimole
mg/dl	Milligrams per Decilitre
g/L	Gram per Litre
kHz	Kilohertz

pF/mm	Picofarad per millimeter
nF	Nanofarad
M	Molar
U/L	Units per litre
h ⁻¹	Specific growth rate

ABSTRACT

Colonization of bacteria from diverse ecological niches adopting a biofilm mode of sustenance on the drinking water framework endangers the quality of potable water. This necessitates an urgency to take up considerable safety measures. Ability of *Sphingomonas*, an oligotroph, to form biofilm play an important role in water borne infections by acting as a reservoir for pathogens and enhanced resistance to chlorine compounds. Growing body of evidences has suggested the role of cell to cell communication system called quorum sensing in formation of biofilm. Interference with bacterial communication system with suitable antagonists can offer a benign yet effective strategy for biofilm prevention. Microbial exopolymers, an attractable class of molecules, present an interesting proposition for exploitation. In view of this, the present study investigated the role of bacterial exopolymers modified chemically as quorum sensing inhibitors to prevent biofilm formation by *Sphingomonas*.

A bacterial isolate with strong biofilm forming ability was isolated from drinking water samples and characterized as *Sphingomonas paucimobilis* strain MG6. *Sphingomonas terrae* MTCC 7766 was used as reference strain. A rapid technique for determining biofilm age by detecting changes in capacitance was developed. The ability of *Sphingomonas* to form a strong biofilm on various commonly used materials and evading chlorination in drinking water networks was studied. To understand the mechanism of biofilm formation, the signal molecule inducing quorum sensing in *Sphingomonas* was isolated and the identity of the later was characterized as a homolog of N-dodecanoyl-L-Homoserine lactone. The same was confirmed by a battery of qualitative and quantitative assays. In an attempt to develop quorum quenchers, we altered the surface properties of biopolymer extracted from *Klebsiella terrigena* to form quaternized biopolymer (QB) and N-

methyl biopolymer (NMB). Their effect on quorum sensing and biofilm formation was investigated. It was observed that they had considerable potential for effectively reducing AHL molecule production, hence, leading to biofilm inhibition. AHL modulation by QB and NMB suggested that QB modulated both quorum sensing genes *luxI* and *luxR* activities particularly Lux R whereas NMB supposedly had no significant effect ($p>0.05$) on either of them. The role of AHL in biofilm formation was substantiated by the lack of biofilm formation of an AHL negative mutant obtained by transposon mutagenesis out of the library of 534 transconjugants. The effect of these compounds on the growth profile of *Sphingomonas* was also analyzed. QB was found to have major effect in reducing the growth of planktonic cells as compared to NMB. This was confirmed by analyzing leakage of potassium ions, UV –absorbing materials, proteins, glucose, Lactate dehydrogenase and ATP content of *Sphingomonas* after treatment with these compounds which inferred about QB in leading to cell damage while, NMB possessing negligible effect on the cell membrane. Based on the structure of the NMB, it was hypothesized to act through lactonolysis of lactone moiety of signal molecule of *Sphingomonas* resulting in ring opened product.

Finally, the effect of QB and NMB on biofilm forming *Sphingomonas* under real time conditions was indicated. These were found to effectively reduce biofilm formation suggesting potential application of these compounds as a safe quorum sensing inhibitors for *Sphingomonas*.

Keywords: *Sphingomonas*; biofilm inhibition; quorum sensing; drinking water; exopolymers; chlorine resistance.

LAYOUT OF THESIS

The thesis is divided into five major chapters.

Chapter 1: Introduction contains the summary of the thesis, gaps, significance and objectives of research.

Chapter 2: Review of Literature discusses the background information on microbial biofilms commonly found in distribution systems. It entails information on current strategies for biofilm removal. Interference of biofilm formation by interrupting quorum sensing has been explained with relevant literature.

Chapter 3: Materials and methods describe the approach and details of all the experiments that were performed in triplicates.

Chapter 4: Results contains detailed results of the study and is divided into sections corresponding to study objectives. **Section 1** describes screening and isolation of microbial strains capable of producing biofilm using *Sphingomonas* selective medium and identification of the most promising isolate. **Section 2** investigates culture and environmental conditions leading to optimal biofilm formation by the selected *Sphingomonas* isolate. Confirming chlorine resistance property of *Sphingomonas* using commonly used plumbing material and preparation of sensor system which detects biofilm age by detecting changes in capacitance values. **Section 3** Establish the role of AHL in biofilm formation by *Sphingomonas* isolate and complete characterization of the involved AHL **Section 4** describes microbial exopolymers as potential AHL blockers, modifying them and elucidating blocking mechanism which leads to the prevention of biofilms

Chapter 5: Discussion discusses the results and outcomes with reports available in literature.

Chapter 6: Conclusion contains a summary of salient findings and conclusions of the present study.

References from all chapters are compiled at the end of the thesis.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Adherence to surfaces is a hallmark of microorganisms. Over the past decade, abundant evidence has established this unique ability of microorganisms to colonize a wide diversity of ecological niches including biotic or abiotic surfaces. Adherence often translates to biofilm- a bacterial lifestyle which involves the expression of extracellular adhesive appendages, secreted adhesions, the production of extracellular polymeric substances (EPS) and enhanced promotion of inter- and intra-bacterial interactions (Hall-Stoodley et al. 2004; Jefferson 2004; Kolter et al. 2006).

The induction of novel survival characteristics in biofilm are exemplified by high tolerance to stress, biocides, antibiotics and host immunological defenses. Needless to say, this form of adaptation is beneficial for bacterial cells to be in biofilm mode for sustaining the environmental rigours (Castonguay et al. 2006). The all pervading existence of biofilms has been demonstrated in human amenities essential for life with both beneficial and detrimental implications. The later includes the drinking water system whose integrity is crucial for sustained water safety and security.

To date, convincing evidences have demonstrated drinking water distribution systems as reservoir of biofilms (Bachmann & Edyvean 2006). The quality of drinking water in distribution systems is adversely affected by microbial contamination which encourages degradation of pipelines despite of high disinfectant dose (Simoes & Simões 2013). Continuous detachment of biomass into the bulk water distribution system causes deterioration of microbiological quality as well as aesthetic water quality through discoloration, turbidity and malodour (Vilchez et al. 2007). Flemming (2002) reported that 95% of the cell biomass is adhered to pipelines, while only 5% is present in aqueous phase.

Of the prominent biofilm forming gram negative bacteria present in water distribution systems, *Sphingomonas* has attracted substantial interest in recent times (Gusman et al. 2012). *Sphingomonas* is hallmarked by its amazing metabolic diversity, oligotrophic and opportunistic nature (ability to survive in low nutrient conditions) which qualifies its potential for biofilm formation. The ubiquity of *Sphingomonas* spp., its resistance to numerous disinfectants and its oligotrophic nature identifies it as key candidate in distribution systems. In fact, *Sphingomonas* sp has been well studied for their effective metabolism of complex hydrocarbons (Story et al. 2004) and potential importance in biotransformation. But evidence contrary to its beneficial applications indicate its role in biofilm formation, leading to metal corrosion in plumbing materials commonly used in drinking water and industrial water distribution systems, regardless of the pipe materials (Rozej et al. 2015). Being a biofilm former, *Sphingomonas* can survive chlorine treated causative agent for biofouling of drinking water (Gusman et al. 2012; Gutman et al. 2014).

The biofilm mode of bacterial living is associated with an increased resistance towards antimicrobial agents, rendering successful treatment of biofilm related contamination a difficult task to achieve thus incurring tremendous economic loss for industries (Costerton et al. 1995). Need for alternative rapid methods for determination of biofilm age or progression assumes utmost importance and require *in situ* measurements. This becomes necessary for purposes of immediate intervention or for adopting or restoring to alternate strategies (e.g. catheters, biomedical implants etc) (Kostakioti et al. 2013). Developments in bioelectric or optical research have offered exciting prospects for applications in this regard (Sadekuzzaman et al. 2015)

The maturation of biofilm is regulated by generation of cell density dependent signal molecules which are known as auto-inducers (N-acyl homoserine lactones i.e. AHL in gram negative bacteria) (Swift et al. 1996; Cha et al. 1998). This process involves autoinducers and is referred to as quorum sensing (Waters & Bassler 2005; Dickschat 2010). The word “quorum” in this population density mechanism reflects the number of individual cells required to formulate a decision. Several Quorum sensing (QS) systems have been identified till date: one using polypeptides for gram positive bacteria (Abraham 2006; Waters & Bassler 2005) and other, N-acyl homoserine lactone (AHL) derivatives for gram negative bacteria (Swift et al. 1996; Cha et al. 1998). The QS system is proposed as a global signaling system for all bacteria (Winans & Bassler 2002; Waters & Bassler 2005). In gram-negative bacteria, the specificity of N-acyl-L-homoserine lactones (AHL) molecules is being conferred by the length of the acyl chain and the substitution at C-3 (Zhu & Winans 1998). The primary elements in this process include *luxI* and *luxR* homologue which encodes AHL synthethases and response regulator, respectively. Synthesized AHL molecules redistribute actively/passively in the environment (Waters & Bassler 2005; Dickschat 2010). When signal molecules reaches a certain threshold value, they bind to LuxR homologue receptor proteins regulating various phenotypes like luminescence, biofilm formation, virulence and other relevant processes (Waters & Bassler 2005; Irie & Parsek 2008). Therefore any agent or process capable of interfering the transmission of signal molecules may possibly lead to a loss of biofilm forming ability.

In fact, recent studies have suggested that non antibiotic molecules naturally formed within bacterial communities for instance signaling molecules, could also interfere with biofilm formation. In contrast to anti-microbial agents, quorum sensing inhibitors (QSI) work at sub-

inhibitory concentrations which will avoid creating a selection pressure for development of resistance. Involvement of QSI as antibiofilm agents is quite promising in biofilm formation, maintenance and dispersal. Quorum sensing inhibition represents a widespread strategy for biofilm eradication (Bauer & Robinson 2002). Interference in quorum sensing systems due to signal molecule generation, its dissemination, its perception or upstream global regulation leads to the decrease of virulence and known as quorum quenching /quorum sensing inhibition/anti-quorum sensing (Kalia 2013).

In general many synthetic compounds have been explored for the disruption of quorum sensing till date which includes 4-Nitro-pyridine-N-oxide (Rasmussen & Givskov 2006), N-(heptyl-sulfanyl acetyl)-L-HSL (Persson et al. 2005) acyl cyclopentylamines (Geske et al. 2008). Biomaterials, especially biopolymers have gained importance as surface applications due to their high biodegradability, chemical stability and high reactivity. The current importance of microbial biopolymers arise from their innate advantages over natural biopolymers (like chitosan, gelatin), primarily based on the time needed to cultivate microorganisms, their cost for cultivation, and the ratio between growth rate and production. Benign nature of biopolymers for human and environment, and their remarkable stability and functionalities, have enabled successful application in various industries and for water treatment (Honarkar & Barikani 2009; Nwodo et al. 2014). Moreover, the structural flexibility of biopolymers has promoted their applicability as suitable primers for modifications by grafting and cationization for improved chelation and adsorption properties. While the enormous possibilities of microbial polymers are being actively explored, few information regarding its application as antagonists for biofilm formation has been reported. The role and possibility of biopolymer based interventions especially through

disruption of signaling system could be a challenging aspect of research and yield newer substitutes and approaches for biofilm control in drinking water systems. Systematic research emphasizing the above facts assumes importance in this regard. In view of these deliberations the current study was aimed to evaluating the microbial biopolymers as potential quorum quenching agents, their application and elucidation of the underlying mechanism for attenuation of *Sphingomonas* using these agents.

SCOPE OF THE STUDY

Despite a rapidly expanding volume of scientific information on biofilms, quorum sensing and exploitation of quorum sensing to curb biofilms in high risk areas, opportunistic pathogens such as *Sphingomonas* sp which frequently reside in drinking water systems have been poorly emphasized. The relevance of *Sphingomonas* is justified from the emerging data on its propensity, survival characteristics, pathogenicity and biofilm forming ability. For developing a viable scientific strategy, a systematic study comprising of the role of AHL in biofilm formation in *Sphingomonas* needs to be established and antibiofilm compounds must have the specificity of either by blocking, binding or degrading the AHL molecules to inhibit biofilm formation. Moreover the behavior of such compounds under environmental conditions, need to be assessed for providing leads to an alternate treatment approach for water.

This study was designed to characterize *Sphingomonas* sp. with biofilm forming ability in drinking water distribution systems and subsequently explore the mechanisms, especially quorum sensing for biofilm formation to develop suitable biopolymer based antibiofilm compounds targeting the quorum sensing molecule(s). Accordingly, a systematic study involving isolation and characterization of *Sphingomonas* from drinking water systems was carried out. Relevant biochemical attributes including cell surface properties were analyzed and biofilm formation investigated on commonly used plumbing materials. The role of quorum sensing and involvement of AHL molecules for biofilm formation was established. In an attempt to develop antibiofilm compounds, biopolymer of microbial origin was quaternized and evaluated extensively under laboratory conditions. To validate the functionality of these biopolymeric antibiofilm compounds, real time studies using environment samples were carried out involving

various doses of these compounds. Additionally, biopolymeric antibiofilm compounds coated on PVC coupons were subjected to environmental conditions as mentioned before. Insights from the study are expected to provide answers to key questions in overcoming challenges in *Sphingomonas* biofilm intervention especially in drinking water and other related systems important for human health.

OBJECTIVES OF THE STUDY

Consequently, the above mentioned study was planned and carried out with following objectives:

1. Isolation of *Sphingomonas* strains capable of biofilm formation and characterization of formation of biofilms
2. Investigation of culture and environmental conditions leading to optimal biofilm formation by the selected *Sphingomonas* isolate(s)
3. Establishing the role of AHL in biofilm formation by *Sphingomonas* isolate(s) and complete characterization of the involved AHL(s).
4. Screening microbial exopolymers as potential AHL blockers and elucidation of blocking mechanism leading to the prevention of biofilms.

CHAPTER 2

REVIEW OF

LITERATURE

REVIEW OF LITERATURE

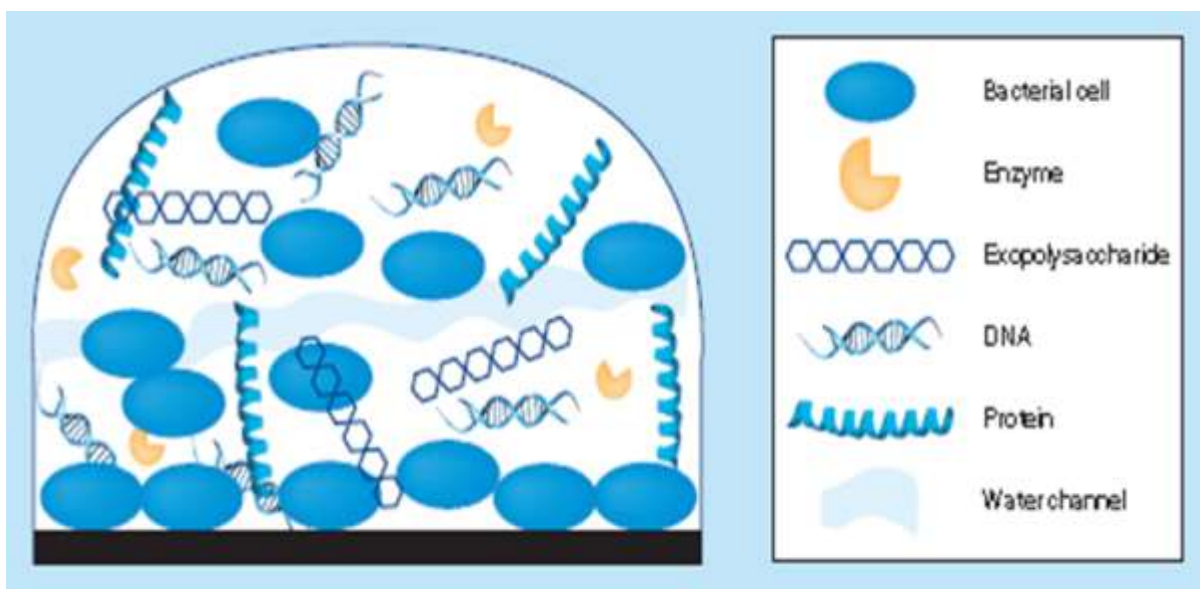
The notable seventeenth century researcher and microbiologist, Antonie van Leeuwenhoek analyzed 'animalcules' swarming on tooth surfaces and investigated the impact of vinegar on microorganisms in dental plaque and published bacterial microscopic visualization for the first time (Costerton et al. 1987). Later, Angst (1923) observed that the number of marine bacteria on hull surfaces of ships was noteworthy as compared to surrounding floating cells and recommended that the corrosion of hulls was due to bacterial biofilms. Zobell (1943) first published a two-step process on microbial colonization, initial reversible adherence of bacteria on substratum followed by irreversible binding of the bacterium. Until 1960s and early 1970s, bacterial biofilms were not much investigated till reorganizations of bacterial biofilms on trickling filters in wastewater treatment plant were made (Jones et al. 1969; Costerton et al. 1978).

2.1 What are biofilms?

A biofilm is an assemblage of surface-linked microorganisms enclosed within self synthesized matrix of extracellular polymeric substance. They are symbiotic communities of microorganisms which adhere to the surface and vary from their planktonic (freely suspended) counterparts by providing up to 1500 times more resistance to antibiotics and antimicrobial agents. Environmental changes lead to the evolution from planktonic to biofilm mode which entail numerous regulatory network translating signals to concerted gene expression changes which reprogrammed the bacterial cell to enable their survival in hostile conditions (O'Toole et al. 2000; Prigent-Combaret et al. 2001; Parsek & Singh 2003; Lenz et al. 2008; Monds & O'Toole 2009). Biofilms are ubiquitous in nature and can be found in distribution systems, waste water channels, hospital settings and frequently occur on firm surfaces submerged in or exposed to water. It can also be formed as floating mats on surface of liquid. Its formation can occur on both

biotic and abiotic surfaces (Costerton et al. 1999). Around 99% of all the bacteria exist as biofilm in their natural habitat. According to The National Institute of Health, about 80% of all chronic infections are related to biofilms (Krivit & Heuertz 2011; Raffa et al. 2004)

2.2 Biofilm structure and composition



Source: Rabin et al. 2015

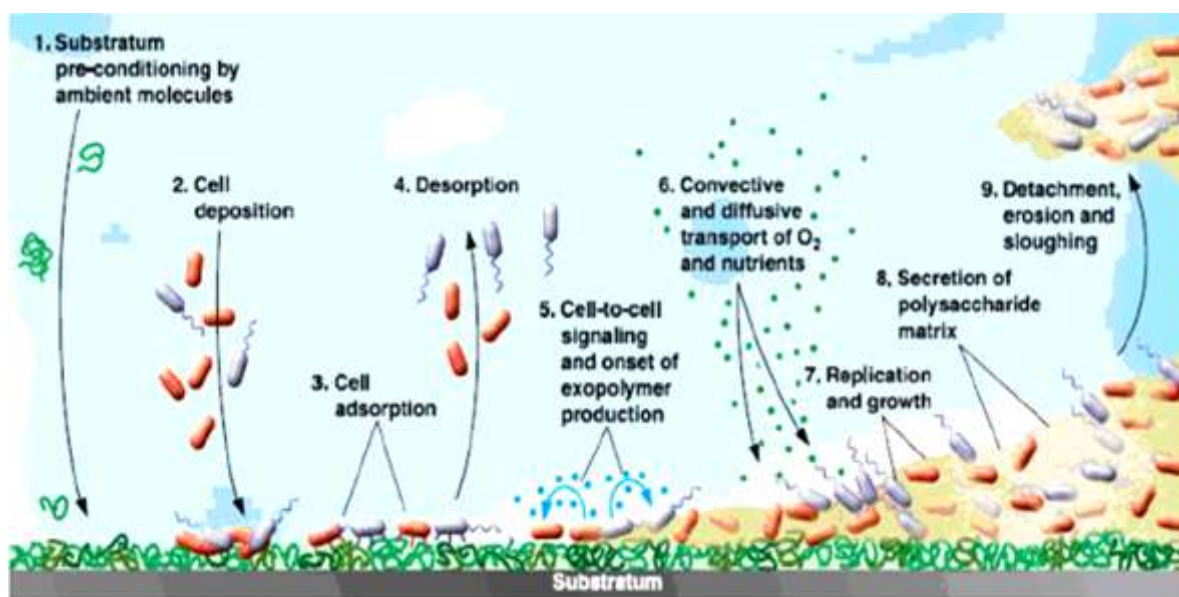
Figure 2.1: Structure and composition of biofilm

This three-dimensional microbial consortia defined as biofilm comprises mostly of hydrated EPS (10-25% cells and 79-90% EPS matrix) (Costerton et al. 1999). The biofilm framework consists of two main components viz. microcolonies and water channels for nutrient transport to microcolonies. The structure and composition of biofilm is shown in Figure 2.1. Microcolonies, the basic structural units of biofilm, are compactly packed communities of cells enclosed into EPS matrix. The matrix protects the bacterial cells from variety of environmental triggers like UV (Ultra violet) radiation, antimicrobial agents, abrupt changes in pH values and nutrient deprivation by forming a protective layer (Flemming 1993).

Several EPS molecules which are neutral or polyanionic are found in gram-negative bacteria. Its anionic property is enhanced by the occurrence of uronic acids or ketal-linked pyruvates which further allow divalent cations like calcium and magnesium to associate and augment the binding force in matured biofilm (Hart et al 2010; Donlan et al. 2002; Sutherland 2001).

2.3 Biofilm Formation

Biofilm formation is a highly complex process by which microorganisms adhere to the substratum, support cell-to-cell interactions and develop as a complicated structure (Bryers & Ratner 2004). Although the exact mechanism of biofilm formation differs from bacterium to bacterium; the stages of biofilm formation highlighted below appear to be conserved for an extensive range of microbes (O'Toole et al. 2000; Simões et al. 2010) (Figure 2.2).



Source: Simões et al. 2010

Figure 2.2: Processes governing the biofilm formation

Processes governing the formation of biofilm have been recognized as consisting of five stages:

1. Formation of a surface conditioning film

2. Movement of planktonic cells from the bulk liquid to the substratum
3. Reversible and irreversible adhesion
4. Growth and replication of the bacterial cells with the colonization of the surface, micro colony and biofilm formation
5. Detachment of bacterial cells.

2.3.1. Formation of a surface conditioning film

In nature, bacterial cells do not adhere directly to the surface as such, but adsorb to the conditioning film that forms on the surface. Conditioning of the surfaces leads to chemical modification of the original substratum and influence the rate and extent of microbial adherence (Prakash et al. 2003). Many variables affect the attachment of bacterial cells to substratum (Table 2.1).

Table 2.1: Variables affecting the attachment of bacterial cells to substratum

Source: Donlan et al. 2002

Adhesion surface	Bulk field	Bacterial Cells
Surface texture	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	pH	Extracellular appendages
Surface chemistry	Temperature	Extracellular polymeric substances
Charge	Cations	Cell-to-cell signaling molecules
Conditioning film	Presence of antimicrobial products	
	Nutrient Availability	

In aquatic or terrestrial environment, the conditioning layer consists of complex polysaccharides, glycoproteins and humic compounds (Compère et al. 2001; Baker et al. 2004; Jain & Bhosle 2009). The conditioning layer also modifies the physico-chemical properties of the surface viz.

concentrated nutrient source, changes in hydrophobicity and important trace elements which in turn affects the biofilm formation.

2.3.2 Movement of planktonic cells from the bulk liquid to the substratum

Many fluid dynamic processes like mass transport, Brownian motion, gravitational effects, liquid flow transport bacterial cells to the substratum (Gharechahi et al. 2012). Laminar and turbulent flow conditions are evident in fluid transport pipes. Fluid flow rate determines the stability of bacterial cells and nutrients (Stoodley et al. 2002), whereas mixing of cells and nutrients takes place in turbulent flow which leads to microbial adhesion (Percival & Walker 1999). Also, bacterial motility is shown to increase the chances of adhesion (Tasaki et al. 2017; Wang et al. 2014).

2.3.3 Adhesion of bacterial cells

Adhesion of bacterial cells occurs following conditioning of the surface and transport of cells and nutrients in close proximity to the substratum. Adhesion is a two-step process-reversible and irreversible adhesion. The initial adhesion of bacterial cells to surface is dynamic and reversible in nature, due to the weak interactions like Vander Waals forces, electrostatic forces and hydrophobic interactions. Favorable environmental conditions mediate the transition of bacterial attachment from reversible to irreversible. In irreversible attachment, various short-range forces are involved which includes dipole-dipole interactions, hydrogen, ionic, covalent as well as hydrophobic interactions. Microbial adhesion to the surface is also dependent on cell surface properties like cell surface hydrophobicity, presence of extracellular filamentous appendages like – flagella, pili or fimbriae, prothecae, stalks and hold-fast, specific receptors or proteins responsible for binding, etc (Berne et al. 2010).

2.3.4 Microcolony formation

Following the irreversible attachment of cells to the substratum, cells grow and replicate by convective and diffusive transport of oxygen and nutrients leading to the formation of microcolonies that merge to form a layer of cells covering the surface. Production of EPS takes place during this phase which helps anchor the bacterial cells to surface (Johnson 2008).

2.3.5 Extracellular polymeric substances (EPS)

Extracellular polymeric substances (EPS) are self synthesized slimy layer which protects the bacterial cells from environmental stresses. It comprises 50% to 90% of the total organic carbon of biofilms (Flemming et al. 2000). It is highly hydrated; can be both hydrophilic and hydrophobic (Sutherland 2001) and can vary in its solubility. Sutherland (2001) reported two imperative properties of EPS which affect biofilm formation. Firstly, its composition and structure establish its primary conformation. Secondly, EPS is generally not uniform and can vary spatially and temporally. EPS affects the physical properties of biofilm like diffusivity, conductivity and rheological properties. It is dependent on nutrient status of the growth medium; excess carbon and nitrogen, potassium or phosphate limitation promote EPS secretion. It contributes to antimicrobial resistance properties of biofilm.

2.3.6 Biofilm formation

Attachment of macromolecules and bacterial cells to the surface is usually followed by growth and replication of microorganisms and development of microcolonies. Attachment of microorganisms is followed by its immobilization in hydrated matrix. A spatially separated but interactive group of microorganisms are established which physiologically cooperate. Biofilm formation is a slow process with highly heterogeneous composition due to the colonization of

varied microorganisms with varying nutritional requirements (Jamal et al. 2015). After the achievement of thickness of 10–25 μm , the base of biofilm become anaerobic indicating a matured biofilm with high species diversity. Medical biofilms are thinner than those found within industrial environments.

2.3.7 Microbial interactions in biofilms

Interactions between the microorganisms are complex and mixed, wherein more than one type of interactions take place between different species (Braga et al. 2016). These interactions of different microorganisms during initial attachment play a significant role in determining the structure and physiology of biofilm. The pioneer colonizers encourage the attachment of other compatible species and form a thicker biofilm than monospecies biofilm with more stability.

Cells living in biofilms interact with each other in variety of ways. Cell-to-cell communication system for regulating the colonization within the biofilm by involving extracellular signaling molecules (autoinducers) is known as quorum sensing (Ng & Bassler 2009). The concentration of autoinducers correlates with the population density. The signal molecules are secreted during microbial growth. When the concentration of autoinducers becomes high in the surrounding environment, they reach a threshold value (quorum level) and regulate the quorum sensing dependent gene expression. Lateral gene transfer is improved in biofilms as compared to planktonic cells (Madsen et al. 2012).

2.3.8 Detachment and dispersal of biofilms

Bacterial cells detach and colonize new niches for its survival after the biofilm ages. Three different modes of biofilm detachment have been recognized: sloughing, erosion and abrasion. Erosion is the constant discharge of bacterial cells from biofilm at minimum levels over the

duration of biofilm formation. Sloughing is rapid detachment of large segments of biofilm, frequently throughout later stages of biofilm. Erosion and sloughing can be either active or passive, while, seeding dispersal can only be an active process (Stoodley et al. 2002; Wilson et al. 2004). Different factors affecting biofilm detachment are pH, temperature, surface roughness (Gjermansen et al. 2005; Sauer et al. 2004; Thormann et al. 2005). Some of the released cells float in the surface while others can be relocated and enable to restart the biofilm formation (Prakash et al. 2003).

2.4 Biofilms in drinking water distribution system (DWDS)

Over the years, a major concern experienced around the world is the deterioration of drinking water quality causing many health risk and death to consumers. Biofilm is present on exposed surfaces in potable water production and supply involving abstraction from source waters, coagulation, filtration, disinfection, distribution and storage. The quality of water available for consumption may vary from the quality at the time of treatment in the waterworks (Pepper et al. 2004). Biofilm in drinking water causes changes in odour, colour taste, and turbidity of the water and pathogenic bacteria can proliferate in biofilms of drinking water systems to survive (Kerr et al. 1998). Biofilms possess the ability to act as an environmental reservoir for pathogenic microorganisms and hence are probable sources of contamination through resident, opportunistic pathogens.

2.5 Factors affecting biofilm development in drinking water distribution system (DWDSs)

Few bacteria can survive the water treatment processes in the distribution system and can multiply given the right condition for growth. The several factors that enhance the growth of bacteria include use of disinfectants, maintenance of residual concentration in system, nature of

biodegradable compounds in treated drinking water, resistance of bacteria to disinfectants, plumbing materials used and water temperature.

2.5.1 Ineffective disinfectants

Disinfectants in appropriate concentration can be effective in the treatment of distribution systems and can contribute to the production of easily biodegradable organic substances which could be utilized by bacteria as energy source and promote biofilm production in DWDSs (Momba et al. 1998; Momba et al. 2000) reported that contamination of microorganisms declines after chlorine treatment but the regrowth is regained if residual chlorine decreases with time. Lu et al (1999) reported that fixed biomass chlorine demand in DWDS is dependent upon incubation time and water quality. Many disinfectants like chloramine maintain a high disinfectant residual throughout distribution systems leading to penetration of biofilm effectively, thereby, controlling biofilm formation better than chlorine.

It is known that many microorganisms survive and grow in the presence of measurable concentration of commonly used disinfectants in distribution systems due to the development of resistance towards these compounds. Biofilm cells are less susceptible to disinfectants as compared to their planktonic counterparts (Donlan & Costerton 2002; Davies 2003; Steed & Falkinham 2006). This resistance is due to the nutrient limitation which may alter cell surface properties and membrane function (Poole 2012).

2.5.2 Enviromental factors (pH and temperature)

pH and temperature affect the microbial life by altering the electrostatic interactions between substratum and microorganisms, microbial metabolism, enzymatic activity, kinetics and

equilibrium of reactions, diffusion and solubility. Residues of disinfectants present in distribution system are severely reduced when temperature increases and pH decreases.

2.5.3 Plumbing materials

The direct relationship between the plumbing materials used for the manufacture of drinking water distribution systems and the quality of water was reported by Lehtola et al (2005). Recently, polymeric materials have been preferred in the distribution systems, mainly polyvinyl chloride (PVC) polyethylene (PE), polypropylene (PP) because they are easy to handle and implement. The effect of support materials on biofilm development has been well documented (Momba et al. 2000; Schwartz et al. 1998; Kerr et al. 1998; Niquette et al. 2000; Cloete 2003; Yu et al. 2010). It has been reported that most plumbing materials in distribution systems might hold biofilms with bacterial densities as high as 10^9 bacteria·cm⁻² (Ridgway and Olson 1982). *Sphingomonas paucimobilis*, an oligotroph, form biofilms in plumbing systems which causes accumulation of biological materials in water distribution systems. Ryan & Adley (2010) reported that sterilization problem occurs as this organism can pass through 0.2 µm filters and contaminate several medicinal products.

2.6 Problems associated with biofilm development in DWDSs

Biofilm development eventually impacts the quality of potable water to hydraulic performance of the distribution network. Problems associated with presence of biofilm in distribution system are discussed below:

2.6.1 Health risk

Water- based pathogens have capability to grow to high densities within biofilms on pipelines and sediments; consequently, control above some critical concentration is mandatory to manage pathogens (Ashbolt 2015). Biofilm acts as an environmental reservoir to microorganisms, present below their detection limit (Wingender & Flemming 2011). Many pathogens can utilize metabolic products formed by non pathogenic members of biofilms in distribution systems (Steiner et al. 1998). The most studied example is *Legionella*. It has been revealed that *Legionella* thrive in biofilms and interacts with other microorganisms in biofilms for their proliferation in water (US EPA 2001). Moreover, interactions with certain algae and bacteria in biofilms also support the growth of *Legionella* (US EPA 2001). In the same way, although most of the microorganisms prevalent in distribution systems biofilm are not pathogenic (Szewzyk et al. 2000), it is vital that in few cases a extended treatment can select chlorine resistant-bacterial sub-populations. Pathogenic bacteria are differentiated into primary and secondary ones. Primary bacteria can cause disease inhost by themselves. They are present in lower concentration in distribution systems while secondary pathogens (like opportunistic pathogens) cannot cause disease by themselves. Opportunistic pathogens have significance in distribution systems because they can cause diseases in people with weak immune system.

2.6.2 Deterioration of water quality

Presence of biofilm in water distribution system deteriorates the quality of water (Lee et al. 2006; Wingender & Flemming 2011) and corrodes the structural surfaces (Beech & Flemming 2000). It also results in water treatment yield loss, reduction in efficiency of cooling or heating exchange transport, as well as in membrane processes. These naturally occurring biofilms in drinking or industrial water may induce depletion of residual disinfectants; biodegradation of nutrients can

cause change in color, odor and taste. Moreover, by acting like a reservoir to pathogenic organisms like bacteria, viruses, protozoa, algae, fungi and other invertebrates, it is hazardous for human and animal health (Riley et al. 2011). Corrosion of inner walls of pipelines leads to the detachment of iron and magnesium salts which causes water discoloration. Moreover, some microorganisms in biofilm, such as the sulphate-reducing bacteria (SRB) can metabolize to favor corrosion processes. This occurrence is predominantly significant in U.S. and in few European countries where cast iron was the primary material used in DWDSs. The predicament of discolouration has a significant impact to the consumer, and is mainly aesthetic. However, a more imperative outcome may be the loss of residual disinfectant and the resulting enhancement of biofilm development.

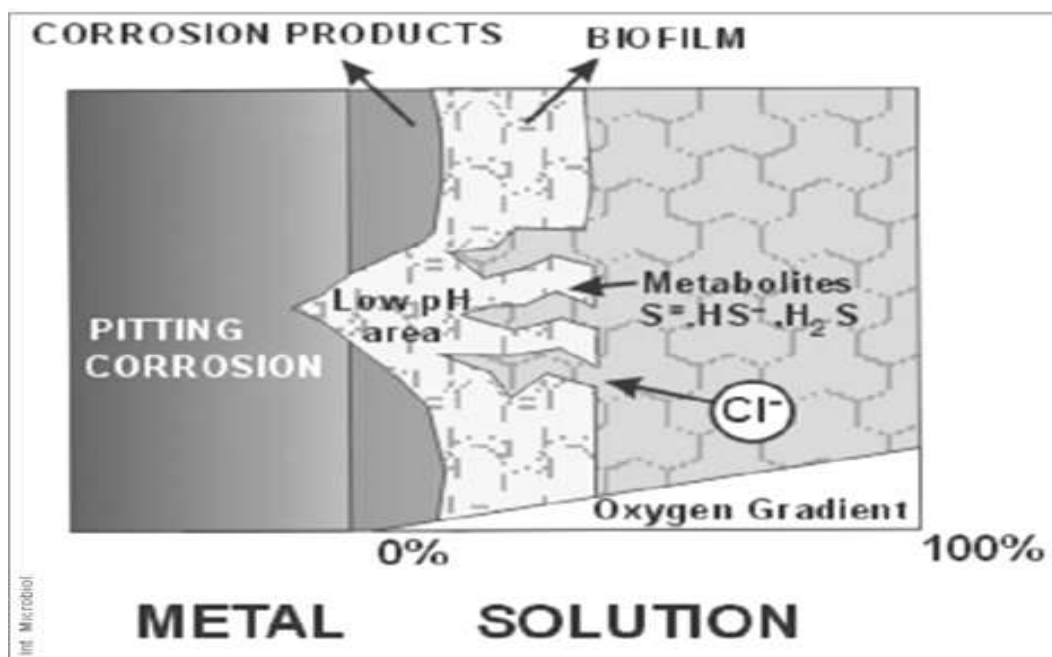
2.6.3 Disinfectant decay

To maintain the quality level of drinking water, disinfectants are applied at the inlet of distribution system. It has been reported that the residual disinfectants are consumed in the distribution systems due to the occurring reactions in circulating water mass and inner pipe lines of distribution systems (Kiene et al. 1998). Parameters such as diameter and material of pipe lines, microorganisms, deposits, corrosion products and biofilms influence the decay of disinfectants (Kiene et al. 1998). Even though disinfectant decay due to the existence of biofilm is inadequately understood, but it is recognized that biofilm reacts with disinfectant consuming it. It has been observed that chlorine can be consumed by diffusing into biofilms. The limited penetration of chlorine into biofilm is caused by transient diffusion as well by the neutralization of chlorine in the biofilm matrix (De Beer et al. 1994; Stewart et al. 2001). The rate with which chlorine penetrates into the biofilm varies suggesting the occurrence of local difference in biofilm (De Beer et al. 1994; Stewart et al. 2001). EPS in biofilm consumes the disinfectant,

retard membrane permeabilization of bacteria and hence decrease the susceptibility of bacteria (Seo 2009). The development of biofilm development on pipewalls in distribution systems with disinfectant consumption has been proposed as an important parameter in distribution system modeling.

2.6.4 Biocorrosion/ Microbiological Influenced Corrosion (MIC)

It is defined as the influence of microorganisms on the kinetic processes of metal corrosion. Biocorrosion processes associated with metabolic activity of microorganisms and its products which includes enzymes, exopolymers, ammonia and hydrogen sulfide (Beech & Gaylarde 1999). Electrochemically, corrosion is the chemical reaction where electrons are transferred from zero valence metal to an external electron acceptor which causes the release of metal ions into surrounding medium (Díaz-Ballote et al. 2009).



Source: Videla & Herrera 2005

Figure 2.3: Bioelectrochemical interpretation of the function of biofilm in biocorrosion of pipe walls

The ability of bacteria to replace oxygen for other oxidized compounds as final electron acceptor in respiration and production of various corrosive metabolic products makes them real threat to stability of metals causing corrosion (Figure 2.3).

2.6.5 Operational problems

Impact of biofilm development on performance of pipelines in distribution systems can be significant. It decreases the speed of the flow and the pipes' capacity of circulation (Cowle et al. 2014). Primary cause of reduction of flow capacity within pipelines is increase of friction along the solid-liquid interface with increase of roughness of surface and interface instabilities (Shockling et al. 2006). Biofilm development can also affect maintenance costs (Barton 2006), operational efficiency and energetic efficiency of the system leading to economical and environmental consequences. Various parameters like physical and structural nature of biofilm (i.e. gelatinous and filamentous) helps in change of surface roughness dynamics (Barton 2006). Biofilm formation in pipelines of distribution systems causes pressure drops, frictional drag and need for increased pumping power impact energy requirements of distribution systems.

2.7 Biofilm control in distribution systems

To avoid the negative consequences of biofilm development in pipelines of distribution systems, its successful prevention is need of hour. Antibiofilm approaches have been used to control microbial growth in distribution systems which are as follows.

2.7.1 Disinfection

The primary method to control formation of biofilm is by regular cleaning and disinfectants which includes detaching microbial cells from surfaces (Simões et al. 2006). Different types of disinfection strategies are as follows: (i) disinfection before the formation of biofilm, (ii) using

harsh disinfectants, and (iii) selection of surface materials not supporting adherence or by supplementation of nutrients (Meyer 2003). Most commonly used disinfectants are chlorine, chloramines, ozone, hydrogen peroxide or ultraviolet radiation. They are introduced for the removal of microorganisms which escape the previous treatment procedures. The effectiveness of disinfectants depends on pH, temperature, hardness of water, chemical inhibitors and contact time. It was reported that monochloramine maintained a longer disinfectant residual within water distribution systems by penetrating biofilm more effectively resulting in better biofilm control as compared to free chlorine (Stewart et al. 2001; Kim & Park 2008). Momba & Binda (2002) reported that slight increase in microorganisms was observed in the presence of residual disinfectant concentrations. Even when residual concentration of 12.5 mg l⁻¹ hydrogen peroxide, 0.8 to 1 mg l⁻¹ monochloramine and 0.2 to 0.5 mg l⁻¹ free chlorine was present, biofilm was formed on different coupons. Many biofloculant producing bacterial strains have been identified for water treatment purposes as reported by Zulkeflee et al (2012).

Many detergents containing chelating agents like Ethylenediamine-tetra acetic acid (EDTA) (Finnegan & Percival 2015; Robertson et al. 2012), peracetic acids (Cabeca et al. 2008) were used as chemical agents to control biofilm.

2.7.2 Production of biologically stable water

Biological stability refers to the function of biologically available organic carbon. Production of biologically stable water is implied by inability of drinking water to support growth of microorganisms (Hammes et al. 2010). It is virtually impossible to inhibit biofilm formation by limiting carbon sources as ultra pure water systems still support the development of biofilms.

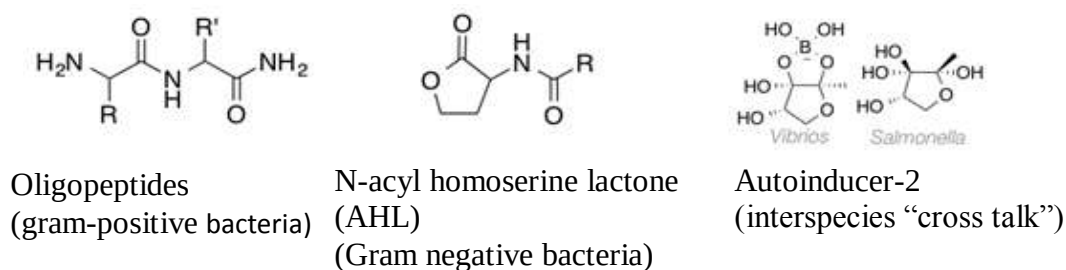
Bacterial biofilm presents substantial resistance towards disinfectants thus making strategies which are imperative towards successful eradication of contamination and infection, ineffective resulting in tremendous economic loss. Recently, for the inhibition of biofilm, compounds have been derived from natural sources such as bioactive component from *Moringa oleifera* seed coat revealed antibiofilm potential against bacteria and yeast (Onsare & Arora 2015); antibiofilm activity of pyocyanin from *Pseudomonas aeruginosa* BTRY1 (Mohandas et al. 2016). Also, mechanism of adhesion of pathogenic bacteria with a probiotic strain of *Lactobacillus* have shown to reduce colony formation. In case of *L. plantarum* and *L. rhamnosus* probiotic strains of the gut, few of the known adhesive molecules produces alfa-enolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and mucus via mannose binding (Msa) and modulator of adhesion and biofilm (MabA). They exhibit adhesion property of bacteria which can lead to antimicrobial properties by reducing biofilm proliferation (Dhanani et al. 2011).

Thus, alternative to disinfection methods, nontoxic and environment friendly biofilm inhibition strategies exploiting chemical signals secreted by bacteria to regulate biofilm developmental processes can be employed. Most significant one is quorum sensing (QS) signaling system (Galloway et al. 2012; Feng et al. 2013) with a wide range of natural and synthetic compounds like halogenated furanones (McDougald et al. 2012), 4-nitropyridine-N-oxide(4-NPO) (Cady et al. 2012), and natural ones like vanillin (Ponnusamy et al. 2009), ajoene (Jakobsen et al. 2012b). This technique enables the inhibition of biofilm on water pipes by coating QS inhibitors onto the surface (Ho et al. 2014).

2.8 Quorum quenching: Exploiting socio-microbiological process as antibiofilm control

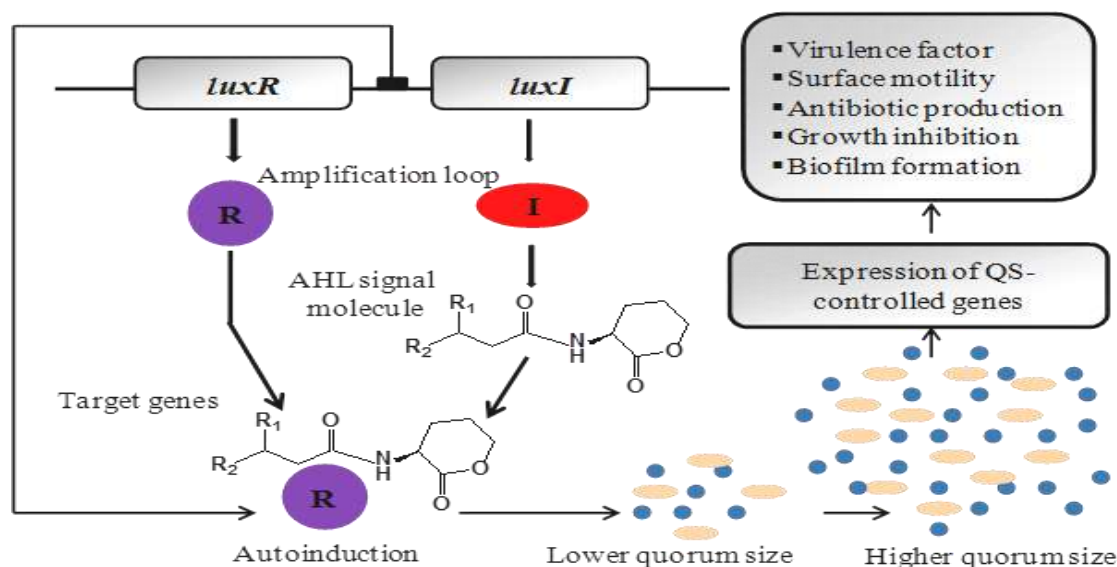
2.8.1 Quorum sensing (QS)

The socio-ecology of microorganisms involve an intricate regulatory process between microcolonies through which bacteria secrete signal molecules that aid in coordinating their behavior in cell density-dependent manner (Waters & Bassler 2005). This process, known as “Quorum Sensing (QS)” involves three types of QS systems - N-acyl homoserine lactone (AHL) QS system in gram negative bacteria, the autoinducing peptide (AIP) or oligopeptide QS system in gram positive system, and autoinducer 2 (AI-2). QS system in both gram negative and gram positive bacteria (Figure 2.4) (Bassler 2002; Li & Tian 2012). There may be more than QS signal molecules employed by bacteria which can be from a same or a different chemical class. Activities of these signal molecules are coordinated by interacting quorum sensing systems, each system possess a signal molecule synthase and a receptor (Fuqua & Greenberg 2002; Lazdunski et al. 2004; Williams 2007). When the intracellular concentration of AHL synthesized by LuxI homologs reach a threshold value, the signal molecules received by LuxR homologs lead to regulation of various phenotypes like luminescence, biofilm formation, virulence and other relevant processes (Figure 2.5) (Waters & Bassler 2005; Irie & Parsek 2008).



Source: <https://sites.tufts.edu/quorumsensing/quorumsensing101/>

Figure 2.4: Different types of QS signal molecules



Source: Lade et al. 2014

Figure 2.5: Quorum sensing process

2.8.2 Bacterial reporters to detect QS

A significant prerequisite for controlling the pathogenicity associated with QS is its rapid detection; the importance of biosensors for detection was comprehended due to the rising variation in the QS signals secreted by diverse microorganisms. These biosensors do not generate AHLs and the cognate target promoter (frequently the promoter of the cognate luxI synthase) is fused to functional LuxR homolog. When exogenous QS signal molecule is provided, promoter is induced, hence activating the receptor which positively regulates the transcription of a reporter gene (e.g. bioluminescence, β -galactosidase, green-fluorescent protein and violacein pigment production). Different types of reporters developed over a period of time are depicted in Table 2.2.

Table 2.2: Different biosensor strains used for detection of quorum sensing

S.No	Biosensors	Major Characteristics	Phenotype	Reference
1.	<i>Agrobacterium tumefaciens</i> A136 [traI-lacZ fusion (pCF218)(pCF372)]	Detects wide range of AHLs (C6-C14 AHLs)	β -galactosidase activity	Fuqua et al. 1996; Zhu & Winans 1998
2.	<i>A. tumefaciens</i> strain NT1 (pDCI41E33 containing a traG::lacZ fusion)	AHLs with 3-oxo-, 3-hydroxy-, and 3-unsubstituted side chains of all lengths, (C6-C14AHLs)	β -galactosidase activity	Shaw et al. 1997
3.	<i>Chromobacterium violaceum</i> strain CV026	Detects C4-C8 AHLs	Violacein pigment production	McClellan et al. 1997
4.	<i>Pseudomonas aureofaciens</i> 30–84	wt pigmented strain used in QSI assay	Phenazine production	McLean et al. 2004; Wood & Pierson 1996
5.	<i>Vibrio harveyi</i> BB170	Autoinducer-2 (AI-2) detection	Bioluminescent	Bassler et al. 1997
6.	<i>Escherichia coli</i> plasmid carrying a luxCDABEcassette activated by AhyR receptor of <i>A. hydrophila</i> (pSB403)	Wide host range of AHLs	Bioluminescent	Winson et al. 1998
7.	<i>A. tumefaciens</i> KYC55 (pJZ372)(pJZ384)(pJZ410)	Increased detection of AHLs (C4-C18 AHLs)	β -galactosidase activity	Zhu et al. 2002
8.	<i>Pseudomonas putida</i> 117(pAS-C8)-CepR receptor	C8AHL	Green Fluorescent Protein	Steidle et al. 2001
9.	<i>Staphylococcus aureus</i> pRN6683 in lab strain RN6390	RNAIII-blaZ transcriptional fusion	Beta-lactamase activity	Novick et al. 1995
10.	<i>Sinorhizobium meliloti</i> Rm41 sinI::lacZ(pJNSinR)a	AHL reporter for long chain (up to C16) HSLs	β -galactosidase activity	Llamas et al. 2005

2.8.3 Quorum sensing and biofilm formation

Each developmental stage in the form of biofilm requires the expression of specific genes. For regulating gene expression between microcolonies, a cell- to-cell communication system known as quorum sensing is involved. Genes which control surface attachment and synthesis of matrix components are under the regulation of QS (Mack et al. 2004). It can induce the transition in behavior of biofilm cells altering the course of its formation. Alternatively, QS might induce or repress activities like surface motility, which have an important impact on biofilm architecture. The role of quorum sensing in the formation of biofilms was first reported by Davies et al (1998). It was reported that *Pseudomonas aeruginosa* mutants which lack synthesis of AHL signal molecules are unable to form proper biofilm architecture (Davies et al. 1998). This cell-to-cell relationship is more complex in case of gram positive bacteria like *Bacillus subtilis*. Surface attachment is triggered by QS-associated genes and subsequently formation of biofilm (Singh et al. 2015). In *Staphylococcus aureus*, agr QS system is repressed which is important for formation of biofilm (Ganin et al. 2012). QS has also been reported to regulate the biofilm formation in simple eukaryotes such as *C. albicans*. Alem et al. (2006) reported that in early stages of biofilm formation, the QS signal tyrosol promotes the hyphal formation, whereas during the later stages, another QS signal molecule, farnesol, and supports dispersal of yeast cells from the biofilm. This suggests that the synthesis of tyrosol and farnesol are differential throughout biofilm formation, according to which QS signal accumulates at particular developmental stages. Although QS is significant during biofilm formation for a diverse variety of species, but in many cases the QS-regulated functions accountable for observed biofilm phenotypes remains to be identified as a challenge for researchers.

2.8.4 Quorum sensing Inhibition

Inhibition of Quorum sensing or quorum quenching is a promising approach to the control of biofilms without noteworthy planktonic cell death. QS inhibitors (QSIs) have been recommended as novel antibiofilm agents. Three points of attack offered by QS system are the signal molecule-generating LuxI homolog, AHL molecule itself, and the signal receptor LuxR homolog. If AHL molecule would not be produced by bacteria, then the genes controlled by QS will not be expressed. Also, if signal molecules are inhibited after their production, then, they will not be able to bind with LuxR homolog, hence, QS-regulated virulence factors will not be produced. Another way to inhibit QS is by preventing the AHL molecules from activating LuxR homolog. To achieve this goal, AHL binding pocket can be blocked either by AHL homolog or by introduction of conformational change in R-protein by binding a small molecule to another part, thereby, disrupting signal molecules from reaching binding pocket. Also, molecules causing LuxR protein to misfold, thereby preparing it for degradation by host proteases (Ren et al. 2010). AHL molecules were synthesized in gram negative bacteria by *luxI* homologue genes (Defoirdt et al. 2004), while its precursors are acyl-ACP (Acyl carrier protein) and S-adenosylmethionine (SAM). It was reported that 97% of LuxI homologue (RhII) activity in *P.aeruginosa* was inhibited by analogues of SAM (Parsek et al. 1999). The interactions between autoinducer and LuxR homologues can be prevented by differences in length of structure or by introducing substitutions in acyl side chain groups which leads to the inhibition of quorum sensing (Defoirdt et al. 2004; Parsek et al. 1999). Diverse molecules able to disrupt the QS system have been documented and their mechanisms have been extensively analyzed in an effort to control biofilm development; some of these include halogenated furanones produced by seaweed *Delisea pulchra* and synthetic derivatives which target R proteins (Lyon et al. 2000),

synthetic analogues of AHL which compete with corresponding signal molecule (Lyon et al. 2000), and quorum quenching enzymes like AHL-acylase, AHL-lactonase, and oxidoreductases that degrade or modify AHL signal molecule. Also, 2,5-piperazinedione was reported to inhibit QS-dependent factors in *Pseudomonas aeruginosa* PAO1 (Musthafa et al. 2012). Table 2.3 and 2.4 depicts commonly known natural and synthetic quorum sensing inhibitors.

Table 2.3: Synthetic quorum sensing inhibitors

QSI Compound	Mode of Action	Reference
N-arylisatin-based glyoxamide derivatives	Formation of a hydrogen bonding network	Nizalapur et al. 2016
compound (z)-5-octylidenethiazolidine-2, 4-dione (TZD-C8)	high affinity for the LasI activity pocket	Lidor et al. 2015
3-nitro phenylacetanoyl HL (C14)	Inhibitory to LasR-reporter in <i>P. aeruginosa</i> ; Modestly inhibitory to TraR-reporter in <i>A. tumefaciens</i>	Geske et al. 2008
4-phenylbutanoyl HSL	Antagonizes LuxR in <i>V. fischeri</i> ; Binding of OHC6HSL harbouring LuxR in <i>E.coli</i>	Geske et al. 2008
4-nitro-pyridineN-oxide	Blockage of LuxR homologs	Rasmussen & Givskov 2006
Para-benzoquinone	Blockage of LuxR homologs	Rasmussen & Givskov 2006
2,4,5-tri-bromo-imidazole	Blockage of LuxR	Rasmussen & Givskov 2006
3-nitrobenzene- sulfonamide	Blockage of LuxR	Rasmussen & Givskov 2006
Cyclohexanone	Inactive in LasR activation but active against RhlR in <i>P. aeruginosa</i>	Smith et al. 2003
Azithromycin	Lower amount of AHL produced	Tateda et al. 2001
L-S-adenosylcysteine	Blockage of AHL signal generation	Parsek et al. 1999

Table 2.4: Natural quorum sensing inhibitors

QSI Compound	Source	Mode of Action	References
Seed oils	oleaginous plants	QSI activity was restricted to saturated FA of chains 12-18 carbon atoms in length	Pérez-López et al. 2017
Ellagic acid	Fruit extract of <i>Terminalia chebula</i> Retz	Down-regulate the expression of virulence gene in <i>P. aeruginosa</i> PAO1	Sarabhai et al. 2013
Iberin	Many members of the Brassicaceae family.	Blocks expression of QS-regulated genes in <i>P. aeruginosa</i> .	Jakobsen et al. 2012a
Rosmarinic acid	Sweet basil (<i>Ocimum basilicum</i>)	Inhibit protease, elastase, hemolysin production, biofilm formation and virulence factor in <i>P. aeruginosa</i>	Annapoorani et al. 2012
Furanone/ 2(5H)-Furanone/	Macroalga (<i>Delisea pulchra</i>)	Mimics AHL signal by occupying the binding site on putative regulatory protein	Ponnusamy et al. 2010
Cynnamaldheyde (trans-Cinnamaldehyde)	<i>Cinnamomum zeylanicum</i>	Interfere with AI-2 based QS and decreases the DNA-binding ability of LuxR protein to reduce virulence in <i>V. spp.</i> Lowers the LuxR-mediated transcription from the PluxI promoter which influences biofilm formation in <i>P. aeruginosa</i>	Brackman et al. 2008
Patulin	<i>Penicillium sp.</i>	Blockage of LuxR homologs	Rasmussen & Givskov 2006
Penicillic acid	<i>Penicillium sp.</i>	Blockage of LuxR homologs	Rasmussen & Givskov 2006
L-canavanine	Seed exudates (<i>Medicago sativa</i>)	Inhibit the regulation of QS-regulated phenotype exopolysaccharide II production in <i>Sinorhizobium meliloti</i>	Keshavan et al. 2005
Vanillin	Vanilla beans extract	Interfere with AHL receptors	Choo et al. 2006

2.8.5 A Novel strategy for interfering in quorum sensing mechanism using modified biopolymers

Biopolymers are chain like molecules that are made up of repeating monomers and can vary in length. Salehizadah & Shojaosadati (2001) reported biopolymers as suitable primers for alteration for developing new molecular designs. Different groups present on polymeric backbone can be modified by grafting and cationization. Their infrared spectroscopic studies reveal the presence of carboxyl, hydroxyl, methoxyl, amino and phosphate groups in structure (Gao et al. 2006). Amongst various strategies of modification, most common one is graft polymerization. Reaction of a polysaccharide with various reagents which possess positively charged groups like amino, sulphonium and phosphonium groups (Auzély-Velty & Rinaudo 2003). Positive charges that are attached to polymers, particles, surfaces, peptides or bilayers have been used as antimicrobial agents. Diverse modifications have been reported on various polymers over the years to produce new properties; like, methylation improved binding, thickening and stabilization property of polymers (Hiatt & Rebel 1971); conversion to phosphate ester introduced flame resistant and ion exchange properties in polymers (Isogai et al. 1986) and introducing quaternary ammonium groups to polymers which confer adhesive properties (Tan et al. 2013). Development of novel compounds based on biopolymer by altering their chemical structure can open up opportunities in controlling various industrial and environmental concerns (Chassenieux et al. 2013). Vemula et al (2008) synthesized a biobased monomers/polymers influencing environment. The use of poly (anhydride ester) polymers with chemically linked salicylic acid (SA-PAE), a plant-derived antimicrobial, built into the backbone was elucidated (Guinta et al. 2011). These SA-PAE polymers are active-release substances that degrade, subsequently releasing salicylic acid (SA) and eventually affecting a global regulatory change for transition into biofilms. Modified biopolymers formed after the methylation of their amino sugar

groups impart positive charge and hence, improved antibacterial activity (de Britto et al. 2011; Khaira et al. 2013). Some commonly used polymers as Quorum sensing inhibitors are listed in Table 2.5:

Table 2.5: Polymers and their chemically modified forms developed as potential QSIs

Compound	Target organism	Mode of action	Reference
Nonwoven polymer nanofiber coatings	<i>Staphylococcus aureus</i>	Inhibit the production of hemolysins, an important QS-controlled virulence phenotype in <i>S. aureus</i>	Kratochvil et al. 2017
Copolymers based on methyl methacrylate	<i>Vibrio fischeri</i> <i>Aeromonas hydrophila</i>	By adsorption of AHLs reduce QS-phenotypes, decreasing bioluminescence and biofilm formation	Cavaleiro et al. 2015
Methacrylate/acrylate polymers	<i>V. fischeri</i>	Binding between AHLs and these types of monomers has been shown to be mainly due to electrostatic interactions	Hook et al. 2012
Cross-linked polymers monomers itaconic acid (IA) and methacrylic acid (MAA)	<i>V. fischeri</i>	Highest interactions with the AHLs targets	Piletska & Piletsky 2010

The most promising strategy perhaps, for interruption of QS activity is by using these compounds as signaling molecule analogs. These analogs binds to receptor proteins, hence disrupting its activation leading to interruption of quorum sensing. Many of these compounds exhibit anti biofilm and anti bacterial activities. The strategy of attenuation of bacterial virulence by interfering in cell-to-cell communication systems by these compounds rather than killing or inhabiting bacterial growth has started gaining momentum amongst researchers.

2.9 The genus *Sphingomonas*

The genus *Sphingomonas* was defined by Yabuuchi et al. (1990) and emended consequently by Takeuchi et al. (2001), Yabuuchi et al. (2002), Busse et al. (2003) and Chen et al. (2012). In the light of investigations of phylogeny and polyamine patterns profiles, the genus *Sphingomonas* was subdivided into following genera *Sphingomonas*, *Novosphingobium*, *Sphingobium*, *Sphingorhabdus* and *Sphingopyxis* (Takeuchi et al. 2001). These genera are collectively referred as sphingomonads. *Sphingomonas* are referred as gram-negative, chemo heterotrophic, rod-shaped, non-spore-forming, strictly aerobic bacteria producing yellow to off-white pigmented colonies. The uniqueness of *Sphingomonas* is due to its possession of ubiquinone 10 as their major respiratory quinone and the presence of glycosphingolipids instead of lipopolysaccharide in their cell envelopes (Arellano-Reynoso et al. 2005). The taxonomic classification of *Sphingomonas* is given in Table 2.6.

Table 2.6: Taxonomic Classification

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Alphaproteobacteria
Order:	Sphingomonadales
Family:	Sphingomonadaceae
Genus:	<i>Sphingomonas</i>

Source: Yabuuchi & Kosako 2015

2.9.1 *Sphingomonas* in biotechnological applications

The genus *Sphingomonas* hallmarked by their oligotrophic nature and plasticity in manmade environments, have been intensively exploited for the biotechnological significance. Strains of *Sphingomonas* sp., for instance, has been reported as effective degraders of refractory organic

pollutants and simple aromatic and polyaromatic hydrocarbons. It has also been reported to produce exopolysaccharides such as sphingans (Pollock 1993). The carbohydrate moiety is shorter in glycosphingolipids as compared to lipopolysaccharides resulting in its cell surface being more hydrophobic than other gram-negative bacteria. This might be the reason for both *Sphingomonas*' sensitivity to hydrophobic antibiotics and its capacity to degrade hydrophobic polycyclic aromatic hydrocarbons (Balkwill et al. 2006).

2.9.2 *Sphingomonas*: Reservoir of infection

However the fact that they are opportunistic pathogens (Oie et al. 1998; Laskin & White 1999; Perola et al. 2002) and the ability of this strain to survive in oligotrophic niches (offering low nutrient conditions) such as hospital tap water supply systems, reverse osmosis systems have exacerbated their relevance as unappreciated reservoirs of infection. Furthermore, *Sphingomonas* isolated from drinking water has been reported as being capable of developing biofilm on a variety of surfaces (Simões et al. 2010; Jang et al. 2011; Gusman et al. 2012). Different studies carried out till date have concluded *Sphingomonas* as the predominant species in biofilms (Jang et al. 2011; Simões et al. 2010; Kurissery et al. 2010; Rasimus et al. 2011) occurring in water distribution systems as well as industrial waste water. Recent studies by Gusman et al (2012) indicated that biofilms on surfaces exposed to drinking water in distribution systems are due to the presence of *Sphingomonas*. Health risk associated with *Sphingomonas* includes primary bacteremia, intravascular catheter infections, urinary tract infections, biliary tract infection, cutaneous infection, ventilator associated pneumonia (Ensminger et al. 2006), myositis (Pegoraro et al. 2008), diarrheal disease (Cheong et al. 2008), bacteremia in dental unit water lines and tap water (Kanamori et al. 2016), bacteremia in hospital water system (Perola et al. 2002) etc. Also, bacteremia, endophthalmitis, osteomyelitis and arthritis were correlated to

contamination with *Sphingomonas* in hospital drinking water distribution system in Hungary (Felföldi et al. 2010).

