Applications of Capacitance in monitoring of microbial biofilms

A Thesis

submitted in the partial fulfilment of the requirement for

award of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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JULY 2014

CERTIFICATE

This is to certify that the thesis entitled "**Application of Capacitance in monitoring of microbial biofilms**" submitted by Pawandeep Singh in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by him. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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CANDIDATE DECLERATION

I, hereby declare that the work presented in this thesis entitled **"Application of Capacitance in monitoring of microbial biofilm''** in partial fulfilment of the requirement for the award of the degree of Masters of science in Biotechnology, Department of Biotechnology (DBT), Thapar university, Patiala, is an authentic record of my work during the period of six months from January 2014 to July 2014, under the guidance of **Dr. Moushumi Ghosh**, Associate Professor and **Dr. Arun Kumar Chatterjee,** Assistant Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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ACKNOWLEDGEMENT

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. It gives me immense pleasure to acknowledge with attitude. The help and guidance rendered to me by a lot of people.

First of all, I sincerely acknowledge my gratitude to Almighty GOD for His compassion made me to fell to have and to see the moment whose euphoria is not vanishing from the mind will remain till eterny.

I beat the rays of my illuminant gratitude to my honourable guide, Associate Professor, **Dr. Moushumi Ghosh** for her excellent guidance. Her discipline, principles, simplicity, caring attitude, constructive criticism and provision of fearless work environment will be cherished in all walks of my life. I am very much grateful to her for valuable guidance and everlasting encouragement throughout my course.

I am deeply indebted to my co-supervisor, Assistant Professor **Dr. Arun Kumar Chatterjee** from Electronics and Communication Engineering Department, he helped and encouraged me in all the time of research and in thesis writing.

It gives me great pleasure to express my deep sense of gratitude to **Dr. Dinesh Goyal**, Professor and Head, Department of Biotechnology (DBT), Thapar University, Patiala**,** for allowing me to continue my dissertation work by providing necessary laboratory facilities to fulfill of this work.

My vocabulary utterly fails in expressing my accolade to Mrs. Taranpreet Kaur, research scholar, DBT for her support, valuable suggestions, immense affection, invariant encouragement through out this work. I also wish to express my thanks to Ms. Gurpreet Kaur research scholars, DBT for their constant encouragement and help. I am thankful to Ms. Paruland Mr. Arkadeep Mukherjee,research scholars, DBT for their valuable companionship and suggestions.

I am grateful to laboratory staff Mr. Babban, Mr. Mohinder, Mr. Surinder, Mrs. Lalitaand Mr. BP Singhfor their timely help and assistance while completing this project.

I thank all my teachers throughout my journey to build me as strong to face all conditions of life. I will be remaining thankful till my last breath.

Words of reverence, gratification and affection for my beloved Parentswho brought me to this stage and indebted for their big moral support for me in the completion of my higher studies. It was their confidence, affection and prayer for my success and progress which made it possible for me to complete the task relentlessly in time.

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Table of contents

ABSTRACT

Biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and usually enclosed in a matrix of polysaccharide material. Current methods i.e. Fibre optical sensors, Photoacoustic Spectroscopy etc. are used for biofilm monitoring but they are used to monitor thin biofilms. Present study was conducted to develop non-invasive method for measuring biofilm. For this sensor was fabricated using capacitance based methods. Four isolates out of twenty five were screened on the basis of their biofilm forming capability. Architectural differences between EPS and biofilm of each isolate was analysed using Scanning Electron Microscope. Biochemical characterization confirmed the presence of charged functional groups such as sugars, proteins and amino sugars. For analysing biofilm age and EPS concentration, aluminium matrix was used as appropriate conducting material. It was observed that maximum capacitance was measured for EPS BP19 at 6 mg/ml followed by BP10 at 5 mg/ml and BP7 and BP4 at 4 mg/ml concentration. Highest capacitance of 53854pF was observed for BP4 at the time of 72 hours and lowest was observed in case of BP10 of 37894pF. In conclusion we anticipate the developed sensor could be used for in situ biofilm analysis.

List of Abbreviations

°C	Degree(s) Celsius
hr	Hour
mL	Millilitre
mg/ml	Milligram per millilitre
µl	Microliter
nm	Nanometer
rpm	Revolution per minute
pF	Pico Farad
EPS	Extracellur Polymeric Substances
g/l	Gram per litre
mg/L	Milligram per litre
IRE	Internal Reflection Element
FISH	Fluorescence in situ Hybridization
CLSM	Confocal Laser Scanning Microscopy
AFM	Atomic Force Microscopy
EM	Electron Microscopy
TEM	Transmission Electron Microscope
SEM	Scanning Electron Microscope
PAS	Photoacoustic Spectroscopy
FTIR/ATR	Fourier Transformation Infrared Spectroscopy Attenuated Total Reflection
FOS	Fibre Optic Sensors
QCM	Quartz Crystal Microbalance
MHz	Megahertz
C	Capacitance
R	Resistance
L	Inductance
F	Farad
BG	Biofilm Growth media
NB	Nutrient Broth
O.D	Optical Density
BSA	Bovine Serum Albumin
PBS	Phosphate Buffer Saline
UV	Ultra Violet

List of figures

List of Tables

1. INTRODUCTION

Biofilms are sessile microbial communities growing on surfaces, frequently embedded in a matrix of extracellular polymeric substances (Heydorn *et al*, 2000). These can exist on all type of surfaces i.e. glass, plastic, metal, soil particles, wood, medical implant materials, tissue and food products. Biofilm growth in food processing units may lead to increasing opportunity of microbial contamination in processed food. Further biofilm cause chronic infectious diseases, infections on catheters and other biomaterial used in medicines.

One of the most remarkable and best documented features of biofilms is the fact that they can form under extremely diverse conditions, which becomes a dominating consideration once attempted to monitor biofilms. The environment in which biofilms form, can differ in each and every chemical and physical parameter. Monitoring methods that work perfectly in one application not perform well in another. The detection of individual microorganisms in clean environment at bench scale presents different challenges than monitoring the growth of a bacterial lawn with a complex biocenosis in natural systems or technical applications. Therefore detection of biofilm assumes considerable significance especially in medical setups and in industries. Among the available methods, staining assays, electron microscopy, Confocal Laser Scanning Microscopy (CLSM), Fibre Optical Sensors (FOS), Photo acoustic spectroscopy, FTIR/ATR have been employed for monitoring biofilm. Due to their limitations, these methods are not acceptable for routine biofilm measurements. Therefore, non-destructive and non-invasive detection techniques to monitor biofilm age and thickness are of major interest. In addition, the use of sophisticated monitoring systems for monitoring biofilms are still limited and those advocated are either complicated or have high economic considerations. One of the non-destructive method used in monitoring of biofilm includes the use of electrical capacitance as this method is non-invasive and easy to access in the interior without disturbing

the remaining biofilm. Considering these advantage, we developed a sensor to monitor biofilm age and thickness based on electrical capacitance methods.

Objectives

The main objectives of this study are

1) To develop capacitance based method for biofilm monitoring.
2) Comparative study of capacitance profile on maturation of biofilm produced by different bacterial strain.

2. REVIEW OF LITERATURE

Biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and usually enclosed in a matrix of polysaccharide material (Kokare *et al,* 2009). Biofilms are composed primarily of microbial cells and EPS. EPS may account for 50% to 90% of the total organic carbon of biofilms (Fleming *et al,* 2000) and can be considered the primary matrix material of the biofilm. This EPS architecture serves as both structural and protective functions. It forms channels that facilitate the transport of nutrients, enzymes, metabolites, and disposal of waste products within and outside of the biofilm matrix. This architecture is essential in terms of supporting the needs of a multi-cellular biofilm, allowing for each individual cell's requirements to be met in order to sustain viability. In addition to the EPS, there are proteins, nucleic acids, peptidoglycan, lipids, phospholipids, and other cell components are also present in the matrix of biofilm communities (Fleming *et al,* 2000).

2.1 Occurrence of biofilm

Biofilms are ubiquitous, nearly every species of microorganism, not only bacteria and archaea, have mechanisms by which they can adhere to surfaces and to each other. Biofilms are known to form on virtually every non-shedding surface in a non-sterile aqueous (or very humid) environment are more commonly associated with the colonization of microbes on indwelling medical devices. E.g. implant biofilm-mediated diseases: Prosthetic valve- endocarditis (*Staphylococcus epidermidis*) Contact lenses- Keratitis (*Pseudomonas aeruginosa)* (Kokare *et al,* 2009).Biofilm growth in food processing units leads to increase opportunity for microbial contamination of processed food. Bio-surfactants are reported to be produced by bacteria yeasts and fungi can serve as green surfactants. These are considered to be less toxic and eco-friendly and thus have the potential to be commercially produced for use in pharmaceutical cosmetics and food and agriculture industries. Many rhizosphere and plant associated microbes produce bio-surfactant which play a vital role in motility signalling and biofilm formation indicating that bio-surfactant governs plant microbe interaction.

2.2 Biofilm formation

Although the mechanism of biofilm formation varies from species to species yet the stages of biofilm formation appear to be conserved for a wide range of microbes which are as follows (Stoodley *et al*, 2002): Reversible attachment, irreversible attachment, aggregation of cells, maturation of biofilm and detachment.

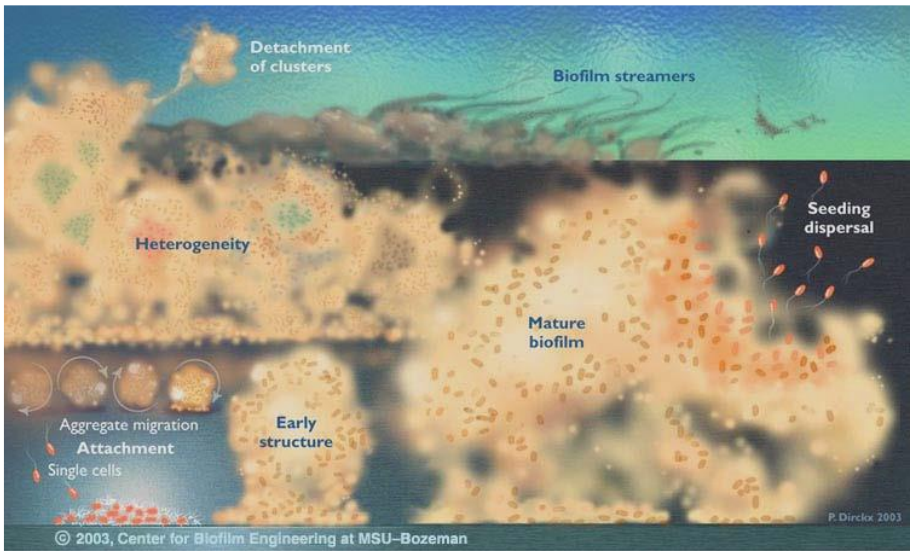


Figure 2.1 Different stages involved in process of Biofilm formation

(courtesy: http://www.pnas.org/content/101/49/16983/F1.large.jpg)

Biofilm formation begins with adsorption of macromolecules (proteins and polysaccharides) and smaller molecules (fatty acids and lipids) at surfaces resulted in formation of film known as conditioning layer that alter physiochemical characteristics of the surface, including surface hydrophobicity and electrical charge (Garret *et al*, 2008). Bacterial transition from reversible to irreversible attachment occurs through pili, curli, fimbriae and other cell surface proteins. Irreversible attachment is further marked by production of extracellular polymeric substances. At some point following initial contact, the cells become encased in a polymeric matrix that the cells produce and thus are anchored to the substratum. The more stable attachment is due to the production by the bacteria of a complex array of extracellular polymeric substances

including polysaccharides, proteins and in some cases, DNA, lipids (Allison *et al,* 2000, O'Toole *et al,* 1998, Finelli *et al,* 2003).As the cells divides, daughter cells spread outward and upward to form clusters (Stoodley *et al,* 2002) which in turn developed in mushroom like structures in the developing biofilm. These mushroom structures are believed for the passage of nutrients to cells deep inside the bacterial biofilm.

The synthesis of EPS around mushroom like structures matures the biofilm formation to three dimensional structures having pores and channels for the movement of water and nutrients so that they may reach the cell deeper inside the biofilm. The genomic and proteomics study of the biofilm has revealed that biofilm bacteria are fundamentally different from its counterpart in terms of genetic level and gene expression (Camper *et al,* 2002). Finally, individual cells or cell masses separate from the substrate to which the biofilm is attached. Stewart (1993) divided biofilm detachment processes into four categories: abrasion, erosion, sloughing and predator grazing. It is known sudden nutrient limitation may be followed by sloughing events in which fragments of the biofilm may become detached and even the growth rate of the biofilm can influence the rate of detachment, additionally rapidly growing biofilms exhibit a greater rate of detachment than slow growing ones (Sauer *et al,* 2008).

2.3 Factors affecting biofilm formation

In nature, biofilms are found on number of surfaces from surgical implants, dental plaque to natural aquatic system as well as in different environmental conditions. Biofilm formation depends on number of factors which are discussed below:

2.3.1 Properties of cells

The rate and extent of attachment of microbial cells depends on cell surface hydrophobicity, presence of fimbriae or flagella and EPS. Most bacteria are negatively charged but still contain

some hydrophobic surface components (Rosenberg and Kjelleberg, 1986). So the hydrophobicity of the cell surface is important for adhesion because hydrophobic interactions tend to increase with an increasing non polar nature of one or both surfaces involved. Fimbriae contribute to cell surface hydrophobicity as they contain a high proportion of hydrophobic amino acid residues (Rosenberg and Kjelleberg, 1986). Rosenburg (1982), Bullitt and Makowski (1995) provided evidence for the role of fimbriae in bacterial attachment to surfaces. The evidence for the attachment of bacteria with surfaces via extracellular polymeric substances was reported by Marshall *et al*, (1971). Zottola (1991) further confirmed the role of polysaccharides in attachment in studies with *Pseudomonas fragi*. The role of flagella in cell attachment was recognised by Korber *et al*, (1989) who used motile and nonmotile strains of *P. fluorescens* to show that motile cells attach in greater numbers and attach against the flow (backgrowth) more rapidly than do nonmotile strains.

2.3.2 Conditioning Films

Microbial attachment to surfaces are greatly affected by the formation of conditioning film on surfaces as they are exposed in the aqueous medium, the polymers are almost immediately conditioned and causes chemical modification (Figure 2.2). The formation of these conditioned films were first time reported on the surfaces exposed to sea water (Loeb and Neihof, 1975). These films were organic in nature, formed within minutes of exposure, and continued to grow for several hours.

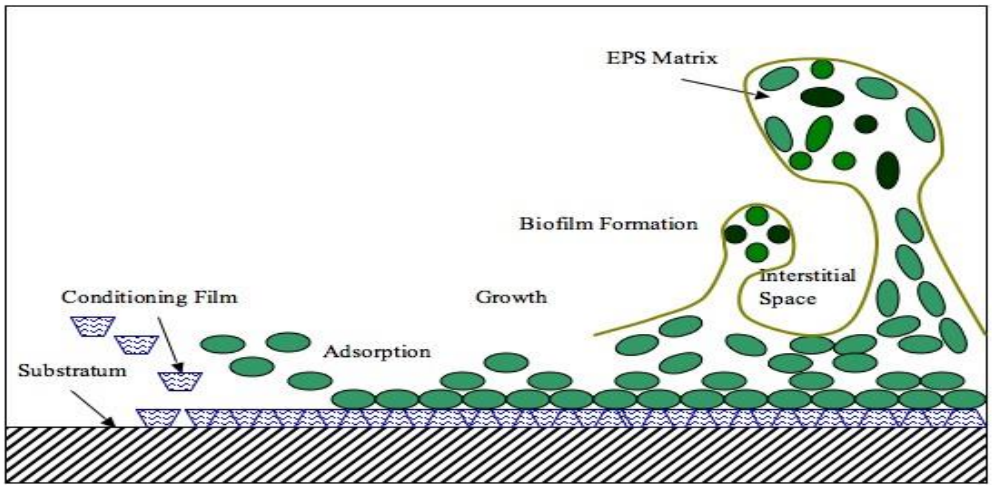


Figure 2.2 The effect of conditioning film on biofilm growth and formation on substratum.(courtesy:http://en.wikipedia.org/wiki/Chemistry_of_biofilm_prevention)

2.3.3 Effect of substratum

There are some characteristics that a substrate must possess to facilitate bacterial attachment. It was noted that the extent of microbial colonization increases as the surface roughness increases as the surface area is higher on rougher surfaces, thereby prevents the microbes from the effect of the shear forces (Characklis *et al,* 1990). The physicochemical properties of the surfaces also effect the attachment of bacteria to the surfaces. The interactions that are primarily involved in bacterial cell wall and surfaces are interfacial electrostatic (attraction, repulsion) and van der wall forces (McClaine *et al,* 2002) as shown in figure2.3.

The chemical properties of substrates can be used for regulating the adhesion of bacteria to surfaces and their growth in biofilm by modifying these surfaces. General strategies for the design of substrate surface chemistry include covalent modification, non-covalent modification, controlled release of small molecules, and degradation of polymeric surfaces (Qiu *et al,* 2007).

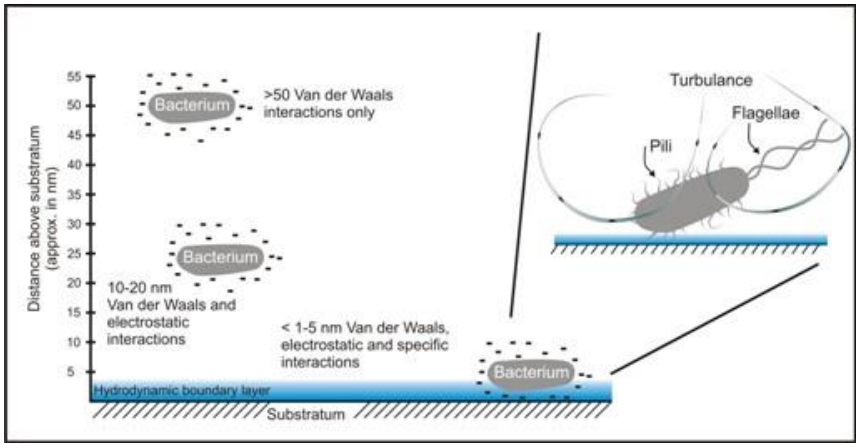


Figure 2.3 The role of Van der Waals and electrostatic interaction on the attachment of bacterium to the substratum. (courtesy: http://www.hypertextbookshop.com/biofilmbook)

2.3.4 Hydrodynamics

The flow velocity immediately adjacent to the substratum and liquid interface is negligible and is termed as hydrodynamic boundary layer. Its thickness is dependent on linear velocity as higher the velocity, thinner is the hydrodynamic boundary layer. Depending upon flow types i.e. whether the flow is laminar or turbulent, the hydrodynamic boundary layer may substantially affect cell-substratum interactions. Laminar flow is characterised by parallel smooth flow patterns with little or no lateral mixing and fastest flow in the centre (Fletcher and Marshall 1982, Lappin-Scott *et al,* 1993). The biofilm developed into thick multi-layered structure in laminar flow. On the other hand, turbulent flow is random increasing the mixing of bacteria and nutrients (Characklis *et al*, 1990) and microbial adhesion (Percival *et al*, 1999). Biofilm developed under turbulent flow is thin monolayer structures.

2.4 Methods for biofilm detection

Some methods for biofilm detection are discussed below:

2.4.1 Staining assays for biofilm detection

Different staining methods are used to quantify biofilm matrix as well as both living and dead cells or quantify viable cells in the biofilm. Crystal violet staining assay is one of the first method adopted for biofilm biomass quantifications because of its ability to indifferently bind to negative charged bacteria and polysaccharides in EPS (Li *et al,* 2003). After staining, the adsorbed crystal violet is eluted using a solvent and the amount of dye solubilised by solvent is directly proportional to biofilm size. But the main limitation of this method is both living and dead cells as well as biofilm matrix are stained by crystal violet and does not provide information on actual number of living bacteria.

There are some assays which are able to detect specifically live cells in EPS matrix. One of such assays involve the use of colourless fluorescein-di-acetate (FDA), a cell membrane soluble dye. As the bacterial cells uptake FDA, it is hydrolysed by cellular esterase to fluorescein which fluorescent yellow and signal is measured spectophotometrically. Dead cells are not able to metabolise FDA, so there is no florescent signal. This methods is used to quantify the growth of *candida albicans* on the surface of silicone disks (Pantanella et al, 2013). Another method involve the use of two nucleic acid binding stains green fluorescent (Syto9) and red fluorescent propidium iodide. Green fluorescent (Syto9) is able to cross all bacterial membranes and binds to DNA of both gram positive and gram negative whereas red fluorescent propidium iodide only crosses damaged bacterial membranes. After staining live bacteria fluorescent green and dead bacteria fluorescent red/orange and samples were observed under fluorescent optical microscope. The main drawback of this method is that it is impossible to

count total number of bacterial cell, so only provide semi quantitative results (Boulos *et al*, 1999, Jin *et al*, 2005).

Another most common method involve the use of resazurin, a biological dye that does not damage living cells. The blue non fluorescent dye also known as Alamar Blue, is reduced by cellular metabolic activity and converted into pink fluorescent resorufin which is measured spectophotometrically. For these properties, Resazurin is being widely used to detect viable microorganism as well as to quantify number of cells in biofilm.

2.4.2 Fluorescence in Situ Hybridization (FISH)

Fluorescence in situ Hybridization (FISH) technique is a genetic approach in which oligonucleotide probes labelled with fluorescent dyes are used. This technique is highly specific for detecting specific bacteria and analysing the spatial organization of a complex microbial community. By the combination of FISH and denaturing gradient gel electrophoresis (DGGE) the spatial organization of unknown and uncultivable bacteria has been analysed. By combining FISH and confocal laser scanning microscopy, it is possible to identify and topographical visualization of different species in multi species biofilm.

2.4.3 Confocal Laser Scanning Microscope (CLSM)

Confocal Laser Scanning Microscopy (CLSM) is an optical microscope equipped with a laser beam, particularly useful in biology to study thick samples such as microbial biofilms, by processing images. Fluorescent probes are used in order to detect various constituents of biofilm (Staudt *et al*, 2003). This technique is applied to study nascent biofilm without affecting structure or architecture of biofilm. The main limitation is that it is expensive and it is not possible to observe motile bacteria and eukaryotic grazers within biofilm (Cloete *et al*, 1998).

2.4.4 Atomic Force Microscopy (AFM)

Recently atomic force microscopy has been used to measure surface tomography on a scale from angstroms to microns. AFM enables high resolution, non-destructive analysis and does not require complex sample preparation methods. Due to these characteristics, AFM is used to visualised surface of biofilm (Dufrene, 2001, Volle *et al*, 2008), bacterial multiplication (Beech *et al*, 2002), EPS production (Pantanella *et al*, 2011, Dufrene *et al*, 2002) and predation (Nunez *et al*, 2005, Martin *et al*, 2003). AFM is used to observe specimen at molecular resolution and also to quantify the surface feature information (Xu *et al*, 2002). The adhesion properties of bacteria can be quantitatively studied using AFM but sometimes AFM imaging of live bacteria is difficult due to motion of bacteria in growth medium as the cells get rapidly attach and detach from the AFM substrate and swim away and if cells are non-motile, they rely on brownian motion for their contact with AFM substrate.

2.4.5 Electron Microscopy (EM)

In electron microscopy high energy electron beam are used for higher resolution of the sample. Surface characterisation of biofilm is best visualized by Scanning electron microscope (SEM) and images of ultrastructure features of biofilm has been visualised by Transmission Electron Microscope (TEM) (Pantanella *et al*, 2013). The main limitation of SEM is tedious samples preparation to dehydrate and make them suitable for vacuum operations. These limitations have been, at least partially, overcome by cryo SEM and environmental SEM (ESEM). In cryo SEM, the sample is rapidly frozen so that vulnerable bacterial structures are preserved (Alhede *et al*, 2012, Karcz *et al*, 2012). Conversely, ESEM enabled the imaging in gaseous environment of hydrated and non-conductive bacterial biofilms (Alhede *et al*, 2012, Karcz *et al*, 2012).

2.6 Sensors systems for monitoring biofilm

One of the most remarkable features of biofilms is the fact that they can form under extremely diverse conditions, which becomes a dominating consideration once we attempt to monitor biofilms. Biofilms can be desirable, unwelcome or even harmful therefore it is important to research on biosensor to detect biofilm initiation, biofilm maturation and final biofilm disposal for the online monitoring of biofilm so that preventive measures should be taken against the biofilm. For the monitoring of biofilm different analytical methods are used which are given below.

2.6.1 Fourier Transformation Infrared Spectroscopy/Attenuated Total internal Reflection (FTIR /ATR)

FTIR is an infrared technique that simultaneously scans all IR frequencies. However, it has a limited capability for monitoring aqueous solutions, as water strongly absorbs in the IR range (Flemming *et al*, 1998). The attenuated total reflection sampling technique in conjunction with FTIR overcomes this problem, as biofilms are grown and sampled on top of a special substratum (internal reflection element, IRE), so that the contact with water is reduced. Generally, FTIR/ATR is not suited for thick biofilms.

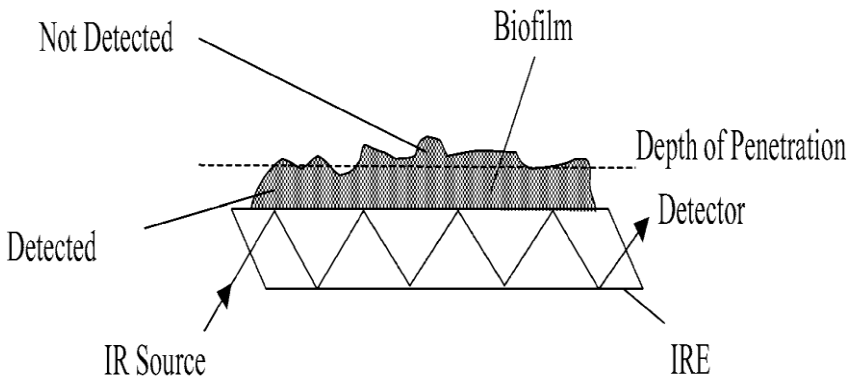


Figure 2.4FTIR ATR monitoring principle

2.6.2 Photo Acoustic Spectroscopy (PAS)

In photoacoustic spectroscopy a short radiation impulse, usually from a laser is used. This radiation impulse is absorbed by a suitable substance inside the sample generate heat, which in turn causes a physical expansion and a subsequent pressure or acoustic wave that can be detected e.g. by a piezoelectric film acting as a microphone. The sensor utilized for PAS-biofilm monitoring consists of a prism which simultaneously serves as a surface for biofilm growth and a transmitter, both for the radiation impulse to the biofilm and the acoustic wave traveling back to the piezoelectric film attached at the back of the prism.