2.9.3 *Sphingomonas* biofilm in municipal water and its inhibition

Of the better known strains of *Sphingomonas*, *Sphingomonas paucimobilis* is a frequently occurring microorganism with an ability to form a biofilm causing metal corrosion of plumbing systems and biofouling of water distribution systems. The biofilm-forming property of *S. paucimobilis* possibly explains its capability to survive in chlorinated water and other biocides. Hence, the water systems can be susceptible to contamination by these bacteria (Gusman et al. 2012). To prevent all the detrimental effects of biofilm produced by *Sphingomonas*, it is indispensable to employ novel strategies for inhibition of biofilm. As biofilm formation is governed by quorum sensing, a long term solution to this problem is by application of agents that interfere and block quorum sensing signals responsible in biofilm formation. Biopolymers with several novel functions have gained importance as surface applications in recent times. Their role in interference with quorum sensing signals such as N-acylhomoserine lactones (AHLs) in gram negative bacteria have not been reported, and hence, will be presumably important for circumventing biofilms in water distribution systems.

CHAPTER 3

MATERIALS

&

METHODS

MATERIALS & METHODS

3.1 Chemicals and media

All the chemicals and reagents used were of highest analytical grade and procured from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified. N-acyl homoserine lactones were procured from Cayman Chemicals (Michigan, USA). Standard media components were purchased from Fischer Scientific (USA) or Sigma Aldrich (St. Louis, MO, USA) and Hi-Media (Mumbai, India). Chemicals of molecular biology grade were procured from New England Bio Labs, UK. The following media were used for the study: Luria- Bertani (LB) agar, FIB (Flocculant isolation broth) media, Nutrient Agar, Brain Heart Infusion (BHI) agar and *Sphingomonas*-selective growth (SSG) medium (Appendix I). Media solutions were sterilized by autoclaving at 121°C and 15 psi for 20 minutes and were allowed to cool below 50°C prior to use.

3.2 Bacterial strains used in this study

The culture conditions and the characteristics of bacterial strains used in the present study are given in Table 3.1. All the cultures were checked to purity by gram staining.

Table 3.1: Bacterial strains employed in the study

Strain	Relevant Characteristics	Procured from	Growth Conditions	Reference
<i>Sphingomonas terrae</i>	Collection Acc. No 7766	Microbial Type Culture Collection and Gene Bank (MTCC)	Nutrient Agar at 30°C	Gulati et al. 2017
<i>Escherichia coli</i> O157:H7 ATCC 32150	Water borne pathogen	Dr. Moushumi Ghosh, Thapar University, Patiala, India	Brain Heart Infusion (BHI) agar at 37°C	Khaira et al. 2014

<i>Shigella flexneri</i> 2a	Water borne pathogen	Dr. Moushumi Ghosh, Thapar University, Patiala, India	Brain Heart Infusion (BHI) agar at 37°C	Khaira et al. 2014
<i>Salmonella typhimurium</i> ATCC 25315	Water borne pathogen	Dr. Moushumi Ghosh, Thapar University, Patiala, India	Brain Heart Infusion (BHI) agar at 37°C	Khaira et al. 2014
<i>Chromobacterium violaceum</i> 026	Quorum sensing biosensor strain Mini Tn5 mutant of CV31532	Dr. Prince Sharma, Department of Microbiology Dr. Neena Capalash, Department of Biotechnology, Panjab University, India	LB at 30°C; Kanamycin 30 µg/ml	McClellan et al. 1997
<i>Agrobacterium tumefaciens</i> A136	Quorum sensing biosensor strain A136 pCF372 with traI::lacZ and pCF218 with traR	Dr. Prince Sharma, Department of Microbiology Dr. Neena Capalash, Department of Biotechnology, Panjab University, India	LB medium with 50 µg/ml Spectinomycin and 4.5 µg/ml tetracycline at 28°C	Fuqua et al. 1996
<i>Chromobacterium violaceum</i> ATCC 12472	AHL over-producer	Microbial Type Culture Collection and Gene Bank (MTCC)	Nutrient broth at 37°C.	Chen et al. 2013
<i>Klebsiella terrigena</i>	Accession number EU082029	Dr. Moushumi Ghosh, Thapar University, Patiala, India	LB at 37°C	Ghosh et al. 2009

3.3 Isolation and screening of biofilm producing strains

3.3.1 Collection of water samples

For the isolation of biofilm producing strain, sample collection was carried out from twenty different municipal sites. Water samples were collected in wide-mouthed presterile, screw capped bottles, kept in an ice box, and transported to laboratory for analysis (Table 3.2). The samples were stored at 4°C till isolation which was usually done within 8 hours of collection.

Table 3.2: Number of bacterial isolates from different sites

S. No.	Sampling sites	Water source	Number of isolates
1.	Thapar University, Patiala	Tap	8
2.	Ablowal Village in Patiala	Storage Tank	5
3.	Sirhind Canal	Canal Surface	7
4.	Leela Bhavan, Patiala	Municipal pipeline	6
5.	New Jawahar Nagar, Jalandhar	Filter Tap	8
6.	Urban Estate, Patiala	Tap	9
7.	Patiala Railway station	Tap	8
8.	Bhakhra Canal, Patiala	Surface	7
9.	Model Town, Patiala	Reservoir	7
10.	Adaalat Bazar, Patiala	Tap	8
11.	Yadwinder Enclave, Patiala	Tap	9
12.	Sirhind Bus Stand	Municipal pipelines	9
13.	Passi Road , Patiala	Storage	9
14.	Drain, Rajpura road, Patiala	Surface	10
15.	Patiala Gate, Nabha	Municipal pipelines	6
16.	Dhuri, Nabha	Tube well	8
17.	Thapar University, Patiala	Overhead Tanks	7
18.	R.C.F Colony, Jalandhar	RO	6
19.	Adarsh Colony, Patiala	Tap	6
20.	Mai Hiran Gate, Jalandhar	Reservoir	7

3.3.2 Isolation and Screening on *Sphingomonas*-selective medium

For isolating *Sphingomonas* sp. from collected water samples, *Sphingomonas* selective growth (SSG) medium (Appendix I) was used. It is comprised of L9 minimal medium supplemented with 1% glucose and 1% sodium glutamate as carbon and nitrogen sources (Yim et al. 2010). The media was sterilized by autoclaving at 121°C for 20 minutes, allowed to cool below 50°C prior to use and glucose and sodium glutamate were filter sterilized. Optimal concentration for Streptomycin and Piperacillin was determined by performing antibiotic susceptibility test from 50 to 400 µgml⁻¹ on standard *Sphingomonas* strain (*S. terrae* 7766) by replica plating on SSG agar plates containing the above mentioned concentrations of antibiotics and selected the optimal concentrations.

One ml of sample was withdrawn and mixed with 9 ml of 0.85% NaCl and labeled as 10⁰. Similarly, 10-fold dilutions were prepared up to 10⁻⁷. Hundred microliters from each dilution was spread evenly on SSG agar plates containing 100 and 50- µg/ml Streptomycin and Piperacillin, respectively. The plates were incubated at 30°C for 24-48 hours. Maximum biofilm producing strains were screened by crystal violet assay as described by Merritt et al (2005).

3.3.3 Crystal Violet Assay

For crystal violet assay, aliquots (300 µl) of cell suspension were dispensed into wells of a sterile 96-well polystyrene plate (Costar, Corning, NY, USA). 300 µl of media (without inoculums) served as a control. The plates were incubated at 30 °C without agitation. After every 4 hours, the suspension was decanted and the wells were washed thrice in 1 × phosphate-buffered saline (PBS) till the period of 72 hours. The plates were allowed to air dry for 15 min. 300 µl of 1% Crystal violet was added to each well and incubated at room temperature for 5 minutes. After incubation, the wells were washed with deionized water extensively and the plates were allowed

to dry. 200µl of 30% (v/v) acetic acid) was added to each well and gently swirled to release the dye from the cells. Aliquots of 100µl were then transferred to fresh micro titer plate and the absorbance measured at 585 nm. This method was followed for evaluating biofilm formation in the entire study unless otherwise stated. Strain forming maximum biofilm was selected for further studies and designated as MG6.

The selected strain was characterized according to Bergey's Manual of Systematic Bacteriology (Holt et al. 1994).

3.4 DNA Isolation and Polymerase chain reaction (PCR) Purification using *Sphingomonas*-specific *spt* primers

Total genomic DNA was isolated from strain MG6 by phenol-chloroform precipitation method given by Sambrook & Russell (2001). The exponential phase cells were washed twice with saline (0.85% NaCl), resuspended in saline EDTA and treated with cell lysis solution (Appendix I) at 37°C for 10 min. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 8,000 g, for 10 min. Upper aqueous layer was separated and equal volume of isopropyl alcohol was added to it. It was further centrifuged at 12,000 g for 30 min followed by washing with 70% ethanol at 10,000 g for 10 min. Then, the pellet was air dried and dissolved in 30 µL of Tris EDTA (TE) buffer. The isolated DNA was resolved on 0.8% agarose gel by electrophoresis.

Two sets of primers were designed targeting Serine palmitoyltransferase gene (*spt*), crucial sphingolipid biosynthesis gene (Yim et al. 2010). The first set of the *Sphingomonas spt* specific primers was Sph-*spt* 295f and Sph-*spt* 713r; second primer set contained Sph-*spt* 694f and Sph-*spt* 983r (Table 3.3). They were targeted for 408 and 289bp DNA fragments, respectively. The reaction mixture is shown in Table 3.4. The PCR was performed at 94 °C for 5 min, 94 °C for 1 min, followed by 30 amplification cycles consisting of 55°C for 1 min, and 72 °C for 1 min. A

final extension was performed at 72 °C for 3 min to complete the amplification (Table 3.5). The PCR products were examined by electrophoresis in 1% agarose gels containing TAE (Tris-acetate-EDTA) (Appendix 1) and ethidium bromide (1 µg/ml).

Table 3.3: Primers used in the study

Primer	Sequence
Sph- <i>spt</i> 295f (forward)	5' -CGATCCCTTCGCGATCGTG-3'
Sph- <i>spt</i> 713r (reverse)	5' -TGGCGGAAGCGGACGATC-3'
Sph- <i>spt</i> 694f (forward)	5' -GAGATCGTCCGCTTCCGC-3'
Sph- <i>spt</i> 983r (reverse)	5' -CCGACCGATTTGGAGAAG-3'
27F	5' -AGAGTTTGATCMTGGCTCAG-3'
1510R	5' -TACGGYTACCTTGTTACGACTT-3'
Tn5-specific forward primer	5' -CATTGACCACACCACCTCT-3'
Tn5-specific reverse primer	5' -CAGCAACAACCATTTCACG-3'

Table 3.4: PCR reaction mixture for *Sphingomonas*-specific amplification

Component	Stock Concentration	Working Concentration
PCR buffer	10X	1X
MgCl ₂	15 mM	2.5 Mm
DNTPs	10 mM each	0.2 mM each
Forward Primer	10 µM	1.0 µM
Reverse Primer	10 µM	1.0 µM
DNA Template	200ng/ µl	100 ng/ µl
Taq DNA polymerase	3U/ µl	1.0 U/µl
Sterile MilliQ Water	----	Final volume upto 25µl

Table 3.5: Amplification conditions for *Sphingomonas*-specific PCR

Steps	Temperature (°C)	Time (minutes)	Number of cycles
Initial denaturation	94	5	
Denaturation	94	1	30
Primer annealing	55	1	
Primer extension	72	1	
Final Extension	72	3	

3.5 16S molecular characterization of MG6

Total genomic DNA from MG6 was isolated as described in section 3.4. DNA was amplified using set of universal primers: 27F and 1510R (Table 3.3). Reaction mixture is shown in Table 3.6. PCR was performed at 94°C for 5 minutes, 94°C for 1 minute, followed by 30 amplification cycles of 54°C for 1minute, 72°C for 1 minute with the final cycle of 73°C for 3 minutes (Table 3.7) on a Bio Rad Thermal cycler (Bio Rad, MyCycler, USA).

Table 3.6: PCR reaction mixture for 16S rDNA PCR reaction

Component	Stock Concentration	Working Concentration
PCR buffer	10X	1X
MgCl ₂	15 mM	1.5 mM
dNTPs	10 mM each	0.2 mM each
Forward Primer	10 µM	0.5 µM
Reverse Primer	10 µM	0.5 µM
DNA Template	200ng/ µl	100 ng/ µl
Taq DNA polymerase	3U/ µl	1.0 U/µl
Sterile MilliQ Water	----	Final volume upto 25µl

Table 3.7: PCR amplification conditions for 16S rDNA PCR reaction

Steps	Temperature (°C)	Time(minutes)	Number of cycles
Initial denaturation	94	5	
Denaturation	94	1	30
Primer annealing	54	1	
Primer extension	72	1	
Final Extension	73	3	

The amplicon so obtained was sequenced by Universal Scientific Pvt. Ltd (New Delhi, India) using an automated DNA Sequencer AB3500xL (Applied Biosystem, USA). The sequence of isolate was searched using NCBI BLAST (National Center for Biotechnology Information - Basic Local Alignment Search Tool) and the related sequences showing similarity were retrieved and aligned using CLUSTALW (Thompson et al. 1997). The resulting multiple alignments were optimized visually and the evolutionary distances were calculated using the Kimura 2-parameter method. Phylogenetic dendrogram was constructed by neighbor-joining method using MEGA 4 software (Tamura et al. 2011). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses. 16S sequence of the isolate was submitted in genbank database as *Sphingomonas paucimobilis* strain MG6 (Accession No KX594380).

3.6 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

For verifying the purity of MG6, Amplified Ribosomal DNA Restriction Analysis (ARDRA) was used. Following amplification as described above, restriction digestion of the PCR amplicon of MG6 was performed. Restriction was executed separately with each enzyme in a. Digest reaction mixture included: 16 µL PCR reaction product, 1.0 µL either *AluI* or *Sau3A* and 2.0 µL Buffer C (Promega). All restriction enzyme concentrations were 5 U/µL (final volume of 25 µl),

and allowed to incubate at 37°C for 1 hour. The restriction patterns were acquired by 1.4% (w/v) agarose gel.

3.7 Optimization of culture conditions for maximum biofilm production by *S. paucimobilis* MG6

3.7.1 Temperature

To determine the effect of incubation temperature on biofilm production, 1ml of log phase culture (O.D. $\approx$ 0.3) was inoculated in 100 ml of SSG media and incubated at temperatures ranging from 25°C to 42°C. Biofilm production was measured using the Crystal violet assay. Experiment was conducted in triplicates.

3.7.2 pH

The effect of pH of culture media on biofilm production was observed in pH range of 5 to 9. The pH of Culture media was adjusted with 0.1 N Sodium hydroxide or 0.1 N Hydrochloric Acid. One milliliter of log phase culture (O.D. $\approx$ 0.3) was inoculated in 100 ml of media and incubated at 30°C. *S. paucimobilis* MG6 was inoculated in micro titer plates was assessed by crystal violet assay as described before. Experiment was conducted in triplicates.

3.7.3 Carbon sources

Different carbon sources such as glucose, sucrose, acetate, succinate, galactose and aspartate were used separately of different concentrations (0.5, 1 and 2%) as a constituent in growth medium to measure their effect on biofilm production of *Sphingomonas* sp. The stock solution of various carbon sources were filter sterilized through 0.22 μ m filter membrane and added to autoclaved media. Crystal violet assay determined the effect of concentrations and the carbon sources on biofilm production.

3.7.4 Nitrogen sources

Different nitrogen sources like inorganic (sodium glutamate, ammonium nitrate, ammonium chloride) and organic (peptone, tryptone and yeast extract) used separately in different concentrations (0.5, 1 and 2%) as a constituent of growth medium. Different solutions of various nitrogen sources were filter sterilized and added in medium. Their effect on biofilm production was analyzed by inoculating the culture medium (100 ml) containing the desired nitrogen sources with 1 ml of log phase bacterial culture (O.D. $\approx$ 0.3) and incubated at 30°C and 120 rpm. Crystal violet assay was used to determine the concentration and nitrogen source for maximum biofilm production.

3.7.5 Statistical optimization of parameters by Response Surface Methodology (RSM)

Central Composite design (CCD) is one of the Response Surface Methodologies (RSM) adopted to optimize the settings and study interactions between significant variables. The central composite designs involve 5 levels of each variable ($-\alpha, -1, 0, +1, +\alpha$). Center points are implied by the points with levels set to coded value zero while combination of experimental variables consists of its lowest (-1) and highest (+1) levels. The total numbers of experimental combinations equals to $2^k + 2k + n$, where k signify the number of independent factors and n is the number of repetitions of the experiments at the central point. A total of 13 experiments were carried out using two independent variables (glucose and sodium glutamate concentration). Experiments were conducted as per CCD for biofilm production by crystal violet assay.

The model was built and experimental data was fitted into the second order polynomial equation (eq 1). Data was analyzed using statistical software Design Expert (Stat-Ease, USA).

$$y = \beta_0 + \beta_1 x_1 + \cdots + \beta_k x_k + \varepsilon$$

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i < j} \beta_{ij} x_i x_j + \varepsilon \longrightarrow \text{Equation 1}$$

Where I is linear coefficient and j is quadratic coefficient; x_i and x_j are dimensionless value of independent variables; k is the number of factors studied; β is the regression coefficient and ε is the random error. The statistical significance of the model was determined by F test and ANOVA and R-Square were used to evaluate the quality of the fit of model; p Values were used as a tool to measure the interactions among the significant variables.

3.8 Growth and biofilm production kinetics of *S. paucimobilis* MG6

3.8.1 Growth Kinetics of *S. paucimobilis* MG6

The growth kinetics of the *S. paucimobilis* MG6 and *S. terrae* MTCC 7766 was studied by growing it in *Sphingomonas* specific media (Appendix I) by inoculating with a 1% inoculum (Optical density, O.D. $\approx$ 0.03), incubated at 30°C with shaking at 150 rpm. Aliquots of 1 ml in triplicates were withdrawn aseptically after every 4 hours, centrifuged at 8000 rpm for 5 min and cells resuspended in 1ml of 0.85% saline. Cell density was estimated by measuring the optical density of cell suspension at 600 nm spectrophotometrically (U-2800, Hitachi, Japan). Specific growth rate was determined from the slope of the natural log of OD₆₀₀ measurements which corresponds to the linear portion of the curve during exponential growth phase.

3.8.2 Determination of biofilm formation

(a) Crystal Violet Assay

Biofilm formation of *S. paucimobilis* MG6 and *S. terrae* 7766 was evaluated using crystal violet assay.

(b) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT Assay is based on the reduction of a tetrazolium salt according to Kairo et al. (1999) with modification of Walencka et al. (2005) for determining cell metabolic activity. Mid –log grown bacterial suspension of 300 μ l (O.D. $\approx$ 0.03) was dispensed in each well of the

polystyrene plates and incubated at 30°C. After removing the bacterial suspension, 150 µl of Phosphate buffered saline (PBS) and 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added at a concentration of 0.3 % and incubated for 2 h at 37°C. After removing MTT solution from the wells; 150 µl of DMSO (Dimethyl sulfoxide) and 25 µl of 0.1 M glycine buffer (pH 10.2) were immediately added to dissolve the formazan crystals created in the wells. It was incubated for 15 min at room temperature. The absorbance was measured at a wavelength of 550 nm using ELISA Microplate Reader (ELx 800, USA).

3.8.3 Biofilm production on commonly used matrices

Various commonly used matrices like glass slides, microtiter plates and stainless steel coupons were placed in sterilized Petri plates containing SSG medium (Appendix I) and 1% inoculum (O.D. $\approx$ 0.03) of *S. paucimobilis* MG6 and *S. terrae* 7766. The coupons were incubated at 30°C and were removed from petri plates after every 12 hours over a span of 72 hours; washed with 1xPBS for removing the unbound cells and left for drying. 1% crystal violet and calcofluor fluorescent brightener (disodium-4, 4'-bis ((4,6-dianilino-1,3,5-triazin-yl)amino) stilbene-2,2'-disulphonate) (Sigma) were used to visually observe biofilm production. An 10× stock solution of calcofluor was prepared at a final concentration of 80 mg/L.

3.8.4 Biofilm production on plumbing materials

Whole plumbing materials like polyvinyl chloride (PVC), Polypropylene (PP), Polyethylene (PE), Aluminium (Al), Rubber (R) and Copper (Cu) (1 × 1 cm²) were taken out using sterile forceps and washed with 50 ml of 1xPBS to remove unadhered cells. 80 mg/l calcofluor was added for staining afterwards and washed with PBS to remove unbound dye. A hand-held UV-lamp Long wave (Blak-Ray Lamp model UVL- 2 1, UV-366 nm); was used to detect the presence of biofilm on each surface (Lehtola et al. 2005).

3.9 Antibiotic susceptibility test (Bauer et al. 1966)

Inoculum for the antimicrobial susceptibility test was prepared by transferring single colony from an agar plate into a tube containing 4 to 5 ml of *Sphingomonas* specific media. The broth culture was incubated at 30°C until it achieved the optical density of 0.03. Antibacterial activity of antibiotics (Polymyxin, Gentamycin, Amikacin, Ampicillin, Ciprofloxacin, Tetracycline) was assayed by agar disc diffusion assay. Ten millilitres of the melted BHI agar medium was seeded with 100 µl broth culture of the test organism in 100 mm sterile petri dishes. When the agar had hardened, uniform sized discs (diameter 6 mm) prepared from Whatmann filter paper no. 1 soaked in sterile solution of different antibiotics were placed on the agar surface. After incubating overnight at the appropriate temperature for each organism tested, diameter of zone of inhibition was determined. All the tests were performed in triplicates.

3.10 BATH (Bacterial adhesion to hydrocarbon) assay and exopolymer production

Cell surface hydrophobicity was estimated by bacterial adherence to hydrocarbons (BATH) assay described by Rosenberg (1984). The supernatant was removed, pellet washed twice and resuspended in 1X PBS (Appendix I) to an optical density (OD) $\approx$ 0.5 at 610 nm. To the cell suspension (2 ml) in test tube, 100 µl of diesel oil was added and vortexed for 3 min. After shaking, crude oil and aqueous phases were allowed to separate for 1 h. Optical density of the aqueous phase was then measured at 610 nm in a spectrophotometer (UV 1800, Shimadzu). From the absorbance (OD) values, percentage of cells attached to crude oil was calculated using the following formula:

$$\% \text{ of bacterial cell adherence} = (1 - (\text{OD}_A / \text{OD}_B)) \times 100$$

Where, OD_A was the absorbance of the cell suspension after addition of oil OD_B is the absorbance of cell suspension in buffer solution.

The exopolymer of *S. paucimobilis* MG6 and *S. terrae* 7766 was isolated and purified by method given by Ghosh et al (2009). Overnight grown strains were inoculated in FIB media, pH 7±0.2 at 30°C (Labcon, 5081U, USA) for 48 hours and was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant fraction was then separated from the cellular debris and two volumes of ethanol were added to 100ml of concentrated supernatant. It was allowed to precipitate at 4°C for 24h. The precipitated polymer was filtered (Whatman GF Filter) and dialyzed using a dialysis tubing (Cellulose, Molecular weight cut-off, MWCO 12000 Da). The exopolymer was re-precipitated by adding 10% cetylpyridinium chloride. The precipitated polymer complex was collected by centrifugation at 10,000 rpm for 20 min at 4°C and re-dissolved in 10% NaCl (Sodium Chloride) solution. Three volumes of ethanol were added to recover the purified biopolymer, dialyzed and lyophilized.

3.11 Determination of aggregation by *S. paucimobilis* MG6

3.11.1 Preparation of samples for aggregation

Both *S. terrae* MTCC 7766 and *S. paucimobilis* MG6 were further explored for their aggregation property. Overnight grown cultures (50 ml) were harvested in their growth phases at 8000 rpm for 5 minutes at 4°C. The supernatant was discarded; 10 ml of 1x Phosphate Buffered Saline (PBS) was added to the pellet and vortexed for 10 seconds. This was centrifuged thrice to wash the pellet. The pellet was resuspended in PBS and OD₆₀₀ was adjusted to 1.0. The adjusted stock was used for aggregation analysis.

3.11.2 Qualitative determination of Aggregation (Cisar et al. 1979)

The degree of auto- and co- aggregation was determined qualitatively by the scoring method of Cisar et al (1979). To determine auto aggregation, both the strains were transferred to different test tubes, while in the case of co-aggregation, the cultures were mixed in pairs with each of the

three pathogen (2 ml each), i.e. *Escherichia coli* O157:H7 ATCC 32150, *Salmonella typhimurium* ATCC 25315 and *Shigella flexneri* 2a. The pure and mixed cultures in pairs were vortexed for 30 sec and incubated at room temperature for 72 hours. The scoring range used is as follows: 0 represents no aggregation and turbid suspension; 1 represents small uniform aggregates in a turbid suspension; 2- easily visible aggregates in a turbid suspension; 3 -clearly visible aggregates which settles leaving a clear supernatant; 4 - large flocs of aggregates that settle instantaneously (Cisar et al. 1979; Simoes et al. 2008).

3.11.3 Quantitative determination of Aggregation

In order to quantitatively determine aggregation, test cultures in triplicates were prepared as described above for auto- and co-aggregation and incubated at room temperature. One hundred microlitre of the aqueous phase was transferred to microtitre plate and absorbance was recorded at 0, 2, 4, 24, 48 and 72 hours. Percentage of aggregation was measured using the following formula:

$$\text{Percentage of Aggregation} = [(A_{600} \text{ initial} - A_{600} \text{ after incubation}) / A_{600} \text{ initial}] * 100$$

3.11.4 Aggregation studies by Fluorescence Microscopy

Cell aggregates were fixed in 10 ml of a 1% paraformaldehyde solution in 1X PBS for 2 hours at room temperature. The aggregates were mounted on a sterile glass slide and allowed to dry. Stained the slides with DAPI (4',6'-Diamidino-2-phenylindole) and observed under epifluorescence microscope (Zeiss Axio Scope A1 Microscope, USA).

3.11.5 Screening for lectin-polysaccharide like interaction in aggregation

Assessment of Lectin-polysaccharide interactions affecting coaggregation can be performed by series of pre-treatments like addition of simple sugars or protease pre-treatment (Cisar et al. 1979; Buswell et al. 1997).

(a) Heat Treatment

To study the effect of heat treatment on the aggregation ability of *Sphingomonas* and the waterborne pathogens, both treated and untreated auto- and co-aggregating bacterial pairs were used for visual and spectrophotometric analysis. The bacterial cultures were incubated at 85°C for 30 minutes for examining co-aggregation reversal by heat treatment (Kolenbrander 1988).

(b) Protease Treatment

The method described by Rickard et al. (2003) and Simoes et al. (2008) was used to understand the protease sensitivity of the aggregation. The protease type XVI from *Streptomyces griseus* (Himedia, India) was added at the final concentration of 2 mg/ml and incubated at 30°C for 2 hours. Following incubation, cells were harvested, washed twice and O.D. was adjusted to 1.0 at 600 nm. The untreated and treated strains were mixed in equal ratios. The experiment was carried out in triplicates (Ramalinganam et al. 2013).

(c) Sugar Treatment

The ability of sugars to reverse aggregation was observed by adding D (+)-lactose and D (+)-galactose following the method given by Simoes et al (2008). The cultures were harvested and the O.D. was adjusted to 1.0 at 600 nm. Different sugars were filter sterilized and added individually to coaggregation pairs of *Sphingomonas* with each of the waterborne pathogens to final concentrations of 50 mM. Mixtures were vortexed and tested for coaggregation using the method of Rickard et al. (2003). The experiment was carried out in triplicates.

3.12 Monitoring of biofilm by capacitance based technique

3.12.1 Capacitance measurement of biofilm

The capacitance of biofilm and EPS was measured using parallel plate capacitors made of aluminium sheet. The capacitors were UV and flame sterilized prior to the experiments. These sterilized capacitors were then placed in glass petriplates with media in which 1% inoculum of overnight grown standardized suspension of bacterial strains was added. After periodic interval of 12 hours the biofilm coated coupons were taken out and capacitance was directly measured on (LCR) meter (Gwinstek, model no 821, Taiwan) [(inductance (L), capacitance (C), resistance (R))] over the span of 72 hours.

3.12.2 Dry weight of biofilm samples

Aluminum coupons were used to study dry weight of biofilm. Weights of coupons were recorded in terms of milligram (mg) prior to the experiment. The coupons were then sterilized and aseptically inoculated in media. The coupons were removed using sterilized forceps at regular interval of 12 h till 120 h and dried in an oven preset at 100 °C. At the regular interval of 30 minutes, coupons were removed from the oven, cooled and weighed, till a constant weight was achieved.

3.12.3 Capacitance measurement in Reverse Osmosis (RO) membranes (Al Ashhab et al. 2017)

S. paucimobilis MG6 biofilms were developed under cross flow conditions on flat sheet reverse osmosis membranes [(21 x 13.2) cm, 83% porosity] excised from commercial spiral wound membrane element. The membranes were directly placed between the sterilized capacitors after fixed time intervals. Capacitance was directly measured using LCR meter [(inductance (L), capacitance (C), resistance (R))] (Gwinstek, model no 821, Taiwan).

3.13 Determination of chlorine resistance of *S. paucimobilis* MG6 biofilm

3.13.1 Determination of Bacterial Chlorine Resistance

S. paucimobilis MG6 and *S. terrae* 7766 were incubated at 30 °C at 120 rpm. Overnight grown cultures were harvested by centrifugation at 8000 rpm for 5 minutes and washed twice in sterile 1x Phosphate buffered saline (pH 7.2). The cultures were resuspended in 1x PBS to an optical density of 1.05 (at 580 nm); 10µl and 20µl of this suspension were added to each of the tubes containing 10ml of the PBS. The final cell concentration in each tube was approximately 4.0×10^6 per ml and 8.0×10^6 per ml respectively. One tube served as an untreated control with no chlorine but instead equal volume of sample and sterile chlorine free distilled water was added. The applied sodium hypochlorite concentrations in the tubes varied from 2 to 8 mg/l. After time (t) = 0, 30, 60, 90 and 120 minutes at 30°C, 50 µl of the sample was plated and were incubated at 30°C for 48 hours, and colonies were enumerated (Ridgway & Olson 1982).

3.13.2 Biofilm formation on different plumbing materials under static conditions using calcofluor

(a) Effect of chlorine treatment on biofilm formation ability of *Sphingomonas*

Effect of chlorine treatment was observed by inoculating standardized suspension of *S. paucimobilis* MG6 in two setups: one containing rich medium (Luria broth) and other containing filter sterilized water from municipal distribution system. Sterilized PVC coupons were added. After every 12 hours, three coupons from each setup were taken out and incubated in 8 mg/l sodium hypochlorite solution for 1, 3 and 5 hour, respectively. After staining with calcofluor, it was visualized under UV lamp (Blak-Ray Lamp model UVL- 2 1, UV-366 nm). Duplicate of each coupon was placed in fresh medium/water and visualized by calcofluor staining after 96 hours.

(b) Effect of chlorine treatment on combination of *Sphingomonas* with waterborne pathogens

Effect of chlorine resistance was observed by inoculating the standardized suspension of *Sphingomonas* in combination of waterborne pathogens *Escherichia coli* O157:H7 ATCC 32150, *Shigella flexneri* 2a and *Salmonella typhimurium* ATCC 25315 respectively, in both medium (luria broth) and filter sterilized water. Sterilized PVC coupons were added in both setups and the experiment was performed as described above.

3.13.3 Flow through simulation of biofilm formation in plumbing materials

A laboratory-scale simulated distribution system was fabricated comprising of 6 different sets of identical drinking water distribution pipes normally used. The pipe materials chosen were polyvinyl chloride (PVC), Polypropylene (PP), polyethylene (PE), Aluminium (Al), copper (C) and rubber (R). The length of distribution pipe was 1 m; with an internal diameter of 3 cm. Tap water was pumped from the contact tank (30 liters of tap water) with a flow rate of 1.0 L/min into the simulated drinking water distribution pipe using a peristaltic pump. Overnight grown *S. paucimobilis* MG6 culture was used as inoculum, the concentration of viable cells was (10^5 CFU/ml) of pumped water in the contact tank.

3.13.4 Scanning Electron Microscopy (SEM) examination

Biofilm of *S. paucimobilis* MG6 developed on different pipe materials after 4 days was examined by scanning electron microscopy (SEM) following method by Asahi et al (2015). All biofilm samples for SEM analysis were fixed using 2.5% glutaraldehyde solution overnight at 4°C. Washing was performed with 1x Phosphate Buffer Saline (PBS) to remove excess of the glutaraldehyde. Dehydration of biofilm samples was done by washing with 30% ethanol followed by 50%, 60%, 70%, 80%, 90%, 95% to 100% ethanol respectively. The samples were air dried and 2 drops of hexamethyldisilazane were added and samples covered for 5 minutes.

After 5 minutes samples were again air dried at 37⁰C. Biofilm samples were then coated with gold and analysed by SEM (JSM541-V, JOEL, Japan).

3.13.5 Energy Dispersive X-ray Spectroscopy (EDX)

Scanning electron microscope (SEM) was equipped with Energy dispersive X-ray Spectroscopy analyzer. It provided the elemental analysis of biofilm developed on different pipe materials.

3.13.6 Mixed Biofilm profile by *S. paucimobilis* MG6

The mixed biofilm of *S. paucimobilis* MG6 and *S. terrae* 7766 with water borne pathogens was formed by dispensing their cell suspension into 96-well polystyrene plates and quantifying biofilm production using crystal violet assay as described before. The variation from control *S. paucimobilis* MG6 was further confirmed by SEM.

3.14 N-Acyl Homoserine Lactone (AHL) Detection

3.14.1 AHL Extraction

The OD₆₀₀ of overnight grown *S. paucimobilis* MG6 was adjusted to 1.80. The culture was centrifuged at 10,000 rpm for 15 minutes and cell pellet was discarded. The supernatant was filtered through 0.2 µm membrane filter to remove cell debris. The filtrate was mixed with acidified ethyl acetate (0.01% Glacial Acetic Acid) and extracted for 15 minutes. Mixture was set aside for 10 minutes in a separating funnel to get upper organic and lower aqueous layer. Aqueous layer was extracted twice; the entire organic layer was pooled and dried by Rota-vapour instrument under vacuum at 40-50°C. The dried residues were dissolved in 1 ml of 20% acetonitrile (Yin et al. 2012).

3.14.2 Disc diffusion assay for quorum sensing inhibitory activity

Two biosensor strains were used detect the presence of N-Acyl Homoserine Lactone (AHL) in *S. paucimobilis* MG6. The biosensor strains used has been used extensively:

C. violaceum 026 is the mini Tn5 double mutant defective in the synthesis of violacein pigment, but retains the ability to respond to C4-C6 AHLs (McLean et al. 1997). Five milliliter of molten semi-solid Luria agar (0.3% w/v) was inoculated with 50 µl of an overnight grown culture of *C. violaceum* 026 and poured immediately over the surface of LB agar plates. When the overlaid agar solidified, wells were punched in the agar and filled with the cell-free supernatant and adjusted to a final solution of 50 µl by addition of sterile LB. Petri dishes were incubated in upright position overnight (28°C), then examined the same for induction or inhibition of violacein synthesis (McLean et al. 1997).

For *A. tumefaciens* bioassay, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal (20 mg/ml) was added to the molten Luria agar before pouring the media into sterile petri plates. After solidification, 0.5% LA containing overnight grown A136 was poured onto the surface of agar. The wells were punched with the help of sterile cork borer and samples were added into the wells. The plates were incubated overnight at 30°C.

3.14.3 Thin Layer Chromatography (TLC)-overlay assay with biosensor

50 µl of the AHL extract was applied on a Silica Gel G F₂₅₄ TLC plate (Merck, Darmstadt, Germany) and developed with a mixture of chloroform: methanol (95:5). After the migration of solvent front to within 2 cm from the edge, the plate was air dried and then overlaid with a thin film of 0.5% LA containing the AHL biosensor (*A. tumefaciens* A136).

3.14.4 High Performance Liquid Chromatography

AHL extract resuspended in 20% HPLC-grade acetonitrile was separated by reverse-phase C₁₈ HPLC, using a column (250×4.6 mm; particle size 5 µm, Shimadzu, USA) at a flow rate of 0.5 ml min⁻¹ and UV detector set at 210 nm. 20 ml of the sample was injected onto the HPLC column. The mobile phase comprised of a linear gradient (20-95%) of solvent A (acetonitrile)

and solvent B (0.1% formic acid). The column was equilibrated for 5 min, and a blank run was performed between each analysis (Gould et al. 2006). AHLs were identified based on their retention times.

3.14.5 Gas Chromatography-Mass Spectrometry

Extract of AHL isolated from *S. paucimobilis* MG6 was analyzed by gas chromatography-mass spectrometry (GC-MS) (PerkinElmer Clarus 680 GC, India) according to Burton et al (2005) . Samples were injected into ELITE-5MS capillary column (30.0 m, 0.25 mm, 250 μ m df). Operation parameters are as follows: Helium as carrier gas; injector temperature: 260°C; 1 ml/min flow rate; oven temperature was programmed as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C /min and 300 °C, where it was held for 6 min. MS detector conditions were: transfer line temperature 240 °C; ion source temperature >240 °C; and ionization mode impact at 70 eV.

3.15 Transposon mutagenesis for development of AHL negative mutants

3.15.1 Generation of spontaneous mutants

For obtaining an antibiotic selection marker for *S. paucimobilis* MG6, plating of log phase culture of MG6 was carried on kanamycin and nalidixic acid (25 to 100 μ g/mL) containing LA plates to generate spontaneous mutants. The survivor colonies which appeared after overnight incubation were further grown on antibiotic-containing LA plates for ten subsequent generations to confirm resistance against the same. This colony was referred as spontaneous mutant (Wang et al., 2001).

3.15.2 Selection of AHL Negative mutant

Transposon mutagenesis was carried out by conjugation between *Escherichia coli* JB110 harboring Tn5-containing pG59 (Suicidal vector) and recipient spontaneous mutant *S. paucimobilis* MG6 (Ghosh et al. 2006). The antibiotic resistant strains were selected using

kanamycin (50µg/ml) for donor and nalidixic acid (25 µg /ml) and kanamycin (25µg/ml) for recipient. Mid log phase cultures were harvested, washed twice with saline and mixed in different ratios (1:1, 1:2, 2:1 and 1:3). The mixed cells were placed on filter paper discs and incubated at 30°C for 24 hours. The discs were transferred to 50 ml LB containing nalidixic acid (25 µg/ml) and kanamycin (25µg/ml). The cells were incubated in selection medium for 8 h at 30°C. 0.1 ml was withdrawn from antibiotic containing LB and spread on LA plate. AHL-negative mutants were selected from library of 534 mutants on the basis of inability of inducing blue color by using indicator organism *A.tumefacies* A136. Subsequently, the mutant bank was replica plated onto indicative LA plates containing approximately 1% (v/v) overgrown culture of the monitor bacterium *A. tumefaciens* A136. Putative AHL-lacking clones were not capable to induce blue color production after 12 h of incubation at 28°C.

3.15.3 Confirmation of Tn5 insertion

Total genomic DNA was extracted from *S. paucimobilis* MG6 and its mutant strain *S. paucimobilis* MG12 as described in section 3.4. PCR reactions were conducted using Tn5-specific forward primer and reverse primer (Table 3.3). The PCR mixture was prepared as given in table 3.8. The reaction mixture volume was adjusted to 100µl with milliQ water. Amplification conditions followed are given in table 3.9. The amplified Tn5 DNA was resolved on 1.2% agarose gel.

Table 3.8: PCR reaction mixture for Tn5 insertion

Component	Stock Concentration	Working Concentration
PCR buffer	10X	1X
MgCl ₂	15 mM	1.5 mM
dNTPs	10 mM each	150 µM each
Forward Primer	10 µM	10 pmol
Reverse Primer	10 µM	10 pmol
DNA Template	200ng/ µl	100 ng/ µl
Taq DNA polymerase	3U/ µl	2.5 U/µl
Sterile MilliQ Water	----	Final volume upto 100 µl

Table 3.9: PCR amplification conditions for Tn5 insertion

Steps	Temperature (°C)	Time (minutes)	Number of cycles
Initial denaturation	95	5	
Denaturation	94	1	36
Primer annealing	51	1	
Primer extension	72	2	
Final Extension	72	10	

3.15.4 Restoration of AHL in mutant MG12

The extracted AHL from *S. paucimobilis* MG6 was incubated with mutant MG12 on LA plate using *A. tumefaciens* as described before. The plate was observed for color production.

3.16 Extraction and purification of biopolymers from *Klebsiella terrigena*

For extraction and purification of biopolymers, the method described by Ghosh et al (2009) was followed as described in section 3.9. The biopolymer isolated and purified was named as BP. To examine the thermal stability of BP, thermogravimetric analysis (TGA) was carried out. 6.11 mg

of BP was heated from 25 to 900°C under a constant flow of nitrogen gas with an increase of 15°C/ sec. The degradation temperature was observed from the TGA curve.

BP along with chitosan (as control) were used to inhibit biofilm formation at their sub inhibitory concentrations (0.01 mg/ml BP, 0.02 mg/ml BP, 0.03 mg/ml Chitosan and 0.04 mg/ml Chitosan) by using crystal violet.

3.17 Synthesis of quaternized biopolymer and N-methylbiopolymer

3.17.1 Synthesis of quaternized biopolymer by Dimethyl Sulphate (DMS) method (deBritto 2011)

Biopolymer (500mg) was dissolved in dimethyl sulfate (8ml) and deionized water (2ml). The resulting solution was filtered to remove the impurities. Sodium hydroxide (600mg) and sodium chloride (440mg) were added to the solution, followed by stirring at ambient temperature for 6h. The product was precipitated by acetone, filtered and vacuum dried. White precipitates obtained were re-dissolved in deionized water (20ml) and subjected to dialysis using a dialysis tubing (Cellulose, MWCO 12000 Da) overnight. Dialyzed solutions were then lyophilized and the resultant derivative of biopolymer was named as Quaternized biopolymer (QB) (yield 70%; 300mg).

To determine the degree of quaternization of quaternized biopolymers, the quaternized biopolymer was titrated with 1% silver nitrate solution. 5.0 ml of 1% (w/v) quaternized biopolymeric solution and 4.0 ml of 1% (w/v) potassium chromate (K_2CrO_4) solution were added to an Erlenmeyer flask. The silver nitrate solution was added to the quaternized biopolymeric solution drop by drop with swirling the flask until the entire solution in the flask turned a very light orange-red color which indicates the endpoint had been reached. The total volume of the titrant (silver nitrate solution) was recorded and the corresponding amount of chloride on the

quaternized biopolymer was determined. Each sample was titrated three times to obtain an average DQ value.

3.17.2 Synthesis of N-methylbiopolymer (Belalia et al. 2008)

Biopolymer (500 mg; 10 mmole) was mixed with 1% (v/v) acetic acid (50 ml) and formaldehyde (0.5 ml; 0.22 mmole) was added. The suspension was stirred at room temperature for 30 min and sodium borohydride (41.25 mg; 1.12 mmole) was added. After stirring, the pH of the suspension was adjusted to 10 by NaOH (1 M). White precipitates appeared were filtered and washed to reach the pH of 7. Trapped moisture was removed by refluxing it with dry benzene through azeotropic distillation. Evaporation of benzene resulted in N-methylbiopolymer (NMB) (yield 86%; 430 mg).

3.17.3 NMR Analysis

¹H-Nuclear magnetic resonance (NMR) spectra of the QB was obtained with an NMR spectrometer (Bruker Avance II (400 MHz), Switzerland) by dissolving samples of the polymer in deionized water at 80°C with suppression of the water peak.

3.18 Determination of quorum sensing inhibitory activity of QB and NMB

3.18.1 Determination of subinhibitory concentrations of compounds

A paper disc diffusion assay to determine the subinhibitory concentrations of 4-Nitropyridine-N-Oxide, and quaternized biopolymer (Khaira et al. 2014) was carried out. *S. paucimobilis* MG6 was incubated for 16-18 hours and the O.D₆₀₀ was adjusted to 0.1. 100µl of culture was spread on plates and allowed to dry for 30 minutes. Paper discs (10 mm diameter) were placed on agar plates and 20µl of all compounds were added individually on the discs (0.05-1mg/ml). The plates were incubated at 30°C for 24 hours and observed for zone of inhibition. This assay was repeated in three independent triplicates.

3.18.2 Anti-quorum sensing activity of anti biofilm agents

For assessing quorum sensing inhibition by *A. tumefaciens* bioassay, X-gal (20 mg/ml) was added to the Luria agar before pouring the media into sterile petri plates. After solidification, *A. tumefaciens* A136 lawn was prepared by adding 8 mL of overnight grown culture adjusted to O.D of 1.2 (OD₆₀₀) into 40 mL of warm molten 0.5% LB agar. The wells were punched with the help of sterile cork borer and added the samples into the wells. The compounds with final concentrations were loaded into each well. The plate was incubated for 18–24 hours at 30°C to check for inhibition of blue coloration. This assay was performed in three independent triplicates.

3.18.3 Beta -galactosidase assay (Miller 1972)

An overnight grown culture of *A. tumefaciens* A136 was diluted to 0.2 at 660nm. Each tube contained 2ml of A136 bacteria cell suspension and 0.5 ml of test supernatant. The mixture was incubated at 28°C for 2 hours and activity was measured. 500 µl of this mixture was added to 500 µl of Z- buffer. Abs_{650nm} was measured. 50 µl of Sodium dodecyl sulfate (SDS) and 100 µl of chloroform were added to the above. The mixture was incubated at 28°C for 2 minutes. 0.2 ml of ortho-Nitrophenyl-β-galactoside (ONPG) (4 mg/mL in Z buffer) was added and incubated for 30 minutes till yellow color develops. 0.5 ml of 1M sodium carbonate and measured absorbance at 420 and 550 nm. A unit of β-galactosidase activity was determined as micromoles of o-nitrophenol (ONP) produced/min mg cell dry weight (Holt & Ricciardi 2001). The above protocol was repeated with selected concentrations of 4-NPO, NMB and QB.

3.18.4 Biofilm Inhibition

Twenty microlitres of standardized inoculum of *S. paucimobilis* MG6 was added along with varying concentrations of 4-NPO, and QB and made the final concentration upto 0.7 mg/ml

NPO, 1 mg/ml NPO, 0.05 mg/ml QB, 0.07 mg/ml QB, 0.01 mg/ml NMB and 0.03 mg/ml NMB in each well of the polystyrene plates and incubated at 37°C. The plates were taken out after every 4 hours till 72 hours and biofilm formed was measured by crystal violet assay. The percentage of biofilm inhibition was calculated by:

$$\text{Percentage of Biofilm inhibition} = \frac{[\text{Control O.D 585 nm}-\text{Test O.D 585 nm}]}{\text{Control O.D 585 nm}} * 100$$

3.18.5 Motility Assays

Assays for swimming, swarming and twitching motilities were done on luria agar plates. Overnight grown culture of *S. paucimobilis* MG6 was used as inoculum for this study.

For swimming motility, cells were stab-inoculated into the centre of 0.3 % (w/v) luria agar plates using sterile toothpicks. This motility was analyzed qualitatively by examining the circular hazy zone by the bacterial cells migrating away from the point of inoculation within the agar (Murray et al. 2010)

Two microlitres overnight grown *S. paucimobilis* MG6 was point- inoculated onto the swarming motility plates (0.5% LB agar) alone and in combination with different concentrations of 4-NPO, NMB and QB, respectively. The swarming motility was observed based on the movement of growth on the surface of the plate away from the point of inoculation (Kohler et al. 2000).

For twitching motility, overnight grown bacterial cultures were stab-inoculated to underlying petri dish of 1% agar plates. Incubated the plates for 16 h at 37°C with incubation at room temperature for 48 h. Agar was removed after incubation and unattached cells were rinsed off gently by a stream of ultrapure water. The zone of motility was visualized by staining the attached cells with 1% crystal violet (Murray et al. 2010)

3.18.6 Colorimetric method for AHL determination

The procedure described by Yang et al (2006) was used to determine the concentration of AHLs. 40 µl of each sample along with varying concentrations of NPO, NMB and QB were incubated in a 96-well microtitre plate and then mixed with 50 µl of 1:1 ratio of hydroxyl amine (2 M): NaOH (3.5 M). After mixing, the same amount of 1:1 (v/v) ratio of ferric chloride (10% in 4 M HCl): 95% ethanol was added to the above solution. A dark brown colour was produced in the samples containing AHL compounds. The quantities of lactone molecules were determined at 520 nm spectrophotometrically.

3.18.7 Qualitative Modulation of Quorum Sensing (QS) Activity by NMB and QB

To discern whether potential inhibitors target AHL production (via Lux I) or AHL response (via Lux R) (Chenia et al. 2013); two strains were used: an AHL overproducer such as *C. violaceum* 12472 and an AHL biosensor such as *A. tumefaciens* A136. A test compound was placed on a sterile paper disc and the AHL overproducer and biosensor strains were inoculated in proximity to the compound. To test for potential Lux I inhibition, the AHL overproducer was placed in close proximity to the test substance and the biosensor was placed distal. To determine Lux R inhibition, the positions of the AHL overproducer and biosensor strains were reversed. In either case, the potential QSI activity resulted in a lowered signal from the AHL biosensor.

3.18.8 Effect of QB and NMB on time course of *S. paucimobilis* MG6 AHL synthesis

The effect of QB and NMB on AHL synthesis was measured after every 2 hours by β -galactosidase activity as described in section 3.17.3 over the period of 24 hours. The AHL production was measured in Miller units.

3.19 Studies on Physiological, Biochemical and Molecular Responses of microbial inactivation

3.19.1 Preparation of washed cell suspension

Overnight grown *S. paucimobilis* MG6 were centrifuged at 11,000×g for 10 min. Cell pellets were washed in 10mM PBS (pH 7.0) and resuspended with the same buffer and adjusted to $\sim 10^7$ CFU/ml at an absorbance (OD 600nm) of 1.

3.19.2 Electrical conductivity measurement

Cellular leakage of the treated bacterial cells was determined by measuring electrolyte leakage into the incubation medium by conductivity meter (4510 Conductivity meter, Jenway, UK). QB and NMB were added to bacterial cells suspension and centrifuged at 8,000×g for 15min. The supernatant was analyzed for electrical conductance with 10 min interval. All the experiments were performed in triplicates.

3.19.3 Determination of potassium ion leakage

QB and NMB were added to the prepared bacterial cells suspensions. The treated cell suspension was centrifuged at 8,000 rpm for 15min, and the supernatant was analyzed for potassium using an atomic absorption spectrophotometer (AAS 5EA, Analytic Gena, Germany) with an air acetylene flame at wavelength of 324.8 nm. Three replicates per sample were assessed. The intracellular level of the K⁺ ion was expressed in percentage released and plotted against time.

3.19.4 Determination of the leakage of 260-nm-absorbing material

QB and NMB were added to bacterial cells suspensions. The cell suspension was centrifuged at 8,000 × g for 15 min, and the supernatant was then decanted into a plastic UV cuvette (1cm path length). The absorbance was read against a buffer control at 260 nm and graph was plotted against time.

3.19.5 Intracellular Adenosine Triphosphate (ATP) measurements

Cells were harvested and washed as described above and incubated for 10 min at 25°C in 50 mM HEPES (pH 7.0 or 8.0) buffer containing 0.2% (w/v) glucose. Total ATP after treatment with QB was determined with a luminometer (Lumac/3M Biocounter M2010A) as described by Guihard et al. (1993). Aliquots were withdrawn at intervals of 10min for one hour and viable counts determined.

3.19.6 Leakage of glucose, lactate dehydrogenase (LDH) and protein from treated bacterial cells

To analyze the effect of compounds on cellular leakage of the *Sphingomonas*, test organism (1ml) was inoculated into sterile deionized water (10ml) with or without compounds in a culture tube. The mixture was incubated at 37°C with shaking (120rpm) for 12h. At regular interval of 1hour, aliquots were withdrawn to determine protein, glucose and lactate dehydrogenase (LDH) activity. The cell suspension was centrifuged at 8,000×g for 15min, and the supernatant was collected. The glucose content was analyzed by a glucose assay kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). A sample or glucose standard (0.0–3.0mg/dl, 200µl) was added to reagent (1ml) containing glucose dehydrogenase. After incubation at 25°C for 15minutes, the absorbance was recorded at 334 nm. The LDH activity was analyzed by an LDH assay kit (Clontech, USA). A 100µl sample was added to reaction mixture containing 0.5 mL of 100 mM pyruvate, 5 mg Nicotinamide adenine dinucleotide (NADH) in 20 mL of 500 mM potassium phosphate buffer, and pH 7.5 at 30°C. Absorbance was recorded at 340 nm. The protein concentration was determined by measuring absorbance at 280nm.

3.20 Effect of QB and NMB on time dependent growth pattern

To study the effect of QB and NMB on growth kinetics of *S. paucimobilis* MG6 and *S. terrae* 7766, selected concentrations of these compounds were added to respective cultures. Absorbance of strains was measured at 600 nm after every 4 hours.

3.21 Biofilm inhibition studies in real time conditions

3.21.1 Water quality analysis

Collection of water samples: Water samples (from distribution tanks, hand pump, canal, and tap water) were collected from Patiala and nearby regions. For collecting water samples, the sterile containers were first rinsed with source water by holding it at the bottom and plunging it below the water surface. The sample was collected by leaving about about 30mm of empty space to allow mixing during laboratory analysis (Buckalew et al. 2006). The samples were transported to the laboratory on ice and were stored at 4°C until analysis. The water samples were analyzed for parameters such as pH, total dissolved solids, chlorides, dissolved oxygen, total suspended solids, total organic carbon as described in “Standard methods for the examination of water and wastewater American Public Health Association (APHA)” (APHA et al. 1998) (Table 3.2).

(a) Potentiometric measurement of pH

Estimating the pH by dipping the electrode of pH meter into water samples, gently shake it and recorded the value from pH meter (Eutech Instruments, India).

(b) Total Dissolved Solids (TDS)

Dried a clean porcelain dish at 180°C for 1 hour and weighed the dish. Mixed the sample and filtered 50 ml of sample. The filtrates were transferred to evaporating dish, dried for 1 hour, cooled and weighed. The value was calculated in mg/l by following formula::

$$\text{mg Dissolved Solids /l} = \frac{[\text{weight of dried residue + dish (mg)} - \text{weight of dish}]}{\text{sample volume (ml)}} * 1000$$

(c) Total Suspended solids (TSS)

The initial dry weight of filter paper was recorded and 50 ml sample was filtered. The filter paper was placed on evaporating dish, dried, cooled and weighed. The value was calculated in mg/l by the following formula:

$$\text{mg Suspended Solids / l} = \frac{[\text{weight of filter} + \text{dried residue (mg)} - \text{weight of filter (mg)}]}{\text{sample volume (ml)}} * 1000$$

(d) Dissolved Oxygen (DO) measurement

One ml of Manganese sulphate solution was added to 1 ml of alkaline Potassium iodide solution in 300 ml of sample, the sample was mixed well. Allowed the precipitates to settle down and then added 2 ml of conc. sulphuric acid to solution and shaken until the precipitates disappeared. Titrated 203 ml of this sample with 0.025N sodium thiosulphate solution using starch as indicator (End point: blue to colourless). Noted the amount of titrant used. Calculated DO value using the following formula:

$$\text{Dissolved oxygen (DO) (in mg/L)} = \text{mL of sodium thiosulfate (0.025N) consumed}$$

(e) Estimation of chloride concentration

Added one drop of potassium chromate indicator in 100 ml sample. Titrated with 0.028 N silver nitrate (end point: yellow to brick red). Noted the amount of titrant used. Calculated the chloride concentration by:

$$\text{Chloride Ion Concentration (mg/L)} = (A \times N \times 35.45) * 1000 / V_{\text{sample}}$$

where, A = volume of titrant used, N is normality of silver nitrate, and V_{sample} is volume of sample used (ml).

(f) Total Organic Carbon (TOC)

TOC was analyzed by a TOC Analyzer (AJ- Analyzer multi N/C 111 3100; multiwin 4.09, Germany).

3.21.2 Evaluation of biofilm forming efficiency of water samples

The biofilm forming ability of *S. paucimobilis* MG6 in all the water samples was observed by crystal violet assay. The water sample with highest biofilm formed (P7) was selected for further studies.

3.21.3 Biofilm Inhibition by QB and NMB

Biofilm formation by *S. paucimobilis* MG6 was observed by using sterile polystyrene, 96-well round-bottom plate. The culture was grown in different water samples in wells. After every four hours, bacterial suspension was poured off. Crystal violet staining was used to quantify the biofilm produced as described before. Biofilm inhibition was evaluated by using 0.05 mg/ml and 0.07 mg/ml QB & 0.01 mg/ml and 0.03 mg/ml NMB in P7 and SSG media for real time study. In order to investigate the antibiofilm activity of these compounds on already formed biofilm, selected concentrations of these QB and NMB were added to 12 h old biofilm and the effect was checked by crystal violet assay.

Also, to check the inhibition of biofilm formed on plumbing materials in real time conditions, QB and NMB were added to sterile PVC coupons and dried for 1 hour. *S. paucimobilis* MG6 was inoculated on all the coupons in water and SSG medium and biofilm was allowed to form for 72 hours. After every 12 hour, single coupon was stained by calcofluor and intensity of biofilm formation was measured under UV conditions.

To check for leaching of QB and NMB, PVC coupons were pre coated with different concentrations of QB and NMB ranging from 0.1 to 1 mg/ml by dip coating method. The

coupons were allowed to dry for 1 hour and were placed in water sample (P7). This sample was analyzed and monitored for 72 hours for leaching of QB and NMB using calcofluor.

3.22 Degradation of *S. paucimobilis* MG6 AHL by NMB

Inoulation of SSG medium was done with 1% inoculum of *S. paucimobilis* MG6 and incubated with 0.03 mg/ml NMB at 30°C. Supernatant of overnight grown culture was collected and concentrated. AHL was extracted with 0.01% glacial acetic acid as described by Yin et al (2012). The extracted AHL sample was analyzed by GC-MS

CHAPTER 4

RESULTS

RESULTS

4.1 Isolation and Screening of biofilm producing *Sphingomonas* strains

To isolate biofilm forming *Sphingomonas* predominantly from drinking water systems, water samples were collected from twenty distribution networks. A total of 140 isolates were screened on the basis of the morphology of the colonies (yellow pigmented round colonies), thereafter, 60 isolates were further subjected to growth in *Sphingomonas* selective medium containing 100 and 50 µg/ml streptomycin and piperacillin, respectively (Yim et al. 2010). These isolates were assessed for their potential for biofilm production using the crystal violet assay. Out of 60 isolates, 5 (8.33%) were strong biofilm producers, 25 (41.6%) were moderate biofilm formers and 25 (41.6%) were weak or biofilm producers and 5 (8.33%) were non-biofilm producers (Table 4.1). Biofilm production was interpreted as described by according to Stepanovic et al (2007) (Table 4.1). The strains producing strong biofilm were used for further studies and designated as MG1, MG6, MG13, MG20 and MG23.

Table 4.1: Interpretation of biofilm production of *Sphingomonas* isolates. The tentative classification was based on average OD values obtained by Crystal violet assay.

Average OD Value	Biofilm production	Number of Isolates
$OD \leq OD_c$	Negligible	5
$OD_c < OD \leq 2 * OD_c$	Weak	25
$2 * OD_c < OD \leq 4 * OD_c$	Moderate	25
$4 * OD_c < OD$	Strong	5

Optical density Cut –off value (OD_c) = average OD of negative control + 3x standard deviation (SD) of negative control

4.2 Evaluation of biofilm forming ability of isolates

Out of selected five strains, strongest biofilm forming strain was selected by crystal violet (CV) method (Figure 4.1). The CV assay has been described as a reproducible and rapid method for

calculating cell biomass (Beściak & Surmacz-Górska 2011) and was therefore used. The increase in the intensity of crystal violet dye measured at 585 nm signifies the increase in the formation of biofilm. Strain MG6 produced maximal biofilm and rest of the strains follows order:

$$\text{MG6} > \text{MG1} > \text{MG13} > \text{MG20} > \text{MG23}$$

Biofilm formation increased exponentially after 20 hours in strain MG6 with maximum intensity at 48 hours after which a constant rate was attained. The biofilm production peaked between 24 to 48 hours as the cells reached late stationary phase prior to senescence (Figure 4.1). For isolates MG1, MG13, MG20 and MG23, biofilm production peaked at 45-72 hours, the maximal biofilm production was not significantly ($p > 0.05$) different for these isolates.

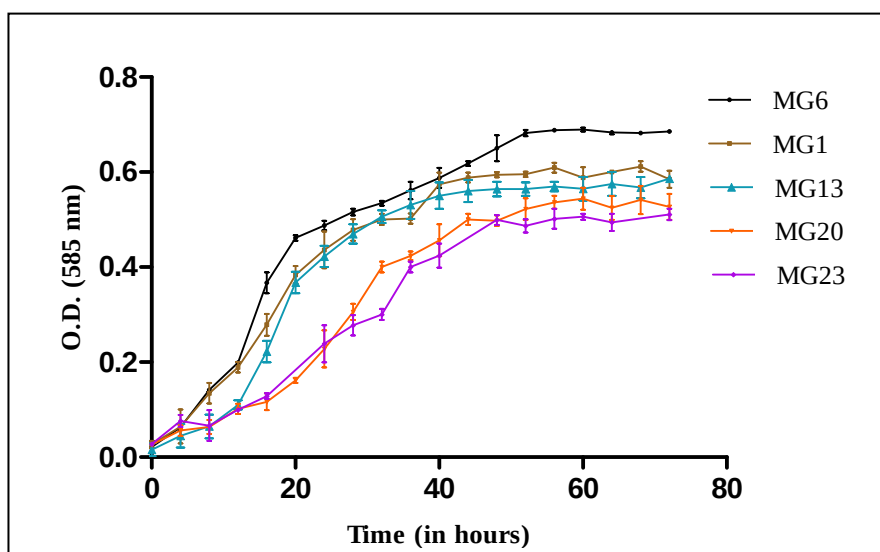


Figure 4.1: Biofilm production kinetics of *Sphingomonas* isolates. The error bars indicates standard deviation from mean of triplicate experiments.

4.3 Morphological and biochemical identification of bacterial strain MG6

The bacterial isolate, MG6, was identified to the genus level by determining the biochemical and morphological properties as described in Bergey's Manual of Systematic Bacteriology (Holt et al. 1994). Morphologically, the strain MG6 formed yellow pigmented circular colonies. The strain

was strictly aerobic, rod shaped, gram negative, motile and non-spore forming. Light microscopic analysis revealed that cells were straight or slightly curved rods with rounded ends. The biochemical analysis of the protein revealed that it is oxidase and catalase positive bacterium, indole negative and can assimilate glucose, L-Arabinose, D-Mannose which places it into the class alphaproteobacteria (Appendix II).