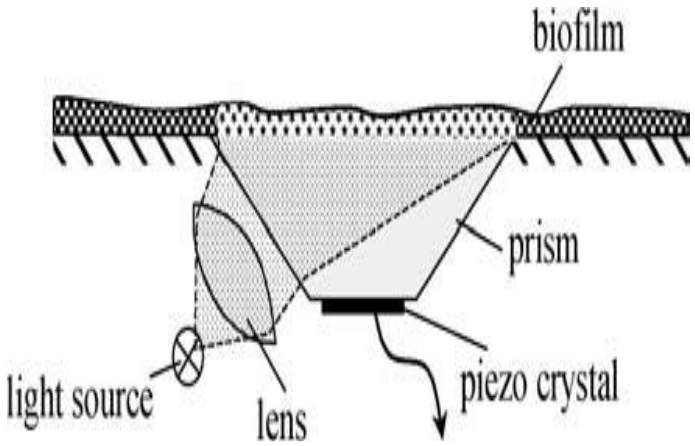


Figure 2.5 Scheme of PAS sensor

By comparing the responses to pulses of different radiation wavelengths different components of biofilm can distinguish such as water, pigments, and carbohydrates and detect their relative distribution within the biofilm (Schmid *et al,* 2003).

2.6.3 Fibre Optical Sensors (FOS)

The Fibre optical sensors are based on light reflection by biofilms. They can be inserted into the water system, and biofilm growth occurs on the tip of the fibre, which is assumed to simulate biofilm growth on the system walls. A light beam is transmitted from the optical fibre to the biofilm surface, where it is reflected by the biofilm material. Part of the reflected light, termed backscattered light, is then collected by the same optical fibre and is transmitted to the photo detector as shown in figure 2.6. The disadvantage of the FOS system is that it is not

selective to microbial deposits, but also incorporates backscattering from abiotic particles (Flemming *et al*, 1998) and is not applicable for thick biofilms.

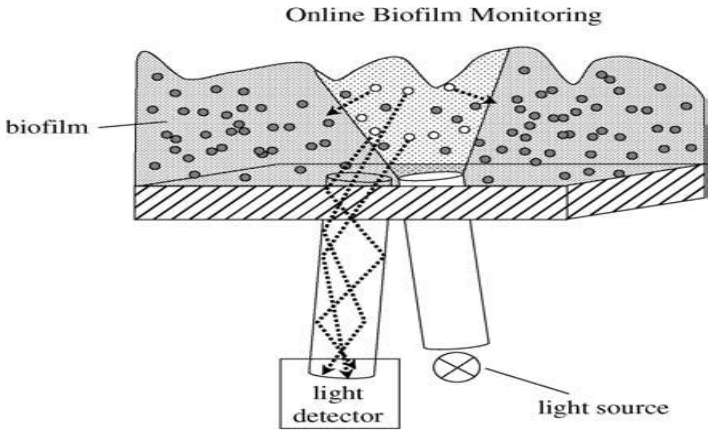


Figure 2.6 Scheme of Fibre Optical Sensors (FOS)

2.6.4 Quartz Crystal Microbalance (QCM)

QCM is a piezo sensors applied for biofilm monitoring (Nivens *et al*, 1993), also designated as thickness-shear mode quartz (TSM, Helle *et al*, 2000). In this device the whole surface vibrates transversally when alternating voltage is applied to the electrodes. Typical resonance frequencies are in the range of 5–10 MHz (Grate *et al*, 1993). Since the rigidity of most biofilms is limited, the higher layers of a thick biofilm would not contribute to that result.

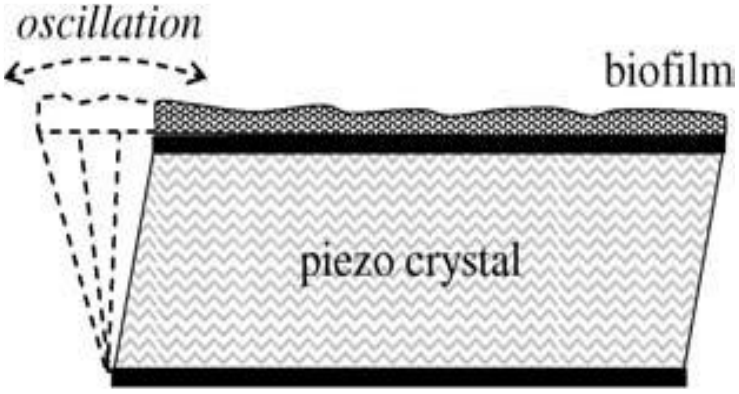


Figure 2.7 Cross section of oscillated QCM

2.7 Drawback of these sensor systems

FTIR/ATE spectroscopy shows limited capacity for monitoring aqueous solution as water strongly absorb in IR range (Flemming *et al*, 1998). Further the geometrical range for the detection is limited to thin layer next to IRE surfaces as a result thick biofilms are not monitored using this technique. The biofilm detection limit of this method was estimated to be 5×10^5 cells cm^{-2} and is only used in lab scale so far. The main disadvantages of Fibre optical sensors (FOS) is that scattering of light is not selective to microbial deposits, but abiotic particles also cause scattering of light (Flemming *et al*, 1998) resulted in false results of detection and is not applicable to thick biofilms. In QCM, the environmental temperature and liquid phase pressure effect the QCMs as reported by Nivens *et al*, in 1993. The detection limits for QCM was reported to be 3×10^5cells cm^{-2} for *Pseudomonas cepacia* biofilm. So this method is only applicable to thin biofilms.

The main drawbacks of the above method are that they are not applicable to monitor thick biofilms and some of them require equipment or operating conditions that limits their practical applicability. As a result a simple, economic and sensitive sensor based detection system is required. So for the online monitoring of biofilm capacitance based methods was discussed. This technique has the advantage that it detect cells with intact plasma membrane and gives values that correlate with biomass rather than necromass

2.8 Capacitance measurement

Capacitance is a property that opposes a change in voltage or electric potential across an object and acts to store energy. A capacitor consists of two conductors, each oppositely charged and separated by a dielectric material. Permittivity is a property of the dielectric material and reflects the ability of charges in the material to move in response to an electric field. Capacitance is a function of the permittivity and the physical geometry of the object. The capacitance formula for a two-plate capacitor is $C = \varepsilon A / d$, where A is the area of each plate, d is the distance between them and ε is permittivity of dielectric.

The living cells contain cell cytoplasm and a membrane enclosing the cytoplasm of cells. The cytoplasm is a highly complicated, consist of salts, proteins, nucleic acid and smaller molecules (Clegg, 1984). The internal membrane bound structures present effects the dielectric properties (Foster and Schwann, 1989, Asami *et al,* 1996). The cell conducting core is surrounded by plasma membrane, which is essentially non conducting (Takashima *et al,* 1988). When an electric current is applied to a suspension of cells in an aqueous ionic solutions, the ions in that solutions are force to move. The positive charged ions are pushed in direction of field, while negative charged ions are push against the direction of field. The ions start moving inside and outside the cells before they encounter the plasma membrane which act as insulating physical barrier. This result in charge separation or polarization at the poles of cells as shown in figure 2.9. The magnitude of suspension field induced separation is measured by its capacitance (C) in Farads (F) but farads is very large capacitance so one normally see its values expressed in terms of pico Farad (pF). Dead cells (Kell *et al,* 1998, Barer *et al,* 1998) do not possess intact plasma membrane and so do not polarise, therefore do not contribute to capacitance of cell suspension (Harris *et al,* 1987, Stoicheva *et al,* 1989). Thus by measuring the capacitance of suspension at one or more appropriate frequency its biomass can be estimated because, as the fraction volume of the cell increase, there are more polarised membrane which in turn give high measured capacitance (Kell and Todd, 1998, Harris *et al,* 1987).The formation of biofilm can be measured using dielectric spectroscopy as an online method as Mark and Kell (1990) observed the biofilm formation of *Klebsiella rubiacearum.* The biofilm was grown in plate system at a constant flow of medium with the tip of BM probe flush to the plate wall.

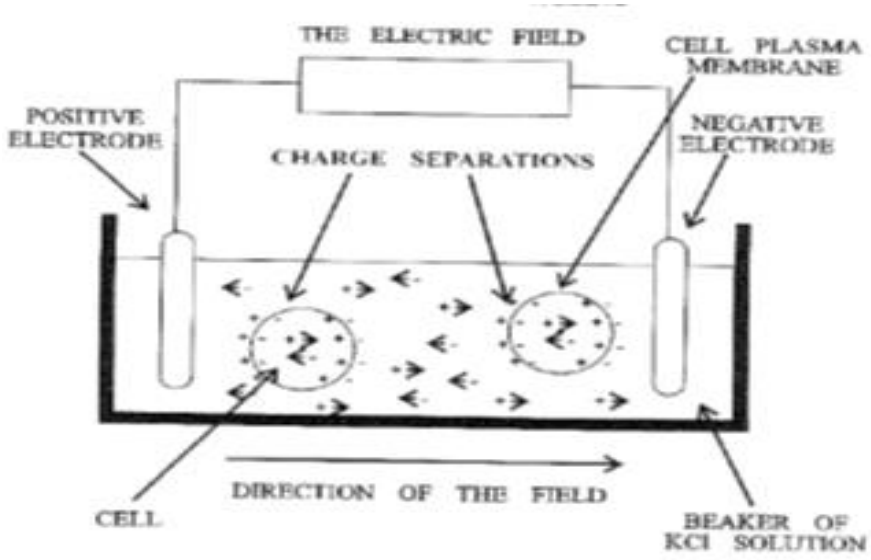


Figure 2.8: The effect of applied electric field to a suspension of cells in aqueous ionic solution. The positive ions are pushed in direction of field and negative ions in counter directions until they encounter cell plasma membrane result in charge separation or polarization at the poles of cells.

Biomass monitor measure the change in capacitance of the culture in a frequency range of 0.1 MHz to 10 MHz. Davey *et al*, (1991) measures the growth of Filamentous fungus *Rhizopus oligosporus* during solid state fermentation using biomass monitor electrode to measure change in capacitance. The change in capacitance was observed as the fungus grows and as the cell lysed the loss of plasma membrane result in fall of capacitance. The growth of yeast in air lift fermenter was studied by Harris *et al*, (1987) use Biomass probe to measure change in capacitance as a function of growth. Further the dielectric properties of chinese hamster ovary cells and Hela cells (Cerkel *et al*, 1993) were studied in suspension cells and a linear relationship was observed between growth of cells and capacitance.

3. MATERIALS AND METHODS

3.1 Media and Reagents

The Biofilm Growth (BG) media was used to study EPS production kinetics and for biofilm growth kinetics. Composition of BG media is bacteriological peptone 5.0 g/l, diammonium sulphate 2.0 g/l, yeast extract 1.0 g/l, $CaCl_2.2H_2O$ 0.7 g/l, NaCl 0.1 g/l, $MgSO_4$ 0.2 g/l, K_2HPO_4 1.0 g/l, Dextrose 1.0 g/l. Nutrient Broth (NB) was used for routine maintaince and culturing of isolates. All of media were routinely sterilised by autoclaving at 121°C and 15 psi pressure for 15 minutes and allow to cool below 50°C before use. All reagent and chemical used for biochemical characterization were of analytical grade were purchased from Himedia (Mumbai). Stains used for staining assays were purchased for Sigma (USA).

3.2 Bacterial strains and culture conditions

Bacterial strains used for the study were previously isolated from various environment and industrial sites. The twenty five strains used in the study. These isolates were grown in LB media at 37°C and were cultured in BG media for EPS and biofilm formation.

3.3 Determination of biofilm formation

The biofilm formation kinetics of the strains were studied using crystal violet assay. In each well of micro titre plate, 1% inoculum was added to BG media and the plates were incubated at 37°C in static conditions. The control wells in micro titre plates contain sterile BG media. The biofilm formation kinetics were studied at every 12 hours over the span of 72 hours.

3.4 Determination of growth profile of strains

The growth profile of biofilm producing strains was studied in BG media. 50 ml of BG media were added in 250 ml flask, inoculated with overnight growth culture (OD 0.02-0.03) and placed in incubator shaker at 37°C with agitation speed of 130 rpm. At every 2 hour interval samples were taken and absorbance was measured at 660 nm.

3.5 Quantification of EPS

EPS production kinetics of each strain was studied over a span of 72 hours. 50ml of BG media were added in 250 ml flask, inoculated with overnight growth culture (OD 0.02-0.03) and placed in incubator shaker at 37°C with agitation speed of 130 rpm. At every 12 hours interval samples were taken and concentration of EPS were measured using alcian blue assay at 610 nm.

3.6 Qualitative estimation of biofilm formation

The glass slides and aluminium stubs were used for the qualitative estimation of biofilm by crystal violet staining assay. The glass and aluminium were first washed with distilled water, wiped with ethanol and then autoclaved. These sterile matrices were then placed into a 1% inoculum containing medium and at the every 12 hours intervals they were observed for biofilm formation over a span of 72 hours. After removing they were washed with sterile distilled water to remove unbound cell and left for drying. Then the matrices were stained with 1% crystal violet solution for 5 minutes. After staining the matrices were washed with distilled water and biofilm so formed was observed qualitatively.

3.7 Biochemical characterization of EPS

3.7.1 Estimation of total protein content

Folin-Lowry method (Lowry *et al*, 1951) was used to determine the total protein content in the EPS. Bovine serum albumin (BSA) was used as standard and EPS stock was mg/mL. 1 mL of freshly mixed complex-forming reagent (annexure I) was added to 0.2 mL of the sample or standard. The solution was left undisturbed for 10 min at room temperature, then 0.1 mL of folins reagent was added and again left undisturbed for 30 minutes. The absorbance was taken at 750 nm. The amount of total proteins present in the sample were calculated from the standard curve prepared by using pure BSA (10-100 µg) as standard.