4.4 Molecular characterization of strain MG6

4.4.1 PCR Purification using *Sphingomonas*-specific *spt* primers

Two sets of PCR primers targeting the serine palmitoyltransferase gene (*spt*) were developed which play a crucial role in lipid biosynthesis pathway (Ikushiro et al. 2003). Presence of sphingolipids on the outer membrane of *Sphingomonas* distinguishes it from other bacteria and is currently considered important for molecular identification of *Sphingomonas*. Therefore, these 408 and 289bp DNA fragments for *Sphingomonas spt* gene was targeted. Figure 4.2 depicts the *spt*-PCR products of strain MG6.

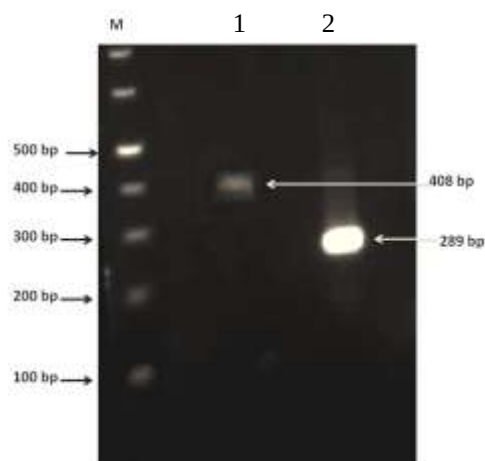
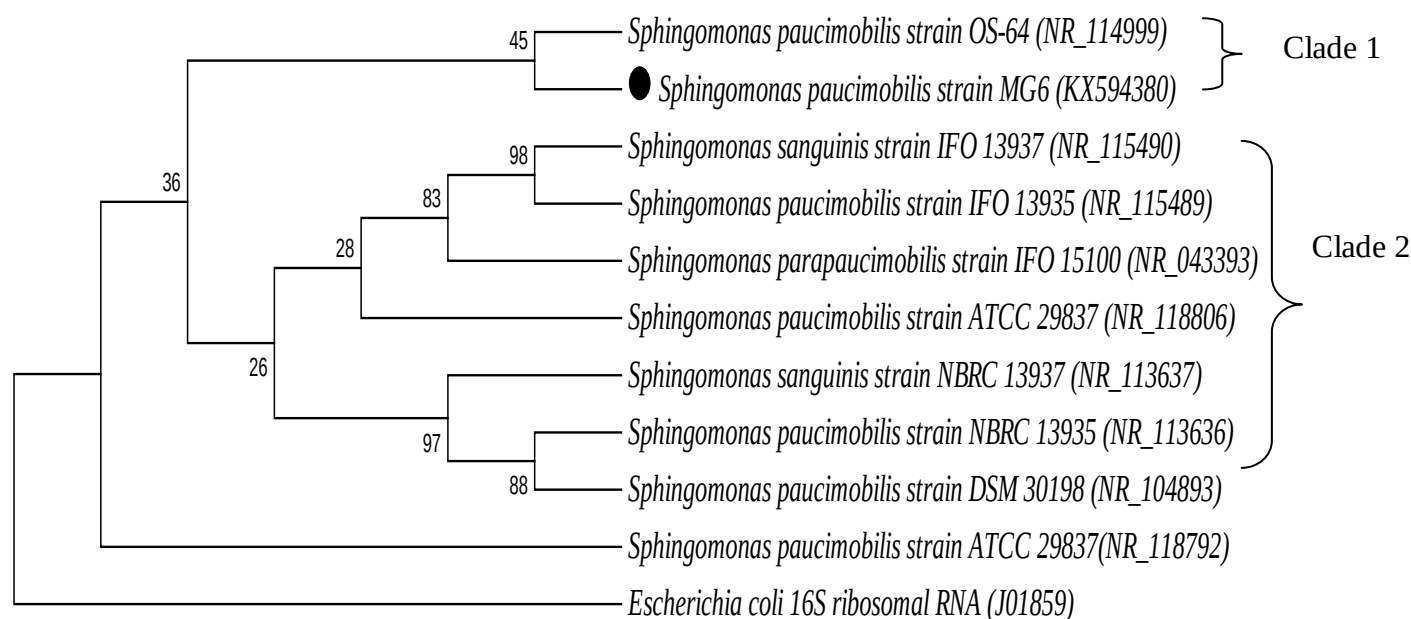


Figure 4.2: Agarose gel electrophoresis of *spt*-PCR amplicons. Lane 1: 408 bp product amplified by Sph-spt295f and Sph-spt713r primer set; Lane 2: 289 bp product amplified by Sph-spt 694f and Sph-spt 983r primer set; M – DNA ladder (100bp)

4.4.2 16S rDNA Sequencing

16S rDNA sequence obtained was compared with sequences in National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST). The sequences with maximum identity score was aligned using the program CLUSTALW (Thompson et al. 1997). Visual optimization of resultant multiple alignments was performed. The phylogenetic tree was constructed with the sequences of representative bacteria by neighbor-joining method using MEGA 5.0 software (Tamura et al. 2011). Gaps are treated as missing data and only unambiguous alignments were utilized in this analysis. MG6 formed evolutionary lineage within the radiation cluster which comprise of *Sphingomonas* spp., the strain type MG6 was phylogenetically closely related to the type strain *Sphingomonas paucimobilis*. The phylogenetic tree based on 16S rDNA sequence was partitioned into two Clades – Clade I and Clade II.



(a)

>KX594380.1 *Sphingomonas paucimobilis* strain MG6 16S ribosomal RNA gene, partial sequence

```
GAAGGCGCTCTGGCTGATGACCTGAGGGCGAAAGCGTGGGGAGCACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGACTATGTGGTTGCTTGTGGATAACGCATTAAGTCCCCTGGGGAGTACGGCCCAAGGTAAACTCAAAGGAA
TTGCGGGGGCCGCACAAGCGGTGGAGCATGTGGTTTAATTCTGAAGCAACGCGAGAACCTTACCAGGTTTGACATTGA
CACCCAGAGATGGTTCTCTTCGGGACAACAGGTGTGCATGGGTGCTCAGCTCGTGTCTGTGAGATGTTGGGTAAGTCC
CGCAACGAGCGCAACCCTGTTAGTTCCATCATTTGTTGGGACTCTAAGGACGCCGGTGAAACCGGAGGAAGGTGGGG
ATGACGTCAATCTCATGCCCTTAGTGGGCTACACACGTGCTACAATGGACTACAGGCGCGACCGCGAGGGAGCTAAT
CCAAAGTTCTCAGTTCGGATTGTCTGCAACTCGCATGAAGGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTG
AATAC
```

(b)

Figure 4.3: (a) Phylogenetic tree of *Sphingomonas paucimobilis* strain MG6 based on bacterial 16S rDNA sequence. A neighbor-joining tree was constructed by aligning the sequences with other selected members. (b) Aligned sequence data of *Sphingomonas paucimobilis* strain MG6.

Isolate MG6 was clustered within clade I with significant bootstrap support values. *Escherichia coli* was used as out-group to root the tree (Figure 4.3); the sequence of MG6 has been deposited in GenBank database (Accession No KX594380).

4.4.3 Genetic fingerprinting of *S. paucimobilis* by ARDRA

The amplified ribosomal DNA restriction analysis (ARDRA), a PCR-based genomic fingerprinting method (Arturo et al., 1995) determined the authenticity of *Sphingomonas paucimobilis* strain MG6. Amplicon of size 1.5 kb of 16S rDNA was digested with tetra nucleotide cutting restriction endonucleases *AluI* and *Sau3A* to observe strain specific restriction fragment pattern. Same pattern was observed when this strain was analyzed for ten generations.

As observed in Figure 4.4, the three bands were generated by *Sau*3A and three after digestion with *Alu*1 confirming the purity of the strain.

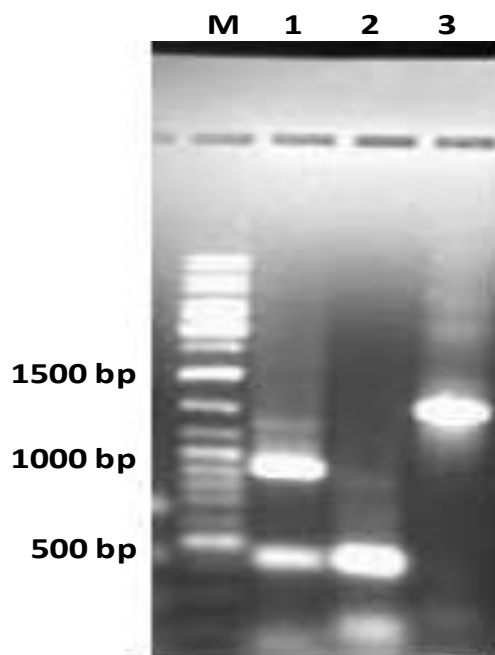


Figure 4.4: ARDRA profile of *S. paucimobilis* MG6 strain; M: Molecular weight DNA ladder (100 bp), Lane 1: Amplicon digested with *Sau*3A, Lane 2: Amplicon digested with *Alu*1, Lane 3: Undigested ~1.5 kb 16S DNA amplified product.

4.5 Optimization of culture conditions of *S. paucimobilis* MG6 for biofilm production

Biofilms create microenvironments for a consortium of bacteria residing on a substrate. Therefore, various physical parameters such as temperature, oxygen, variations in pH and nutritional parameters such as carbon and nitrogen sources are involved in biofilm production by *S. paucimobilis* MG6.

It was deemed important to elucidate the temperature, pH, carbon and nitrogen sources to gain insights leading to optimal production of biofilm by *S. paucimobilis* MG6.

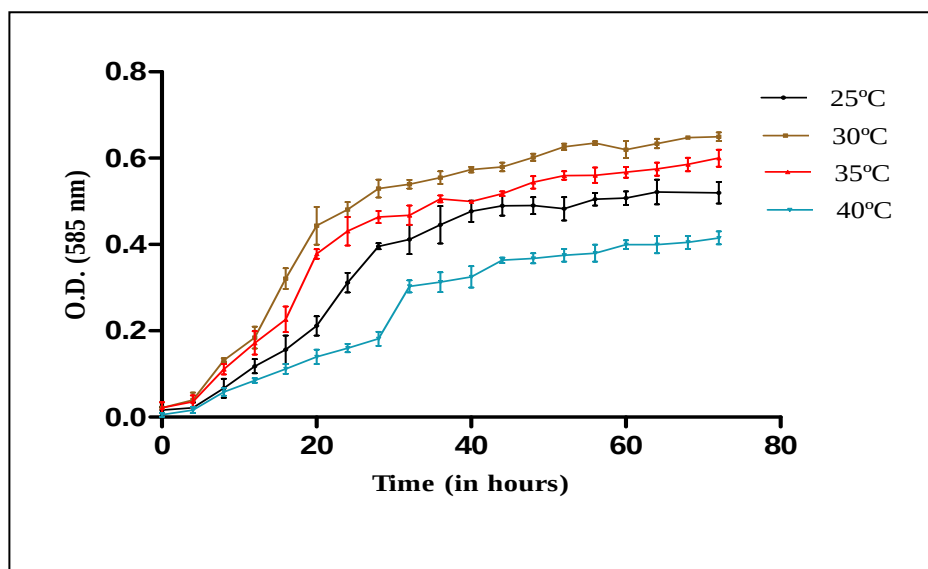
4.5.1 Temperature

Koskinen et al (2000) reported that *Sphingomonas* can produce more exopolysaccharides at temperatures lower than optimal growth temperature of 30-35°C. In the present study, increase in the biofilm mass was observed from 25°C to 30°C, with maximum biofilm production at 30°C (Figure 4.5 (a)) in line with earlier reports. Higher temperatures did not lead to increase in biofilm presumably due to the fact that biofilm production had already achieved the maturation stage at 30°C as suggested by Morimatsu et al (2012).

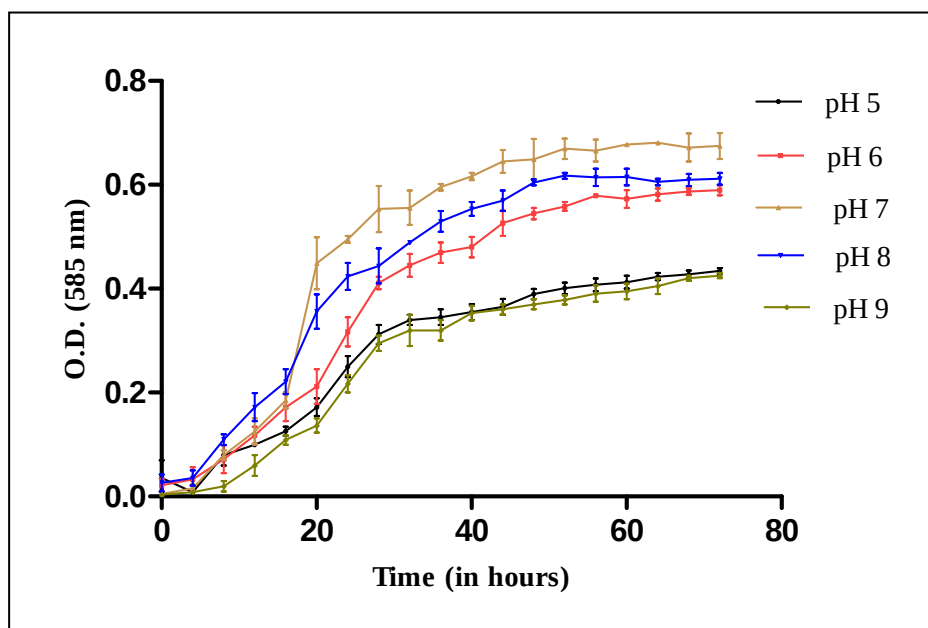
4.5.2 pH

Since the external environment is subject to fluctuations in pH, the interrelation of biofilm formation with this variable was important to determine. An increase in biofilm profile was observed from pH 5 to 7 followed by decrease in biofilm production at higher pH values. The optimal pH for maximum biofilm production was found to be 7.0 (Figure 4.5 (b)).

Biofilm formation in *Salmonella enterica* was found to be pH independent (Giaouris et al. 2005) however, few studies have specifically evaluated the role of pH on *Sphingomonas*, therefore an explanation based on critical comparison cannot be made. The pH value of 7 is most conducive for cellular metabolism, therefore it is possible that optimal metabolism of the cells resulted in high biofilm production. At higher pH values viz 8 and 9, the cell metabolism is affected with notable decline of biofilm formation.



(a)



(b)

Figure 4.5: Biofilm production kinetics of *S. paucimobilis* MG6 with varying (a) Temperature (b) pH. Data is average $\pm$ Standard deviation (SD) of triplicate samples.

4.5.3 Carbon sources

Exopolysaccharide production has been reported as crucial for initial adherence and biofilm formation in *Sphingomonas* (Oliveira et al. 2003). The type and amount of carbon sources therefore, is expected to play an important role in the optimum production of EPS and facilitating biofilm formation. The biofilm production was maximum in glucose followed by sucrose and galactose for *S. paucimobilis* MG6. Lower biofilm production was observed in acetate and aspartate. Though change in concentrations failed to cause significant ($p>0.05$) decline or enhancement of cell, the biofilm production was highest when glucose concentration was 1% and lowest with 0.5%. Therefore, 1% glucose was considered as an optimal concentration of glucose as carbon source (Figure 4.6). In general, other than glucose, a wide variety of other sugars such as arabinose, fucose, galactose, lactose, mannose, melibiose, sucrose, trehalose, and xylose also are frequently assimilated by *Sphingomonas*. Besides, many members can degrade and utilize mono- and disaccharides. For *S. paucimobilis*, in particular, glucose has been reported to be the most preferred carbon source for metabolism and exopolysaccharide secretion. The results thus obtained are in concurrence with as reported by Wang et al (2006).

4.5.4 Nitrogen sources

To date, various complex and simple nitrogenous compounds have been reported to be utilized by *Sphingomonas*. Results showed that the maximum biofilm production was supported by sodium glutamate followed by peptone, tryptone, ammonium nitrate and yeast extract. Sodium glutamate at the concentration of 1% was observed to be the optimal concentration for biofilm formation (Figure 4.7). An optimal C: N ratio is known to favor EPS production a key factor for biofilm formation. It is possible that sodium glutamate at the noted concentration afforded the criteria enabling the cells to produce sufficient EPS for optimal biofilm production.

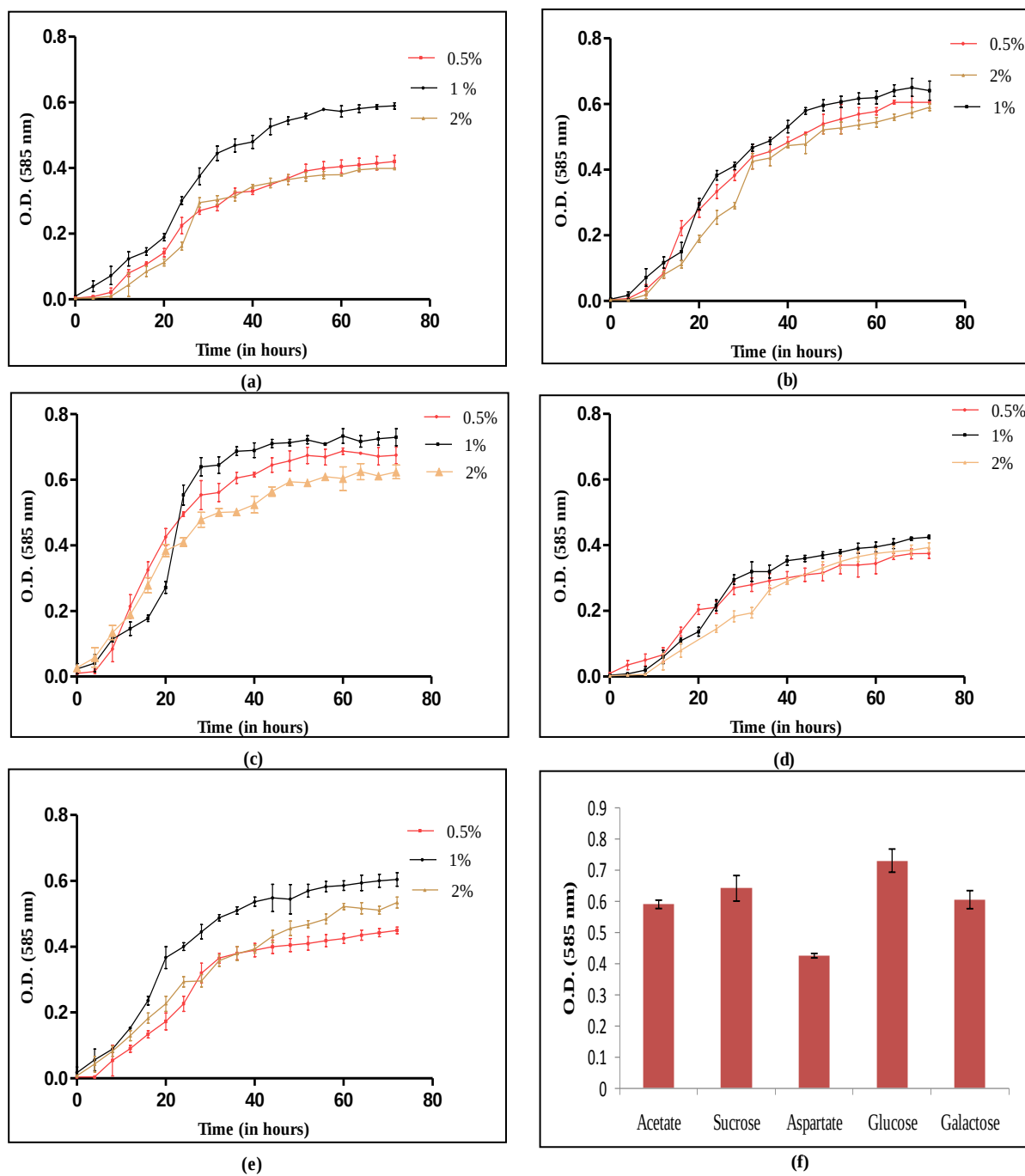


Figure 4.6: Biofilm production kinetics of *S. paucimobilis* MG6 with different carbon sources (a) acetate (b) sucrose (c) glucose (d) aspartate (e) galactose (f) Comparative account of biofilm production using different carbon sources at 72 hours (1%)

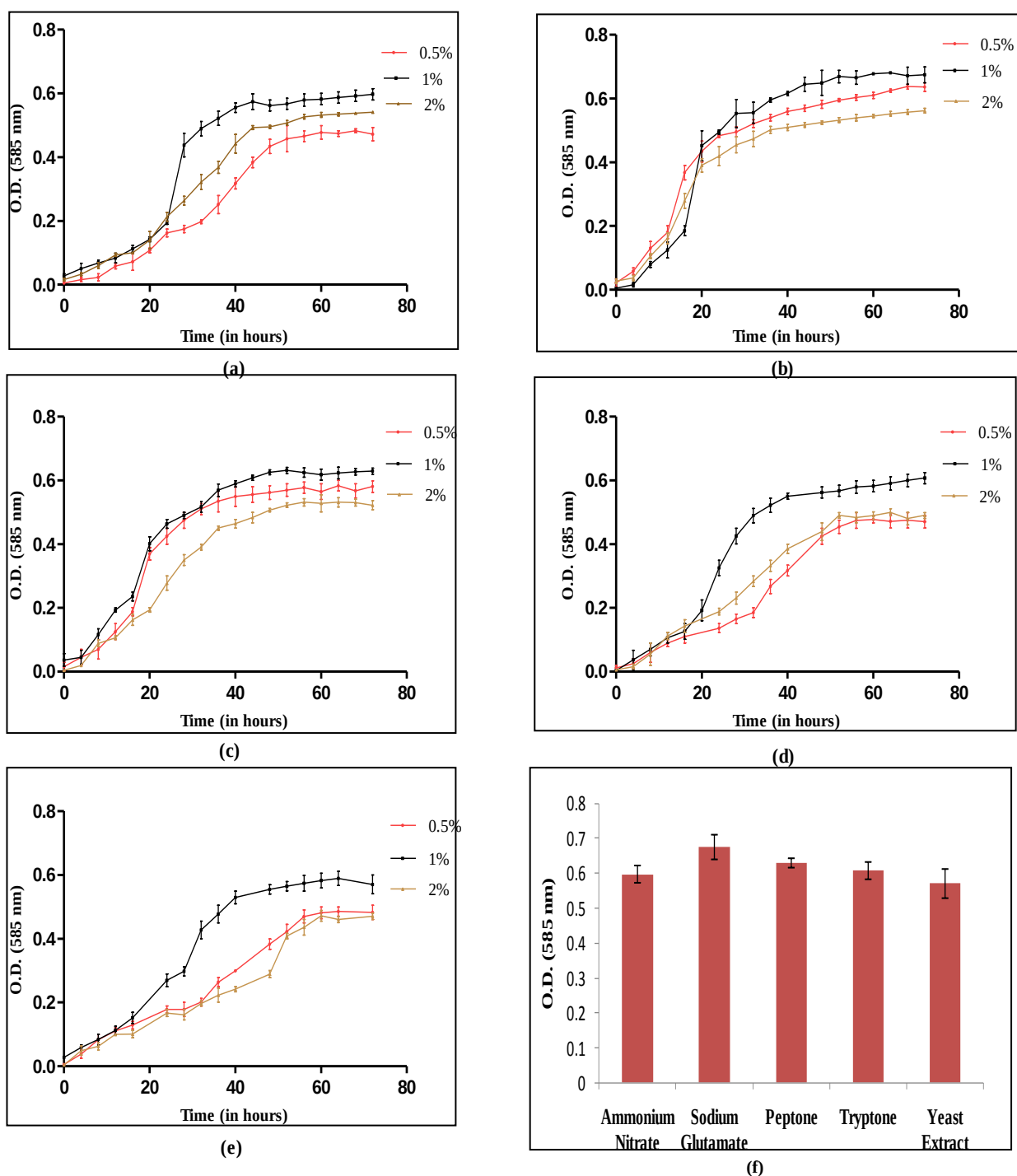


Figure 4.7: Effect of nitrogen sources at different concentrations on biofilm production (a) ammonium nitrate (b) sodium glutamate (c) peptone (d) tryptone (e) yeast extract (f) Comparative account of biofilm production using different nitrogen sources at 72 hours (1%)

4.5.5 Optimization of carbon and nitrogen sources by Response Surface Methodology

The optimal concentration of glucose and sodium glutamate was determined using Central Composite Design (CCD) of Response Surface Methodology (RSM). The coded values of the independent variables (A: Glucose; B: Sodium Glutamate) along with its experimental results of each independent variable are listed in Table 4.2. The CCD matrix was analyzed by Analysis of Variance (ANOVA) and Fisher's F test (Table 4.3). The best fitting model was observed by applying quadratic regressions. Quadratic model was found to be highly significant ($P < 0.05$) to represent the relationship between responses and the significant variables. In model for all responses, small P values (< 0.05) with suitable R^2 (> 0.9) were obtained in the created model for all the responses. Second level polynomial equation provided the level of biofilm production in terms of coded factors as given in the following equations:

$$\text{Optical Density (O.D.) at 12 hours} = +0.24 + 0.016*A + 7.945E-003*B - 0.013*A*B - 0.044*A^2 - 0.053*B^2$$

$$\text{O.D. at 24 hours} = +0.54 + 0.021*A + 0.030*B + 0.015*A*B - 0.056*A^2 - 0.068*B^2$$

$$\text{O.D. at 36 hours} = +0.57 + 0.041*A + 0.037*B + 9.250E-003*A*B - 0.053*A^2 - 0.054*B^2$$

$$\text{O.D. at 48 hours} = +0.68 + 0.064*A + 0.034*B + 0.024*A*B - 0.065*A^2 - 0.097*B^2$$

$$\text{O.D. at 60 hours} = +0.69 + 0.051*A + 0.034*B + 0.033*A*B - 0.071*A^2 - 0.079*B^2$$

Where A: Glucose, B: Sodium glutamate

The 'lack-of-fit' value was insignificant for biofilm production and the goodness of fit of the model was determined by coefficient of determination (R^2). Predicted R^2 values were in reasonable agreement with adjusted R^2 values for all the responses (Table 4.4). Adequate precision measures the signal to noise value. A ratio greater than 4 is desirable. Values obtained in all responses (26.608, 23.927, 50.776, 37.697 and 42.582 at 12, 24, 36, 48 and 60 hours, respectively) leading to an adequate signal indicating that quadratic equation can be used within the range of factors in the design space. Coefficient of variance i.e. CV in all responses was

observed to be 4.13, 2.39, 1.01, 1.63 and 1.23 %, respectively at different time intervals (12, 24, 36, 48 and 60 h), which indicated that model could be reproduced as its value was $< 10\%$.

The response surface plots of the significant interactions are shown in Figure 4.8. Biofilm production was greatly affected by interactions among significant variables. It was observed that the optimal concentration of both glucose and sodium glutamate laid between the range of 0.1 to 2%. Validation was done by performing number of experiments and by confirming the suitability of the model. The predicted responses obtained were verified with experimental data to maximize biofilm production at different time intervals with both glucose and sodium glutamate at the concentration of 1.05% (Table 4.5) .

Table 4.2: Experimental variables and results for biofilm production in terms of coded values.

Run	A: Glucose	B: Sodium Glutamate	O.D _{585 nm} (12 hours)	O.D _{585 nm} (24 hours)	O.D _{585 nm} (36 hours)	O.D _{585 nm} (48 hours)	O.D _{585 nm} (60 hours)
1	0.000	0.000	0.25	0.55	0.567	0.68	0.686
2	0.000	0.000	0.243	0.543	0.57	0.68	0.686
3	1.414	0.000	0.175	0.45	0.523	0.65	0.623
4	0.000	-1.414	0.129	0.35	0.413	0.45	0.48
5	0.000	0.000	0.242	0.52	0.57	0.69	0.7
6	-1.414	0.000	0.13	0.4	0.403	0.46	0.47
7	0.000	1.414	0.14	0.45	0.51	0.53	0.58
8	0.000	0.000	0.234	0.534	0.56	0.682	0.692
9	-1.000	1.000	0.15	0.401	0.451	0.472	0.5
10	1.000	1.000	0.158	0.48	0.548	0.64	0.66
11	0.000	0.000	0.23	0.543	0.572	0.672	0.693
12	-1.000	-1.000	0.1	0.38	0.39	0.44	0.5
13	1.000	-1.000	0.16	0.4	0.45	0.512	0.53

Table 4.3: Statistical analysis showing ANOVA for biofilm production by *S. paucimobilis* MG6

Where, A= Glucose; B= Sodium Glutamate

	12 hours			24 hours			36 hours			48 hours			60 hours		
Source	df	F-Value	p-value Prob>F	df	F-Value	p-value Prob>F	df	F-Value	p-value Prob>F	df	F-Value	p-Value Prob>F	df	F-Value	p-value Prob>F
Model	5	118.04	<0.0001 Significant	5	98.18	<0.0001 Significant	5	462.14	<0.0001 Significant	51	287.03	<0.0001 Significant	5	380.13	<0.0001 Significant
A	1	39.16	0.0004	1	29.53	0.0010	1	515.99	<0.0001	1	361.26	0.0001	1	381.08	<0.0001
B	1	9.13	0.0194	1	60.26	0.0001	1	424.07	<0.0001	1	104.15	0.0001	1	170.00	<0.0001
AB	1	12.22	0.0101	1	7.14	0.0319	1	13.24	0.0003	1	25.73	0.0014	1	78.00	<0.0001
A ²	1	243.72	<0.0001	1	177.75	<0.0001	1	756.44	<0.0001	1	323.47	<0.0001	1	638.97	<0.0001
B ²	1	353.56	<0.0001	1	266.28	<0.0001	1	777.99	<0.0001	1	731.38	<0.0001	1	797.18	<0.0001

Table 4.4: Model Statistics for biofilm production by *S. paucimobilis* MG6

	12 hours	24 hours	36 hours	48 hours	60 hours
Std. Dev.	7.438E-003	0.011	5.08E-003	9.463E-003	7.360E-003
Mean	0.18	0.46	0.50	0.58	0.60
C.V.%	4.13	2.39	1.01	1.63	1.23
PRESS	3.17E-003	3.106E-003	7.94E-004	3.54E-003	2.11E-003
R-Squared	0.9883	0.9859	0.9970	0.9951	0.9963
Adj R-Squared	0.9799	0.9759	0.9948	0.9917	0.9937
Pred R-Squared	0.9584	0.9488	0.9867	0.9726	0.9795
Adeq Precision	26.608	23.927	50.776	37.697	42.582

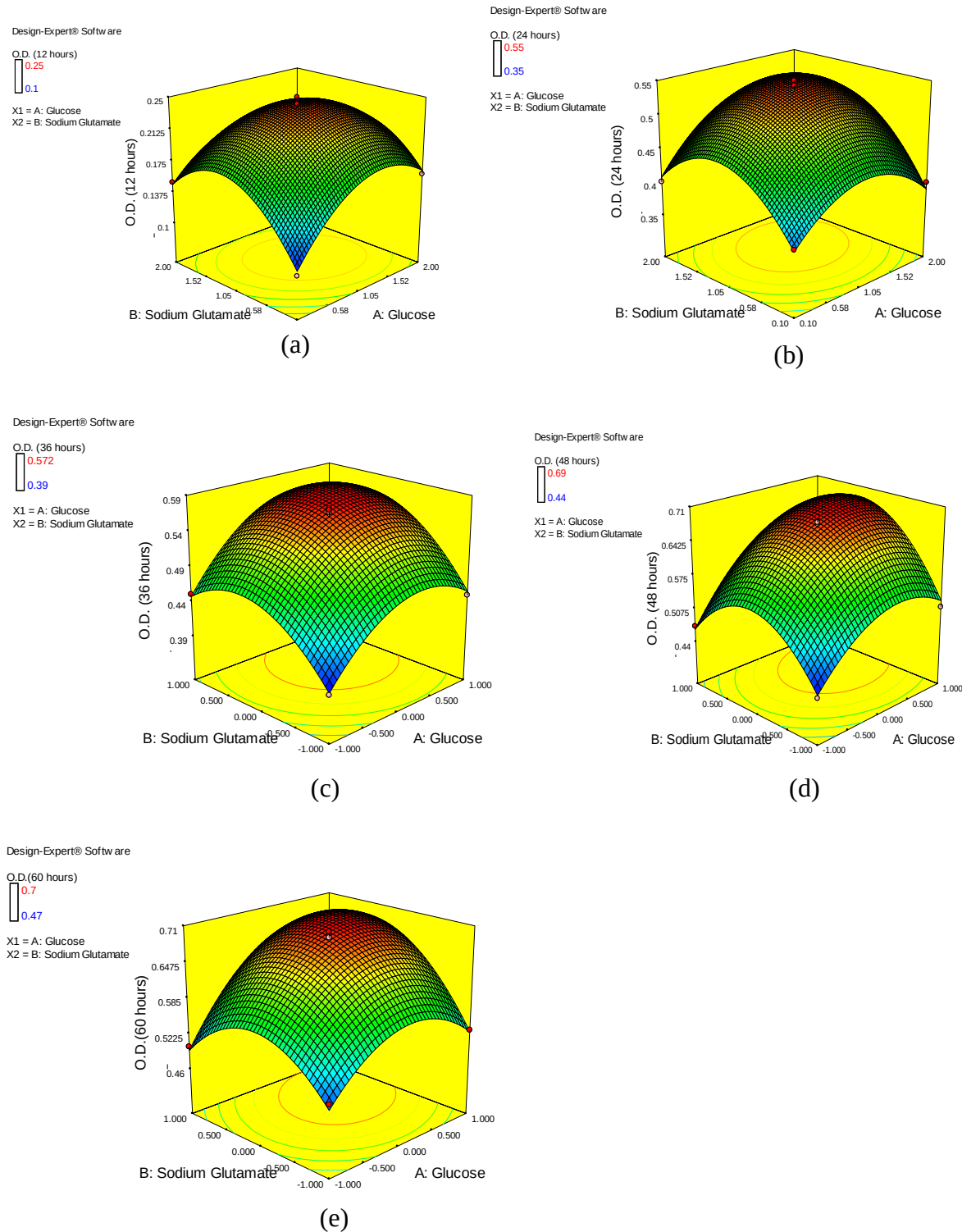


Figure 4.8: 3D Surface plots showing interaction between glucose and sodium glutamate for different time intervals (a) 12 hours (b) 24 hours (c) 36 hours (d) 48 hours (e) 60 hours

Table 4.5: Predicted responses of biofilm formation at different time intervals

Time Intervals (hours)	Prediction
12	0.2398
24	0.538
36	0.5678
48	0.6808
60	0.6922

4.6 Effect of nutritional and cultural factors on biofilm formation by *S. paucimobilis* MG6

Media composition plays a vital role in the ability of bacteria to form biofilm (Hood & Zottola 1997; Stepanović et al. 2004). Since, *Sphingomonas* has the ability to grow on low nutrient conditions i.e. oligotrophic conditions, the medium chosen was appropriate for the study and the growth and biofilm kinetics (Gusman et al. 2012). Growth pattern of planktonic *S. paucimobilis* MG6 depicted in Figure 4.9 indicated a lag phase which continued till 6 hours followed by exponential phase up to 24 hours. The specific growth rate was 0.18 h^{-1} . Biofilm formation by the *Sphingomonas terrae* MTCC 7766 used for comparison demonstrated a similar trend.

Both biomass quantity and microbial activity of the bacterial strain are the parameters suggested for estimating the amount of biofilm formed (Characklis & Marshall 1990). The biofilm biomass and metabolic activity of cells can be easily determined by CV assay (Merritt et al. 2005) and MTT assay (Trafny et al. 2013). It was observed that biofilm production initiated in the early stationary phase and maximum biofilm production was recorded in the late stationary growth phase. Maximum biofilm production occurred between 24 to 48 hours before senescence phase starts in both the strains (Figure 4.10 and Figure 4.11). Since cells in the stationary phase start synthesizing the EPS which facilitates the attachment of cells to the surface (Dunne 2002) high

biofilm production is expected. The *S. paucimobilis* MG6 in the present study could produce maximum biofilm in the late stationary growth phase and further increase was not observed.

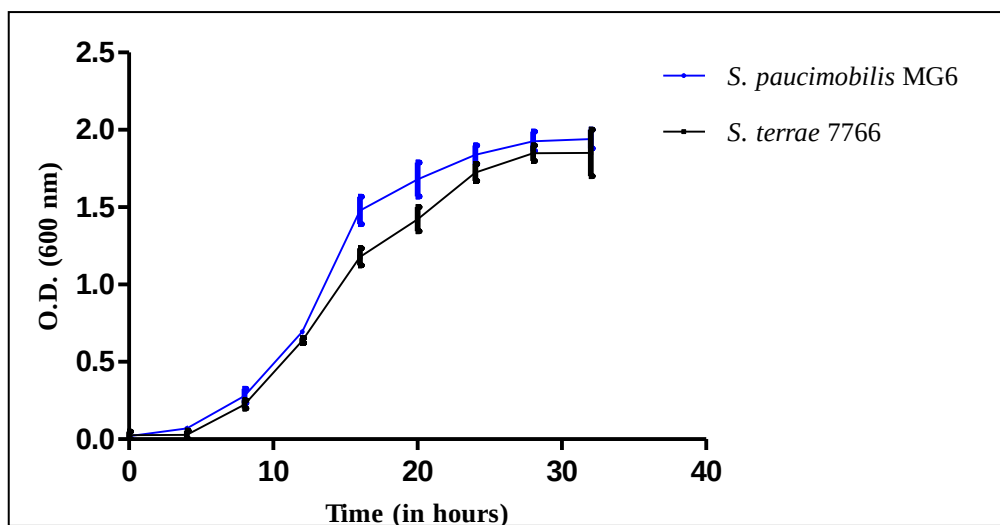


Figure 4.9: Comparison of growth kinetics between planktonic *S. paucimobilis* MG6 (isolate) and standard *S. terrae* (MTCC 7766)

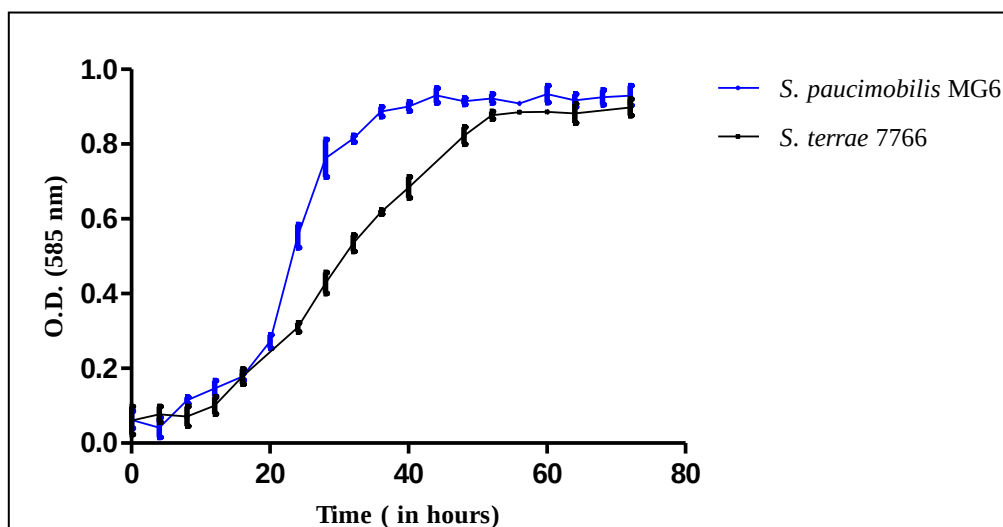


Figure 4.10: Comparison of biofilm profile between *S. paucimobilis* MG6 sp. (isolate) and standard *S. terrae* (MTCC 7766)

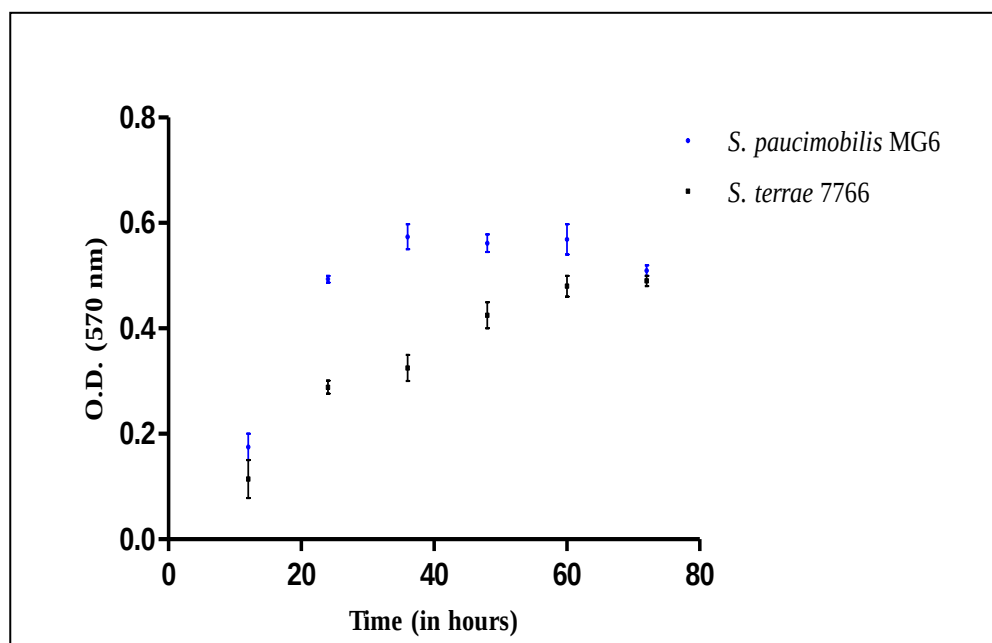


Figure 4.11: Biofilm production kinetics of *S. paucimobilis* MG6 (isolate) and standard *S. terrae* MTCC 7766 by MTT

EPS production by *Sphingomonas* have been reported in earlier studies (Seo et al. 2004) and a sudden increase in EPS production marks the advent of the maturation phase in a *Sphingomonas* sp. biofilm. This may be a possibility, as observed from our results, since EPS production was found to increase sharply after 35 hours (results not shown). Possibly, the environmental influence, which results in complex biofilm architecture, takes effect during the maturation phase (55-60 hours) which is further contributed by increased EPS production. Both *S. paucimobilis* and *S. terrae* produced exopolymers with yield 0.246 g/l and 0.231 g/l after 40 hours. A slight difference in biofilm maturation time was noted in case of *S. terrae* (44-50 hours). Biofilm development in *Sphingomonas* has been explicitly described by Venugopalan et al (2005). The *Sphingomonas* biofilm is thought to be complicated with the presence of micro-colonies. However, the architectural nature was not studied in detail since it was beyond the scope of the present study.

4.7 Biofilm production on common matrices

The ability to construct and maintain a structured multicellular bacterial community or biofilm depends critically on the production of extracellular matrix components such as exopolysaccharides, proteins etc. The nature of the surface is an important external factor which determines the biofilm strength since adherence and bacterial survival within biofilm are linked with cell interactions with surface (Mohammad et al. 2010). Therefore evaluation of biofilms on different matrices can provide valuable insights on the biofilm characteristics.

The efficiency of attachment and biofilm formation of the isolated strain *S. paucimobilis* MG6 was studied on microtiter, glass, stainless steel and on plumbing materials by crystal violet and calcofluor staining assays. These assays are recognized as rapid methods for qualitative determination of formation of biofilm without effecting its stabilization. The biofilm development on these surfaces was measured through a time span of 72 hours (Figure 4.12 and 4.13).

A high degree of biofilm with an increasing gradient with respect to consistency was noted; the degree of attachment was highest on glass and microtiter plates followed by stainless steel. This visible difference in degree of adherence is attributed to the surface roughness (Characklis & Marshall 1990; Rogers et al. 1994; Meyer 2003) and similar observations have been reported in various other studies (Azeredo et al. 1999).

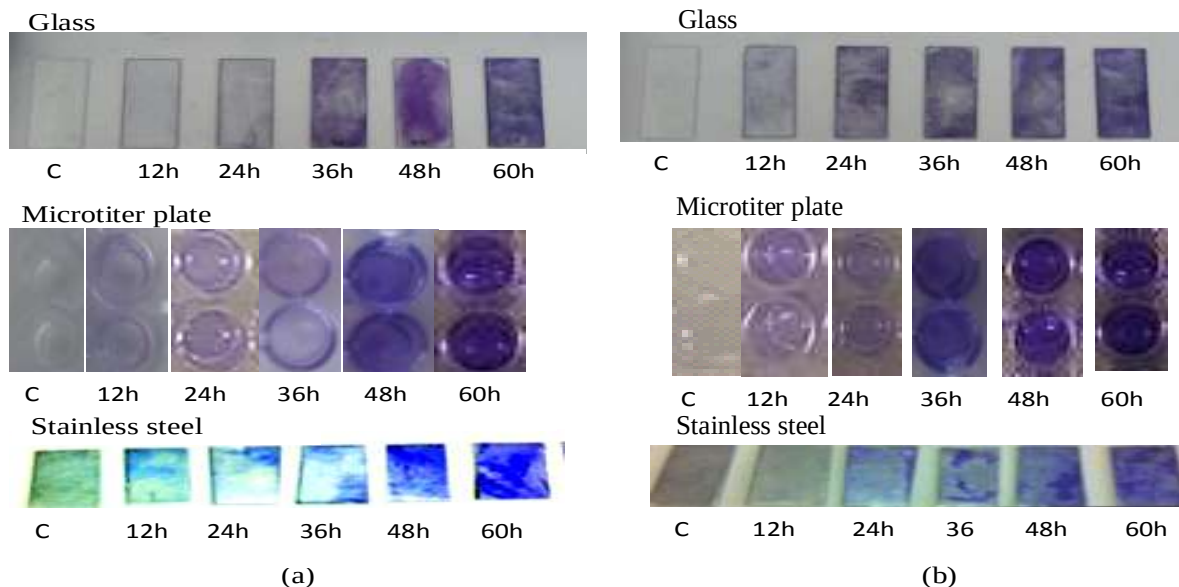


Figure 4.12: Biofilm production by (a) *Sphingomonas paucimobilis* MG6 (b) *Sphingomonas terrae* MTCC 7766 on different matrices determined by crystal violet assay

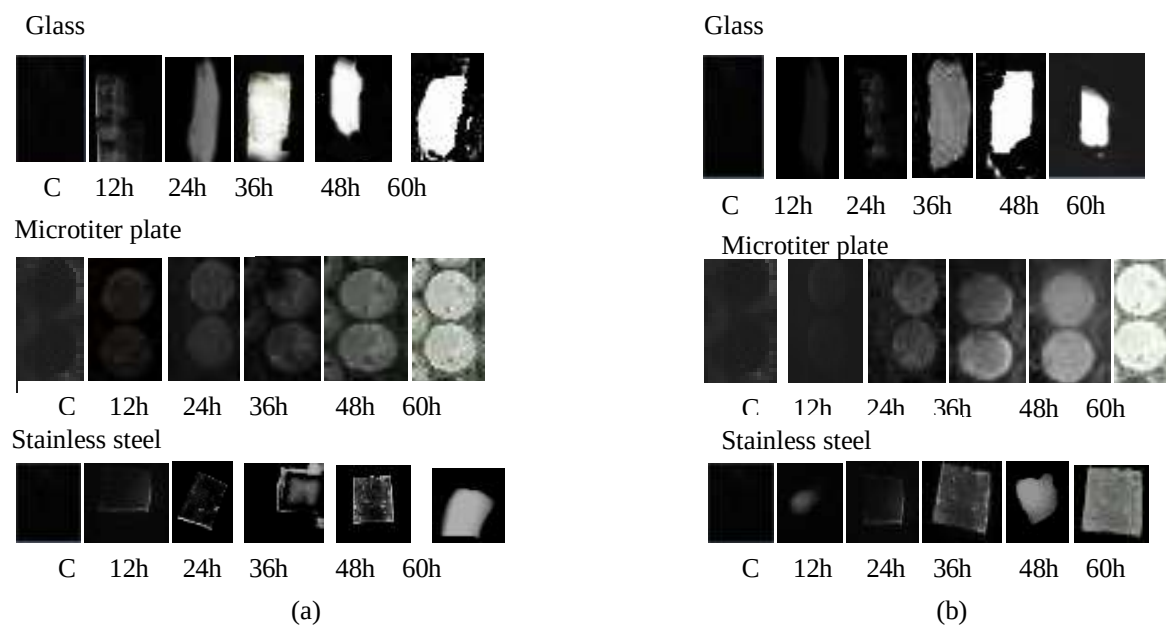


Figure 4.13: Biofilm production by (a) *S. paucimobilis* MG6 (b) *S. terrae* MTCC 7766 on different matrices determined by calcofluor assay

4.8 Biofilm production on different plumbing materials

An important consequence of biofilm production is manifested in plumbing materials used for water distribution. Therefore evaluation of biofilm formation on these materials is important for translating the outcomes of the study. Consequently, different plumbing materials like polyvinyl chloride (PVC), Polypropylene (PP), Polyethylene (PE), Aluminium (Al), Rubber (R) and Copper (Cu) were used to develop biofilms. Results were recorded by visualizing the biofilm under UV lamp after equal interval of 12 hours for up to 72 hours with each material. The calcofluor assay revealed the biofilm formation on pipe materials such as PVC, PP, PE and Al; however, variation was observed with increase in intensity (Figure 4.14). Also, increase in intensity was observed with the progress of time. The intensity was negligible in case of copper and rubber suggesting the possibility of less biofilm formation on these materials.

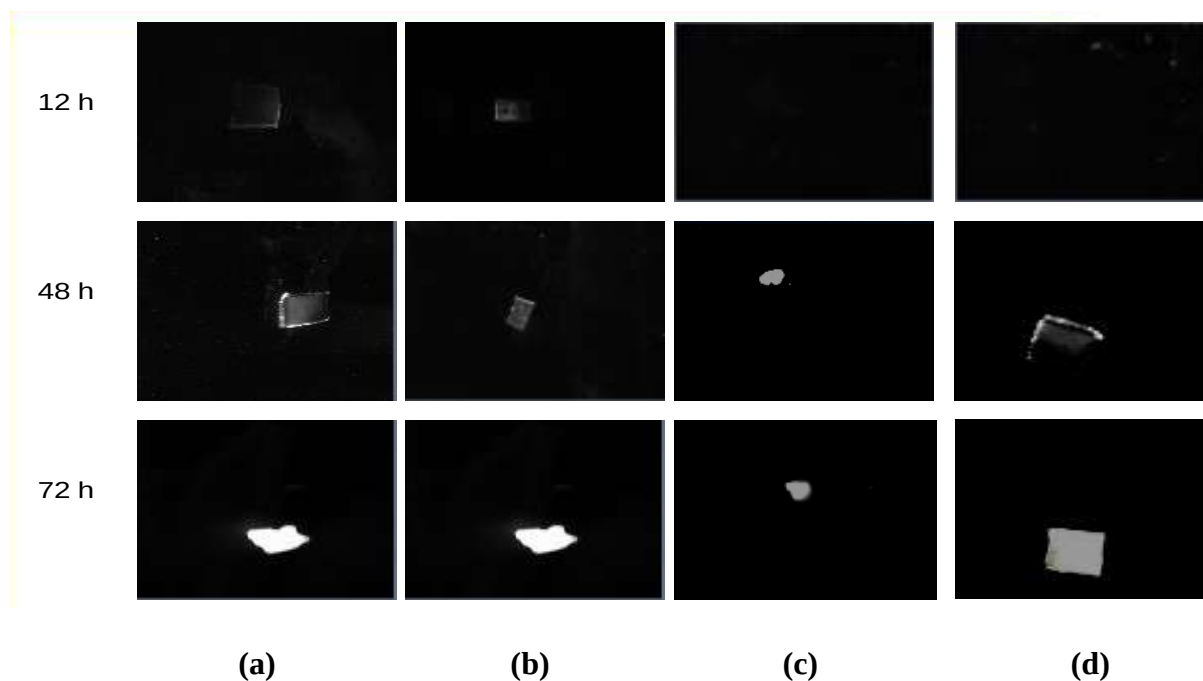


Figure 4.14: Biofilm formation by *Sphingomonas paucimobilis* MG6 on different plumbing materials: (a) Polyvinyl Chloride (PVC), (b) Polypropylene (PP), (c) Aluminium (Al), (d) Polyethylene (PE) determined by calcofluor assay

4.9 Antibiotic susceptibility test

It was important to determine antibiotic susceptibility pattern of *S. paucimobilis* MG6 was important to obtain insights on its antibiotic susceptibility, since the isolate originated from public drinking water system. The Kirby Bauer test was used to determine the susceptibility of *Sphingomonas* against different antibiotics. The results of different antibiotics against *Sphingomonas* at ambient temperature are depicted in Table 4.6. Sensitivity to Gentamicin, Tetracycline and resistance to Ampicillin, Polymyxin B, Amikacin, Piperacillin, Streptomycin was observed. The cell surface properties of *Sphingomonas* are intrinsically related to susceptibility of hydrophobic drugs such as Tetracycline and Gentamycin. It is likely that the *S. paucimobilis* MG6 did not acquire resistance from antibiotic resistant strains.

Antibiotic resistance in clinical isolates of *S. paucimobilis* responsible for bacteraemia in hospitalized patients has been of increasing concern and in most cases *Sphingomonas* contamination has been traced to water supplied to the clinical units. For instance, Meric et al (2009) reported *Sphingomonas* isolates from hospital water samples. All of the isolated strains were resistant to ceftazidime and susceptible to ampicillin/sublactam, cefazolin, cefepime cefotaxime, ciprofloxacin, norfloxacin, amikacin, gentamicin, tobramycin, meropenem, imipenem, piperacillin, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, cefoperazone/sulbactam, and tetracycline. Multiple antibiotic resistant *S. paucimobilis* was reported by Maragakis et al (2009).

Table 4.6: Antibiotic susceptibility of *S. paucimobilis* MG6

Antibiotics	Potency (μ g)	General criterion			Inhibition diameter (mm)	Comments
		Resistant ($< \text{ or } =$ mm)	Intermediate (mm)	Susceptible ($= \text{ or } >$ mm)		
Ampicillin	10	11	12-13	14	8.9 $\pm$ 0.09	Resistant
Polymyxin B	300U	8	9-11	12	9 $\pm$ 0.07	Intermediate
Amikacin	10	11	12-13	14	12 $\pm$ 0.05	Resistant
Tetracycline	30	14	15-18	19	16 $\pm$ 0.08	Intermediate
Gentamicin	10	12	13-14	15	15.7 $\pm$ 0.09	Susceptible
Piperacillin	100	10	11-18	15	16 $\pm$ 0.05	Resistant
Streptomycin	10	11	12-14	15	9.2 $\pm$ 0.04	Resistant

4.10 Cell surface properties of *S. paucimobilis* MG6

4.10.1 Surface hydrophobicity and exopolymer production

Bacterial adherence to hydrocarbons (BATH) is a technique for the determination of the hydrophobic surface characteristics of bacterial cells. To measure the cell surface hydrophobicity, this test was conducted with both *S. paucimobilis* MG6 and *S. terrae* 7766. This technique is used to measure the surface area of two liquid phases of diesel droplets in aqueous phase and the formation of droplets is dependent on many parameters like conditions of mixing, type of vessel in which mixing were carried out etc. The assay results revealed adhesion of *S. paucimobilis* to diesel as 46% and that of *S. terrae* to be 44.4%. Adhesion of *Sphingomonas* cells towards hydrocarbon molecules is considered to be an indigenous property and not significantly altered through cell surface modification upon exposure to hydrophobic chemicals. The cell wall of *Sphingomonas* completely lacks lipopolysaccharides (LPS) in the cell membrane which consists of proteins, phospholipids, and respiratory quinones and an outer membrane that

contains glycosphingolipids (GSLs). The GSLs occupy a position similar to that of LPS in other Gram-negative bacteria but presence of the carbohydrate part of a GSL is shorter than that of LPS. The latter renders the cell surface of *Sphingomonas* more hydrophobic than that of other Gram-negative bacteria (Balkwill et al. 2006) and confers the indigenous capability of adhesion. Association of exopolymers with the initial attachment of bacteria has a significant effect on physical and physiological properties of biofilms necessitating the estimation of exopolymer of *S. paucimobilis* MG6 and *S. terrae* 7766. In both exopolymer with yield 0.246 g/l and 0.231 g/l were produced after 48 hours in the growth medium (Table 4.7).

Table 4.7: Exopolymer yield produced by *S. paucimobilis* MG6 and *S. terrae* MTCC 7766 after 48 hours incubation.

Strain	Yield of exopolymer (g/l)
<i>S. paucimobilis</i> MG6	0.246
<i>S. terrae</i> MTCC 7766	0.231

4.11 Co-aggregation studies of *S. paucimobilis* MG6 with water borne pathogens

4.11.1 Qualitative Analysis

Based on the visual method of scoring, no aggregation of pure cultures as well as combinations of the cultures after zero hour of incubation was observed resulting in increasing the incubation time of cultures. Aggregation was visible as turbid suspension after 24 hours and clear supernatant with aggregates after 72 hours (Figure 4.15). The scoring range used in literature (Cisar et al. 1979; Simões et al. 2008) was used to compute the visual aggregation scores exhibited by culture (Table 4.8). Pure cultures of both *S. paucimobilis* MG6 and *S. terrae* 7766 exhibited maximum score of 3 after 72 hours. Increase in this trend was observed in aggregation of *S. paucimobilis* MG6 with *Shigella flexneri* 2a.