3.7.2 Estimation of total sugar content

The total sugar or carbohydrate content of EPS was determined by phenol sulphuric acid method (Dubois *et al,* 1986). Glucose was used as standard. 200 µl of phenol reagent (5 % v/v in water) was added to standards as well as samples. After the addition of phenol reagent 1.0 mL of concentrated sulphuric acid was rapidly added to the surface of the solution without touching the sides of the test tube. The tubes were left undisturbed for 10 min at room temperature. After incubation the tubes were shaken vigorously and absorbance was taken after 30 min at 490 nm. The total sugar content present in the sample were calculated from standard curve prepared by using glucose (10-100 µg) as a standard.

3.7.3 Estimation of hexosamine content

The hexosamine content in EPS was determined using Elson-Morgan method (1934). Galactosamine was used as standard. 250 µl of standards and samples were added to 50 µl of reagent A (annexure I). Then each mixture was heated to 100ºC for 3 min. After cooling rapidly to room temperature 1.5 mL of reagent B (annexure I) was added, washing down any condensate formed. The mixture was incubated 37ºC for 20 min. After cooling to room temperature, the absorbance was determined at 585 nm. The hexosamine content present in the sample were calculated from standard curve of galactosamine (10-100 µg).

3.7.4 Estimation of uronic acid content

The uronic acid content of EPS was determined by method described by Haug and Larsen (1962). D-glucouronic acid was used as a standard and EPS stock was 1 mg/mL. In 250 µl of sample or standards 1.5 mL of ice cold reagent A was carefully added with mixing and cooling in ice bath. Then mixture was heated at 100°C for 10 minutes. The mixture was rapidly cooled in the ice-bath and 50 µl of reagent B was added and mixed well. Reheating was done at 100ºC for 15 min. The mixture was cooled rapidly to room temperature and the absorbance was

determined at 525 nm. The uronic acid content present in the samples were calculated from standard curve of D-glucouronic acid (10-100 µg).

3.7.5 Estimation of pyruvic acid content

The pyruvic acid content of EPS was determined by method described by Friedman and Haugen (1943). Pyruvic acid was used as standard EPS stock was 1 mg/mL. Samples were firstly treated with per chloric acid (50%) for deproteinization and were kept at 30ºC for 30 min. Then 1 mL of DNP reagent was added to the extract. Further, 4 mL of water and 10 mL 2.2 N NaOH were added. The tubes were shaken and absorbance was taken at 416 nm. A standard curve was made by using pyruvic acid (10-100 µg).

3.8 Methods of detection

3.8.1 Alcian blue assay

The estimation of EPS concentration in samples were estimated using alcian blue assay (Bobber, 2005). 200 µl of the supernatant were added in eppendorf tube containing 700 µl of 0.5M acetic acid to which 100 µl of alcian blue was added and incubated for 2 hours at room temperature. After incubation, it was centrifuged at 8000 rpm for 10 minutes and absorbance were measured at 610 nm.

3.8.2 Crystal violet assay

The biofilm formation capacity of isolates were estimated using crystal violet assay (Li *et al*, 2003). After respective incubation period, content of each well was gently removed by slightly tapping the plates. The wells were then washed with 300 µl of phosphate buffer saline (PBS pH 7.3) to remove free-floating ‘planktonic’ bacteria. The plates were then stained with 0.1% (w/v) crystal violet solution for 5 minutes. Excess stain was washed off thoroughly and rinsed with deionised water and plates were kept for drying for 30 minutes. Then 200 µl of 30% acetic acid was added, and then again incubated for 15 minutes at room temperature. Then 100 µl of this solution is transferred in fresh microtitre plate and absorbance was measured at 595 nm.

3.8.3 Capacitance measurement of biofilm and EPS

The capacitance of biofilm and EPS were measured using parallel plate capacitor made of aluminium sheet. The capacitors were UV and flame sterilised before every experiment. These sterilised capacitors were then placed in glass petriplates having BG media containing 1% inoculum and at periodic interval of 12 hours the capacitance of the biofilm coated capacitors were measured on (LCR) meter (inductance (L), capacitance (C), resistance (R)) over the span of 72 hours. For capacitance measurement of EPS 10µl of EPS solution (1mg/mL) was coated on capacitor and then dried in oven. As the drop dried, the capacitance of the EPS coated capacitor was measured on LCR meter (Gw instek, model no 821, Taiwan).

3.8.4 Scanning Electron Microscopy (SEM) of biofilm and EPS

SEM analysis was done 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. The samples were washed with 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. Then samples were air dried and 2 drops of hexamethyldisilazane were added and samples were covered for 5 minutes. After 5 minutes samples were air dried at 37°C. Biofilm samples were coated with platinum and EPS samples were coated with gold and analysed by SEM (JSM541-V, JOEL, Japan).

4. RESULTS AND DISCUSSION

Biofilm are ubiquitous in nature as every species of bacteria have mechanism to adhere to surfaces and to each other. Biofilms are found on medical devices such as surgical implants, contact lenses and in industrial machinery such as heat exchangers, cooling towers etc. Various preventive step have been taken to avoid unnecessary effects of biofilms. Therefore it is necessary to monitor the biofilm for adopting timely interventions. Numerous methods for

qualitative and quantitative analysis have been employed in the study of biofilms. However available methods have certain limitations as they may lead to destruction of biofilm surfaces often resulting in discrepancies. In present work, a non-destructive technique was developed for monitoring biofilm by measuring the capacitance of biofilm. The capacitance measurement is a rapid detection method since it only monitors the viable cells in the suspension as already reported in literature (Cerkel *et al*, 1993).

4.1 Screening of biofilm producing bacteria

A total of 25 previously isolated bacterial strains were screened for the biofilm formation ability. Crystal violet staining assays was used for estimation of biofilm formation by these isolates. Of these 25 isolates, 4 isolates i.e. BP4, BP7, BP10 and BP19 were selected on the basis of robustness of the biofilm formation in shorter duration.

4.2 Growth profile of bacterial strains

Growth profile of four bacterial strains were studied in BG media and growth were monitored for 24 hours. The experiment were run in shake flask level at 37°C temperature and agitation speed of 130 rpm. The growth profile of four bacterial strains were shown in figure 4.1. The growth profile of BP4 indicated log phase of 4 hours with specific growth (μ) rate of $0.275h^{-1}$; was maximum of all four strains was achieved. Between 6 hours to 14 hours the cells were in late log phase. After 14 hours the cells reached stationary phase with no significant change in absorbance thereafter. The growth profile of BP7 indicated the log phase of 2 hours followed by stationary phase of 8 hours with specific growth rate (μ) of 0.162 h^{-1}. After 16 hours the cells reached the stationary phase of their growth. A specific growth rate (μ) of 0.187 h^{-1} was observed for BP10. The growth profile of BP19 indicated a log phase of 6 hours with specific growth rate (μ) of 0.25 h^{-1}. Cells reached the stationary phase after 14 hours of their growth. It was found that rate of biofilm formation depends upon the EPS production during the stationary

phase (Kokare *et al*, 2009) and cells in stationary phase is more adhesive than exponential phase are also found in literature (Rosenberg *et al*, 1986). Our results indicated that the stationary phase usually began between 14 to 16 hours of growth of strains. Therefore EPS production may be presumed to occur during this time leading to a subsequent development of biofilm

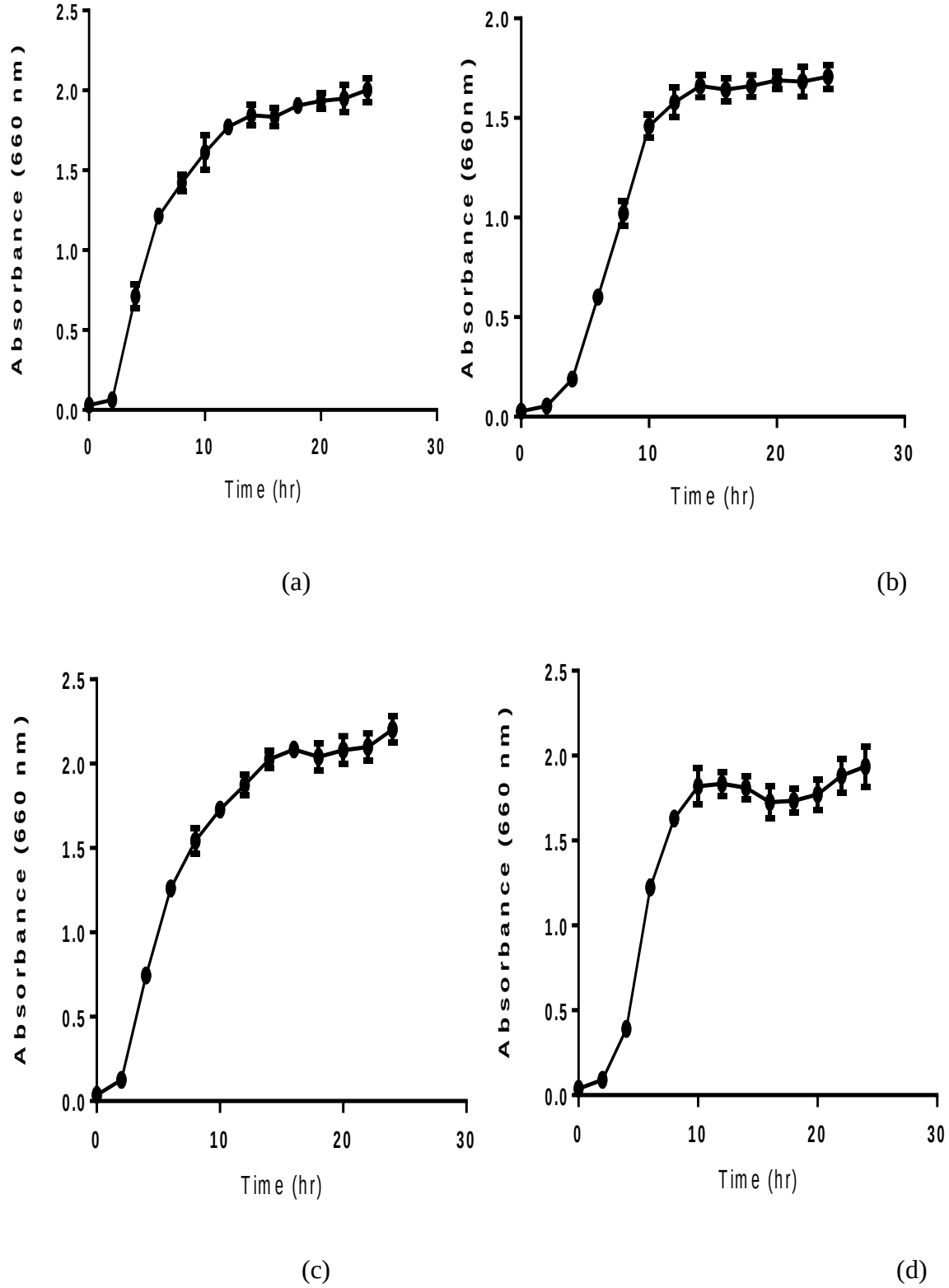
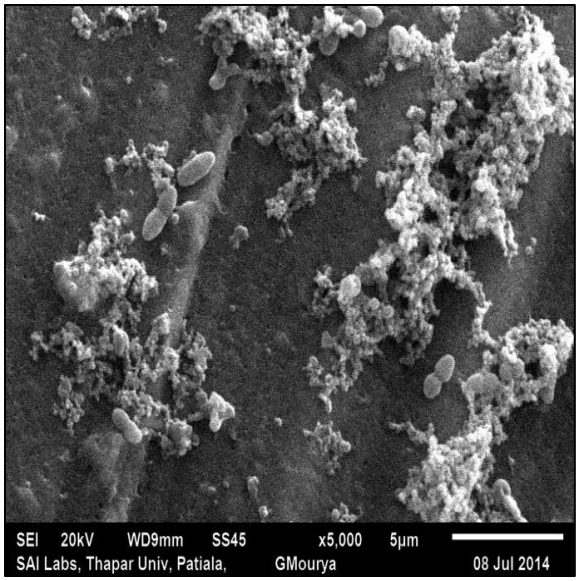


Figure 4.1: Growth profile of (a) BP4 (b) BP7 (c) BP10 and (d) BP19. Error bars represents standard deviation from the mean of 3 replicates.

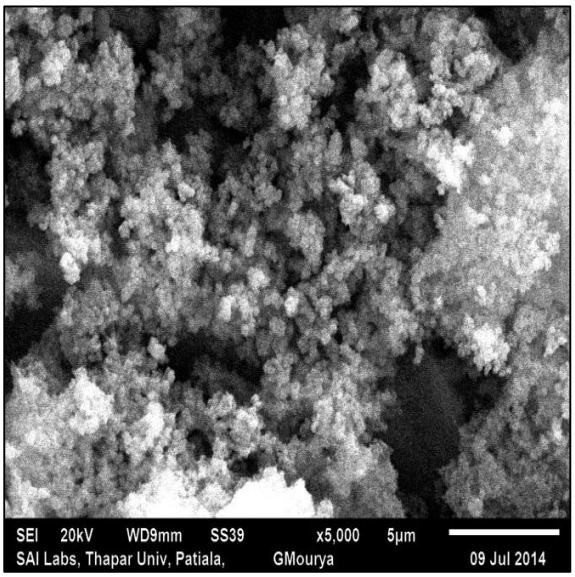
4.3 Biofilm formation

Biofilm kinetics was carried out to infer the influence of bacterial growth phase on the adhesion of cells to the surface. The experiment was conducted in 96 well microtitre plates. Figure 4.2-4.5 depicts the growth kinetics and SEM micrographs of the four bacterial strains. The biofilm kinetics indicated that after 12 hours no significant change ($P>0.05$) in absorbance was occurred, for upto 24 hours suggesting that the cells to have reached the stationary phase of their growth. Cells in the stationary phase starts synthesising the EPS which facilitates the attachment of cells to the surface (Dunne, 2002); SEM micrographs of 24 hours biofilm revealed the synthesis of EPS by bacterial strains as shown in figure 4.2-4.5 (a). After 24 hours, increase in absorbance was observed over a span of 72 hours for all bacterial strains indicated the synthesis of EPS matrix increases around cells. SEM micrograph indicated that at 36 hours of biofilm formation the density of the EPS in the matrix increases which further strengthen the connection between cells and substrate. The cells in the EPS matrix divides, spread outward and upward to form clusters (Stoodley *et al*, 2002) which in turn developed into mushroom like structures in developing biofilms as shown by the SEM micrograph in figure 4.2-4.5 (c).

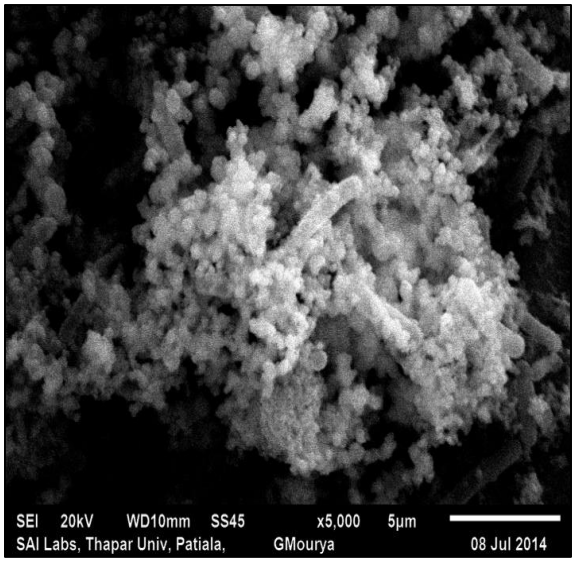
The synthesis of EPS around mushroom like structures helps in the maturation of biofilm and leads to the formation of three dimensional structures. The mushroom like structures in maturing biofilm contain pores and water channel for the transportation of water and nutrient to the cells deeper inside the biofilm and to remove the toxic substances from the biofilm. Biofilm formation kinetics revealed that maximum biofilm formation was observed for BP10 followed by BP19, BP4 and minimum biofilm formation in BP7 at 48 hours of their growth. SEM micrograph of 48 hour biofilm indicated the maximum biofilm formation for BP 10 and minimum biofilm formation for BP7. These results indicates that after 24 hours biofilm formation initiated and BP10 is a strong biofilm producer and BP7 is the least biofilm producer among the four bacterial strain examined.



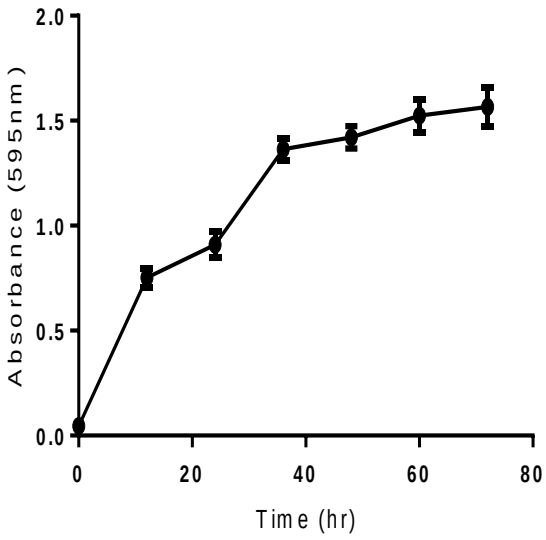
(a)



(b)

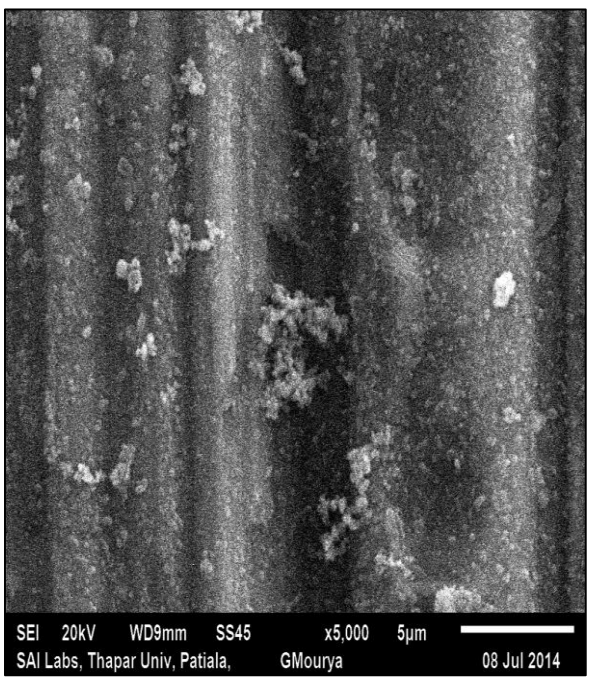


(c)

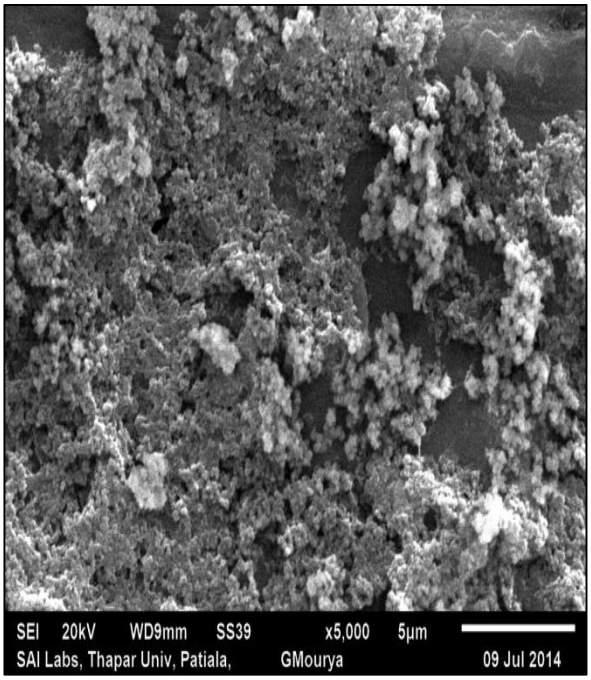


(d)

Figure 4.2 SEM micrographs of BP4 biofilm at (a) 24 hours (b) 36 hours and (c) 48 hours and (d) represents biofilm formation kinetics. Error bar represent standard deviation from the mean of 3 replicates



(a)



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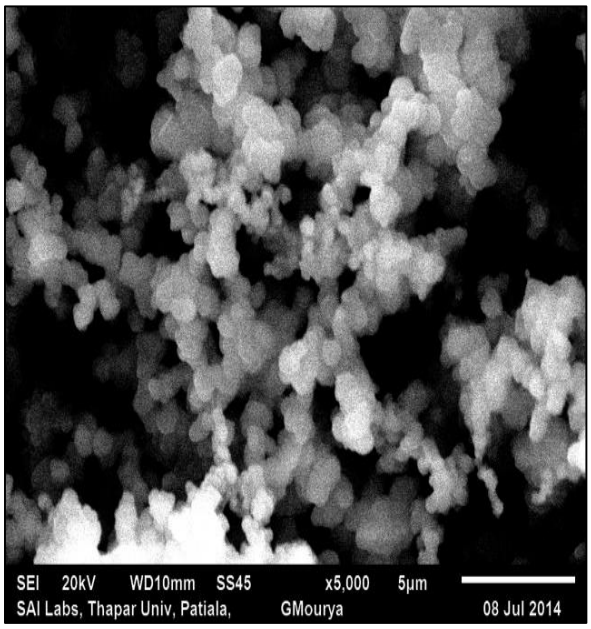
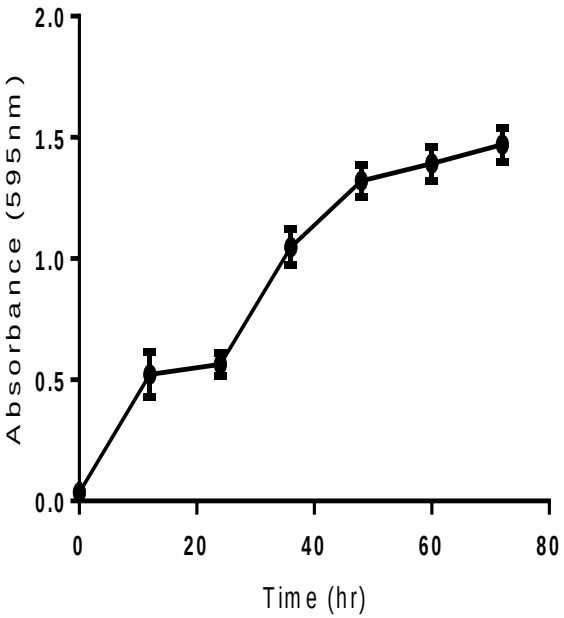
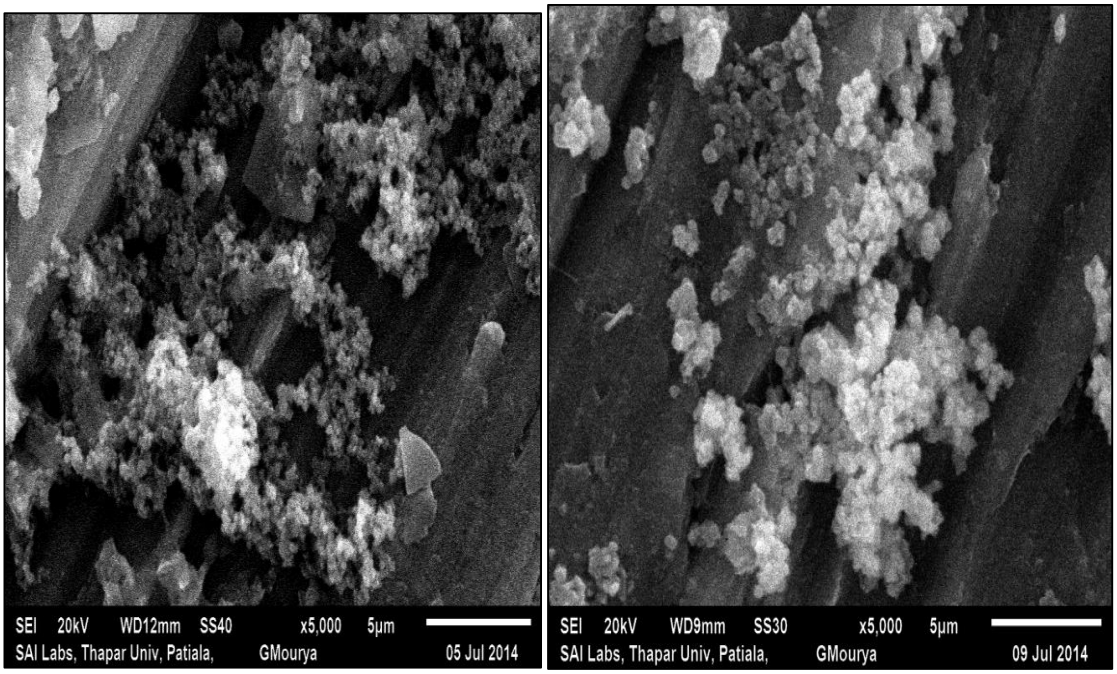


(c)

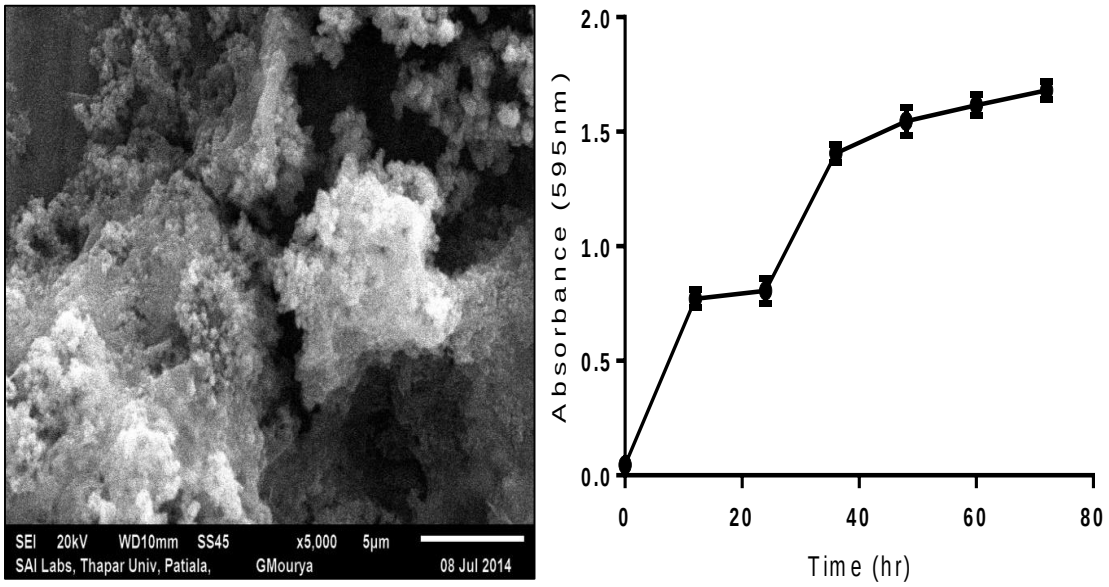


(d)

Figure 4.3 SEM micrographs of BP7 biofilm at (a) 24 hours (b) 36 hours and (c) 48 hours and (d) represent biofilm formation kinetics. Error bar represent standard deviation from the mean of 3 replicates