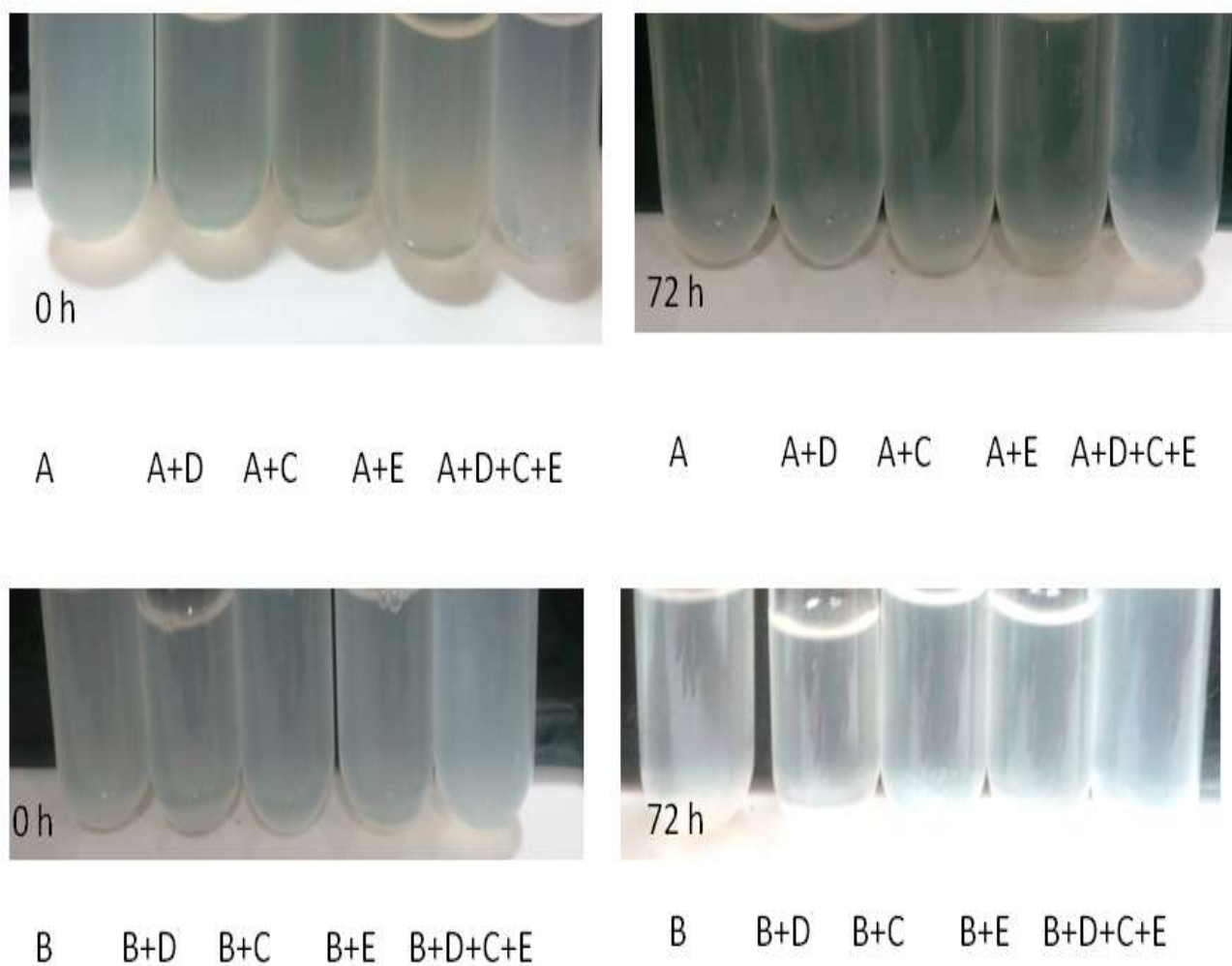


Figure 4.15: Qualitative analysis of auto- and co-aggregation in (a) *S. paucimobilis* strain MG6
(b) *S. terrae* MTCC 7766

A=*S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C=*Salmonella typhimurium* ATCC 25315;
D=*Escherichia coli* O157:H7 ATCC 32150; E=*Shigella flexneri* 2a

Table 4.8: Visual aggregation score exhibited by cultures during auto- and co-aggregation. (a) *S. paucimobilis* MG6 (b) *S. terrae* MTCC 7766

A= *S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

Time (hours)	A	A+C	A+D	A+E	A+C+D+E
0	0	0	0	0	0
2	1	0	1	1	0
4	1	1	1	1	1
24	2	2	2	2	1
48	3	3	2	2	2
72	3	3	3	4	3

(a)

Time (hours)	B	B+C	B+D	B+E	B+C+D+E
0	0	0	0	0	0
2	1	0	1	1	1
4	1	1	2	2	2
24	2	2	2	2	2
48	3	3	2	3	3
72	3	3	3	4	4

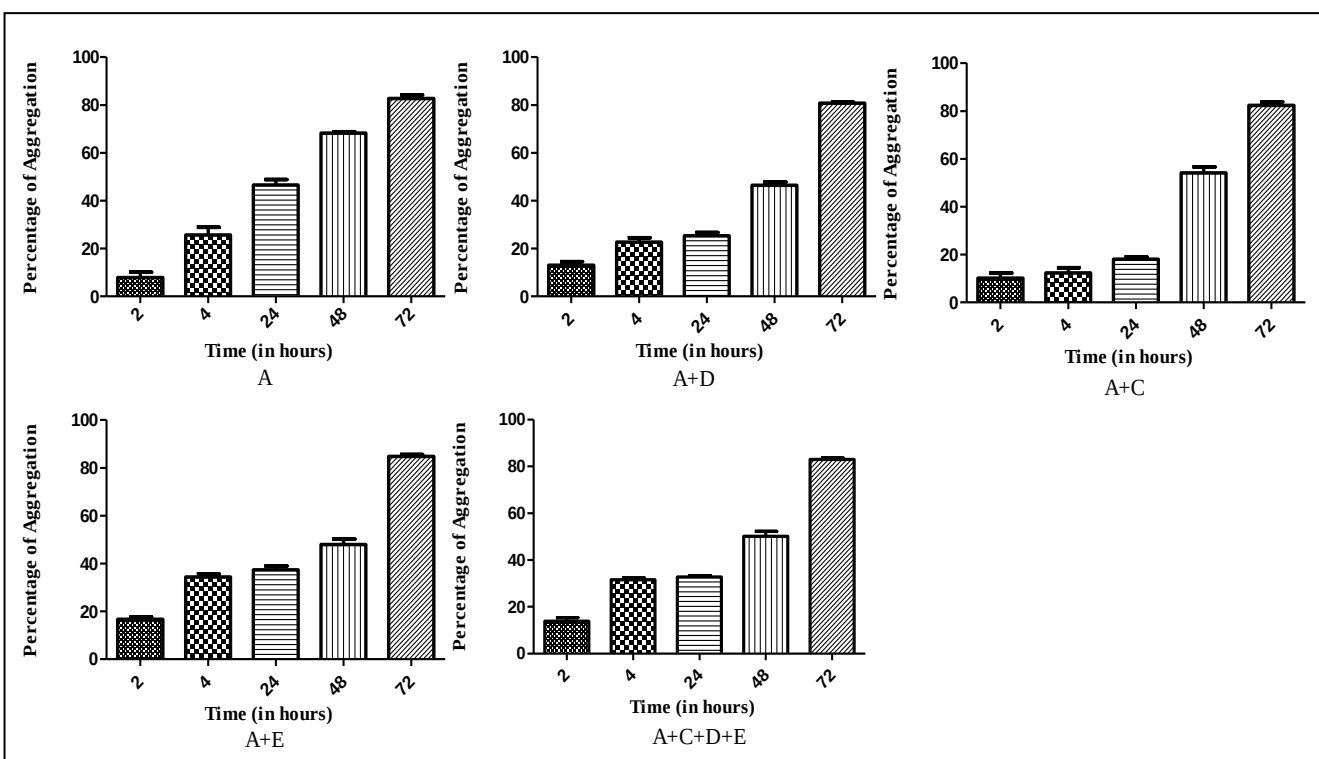
(b)

The scoring range used: 0 - no aggregation and turbid suspension; 1 - small uniform aggregates in a turbid suspension; 2- easily visible aggregates in a turbid suspension; 3 -clearly visible aggregates which settles leaving a clear supernatant; 4 - large flocs of aggregates that settle instantaneously

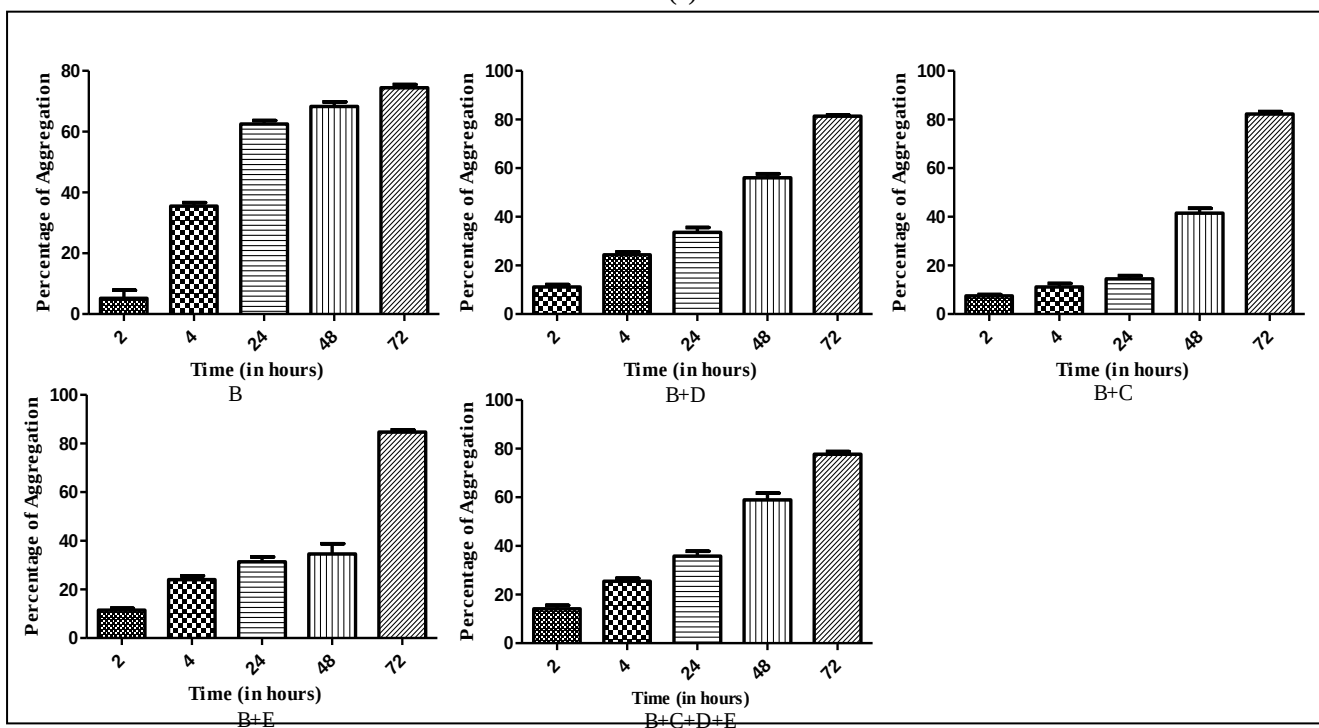
4.11.2 Aggregation Index/Percentage of Aggregation

Spectrophotometric analysis is a more sensitive method to assess the aggregating ability of cultures; therefore aggregation ability was assessed spectrophotometrically. The incubation time is an important requisite for determining the aggregation index of auto-aggregating bacteria as well as co-aggregating bacterial pairs as reported by Saravan et al. (2014). The aggregation index was determined for all the cases at 0, 2, 4, 24, 48 and 72 hours. Aggregation indices of *S. terrae* MTCC 7766 and *S. paucimobilis* MG6 were calculated to be 74.5% and 82.75% respectively after 72 hours of incubation (Figure 4.16).

Strong coaggregation of *Sphingomonas* with the waterborne pathogens *Escherichia coli*, *Shigella flexneri* 2a and *Salmonella typhimurium* was also observed. Notable coaggregation occurred with a cocktail of the above three water borne pathogens; highest aggregation index of *S. paucimobilis* MG6 was observed with *S. flexneri* 2a with 84.75% of aggregation index after 72 hours of incubation followed by *S. typhimurium* and *E. coli* with aggregation index of 82.4% and 80.8%, respectively. Similar trend was observed with coaggregation of *S. terrae* 7766 with different pathogens; highest being with *Shigella* (84.8%) followed by *Salmonella* (82.25%) and *E.coli* (81.4%). Co-aggregation by bacteria promotes biofilm development by facilitating the attachment to the partner species- this being the reason for *S. paucimobilis* MG6 to form biofilms with *S. flexneri* (Min & Rickard 2009).



(a)



(b)

Figure 4.16: Aggregation Index at different interval of time (a) *Sphingomonas paucimobilis* MG6 and its various combinatons (b) *S. terrae* MTCC 7766 and its combinations; where, A=*S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

4.11.3 Visualization of aggregates by DAPI

The visualization of adherent bacteria is of prime importance in studying microbial biofilm (Hannig et al. 2010). Direct staining by DAPI (4',6-diamidino-2-phenylindole) is a method of choice, the DAPI molecules bind to DNA and fluoresce intensely. The enhancement in aggregation ability of *S. paucimobilis* MG6 in the presence of three pathogens could be clearly depicted by DAPI staining- furthermore, a cocktail of all three pathogens used to coaggregate indicated a similar result. In fact the aggregating pairs showed a higher level of interactions as compared to the visual aggregation test for these strains when observed by fluorescence microscopy (Figure 4.17 and Figure 4.18).

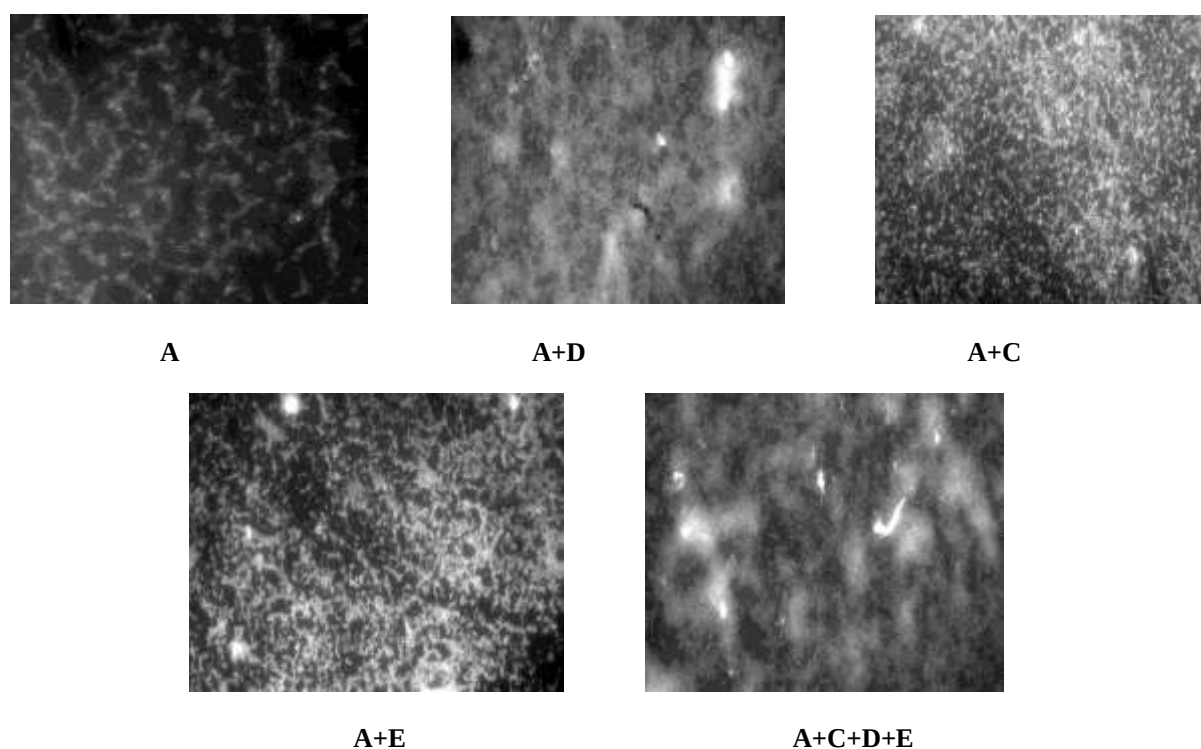


Figure 4.17: Micrograph of DAPI stained cells of auto- and co aggregating *S. paucimobilis* MG6 and its various combinations with pathogens, where, A=*S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

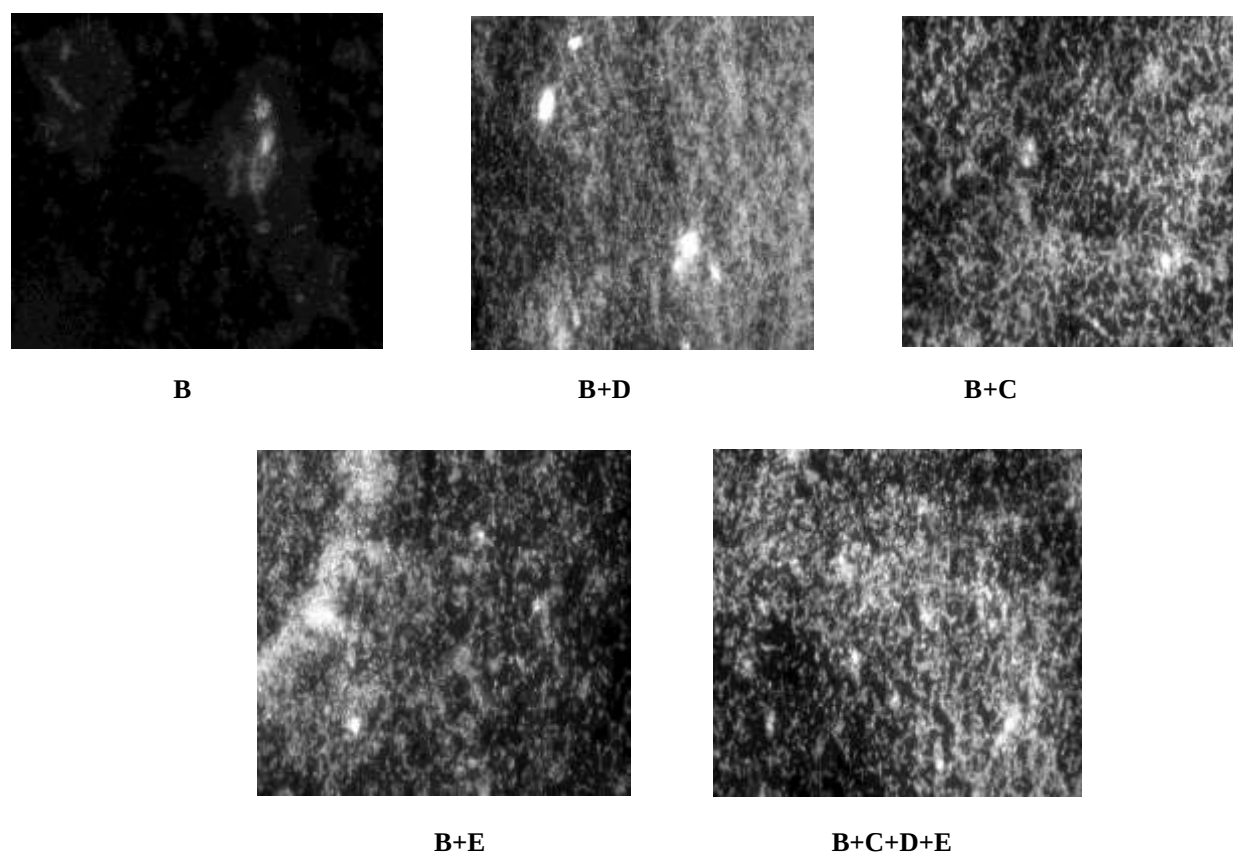


Figure 4.18: Micrograph of DAPI stained cells of auto- and co aggregating *S. terrae* MTCC 7766 and its various combinations, where, A=*S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

4.11.4 Nature of interactions between bacterial strains

The heat treated and untreated pure cultures did not aggregate either after zero hour of incubation or following 2 hours of incubation. Coaggregation between different combinations was significantly affected ($p < 0.05$) upon heat treatment with the exception of *E. coli* O157:H7, since aggregation index remained at 80% in both treated and untreated samples (Figure 4.19).

Several *E. coli* O157:H7 surface proteins are thought to be important for adhesion and/or biofilm formation, therefore it is possible that the treatment processes failed to exert a substantial effect on binding. Visual aggregation scores of heat treated auto- and co-aggregating cultures are depicted in Table 4.9.

The protease treated culture combinations failed to show any visual aggregation after 0 and 2 hours (Table 4.10). The inhibition of aggregation of *S. paucimobilis* MG6 and *S. terrae* 7766 with its aggregating partners might be due to surface protein like attachment (Figure 4.20). *Sphingomonas* with its aggregating partner, *E.coli* O157:H7 showed no decrease in aggregation index.

Treatment with sugars-lactose and galactose failed to revert coaggregation in *Escherichia coli* O157:H7 (Figure 4.21 and Figure 4.22). The visual aggregation scores after lactose and galactose treatment are given in Table 4.11 and 4.12. The differential behavior observed may be attributed to the enzyme type and concentration (Goldhar 1995) important for affecting lectins during the treatment process, in addition to the nutritive media which modulates lectin production (Gilboa-Garber 1988; Goldhar 1995). However, further possibility of coaggregation reversal need also be examined with other sugars.

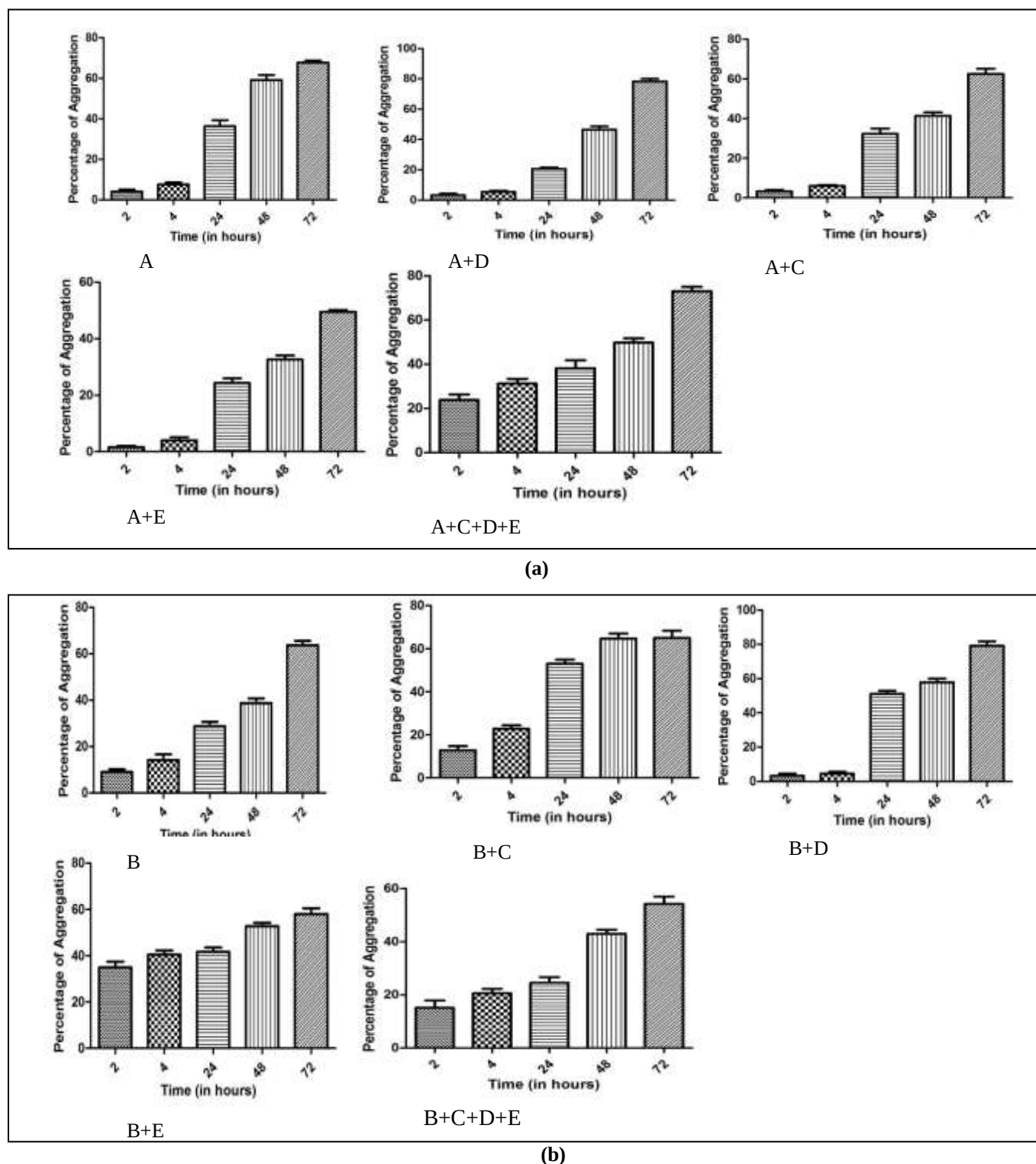


Figure 4.19: Aggregation Index observed at different time intervals after heat treatment (a) *S. paucimobilis* MG6 and various combinations (b) *S. terrae* MTCC 7766 and various combinations, where, A=*S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

Table 4.9: Visual Aggregation Scores after heat treatment (a) *S. paucimobilis* MG6 and various combinations (b) *S. terrae* MTCC 7766 and various combinations

A= *S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *Escherichia coli* O157:H7 ATCC 32150; E= *Shigella flexneri* 2a

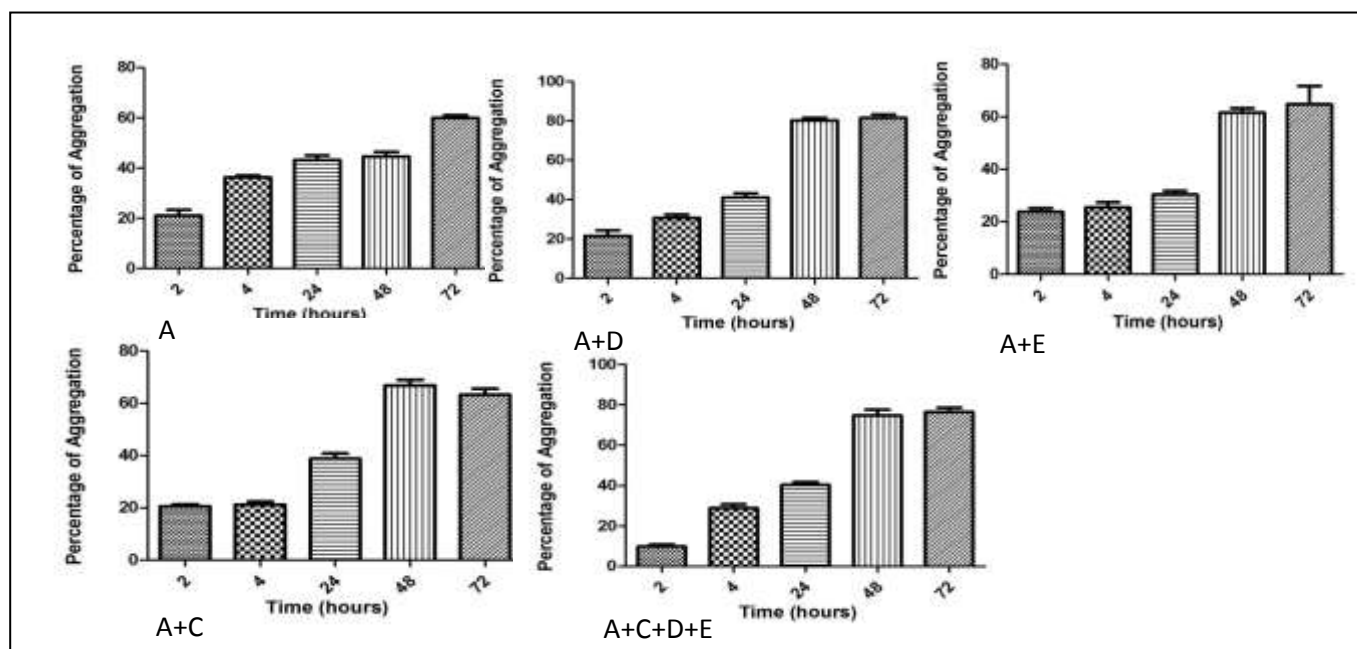
Time (hours)	A	A+C	A+D	A+E	A+C+D+E
0	0	0	0	0	0
2	0	0	0	0	0
4	0	0	1	0	1
24	1	1	1	1	2
48	2	2	2	1	2
72	2	2	3	2	3

(a)

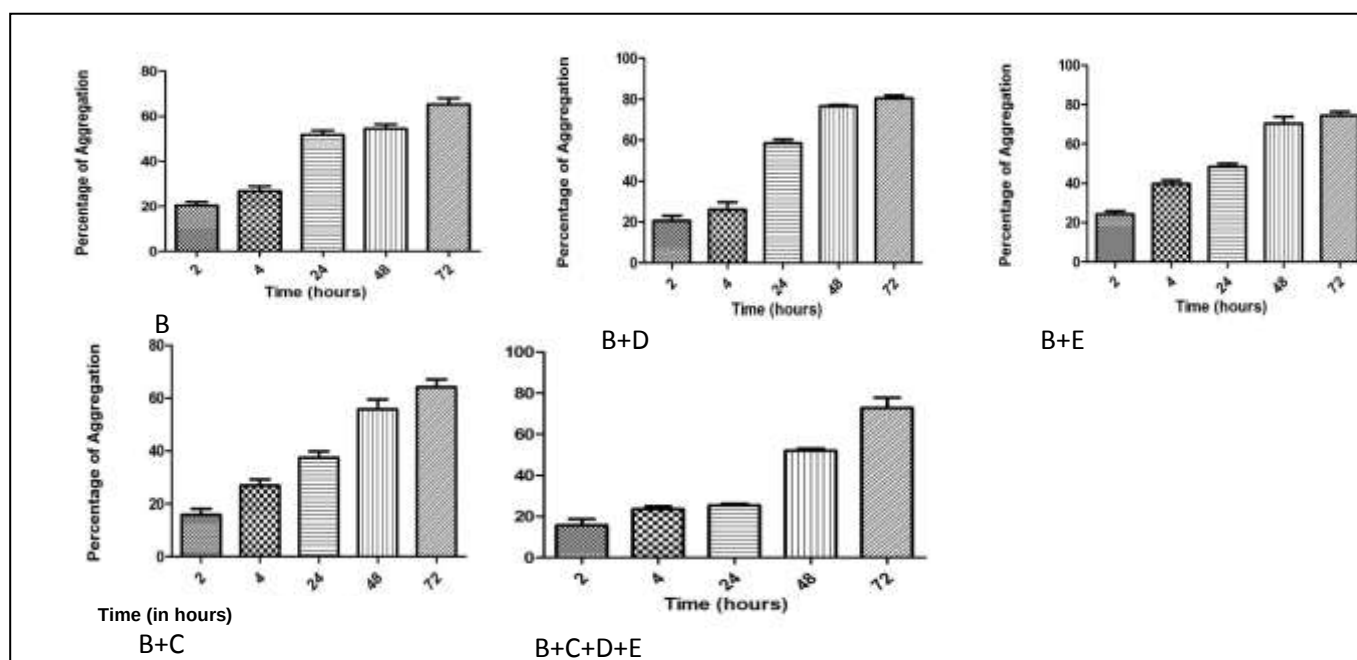
Time (hours)	B	B+C	B+D	B+E	B+C+D+E
0	0	0	0	0	0
2	0	0	0	0	0
4	1	1	1	1	1
24	1	2	2	1	1
48	2	3	3	2	2
72	3	3	4	3	3

(b)

The scoring range used: 0 - no aggregation and turbid suspension; 1 - small uniform aggregates in a turbid suspension; 2- easily visible aggregates in a turbid suspension; 3 -clearly visible aggregates which settles leaving a clear supernatant; 4 - large flocs of aggregates that settle instantaneously



(a)



(b)

Figure 4.20: Aggregation Index observed at different time intervals after protease treatment (a) *S. paucimobilis* MG6 and its various combinations (b) *S. terrae* 7766 and its various combinations, where, A=*S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

Table 4.10: Visual Aggregation Scores of bacterial isolates treated with protease (a) *S. paucimobilis* MG6 and various combinations (b) *S. terrae* MTCC 7766 and various combinations

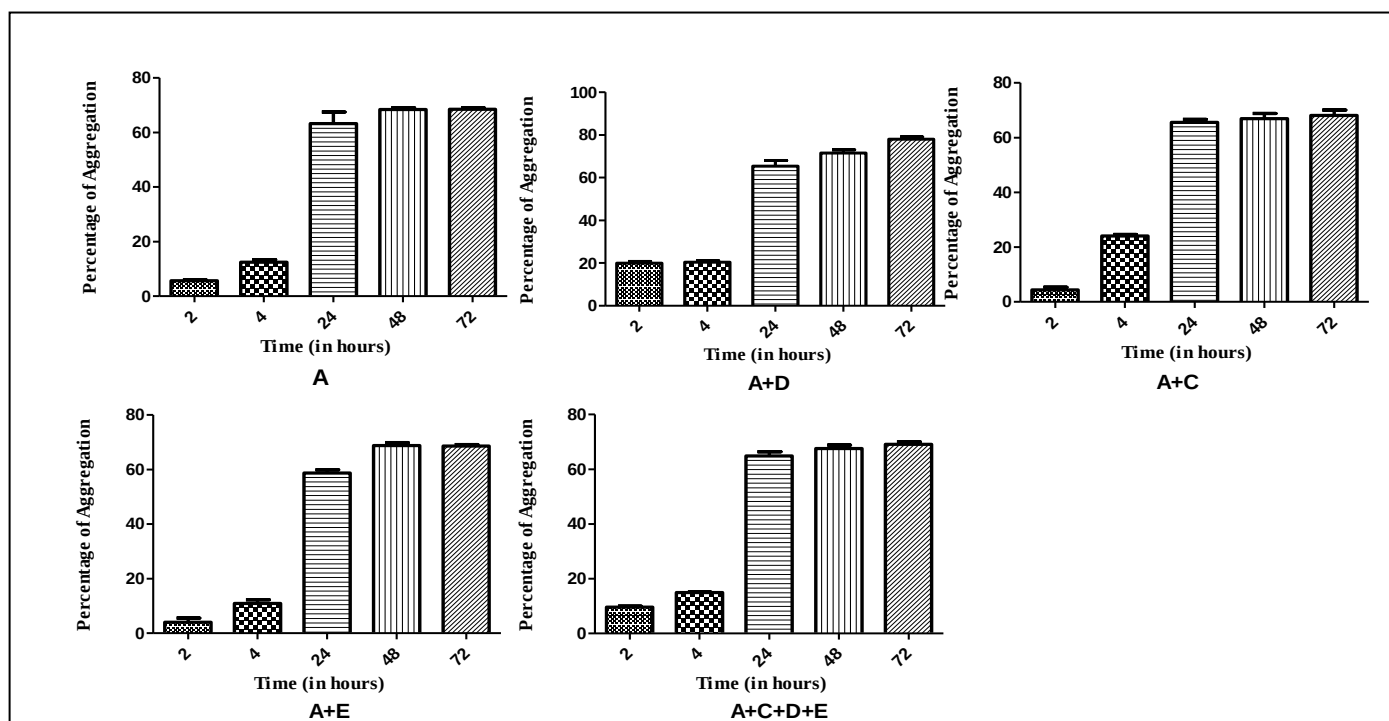
A= *S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

Time (hours)	A	A+C	A+D	A+E	A+C+D+E
0	0	0	0	0	0
2	0	0	0	0	0
4	1	1	1	1	1
24	2	2	2	1	2
48	2	2	3	2	2
72	3	2	4	3	3

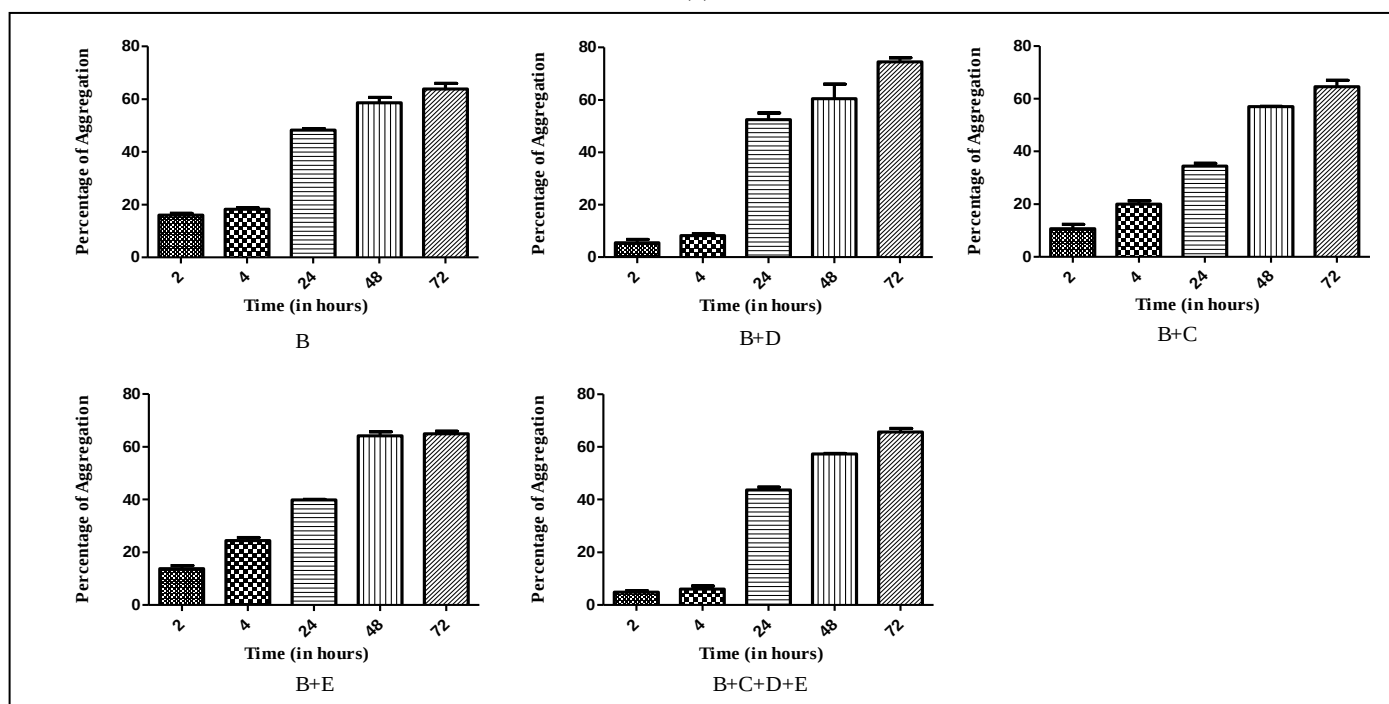
(a)

Time (hours)	B	B+C	B+D	B+E	B+C+D+E
0	0	0	0	0	0
2	0	0	0	0	0
4	0	0	1	1	0
24	1	1	2	2	1
48	2	2	3	3	2

The scoring range used: 0 - no aggregation and turbid suspension; 1 - small uniform aggregates in a turbid suspension; 2- easily visible aggregates in a turbid suspension; 3 -clearly visible aggregates which settles leaving a clear supernatant; 4 - large flocs of aggregates that settle instantaneously



(a)



(b)

Figure 4.21: Aggregation Index observed at different time intervals after treatment with lactose
 (a) *S. paucimobilis* MG6 and various combinations (b) *S. terrae* MTCC 7766 and various combinations, where, A=*S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

Table 4.11: Visual Aggregation Scores of bacterial isolates treated with lactose (a) *S. paucimobilis* MG6 and various combinations (b) *S. terrae* MTCC 7766 and various combinations

A= *S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

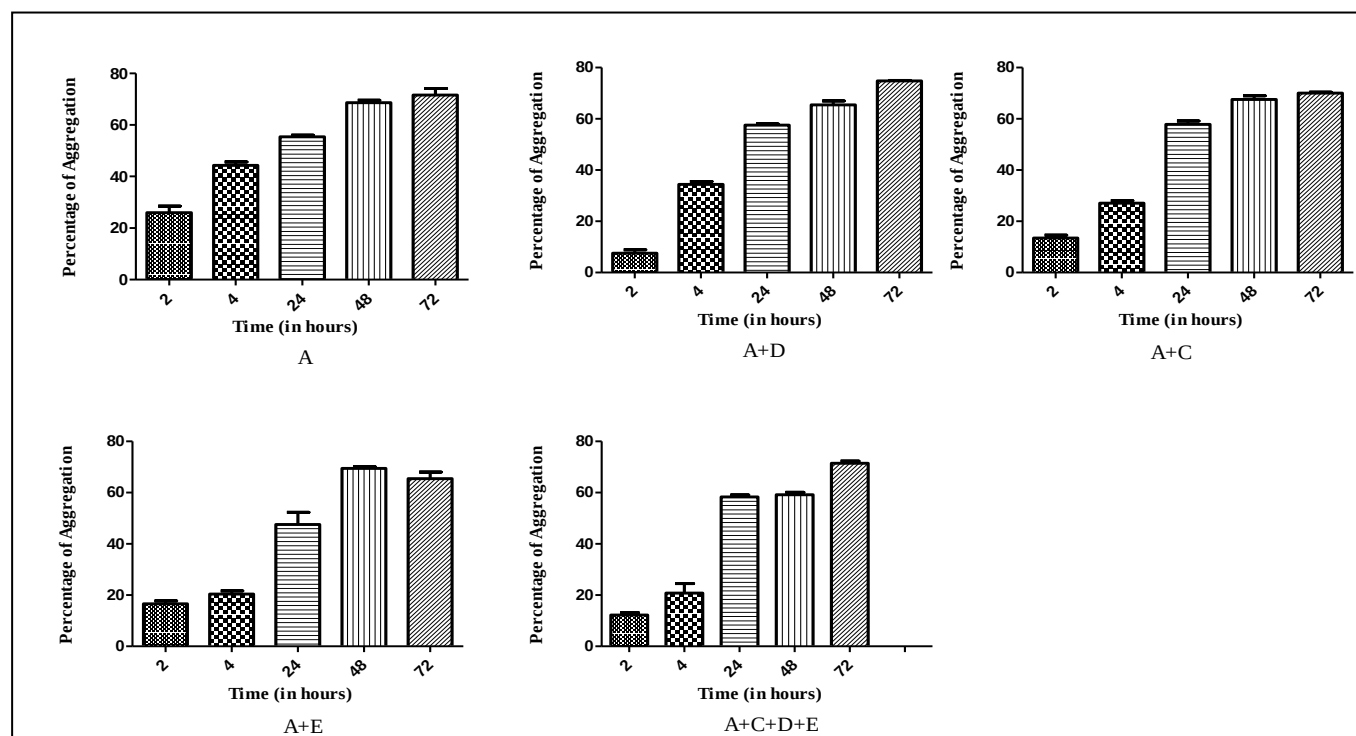
Time (hours)	A	A+C	A+D	A+E	A+C+D+E
0	0	0	0	0	0
2	0	0	0	0	0
4	2	2	2	1	2
24	2	2	3	2	3
48	3	2	4	3	3
72	3	3	4	3	3

(a)

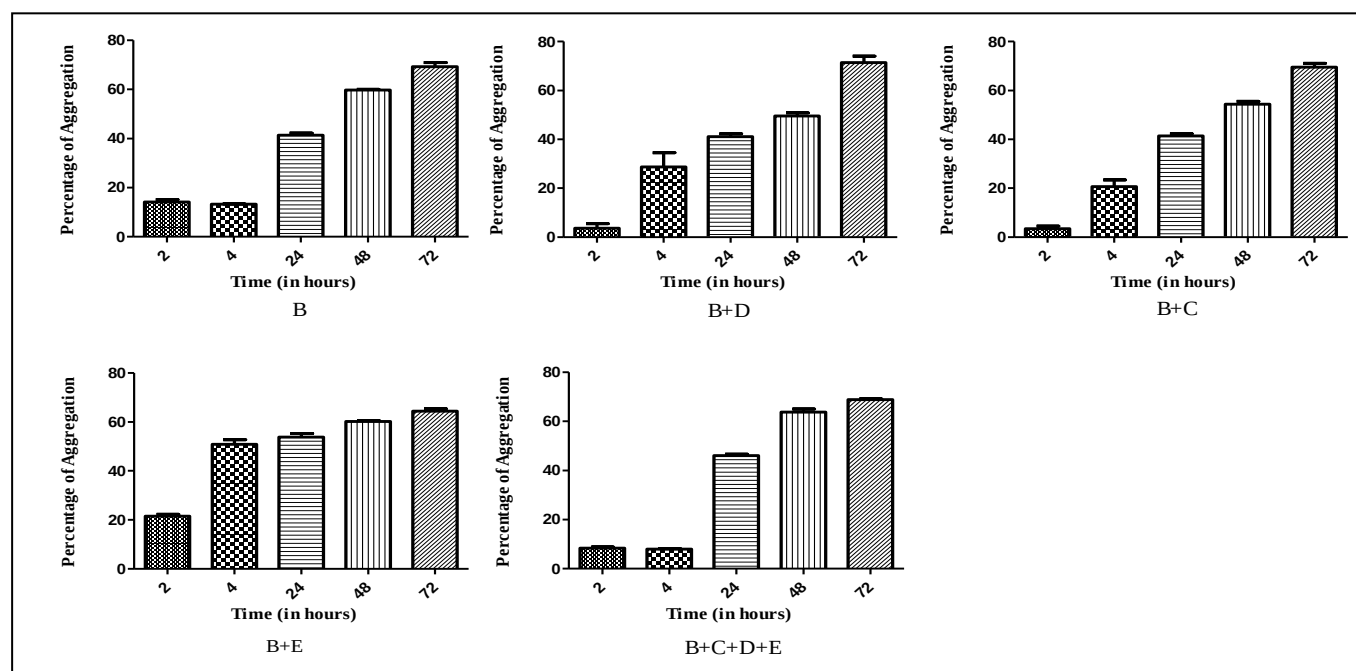
Time (hours)	B	B+C	B+D	B+E	B+C+D+E
0	0	0	0	0	0
2	0	0	0	0	0
4	1	0	1	1	1
24	2	1	2	2	2
48	2	2	3	3	2
72	3	2	4	3	3

(b)

The scoring range used: 0 - no aggregation and turbid suspension; 1 - small uniform aggregates in a turbid suspension; 2- easily visible aggregates in a turbid suspension; 3 -clearly visible aggregates which settles leaving a clear supernatant; 4 - large flocs of aggregates that settle instantaneously



(a)



(b)

Figure 4.22: Aggregation Index observed at different time intervals after galactose treatment (a) *S. paucimobilis* MG6 and various combinations (b) *S. terrae* MTCC 7766 and various combinations, where, A=*S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

Table 4.12: Visual Aggregation Scores of bacterial isolates treated with galactose (a) *S. paucimobilis* MG6 and various combinations (b) *S. terrae* MTCC 7766 and various combinations

A= *S. paucimobilis* MG6; B=*S. terrae* MTCC 7766; C= *S. typhimurium* ATCC 25315; D= *E. coli* O157:H7 ATCC 32150; E= *S. flexneri* 2a

Time (hours)	A	A+C	A+D	A+E	A+C+D+E
0	0	0	0	0	0
2	0	0	0	0	0
4	2	2	2	1	2
24	2	2	3	2	3
48	3	2	4	3	3
72	3	3	4	3	3

(a)

Time (hours)	B	B+C	B+D	B+E	B+C+D+E
0	0	0	0	0	0
2	0	0	0	1	0
4	1	0	1	1	1
24	2	1	2	2	2
48	2	2	3	3	3
72	3	2	4	3	3

(b)

The scoring range used: 0 - no aggregation and turbid suspension; 1 - small uniform aggregates in a turbid suspension; 2- easily visible aggregates in a turbid suspension; 3 -clearly visible aggregates which settles leaving a clear supernatant; 4 - large flocs of aggregates that settle instantaneously

4.12 Monitoring of *Sphingomonas* biofilm

4.12.1 Development of a Capacitance based quantification technique for *S. paucimobilis* MG6 biofilms

Living cells are considered as ‘electric capacitors’ which store energy in the form of charge due to the movement of ions within the electric field. A capacitor comprises of two or more parallel plates electrically separated by insulating layer commonly called as ‘dielectric’. To understand the correlation between biofilm age and change in capacitance, parallel plate capacitors were used. Since biofilm acts as a dielectric material between the plates, modification in the composition of biofilm is expected to bring changes in the capacitance values with time.

For the experimental procedure, measurements were made on aluminum coupons on which the biofilms were grown; the distance between the coupons was fixed at 0.14 cm. The plates used were 2.5 cm long and 2.5 cm wide (i.e. 6.25 cm²). An LCR [inductance (L), capacitance (C), resistance (R)] meter (Gwinstek, model no 821, Taiwan) was used for the measurement; its electrical capacitance measurement range is between 0.00001pF and 99999 μ F, with the exciting frequency between 120 Hz and 200 kHz. In all experiments, the voltage applied between plates was 1V. The capacitance values of biofilm were finally recorded on three different frequencies i.e. 1 kHz, 100 kHz and 200 kHz.

Changes in frequencies caused variation in capacitance values irrespective of the slope of the graph (Figure 4.23). As observed from the graph, the initiation of biofilm resulted in an exponential increase in capacitance values of biofilm. This increase in capacitance values may be directly correlated to increase in biomass and EPS content of the biofilm. As the biofilm formation reached the stationary phase i.e. after 96 hours (4 days) a marked decrease in capacitance value was observed. This decrease in capacitance values was either due to cell death or damaged cell having disrupted membrane as both of these resulted in no significant changes

($p > 0.05$) in capacitance values. These results suggested that the capacitance measurements at 1 kHz was satisfactory to determine the age of the biofilm. The biofilm age and its correlation to capacitance were based on dry weight measurements of the biofilm. The visible variations in values of electrical capacitance occurred only after 36 hours as observed in Figure 4.24. This fact is directly related with the small growth of the biomass in the first period and with the inherent detection limit of the method. The biofilm dry weight increased over time with biomass growth which leads to increase in electrical capacitance; growth was accentuated between the 120 and 150 hours and electrical capacitance decreased post 120 hours, possibly on account of cessation of actual cell mass and accumulation of EPS. In fact, when a linear regression of the data (electrical capacitance with biofilm weight) was performed, the correlation coefficient was 0.9613.

From the regression values it was also concluded that the sensitivity of this type of measurement was -0.2065 pF/mm . A statistical analysis was performed to validate the correlations between biofilm weight and electrical capacitance according to the data obtained in assays (Table 4.13). The results were reproducible with correlation coefficients greater than 98%.

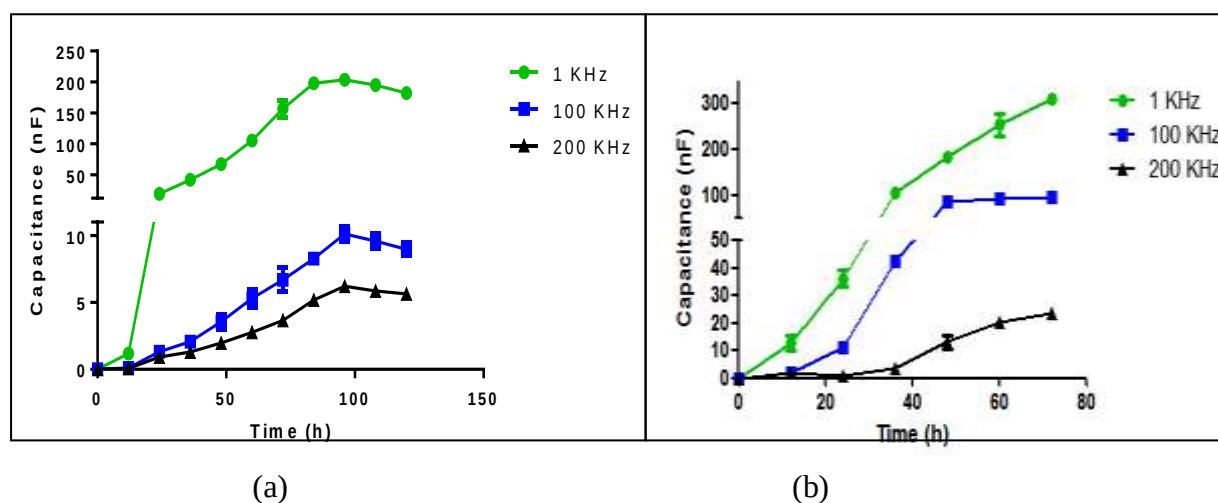
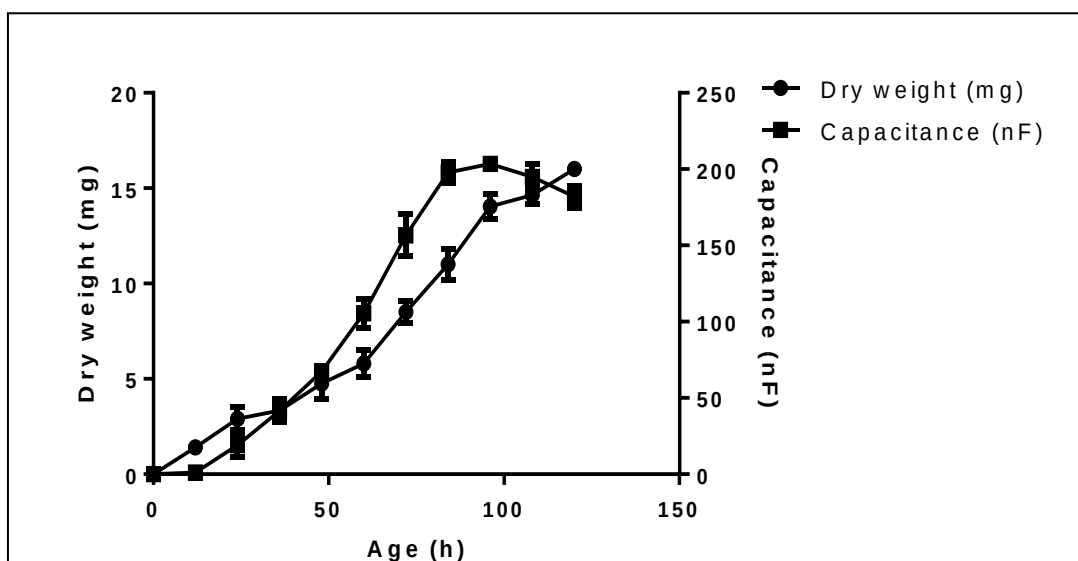
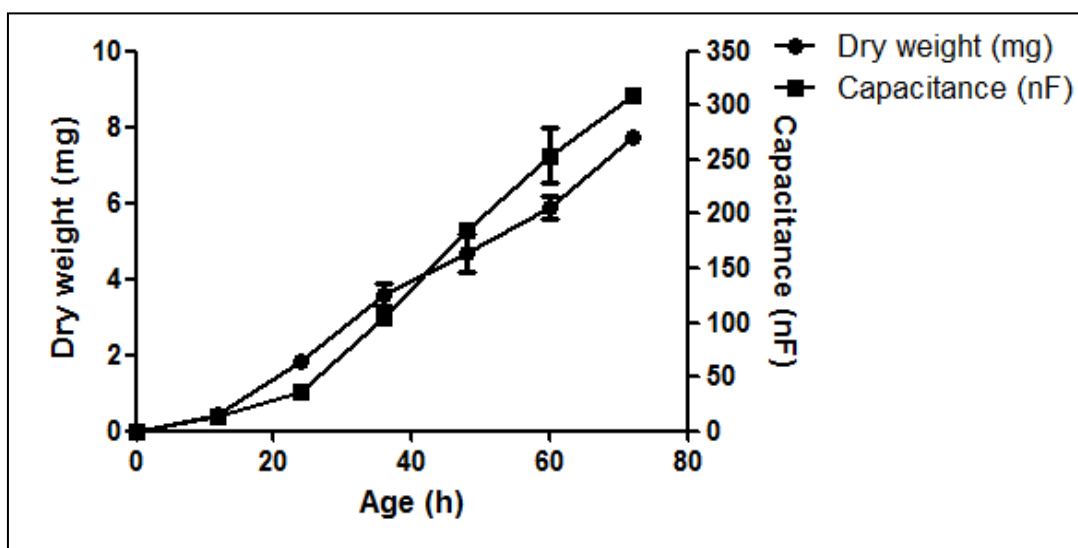


Figure 4.23: Capacitance changes measured during (a) *S. paucimobilis* MG6 biofilm growth and (b) standard *S. terrae* 7766 growth at 30°C ; All values are average of three independent replicates



(a)



(b)

Figure 4.24: Correlation between biofilm age and capacitance (1kHz) . Values are average of three independent replicates. (a) *S. paucimobilis* MG6. (b) Standard *S. terrae* MTCC 7766

Table 4.13: Dry weight and capacitance of *S. paucimobilis* MG6 measured over 120 hours. Data is Average $\pm$ Standard Deviation (SD) of triplicate samples

Time (hr)	Dry weight (mg)	Capacitance (nF)
0	0	0
12	1.1 $\pm$ 0.02	1.7 $\pm$ 0.01
24	3.5 $\pm$ 0.34	2.3 $\pm$ 0.03
36	2.8 $\pm$ 0.32	3.9 $\pm$ 0.03
48	3.9 $\pm$ 0.24	5.6 $\pm$ 0.06
60	5.1 $\pm$ 0.04	6.5 $\pm$ 0.05
72	7.9 $\pm$ 0.05	9.1 $\pm$ 0.14
84	11.8 $\pm$ 0.1	10.2 $\pm$ 0.13
96	13.4 $\pm$ 0.21	14.7 $\pm$ 0.11
108	15.1 $\pm$ 0.33	14.2 $\pm$ 0.15
120	16.1 $\pm$ 0.41	15.9 $\pm$ 0.20

4.12.2 Capacitance measurement in RO membranes

Since *Sphingomonas* have been reported as predominant members of RO membranes leading to biofouling (Bereschenko et al. 2010), it was envisaged that validation of this technique using RO membranes may provide a real time validation of the method developed. Consistent values were obtained (Table 4.14) over a four day period, these values did not differ significantly ($p>0.05$) from the capacitance values observed on coupons.

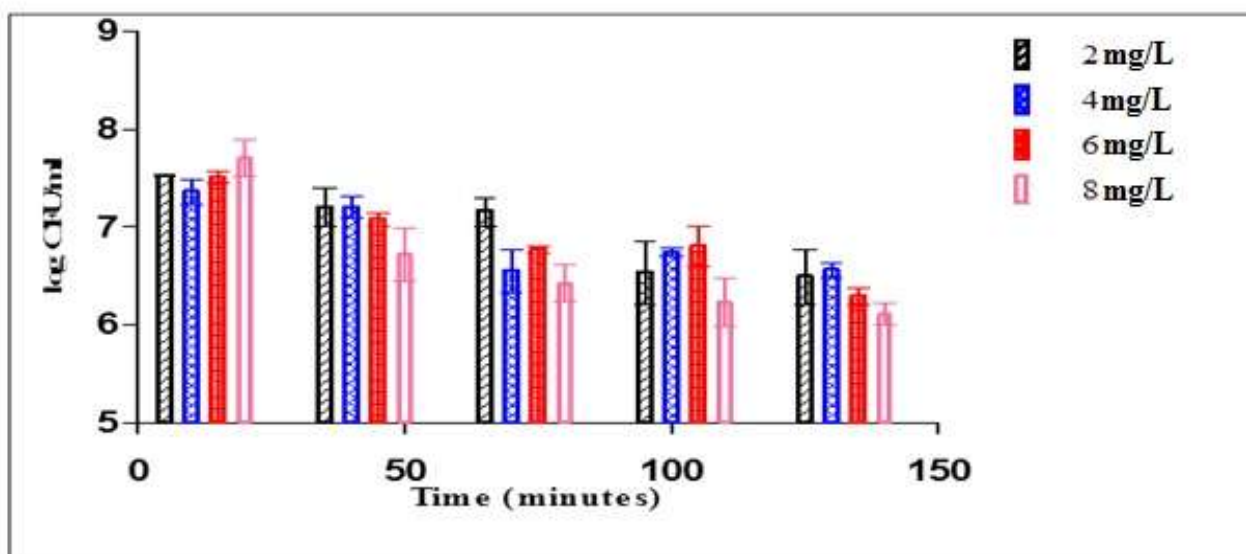
Table 4.14: Capacitance value in RO membrane by *S. paucimobilis* MG6. Data is Average $\pm$ Standard Deviation (SD) of triplicate samples

Time (hours)	Capacitance (RO) (mg)
0	0
12	1.6 $\pm$ 0.04
24	2.0 $\pm$ 0.05
36	3.7 $\pm$ 0.03
48	5.0 $\pm$ 0.02
60	6.1 $\pm$ 0.03
72	9.3 $\pm$ 0.10
84	9.8 $\pm$ 0.12
96	13.8 $\pm$ 0.11
108	14.8 $\pm$ 0.10
120	16.3 $\pm$ 0.22

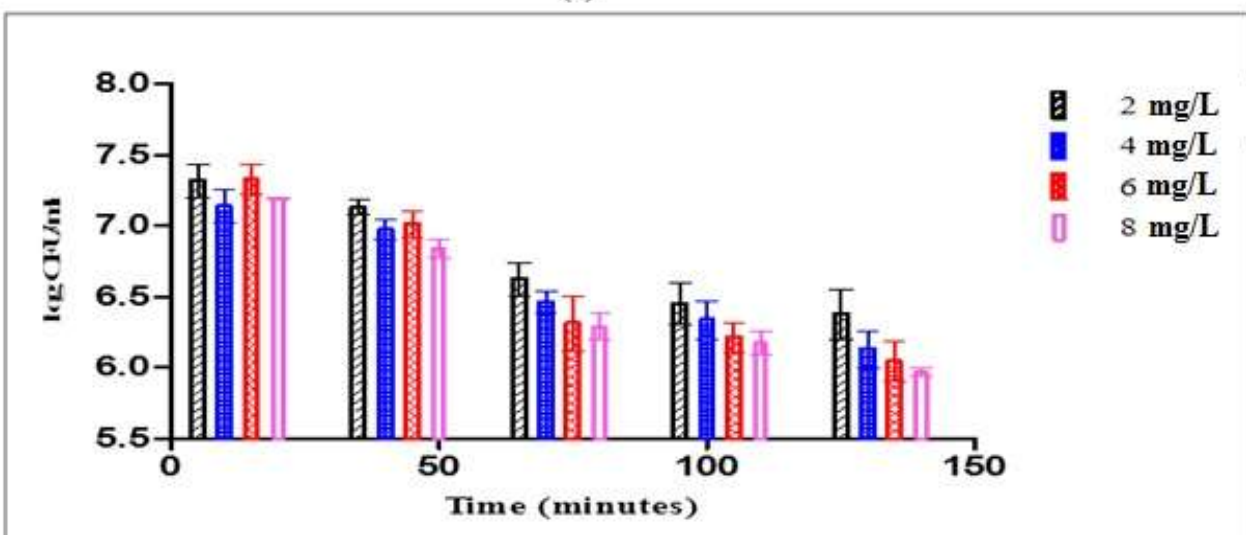
4.13 Chlorine resistance capability of *S. paucimobilis* MG6 biofilm in water distribution systems

4.13.1 Effect of residual chlorine on growth of *S. paucimobilis* MG6

Earlier studies have reported the importance of analyzing the effect of disinfectants especially chlorine on *Sphingomonas*. The various circumstantial variations in distributed water for instance, residual chlorine depletion, nutritional intrusion over long residence periods when water flows through a supply system can lead to regrowth and resistance in *Sphingomonas* (Lee & Park 2013) ultimately leading to poor water quality. Therefore, the effect of residual chlorine on the growth of *S. paucimobilis* MG6 at different contact times was estimated. The dosage of Sodium hypochlorite led to the reduction of log CFU/ml for both the strains. Four different concentrations of Sodium hypochlorite i.e. 2, 4, 6 and 8 mg/L were used (Gulati & Ghosh 2017).



(a)



(b)

Figure 4.25: Viability (log CFU/ml) of *S. paucimobilis* MG6 at different concentrations of Sodium hypochlorite on concentrations used were (a) 4×10^6 cells/ml (b) 8×10^6 cells/ml

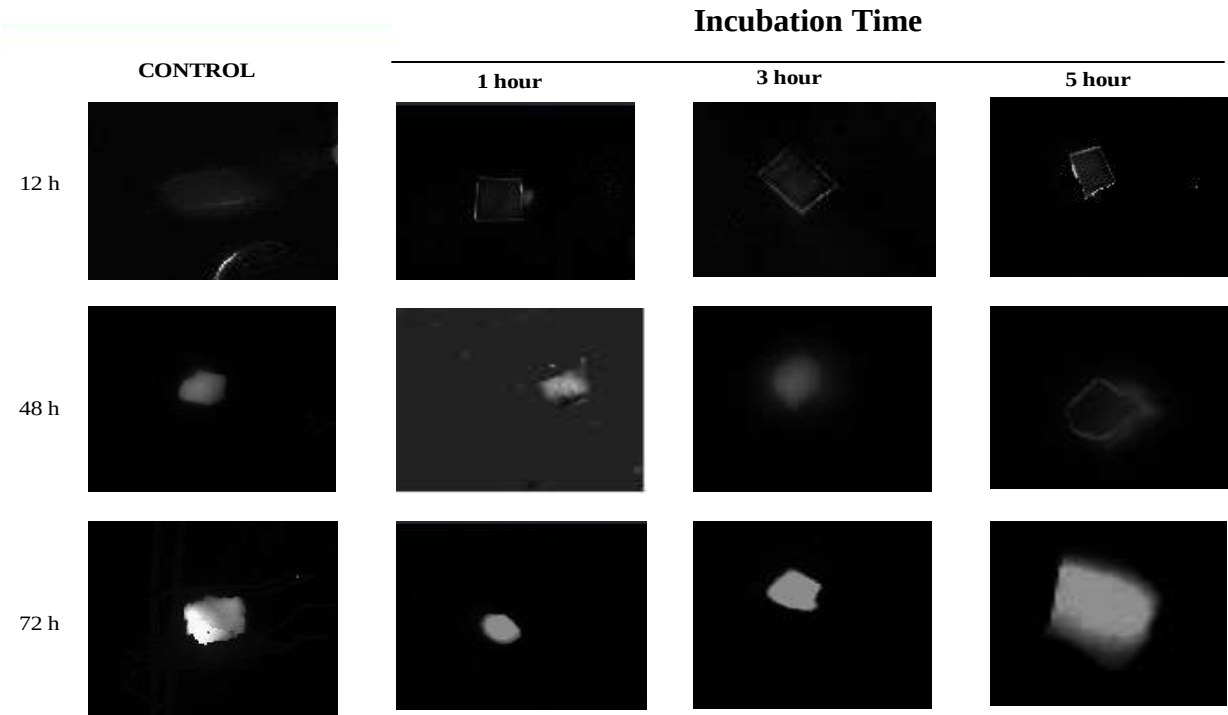
S. paucimobilis MG6 was found to be chlorine resistant at the concentration of 2 mg/L at the contact time of 90 minutes for different microbial loads (Figure 4.25). At higher concentrations, viability was reduced resulting in approximately 1.5 log reduction after 150 minutes.

The relative abundance of *Sphingomonas* in drinking water systems has been amply demonstrated in a large number of preceding studies. Barring a few reports, recent observations have indicated the elevated tolerance of *Sphingomonas* isolates to residual chlorine levels (Zhang et al. 2012). It has been emphasized that disinfectant resistance in predominant water microorganisms be critically reviewed. Based on the results of this study, it may be inferred that *S. paucimobilis* MG6 certainly deserves attention with regards to the observed resistance profile to chlorine.

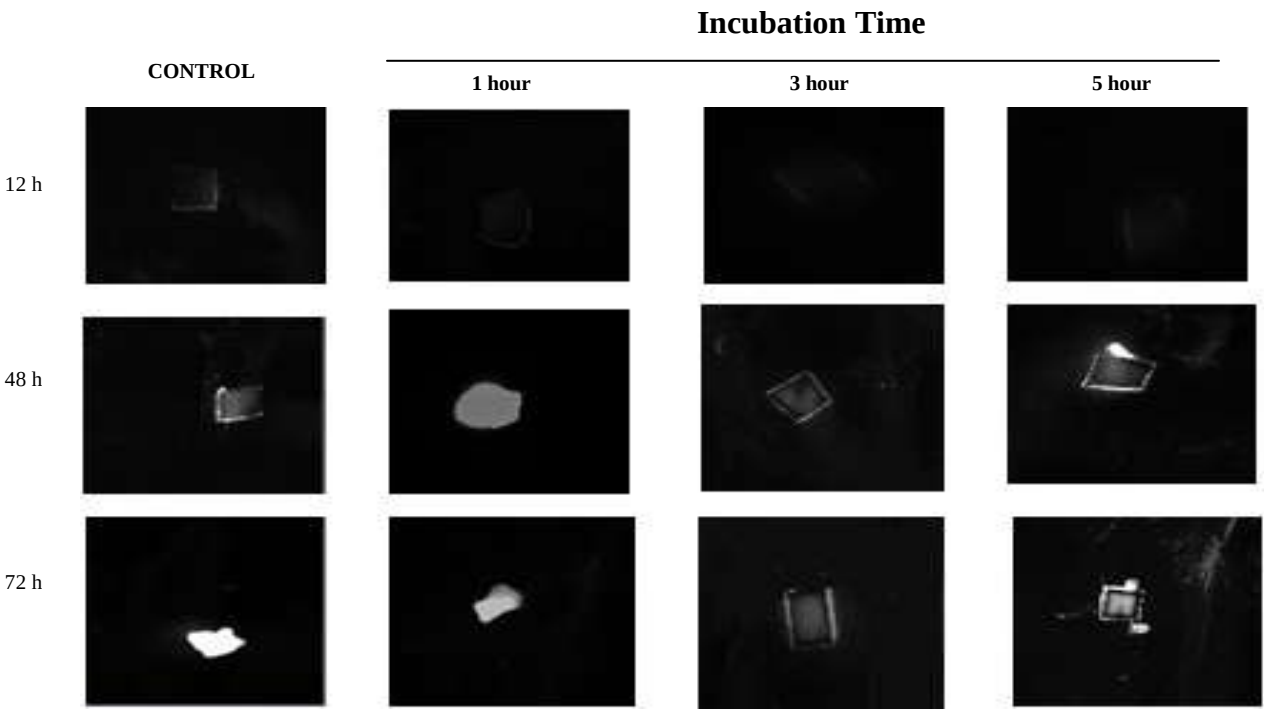
4.13.2 Qualitative estimation using calcofluor

(a) Effect of chlorine treatment on biofilm formation ability of *S. paucimobilis* MG6

The intensity of biofilm formation on PVC coupons recorded varied with time as depicted in Figure 4.26, with the increase of time span of chlorine treatment, little or no decrease in intensity of biofilm development was observed both with medium and water. Replicates of coupons were placed in fresh medium/water after each interval till 96 hours. Visualization indicated a slight increase in intensity possibly marking the biofilm development, indicating little or no reduction of biofilm cells. Le Chevallier et al (1988) described that adherence of bacteria to surfaces provided the greatest increase in disinfection resistance.



(a)



(b)

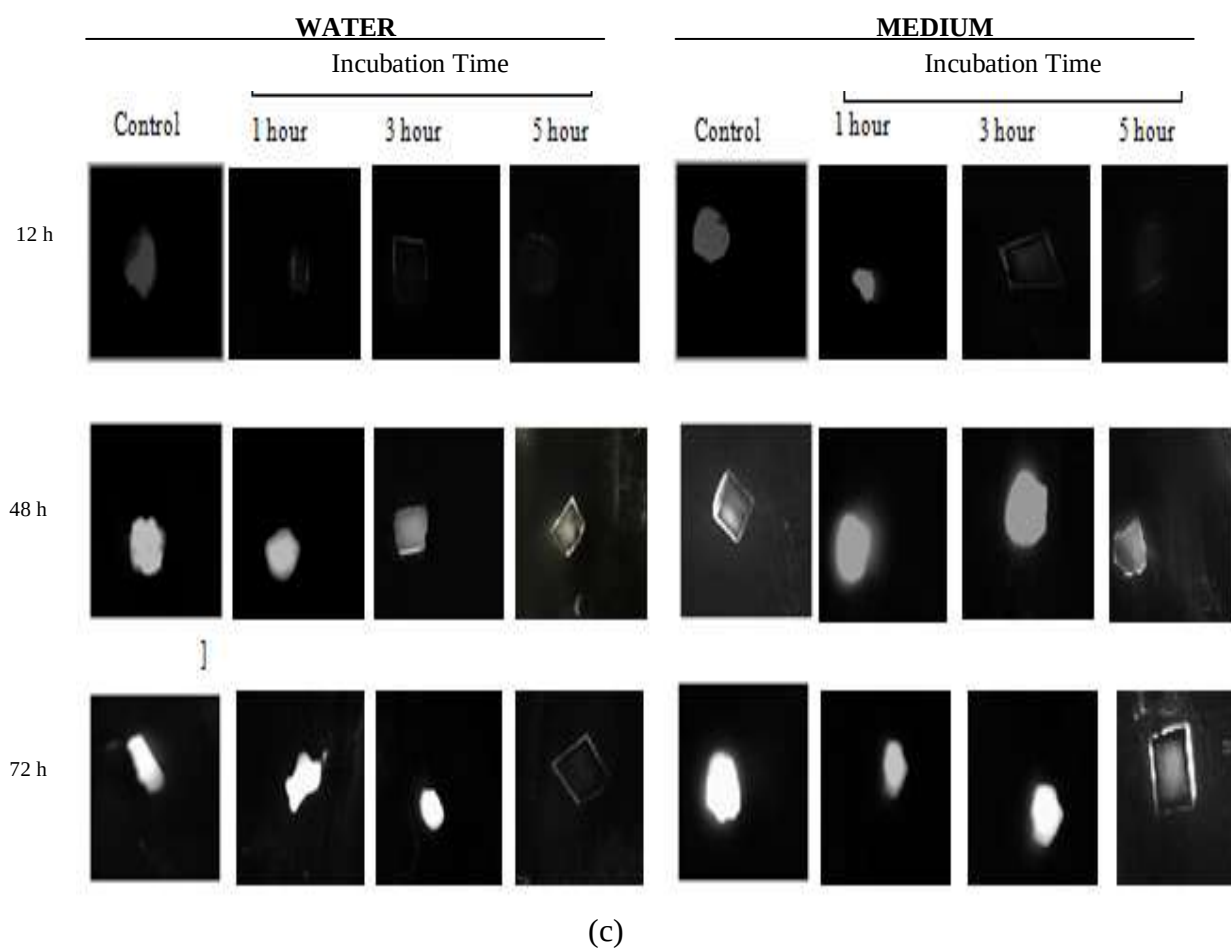


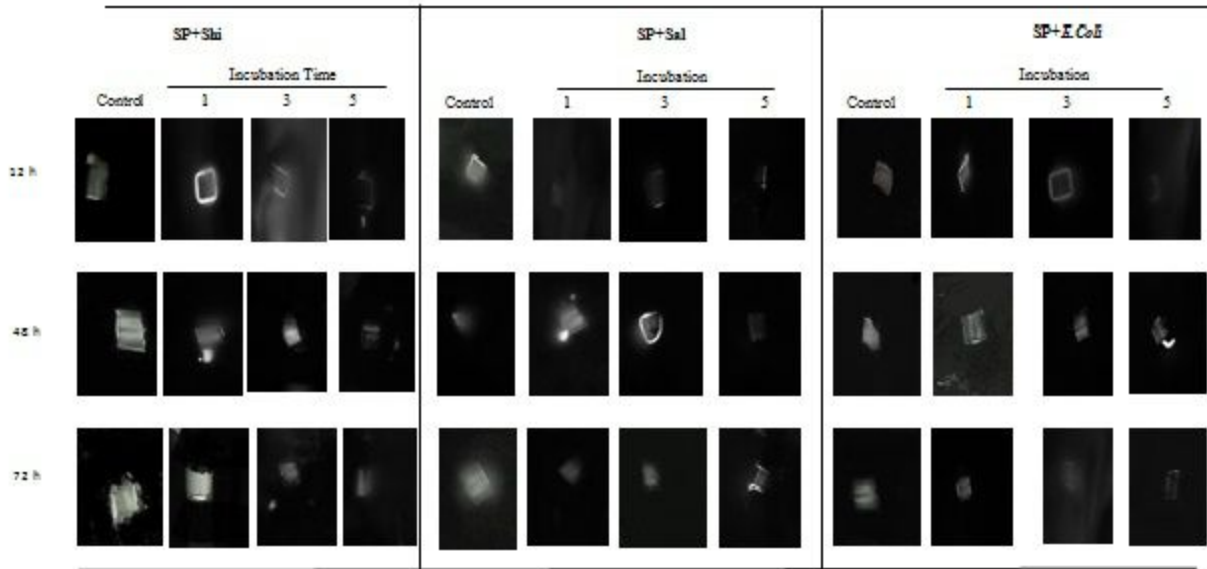
Figure 4.26: Effect of chlorine treatment on biofilm development on PVC using (a) Water (b) SSG Medium (c) after 96 hours of placing coupons in fresh media/water

Chlorination is an important intervention for controlling biofilm development in drinking water. It has been demonstrated that planktonic drinking water bacteria are inactivated following chlorination but biofilms, especially on the pipe walls are least affected with insignificant loss of viable biofilm cells (Lin et al. 2017). Effect of chlorination on *S. paucimobilis* MG6 biofilm were similar to those observed by other authors with enhanced resistivity (Wang et al. 2013, Lin et al. 2017).

(b) Effect of chlorine treatment on mixed biofilm of *S. paucimobilis* MG6 with waterborne pathogens

To evaluate the effect of sodium hypochlorite on the combination of *S. paucimobilis* MG6 with different waterborne pathogens, mixed biofilm was prepared on PVC coupons. After 12, 48 and 72 hours, PVC coupons were removed, incubated in 8 mg/L of residual sodium hypochlorite for 1, 3 and 5 hour and intensity were recorded. The intensity of the mixed biofilm of *S. paucimobilis* MG6 and *Shigella flexneri* 2a, was maximum indicating high resistance to chlorine followed by *Salmonella typhimurium* and *Escherichia coli* O157:H7 (Figure 4.27). It was also observed that waterborne pathogens enhanced the resistance ability of *Sphingomonas* biofilm to chlorine treatment.

An alarming consequence of synergistic interactions between microorganisms is associated with resistance to antimicrobial agents (Bennett 2008). Within mixed biofilms, such resistance does not necessarily involve direct acquisition or transfer of genes encoding antibiotic resistance, but rather relies on the ability of the community to cooperate in such a way that it can survive exposure to the antimicrobial agent. Mixed-species biofilms have been reported to be significantly more resistant to antimicrobial treatment or disinfection (Whiteley et al. 2001; Leriche et al. 2003; Al-Bakri et al. 2005; Burmolle et al. 2006; Kara et al. 2007).



(a)

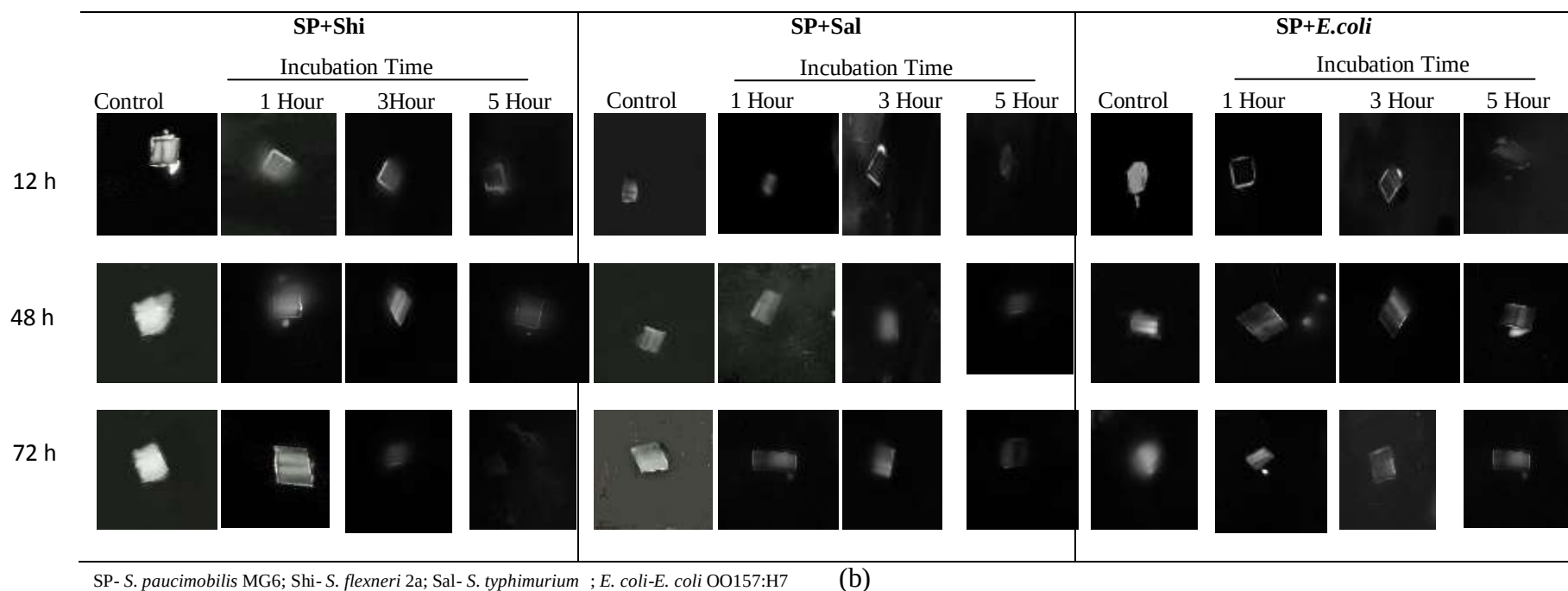


Figure 4.27: Effect of chlorine treatment on mixed biofilm of *S. paucimobilis* MG6 with waterborne pathogens (*E.coli*, *S. flexneri* 2a and *S. typhimurium*) on PVC coupons in (a) SSG Medium (b) Water

4.14 Flow through simulation of biofilm formation in plumbing materials

Surrounding environmental conditions are key factors to the growth of biofilms in biotechnological systems, in the human body, and in nature. Several studies have demonstrated that the microbial growth is strongly influenced by the nutrient availability, physical transport, and biofilm structure (Boessmann et al. 2004; Kuehn et al. 2001; Venugopalan et al. 2005). For example, biofilms have been observed to develop different morphologies under different flow conditions (Besemer et al. 2007; Chang et al. 2003; Purevdorj et al. 2002; Teodosio et al. 2011), hydrodynamic shear forces significantly affect the formation of biofilms in wastewater treatment plants (Liu & Tay 2002), and internal transport limitations are among many factors that cause biofilms to be highly resistant to chemical stresses (Kim et al. 2008).

It has been realized that distributions of flow, hydrodynamic shear on surfaces, and important chemical constituents define habitat conditions for microbial growth in biofilm (Battin et al. 2001; Kreft et al. 2001; Lapidou & Rittmann 2004; Rittmann 1982). Several flow through systems has been experimented to understand interactions between the biofilm growth and spatial patterns of key environmental conditions (de la Rosa & Yu 2005; Singer et al. 2010).

Although the objective of this study was not the evaluation of the physical effects during biofilm formation, it was important to understand whether the different plumbing materials used, actually supported biofilm formation by *S. paucimobilis* MG6 in the same fashion as observed under static conditions. The observations of the flow through simulation experiment corroborated the fact that plumbing materials supported biofilm development. Viable cells of *S. paucimobilis* MG6 were recorded from different scrapings of plumbing materials at different ages (Table 4.15).

A distinct variation was observed between different plumbing materials with log count of biofilm cells on polyvinyl chloride (PVC) - being the highest supported growth followed by polypropylene and polyethylene. A similar pattern and affinity was evident under static conditions as well. These results suggested the propensity of *S. paucimobilis* MG6 to exhibit stable biofilms in PVC, PP and PE based water distribution systems.

Table 4.15: The relationship between log counts of biofilm cells which formed on different pipe materials at different ages. PVC: polyvinyl chloride; PP: polypropylene; PE: polyethylene; Al: Aluminium; Cu: Copper; R: Rubber

Tested Pipe Plumbing materials	Biofilm ages (hours)		
	24	48	96
PVC	92.7	92.6	94.9
PP	88.1	84.2	91.7
PE	84.7	84.6	73.3
Al	74.5	79.0	85.9
Cu	75.8	74.4	84.1
R	74.1	73.2	72.3

*The values represented are log CFU /ml of *S. paucimobilis* MG6

4.15 Scanning Electron Microscope (SEM) and Energy Dispersive X-ray spectroscopy (EDS) of biofilms

Scanning photomicrographs enabled clearer interpretation of biofilm structure by *S. paucimobilis* MG6 on different pipe materials (Figure 4.28). Photomicrographs acquired at 12, 48 and 72 hours revealed that EPS secretion by cells began at 12 hours and biofilm development progressed actively till 48 hours. Fully matured biofilm occurred at 72 hours.

The cells were embedded in a polymer matrix and exopolysaccharides; *S. paucimobilis* MG6 growing on copper and rubber pipe produced lower amount of exopolysaccharides than the other types of pipes, hence, low biofilm formation.

Elemental composition of biofilm formed on different pipe materials by energy dispersive X-ray spectroscopy (Table 4.16) represented the weight percentage of different elements found at 12 and 72 hours on different pipe materials. It was observed that carbon and oxygen are the dominant elements in biofilm formed on all the plumbing materials followed by calcium and chlorine. Carbon and oxygen are interdependent and inversely related to each other as increase in carbon content is followed by consumption of oxygen by cells in PVC as cells are more metabolically active in this case. Inverse is the case with Polyethylene. Little variation was observed in polypropylene and Aluminium. Weaker signals from other elements present in biofilm like Magnesium, Potassium, Chromium and Iron were also obtained. The EDX analysis allowed better knowledge of observed structures.

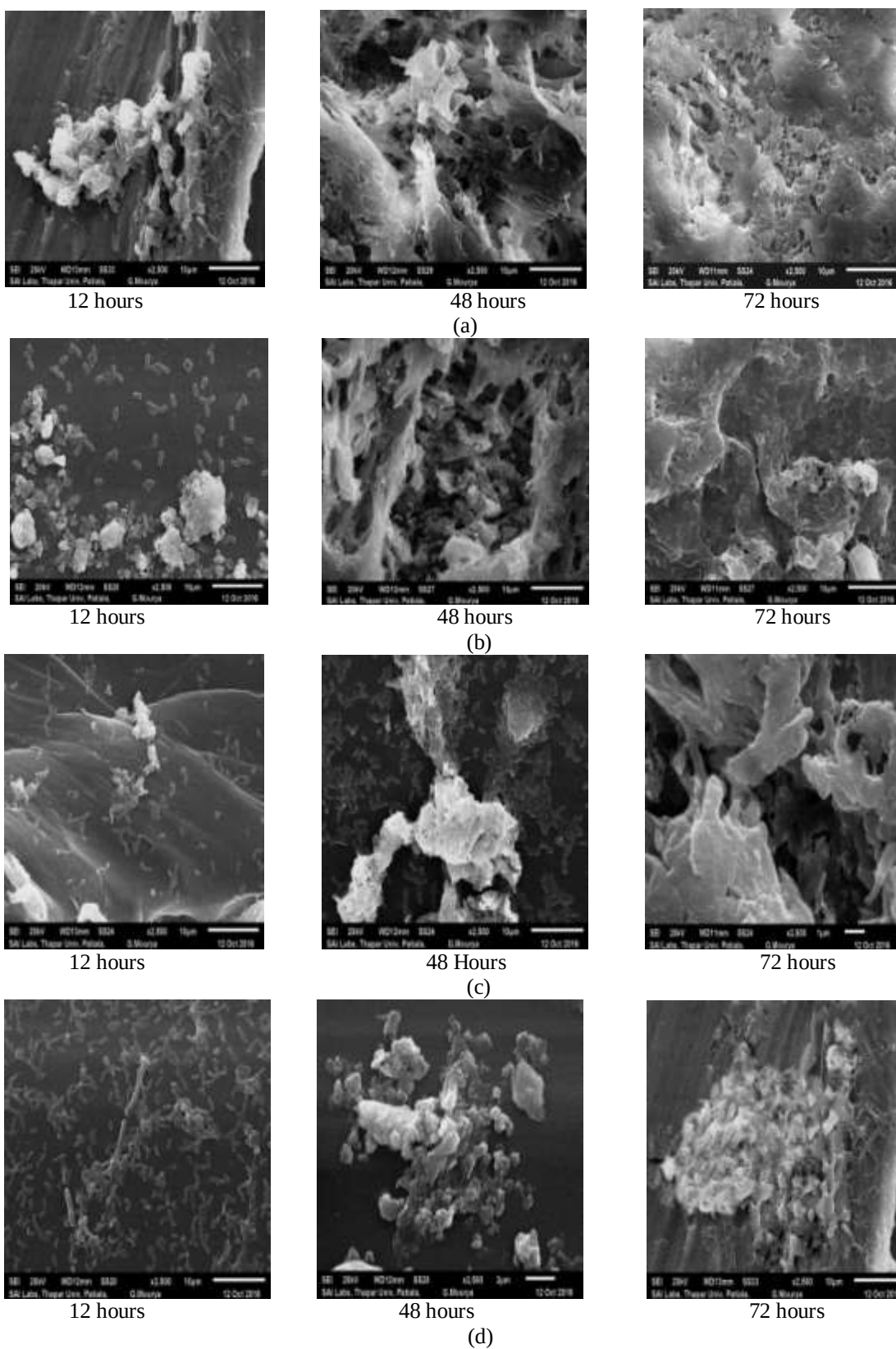


Figure 4.28: Scanning electron micrographs of *S. paucimobilis* MG6 biofilm progression at different time intervals on (a) PVC (b) PP (c) Al (d) PE (Magnification: x 2500)

Table 4.16: EDX Analysis elemental composition (Weight percentage) of biofilm at different time intervals

Elements	PVC		PP		Al		PE	
	12 hours	72 hours	12 hours	72 hours	12 hours	72 hours	12 hours	72 hours
Carbon	24.86	68.12	90.87	82.11	90.26	89.26	77.56	58.69
Oxygen	38.07	5.17	8.91	16.82	7.00	9.91	6.95	32.34
Magnesium	0.04	0.20	0.11	----	0.49	0.19	0.10	0.19
Chlorine	12.46	22.62	----	0.62	----	----	14.71	1.61
Potassium	0.01	----	----	0.07	----	0.03	----	0.37
Calcium	24.51	3.53	0.03	0.13	0.31	----	0.57	4.20
Chromium	0.05	0.29	----	----	----	0.07	----	0.26
Iron	----	----	0.09	0.10	----	0.17	----	----
Sodium	----	0.08	----	0.15	----	0.07	0.11	0.57
Silicon	----	----	----	----	----	----	----	----
Phosphorus	----	----	----	----	1.94	0.30	----	1.78
Total	100	100	100	100	100	100	100	100

4.16 Mixed biofilm profile by *Sphingomonas*

Scanning electron microscopy (SEM) was used to visualize the cell-surface of biofilm forming *Sphingomonas* with adhered pathogens on PVC coupons. Mixed biofilms (*S. paucimobilis* MG6/*S. terrae* 7766 + *E. coli*, *S. flexneri* 2a and *S. typhimurium*) exhibited variation than that observed in the controls and reached maturation with 65-70 hours by the kinetics (Figure 4.29). Tight adherence of the biofilm cells with PVC was observed for *S. paucimobilis* with the cells being enmeshed in exopolysaccharides (Figure 4.30).

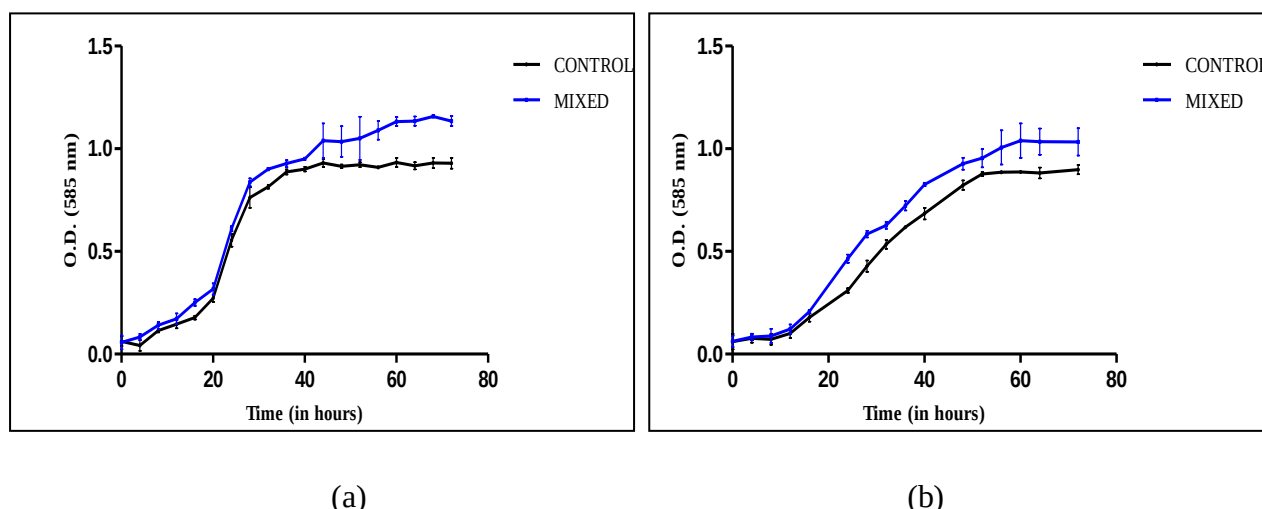


Figure 4.29: Kinetics of mixed biofilm formation of (a) *S. paucimobilis* MG6 (b) *S. terrae* 7766 with water borne pathogens (*E. coli*, *S. flexneri* 2a and *S. typhimurium*).

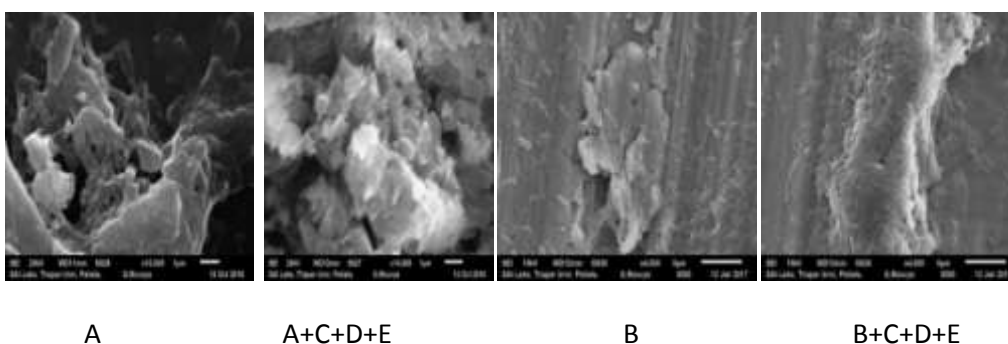
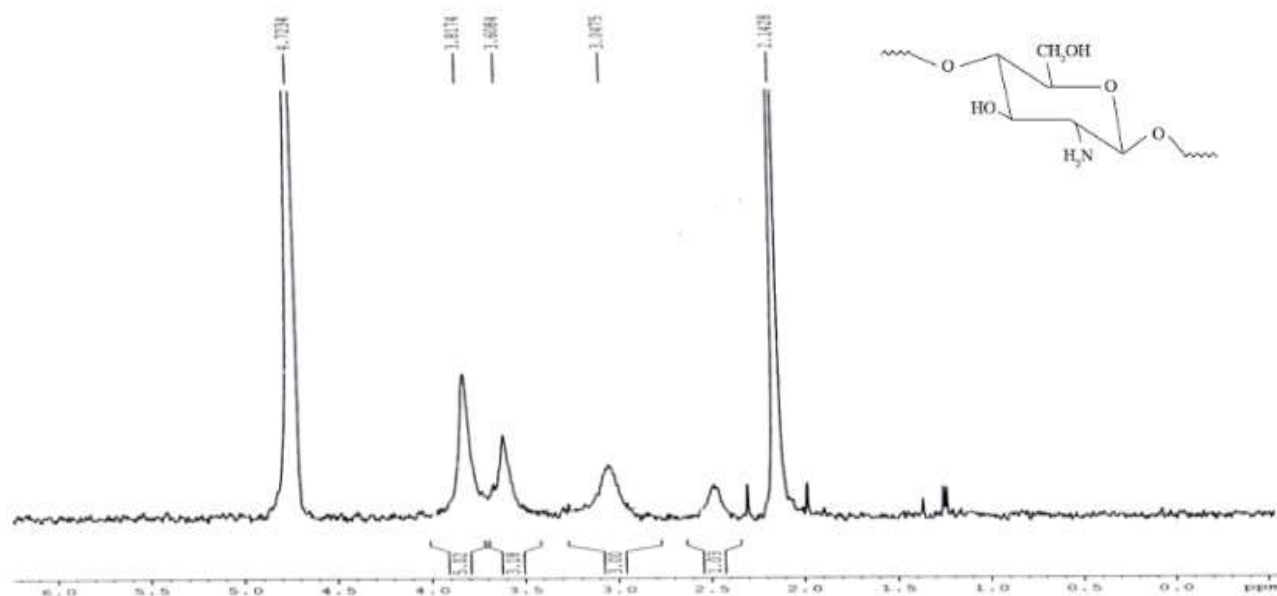


Figure 4.30: Scanning electron Micrographs of mixed biofilms of (a) *S. paucimobilis* MG6 (b) *S. terrae* 7766, where, A=*Sphingomonas* isolate; B=*Sphingomonas terrae* MTCC 7766; C= *Salmonella typhimurium* ATCC 25315; D= *Escherichia coli* O157:H7 ATCC 32150; E= *Shigella flexneri* 2a. Mixed biofilms were developed on PVC coupons.

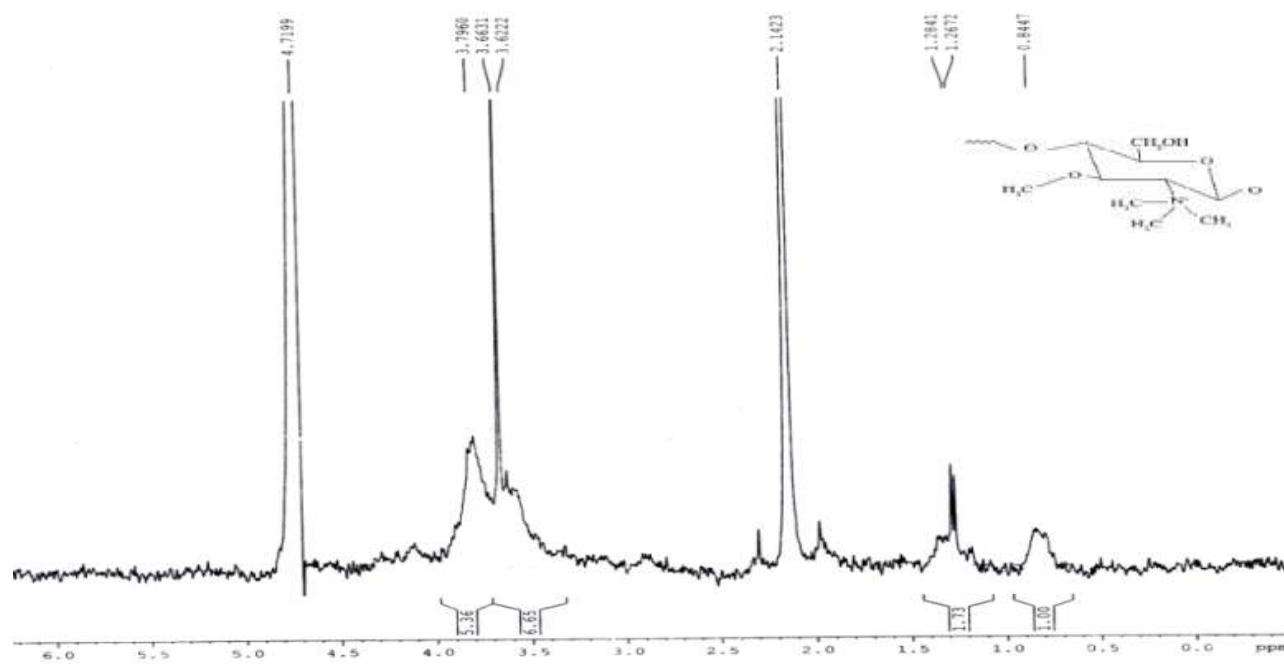
Though *S. terrae* produced less exopolysaccharides, adherence was observed on PVC. The results suggested stable attachment of pathogen-*S. paucimobilis* biofilm on PVC matrix.

4.17 Development of Quaternized biopolymer (QB) and N-methyl biopolymer (NMB) as quorum quenchers

Amino group present in the polysaccharide backbone of the biopolymer were methylated at C-2 position to form quaternary amino groups having fixed positive charges on the repeating units of the quaternized polymer chain. Quaternization process involving dimethyl sulphate or methyl iodide has been reported for producing quaternary ammonium compounds of chitosan (de Britto et al. 2011; Belalia et al. 2008; Tan et al. 2013). Thus, the suitability and applicability of the same was attempted in case of the selected biopolymer in the present study. Degree of quaternization (DQ) was measured after 6 hours of stirring with dimethyl sulphate yielding (DQ) of 71%. The N-methylbiopolymer was obtained with yield of 460 mg per 500 mg of biopolymer (87%). The singlet peak at 3.66ppm of ¹H NMR spectra represents the trimethylated product of biopolymer (quaternized biopolymer). The peak at 2.76ppm is related to monomethylation on the basis of ¹H NMR spectral analysis of modified chitosan was similar to that reported by Sieval et al. (1998). The NMR results in this study indicated methylation of the native biopolymer (Figure 4.31).



(a)



(b)

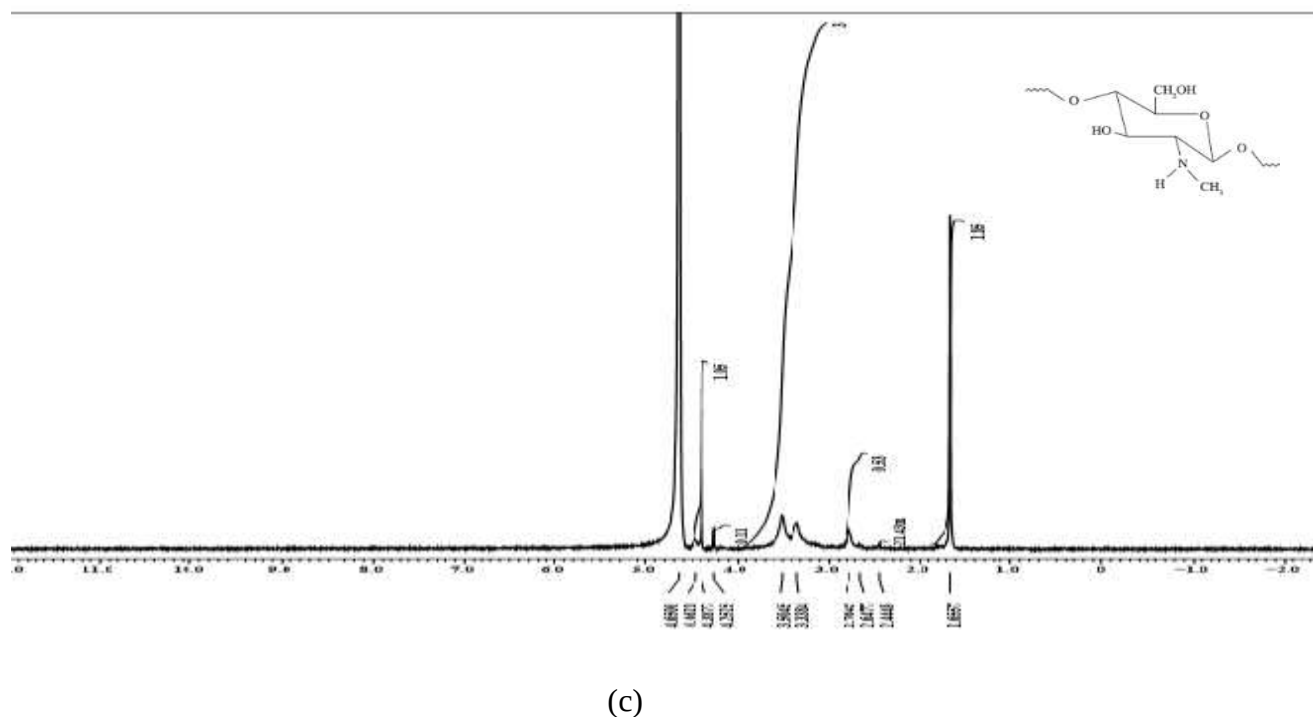


Figure 4.31: ^1H NMR spectrum of (a) Biopolymer (BP) (b) Quaternized Biopolymer (QB) (c) N-Methyl Biopolymer dissolved (NMB) in D_2O

4.18 Determination of AHL production in *Sphingomonas* spp.

AHL signaling has been found to be important for biofilm formation; the biofilms consists of mushroom-like microcolonies separated by water channels (Davies et al. 1998; Huber et al. 2001; Lynch et al. 2002; Steidle et al. 2002). Until now, there has been little evidence for AHL regulation of biofilm development in *Sphingomonas*. Therefore an attempt was made to detect AHL in the isolate. For this, the CV026 inhibition test was carried out since these molecules are expected to inhibit the induced CV026 and produce colorless colonies in purple background (Figure 4.32). Moreover, this particular assay is useful for detecting the acyl chains ranging from C_{10} to C_{14} (McClellan et al. 1997). The *A. tumefaciens* A136, a broad range biosensor, was employed thereafter to confirm the presence of the C_8 to C_{12} -HSLs including oxo- C_6 -HSL and is quite sensitive to even low level of longer acyl side chain AHLs (Farrand et al. 2002).

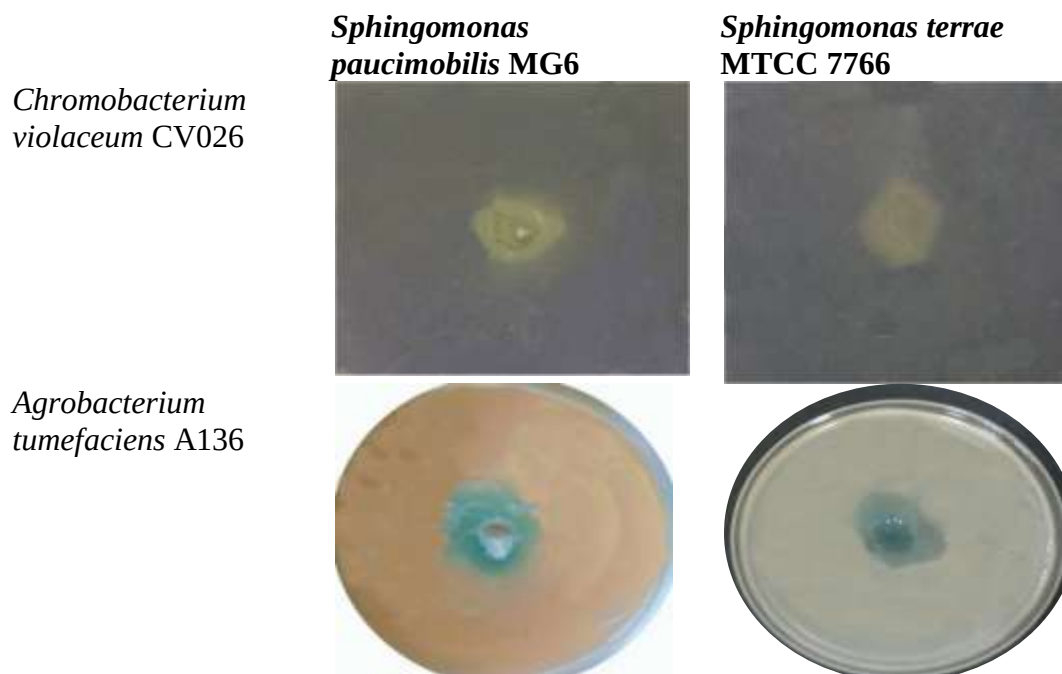


Figure 4.32: Detection of acyl homoserine lactones (AHL) production in *Sphingomonas paucimobilis* MG6 and *Sphingomonas terrae* MTCC 7766. *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136 bioreporter strains were used.

4.19 Bioactivity based Thin Layer Chromatography for detecting AHLs

TLC when coupled with a biosensor proves to be a simple and rapid method for assessing the minimum concentration of AHLs produced. TLC separation of bacterial extracts of *S. paucimobilis* MG6 displayed autoinducer activity with *Agrobacterium tumefaciens* bioreporter strain. Figure 4.33 shows signal molecule which corresponds to retention factor (R_f) of 0.51 both in *S. paucimobilis* MG6 and *S. terrae* 7766. However it has been realized that the specific structure of AHL signals cannot be determined by TLC, though their chromatographic properties are exhibited fairly well and has been widely used to assign a tentative identity. Therefore further analytical confirmation is usually deemed necessary.

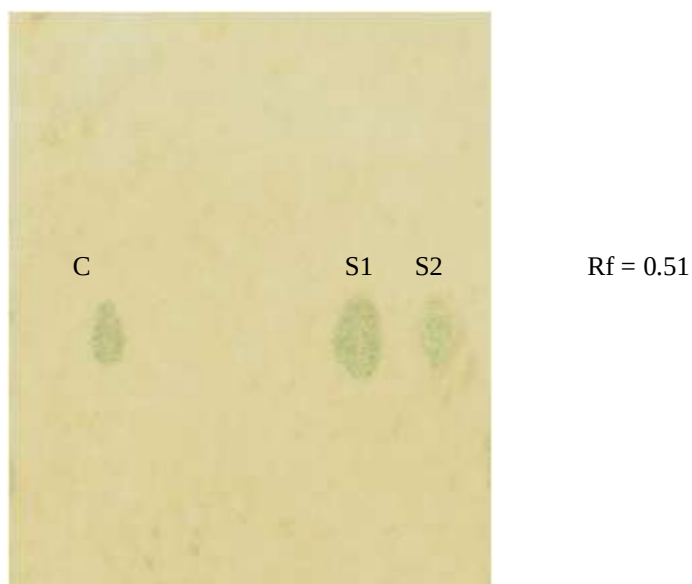
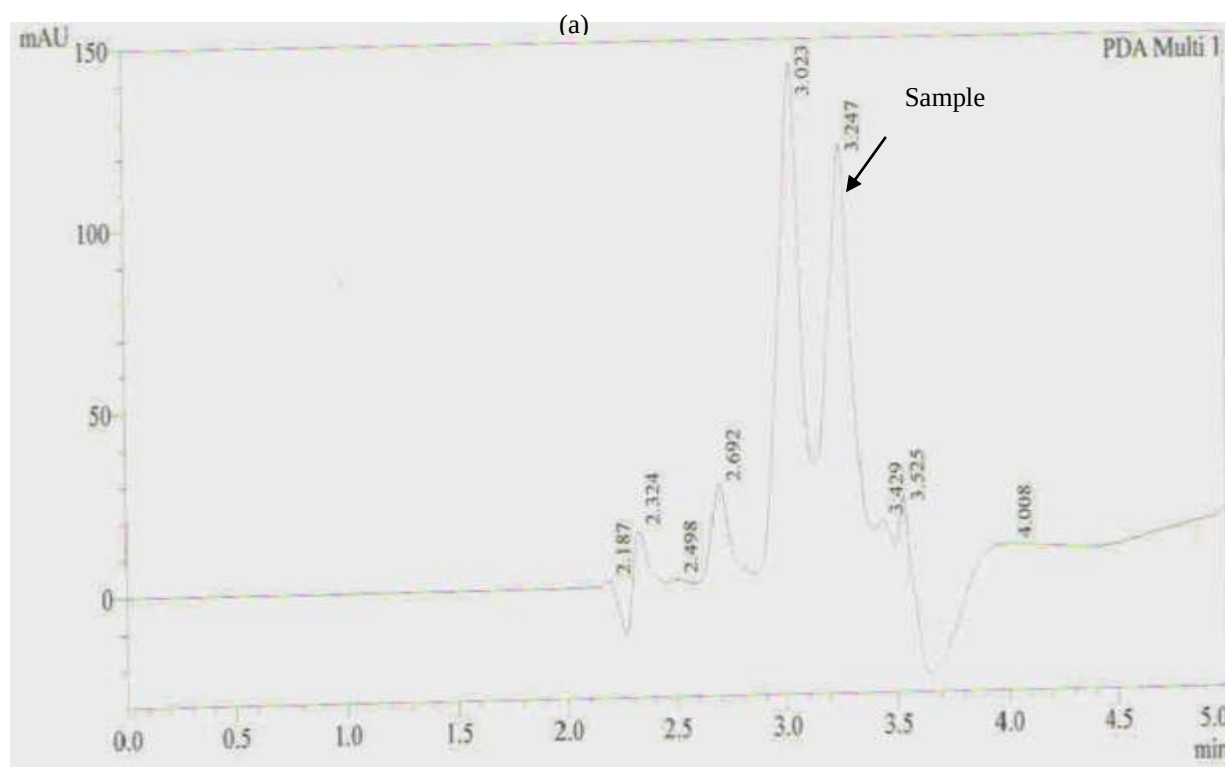
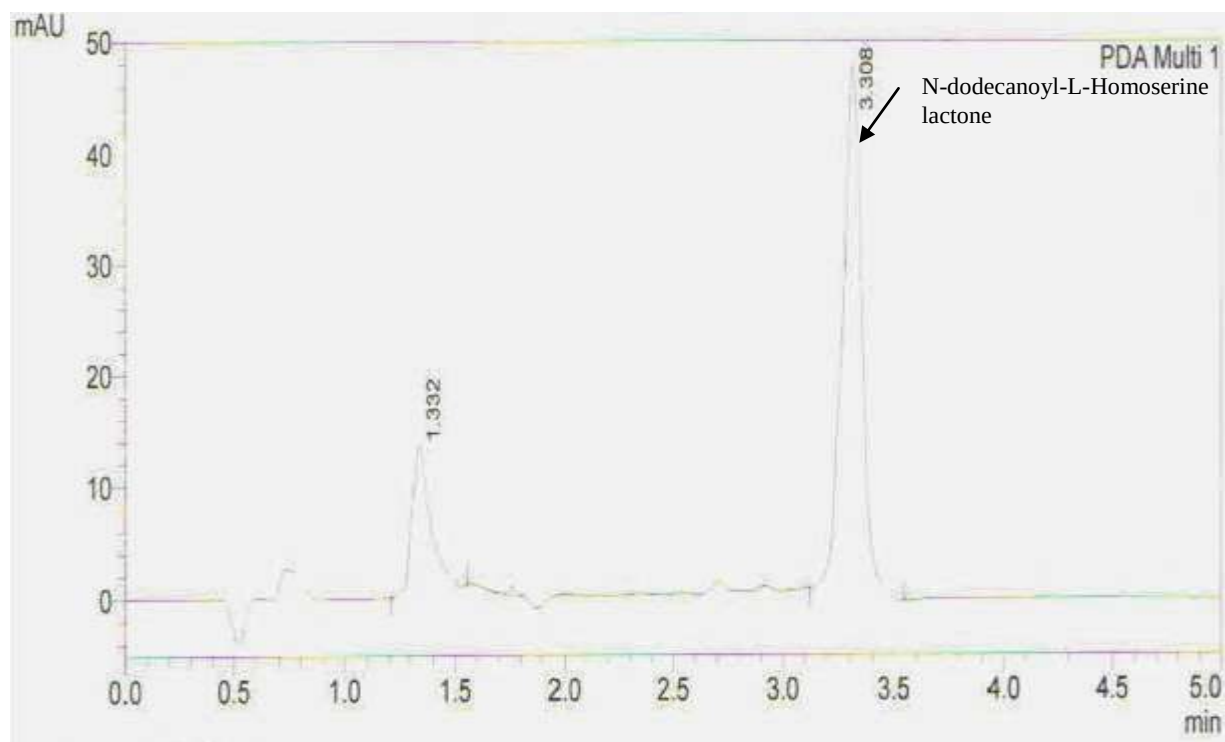


Figure 4.33: Thin layer chromatography (TLC) of AHL extracts of (a) S1= *S. paucimobilis* (b) S2= *S. terrae*; C= Standard AHL (N-dodecanoyl-L-Homoserine lactone)

4.20 AHL Extraction and HPLC analysis

HPLC analysis was carried out to compare the retention time of AHL extracted from *S. paucimobilis* MG6 with that of reference compounds (N-Hexanoyl-L-homoserine lactone, N-dodecanoyl-L-Homoserine lactone and N-3-oxo-dodecanoyl-L-Homoserine lactone). Retention time of N-dodecanoyl-L-Homoserine lactone was found to be 3.308 minutes while that of the sample was 3.247 minutes (Figure 4.34). Statistical analysis by p-test indicated no significant difference in retention time between reference and the compound from extracts.



(b)

Figure 4.34: High performance liquid chromatograms of (a) dodecanoyl-L-Homoserine lactone (Standard compound) (b) AHL sample of *Sphingomonas paucimobilis* MG6

4.21 Identification of AHLs using GC-MS

Detection and characterization of AHLs has been attempted recently using advanced analytical methods including GC-MS. The structural conformation of AHL was carried out by GC-MS, AHL was predicted at the retention time of 14.2 minutes (Figure 4.35). The mass spectrum of each compound showed ion fragments which are characteristic of homoserine lactone molecule. MS showed three specific ions at m/z 156, 57 and 71 which are similar to that observed by Park et al (2001). This can be related to the presence of C_{12} -AHL. The TIC trace with common precursor ions are depicted in Figure 4.35.

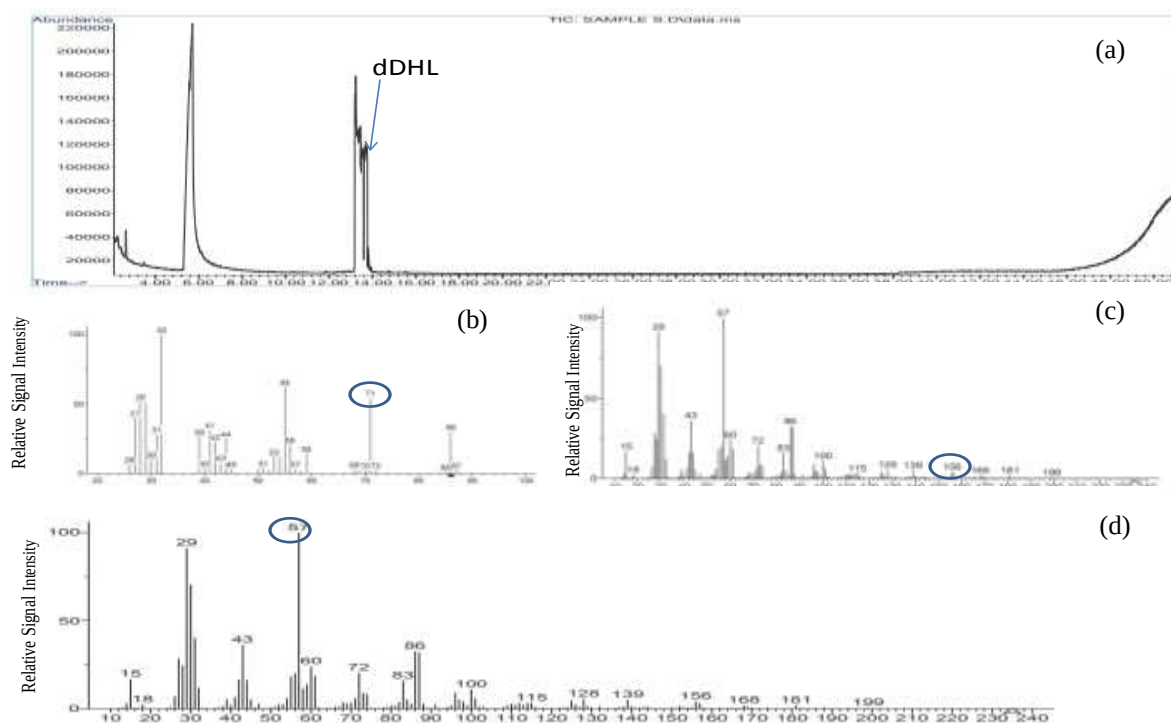


Figure 4.35: GC-MS spectra of the AHLs extracted from the cell-free supernatants of *S. paucimobilis* MG6 in full scanning positive mode (a) Total Ion Chromatogram (b)-(d) Ion-fragments at different m/z . The highlighted m/z values represent diagnostic fragment ions.

4.22 Acyl homoserine Lactone(AHL) synthesis and its correlation to biofilm production in *S.paucimoblis* MG6

4.22.1 Transposon mutagenesis of *S. paucimobilis* MG6 for AHL negative mutants

Transposon mutants have been used to decipher several regulatory aspects in biofilm production therefore transposon mutagenesis of *S. paucimobilis* was attempted with a view of generating mutants incapable of AHL production. It was anticipated that these mutant(s) could help establish a correlation of AHL with biofilm formation.

A total of 534 transconjugates were screened after culturing on antibiotic containing plates which confirm resistance against kanamycin and nalidixic acid. *S. paucimobilis* MG6 was used as control which induces blue color; the AHL-negative mutant was selected on the basis of inability of inducing blue color by using biosensor strain *A. tumefaciens* A136 and it was designated as MG12 (Figure 4.38). The Tn5 element in chromosome of *S. paucimobilis* was confirmed by PCR amplification using Tn5 specific primers. A 625 bp amplicon was generated using MG12 and positive control (pGS9 vector); no amplification was observed in wild MG6 (Fig. 4.36).

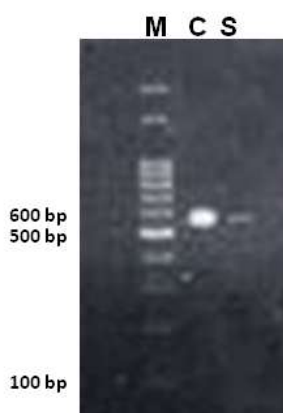


Figure 4.36: Presence of transposon (Tn5) in mutant of *S. paucimobilis* MG6

M –DNA ladder (100bp); C – Positive Control (pGS9 vector); S – Sample

Evaluation of biofilm formation by the *S. paucimobilis* MG12, indicated its failure to form biofilm as revealed by the crystal violet assay (Figure 4.37). *N*-Acylhomoserine lactone (AHL)-mediated quorum sensing (QS) controls diverse phenotypic traits in various gram-negative proteobacteria (Lade et al. 2014) However, despite the proposed role of QS systems in bacterial colonization of surfaces, only a limited number of organisms are known to require a functional AHL-mediated QS system for formation of biofilms for instance, in *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Aeromonas hydrophila*. Therefore it was of relevance to elucidate both existence and functionality of AHL in the regulation of biofilm formation.

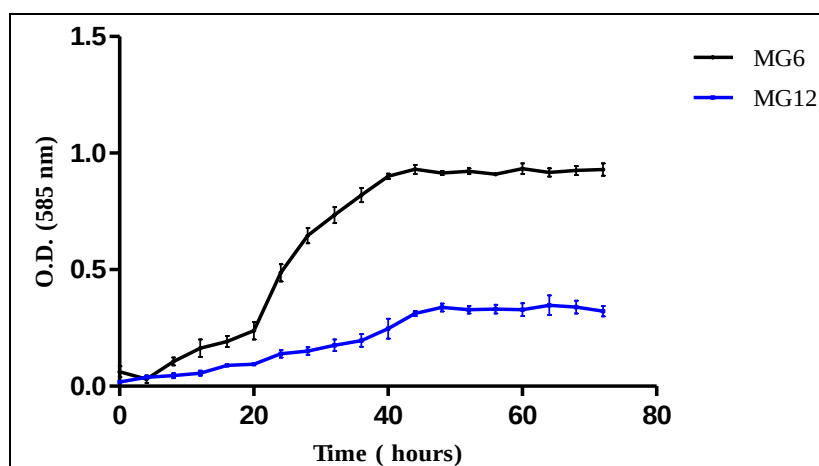


Figure 4.37: Biofilm formation observed in wild type strain *S. paucimobilis* MG6 and its mutant counterpart MG12

Mixing experiments were further conducted, wherein concentrated culture supernatants of *S. paucimobilis* MG6 were supplemented to growing MG12 in microtitre plates. Since the wild type culture supernatants contained AHL molecules, it was envisaged that supplementation should restore the ability to form biofilms by the mutant. Detection of biofilms in the AHL supplemented mutant clearly indicated the role of AHL in *S. paucimobilis* MG6 for biofilm

formation; this observation also suggested that antibiofilm compound developed should target AHL for inhibiting biofilm formation in *S. paucimobilis* MG6 (Figure 4.38).

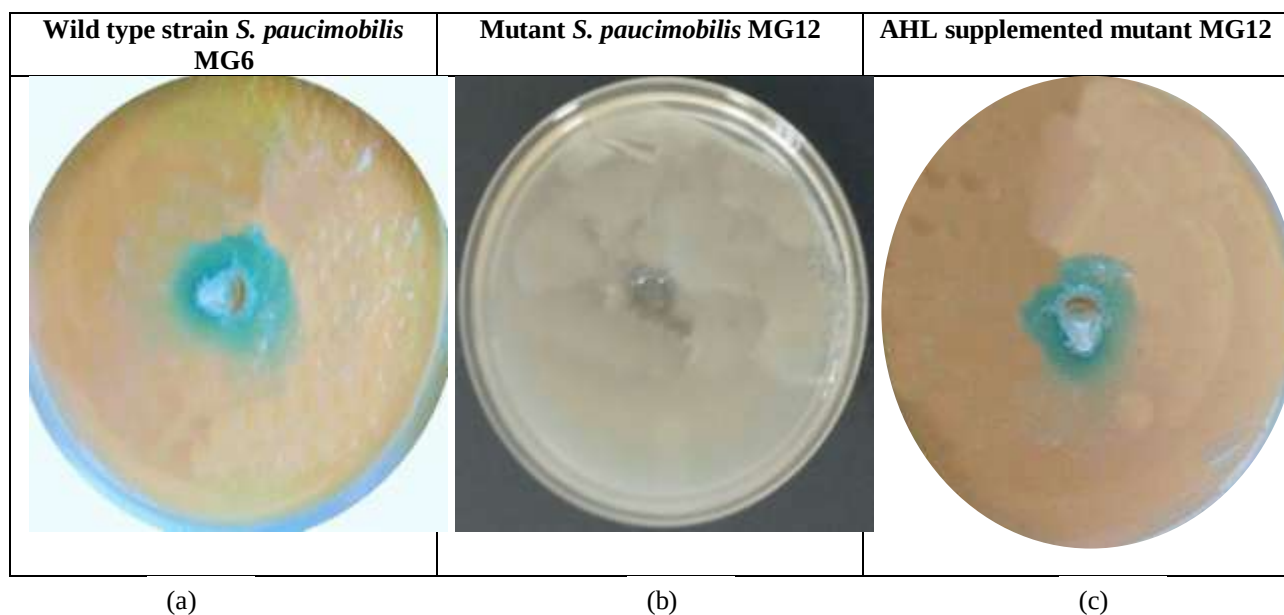


Figure 4.38: Detection of AHL production using *A. tumefaciens* A136 in (a) *S. paucimobilis* MG6 (wild type strain) (b) *S. paucimobilis* MG12 (mutant) (c) AHL supplemented mutant MG12

4.23 Development of biopolymer anti-biofilm agents

Biofilm development has been the focus of intense interest recently, and the ultimate goal is to develop methods for biofilm prevention, control, or eradication. Biopolymers with ability to interfere quorum sensing mechanism and thereby disable biofilm production has however received less emphasis. The biopolymer producing environmental isolate, *Klebsiella terrigena* was reported by Ghosh et al (2009) and Khaira et al (2014). Several desirable characteristics, safety and functionality of the exopolymer prompted evaluation of this for chemical modification. The biopolymer was extracted and purified from *Klebsiella terrigena* and designated as BP. Thermal behavior is an important attribute which dictates the performance and fate of the biopolymer during further processing. Therefore, it was important to determine this in

the *K. terrigena* biopolymer. Pyrolysis characterization carried out using thermogravimetric analysis (TGA) (Figure 4.39) indicated a degradation temperature (T_d) of around 250°C as estimated from the curve; the biopolymer showed an initial weight loss due to moisture content. The initial moisture content in the sample may be ascribed to the increased level of carboxyl groups in the biopolymer. Decomposition was evident at about 220°C and the recorded mass loss was 22%. The polymer lost weight around 270°C and retained about 15% of the weight after exposure at 750°C . These results indicated the thermal stability of the biopolymer (BP) and amenability to further processing.

The biofilm inhibitory activity of the biopolymer was evaluated by crystal violet assay. It was observed that 0.01 mg/ml BP and 0.02 mg/ml BP inhibits biofilm by 35.5% and 40%. 0.03 mg/ml and 0.04 mg/ml Chitosan (used as control) inhibits 26.2% and 39% biofilm (Figure 4.40). However, the observed inhibition of biofilm by BP was fairly low, thus it was deemed appropriate to further modify it to enhance its antibiofilm property..

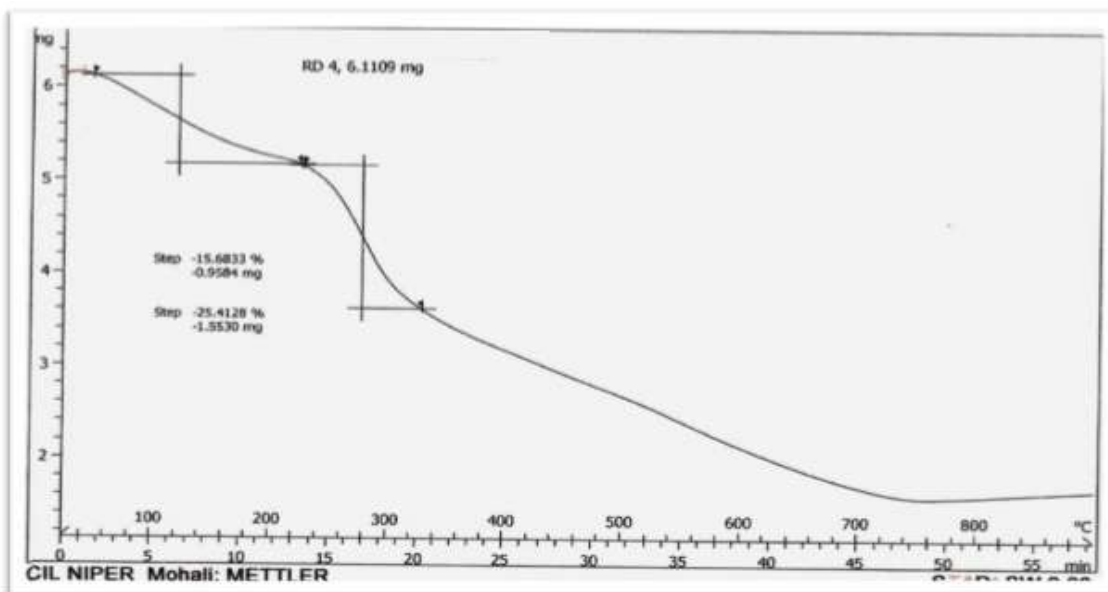


Figure 4.39: Thermal gravimetric analysis of the purified biopolymer from *Klebsiella terrigena*

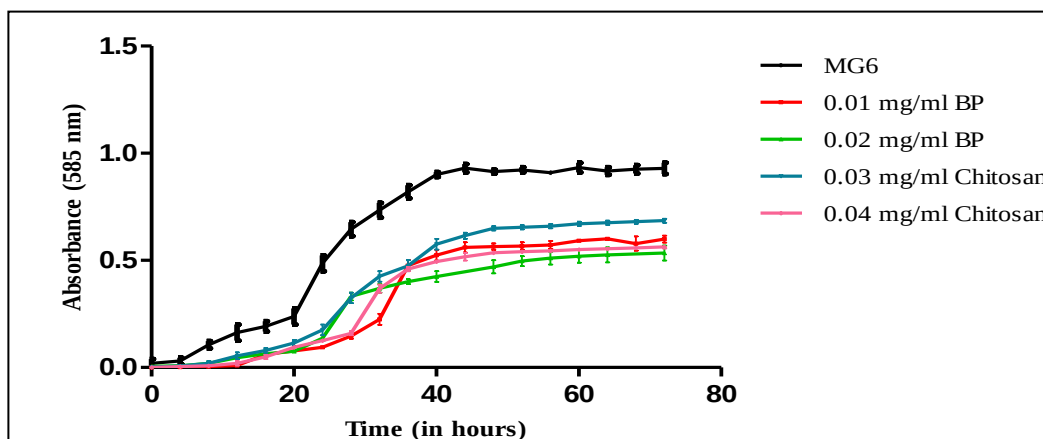


Figure 4.40: Comparison of inhibition of *S. paucimobilis* MG6 biofilms by Biopolymer (BP) and Chitosan.