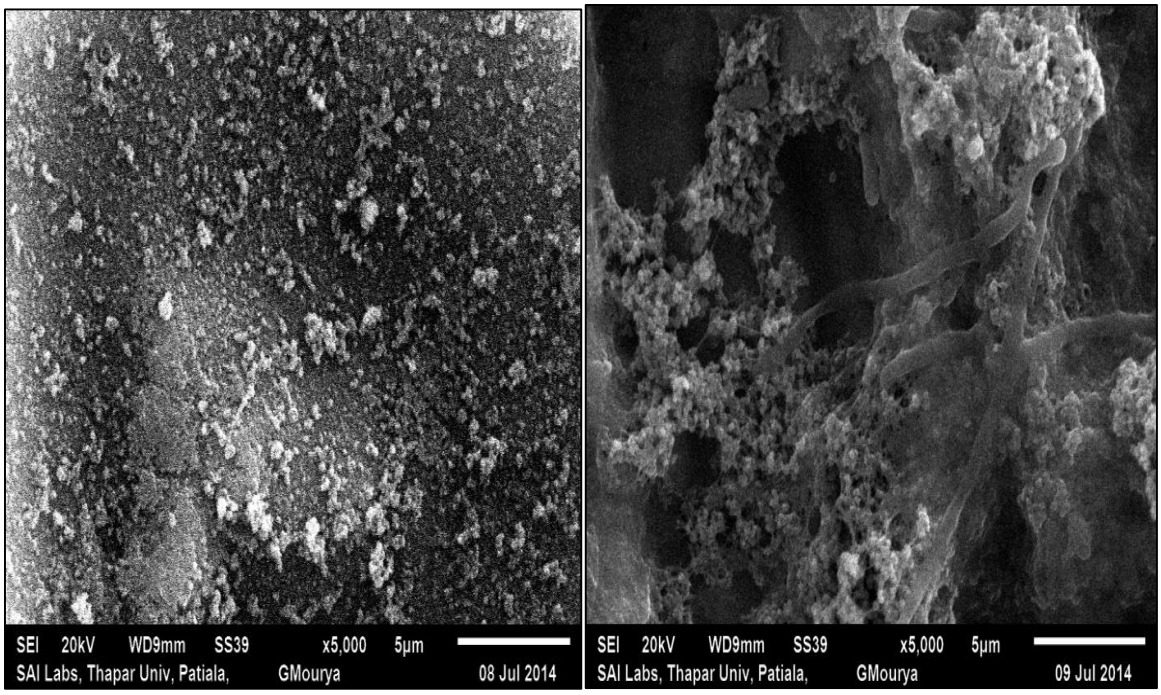


(a) (b)

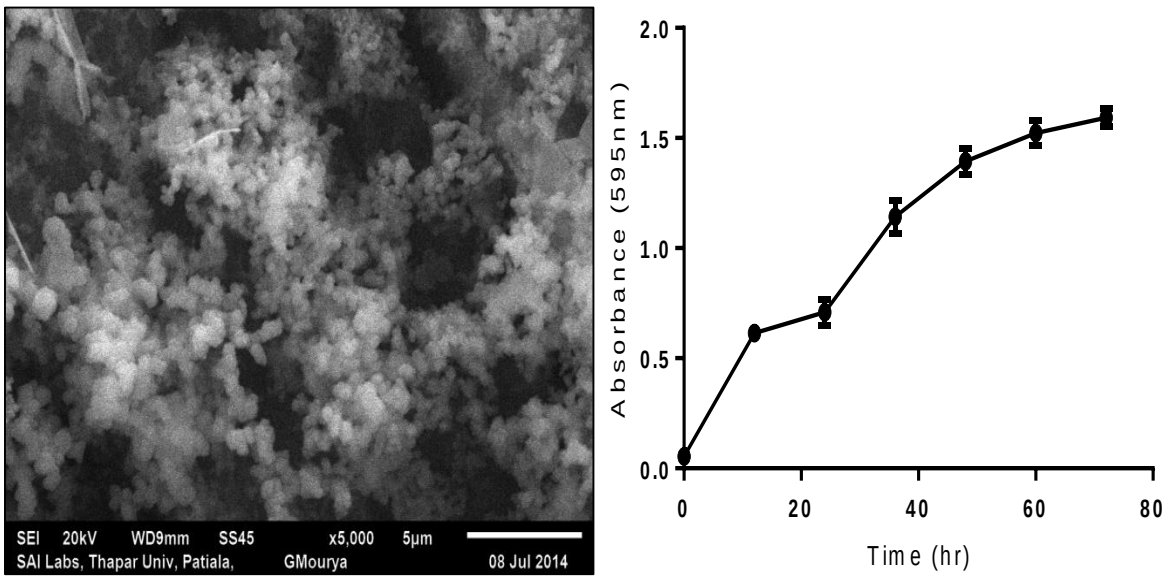


(c) (d)

Figure 4.4 SEM micrographs of BP10 biofilm at (a) 24 hours (b) 36 hours and (c) 48 hours and (d) represents biofilm formation kinetics. Error bars represents standard deviation from the mean of 3 replicates



(a) (b)

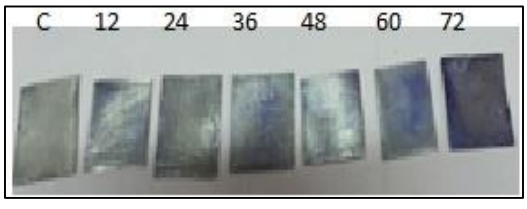


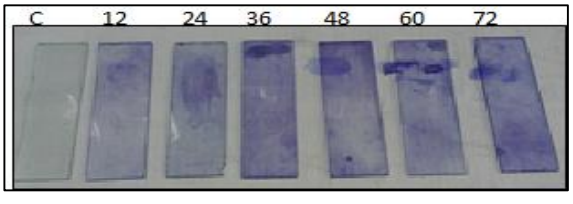
(c) (d)

Figure 4.5 SEM micrographs of BP19 biofilm at (a) 24 hours (b) 36 hours and (c) 48 hours and (d) represents biofilm formation kinetics. Error bars represents standard deviation from the mean of 3 replicates

4.4 Effect of biofilm formation on two different matrices

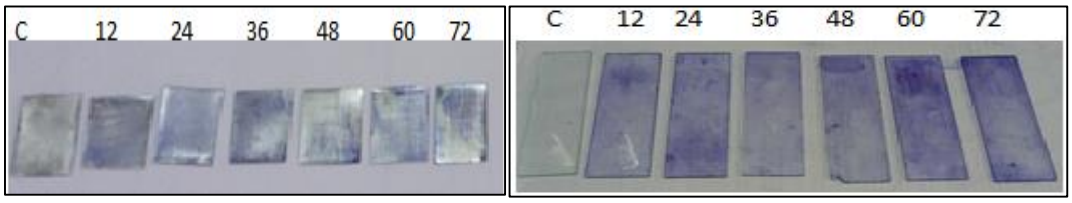
The biofilm formation capabilities of the bacterial strains were studied on glass and aluminium surfaces. The efficiency of attachment of the strains in these matrices were determined using crystal violet staining assay a rapid method for the qualitative estimation of biofilm formation (Li *et al*,, 2003). The biofilm formation capability of the isolates was studied over a span of 72 hours. The biofilm grown on different substrata were shown in Figure 4.6-4.9. Results showed differences in biofilm formation efficiency on different substrates. The biofilm formation increases with respect to time. The bacterial cells generally adhere to rough surfaces due to abundant surface area (Characklis *et al*,, 1990) and cells generally adhere to low energy hydrophobic surfaces since hydrophilic surfaces are generally negatively charged (Fletcher and Pringle, 1983). It was observed that more cell attachment and biofilm formation was observed on glass surfaces as compared to aluminium stubs. This difference in attachment and biofilm formation may be attributed to differences in the properties of the surfaces i.e. hydrophobicity and roughness. The glass surface is less hydrophobic as compared to aluminium surface as more biofilm formation was occurred on glass surface.





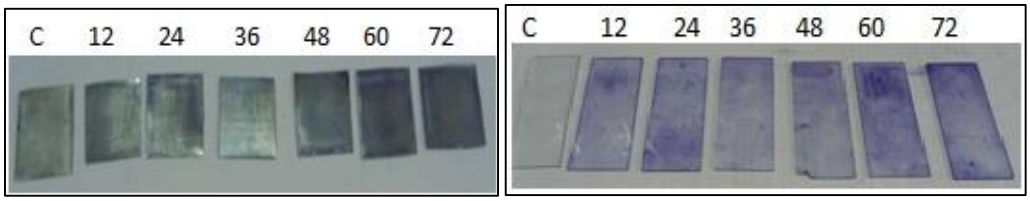
(a) (b)

Figure 4.6 Biofilm formation by BP4 on (a) aluminium stubs and (b) glass slides on different time interval.



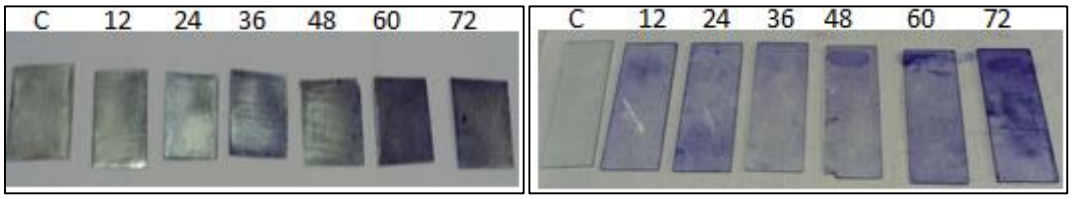
(a) (b)

Figure 4.7 Biofilm formation by BP7 on (a) aluminium stubs and (b) glass slides on different time interval.



(a) (b)

Figure 4 .8 Biofilm formation by BP10 on (a) aluminium stubs and (b) glass slides on different time interval.



(a) (b)

Figure 4.9 Biofilm formation by BP19 on (a) aluminium stubs and (b) glass slides on different time interval.

4.5 EPS production

EPS production kinetics of four bacterial strains were studied in order to measure the biofilm production capability of the strain. Table 4.1 indicated the yield of EPS at time interval of 24 hours and 48 hours of incubation and EPS production profile of bacterial strain were shown in

figure 4.10. The EPS production profile of bacterial strain indicated that EPS synthesis initiated after 12 hours as the cell reaches their stationary phase as discussed in their growth profile. After 24 hours exponential increase in EPS concentration was observed till 36 hours indicated the log period for EPS production. At 24 hours maximum yield of EPS was observed for BP10 of 0.45mg/ml, while minimum yield of EPS was observed for BP7 of 0.40 mg/ml. The yield of EPS is directly correlated with the biofilm formation capability of the strain and strengthens the binding of cells with the substrata. At 24 hours no significant differences in the EPS yield of bacterial strain was observed. EPS production profile indicated after 36 hours less increase in the concentration of EPS was observed.

The maximum yield of EPS was observed for BP4 of 1.98 mg/ml, followed by BP10, BP7 and minimum in case of BP19 of 1.46 mg/ml. After 48 hours no significant increase in EPS concentration was observed for BP4 and BP7, however slight increase in EPS was observed for BP19 and BP10. The maximum EPS yield was observed at 48 hours for BP4 and BP7, at 60 hours for BP10 and 72 hours for BP19. These results suggest that EPS production occurs between late log phase to late stationary phase of their growth (Mahmoudi *et al*, 2010). Additional evidences that the EPS production occurs during stationary phase has been reported in literature (Othman *et al*, 2012). The rate of EPS production was directly correlated with the biofilm formation capability of strain and EPS profile indicates the increase in concentration of EPS with time

Table 4.1 Yield of EPS at 24 hours and 48 hours of incubation of bacterial strain