4.24 Anti quorum sensing activity of QB and NMB on *S. paucimobilis* MG6

Since Quorum sensing inhibition depends on the disruption of bacterial signaling and not on antibacterial activity, it is pertinent to ensure that anti-quorum sensing activity is not a reflection of antibacterial activity. Quorum sensing inhibitory activity is usually measured at sub-inhibitory concentration of a compound (Williams 2007). Paper disc diffusion assay was carried out to rule out this possibility. Sub-inhibitory concentrations were selected (0.7 mg/ml NPO, 1 mg/ml NPO, 0.05 mg/ml QB, 0.07 mg/ml QB, 0.01 mg/ml NMB and 0.03 mg/ml NMB) for further analysis. Production of blue coloration by quorum sensing signal communications in A136 provides a naturally occurring, readily observable phenotype and eases the determination of quorum sensing inhibition of compounds in *Sphingomonas*. Quorum sensing inhibition was checked by disc diffusion assay using the biosensor strain *A. tumefaciens* A136. The results of this analysis are depicted in Figure 4.41. Exposure to the compounds significantly reduced the quorum sensing both in isolate and standard strain. It was observed that as the concentration of the compounds increased, the blue zone determining the intensity of AHL decreased. 4-NPO, a known quorum sensing inhibitor was used as positive control in this study.

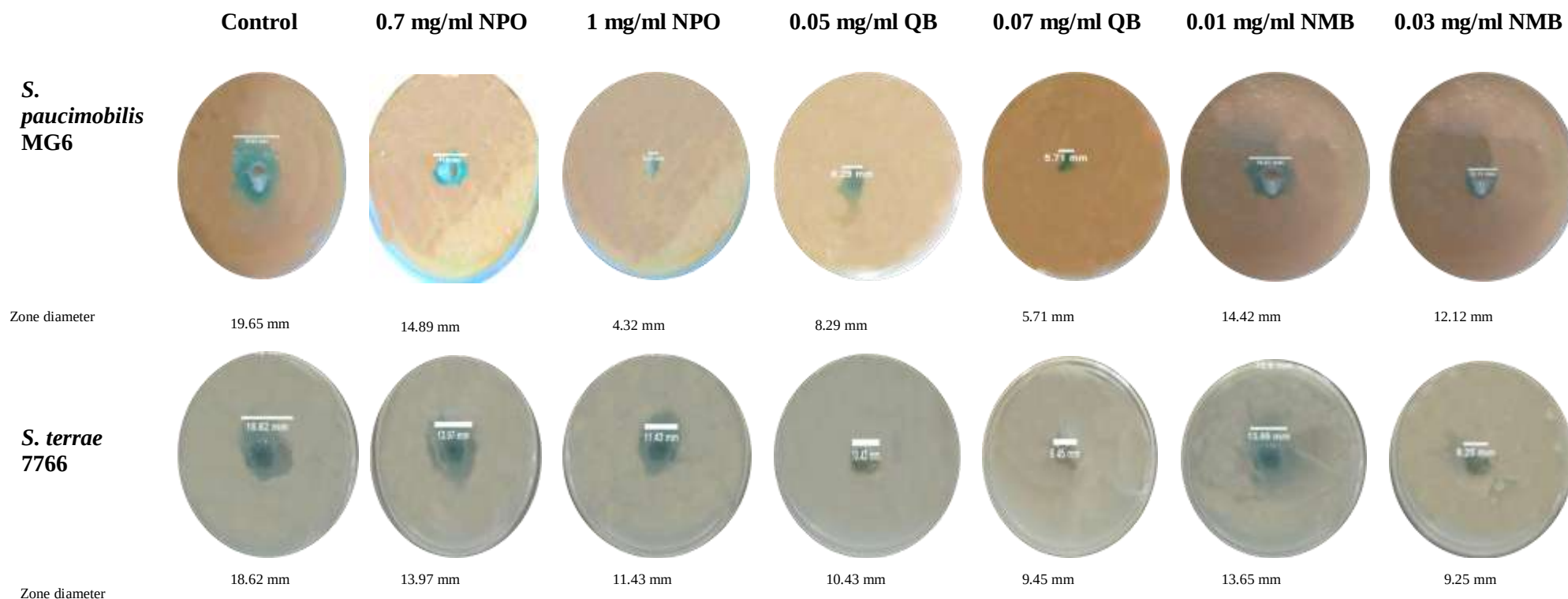


Figure 4.41: Quorum sensing inhibition in *S. paucimobilis* MG6 and *S. terrae* 7766 using varying concentrations of 4-NPO, Quaternized biopolymer (QB) and N-methylbiopolymer (NMB). The zone of diameter indicates the extent of blue color produced (AHL) using *A. tumefaciens* A136 (bioreporter strain)

4.25 Effect of QB and NMB on the expression of *LacZ* in *E.coli* (Miller 1972)

AHL can be quantified by its ability to activate *traR* gene in the β -galactosidase reporter strain *A. tumefaciens* A136 (Zhu et al. 2002). Therefore interferences leading to loss or reduction in AHL should be reflected in β galactosidase production; besides the extent can be quantified. Both, QB and NMB caused AHL mediated reduction in β -galactosidase activity of *A. tumefaciens* A136. Miller Units were calculated for control (only *Sphingomonas*) and by adding selected concentrations of inhibitors viz. 0.7 mg/ml NPO, 1 mg/ml NPO, 0.05 mg/ml QB, 0.07 mg/ml QB, 0.01 mg/ml NMB and 0.03 mg/ml NMB. Miller Units for *S. paucimobilis* MG6 and *S. terrae* 7766 were calculated to be 172.35 and 135.36. 4-NPO being the known quorum sensing inhibitor significantly reduced the Miller Units. 0.05 mg/ml and 0.07 mg/ml QB. was able to lower the Miller units from 172.35 to 72 and 45.4 respectively, in *Sphingomonas paucimobilis*. (Figure 4.42). NMB was also able to lower the value of Miller units to 84.3 and 58.2 for 0.01 mg/ml & 0.03 mg/ml NMB in *S. paucimobilis*, however, in *S. terrae*, value observed were 80% and 52% for 0.01 mg/ml and 0.03 mg/ml NMB.

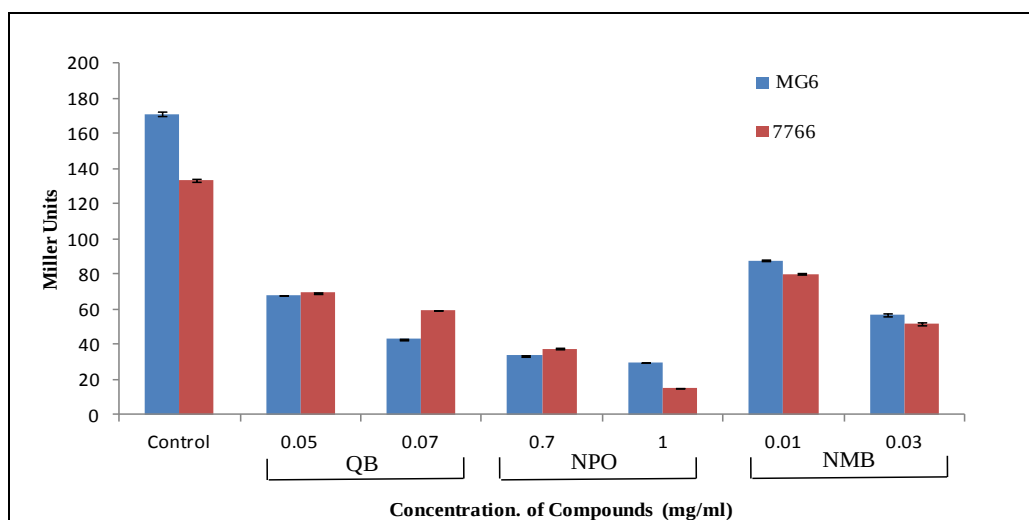


Figure 4.42: Quantitative determination of quorum sensing levels in the culture supernatants of *S. paucimobilis* MG6 and *S. terrae* 7766 by measuring β -galactosidase activity expressed in miller units (MU)

4.26 Effect of QB and NMB on *S. paucimobilis* MG6 biofilm

We hypothesized that the QS inhibitory effect of QB could profoundly alter the biofilm formation. Figure 4.43 depicts the influence of QB and NMB in biofilm progression over time. Biofilm production was reduced sharply and with lower (0.03mg/ml) doses NMB (as compared to NPO, a standard quorum quencher) 45% reduction of biofilm was noted. A similar trend was observed in case of *S. terrae*. The selected concentrations viz. 0.7 mg/ml NPO, 1 mg/ml NPO, 0.05 mg/ml QB, 0.07 mg/ml QB, 0.01 mg/ml NMB and 0.03 mg/ml NMB were sufficient to inhibit *Sphingomonas* biofilm (Figure 4.43). Percentage of biofilm inhibition by compounds given in Figure 4.44 was calculated by:

$$\text{Percentage of Biofilm inhibition} = \frac{[\text{Control O.D 585 nm} - \text{Test O.D 585 nm}]}{\text{Control O.D 585 nm}} * 100$$

Very recently, the development, characterization, and biological testing of a new type of linear molecularly imprinted polymer (LMIP) were reported. The designed polymer acted as an anti-infective by blocking the quorum sensing (QS) mechanism and thus the virulence of the pathogen *Streptococcus pneumonia* (Motib et al. 2017). However there is no documentation of either chemically modified biopolymers or biopolymers alone as antiquorum sensing molecules as yet. Although, preceding experiments established the role of QB and NMB in biofilm inhibition, their effect on cellular integrity of *S. paucimobilis* MG6 and the possible mechanism of action, remained questionable.

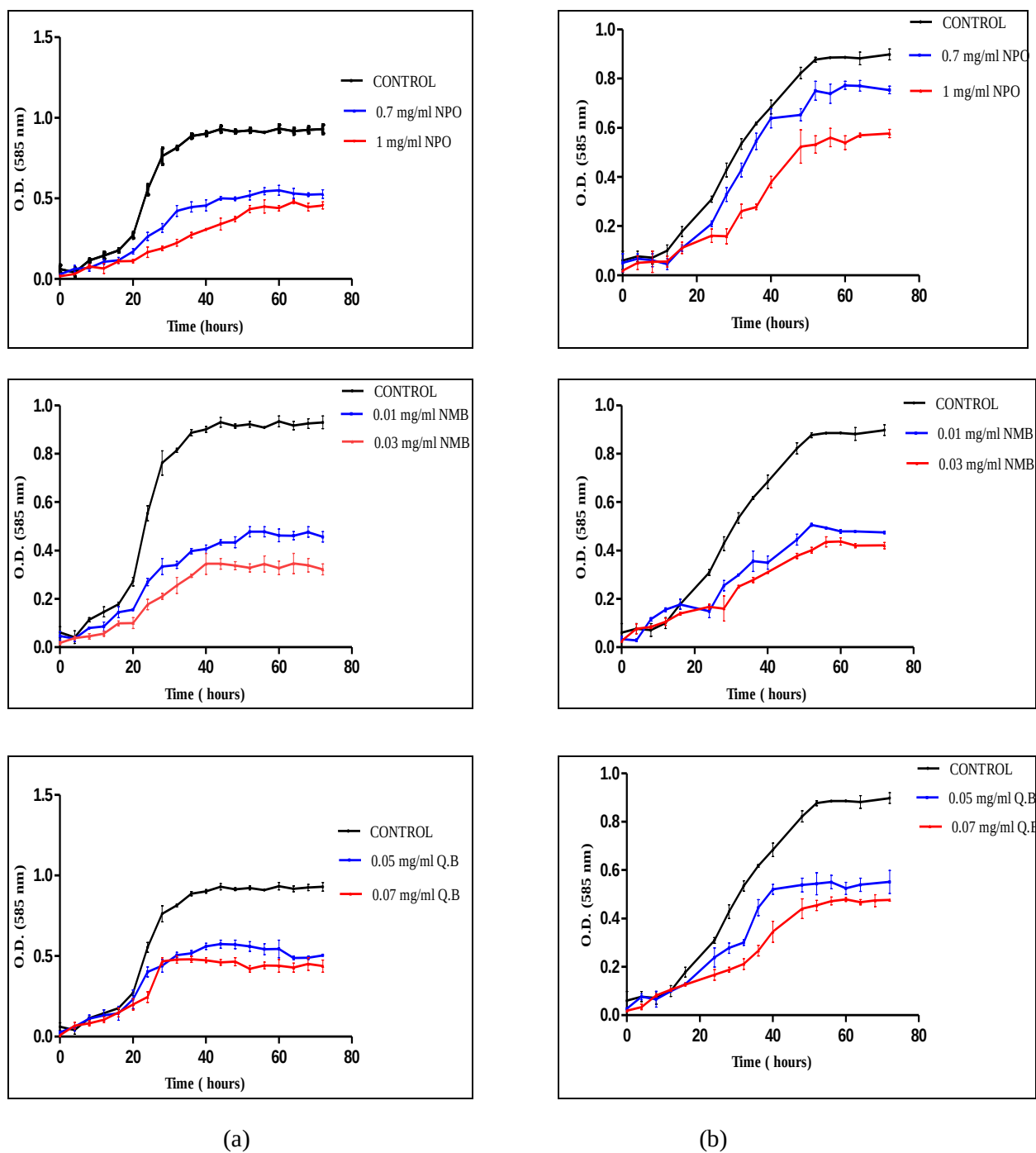


Figure 4.43: Inhibition of biofilm formation by (a) *Spingomonas paucimobilis* MG 6 and (b) *Spingomonas terrae* 7766 observed with varying concentrations of QB and NMB

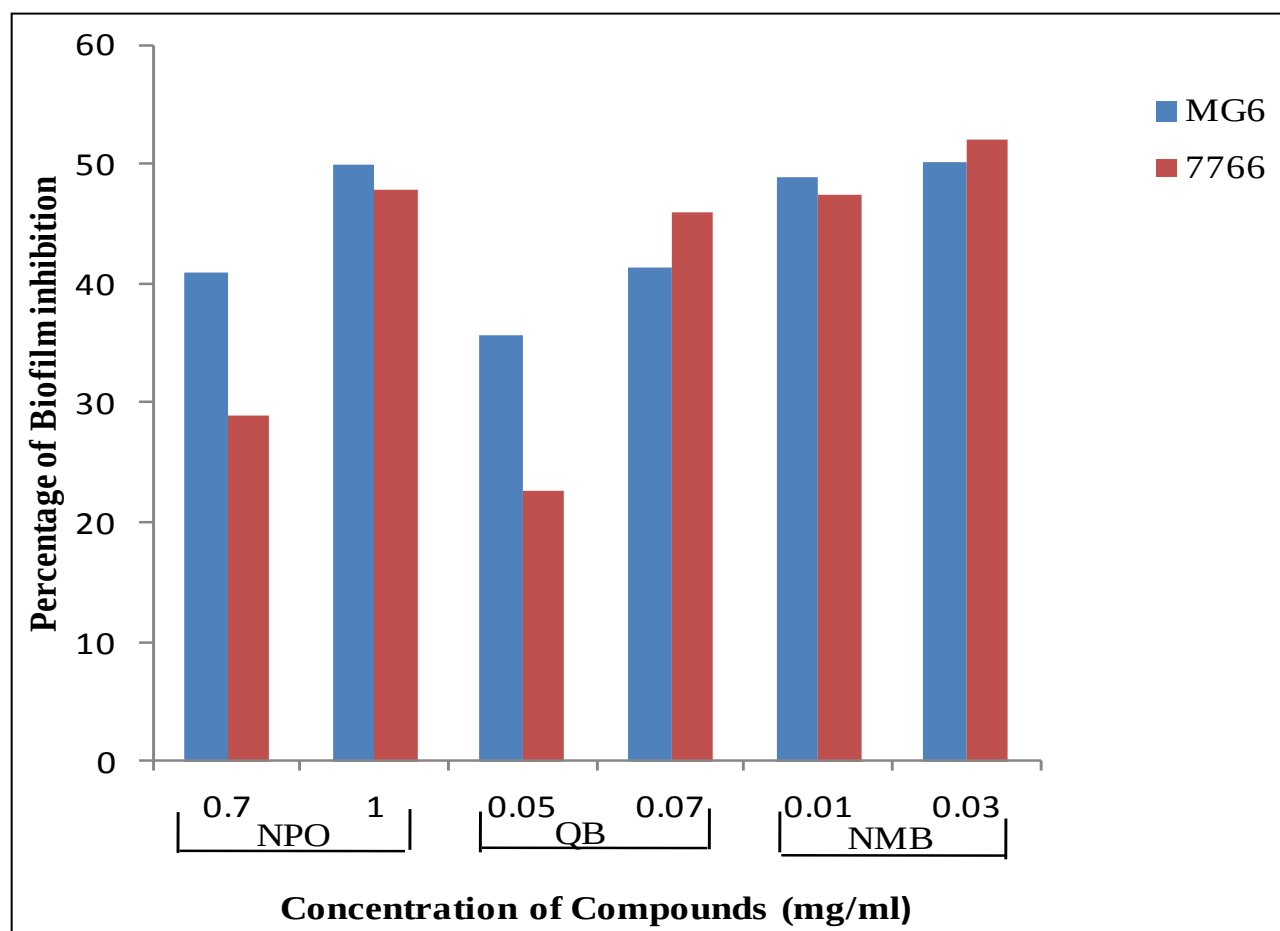
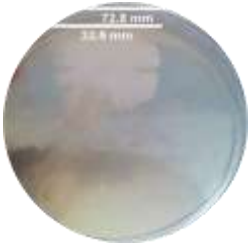


Figure 4.44: Biofilm inhibition in *S. paucimobilis* MG6 and *S. terrae* 7766 by quorum quenchers 4-Nitropyridine –N- oxide (4-NPO), Quaternized Biopolymer (QB) and N-MethylBiopolymer (NMB)

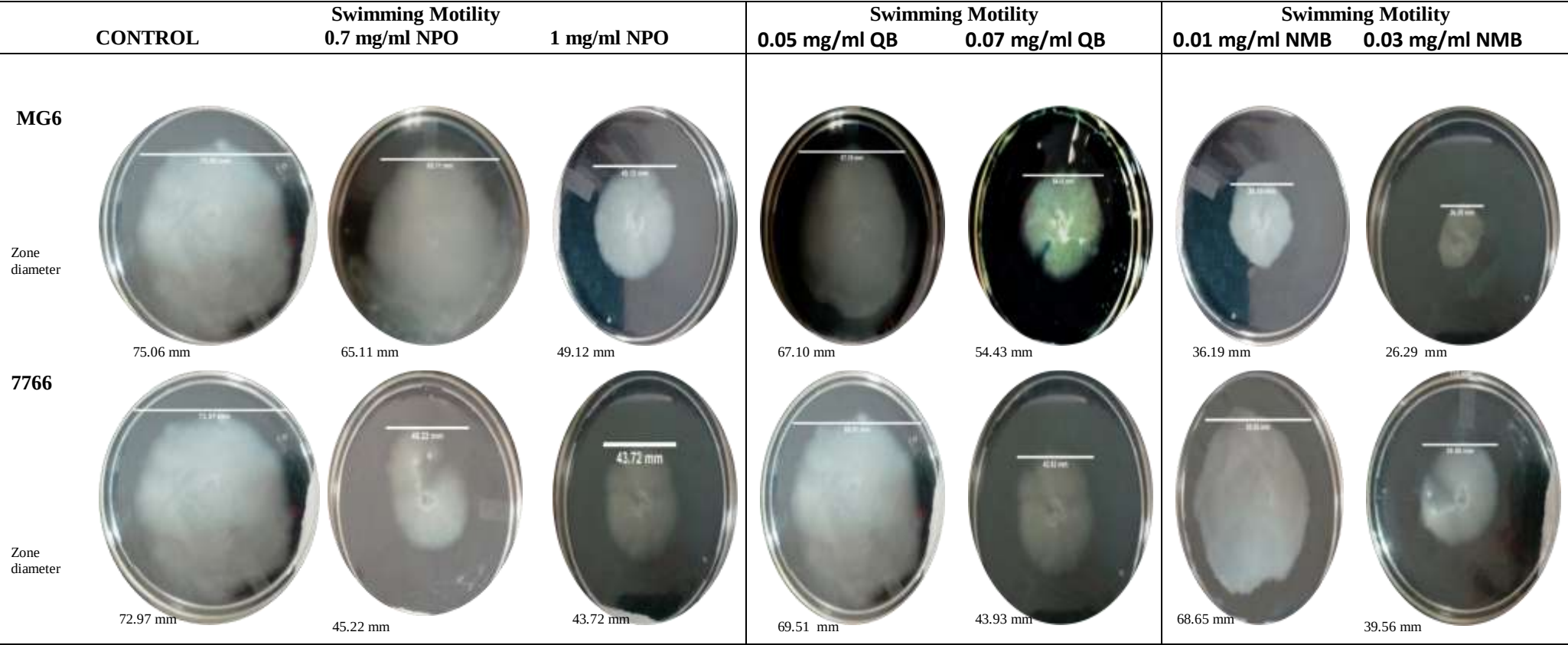
4.27 Impact of QB and NMB on surface motilities of *S. paucimobilis* MG6

Many bacterial physiological functions are under the regulation of quorum sensing, for instance, virulence, luminescence, motility, sporulation and biofilm formation. Swarming is one essential attribute by which bacteria can spread as a biofilm over the surface (Daniels et al. 2004). Swimming motility is type of bacterial movement by rotating flagella similar to swarming. But, in swimming motility individual cells move in liquid environments. Twitching motility is dependent on the extension and retraction of type IV pili that confers slow cell movement (Kearns 2010). It was logical therefore to investigate whether the designed molecules QB and NMB could inhibit the swarming motility of *S. paucimobilis* MG6.

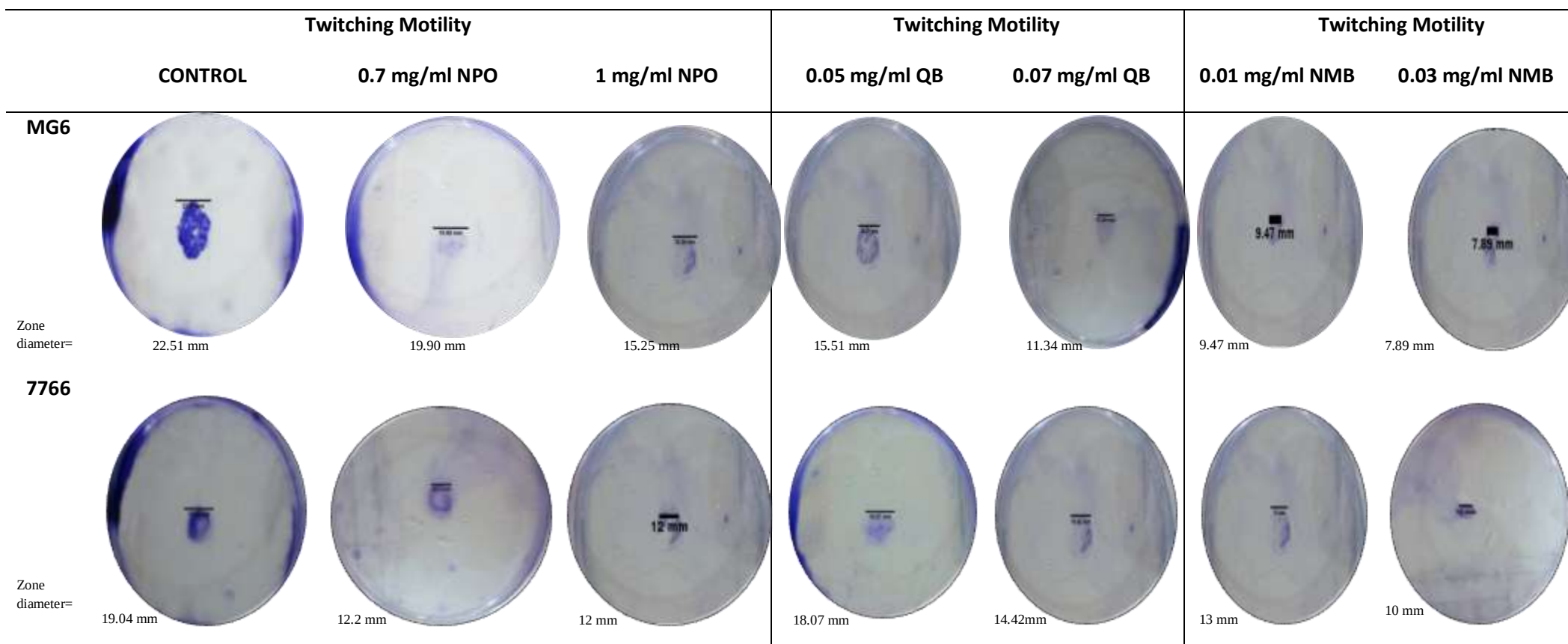
Plates supplemented with selected concentration of compounds displayed inhibition against swarming of *Sphingomonas*. 0.7 mg/ml NPO showed more than 20% reduction in swarming motility while in case of 1 mg/ml NPO, reduction increased to 77% in both the strains. 0.05 mg/ml and 0.07 mg/ml QB reduced for up to 80 % and 82.8% swarming motility in *S. paucimobilis* MG6; 0.01 and 0.03 mg/ml NMB reduced upto 20% and 50% swarm motility in *S. paucimobilis* (Figure 4.45 (a)). In swimming motility, 0.7 mg/ml and 1 mg/ml NPO can reduce the diameter of *Sphingomonas* spreading by 15% and 30%, respectively in *Sphingomonas paucimobilis* (Figure 4.45 (b)). 0.05 mg/ml and 0.07 mg/ml QB. can reduce 30% and 40% and 0.01 mg/ml; 0.03 mg/ml NMB can reduce upto 52% and 65 % in *Sphingomonas paucimobilis*. In twitching motility, reduction can be up to 11% and 30% by 0.7 mg/ml NPO and 1 mg/ml 4-NPO. 30% and 40% reduction was observed by 0.05 % QB and 0.07 mg/ml QB with These values are average of triplicates of each experiment (Figure 4.45 (c)).

Swarming Motility			Swarming Motility		Swarming Motility	
CONTROL	0.7 mg/ml NPO	1 mg/ml NPO	0.05 mg/ml QB	0.07 mg/ml QB	0.01 mg/ml NMB	0.03 mg/ml NMB
MG6						
Zone Diameter	32.5 mm	26.36 mm	8.24 mm	12 mm	10.8 mm	26.29 mm
7766						
Zone diameter	46 mm	34.17 mm	12.43 mm	35.32 mm	11.08 mm	19.79 mm

(a)



(b)



(c)

Figure 4.45: Surface Motility assays of *S. paucimobilis* MG6 and *S. terrae* 7766 with varying concentrations of QB, NMB and NPO (a) Swarming motility (b) Swimming motility (c) Twitching motility. The motilities were determined by calculating the diameters of zone of bacterial strain on the plates from three independent experiments

4.28 Effect of QB and NMB on *S. paucimobilis* MG6 AHL production

To investigate the effect of QB and NMB on AHL, the colorimetric method of Yang et al (2006) was used. As observed in Figure 4.46, addition of both QB and NMB compounds led to significant ($p < 0.05$) decrease in AHL production, the decrease being directly proportional to the concentration of compounds used. Both *S. paucimobilis* MG6 and *S. terrae* 7766 exhibited a similar trend. Threshold considered for significant AHL activity ≥ 0.98 . Increasing the concentrations beyond 0.7 mg/ml NPO, 0.05 mg/ml QB and 0.01 mg/ml NMB decreased the AHL activity of *Sphingomonas* below threshold. These findings indicated that both compounds were able to interfere with AHL and therefore valuable for further analyzing their role as quorum sensing inhibitors.

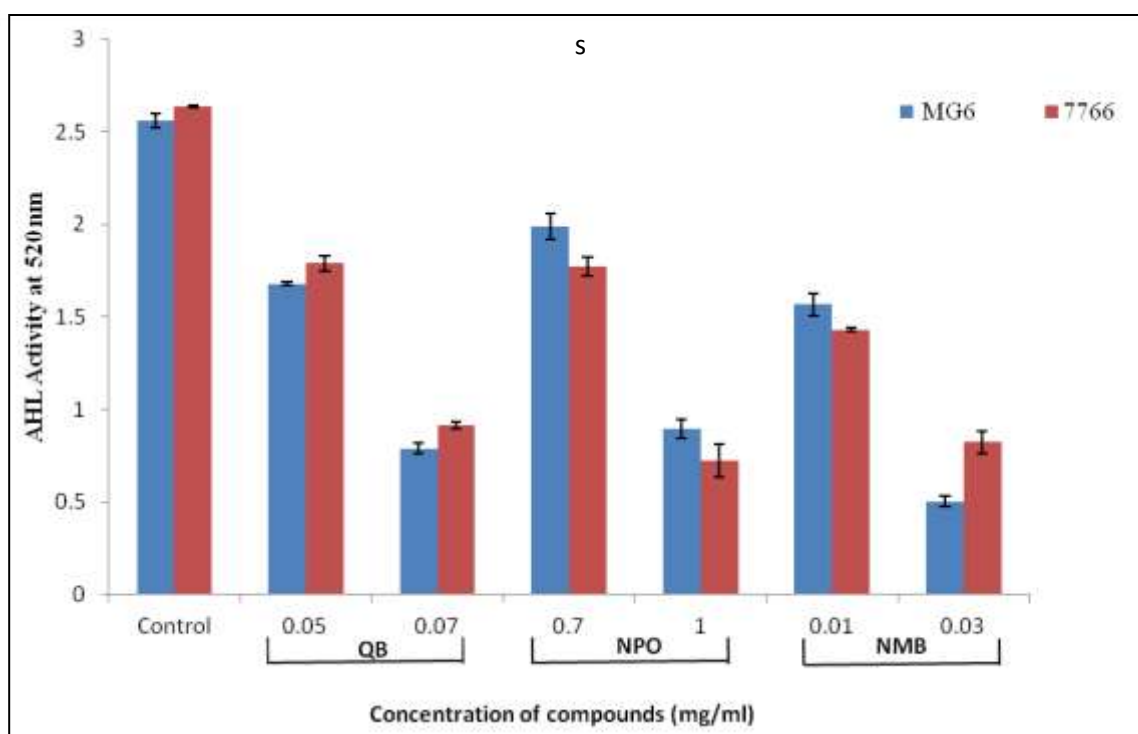


Figure 4.46: Effect of quorum sensing inhibitors (a) 4-Nitropyridine-N-oxide (4-NPO) (b) Quaternized biopolymer (QB) (c) N-methylbiopolymer on AHL activity by colorimetric method. Threshold for AHL activity ≥ 0.98 . The results are means of three replicates

4.29 Qualitative modulation of AHL

The signal-producing LuxI homolog, the *N*-acylhomoserine lactone molecule, and the signal receptor LuxR are potential targets in the AHL system. Many compounds hinder QS by interfering with the AHL activity either by competing with them due to their structural similarity or by degrading the LuxR receptors of the AHL molecules (Vattem et al. 2007). To determine whether potential inhibitors target AHL synthesis (via LuxI) or AHL response (via LuxR), a double ring bioassay was carried out using the *A. tumefaciens* biosensor system, at a sub-inhibitory concentration of QB. It was observed that QB displayed both LuxI and LuxR modulation as compared to the control (disc impregnated with sterile water). QB decreased the blue pigment production by *A. tumefaciens* A136, a phenomenon controlled by quorum sensing, leading to decrease in both LuxI and LuxR activity (Figure 4.47). While, the coloration was not affected by NMB due to the negligible effect to LuxI or LuxR activity.

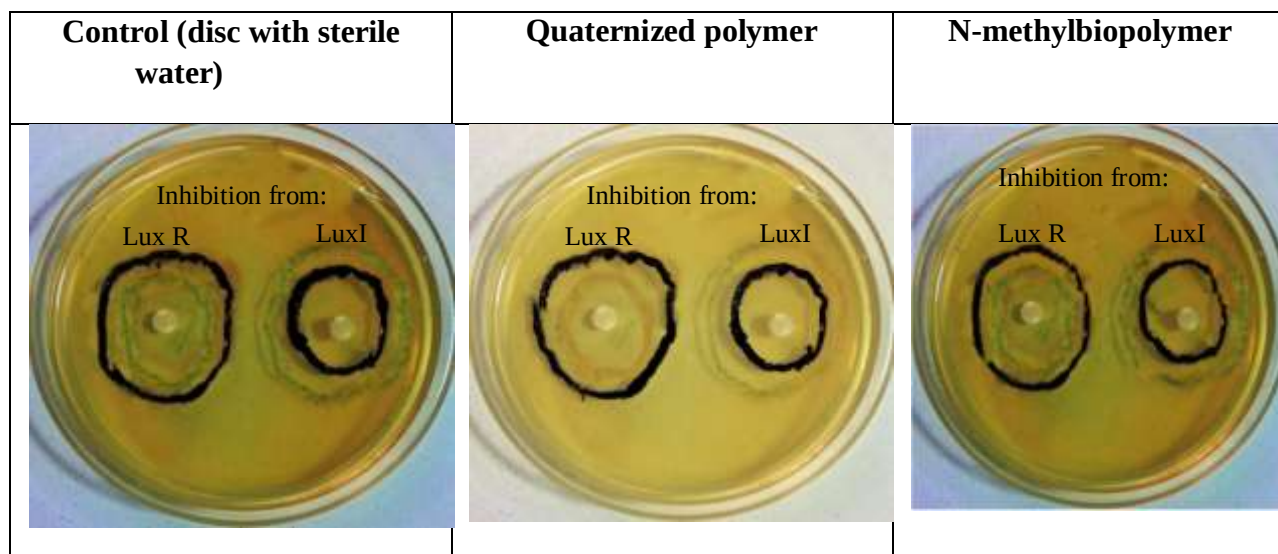
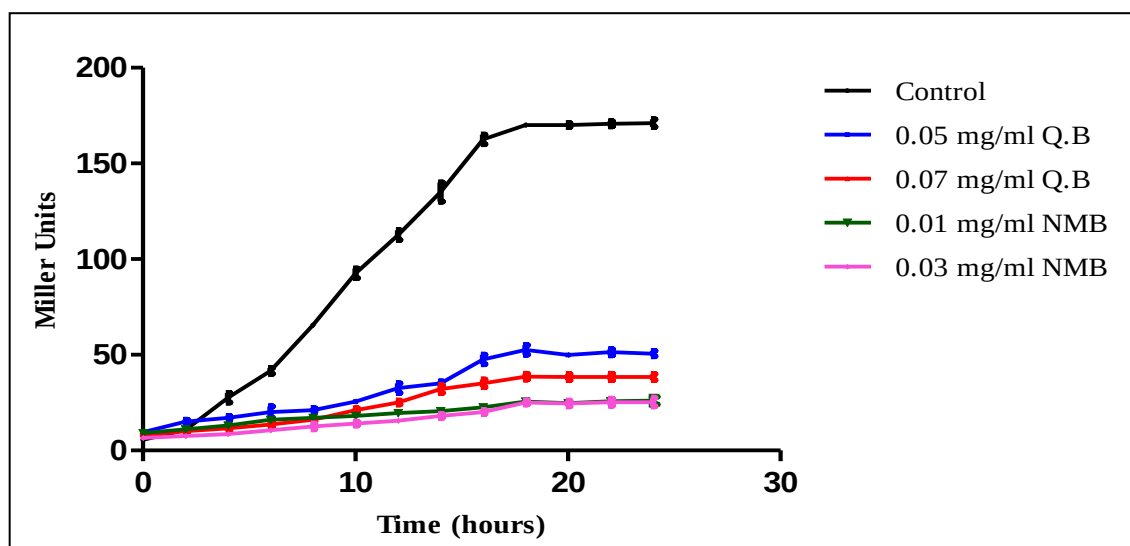
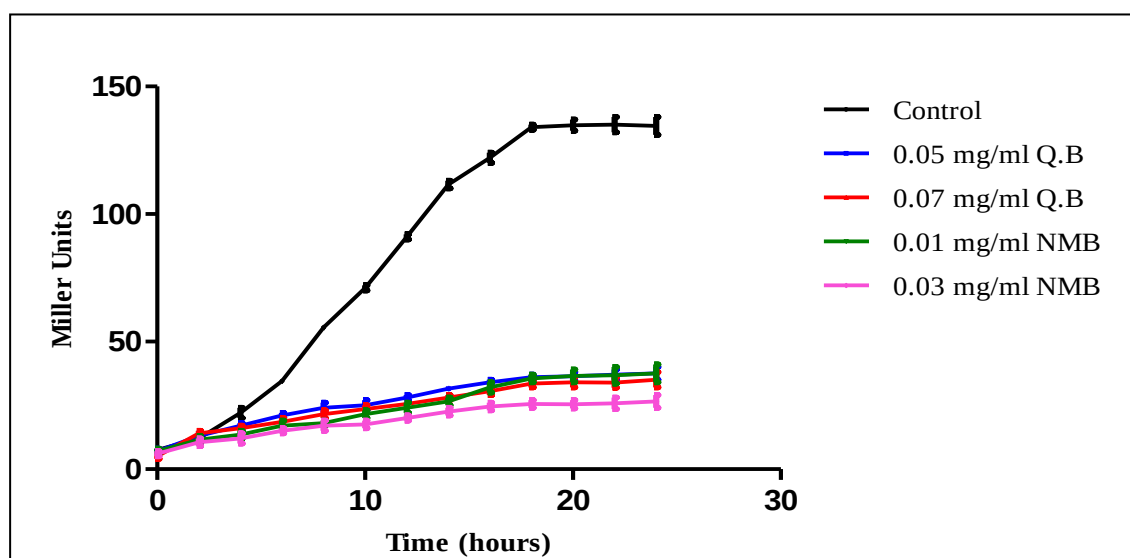


Figure 4.47: Quorum Sensing inhibition by quaternized biopolymer at sub-inhibitory concentration demonstrating modulation of AHL synthesis and activity

Effect of quaternized biopolymer (QB) and N-methyl biopolymer (NMB) was studied on kinetics of AHL production by *S. paucimobilis* MG6. It was observed that AHL production peaked at 18 hours. The decrease in autoinducer production was significant ($p < 0.05$) using QB and NMB. The kinetics of AHL production by measuring β -galactosidase activity (in Miller Units) is shown in Figure 4.48.



(a)



(b)

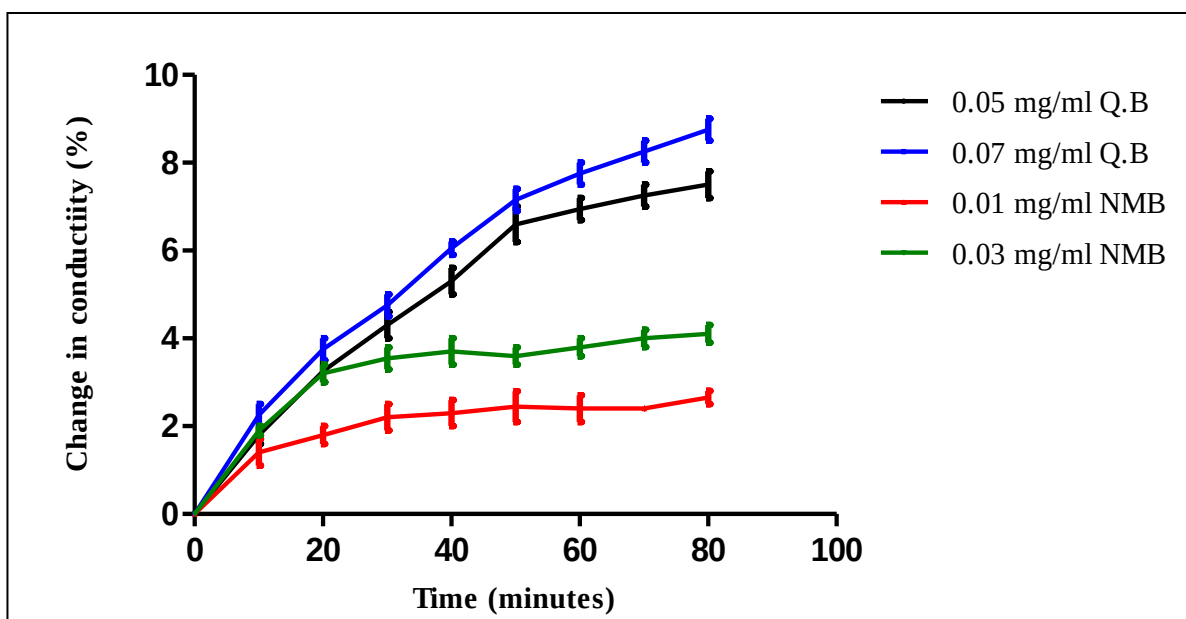
Figure 4.48: Effect of QB and NMB on AHL synthesis in (a) *S. paucimobilis* MG6 (b) *S. terrae* 7766 determined by assaying β -galactosidase activity of culture supernatants

4.30 Biochemical responses of QB and NMB treated *S. paucimobilis* MG6

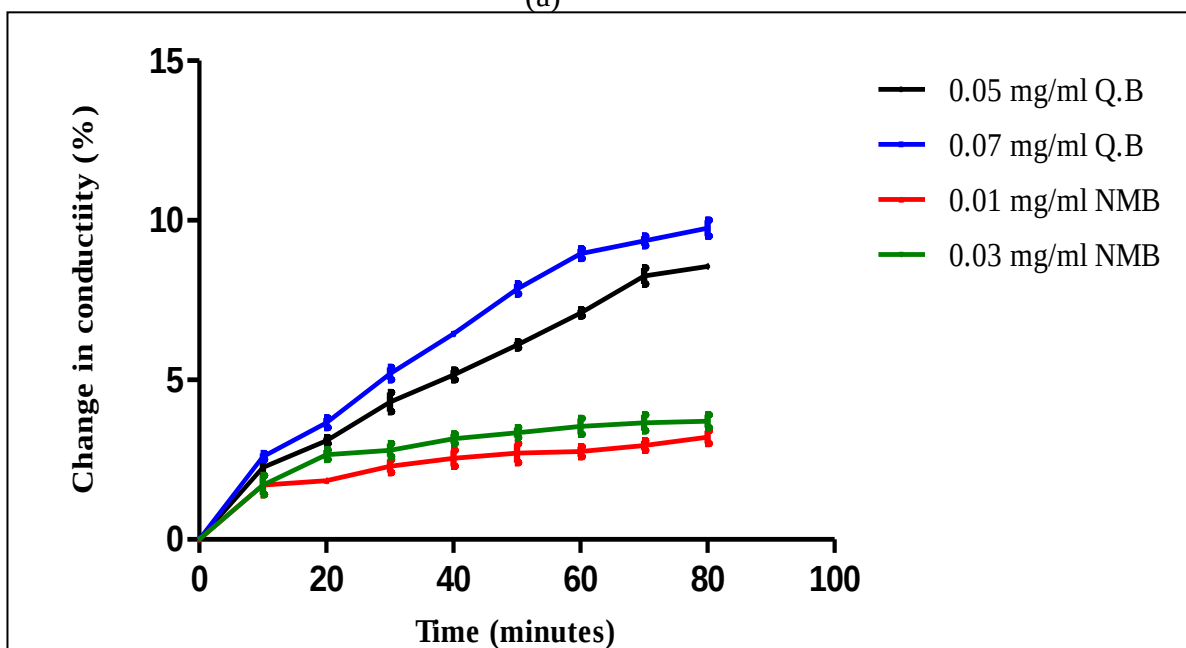
Insights on mode of action of antibiofilm agents are significant both for structure refinement of these agents and the fate of bacterial strains following exposure to the agents. A number of assays are usually carried out for evaluating the overall biochemical response of target bacterial cells. In order to elucidate the possible mechanism of the inhibitory action of QB against *Sphingomonas*, the membrane potential, conductivity, cellular leakage estimation (intracellular K⁺ content), intracellular ATP concentration, measurement of glucose and lactate dehydrogenase activity was carried out.

4.30.1 Electric conductivity of treated bacterial cell suspensions

Relationship between antibiofilm activity of compounds and membrane permeability of *S. paucimobilis* MG6 was evaluated by measuring electrical conductivity (Figure 4.49). *S. paucimobilis* MG6 and *S. terrae* 7766 treated with NMB and QB. The electrical conductivity of cell suspensions treated with QB showed time-dependent increase. While, bacterial strain treated with NMB showed little to no increase in conductivity. In NMB treated cell suspension, conductivity was observed to increase by 2.5% and 3.9% for 0.01 mg/ml and 0.03 mg/ml in *S. paucimobilis*. In *S. terrae*, 3% and 3.5% was observed for 0.01 mg/ml and 0.03 mg/ml NMB. In case of QB treated suspensions, electrical conductivity was supposed to increase by 7.2% and 8.5% for 0.05 mg/ml and 0.07 mg/ml QB in *S. terrae* MG6 while in case of *S. terrae* 7766, 3.0 % and 3.5% increase was observed for 0.01 mg/ml and 0.03 mg/ml NMB and 8.5 % and 9.5% increase for 0.05 mg/ml and 0.07 mg/ml QB. Differences in changes in electrical conductivity between the two treatments showed that the effect of QB on membrane permeability of *S. paucimobilis* MG6 was greater as compared to NMB.



(a)



(b)

Figure 4.49: Change in conductivity in suspension of 10^7 CFU/ml of (a) *S. paucimobilis* MG6 (b) *S. terrae* 7766 . Data is Average $\pm$ Standard Deviation (SD) of triplicate samples

4.30.2 Potassium Leakage

Membrane permeability and leakage of cellular contents from bacterial cells was detected by using K⁺ specific ion electrode after exposure to different concentrations of QB and NMB. Table 4.17 represents the effect of 0.05 mg/ml and 0.07 mg/ml QB and 0.01 mg/ml & 0.03 mg/ml NMB upon membrane of target bacteria which is determined by Potassium (K⁺) leakage. Bacterial suspension at 0 hours contact time showed similar results to that of the highest concentration of NMB (0.03 mg/ml) at the longest contact time (1 hour), while increase in potassium leakage was observed by 30 % and 38% when 0.05mg/ml and 0.07 mg/ml QB was used in case of *S. paucimobilis* MG6. While, 22% and 31% increase was observed in *S. terrae* 7766 using 0.05 and 0.07 mg/ml QB, respectively. Membrane damage was not apparent with NMB as indicated by minimal leakage of potassium ions while QB treated cell suspensions indicated possible membrane leakage.

Table 4.17: Potassium leakage after 1 hour incubation from (a) *S. paucimobilis* MG6 (b) *S. terrae* 7766 after treatment with QB and NMB. Data is Average $\pm$ Standard Deviation (SD) of triplicate samples

	Control (0h)	Potassium leakage (mg/l)			
		0.05 mg/ml QB	0.07 mg/ml QB	0.01 mg/ml NMB	0.03 mg/ml NMB
<i>S. paucimobilis</i> MG6	4.2	6.0	6.8	4.4	4.3
<i>S. terrae</i> 7766	4.8	6.2	7.0	4.9	5.1

4.30.3 Leakage of UV-absorbing material

The UV-absorbing materials released from bacterial cells after treatment with compounds were indicated as an indicator to membrane permeability. If the membrane is damaged, the release of cytoplasmic constituents of the cells such as DNA, RNA, and other materials can be determined. Since nucleotides have strong UV absorption at 260 and 280 nm wavelengths, membrane integrity can be monitored through the measurement of absorbance at these wavelengths. UV absorption of the supernatant of *S. paucimobilis* MG6 and *S. terrae* 7766 with and without QB and NMB was measured for a period of time. Treatment of *Sphingomonas* cell suspensions treated with QB led the gradual leakage of UV-absorbing substances, followed by plateau for up to 2 h (Figure 4.50). This was found to be time and concentration dependent. However, treatment of cell suspension with NMB leads to negligible leakage of UV-absorbing substances.

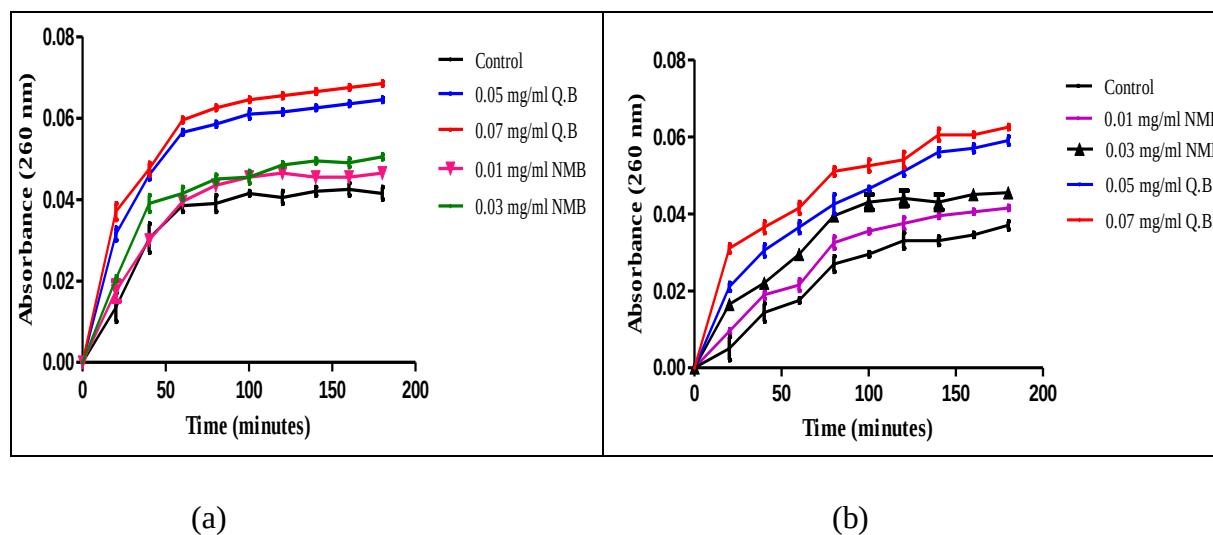


Figure 4.50: Leakage of UV absorbing substances from (a) *S. paucimobilis* MG6 (b) *S. terrae* 7766 upon treatment with QB and NMB in different doses

4.30.4 ATP leakage

An ATP bioluminescence test was used to establish whether cell envelope perturbation was implicated in the antibacterial effect. The disinfection of *S. paucimobilis* with 0.05 & 0.07 mg/ml QB and 0.01 and 0.03 mg/ml NMB resulted in low levels of ATP leakage from NMB treated cell suspension (Table 4.18). Upon the addition of different concentrations of QB and NMB, samples were incubated for 45 minutes at ambient temperature and minimal variation in ATP content was observed in NMB treated *Sphingomonas* suspension while significant change ($p < 0.05$) was observed in suspension treated with QB as compared to the control. ATP is a marker for cell viability which is present in all the metabolically active cells (Kangas et al. 1984). The reliability and sensitivity of this ATP based assay makes it appropriate technique for assessing efficacy of antibiofilm agents.

Table 4.18: ATP content in the extracellular media of *S. paucimobilis* MG6 and *S. terrae* 7766 after treatment with QB and NMB.

	Control	ATP Content (RLU)			
		0.01 mg/ml	0.03 mg/ml	0.05 mg/ml	0.07 mg/ml
		NMB	NMB	QB	QB
<i>S. paucimobilis</i> MG6	2.34±0.06	2.22±0.07	2.19±0.04	8.79 ± 0.01	8.85 ± 0.03
<i>S. terrae</i> 7766	2.78±0.05	2.5±0.05	2.45±0.06	8.66 ± 0.06	8.7 ± 0.04

4.30.5 Leakage of proteins, glucose and Lactate dehydrogenase (LDH)

For the elucidation of the possible mechanism of anti biofilm activity of modified biopolymers, leakage of protein and glucose from *Sphingomonas* was measured (Table 4.19 and 4.20). For the

determination of oxidative stress-induced damage of the respiratory system of the cells, LDH activity was measured. The effects of QB on LDH activities of *S. paucimobilis* MG6 and *S. terrae* 7766 cells are shown in Table 4.21. Treatment of *Sphingomonas* with 0.05 mg/ml and 0.07 mg/ml QB led to leakage of proteins and glucose after a period of 3 hours. However, treatment of cell suspension with 0.01 mg/ml and 0.03 mg/ml NMB led to minimal leakage of proteins, glucose and lactate dehydrogenase activities. A stronger correlation have been observed between leakage and duration of exposure (3h) in Gram-negative than in Gram-positive organisms (Khaira et al. 2014). Judging by the time of exposure and observed marker enzyme activities for cell leakage, it was inferred that NMB possessed negligible role in compromising cellular structure of *S. paucimobilis* MG6.

Table 4.19: Effect of different concentrations of QB and NMB on the leakage of protein in the extracellular media of *S. paucimobilis* MG6 and *S. terrae* 7766 after 3 hours

Control		Absorbance (280 nm)			
		0.01 mg/ml NMB	0.03 mg/ml NMB	0.05 mg/l QB	0.07 mg/ml QB
<i>S. paucimobilis</i> MG6	0.100±0.04	0.123±0.07	0.165±0.03	0.616 ±0.03	0.66 ± 0.05
<i>S. terrae</i> 7766	0.101±0.06	0.102±0.06	0.112±0.06	0.520 ±0.02	0.57 v0.07

Table 4.20: Effect of QB and NMB on leakage of glucose concentration in the extracellular media of *S. paucimobilis* MG6 and *S. terrae* 7766 after 3 hours

	Control	Glucose (mg/dl)			
		0.01 mg/ml NMB	0.03 mg/ml NMB	0.05 mg/l QB	0.07 mg/ml QB
<i>S. paucimobilis</i> MG6	0.09±0.02	0.109±0.04	0.134±0.09	0.48±0.02	0.5±0.06
<i>S. terrae</i> 7766	0.1±0.05	0.112±0.09	0.121±0.02	0.4±0.03	0.45±0.08

Table 4.21: Effect of QB and NMB on leakage of lactate dehydrogenase in the extracellular media of *S. paucimobilis* MG6 and *S. terrae* 7766 after 3 hours

	Control	LDH Activity (U/L)			
		0.01 mg/ml NMB	0.03 mg/ml NMB	0.05 mg/ml QB	0.07 mg/ml QB
<i>S. paucimobilis</i> MG6	11.6±0.03	11.9±0.06	12.4±0.01	18.28±0.03	20.29±0.02
<i>S. terrae</i> 7766	12.3±0.07	13.0±0.1	13.5±0.05	17.44±0.05	18.9±0.01

4.31 Effect of QB and NMB on survival of *S. paucimobilis* MG6 and *S. terrae* 7766

To analyze the effect of QB and NMB on survival and growth of planktonic cells of *S. paucimobilis* MG6 and *S. terrae* 7766, a kinetic study with different concentrations of QB and NMB was carried out. NMB failed to exert notable effect on the reduction of growth of the bacterial strains; however, QB significantly reduced the same by about 3 folds (Figure 4.51). An extended lag phase was also evident. These results and those obtained prior to this (cellular

assays) suggested that the N-methyl biopolymer did not interfere with either the integrity or metabolism of the cells justifying its role as an authentic quorum sensing inhibitor. The quaternized biopolymer (QB) however affected the cellular integrity and therefore was not suitable as antiquorum sensing agent.

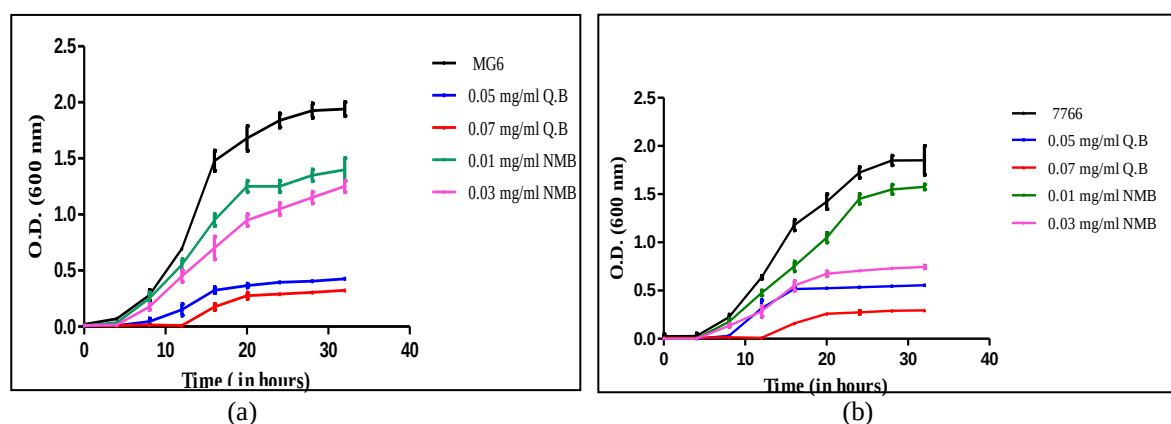


Figure 4.51: Effect of different concentrations of QB and NMB on survival and growth of (a) *S. paucimobilis* MG6 (b) *S. terrae* 7766

4.32 Biofilm inhibition studies in real time conditions

In order to investigate the antibiofilm activity of quaternized biopolymer (QB) and N-methylbiopolymer (NMB) in real time conditions, 20 water samples were collected from various sources nearby regions of Patiala and analyzed for relevant quality parameters (Table 4.22). Biofilm formation in these samples was evaluated by crystal violet assay. Biofilm was formed by *S. paucimobilis* MG6 in all the water samples and it was observed that P2, P6, P7, P16 and P19 samples support highest formation of biofilm (Figure 4.52). Amongst them, P7 supported highest biofilm formation and hence used for studying biofilm inhibition by quorum sensing inhibitors used in the present study. The water quality values did not differ significantly in the samples analyzed; however, in sample P7 residual chlorine levels were lower than the other samples and may explain the maximal biofilm formation by *S. paucimobilis* MG6.

Table 4.22: Analysis of water quality parameters. Data is Average $\pm$ Standard Deviation (SD) of triplicate samples

Parameters	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
	Tap water 1, Patiala	Bhakhra canal surface water	Sewage water (site 1), Patiala	Tap water 2, Patiala	Deionized water	Tap Water 3, Patiala	Sewage water (site 2), Patiala	Drain, Rajpura Road, Patiala	Reverse Osmosis Drinking Water	Tap Water 4, Patiala
pH	7.2 $\pm$ 0.07	7.7 $\pm$ 0.05	6.9 $\pm$ 0.02	7.7 $\pm$ 0.07	8.0 $\pm$ 0.08	7.5 $\pm$ 0.03	7.3 $\pm$ 0.04	7.6 $\pm$ 0.01	7.5 $\pm$ 0.09	7.43 $\pm$ 0.05
TDS (mg/l)	336 $\pm$ 0.04	87 $\pm$ 0.09	115 $\pm$ 0.06	328 $\pm$ 0.05	98 $\pm$ 0.01	100 $\pm$ 0.03	100 $\pm$ 0.06	283 $\pm$ 0.01	48 $\pm$ 0.09	190 $\pm$ 0.01
TSS (mg/l)	18 $\pm$ 0.123	59 $\pm$ 0.1	60 $\pm$ 0.07	63 $\pm$ 0.04	55 $\pm$ 0.08	107 $\pm$ 0.07	66 $\pm$ 0.02	105 $\pm$ 0.01	83 $\pm$ 0.09	72 $\pm$ 0.14
DO (mg/l)	2.35 $\pm$ 0.03	6.83 $\pm$ 0.08	5.41 $\pm$ 0.09	6.4 $\pm$ 0.09	4.92 $\pm$ 0.08	4.43 $\pm$ 0.09	5.23 $\pm$ 0.07	6.1 $\pm$ 0.06	4.75 $\pm$ 0.03	4.9 $\pm$ 0.01
Chlorides (mg/l)	29.9 $\pm$ 0.04	99.9 $\pm$ 0.09	39.9 $\pm$ 0.05	77.9 $\pm$ 0.01	249.9 $\pm$ 0.08	39.9 $\pm$ 0.08	19.9 $\pm$ 0.08	99.9 $\pm$ 0.07	39.9 $\pm$ 0.01	29.9 $\pm$ 0.02
TOC (mg/l)	0.2 $\pm$ 0.08	1.3 $\pm$ 0.08	0.5 $\pm$ 0.05	1.00 $\pm$ 0.08	1.00 $\pm$ 0.02	0.5 $\pm$ 0.01	2.2 $\pm$ 0.09	3.2 $\pm$ 0.09	1.5 $\pm$ 0.02	2.7 $\pm$ 0.03
Parameters	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20
	Tap water 5, Patiala	River Drain 2, Patiala	Passi Road river drain surface water, Patiala	Distilled water	Double distilled water	River drain 1, Patiala	Sirhind Canal	Sidhwan Canal, Ludhiana	Sewage water (site 3), Rajpura	Filtered Water
pH	6.5 $\pm$ 0.01	7.1 $\pm$ 0.02	6.2 $\pm$ 0.12	6.0 $\pm$ 0.06	6.7 $\pm$ 0.01	7.5 $\pm$ 0.08	8.0 $\pm$ 0.06	6.1 $\pm$ 0.05	7.0 $\pm$ 0.06	7.9 $\pm$ 0.09
TDS (mg/l)	123 $\pm$ 0.09	156 $\pm$ 0.07	289 $\pm$ 0.09	200 $\pm$ 0.06	150 $\pm$ 0.08	60 $\pm$ 0.1	145 $\pm$ 0.03	190 $\pm$ 0.01	95 $\pm$ 0.06	178 $\pm$ 0.09
TSS (mg/l)	45 $\pm$ 0.09	98 $\pm$ 0.05	112 $\pm$ 0.09	56 $\pm$ 0.09	78 $\pm$ 0.01	56 $\pm$ 0.05	55 $\pm$ 0.02	109 $\pm$ 0.09	90 $\pm$ 0.01	87 $\pm$ 0.07
DO (mg/l)	3.21 $\pm$ 0.01	4.1 $\pm$ 0.03	3.9 $\pm$ 0.08	3.09 $\pm$ 0.07	4.23 $\pm$ 0.08	7.23 $\pm$ 0.04	6.23 $\pm$ 0.09	3.45 $\pm$ 0.01	6.89 $\pm$ 0.08	6.21 $\pm$ 0.09
Chlorides (mg/l)	20.9 $\pm$ 0.11	39.2 $\pm$ 0.02	189.7 $\pm$ 0.08	132.0 $\pm$ 0.01	56.9 $\pm$ 0.08	39.9 $\pm$ 0.02	109.6 $\pm$ 0.7	98.7 $\pm$ 0.02	54.3 $\pm$ 0.05	91.2 $\pm$ 0.08
TOC (mg/l)	0.5 $\pm$ 0.09	0.8 $\pm$ 0.01	0.5 $\pm$ 0.09	0.4 $\pm$ 0.08	1.7 $\pm$ 0.08	2.0 $\pm$ 0.01	1.1 $\pm$ 0.02	0.3 $\pm$ 0.12	1.0 $\pm$ 0.06	2.9 $\pm$ 0.12

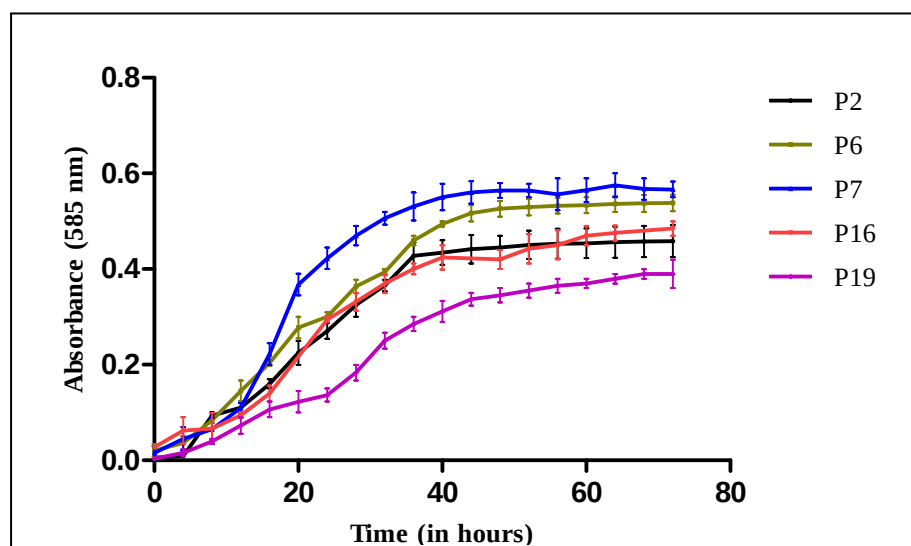


Figure 4.52: Kinetics of *S. paucimobilis* MG6 biofilm in water samples (P2, P6, P7, P16 and P19) allowing maximum biofilm production

Quaternized biopolymer and N-methyl biopolymer were used as antibiofilm agents for inhibiting biofilm formation by *S. paucimobilis* MG6 in SSG medium and P7 water sample. In order to understand whether the anti quorum sensing compounds could exert their effect on growing biofilms, both QB and NMB were added at the time of start of experiment in one second and in the other second to 12 hours grown biofilm (Figure 4.53).

Biofilm formation was reduced in a dose-dependent manner, reaching a maximum of 60.3% inhibition at a concentration of 0.07 mg/ml Quaternized biopolymer in medium while in water sample (P7), maximum inhibition of 58% was observed at the same concentration. When antibiofilm agents were added after 12 hours of growing biofilm in microtiter plates, maximum inhibition of 55.9 % was observed in medium and 43.7% in water at the concentration of 0.07 mg/ml QB. While inhibition of 39% and 46% was observed by 0.01 mg/ml and 0.03 mg/ml NMB in medium. In water, 38% and 41% inhibition was observed by these concentrations. Addition of NMB after 12 hours led to 39% inhibition in medium and 27.7% in water by 0.01

mg/ml NMB (Figure 4.54). This observation indicated that the inhibitory effects of both QB and NMB tends to diminish once biofilm formation is initiated.

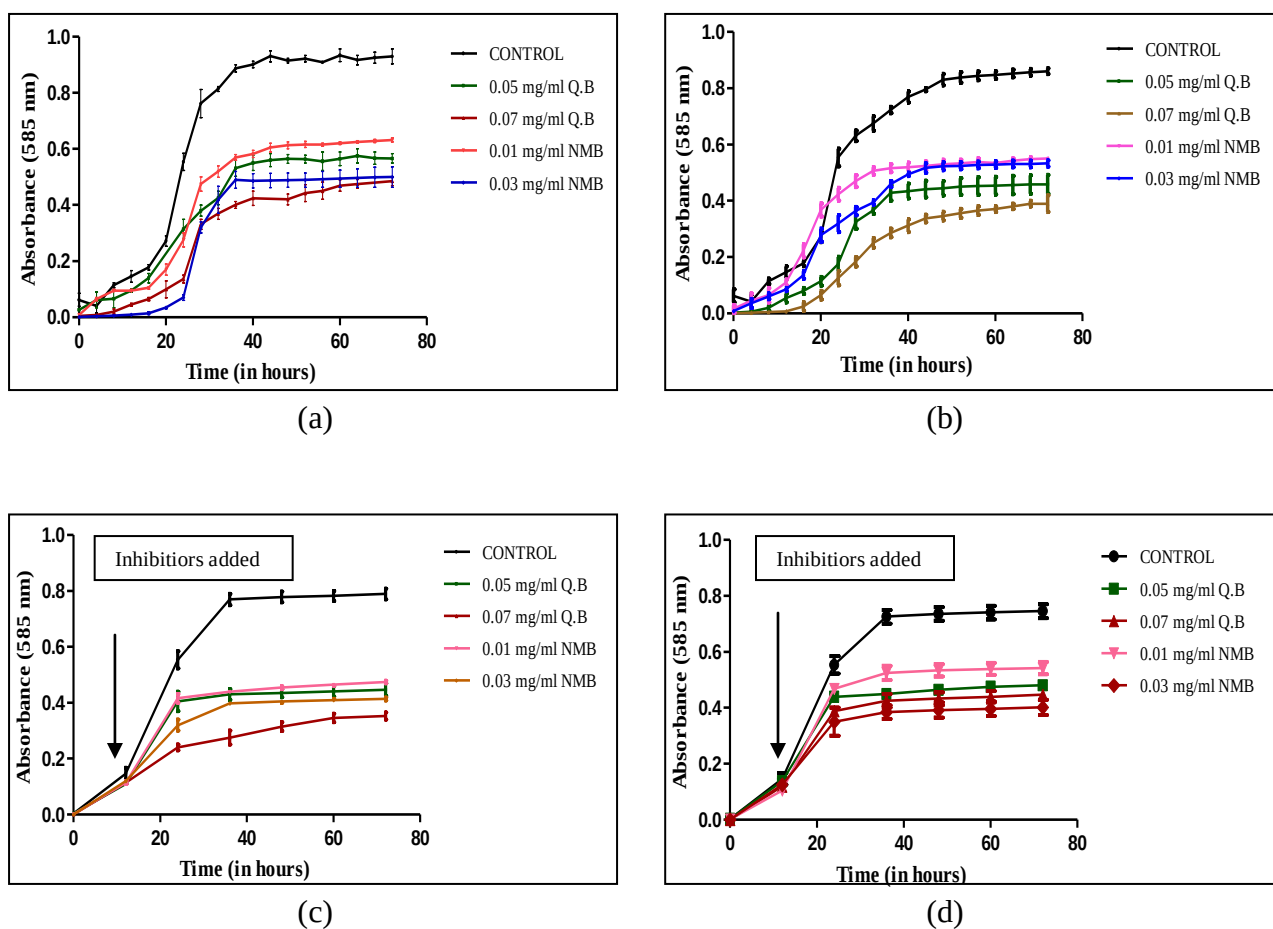


Figure 4.53: Biofilm inhibition by *S. paucimobilis* MG6 in (a) Medium (b) Water (c) Medium (12 h old biofilm) (d) Water (12 h old biofilm)

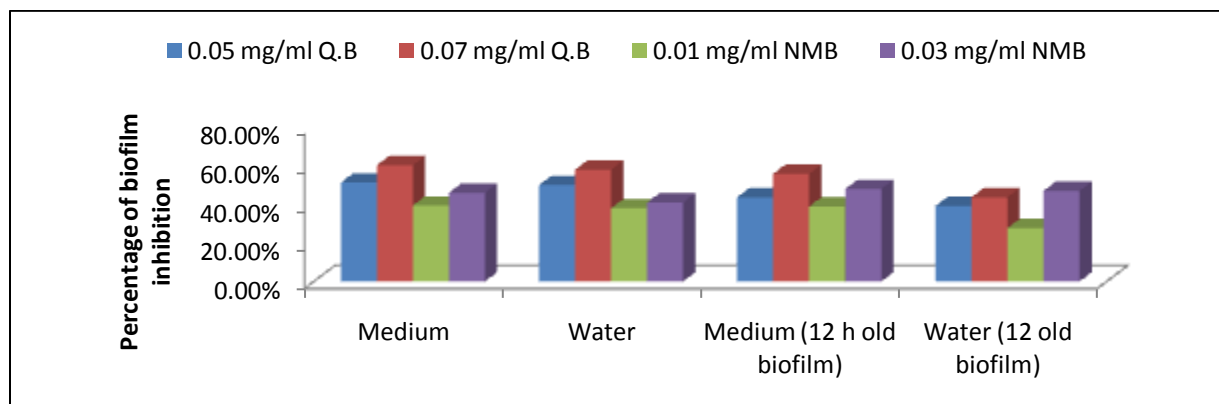


Figure 4.54: Comparison of *S. paucimobilis* MG6 in medium and water by different doses of QB and NMB

Biofilm inhibition of compounds QB and NMB was analyzed on PVC coupons under real time conditions. Biofilm inhibition was observed for up to 72 hours. Since intensity of fluorescence reciprocates biofilm formation, the effect of quorum quenchers on biofilm inhibition could be clearly noted (Figure 4.55). To ensure whether leaching of both biopolymeric compounds occurred from the coatings, the calcofluor assay was carried out in water samples for up to 72 hours; however no fluorescence was observed indicating the stability of the coated antibiofilm compounds. These observations imply the possibility of application of the developed antibiofilm compounds in the form of coatings. Recently, Norcy et al (2017) reported the anti-biofilm effect of biodegradable coatings based on Dibromohemibastadin-1 (DBHB) in marine environment. The concentration (0.02%) used in coating was extremely low compared to commercial coatings which is 20–40% average and could inhibit biofilm formation by marine microorganisms. These results imply the stability and functionality of the developed compounds as a prospective antibiofilm material for coating surfaces vulnerable to *Sphingomonas* biofilm formation.

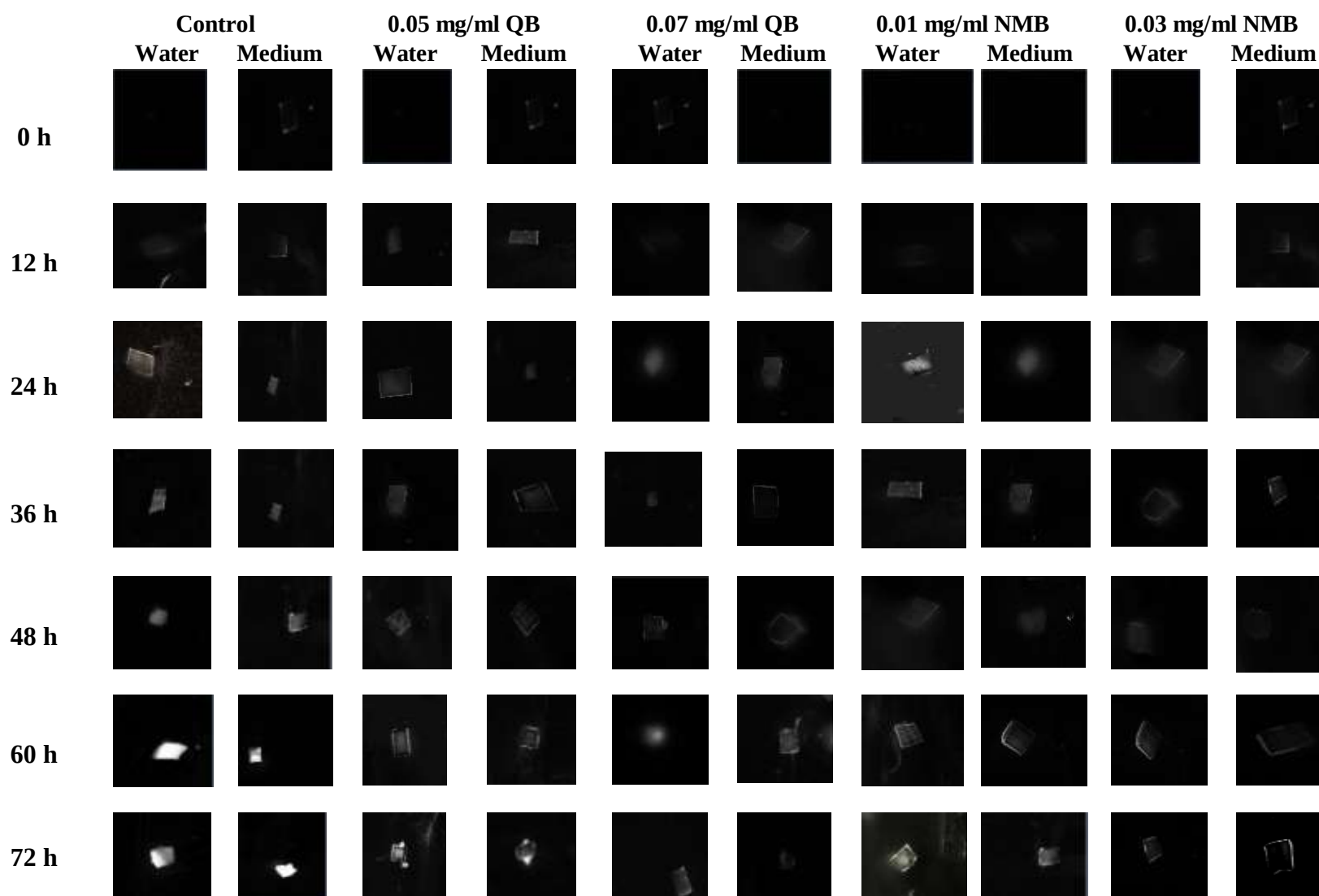


Figure 4.55: Inhibition of *S. paucimobilis* MG6 biofilm on PVC coupons in water and medium by QB and NMB

4.33 Degradation of *S. paucimobilis* MG6 AHL by NMB

To demonstrate whether NMB can degrade AHL signaling molecule of *S. paucimobilis* MG6, GC-MS analysis was performed in concentrated supernatants incubated with NMB. After incubation of NMB with dDHL, it was observed that [M-H] ions which are characteristic of this signaling molecule are absent, while the presence of specific ions at m/z 69, 86 and 128 confirmed the loss of dDHLs (Figure 4.56). These results indicated that NMB could effectively remove AHL molecules and subsequently degrade them.

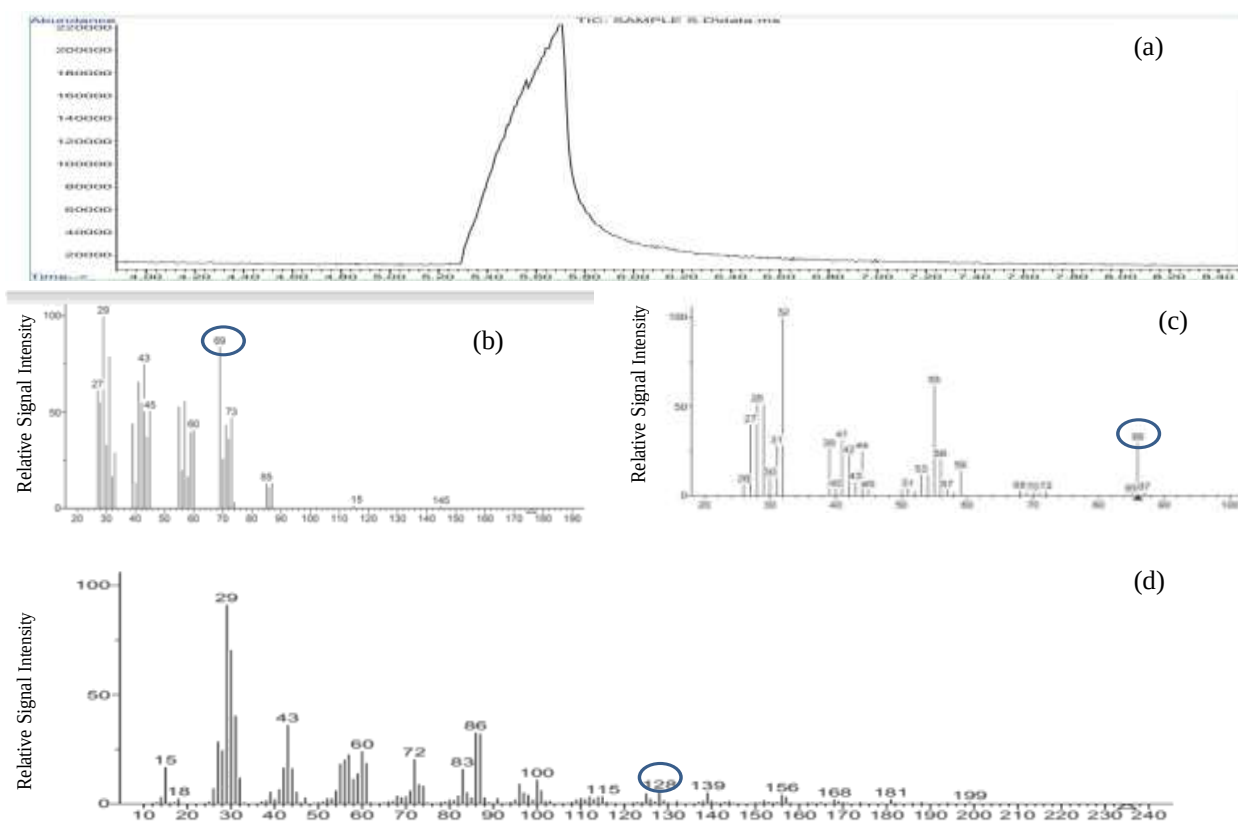


Figure 4.56: GC-MS spectra of extracted AHL (N-dodecanoyl-L-Homoserine lactone) degradation by NMB in full scanning positive mode (a) Total Ion Chromatogram (b)-(d) Ion-fragments at different m/z .

The artificial control of AHL concentration has been regarded as an effective strategy to control the group behavior of bacteria. Recent studies have indicated the enormous applicability of natural or synthetic compounds capable of controlling AHL either by binding irreversibly, trapping or by degradation leading to attenuated virulence or elimination of derogatory outcomes mediated by Q.S (Okano & Kato 2016). It is possible that a trapping effect followed by lactonolysis could be a predominant phenomenon responsible for degradation of dDHL of *S. paucimobilis* MG6 by NMB. Since biofilm formation is mediated by AHL, degradation of AHL lead to the loss of biofilm production. Though further studies may unravel the finer mechanistic aspects of AHL degradation by the modified biopolymeric compound, NMB, the findings presented in this study imply a convincing approach for alternatives to currently used water treatment methods specifically with relevance to *Sphingomonas*.

CHAPTER 5

DISCUSSION

DISCUSSION

Though provision of safe drinking water is possibly a land mark achievement in public health, maintaining the quality of water has presented a constant challenge for microbiologists and engineers (WHO 2004). The fact is profoundly realized by the global morbidity and mortality data related to water safety, for instance, an estimated 3.4 million people die of water-related diseases every year (WHO 2004). In the U.S. alone, annual drinking-water gastrointestinal cases range from 12-19 million.

The drinking water distribution systems (DWDS) are known to support a diverse microbial community comprising of opportunistic pathogens. Environmental growth of several such water-borne opportunistic pathogens occurs in the form of biofilms attached to the pipe walls. Any change in the hydraulic conditions in the distribution network change can lead in overcoming the biofilm adhesive forces thereby rendering detachment of biofilms. As a result, the biofilm forming bacteria, pipe characteristics and hydrodynamic conditions simultaneously influence the physico-chemical attributes and safety of drinking water (Szewzyk et al. 2000; Percival & Walker 1999; Sharma et al. 2003). The health burden resulting from opportunistic pathogens via drinking water has been reported to be largely unregulated worldwide (Ashbolt 2015).

While a number of physical and chemical methods have been suggested as alternatives to conventional treatment for inactivating the opportunistic pathogens in drinking water, few have been viable in view of their applicability. Green approaches for water treatment have infused considerable scientific enthusiasm in this category, newer antagonists or anti-biofilm compounds are being increasingly sought after for a more benign and fail- safe approach for inhibition.

Bacterial cross talk or sensing pathways are particularly favorable for developing such compounds isolated either from microbial or plant sources.

The prevalence of *Sphingomonas* in water distribution systems and the role of this emerging and opportunistic pathogen in biofouling have been reported from across the globe. Though biofilm formation by *Sphingomonas* have been reported, the lack of scientific information on the mechanism of biofilm formation and its relation to quorum sensing has been a bottleneck for developing rational interventions based on non conventional approaches. In view of these deliberations, an attempt was made to characterize *Sphingomonas* in the Indian water distribution system(s) and establish the basis of biofilm formation. The insights of the initial study, was applied for developing biopolymer antagonist(s) effective for disabling biofilm formation.

The ubiquity, clinical importance and anthropological occurrence of *Sphingomonas* have been of a major interest. Therefore, monitoring the presence, biodiversity and dynamics of *Sphingomonas* have been attempted by both culture independent methods such as PCR-DGGE and by culture dependent methods (Leys et al. 2004). A combination of physiological characteristics, biochemical and molecular tools can serve as a fair choice, when simply *Sphingomonas* sp. requires to be characterized. Koskinen et al (2000) reported that *Sphingomonas* sp. can be effectively characterized using cultural and molecular approaches from drinking water systems. A similar approach using cultivation based methods were applied to screen *Sphingomonas* from drinking water samples in this study. The isolates were then identified on the basis of physiological characteristics, biochemical and molecular tools based on established parameters.

A notable proportion of the isolates were biofilm formers, one with the highest ability was completely characterized as *Sphingomonas paucimobilis* MG6 (GenBank database Accession No KX54380). Planktonic cells of this strain possessed a specific growth rate of 0.18 h^{-1} .

To date, *Sphingomonas* has been reported in various environments, including both aqueous (drinking water and seawater) and terrestrial habitats, plant root systems, clinical specimens, and others. Its widespread distribution in the environment is accounted for its ability to utilize a wide range of organic compounds, to grow and also survive under low-nutrient conditions. This qualifies it to compete successfully with indigenous organisms in respective niches (Besner et al. 2002; Flemming 2002; Bachmann & Edyvean 2006; WHO 2011). The prevalence of *Sphingomonas* as an inhabitant of biofilm in water distribution systems (Momba et al. 2000; Flemming 2002; Stopforth et al. 2002) has been extensively documented. However, the presence or characteristics of this microorganism in Indian drinking water systems has not been reported before.

Since biofilm formation relies on an interplay of nutritional components and environmental variables, it may be anticipated that optimization of these variables would be important in identifying relevant parameters responsible for maximum biofilm production. An initial medium optimization and culture conditions provided cues for further rationalizing these parameters by central composite design. This method led in identifying the actual concentrations required for maximum biofilm formation.

Bearing in mind that crystal violet assay provides indication of good biofilm mass but fails to indicate metabolic viability; assay with MTT was carried out for complete determination of

biofilm. The chromophore, calcofluor on the other hand enables a rapid visual method for detection and was used to detect the adherence of cells to the surface. Incorporation of all three assays was crucial for overcoming the individual limitations of each method for characterizing the *S. paucimobilis* biofilm. Besides evidence of biofilm formation, adherence of *S. paucimobilis* onto various matrices like glass, microtiter plate and stainless steel could be clearly observed. The variation in the degree of adherence observed can be explained by considering the surface of matrices i.e. hydrophobicity and roughness. The degree of microbial colonization is directly dependent on the surface roughness (Donlan et al. 2002) which affords a high surface area and hydrophobicity of surfaces (Characklis & Marshall 1990; Rogers et al. 1994; Meyer 2003). Whether specific cellular appendages or proteins are additionally involved in adhesion, remains to be seen. Interestingly a recent study has identified several virulence genes in the genome of *S. paucimobilis* involved in adherence such as flagella genes and Type IV pili genes (Saeb et al. 2014). However, the effects of the protein products of these genes and their contribution for surface attachment remain obscure. Motility in Gram negative bacteria constitutes an important functionality positively correlated to biofilm formation. Surface motilities like swimming, swarming and twitching are regulated by flagella and pili which help in bringing the cells in close proximity to the substratum and enable the organism to migrate across biotic substratum to form cell aggregates. The role of motility in *Sphingomonas* leading to biofilm formation has been demonstrated by Bereschenko et al (2010) and more recently in *Sphingomonas melonis* by Francez-charlot et al (2015).

Quantification of biofilms assume importance in view of either invasiveness, economics or complexity of existing techniques, for instance, Fourier Transformation Infrared

Spectroscopy/Attenuated Total Internal Reflection (FTIR/ATR), Photo Acoustic Spectroscopy (PAS), Fibre Optical Sensors (FOS), Quartz Crystal Microbalance (QCM). Consequently, non-destructive and non-invasive detection techniques for the monitoring of biofilm accumulation are of major interest especially for *Sphingomonas*, since this would render routine analysis of biofilms simpler. In view of this, a method for analyzing biofilm of *Sphingomonas* through the measurement of electrical capacitance was developed. The method, proved to be effective in detecting the age of biofilms by depicting a proportional increase in capacitance value. The latter could be correlated to the increase in biomass and EPS content with age of the biofilm and was subsequently used in rapidly assessing *Sphingomonas* biofilm progression on experimental materials as well as in other experiments.

Attachment of microorganisms to surfaces or matrices in a step wise manner constitutes the initial stage of biofilm development. Bacterial aggregation is recognized to be an important event and a prelude to biofilm development (Voloshin & Kaprelyants 2004). Since aggregation depends on the range of interactions like synergistic, mutualistic, antagonistic, competitive, and commensalism interactions (Simões et al. 2007), it becomes important to analyze the ability of the candidate bacteria in recognizing members of other species, likely in a natural environment. Such recognition, if positive can eventually lead to the development of multispecies biofilm communities. The detailed analysis of aggregation and coaggregation capabilities of *S. paucimobilis* MG6 indicated its profound capacity to coaggregate water borne pathogens like *E.coli* O157:H7, *Shigella* and *Salmonella*. The aggregation index with *Shigella flexneri* 2a was highest followed by *Salmonella typhimurium* and *Escherichia coli* O157:H7; these results also indicated that *S. paucimobilis* MG6 could form flocs. Visualization experiments using

microscopic methods and DAPI staining clearly demonstrated the coaggregate formation. It may be inferred that ability to coaggregate confers protection of the *Sphingomonas* cells from directed shear forces and enables them to proceed to a multigeneric biofilm. Biofilm formation in water distribution system is expected to occur on the piping or plumbing material. Therefore it was deemed judicious to investigate the biofilm formation on different commonly used plumbing materials. The colonization of plumbing materials like polyvinyl chloride (PVC), polypropylene (PP), polyethylene (PE), aluminium (Al), copper (Cu) and rubber (R) by *Sphingomonas* was studied using the fluorescent probe, calcofluor. The quantity of fluorescence is a function of the calcofluor affinity for capsular polysaccharides (CPS) and the amount of adherent CPS. Thus, a negative or weak result denotes minimal biofilm formation, which is exactly what the probe is intended to detect (Singla et al. 2014). The variation in biofilm growth was consistent and could be correlated to that obtained in the flow through simulation system designed with the different plumbing materials. Piping or plumbing material plays a significant role in the maintenance of water quality as it can provide the bacteria with nutrients in water through the leaching of organic compounds. Lesser bacteria are supported by those materials with minimal leaching, ultimately lowering the biocorrosion level and ensuing better water quality. Lehtola et al (2005) observed that the biofilm formation was slower in copper pipes as compared to PE pipes. Besides, copper ions lower the number of microorganisms in water; while plastic pipes (PE), which have been used as cost-effective replacements of traditional plumbing materials, may release biodegradable organic compounds and phosphorus which promotes microbial regrowth and formation of biofilm. Our observations were in line with those reported earlier; PVC being able to promote highest biofilm formation.

Antimicrobial products are extensively used to combat biofilm contamination in various settings including water meant for distribution and consumption. These active chemical agents or biocides act on multiple intracellular targets. Regardless of the particular chemistry or mechanism, the biocide must be able to reach the target cell to cause damage. At the multicellular level, therefore, the effective biocide must penetrate into extracellular matrices of biofilm, which acts as a diffusion barrier and as a charged binding filter. The biofilm extracellular matrices, in addition complement enzymes and multidrug resistance pumps on cells that remove antimicrobials (Singh et al. 2017). Given the wide compositional variability in composition among species, biofilms pose a significant threat in terms of resistance to disinfection treatments using liquid antimicrobials. For instance, biofilms exposed to chlorine bleach for 60 min were reported to still harbor live cells (Liu et al. 2017). It was therefore relevant in this study to evaluate the chlorine resistance of the isolated *Sphingomonas*. Lack of significant ($p>0.05$) reduction of viability at a concentration of 2 mg/L over a contact time of 90 minutes indicated resistance to low concentrations of chlorine, higher levels of 8mg/L led to significant ($p<0.05$) reduction. Le Chevallier et al (1988) suggested that low concentration of chlorine may not be effective for *Sphingomonas*. The chlorine resistance of *Sphingomonas* has been ascribed to its ability to aggregate (Gauthier et al. 1999), the *S. paucimobils* MG6 possesses a high aggregation index, which can account for its chlorine resistance. However, *Sphingomonas* strains with higher chlorine resistances have been reported. Sun et al (2013) reported a strain of *Sphingomonas* tolerant to 4 mg/ L of chlorine with 240 min retention time and as low as 5% viability reduction. In view of the foregoing deliberations, it may be argued that for

Sphingomonas, a fail-safe prediction of inactivation would require either a combination of inactivation methods or a non conventional approach.

Strategies, especially those which adopt a non conventional cellular target have evoked considerable scientific interest for developing antimicrobials. The essential approach herein lies in targeting bacterial cell-to-cell interactions or quorum sensing (QS), interruption of this network using suitable antagonist molecules can attenuate microbial virulence and biofilm production (Bauer & Robinson 2002). Therefore, the elucidation of signaling molecules and their role in biofilm formation by *S. paucimobilis* MG6 was cardinal in the study.

Quorum sensing bioreporter/biosensor strains are commonly used as a reliable detection method for AHL production by Gram negative bacteria. The use of reporter strains to perform thin layer chromatography (TLC) -overlays of AHLs provides a simple and rapid technique used to identify AHL activity and can furnish insights into the physicochemical properties of AHLs. Of the two biosensor strains *A. tumefaciens* A136 and *C. violaceum* CV026 employed in this study, *A. tumefaciens* is generally used for detecting C6-C12 AHLs, while C14-C18 AHLs could be detected at micromolar concentrations, while *C. violaceum* CV026 is useful for detecting C4- and C6-AHLs on the basis of pigment production. Long chain AHLs molecules detected were confirmed using TLC overlay assay with A136 as biosensor strain, and a single spot displaying autoinducer activity provided a straightforward visual index for the presence of AHL. Structural identity was deemed important at this stage, therefore extracted samples were subjected to HPLC analysis. Sharp eluted peaks were observed when linear gradient of acetonitrile and formic acid were used as the mobile phase (Pearson et al. 1995). It is known that the side-chains of AHLs affect the polarity, and the polarity determines the retention time of AHLs in the

chromatographic column. Earlier results indicate that the log P value (P is the retention time) is proportional to the length of the side-chain. Therefore, the residence time for the unknown AHL molecule obtained from the HPLC column was used to determine the length of the side-chain. The signal molecule of *Sphingomonas* appeared to be the homolog of N-dodecanoyl-L-Homoserine lactone. The mass spectrum of the eluted compounds from HPLC showed ion fragments characteristic of homoserine lactones. MS showed three specific ions at m/z 156, 57 and 71; these results were similar that observed by Park et al (2001). This can be related to the presence of C12-AHL.

Kawaguchi et al (2008) proposed as simple high throughput method for quantitating AHL autoinducers in cell free assay system. The method based on β -galactosidase expression by the bioreporter strain of *A. tumefaciens* was considered to be ideal for determining the kinetics of AHL production by *S. paucimobilis*. Maximum AHL production was observed at 18 hours and judged to be growth associated. Biswa & Doble (2014) reported that highest AHL production occurred at 24 hours in a marine strain of *P. vulgaris* with an onset of production at the initiation of stationary phase. It is likely that *S. paucimobilis* produced highest AHL from late log phase for up to mid stationary phase during its growth. For Gram negative bacteria, QS systems have been extensively studied, especially those possessing N-acylhomoserine lactone (AHL) signal molecules. It has been suggested that when the cell number reaches a quorum, the N-acylhomoserine lactone (AHL) concentration increases enough to form a stable complex with its receptor protein, the complex then interacts with the QS-target gene for the transcriptional activation (Whitehead et al. 2001; Lazdunski et al. 2004). Though biofilm and AHL production kinetics indicated that biofilm in *S. paucimobilis* MG6 was correlated to AHL, a positive

correlation was sought. It was speculated that if AHL production is positively correlated to biofilm production in *S. paucimobilis* MG6, its deficiency should disable biofilm formation.

Transposon mutagenesis of *S. paucimobilis* MG6 was subsequently attempted for generating mutants lacking AHL producing ability. *S. paucimobilis* MG12, which failed to qualify as an AHL producer and more importantly lacked the ability to form biofilms. Mixing experiments to ensure whether AHL could favor biofilm production were carried out by supplementing *S. paucimobilis* MG12 with cell free supernatants of native *S. paucimobilis* MG6. The observations that *S. paucimobilis* MG12 resorted to biofilm formation upon AHL supplementation, indicated that AHL was instrumental in biofilm formation. It was therefore apparent that antagonists designed should affect AHL in order to effectively disrupt biofilm formation.

Recent studies on exopolysaccharides with anti-biofilm functionality, both from bacterial and non bacterial sources have indicated exciting possibilities. For instance, Jiang et al (2011) demonstrated that exopolysaccharides from a marine strain of *Vibrio* sp. Ky101 could effectively inhibit biofilm synthesis whereas Bendaoud et al (2011) showed the efficacy of exopolysaccharides from *Kingella kingae* in inhibiting biofilm by the producer bacteria. The microbial exopolymer produced from an environmental isolate of *Klebsiella terrigena* has been extensively investigated by Khaira et al (2013). The availability of the exopolymer producing bacterial strain, functional and structural prospects and safety of this exopolysaccharide biopolymer was important leads for developing the quorum sensing inhibitor(s). The mono- and tri-methylated biopolymers, designated as quaternized biopolymer (QB) and N-methylbiopolymer (NMB), consistently inhibited (at sub-inhibitory concentrations) the blue coloration produced by *A. tumefaciens* A136, besides significantly ($p < 0.05$) lowering β -

galactosidase activity. Motility is an important function for *Sphingomonas* and its absence should render the cells incapable to attach or aggregate. The swarm plate assays conclusively proved a complete loss of motility of *S. paucimobilis* MG6 following exposure to both QB and NMB. It was presumed that the modified biopolymers were able to effectively inhibit QS-gene expression possibly by blocking the auto-inducer signaling molecules or AHL with a consequent loss of bacterial motility. However, it was equally important to understand whether the anti QS compounds affected the viability of the cells.

Prominent cellular leakage by QB but none by NMB was noted. These results confirmed that NMB lacked ability to compromise the cellular viability though it could inhibit several functions of QS, but QB was responsible for anti QS effect by inactivating the cells. Quaternary antimicrobials either traverse the outer membrane or compromise it. Besides they can completely alter the lipopolysaccharide (LPS) structure of bacterial strains affecting its virulence by amount of LPS produced, the O side chain length, composition, and the degree of glycosylation (Murray et al. 2006; Patel et al. 2010). The fact that increased membrane permeability leads to destabilization of the cell membrane and leakage of intracellular materials ultimately leads to cell death (Raafat & Sahl 2009) has been the most prevalent observation. The results obtained in this study were similar to those reported in other studies, suggesting membrane damage by QB to be the principal mechanism for inactivation. NMB, on the other failed to affect the outer membrane as evident from the results.

It has been proposed that the signal-inducing LuxI homolog, the N-acylhomoserine lactone signaling molecule, and the receptor LuxR homolog are the potential targets for antagonists. To date, several compounds have been documented to have interference with QS due to their

structural similarity with autoinducer or increasing the degradation of the LuxR receptors of the system leading to the interference with quorum sensing (Vattem et al. 2007). A process involving modulation of AHL molecule synthesis by lowering the expression of the LuxI/Lux R homologs or the ability of compounds to competitively or non-competitively inhibit their activity would lead to loss of biofilm production. To establish whether potential inhibitors target AHL synthesis (via LuxI) or AHL output (via LuxR), a double ring bioassay was carried out using the *A. tumefaciens* biosensor system, at a sub-inhibitory concentration of QB and NMB. It was observed that QB decreased both LuxI and LuxR activity leading to both LuxI and LuxR modulation, especially Lux R modulation whereas NMB exerted insignificant ($p>0.05$) effect on both of them. It was apparent that ‘Artificial reduction’ of the AHL concentrations, inhibited the AHL complex formation with its receptor inside the cell. It is possible that NMB interacted with AHL outside the cell as would be expected of a ‘trusted QS blocker’. Given the polymeric structure of NMB, a ‘capturing effect’ wherein the AHL molecules are trapped within the polymer seems a strong probability to explain the phenomenon. In the event of such a process, AHL molecules undergo lactonolysis in which the homoserine lactone ring is converted to ring open form of its corresponding signal molecule. The lone pair of electrons on nitrogen atom makes methylamine a potential nucleophile. Its effect on lactone group’s carbonyl carbon results in the formation of an intermediate. Finally, the ester bond of lactone moiety of the signal molecule breaks to yield the ring-opened product, in this process (Figure 5.1). Incubation of NMB with the AHL fractions of *S. paucimobilis* MG6 indicated absence of [M-H] ions, characteristic of this signaling molecule, while the presence of specific ions at m/z 69, 86 and

128 confirmed the anti-quorum sensing ability of NMB, lending credence to this hypothesis.

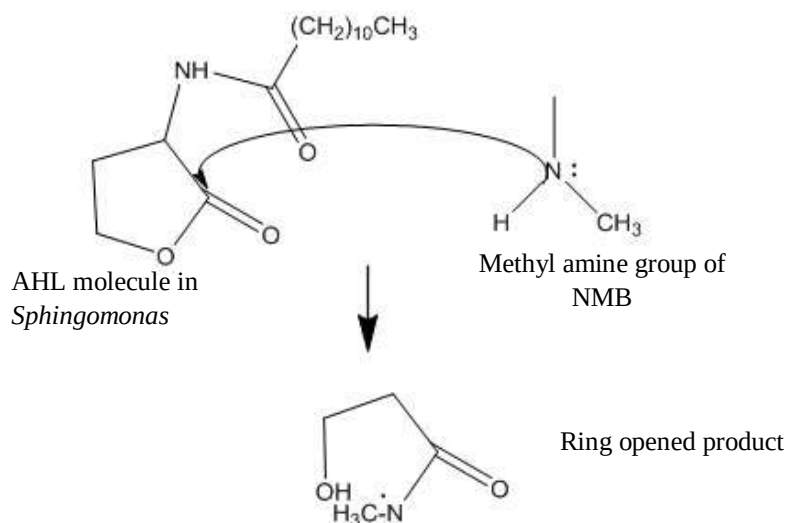


Figure 5.1: Hypothesized mechanism for AHL ring opening by NMB

Molecules with structural similarities to AHLs, like fimbriolides affect the interaction between AHL and putative regulatory protein (LuxR or LuxR homolog), they usually bind to the receptor competitively (Givskov et al. 1996) causing loss of signal reception. Besides, binding of furanones to receptor causes conformational changes that lead the resulting complex into rapid proteolytic cleavage (Manefield et al. 2002). These agents attenuate the expression of QS regulated behavior like biofilm formation in microorganisms. In contrast with the abundant literature on quorum sensing inhibitors targeting *Pseudomonas aeruginosa* (Kalia 2013; Stevens et al. 2010; Galloway & Burbey 2011), *Pseudomonas putida* (Ren et al. 2005) and *Escherichia coli* (Ren et al. 2001), negligible work has been done on deciphering the quorum sensing system in *Sphingomonas* and exploring inhibitor(s) specifically designed to interfere with its quorum sensing systems leading to a loss of biofilm production. Very recently, an interesting new class

of compounds, glycomonoterpenols, derived from monoterpenoids alcohols synthesized using *Candida bombicola* ATCC 22214 by feeding the cells with linalool and alpha terpineol (Patil et al. 2017). The specific advantage of these molecules described was their additional surfactant like property, increased solubility and enhanced QSI potential, the compounds possessed strong anti-QS activity. The findings from the present study expand the existing knowledge of ‘green antagonists’ based on partial chemical modification of biopolymers which could possibly be used for restricting biofilm formation by *Sphingomonas* in water distribution systems. Besides being robust biopolymers from bacteria can provide the inherent advantage of trapping the AHL molecules with subsequent inactivation (lactonolysis) by affording proximity of the surface modified groups.

To understand whether the chemically modified biopolymer antagonist, NMB could successfully inhibit biofilm production of *Sphingomonas* under natural conditions, water samples from diverse locations were collected, analyzed and evaluated for biofilm production. The water sample with maximum biofilm formation was thereafter challenged with different doses of NMB to verify whether biofilm formation still occurred. PVC coated NMB was analyzed for presence of *S. paucimobilis* biofilm formation. In both instances, absence of biofilm production could be observed. The stability of NMB on PVC coated coupons was retained for over a period of 72 hours as indicated by its non leachability and activity. Overall, the current study conclusively established the AHL based mechanism in *S. paucimobilis* MG6, its role in biofilm formation. The study also proved that biopolymers with minor chemical modification could successfully bind to AHL molecules, inactivating them by lactonolysis, thus leading to a loss of biofilm production.

CHAPTER 6

SALIENT FINDINGS

SALIENT FINDINGS

A strain of *Sphingomonas* with high biofilm forming ability was isolated from public drinking water system. The strain was designated as *Sphingomonas paucimobilis* strain MG6, has been submitted to GenBank with accession number KX594380. Biofilm production by this strain was evaluated by different staining assays, SEM and Fluorescence microscopy. The strain was capable of producing biofilms on commonly used plumbing matrices.

- Culture conditions essential for maximum biofilm formation by *S. paucimobilis* MG6 were determined using Central Composite Design (CCD) of Response Surface Methodology (RSM). The optimum concentration of glucose and sodium glutamate as carbon and nitrogen source was found to be 1.05% after verifying predicted responses with experimental data. Temperature and pH for highest biofilm production with the optimal carbon and nitrogen source was found to be 30°C and pH7 respectively.
- To rapidly and effectively ascertain biofilm age, a novel sensor system using changes in capacitance values was designed. Capacitance value of 1 kHz resulted in reproducible values which could be correlated to biofilm age measured, as dry weight depicting the biofilm growth/progression over time. The sensor could successfully measure biofilm age on *S. paucimobilis* MG6 biofilms, experimentally developed on RO membranes. The method can be used for developing an online system to detect biofilms by this opportunistic pathogen in drinking water, important for designing timely interventions.
- The auto- and co-aggregation in case of *S. paucimobilis* with water borne pathogens was found to proficient and it was confirmed by quantitative and qualitative methods. Notable coaggregation occurred with a cocktail of the three water borne pathogens; highest aggregation index was observed with *Shigella flexneri* 2a followed by *Salmonella*

typhimurium and *Escherichia coli* O157:H7. Heat and protease treatment altered aggregation ability suggesting possibility of surface proteins as mediators of aggregation with other pathogens except *Escherichia coli* O157:H7.

- *S. paucimobilis* MG6 was capable of exopolysaccharide production and possessed a fair degree of hydrophobicity. The isolate was found to be resistant to Ampicillin, Amikacin, Piperacillin and Streptomycin.
- Chlorination had insignificant effect on planktonic *S. paucimobilis* as well as on biofilm formed on plumbing materials. Mixed biofilms of *S. paucimobilis* with water borne pathogens, enhanced the resistance of chlorine the maximum being with *Shigella flexneri* 2a, followed by *Salmonella typhimurium* and *Escherichia coli* O157:H7.
- Biosensor guided assays with *A. tumefaciens* A136 and *C. violaceum* CV026 and TLC were used to identify long chain AHLs molecules produced by *S. paucimobilis* MG6. These molecules were further characterized as homologs of N-dodecanoyl-L-Homoserine lactone by HPLC and GCMS.
- Transposon mutagenesis was used to construct an AHL negative mutant. The mutant, designated as MG12, lacked the ability to produce biofilm. Mixing experiments where AHL was supplemented to growing mutants resorted to biofilm formation. This confirmed the fundamental role of AHL for biofilm formation in *S. paucimobilis* MG6.
- The exopolymer from *Klebsiella terrigena*, was chosen for designing antibiofilm compounds. The biopolymer possessed excellent thermal stability and was mono- and tri-methylated to yield (a) N-methyl biopolymer and (b) Quaternized biopolymer. These

compounds were found to inhibit biofilm formation as determined by a number of assays. Absence of motility in the treated cells was noted as well.

- Double ring bioassays with *A. tumefaciens* biosensor system, indicated that QB decreased both LuxI and LuxR activity which led the modulation of Lux R. While NMB had negligible effect on both AHL synthesis and response regulator.
- Physiological and Biochemical Responses such as electric conductivity measurements, leakage of potassium ions, proteins, glucose, Lactate dehydrogenase (LDH) ATP leakage and UV-absorbing substances of *S. paucimobilis* treated with NMB and QB confirmed membrane destabilization with QB. NMB lacked ability to cause disintegrate the cellular barrier thus ascribing it to be a ‘better quorum quencher’.
- The underlying mechanism for NMB as quorum quencher was hypothesized to be a ring opening reaction with the involvement of methyl amine group as nucleophile. The ester bond of lactone group of AHL breaks to yield the open-ring product. The biopolymeric structure could facilitate in trapping the AHL molecules and bringing them to proximity of reactive groups thus facilitating lactonolysis. Failure of AHL molecules to attain desired threshold values through ‘artificial removal led to loss of biofilm production.
- GC-MS analysis of AHL molecules of *S. paucimobilis* MG6 incubated with NMB revealed absence of characteristic [M-H] ions. This finally confirmed that NMB was effective in removing AHL, thus resulting in quorum quenching.

- Evaluation of NMB for understanding its stability and functionality under environmental conditions, water samples collected from 20 different regions and used to determine *S. paucimobilis* MG6 biofilm production. QB and NMB were challenged in water samples (supporting highest biofilm production) inoculated with *S. paucimobilis* MG6. In a similar fashion, PVC coupons coated with NMB were also analyzed in parallel. Significant loss of biofilm production was noted in both cases, indicating functionality of NMB under environmental conditions and also applicability of NMB as a possible anti-biofilm coating material.

Overall, the results of the present study demonstrated the presence of high biofilm forming *S. paucimobilis* in drinking water distribution system, the profound capability of this strain to resist chlorination and form mixed biofilms with water borne pathogens on plumbing matrices. The characteristics of AHL and its role in biofilm formation could be established for this strain. Biopolymer of microbial origin modified partially could artificially trap and inactivate AHL molecules leading to loss in biofilm production. The environmental stability of the developed biopolymeric antibiofilm compound and its functionality under real time conditions suggest an exciting potential for commercial exploitation.

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APPENDICES

APPENDIX I

Media

FIB media

<i>Composition</i>	<i>Quantity (g/L)</i>
Ammonium sulphate	1.0
Calcium chloride dehydrate	0.7
Dextrose	1.0
Dipotassium hydrogen phosphate	1.0
Magnesium sulphate heptahydrate	0.3
Peptone	5.0
Potassium dihydrogen orthophosphate	1.0
Sodium chloride	0.1
Agar	3.0
pH	7.2 ± 0.2

LB agar

<i>Composition</i>	<i>Quantity (g/L)</i>
NaCl	10.0
Beef extract	5.0
Tryptone	10.0
Agar	10.0

BHI agar

<i>Composition</i>	<i>Quantity (g/L)</i>
Casein peptone	14.5
Meat peptone	7.0
Brain Heart Infusion Solids	6.0
Sodium chloride	5.0
Disodium phosphate	2.5
Dextrose	2.0
Sheep blood (Optional)	6%
Agar	15.0
Distilled Water	1000.0 ml

pH	7.4 ± 0.2
----	-----------

Nutrient broth

<i>Composition</i>	<i>Quantity (g/L)</i>
Beef Extract	1
Yeast Extract	2
Peptone	5
Sodium chloride	5
Distilled Water	1000 ml

Sphingomonas selective growth (SSG) medium (Yim et al. 2010)

L9 minimal salt medium

<i>Composition</i>	<i>Quantity (g/L)</i>
Disodium hydrogen phosphate dihydrate	8.8
Potassium dihydrogen phosphate	3.0
Ammonium chloride	1.0
Sodium chloride	0.5
Magnesium sulfate	0.2
Trace Element solution	2.5 ml

Trace element stock solution (per liter)

<i>Composition</i>	<i>Quantity (g/L)</i>
Manganese (II) Chloride dihydrate	23 mg
Manganese chloride hydrate	30 mg
Boric acid	31 mg
Cobalt (II) chloride hexahydrate	36 mg
Cupric Chloride dihydrate	10 mg
Nickel (II) Chloride hexahydrate	20 mg
Sodium molybdate dihydrate	30 mg
Zinc chloride	50 mg
Glucose	1%
Sodium Glutamate	1%
Streptomycin	100 µg/ml
Piperacillin	50 µg/ml

Reagents and Buffers

50X TAE Electrophoresis buffer

Composition

Quantity (g/L)

Tris free base	242 g
Disodium EDTA	18.61 g
Glacial Acetic Acid	57.1 ml
Distilled water	to 1000 ml

Add the Tris free base and EDTA to approximately 700 ml distilled water and stir until the Tris and EDTA are dissolved. Add the acetic acid and adjust the volume to 1 liter.

The 1x TAE solution is 40 mM Tris, 20 mM Acetate and 1 mM EDTA.

Lysozyme

Stock solution	10 mg/ml
Working solution	300 - 400 mg/mL

Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Glycerol in water	30.0%

TE buffer 10X

Tris-HCl	10 M (pH 8)
EDTA disodium salt	100 mM

Genomic DNA Extraction buffer

Sodium acetate	50 mM
EDTA disodium salt	500 mM
Sodium Chloride	1%
Sodium dodecyl sulfate	1M
	0.15M

Saline EDTA Solution

Ethylenediaminetetraacetic acid , EDTA	137 mM
Sodium Chloride, NaCl	2.7 mM
	10 mM

1 × phosphate-buffered saline (PBS)

Sodium Chloride	2 mM
Potassium Chloride	40mM
	60mM
Sodium hydrogen phosphate	10mM

Potassium dihydrogen phosphate	1mM
<u>Z-Buffer</u>	50mM
Sodium Monohydrogen Phosphate Heptahydrate,	1000ml
Sodium Dihydrogen Phosphate Monohydrate	0.25%
Potassium Chloride	30.0%
Magnesium Sulfate Heptahydrate	0.1 M (pH 8)
2-Mercaptoethanol	10 M (pH 8)
Distilled Water	100 mM
Adjust pH to 7	

Make a 5x stock without the 2-Mercaptoethanol and add 2-Mercaptoethanol to 1x solution right before conducting assay.

1M Potassium Phosphate Buffer

To make 100 mL of 1M potassium phosphate:

1M Monopotassium phosphate	13.4 ml
1M Dipotassium phosphate	86.6 ml
pH	7.6±0.2

20 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside)

Mix 200 mg of X-gal with 10 ml of dimethylformamide (DMF)

Alkaline Potassium Iodide Solution

Add 150g of KI and 700g of KOH to 1L distilled water

APPENDIX II

Morphological and biochemical tests of the bacterial isolate MG6

Morphological Tests		Biochemical Tests	
Configuration	Circular	Indole Test	-
Cell Size	0.7 x 1.4 µm	Methyl red test	-
Margin	Entire	Voges Proskauer test	-
Elevation	Convex	Citrate utilization	-
Surface	Smooth	Casein hydrolysis	-
Pigment	Deep Yellow	Esculin hydrolysis	+
		Gelatin hydrolysis	-
Gram's Reaction	Negative	Starch hydrolysis	+
Cell shape	Rod shaped	Urea hydrolysis	-
Spore(s)	-	Catalase test	+
		Oxidase	+
Motility*	+	Tween 20 hydrolysis	+
		Tween 80 hydrolysis	-
		Polymyxin B	Susceptible
		Growth on	-
		MacConkey agar	
Physiological Tests		Assimilation of :	
Growth on NaCl (%)		Glucose	+
		L-Arabinose	+
5.0	-	D-Mannose	+
6.5	-	D-Mannitol	-
Anaerobe	-	N-acetyl-D-glucosamine	+
Acid production from sugar		Maltose	+
Trehalose	+	Potassium gluconate	+
Lactose	-	n-Caprato	-
Sucrose	-	Adipate	+
Glucose	+	DL-Malate	+
		Sodium Citrate	+
		Phenylacetate	-

*Motility occurs at 18°C to 22°C. Cannot be detected at 37°C.

APPENDIX III

***Sphingomonas paucimobilis* strain MG6 16S ribosomal RNA gene, partial sequence**

GenBank: KX594380.1

OCUS KX594380 544 bp DNA linear BCT 30-JUL-2016

DEFINITION *Sphingomonas paucimobilis* strain MG6 16S ribosomal RNA gene,
partial sequence.

ACCESSION KX594380

VERSION KX594380.1

KEYWORDS .

SOURCE *Sphingomonas paucimobilis*

ORGANISM *Sphingomonas paucimobilis*

Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonadaceae; *Sphingomonas*.

REFERENCE 1 (bases 1 to 544)

AUTHORS Ghosh,M., Gulati,P. and Sharma,V.