EPS of strain	Yield (mg/ml) at 24 hours	Yield (mg/ml) at 48 hours
BP4	0.43	1.98

BP7	0.40	1.48
BP10	0.45	1.65
BP19	0.42	1.46

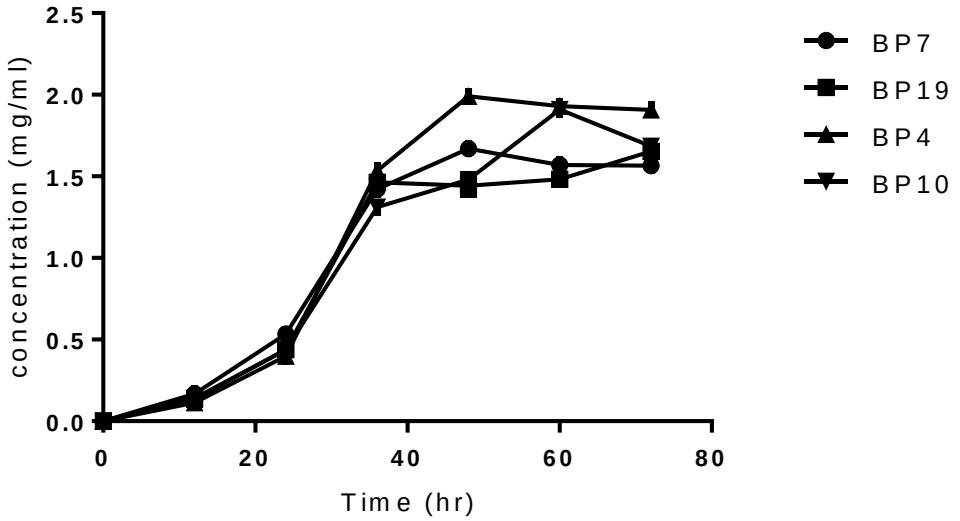
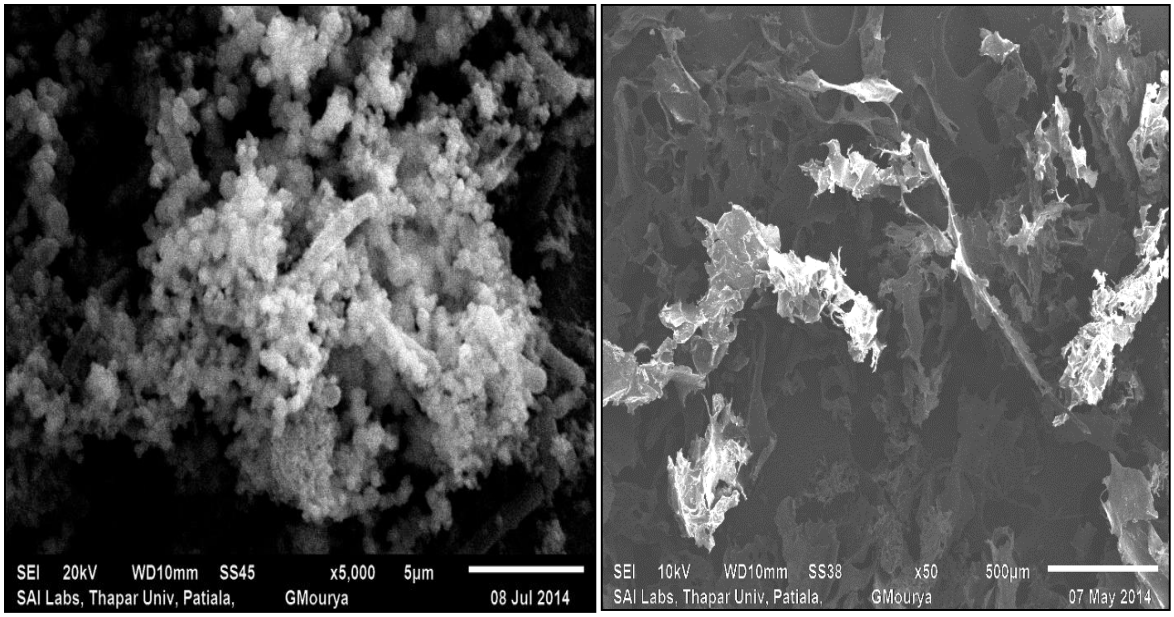


Figure 4.10 EPS production profile of bacterial strains

4.6 Surfaces characterisation of biofilm and EPS

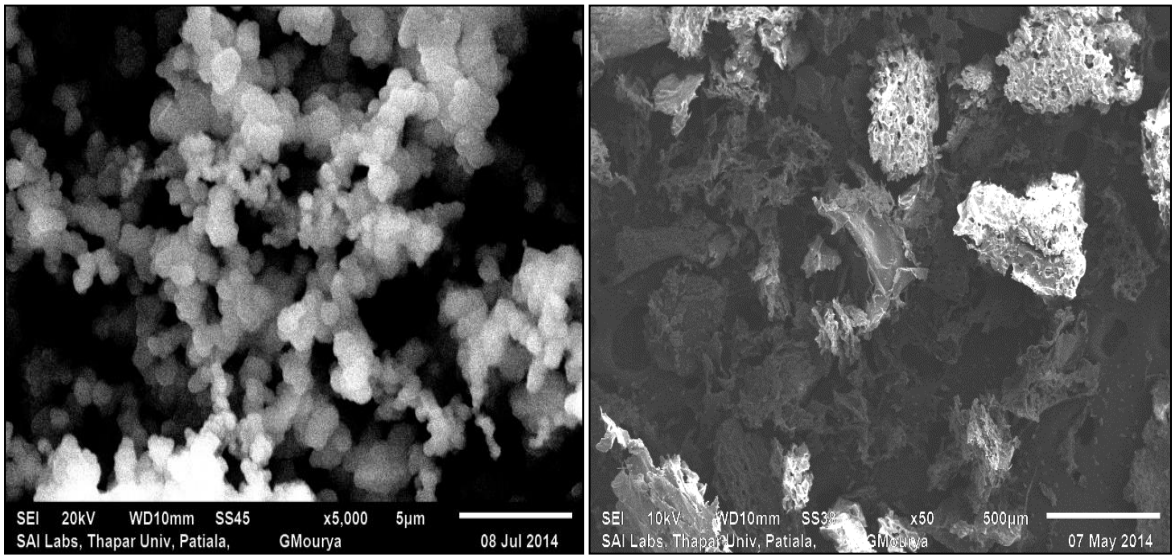
The SEM micrographs of 48 hour biofilm and EPS are shown in figure 4.11 to 4.14. The EPS is the matrix extracted from biofilm and composed primarily of polysaccharides, proteins and other charged ions, whereas biofilm is comprised of EPS as with the cells embedded in the

matrix. SEM micrograph of extracted EPS from all biofilm producing bacterial strains shows the porous and rough surface of EPS. Porosity of the EPS is directly correlated with water retention capacity to form gel like structures (Takashami *et al*, 1998). The porosity was found maximum for BP7 followed by BP19, BP4 and BP10 respectively in the present study. Biofilm producing bacterial strains were embedded in the EPS that forms a protective covering around the cells (Kokare *et al*, 2008). The SEM micrographs of biofilm, differences in the EPS matrix structure of all the strain. Moreover porosity in EPS was observed in BP7 was higher in comparison to all other EPS matrices of other strains (figure 4.16-4.19 (a)). High porosity in EPS resulted in formation of greater number of water channels for the better exchange of water and nutrients and removal of toxic metabolites from the biofilm.



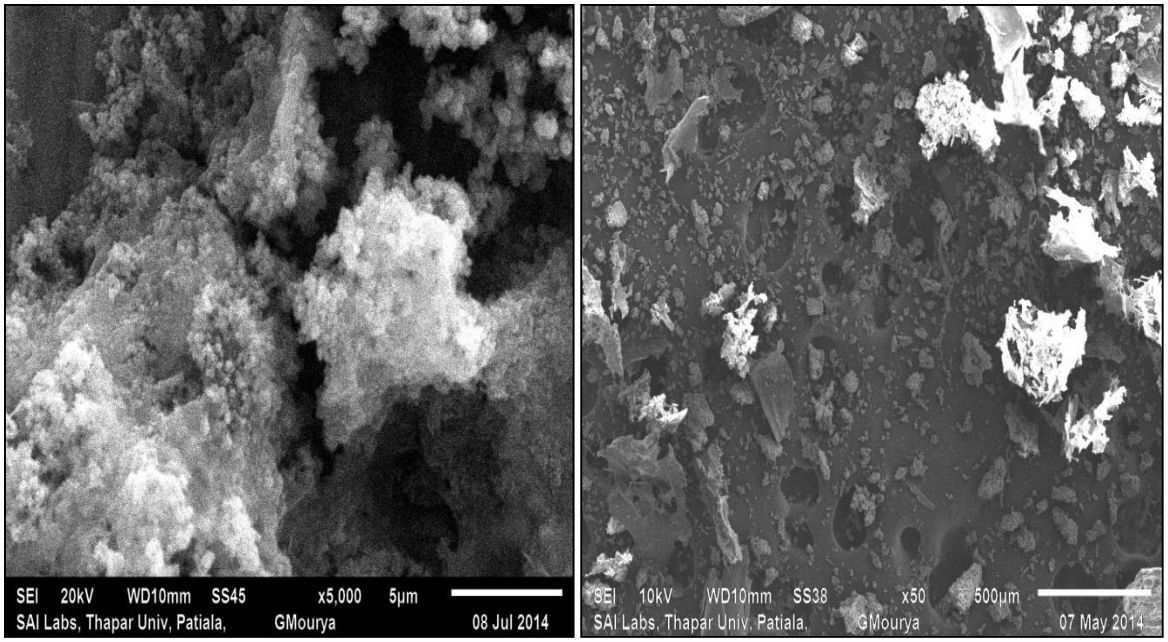
(a) (b)

Figure 4.11 SEM micrograph of 48 hours old (a) biofilm and (b) EPS of BP4 strain



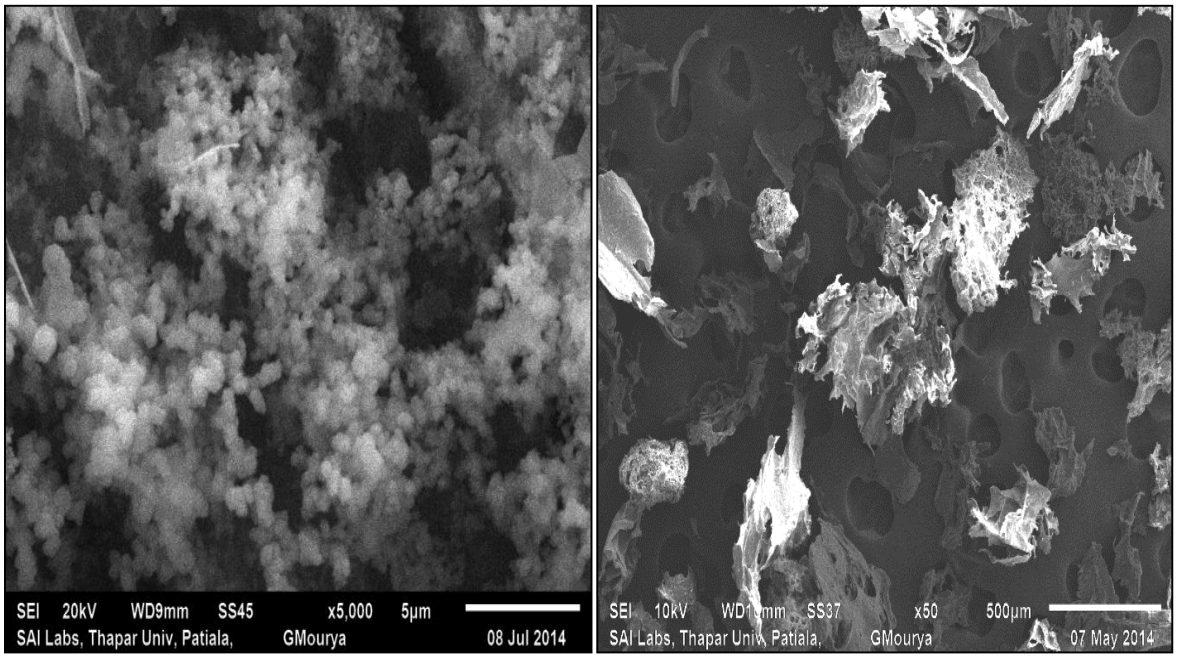
(a) (b)

Figure4.12 SEM micrograph of 48 hours old (a) biofilm and (b) EPS of BP7 strain



(a) (b)

Figure 4.13 SEM micrograph of 48 hours old (a) biofilm and (b) EPS of strain BP10



(a) (b)

Figure 4.14 SEM micrograph of 48 hours old (a) biofilm and (b) EPS of BP19

4.7 Biochemical characterisation and capacitance measurement of EPS

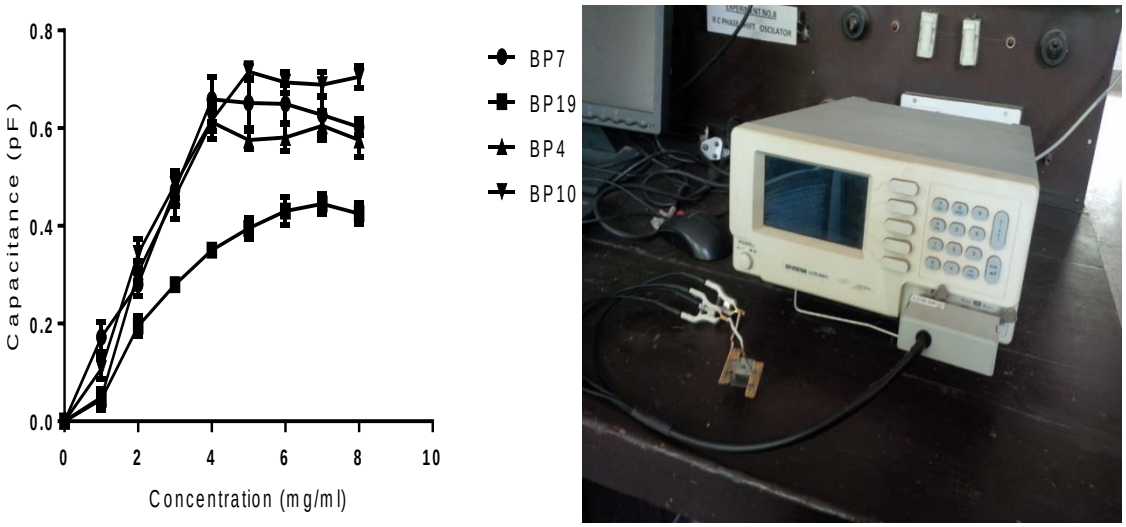
Biochemical analysis of purified EPS was carried out in order to estimate the concentration of charged functional ions present which are responsible for the conductivity of EPS. Different bacterial species produce different and variable amount of EPS (O'Toole *et al*, 2000). The presence of uronic acid and pyruvate confers the anionic properties of the EPS and polysaccharides present in EPS are either neutral or polyanionic (Kokare *et al*, 2009). Biochemical characterisation confirmed that many charged ions i.e. sugars, proteins, amino sugars etc. are present in EPS (Table 4.2). Further EPS of four bacterial strains consist primarily of sugars and minimum amount of proteins. The EPS of strain BP4 contained more sugars and EPS of strain BP7 contained more protein than the other bacterial strains.