TITLE Direct Submission

JOURNAL Submitted (25-JUL-2016) Department of Biotechnology, Thapar
University, Department of Biotechnology, Patiala, Punjab 147001,
India

COMMENT ##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

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/strain="MG6"

/isolation_source="Biofilm scraps from municipal drinking
water supply"

/db_xref="taxon:[13689](#)"

/country="India"

/collection_date="2014"

rRNA <1..>544

/product="16S ribosomal RNA"

ORIGIN

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421 cgtgctacaa tggactacag gcgcgaccgc gagggagcta atccaaagtt ctcagttcgg
481 attgtctgca actcgcatga agggaatcgc tagtaatcgc ggatcagcat gccgcggtga
541 atac
```

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PUBLICATIONS



Monitoring of biofilm aging in a *Sphingomonas* sp. strain from public drinking water sites through changes in capacitance

Parul Gulati, Pawandeep Singh , Arun Kumar Chatterjee & Moushumi Ghosh

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Monitoring of biofilm aging in a *Sphingomonas* sp. strain from public drinking water sites through changes in capacitance

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ABSTRACT

This study reports the applicability of a capacitance-based technique for evaluating the biofilm progression of *Sphingomonas* sp. One hundred and forty isolates of *Sphingomonas* were screened from public drinking water sites, and one potential strain with biofilm-forming ability was used for the study. The biofilm production by this strain was established in microtiter plates and aluminum coupons. The standard biofilm-forming strain *Sphingomonas terrae* MTCC 7766 was used for comparison. Changes in biofilm were analyzed by energy-dispersive X-ray spectroscopy (EDX) and scanning electron microscope (SEM). Capacitance values were measured at 1, 100 and 200 kHz frequency; however, 1 kHz was selected since resulted in reproducible values, which could be correlated to biofilm age measured as dry weight over a time of 96 h (4 days) depicting the biofilm growth/progression over time. The EDX, SEM and capacitance values obtained in parallel indicated the related physiological profile usually displayed by biofilms upon growth, suggesting authenticity to the observed capacitance profile. The results of this study demonstrated the feasibility of a capacitance-based method for analyzing biofilm development/progression by *Sphingomonas* sp. and suggested a simple approach for developing an online system to detect biofilms by this opportunistic pathogen of concern in drinking water.

ARTICLE HISTORY

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KEYWORDS

Biofilm progression;
capacitance; microtiter
plates; opportunistic
pathogen; *Sphingomonas*

Introduction

The genus *Sphingomonas*, hallmarked by their oligotrophic nature and plasticity in man-made environments, has been intensively exploited for their metabolic properties relevant to biotechnological importance. Strains of *Sphingomonas* sp., for instance, have been reported as the effective degraders of refractory organic pollutants and simple aromatic and polyaromatic hydrocarbons. However, their recognition as opportunistic pathogens [1–4], the ability of this strain to survive in oligotrophic niches, such as hospital tap water supply systems and reverse osmosis systems, and their resistance to antibiotics have exacerbated their relevance as unappreciated reservoirs of infection. Furthermore, *Sphingomonas* isolated from drinking water has been reported as being capable of attaching and developing biofilm on a variety of surfaces [5–7]. Biofilm is the microbial consortia embedded in a self-produced exopolymer matrix composed mainly of extracellular polymeric substances (EPS), microbial cells, enzymes, proteins and nucleic acids. Gellan gum, one of the industrially important EPS, is secreted by the members of genus

Sphingomonas [8]. Fialho et al. [9] demonstrated the effectiveness of gellan gum for bacterial adhesion and colonization. Microbes living in these matrixes benefit from nutrient and water supplies, improved lateral gene transfer [10,11] and protection against adverse environmental conditions, such as desiccation and chemicals, including detergents, disinfectants and antibiotics [12–14]. Various environmental factors, such as electrolyte concentrations and medium compositions, have a profound effect on the biofilm development. The role of divalent cations, such as Mg^{2+} and Ca^{2+} , on bacterial colonization was studied by Guvensen et al. [15] either directly or indirectly by affecting electrostatic interactions and physiology-dependent attachment processes, respectively [16,17]. Since biofilm is three-dimensional (3-D) functional consortia of microbial cells [18] its analysis of biofilm structure requires the acquisition of 3-D data, and transmission electron microscopy and scanning electron microscopy (SEM) have been used to assess the architectural changes in biofilm [16,19–20]. In this regard, the age of biofilm is considered to be important since the complexity in architectural pattern

would influence its resistance to biocides and corrosion, and thus quality of water [21]. Although several methods have been attempted for quantifying biofilms, there are no reports on the development of simple methods to quantify *Sphingomonas* biofilms; our ongoing studies have also revealed predominance of this strain in plumbing lines and the capability to form biofilms (unpublished observations). A simple method to monitor biofilms following traditional or interventional treatment procedures in drinking water arguably would be important in rendering water quality and safety. In this study, we attempted to quantify the biofilm produced by a strain of *Sphingomonas* sp. using a simple capacitance-based method. A strong correlation between biofilm dry weights representing different ages which in turn representing progression could be demonstrated for *Sphingomonas terrae* MTCC 7766. The results were in complete concurrence with the *Sphingomonas* isolate, indicating further scope of practically exploring this technique to monitor the biofilm formation by *Sphingomonas* sp.

Materials and methods

Bacterial strains and culture conditions

Tap water samples were collected from 20 different municipal sites in presterile, screw-capped bottles kept in an ice box and transported to laboratory for analysis. One hundred and forty isolates were screened from the water samples on the basis of the morphology of the colonies. *Sphingomonas* selective growth medium is composed of L9 minimal medium, which is supplemented with 1% each of glucose and sodium glutamate as a carbon and nitrogen source, respectively. The L9 minimal salt medium was composed of (per liter) 8.8 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 3.0 g KH_2PO_4 , 1.0 g NH_4Cl , 0.5 g NaCl , 0.2 g MgSO_4 and 2.5 ml of a trace element solution. The trace element stock solution contained (per liter) 23 mg $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 30 mg $\text{MnCl}_4 \cdot \text{H}_2\text{O}$, 31 mg H_3BO_3 , 36 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 20 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 30 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 50 mg ZnCl_2 . The final pH of the growth medium was adjusted to 7.0 [22]. Optimal concentration for streptomycin and piperacillin was determined by performing the antibiotic susceptibility test at 0, 50, 100, 150, 200, 250, 300, 350 and 400 $\mu\text{g}/\text{ml}$. The isolates were further screened on *Sphingomonas*-specific growth medium containing 100 and 50 $\mu\text{g}/\text{ml}$ streptomycin and piperacillin, respectively [23], and characterized based on their biochemical and morphological attributes. One isolate with a demonstrable biofilm-forming ability was selected for further studies and characterized by 16S sequencing. 16S sequence of the isolate was submitted in GenBank database as *Sphingomonas*

paucimobilis strain MG6 (Accession No: KX594380). The standard strain of *S. terrae* MTCC 7766 was used in parallel for the sake of comparison.

Growth and biofilm kinetics

The growth kinetics of the selected strain and *S. terrae* MTCC 7766 was studied by growing it in minimal medium by inoculating the media with a 1% inoculum (O.D. $\approx$ 0.03), incubated at 37°C with shaking. Aliquots of culture samples were withdrawn aseptically after every 4 h and centrifuged at 8000 rpm for 5 min. Pellets were washed twice with sterile phosphate-buffered saline (PBS) and resuspended in 1 ml of the same. Absorbance was recorded at 600 nm with sterile media as reference.

The crystal violet (CV) assay with modifications of [24] was used to estimate biofilm production by *S. terrae* MTCC 7766 and *S. paucimobilis* MG6. Briefly, 300 μl of standardized bacterial suspensions in mid-log phase was revived in biofilm-inducing media and dispensed in each well of the polystyrene plates and incubated at 37°C. The unbound cells were removed by inverting the microtiter plate and vigorous tapping followed by rinsing the wells with phosphate buffer (pH 7.2). The adherent cells were stained with 200 μl of 1% (w/v) CV solution for 5 min. The wells were washed with deionized water extensively, and the plates were allowed to dry. Following washing, 200 μl of 30% (v/v) acetic acid was added to each well for 15 min; 100 μl aliquots were then transferred to fresh microtiter plates and the absorbance was measured at 585 nm.

To further evaluate the biofilm profile of both the *Sphingomonas* strains, the formazan-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on biofilms as described by [25] with modifications of [26]. Biofilm was grown in sterile flat-bottomed 96-well polystyrene microplates. After incubation in the wells of microtiter plates, cells were removed, and 150 μl of PBS and 50 μl of MTT at a concentration of 0.3% were added for 2 h at 37°C. After this time, the MTT solution was removed from the wells, and 150 μl of dimethyl sulfoxide and 25 μl of 0.1 M glycine buffer (pH 10.2) were immediately added to the wells to dissolve the formazan crystals formed in the wells. Incubation was carried out for 15 min at room temperature. The absorbance of the solution was measured at a wavelength of 550 nm using a microplate reader (Teccan, Austria).

Dry weight of biofilm samples

Aluminum coupons were used to study dry weight of biofilm. Weights of coupons were recorded in terms of

milligram prior to the experiment. The coupons were then sterilized and aseptically inoculated in media. The coupons were removed aseptically at a regular interval of 12 h till 120 h and dried in an oven preset at 100°C. At the regular interval of 30 min, coupons were removed from the oven, cooled and weighed, till a constant weight was achieved.

Biofilm characterization

SEM of biofilm

SEM analysis was carried out to observe surface properties of biofilm and EPS. All biofilm samples for SEM analysis were fixed using 2.5% glutaraldehyde solution overnight at 4°C. Washing was performed with PBS to remove the excess glutaraldehyde. Dehydration of biofilm samples was done by washing with 30% ethanol followed by 50%, 60%, 70%, 80%, 90%, 95% to 100% ethanol, respectively. The samples were air dried and two drops of hexamethyldisilazane were added, and samples were covered for 5 min. After 5 min, samples were again air dried at 37°C. Biofilm samples were then coated with platinum and analyzed by SEM (JSM541-V, JOEL, Japan).

Energy-dispersive X-ray spectroscopy. SEM was equipped with an energy-dispersive X-ray spectroscopy (EDX) analyzer. EDX provided the elemental analysis of sample.

Capacitance measurement of biofilm

The capacitance of biofilm and EPS were measured using parallel plate capacitors made of aluminum sheet. The capacitors were UV and flame sterilized prior to the experiments. These sterilized capacitors were then placed in glass petriplates having media containing 1% inoculum, and at a periodic interval of 12 h, the capacitance of the biofilm-coated capacitors was directly measured on an LCR (inductance, capacitance, resistance) meter (Gwinstek, model no. 821, Taiwan) over the span of 72 h.

Statistical methods. Multiple regression analysis using the program Statistic 6.0 was applied to verify the equivalence between the results obtained in the assays.

Results and discussion

Most of the recent works indicate *Sphingomonas* strain to be a biofilm producer and therefore important for human health. Biofilms have been implicated for metal corrosion in plumbing materials commonly used in drinking water and industrial water distribution systems, regardless of

the pipe materials; the biofilm-forming property of *Sphingomonas* may explain its ability to survive in the chlorinated municipal and industrial water distribution systems [27] described in previous studies. As a result, both municipal and industrial water systems can be vulnerable to contamination by this type of bacteria [5,6]. Numerous methods for qualitative and quantitative analysis are employed in the study of biofilms. Many of them require invasive sampling and removal of biofilm material from a surface for the determination of biofilm thickness, total biomass and microbial composition or activity. Therefore non-destructive and non-invasive detection techniques to monitor biofilm accumulation and activity are of major interest especially for *Sphingomonas*. Accordingly, the main objective of this work was to seek a non-invasive, non-destructive online technique for analyzing biofilm of *Sphingomonas* through the measurement of electrical capacitance. We envisaged that this approach could also prove worthy for evaluating biofilm development of *Sphingomonas* following chlorination in water enabling further innovations in the currently used treatment process.

Growth and biofilm profile of *Sphingomonas* sp.

Media composition plays a vital role in the ability of bacteria to form biofilm [28,29]. Since *Sphingomonas* has the ability to grow on low-nutrient conditions, minimal media was used for the study and the growth and biofilm kinetics of the bacterial strain were analyzed by growing the strain in this media [7]. The growth pattern of planktonic *Sphingomonas* is depicted in Figure 1. The lag phase continued till 6 h followed by the exponential phase for up to 24 h followed by the stationary phase. The specific growth rate was 0.18 h⁻¹. Biofilm formation by the *S. terrae* MTCC 7766 demonstrated a similar trend (Figure 2).

Both biomass quantity and microbial activity are the parameters mostly used to estimate the amount of biofilm [30]. The CV assay has been described as a rapid method for calculating biofilm biomass [31], and the MTT assay has been suggested for studying the metabolic activity of cells; hence, cell viability [32] and their difference can effectively depict biofilm profile of the bacterial strain. Maximum biofilm production occurred between 24 and 48 h as the cells are in the late stationary phase before the senescence phase starts. Cells in the stationary phase start synthesizing the EPS, which facilitates the attachment of cells to the surface [33]. With progress of time, the increase in EPS production further accelerates the rate of biofilm formation. In fact, the EPS production determines the immediate conditions of the life of biofilm cell [20] by

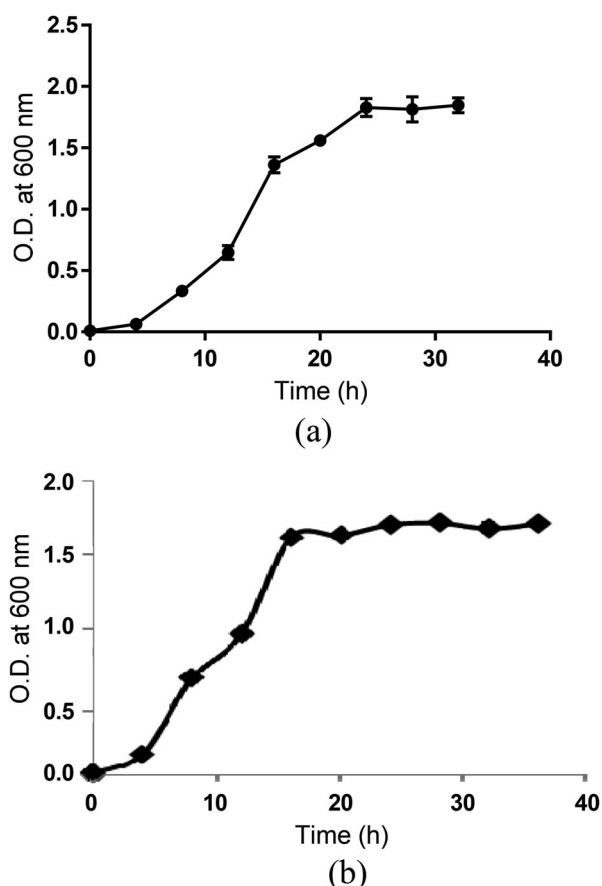


Figure 1. Growth profile of planktonic (a) *Sphingomonas paucimobilis* strain MG6 and (b) standard *S. terrae* MTCC 7766.

providing a variable gel matrix. Our preliminary studies with *Sphingomonas* have demonstrated the capability of this strain to produce EPS in line with earlier observations [34].

Scanning electron microscope

The SEM micrographs show *Sphingomonas* biofilm-coated aluminum coupons at different time intervals

[35]. Figure 3 indicated that biofilm growth experienced three phases: adsorption, logarithmic and stationary phase. In the adsorption phase, the biofilm structure is quite loose because the EPS synthesis has not occurred yet, as shown in sample A. When cells reach their log or exponential phase their structure changes into a denser one, as shown in sample B. Cells in the stationary phase start synthesizing the EPS, which facilitates the attachment of cells to the surface [33]. SEM micrographs indicated that at 36 h of biofilm formation the density of the EPS in the matrix increases, which further strengthens the connection between cells and substrate. The cells in the EPS matrix divide and develop into a mushroom-like structure, which further help in the maturation of biofilm [36].

EDX studies

Elemental composition of biofilm was analyzed using EDX (Table 1). The results clearly indicated a variation in elemental composition of biofilm with growth [37,38].

Capacitance studies of biofilm

The living cells can be regarded as closed, insulating membranes filled with liquid plasma that display dielectric features. Upon their exposure to the electric field, cells behave like electric capacitors, that is, capable of storing charges due to the movement of ions within the electric field [39,40]. The ions rearranged themselves to form dipoles under the electric field that is utilized for the detection of capacitance in the electric field. To understand whether biofilm age is associated with change in capacitance, parallel plate capacitors were used for the capacitance measurement of biofilm. Since biofilm acts as a dielectric material between the plates, alteration in the composition of biofilm is expected to result in changes in the capacitance values with time.

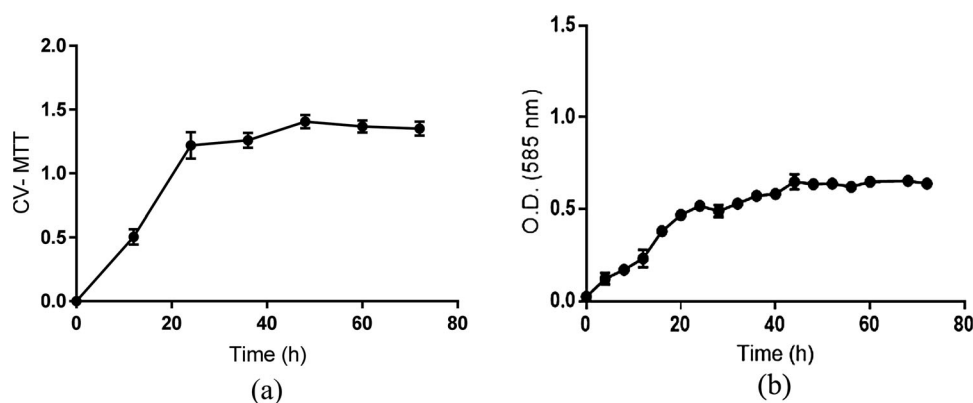


Figure 2. Biofilm profile of (a) *S. paucimobilis* MG6 and (b) standard *S. terrae* MTCC 7766.

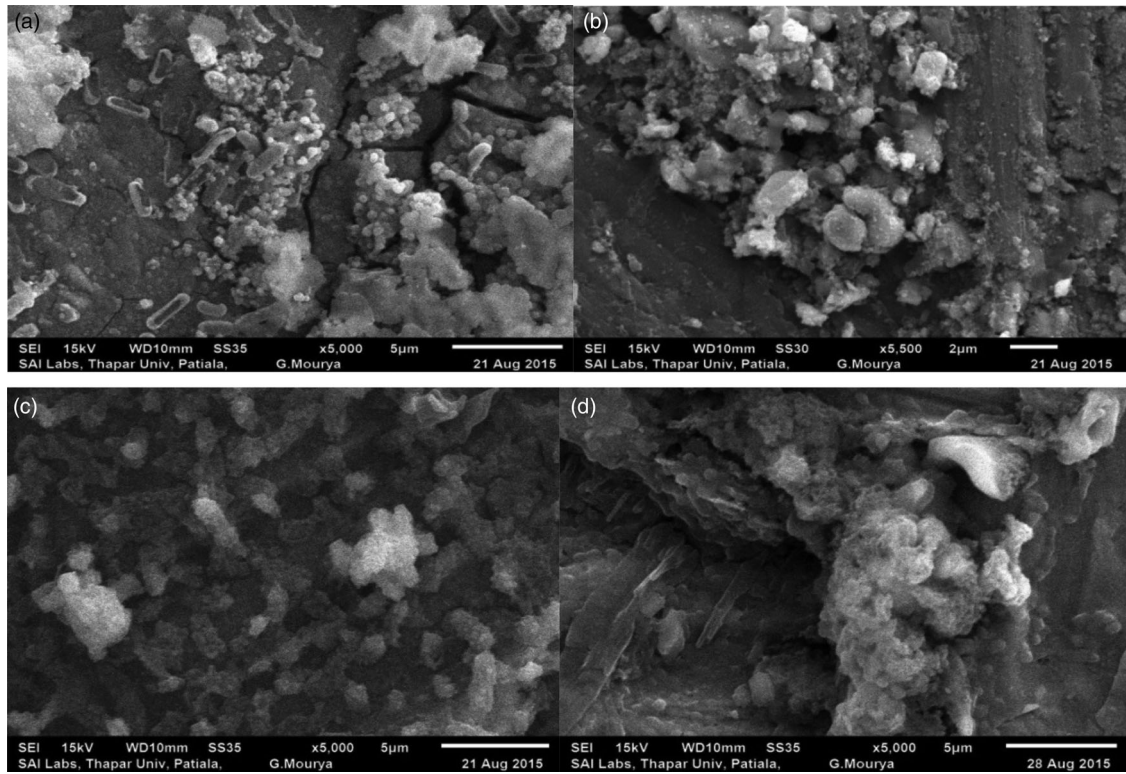


Figure 3. SEM images of the biofilm at different culture times. (a) 12 hours (b) 24 hours (c) 36 hours (d) 48 hours.

The electrical capacitance measurement in our study was based on the application of a voltage between two parallel plates separated by a non-conducting material. The amount of charge in each plate depends on: (1) the voltage applied; (2) the charge separation that occurs in the interior of the material between the electrodes and (3) geometrical factors such as the area of the plates and the distance between them. The ratio between the applied charge to voltage in each plate is the electrical capacitance of the isolating layer [41]. The electrical capacitance (C), measured at a distance between two parallel plates (D) whose surface is S , is given by Equation (1): $C = \epsilon_0 KS/D$, where ϵ_0 is the permittivity of free space (i.e. 8.854×10^{-12} F/m) and K is the dielectric constant of the medium. For the experimental procedure, measurements were done on aluminum coupons on which the biofilms were grown; the distance

between the plates was fixed at 0.14 cm. The plates used were 2.5 cm long and 2.5 cm wide (i.e. 6.25 cm^2). An LCR meter, Gwinstek, model no. 821, Taiwan, was used to measure impedances: its electrical capacitance measurement range is between 0.00001 pF and $99999 \text{ }\mu\text{F}$, with the exciting frequency between 120 Hz and 200 kHz. The frequencies tested were 1 kHz, 100 Hz, 10 kHz, 100 kHz and 1 MHz. In all experiments, the voltage amplitude applied between plates was 1 V. Three capacitance values of biofilm were finally recorded on three different frequencies, that is, 1, 100 and 200 kHz.

It is observed that changes in frequencies caused variation in capacitance values irrespective of the slope of the graph (Figure 4). These variations in capacitance values may be explained on account of relatively slow movement of ions in plasma at high frequencies resulting in a decrease in capacitance values [39]. As observed from the graph, the initiation of biofilm resulted in an exponential increase in capacitance values of biofilm. This increase in capacitance values may be directly correlated to increase in biomass and EPS content of the biofilm. As the biofilm formation reached the stationary phase, that is, after 96 h (4 days), a marked decrease in capacitance value was observed. This decrease in capacitance values was either due to cell death or damaged cell having disrupted membrane, as both of these resulted in no significant changes ($p > .05$) in capacitance values

Table 1. EDX analysis of biofilm elemental composition at different time intervals.

Elements	Time: 24 h	48 h	72 h	96 h	10 h
P	1.36	1.10	0.30	0.52	1.94
Cl	1.63	0.41	0.08	1.28	–
Ca	0.15	–	–	–	0.31
Fe	3.77	1.61	6.84	–	–
Mg	–	0.29	–	–	0.49
K	–	0.25	–	0.24	–
Cr	–	–	0.43	–	–
Total	100	100	100	100	100

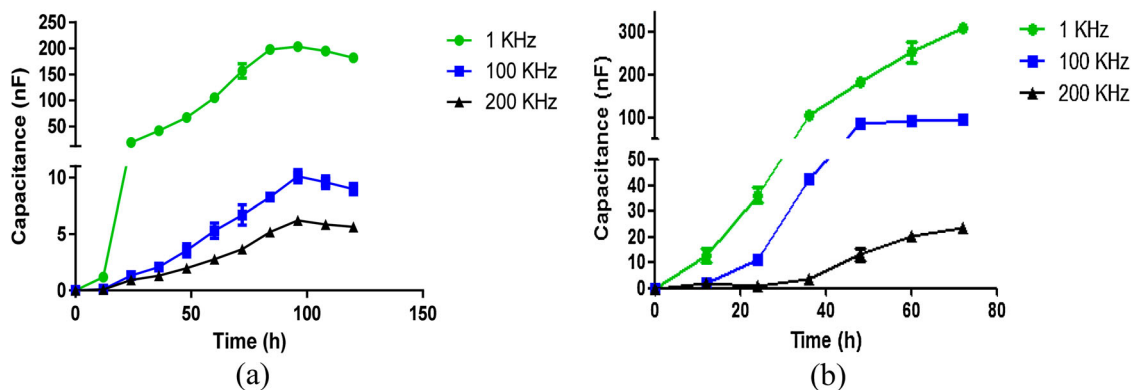


Figure 4. Capacitance changes measured during (a) *S. paucimobilis* MG6 biofilm growth and (b) standard *S. terrae* growth at 30°C; all values are average of three independent replicates.

[42]. The magnitude of the interface capacitance, the parameter of the electrical equivalent circuit capable to monitor the bacterial concentration, has been found to be insensitive to the presence of secreted substances in the medium and only dependent on bacteria concentration. Based on the above results, it may be concluded that the capacitance measurements at 1 kHz were satisfactory to determine the age of the biofilm. An approach to correlate biofilm age with thickness and subsequently with capacitance (results not shown) proved to be unsatisfactory since we observed an inverse relationship of biofilm thickness with capacitance, a phenomenon also noted in earlier studies [43].

The biofilm age and its correlation to capacitance and the predicted age were based on dry weight measurements of the biofilm. Figure 5 indicates that visible variations in values of electrical capacitance occurred only after 36 h. This fact is directly related to the small growth of the biomass in the first period and with the inherent detection limit of the method. The biofilm dry weight increased over time with biomass growth with an increase in electrical capacitance; growth was accentuated between 120 and 150 h and electrical capacitance decreased post 120 h, possibly on account of cessation of actual cell mass and accumulation of EPS. In fact, when a linear regression of the data (electrical capacitance with biofilm weight) was performed, the correlation coefficient was 0.9613.

From the regression values it was also concluded that the sensitivity of this type of measurement was -0.2065 pF/mm . A statistical analysis was performed to validate the correlations between biofilm weight and electrical capacitance according to the data obtained in assays (Tables 2 and 3). The results were reproducible with correlation coefficients greater than 98%.

As evident from the EDX and SEM data (Table 1), changes reflected from capacitance measurements over biofilm age may be explained from the observations

on biofilm architectural changes and ionic dynamics. The observations were also concurrent with earlier findings. To the best of our knowledge, a capacitance-based measurement (for a monoculture biofilm of *Sphingomonas*, assuming no change in external conditions) for detecting biofilm growth (age) has not been attempted, in *Sphingomonas* strain. Considering the ubiquity and importance of *Sphingomonas* in drinking water systems for health, refinement of the capacitance-based method may prove feasible.

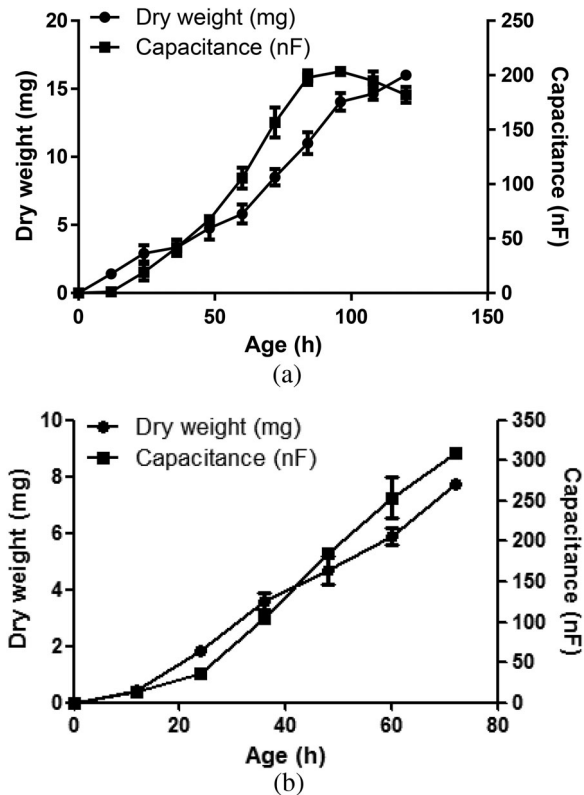


Figure 5. Correlation between biofilm age and capacitance (1 kHz). Values are average of three independent replicates. (a) *S. paucimobilis* MG6 and (b) Standard *S. terrae* MTCC 7766.

Table 2. Dry weight and capacitance of *S. paucimobilis* MG6 measured over 120 h.

Time (h)	Dry wt	Cap
0	0	0
12	1.1 ± 0.02	1.7 ± 0.01
24	3.5 ± 0.34	2.3 ± 0.03
36	2.8 ± 0.32	3.9 ± 0.03
48	3.9 ± 0.24	5.6 ± 0.06
60	5.1 ± 0.04	6.5 ± 0.05
72	7.9 ± 0.05	9.1 ± 0.14
84	11.8 ± 0.1	10.2 ± 0.13
96	13.4 ± 0.21	14.7 ± 0.11
108	15.1 ± 0.33	14.2 ± 0.15
120	16.1 ± 0.41	15.9 ± 0.20

Note: Figures in parentheses indicate SD.

Table 3. Dry weight and capacitance of standard *S. terrae* MTCC 7766 measured over 120 h.

Time (h)	Dry wt	Cap
0	0	0
12	1.3 ± 0.04	1.8 ± 0.02
24	3.3 ± 0.30	2.1 ± 0.02
36	2.6 ± 0.33	4.0 ± 0.04
48	4.0 ± 0.23	5.1 ± 0.05
60	5.2 ± 0.04	6.0 ± 0.02
72	8.1 ± 0.03	10. ± 0.11
84	10.9 ± 0.5	10.5 ± 0.09
96	12.2 ± 0.21	15.2 ± 0.21
108	13.9 ± 0.35	15.0 ± 0.15
120	16.7 ± 0.31	14.9 ± 0.40

Note: Figures in parentheses indicate SD.

Conclusion

The present study demonstrated the screening of 140 isolates from different municipal sites. The isolate with maximum biofilm production was characterized by 16S sequencing and named as *S. paucimobilis* strain MG6. The biofilm development ability of *Sphingomonas* was assessed by the CV assay and further confirmed by the MTT assay. To combat the detrimental effects of *Sphingomonas* biofilm, a capacitance-based method was developed to effectively measure the biofilm progression suggesting that biofilm age, measured as dry weight, could be correlated with the observed capacitance values. Further results of SEM and EDX data acquired from parallel analysis of biofilms could be correlated to its age. We are currently modifying the system in order to obtain a direct online measurement of biofilm growth.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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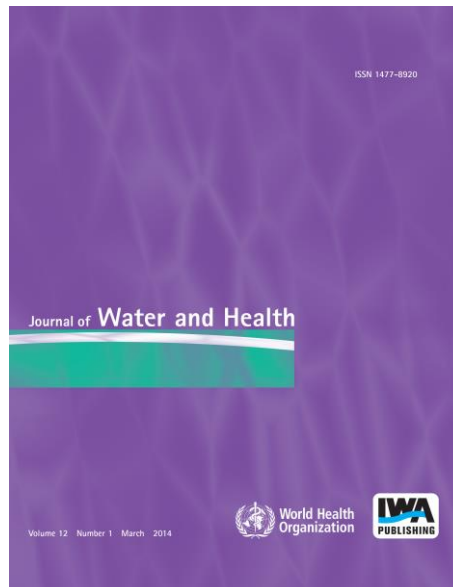
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Biofilm forming ability of *Sphingomonas paucimobilis* isolated from community drinking water systems on plumbing materials used in water distribution

Parul Gulati and Moushumi Ghosh

ABSTRACT

Sphingomonas paucimobilis, an oligotroph, is well recognized for its potential for biofilm formation. The present study explored the biofilm forming ability of a strain isolated from municipal drinking water on plumbing materials. The intensity of biofilm formation of this strain on different plumbing materials was examined by using $1 \times 1 \text{ cm}^2$ pieces of six different pipe materials, i.e. polyvinyl chloride (PVC), polypropylene (PP), polyethylene (PE), aluminium (Al), copper (Cu) and rubber (R) and observing by staining with the chemical chromophore, Calcofluor. To understand whether biofilm formation occurs under flow through conditions, a laboratory-scale simulated distribution system, comprised of the above materials was fabricated. Biofilm samples were collected from the designed system at different biofilm ages (10, 40 and 90 hours old) and enumerated. The results indicated that the biofilm formation occurred on all plumbing materials with Cu and R as exceptions. The intensity of biofilm formation was found to be maximum on PVC followed by PP and PE. We also demonstrated the chemical chromophore (Calcofluor) successfully for rapid and easy visual detection of biofilms, validated by scanning electron microscope (SEM) analysis of the plumbing materials. Chlorination has little effect in preventing biofilm development.

Key words | biofilm, chlorine resistance, drinking water, pipe materials, *Sphingomonas*

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INTRODUCTION

Waterborne contamination in ambient water bodies and its related diseases continue to have a major impact on water quality issues across the globe, pathogen contamination being the most serious issue ascribed for almost all ambient water bodies. Several *Sphingomonas* sp. are ubiquitously distributed in nature and have been isolated from drinking water and drinking water distribution systems. These strains have been well studied for their effective degradation/metabolism of complex hydrocarbons. The ability of *S. paucimobilis* to develop biofilm on surfaces of water distribution systems, for example stainless steel, has been reported as a major concern regarding water quality.

Most microbes in water distribution systems exist in biofilm on the inner surfaces of the pipelines (Bachmann &

Edyvean 2006). The formation of biofilm is influenced by several factors, such as concentration and quality of disinfectants and nutrients, water flow velocity, hydraulic conditions, temperature and/or pipe materials (Zacheus *et al.* 2001). Pipe materials can influence biofilm formation, especially in its early stages (Waines *et al.* 2011; Chowdhury 2012). In addition to that, Flemming *et al.* (2002) estimated that about 95% of bacteria in drinking water distribution systems are attached to the surface of pipe inner walls, while only 5% are in the water phase and detected by sampling as commonly used for quality control. The pipe material used in water distribution systems is an important factor that influences the proliferation of the biofilm (Lehtola *et al.* 2005). Few studies have attempted to characterize

Sphingomonas and evaluated their importance in drinking water systems, moreover their ability to form biofilms on plumbing materials commonly used has not been reported. It may be argued that the outcomes of studies involved in characterization of *Sphingomonas* in community drinking water systems would be important in understanding their role in waterborne infections and enable adequate treatment procedures for assuring water quality. Therefore the objective of this study was to characterize *Sphingomonas* sp. in community water distribution systems and determine their capability for biofilm formation on the plumbing materials commonly used in distribution systems. Biofilm simulation studies with the selected isolate on six types of pipe materials commonly used were designed and a simple visual approach to detect biofilms is also reported.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Tap water samples were collected from 20 different municipal sites and 140 isolates were screened from the water samples on the basis of the morphology of the colonies. *Sphingomonas* selective growth medium is composed of L9 minimal medium which is supplemented with 1% each of glucose and sodium glutamate as carbon and nitrogen source with mineral salts supplementation as described by Yim *et al.* (2010). Optimal concentration for streptomycin and piperacillin was determined by performing an antibiotic susceptibility test at 0, 50, 100, 150, 200, 250, 300, 350 and 400 µg/ml. The isolates were further screened on *Sphingomonas*-specific growth medium containing 100 and 50 µg/ml streptomycin and piperacillin, respectively. One isolate with demonstrable biofilm forming ability was selected for further studies and characterized by 16S sequencing. The 16S sequence of the isolate was submitted in the GenBank database as *Sphingomonas paucimobilis* strain MG6.

Molecular characterization of isolates

DNA of the selected isolate was extracted following the method as described by Moore *et al.* (2004). DNA was

amplified using a set of universal primers: 27F (AGAGTTT-GATCMTGGCTCAG) and 1510R (TACGGYTACCTTGTTACGACTT). The reaction mixture consisted of 1X PCR (Polymerase Chain Reaction) buffer, 0.2 mM each dNTPs (Deoxyribonucleotide triphosphate), 0.5 µM of each primer, 1 unit Taq Polymerase and 2 µl DNA. PCR was performed at 94 °C for 5 minutes, 94 °C for 1 minute, followed by 30 amplification cycles of 54 °C for 1 minute, 72 °C for 1 minute with the final cycle of 73 °C for 3 minutes. The sample was sequenced by automated DNA Sequencer AB3500xL (Applied Biosystem). The phylogenetic tree was constructed by Mega 7.0 software using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method and the 16S sequence of the isolate was submitted in the GenBank database as *Sphingomonas paucimobilis* strain MG6.

Biofilm production

Biofilm formation was estimated by using a modified crystal violet assay (Merritt *et al.* 2005). A volume of 300 µl of a standardized bacterial suspension in mid-log phase was revived in biofilm-inducing media and dispensed in each well of the polystyrene plates and incubated at 30 °C. The unbound cells were removed by inverting the microtiter plate and vigorously tapping followed by rinsing the wells with phosphate buffer (pH 7.2). The adherent cells were stained with 200 µl of 1% (w/v) crystal violet solution for 5 minutes. The wells were washed with deionized water extensively and the plates were allowed to dry. Following washing, 200 µl of 30% (v/v) acetic acid was added to each well for 15 minutes; 100 µl aliquots were then transferred to a fresh microtiter plate and the absorbance measured at 585 nm.

To further evaluate the biofilm profile of *Sphingomonas*, the formazan-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on biofilms as described by Kairo *et al.* (1999) with the modification of Walencka *et al.* (2005). Biofilm was grown in sterile flat-bottomed 96-well polystyrene microplates at 30 °C. After every 12 hours, bacterial suspension was removed till 72 hours, and 150 µl of PBS (phosphate buffered saline) and 50 µl of MTT at a concentration of 0.3% were added for 2 h at 37 °C. After incubation, the MTT solution was removed from the wells and 150 µl of dimethyl

sulfoxide (DMSO) and 25 µl of 0.1 M glycine buffer (pH 10.2) were immediately added to the wells to dissolve the formazan crystals formed in the wells. Incubation was carried out for 15 min at room temperature. The absorbance of the solution was measured at a wavelength of 570 nm using a Microplate Reader (Teccan, Austria).

Determination of chlorine resistance

Overnight grown bacteria were harvested by centrifugation at 8,000 rpm for 5 minutes and washed twice in sterile phosphate buffered saline (pH 7.2). After the final centrifugation, the bacteria were suspended in buffer to an optical density of 1.05 (measured at 580 nm), and 10 µl and 20 µl of this suspension was transferred to each of the reaction tubes containing 10 ml of the PBS. The final cell concentration in each tube was approximately 4.0×10^6 per ml and 8.0×10^6 per ml, respectively. One tube served as an untreated control and received no chlorine but instead an equivalent volume of sample and sterile chlorine free distilled water was added. Active chlorine present in sodium hypochlorite is 46.15 g/L. The applied sodium hypochlorite concentrations in the tubes were varied from 2 to 8 mg/L. After time (t) = 0, 30, 60, 90 and 120 minutes at 30 °C, 50 µl of the sample was plated to rescue surviving bacteria. The plates were incubated at 30 °C for 48 hours, and colonies were enumerated (Ridgway & Olson 1982).

Biofilm formation on individual plumbing materials under static conditions using Calcofluor assay

Biofilm production on different materials

A 10× stock solution of Calcofluor fluorescent brightener (disodium-4,4'-bis((4,6-dianilino-1,3,5-triazin-yl)amino)stilbene-2,2'-disulphonate) (Sigma) was prepared at a final concentration of 80 mg/l. Whole plumbing materials ($1 \times 1 \text{ cm}^2$) were removed using sterile forceps and rinsed with 50 ml of PBS to remove unattached cells and unbound dye. A hand-held UV-lamp long wave (Blak-Ray Lamp model UVL- 2 1, UV-366 nm) was used to detect the presence of biofilm on each surface (Lehtola *et al.* 2005). The images were captured using the CCD (Charge-Coupled Device) detector and stored as separate digital files (Gel

Doc XR+ System, USA). Fluorescence intensity of Calcofluor stained coupons was observed to detect biofilm formation.

Effect of chlorine treatment on biofilm formation ability

The effect of chlorine treatment was observed by inoculating a standardized suspension (8×10^6 cells/ml) of *Sphingomonas* in two setups: one containing rich medium (Luria Broth) and the other containing filter sterilized water from a municipal distribution system. Sterilized PVC coupons were added. After every 12 hours, three coupons from each setup were taken out and incubated in 8 mg/L sodium hypochlorite solution for 1, 3 and 5 hours, respectively. After staining with Calcofluor, it was visualized under a UV lamp (Blak-Ray Lamp model UVL- 2 1, UV-366 nm). A duplicate of each coupon was placed in fresh medium/water and visualized by Calcofluor staining after 96 hours.

Effect of chlorine treatment on combinations of *Sphingomonas* with waterborne pathogens

Biofilm production of *Sphingomonas* in combination with waterborne pathogens *Escherichia coli* O157:H7 ATCC 32150, *Shigella flexneri* 2a and *Salmonella typhimurium* ATCC 25315 was performed as described above by crystal violet assay. Chlorine resistance was observed by inoculating the standardized suspension of *Sphingomonas* in combination with pathogens in both medium (Luria Broth) and filter sterilized water. Sterilized PVC coupons were added in both setups and the rest of the experiment was carried out as described above.

Flow through simulation of biofilm formation on plumbing materials

A laboratory-scale simulated distribution system was fabricated comprising of six different sets of identical drinking water distribution pipes normally used. The pipe materials chosen were polyvinyl chloride (PVC), polypropylene (PP), polyethylene (PE), aluminium (Al), copper (C) and rubber (R). The length of the distribution pipes was one metre, with an internal diameter of 3 cm. Tap water was pumped from the contact tank (30 litres of tap water) with a flow

rate of 1.0 L/min into the simulated drinking water distribution pipe using a peristaltic pump. The water in the tank was inoculated with an overnight culture of *Sphingomonas paucimobilis* to a count of 10^5 CFU/ml.

Electron microscopy examination

Biofilm of *Sphingomonas* developed on different pipe materials after 4 days was examined by scanning electron microscopy (SEM).

Energy dispersive X-ray spectroscopy

The scanning electron microscope (SEM) was equipped with an energy dispersive X-ray spectroscopy (EDX) analyzer. It provided the elemental analysis of biofilm developed on different pipe materials.

Statistical analysis

Regression coefficient (R^2) was applied to obtain the relationship between log count of biofilm cells and the types of pipe materials during different biofilm ages.

RESULTS

Molecular identification of strain

The strain isolated from a municipal water distribution system was characterized by 16S rRNA sequencing using universal primers. The phylogenetic tree was constructed using sequences of representative bacteria. The isolated strain was identified as *Sphingomonas paucimobilis* strain MG6 (Figure 1); the sequence of this strain has been deposited in the GenBank database (Accession No. KX594380).

Biofilm formation kinetics

The crystal violet and MTT assays have been suggested as methods for the estimation of biomass quantity and metabolic activity of the cells, respectively. Figure 2(a) shows that the biofilm formation commences after 24 hours of bacterial growth which is the stationary phase of cells. Maximum biofilm formation was observed after 40 hours. Exopolysaccharides are secreted when the cells reach the stationary phase that facilitates the attachment of cells to the surface (Dunne 2002). With progress of time, increase in exopolysaccharide production further accelerates the



Figure 1 | Phylogenetic tree of *Sphingomonas paucimobilis* strain MG6.

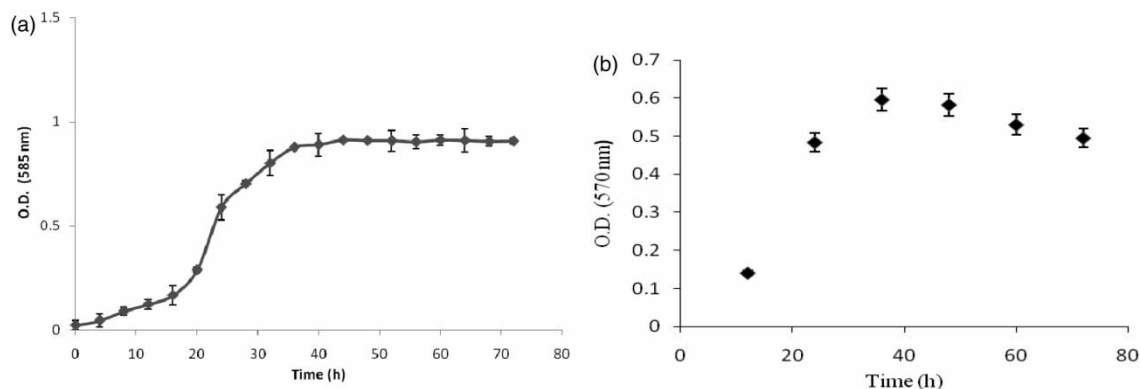


Figure 2 | Biofilm profile of *S. paucimobilis* MG6 by (a) crystal violet assay and (b) MTT assay.

rate of biofilm formation. For the determination of viability of *Sphingomonas* cells in biofilm and number of adhering bacteria, the MTT assay was performed (Figure 2(b)) which further confirmed the biofilm forming ability of *Sphingomonas*.

Determination of chlorine resistance

This study was carried out to estimate the effect of residual chlorine on the growth of *Sphingomonas*. It was found that the dosage of sodium hypochlorite had a profound effect on the reduction of log CFU/ml for *Sphingomonas*. Four different concentrations of sodium hypochlorite, i.e. 2, 4, 6 and 8 mg/L were used. *Sphingomonas* was found to be chlorine resistant at the concentration of 2 mg/L at a contact time of 90 minutes for different microbial loads (Figure 3).

Calcofluor assay

Biofilm production on different materials

Different plumbing materials like PVC, PP, PE, Al, R and Cu were used to develop biofilms. Results were recorded by visualizing the biofilm under a UV lamp after equal intervals of 12 hours till 72 hours with each material. The Calcofluor assay revealed the biofilm formation on PVC, PP, PE and Al pipe materials; however, variation was observed in intensity (Figure 4). Also, an increase in intensity was observed as time increased. The intensity was less in the case of copper and rubber suggesting the possibility of less biofilm formation on these materials.

Effect of chlorine treatment on biofilm formation ability

PVC coupons were removed from filter sterilized surface water/medium at different intervals of time and incubated

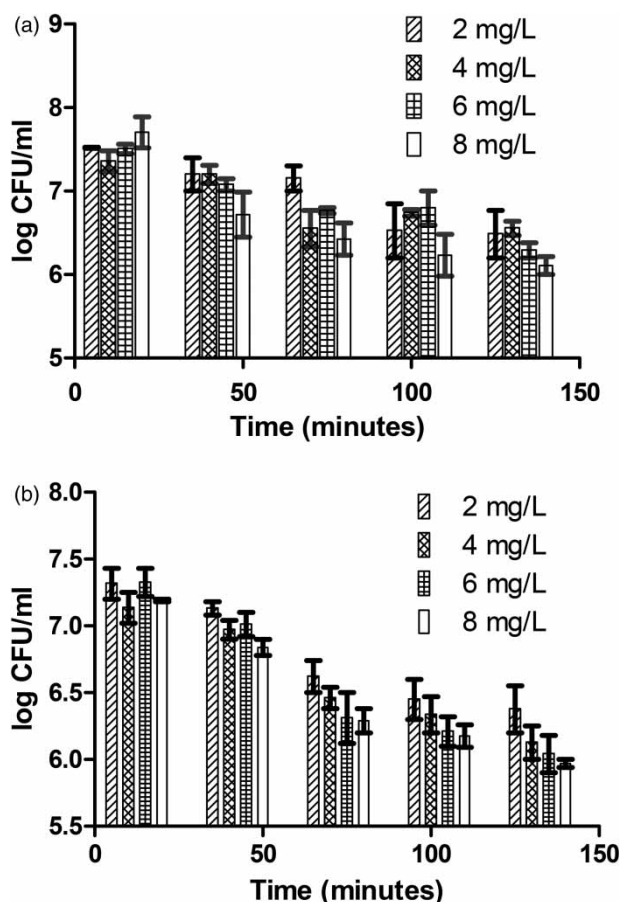


Figure 3 | The log CFU/ml of *Sphingomonas paucimobilis* MG6 at different concentrations of sodium hypochlorite of (a) 4×10^6 cells/ml, (b) 8×10^6 cells/ml.

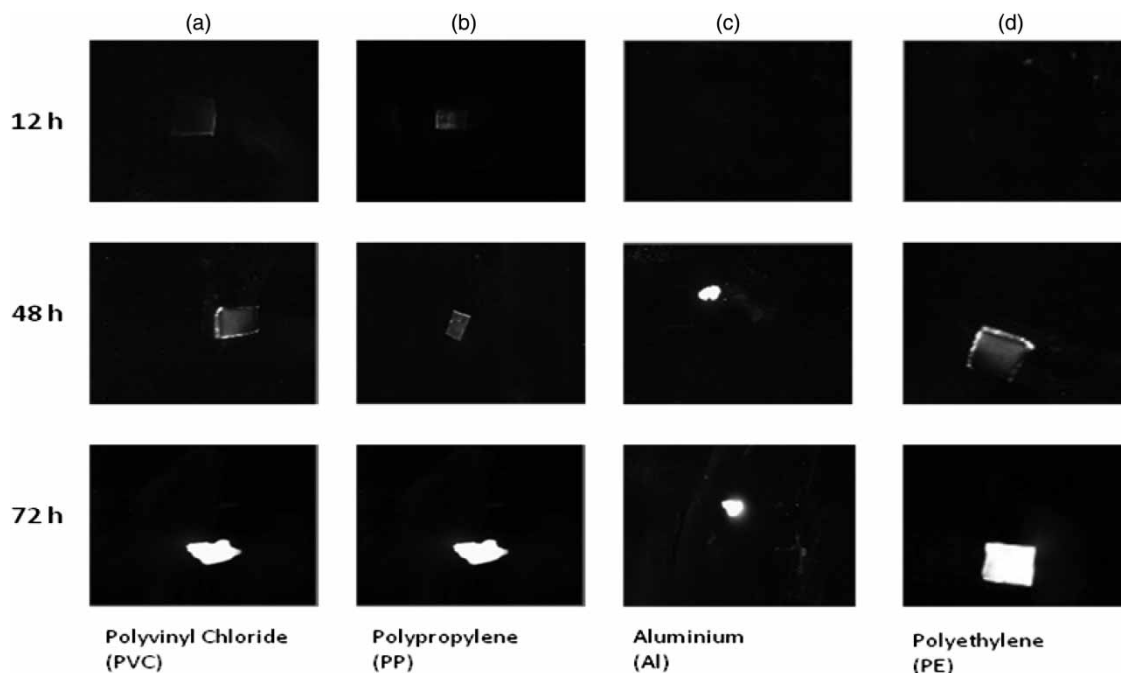


Figure 4 | Calcofluor assay performed by *Sphingomonas paucimobilis* MG6 carried out on (a) polypropylene, (b) polyvinyl chloride, (c) aluminium, and (d) polyethylene coupons.

for 1, 3 and 5 hours, respectively. The intensity recorded varied with time; as depicted in Figure 5, the intensity increased as the time of biofilm development increased. With the increase of the time span of chlorine treatment, little or no decrease in intensity of biofilm development was observed either with medium or with water. Replicates of coupons were placed in fresh medium/water after each interval till 96 hours. After visualization, it was observed that a slight increase in intensity occurred, which marks the biofilm development following chlorine treatment, confirming the chlorine resistance of *Sphingomonas*.

Effect of chlorine treatment on *Sphingomonas* in combination with waterborne pathogens

The detection of various waterborne pathogens from drinking water systems prompted us to study the biofilm production ability of *Sphingomonas* in the presence of three pathogens (Figure 6(a)). Pathogens were found to increase the biofilm production ability of *Sphingomonas*; *Shigella* being the highest followed by *Salmonella* and *E. coli*, making it possible to observe the effect of chlorination on *Sphingomonas* in combination with waterborne pathogens.

PVC coupons were removed, incubated for 1, 3 and 5 hours and the effect of chlorine treatment was visualized by Calcofluor staining. It was observed that *Sphingomonas* with *Shigella flexneri* 2a showed maximum resistance to chlorine as the biofilm formation is maximum in this case followed by *Salmonella typhimurium* and *Escherichia coli* O157:H7. The results can be visualized by observing the variation in the intensity of stained Calcofluor (Figure 6).

Flow through simulation of biofilm formation on plumbing materials

These results were corroborated by observations from the flow through simulation experiment. Viable cells of *Sphingomonas* were recorded from different scrapings of plumbing materials at different ages (Table 1). Variation was observed between different plumbing materials with log count of biofilm cells on polyvinyl chloride being the highest followed by polypropylene and polyethylene.

Scanning electron microscopy micrographs

Biofilm formation of *Sphingomonas* on different pipe materials is explained using scanning electron micrographs.

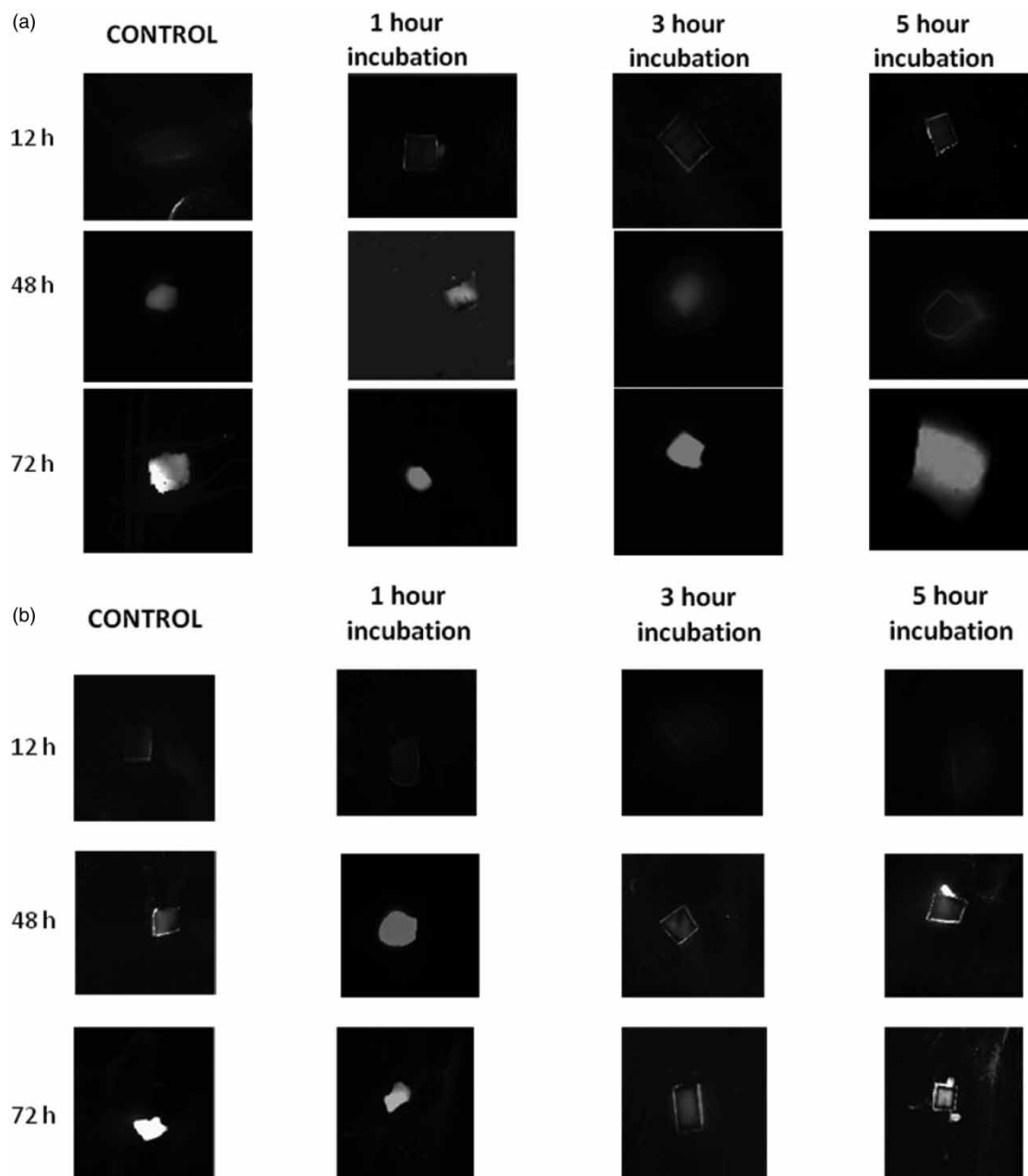


Figure 5 | Effect of chlorine treatment on biofilm development on PVC using (a) water and (b) medium.

Figure 7 shows that a fully matured biofilm was formed at 72 hours. A clear indication of the cells embedded in a polymer matrix and exopolysaccharides could be obtained. *Sphingomonas paucimobilis* growing on copper and rubber pipes produced a smaller amount of exopolysaccharides than on the other types of pipes, hence the low biofilm formation.

Elemental composition of biofilm formed on different pipe materials as determined by EDX is shown in Table 2. It represented the weight percentage of different elements found at 12, 48 and 72 hours on different pipe materials. EDX is more useful when our objective is to compare the composition of biofilm formed on different materials. It was observed that carbon and oxygen are the dominant

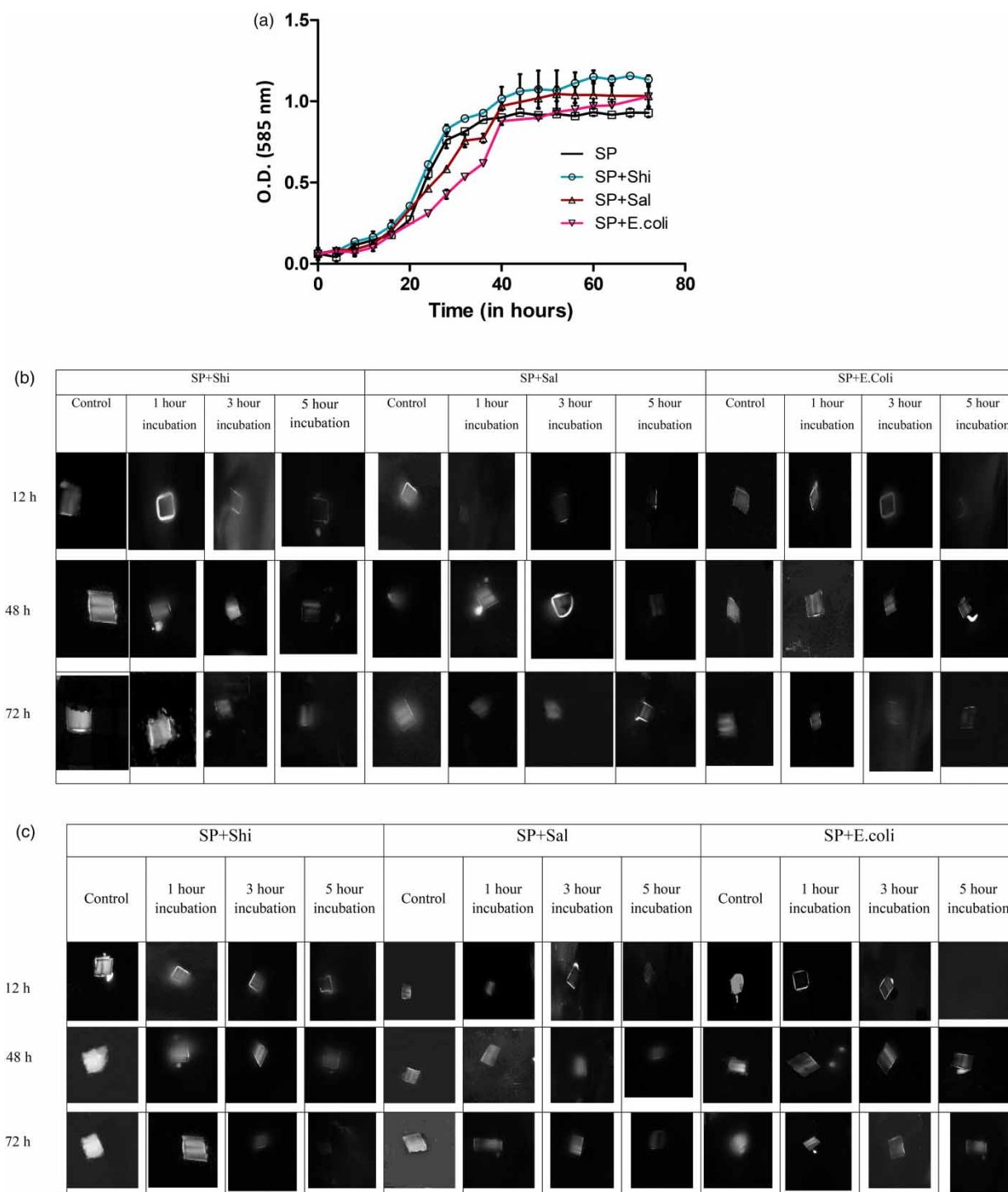


Figure 6 | (a) Biofilm production of *Sphingomonas* in combination with waterborne pathogens, (b) effect of chlorine treatment on PVC in medium, and (c) effect of chlorine treatment on PVC in water.

elements in biofilm formed on all the plumbing materials followed by calcium and chloride. Carbon and oxygen are interdependent and inversely related to each other as

increase in carbon content is followed by consumption of oxygen by cells in PVC as cells are more metabolically active in this case. The inverse is the case with PE. Little

Table 1 | The relationship between log counts of biofilm cells which formed on different pipe materials at different ages

Tested pipe materials	R^2		
	Biofilm ages (hours)		
	24	48	96
PVC	92.7	92.6	94.9
PP	88.1	84.2	91.7
PE	84.7	84.6	73.3
Al	74.5	79.0	85.9
Cu	75.8	74.4	84.1
R	74.1	73.2	72.3

variation is observed in PP and Al. Weaker signals from magnesium, potassium, chromium and iron were also obtained. EDX allowed better knowledge of observed structures.

DISCUSSION

The World Health Organization (WHO) estimated that about 1.2 billion (10^9) people lack access to safe drinking water and 3.4 million people die of water-related diseases every year (WHO 2002). One important factor responsible

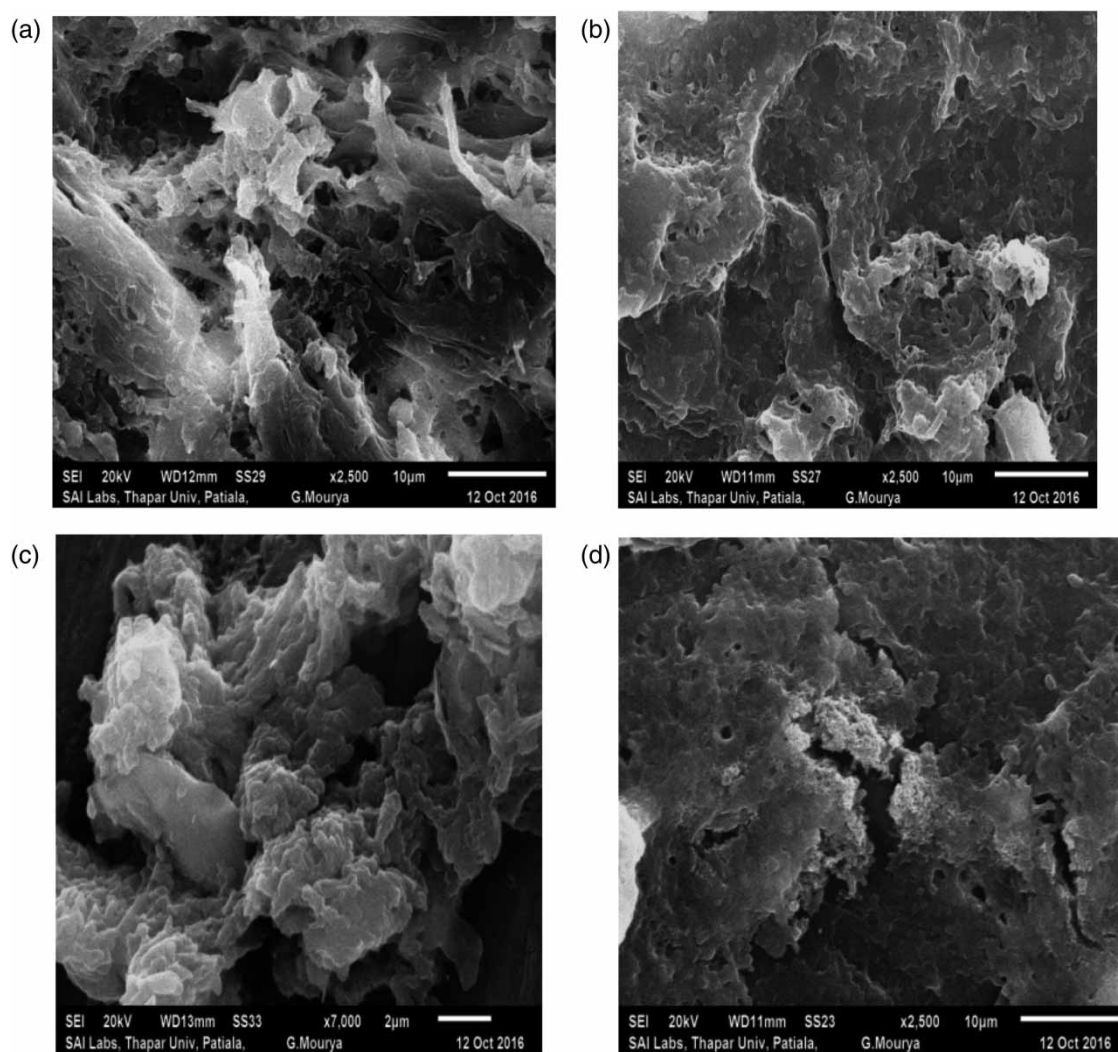
**Figure 7** | Scanning electron micrographs at different time intervals on (a) PVC, (b) PP, (c) Al, and (d) PE.

Table 2 | EDX analysis of biofilm elemental composition (weight percentage) at different time intervals

Elements	PVC		PP		AI		PE	
	12 hours	72 hours	12 hours	72 hours	12 hours	72 hours	12 hours	72 hours
Carbon	24.86	68.12	90.87	82.11	90.26	89.26	77.56	58.69
Oxygen	38.07	5.17	8.91	16.82	7.00	9.91	6.95	32.34
Magnesium	0.04	0.20	0.11	–	0.49	0.19	0.10	0.19
Chloride	12.46	22.62	–	0.62	–	–	14.71	1.61
Potassium	0.01	–	–	0.07	–	0.03	–	0.37
Calcium	24.51	3.53	0.03	0.13	0.31	–	0.57	4.20
Chromium	0.05	0.29	–	–	–	0.07	–	0.26
Iron	–	–	0.09	0.10	–	0.17	–	–
Sodium	–	0.08	–	0.15	–	0.07	0.11	0.57
Silicon	–	–	–	–	–	–	–	–
Phosphorus	–	–	–	–	1.94	0.30	–	1.78
Total	100	100	100	100	100	100	100	100

for sustenance of water-related disease is the treatment process in distribution systems which fail to comply with the appropriate treatment and disinfection specifications. If resistant pathogens survive conventional disinfection methods, these disinfection methods do not comply with appropriate specifications. Bacteria isolated from different environments with resistant phenotypes to various disinfection methods have been reported (Wojcicka *et al.* 2007). *Sphingomonadaceae* are common inhabitants of drinking water worldwide (Momba *et al.* 2000; Flemming *et al.* 2002; Stopforth *et al.* 2002) and have a remarkable capacity to cope with stress conditions and to adapt to new habitats (Besner *et al.* 2002; Flemming *et al.* 2002; Bachmann & Edyvean 2006; WHO 2011). A body of literature has explicitly indicated the risks of *Sphingomonas* contaminated drinking water systems as persistent sources of infection through biofilm formation. Health risks associated with *S. paucimobilis* include primary bacteremia, intravascular catheter infections, urinary tract infections, biliary tract infection, cutaneous infection (Peel *et al.* 1979), ventilator-associated pneumonia (Ensminger *et al.* 2006), myositis (Pegoraro *et al.* 2008), diarrheal disease (Cheong *et al.* 2008), nosocomial urinary tract infection from contaminated water bottles (Crane *et al.* 1981), bacteremia in dental unit water lines and tap water (Kanamori *et al.* 2016), bacteremia in hospital water systems (Perola *et al.* 2002), etc. Also,

bacteremia, endophthalmitis, osteomyelitis and arthritis were associated with hospital drinking water distribution system in Hungary (Felföldi *et al.* 2010). Both staining using chemical probes and SEM indicated that the biofilm cells growing in different pipe materials were embedded in a polymer matrix and exopolysaccharides. Biofilm formation has been shown to be affected by several factors, including disinfectant effectiveness, the nature and concentration of biodegradable compounds in the water, pipe materials used for distribution system construction and water temperature. Proper management of these factors through adequate source water treatment, appropriate materials selection, maintenance of a clean distribution system, and minimization of water age are all important for biofilm control. However, few studies have emphasized the biofilm forming ability of *Sphingomonas* isolates on commonly used water distribution surfaces. We used the fluorescent probe, Calcofluor, to effectively visualize biofilm in the static cultures. The amount of fluorescence is a function of the affinity of Calcofluor for capsular polysaccharides (CPS) and the quantity of adherent CPS. Thus, a negative or weak result denotes minimal biofilm formation, which is exactly what the probe is intended to detect (Singla *et al.* 2014). The variation in biofilm growth was consistent and could be correlated to that obtained in the flow through simulation system. Piping material plays a very important

role in the maintenance of high water quality because it can supply the bacteria in water with nutrients through the leaching of organic compounds. Generally, maintaining a disinfectant residual is thought to limit the growth of organisms within the distribution system and, depending on the residual concentration, contact time, and the pathogens present, a disinfectant residual may afford some protection against contamination from intrusion (Besner *et al.* 2002). Chlorination is the primary recognized method of disinfection of water distribution systems, however, there may be other resistance populations of bacteria (Ridgway & Olson 1982; Goel & Bouwer 2004). The survival of *Sphingomonas paucimobilis* in water distribution systems might be due to its capability to resist chlorine. Drinking water supplies are not expected to be sterile.

Generally accepted specifications for drinking water make provision for heterotrophic plate counts of 100/ml, and in some cases 500/ml and even more, because most of the organisms concerned do not constitute a meaningful health risk. According to the WHO drinking water standard, 2–3 mg/L chlorine should be added to water in order to attain a satisfactory disinfection and maintain residual concentration (WHO 2008). *Sphingomonas* was found to be chlorine resistant at a concentration of 2 mg/L and a contact time of 90 minutes for different microbial loads. LeChevallier *et al.* (1988) suggested that low concentrations of chlorine are not effective in biofilms which prompted us to use a higher concentration of chlorine (8 mg/L) for studying its effect on biofilm development and *Sphingomonas* was found to be chlorine resistant.

Our observations of lower biofilm production on copper plumbing material, may be attributed to the release of copper residuals that have antibacterial properties, expressed by damage to cell membranes and nucleic acid structure (Bruins *et al.* 2000). Copper is the most popular modern plumbing material (Moritz *et al.* 2010) because of its low cost and the ease with which it can be machined into a wide variety of pipes and fittings. Moreover, Lehtola *et al.* (2004) reported that the formation of biofilm was slower in copper pipes than in PE pipes, and that copper ions led to lower microbial numbers in water; while plastic pipes such as PE, which have recently been used as cost-effective replacements of traditional metal plumbing, may release biodegradable organic compounds and phosphorus,

which can promote microbial regrowth and biofilm formation.

Overall, our study provides valuable insights on biofilm formation by *Sphingomonas* in commonly used plumbing materials; the view that chlorine alone can be used as an effective disinfectant needs to be seriously reconsidered due to reasons stated. Alternative methods of disinfection such as application of quorum quenchers or ozone or combinational treatment may prove useful for containing *Sphingomonas* growth and biofilm formation.

CONCLUSION

The present study demonstrated the ability of a *Sphingomonas* isolate from a municipal water distribution system to form biofilms on six different plumbing materials commonly used. Biofilm formation occurred both under static as well as circulatory conditions and could be visualized using the fluorescent chemical probe, Calcofluor, and validated by electron photomicrographs. Viable counts concurrently obtained from pipe materials agreed with these observations. Chlorine resistance of the strain raises concern and suggests an urgent need to develop effective disinfection strategies.

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CONFLICT OF INTEREST

None.

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