Table 4.2: Composition of EPS

EPS of bacterial strains	Sugar (µg/ml)	Protein (µg/ml)	Hexosamine (µg/ml)	Uronic acid (µg/ml)	Pyruvic acid (µg/ml)
BP4	65	39	23	19	27
BP7	64	45	12	15	22
BP10	38	27	16	24	20
BP19	48	21	19	21	18

The capacitance measurement of EPS was carried out using parallel plate capacitors and measurement were recorded with a LCR meter as shown in figure 4.15 (b). The relationship between change in capacitance with concentrations is shown in figure 4.15 (a). A linear relationship between change in capacitance and concentration of EPS was observed as shown in figure 4.15; but after a particular concentration no significant ($P > 0.05$) change in

capacitance was observed. This may be explained by considering a saturation of capacitance due to the presence of charged functional ions present in the EPS as confirmed from the biochemical characterisation of EPS. In case of BP4 and BP7, after 4 mg/ml concentration no significant change in capacitance was observed. But in case of BP10 and BP19, change in capacitance was almost constant after the EPS concentration of 5 mg/ml and 6 mg/ml respectively. This difference in capacitance of EPS was due to difference in concentration of charged functional ions in EPS. This difference in the ionic functional groups in the EPS was confirmed from the biochemical characterization shown in table 4.2. Clearly variation in ionic concentration lead to a difference in capacitance value of the EPS as shown in Figure 4.15 (a). Due to presence of greater number of charged functional ions in BP4 and BP7 the saturation of capacitance occured at lower concentration as compared to BP10 and BP19.



(a) (b)

Figure 4.15 (a) Effect of change in capacitance v/s concentration of EPS and (b) LCR meter. Error bars represents standard deviation from the mean of 3 replicates.

4.8 Capacitance measurement of biofilm

To understand whether biofilm age is associated with change in capacitance, parallel plate capacitors were used for the capacitance measurement of biofilm. At every 12 hours interval, these capacitors were collected and capacitance was measured on LCR meter. The schematic diagram representing fabrication of capacitor is shown in figure 4.16. The calibration curve indicating the age of biofilm with change in capacitance for four bacterial strains is shown in figure 4.17. It was observed that as the biofilm formation initiates after 24 hours the capacitance value of the biofilm increases linearly. This increase in capacitance values may be directly correlated to increase in biomass and EPS content of the biofilm. As the biofilm formation reaches the stationary phase the linear increase in capacitance value is not observed because of constancy in cell density. It was observed that the maximum capacitance value of 53854 pF for BP4 and 44119 pF was observed for BP7 at the time of 72 hours. The capacitance values of 37895 pF and 42156 pF was observed for BP10 and BP19 respectively.

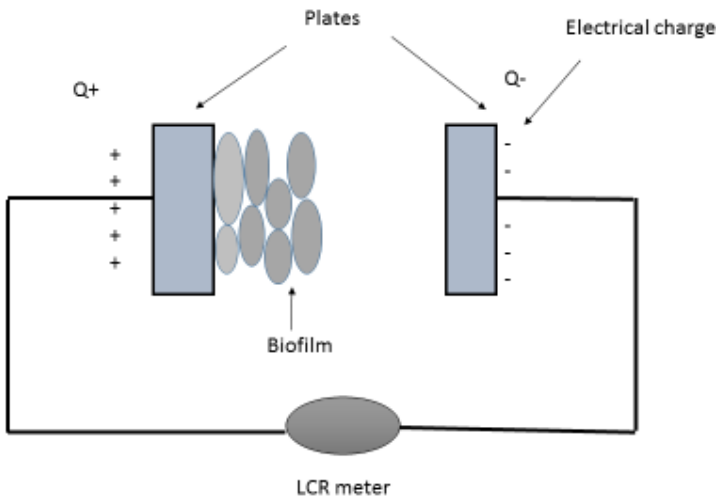


Figure 4.16 Fabrication of capacitor based sensor

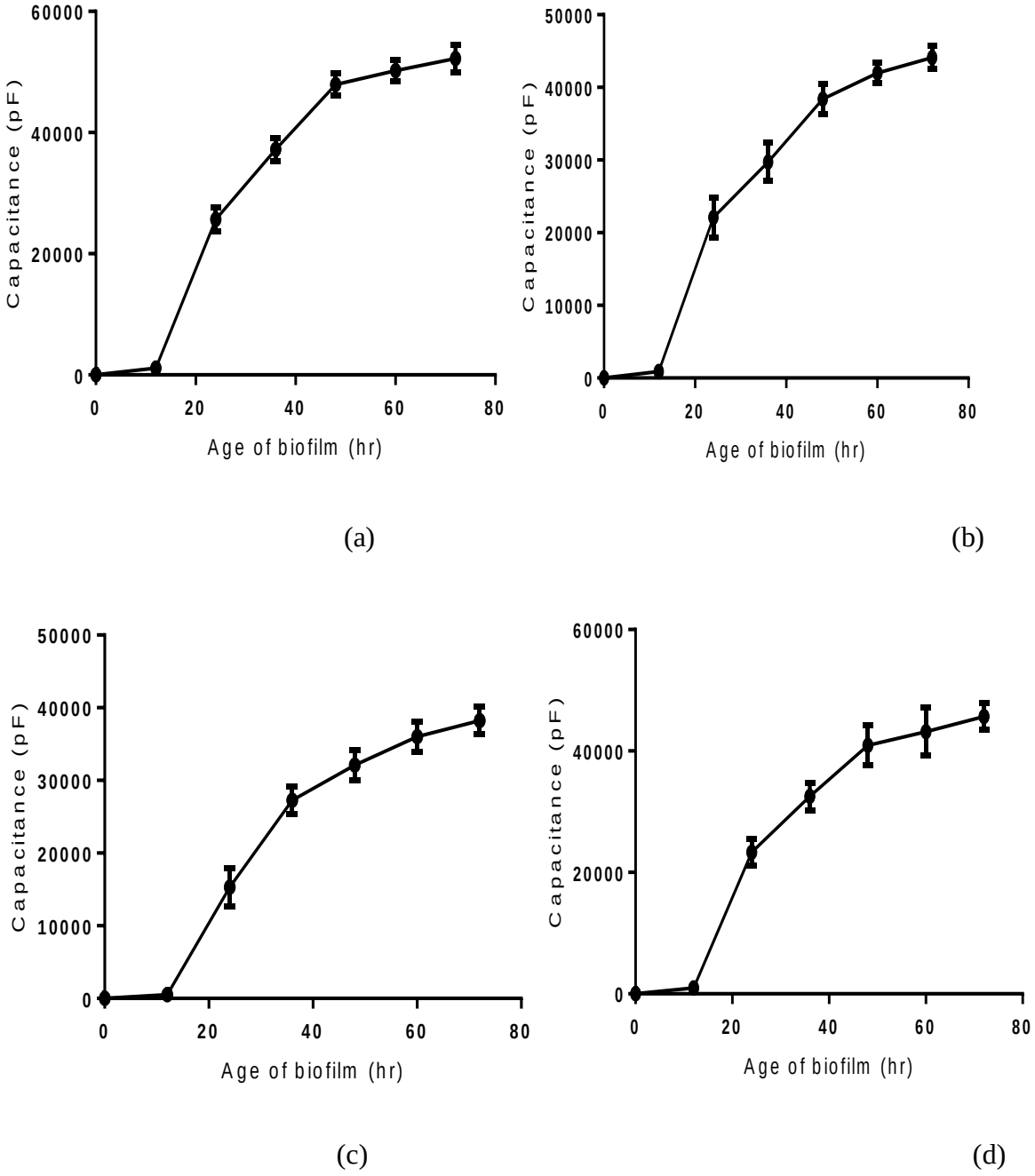


Figure 4.17 Calibration curve of (a) BP4 (b) BP7 (c) BP10 and (d) BP19. Error bars indicate standard deviation from the mean of 3 replicates.

This differences in the capacitance values of the biofilm is due to differences in their EPS content and cell density. The density as well as the presence of charged functional ions in the EPS contributes to the capacitive properties of biofilm. EPS production kinetics as well as

biochemical characterization of EPS revealed that strain BP4 and BP7 has high content of charged ions and EPS content as compared to other strains resulting in high capacitance values. The high cell density was observed in case of case of BP10 and BP 19 but the concentration of charged ion as well in EPS content was found to be low as compared to BP4 and BP7 resulting in low capacitance values. When the capacitance of biofilm and EPS were compared, significant changes ($P > 0.05$) in their capacitance values were observed although they possessed similar physical and chemical properties. However during extraction, presumably due to various conditions used for extraction, the architecture of the EPS changes as a result of which the capacitance values of both biofilm and EPS display notable differences. Further EPS consist of sugars, proteins and other ions which contribute to capacitive properties in contrast to biofilm consisting of cells as well as EPS which contribute to the capacitive properties of biofilms.

5. CONCLUSION

1) A preliminary study was carried out to develop a sensor based on correlation between capacitance and maturation of biofilm.

2) Observation on growth and biofilm formation revealed that biofilm formation initiated at 24 hours of growth for all the bacterial strains and continued forupto 72 hours of incubation. The maximum biofilm formation was observed in BP10 followed by BP19, BP4 and BP7 strains.
3) Biochemical characterisation of EPS of biofilm indicates the presence of charged functional ions such as proteins, carbohydrates, hexosamine etc. Concentration of charged functional ions was observed to be higher in BP4>BP7 >BP19 >BP10 respectively.
4) Capacitance measurement studies revealed that the strain BP4 showed maximum capacitance followed by BP7, BP19 and BP10. The age of the biofilm can be calculated from the calibration curves of respective strains.
5) Capacitance studies on biofilm can be further optimised to develop a biosensor for the online monitoring of biofilm based upon their conducting properties.

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Annexure- I

1) Reagents for Folin- Lowry assay

Reagent A (Alkaline solution 50 mL)

Na_2CO_3	2.0gm
NaOH	0.2gm

Reagent B (50 mL)

$CuSO_4$	0.5gm
Na-K-tartarate	1gm/100mL

Reagent C 50 mL of reagent Aand 50 mL of reagent B) were mixed

2. **Reagent for Elson-Morgsn assay**

Reagent A (100 mL)

Dipotassiumtetraboratetetrahydrate	6.1g

Reagent B

4-N, Ndimethyl-p-aminobenzaldehde	1 g
Glacial Acetic acid	50 mL
HCl (11.5 N)	1.5 mL
Standard solution (galactosamine)	0-1 $mgmL^{-1}$

3. **Carbazole Assay (Uronic Acid)**

Reagent A (100 mL)

Sodium tetraboratedecahydrate	0.9 g
Distilled water	10 mL
Concentrated H_2SO_4 (ice cold) (98%)	90 mL

Reagent B

Carbazole	100 mg
Absolute Ethanol	100 mL
Standard solution (glucuronic acid)	0-1 $mgmL^{-1}$

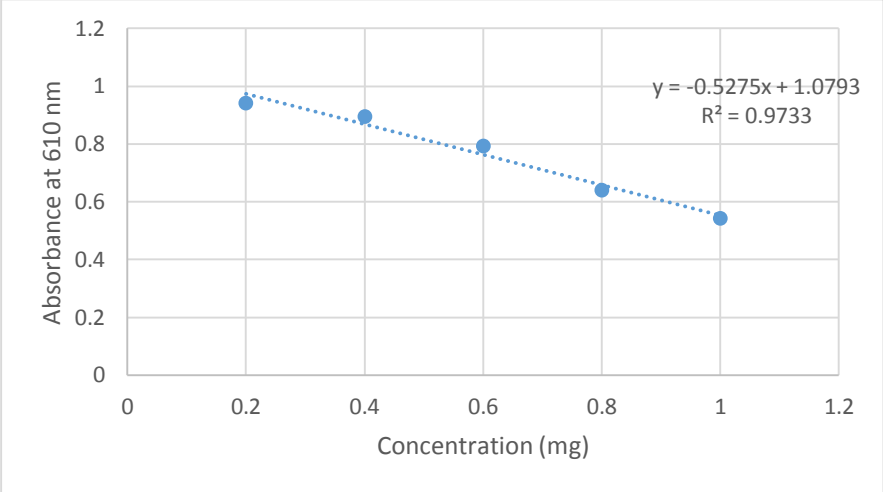
4. Friedman method (Pyruvic Acid)

Perchloric acid	50%
DNP reagent	
(2, 4-dinitrophenylhydrazine)	500 µM
NaOH (2.0 N)	10 mL
Sodium hydroxide	2.2 N
Standard solution	0-3 $mgmL^{-1}$

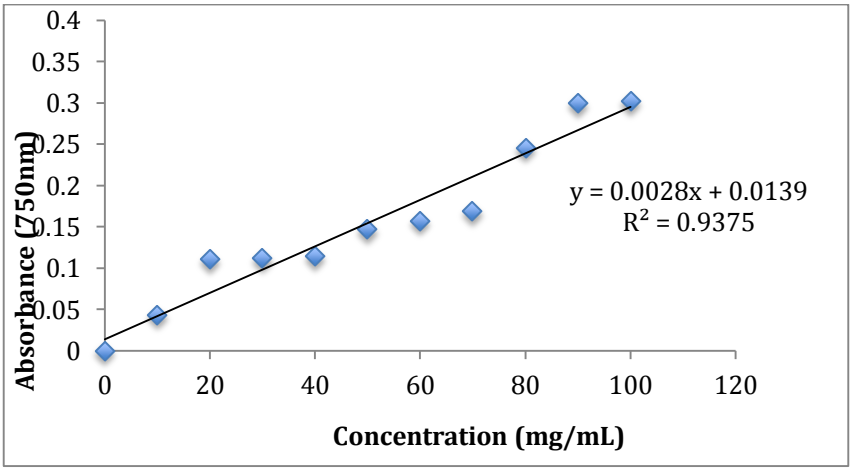
5. Total sugars

Phenol	5%
Sugar standards (glucose, galaxies, xylems,	
Maltose, mannose)	1 $mgmL^{-1}$

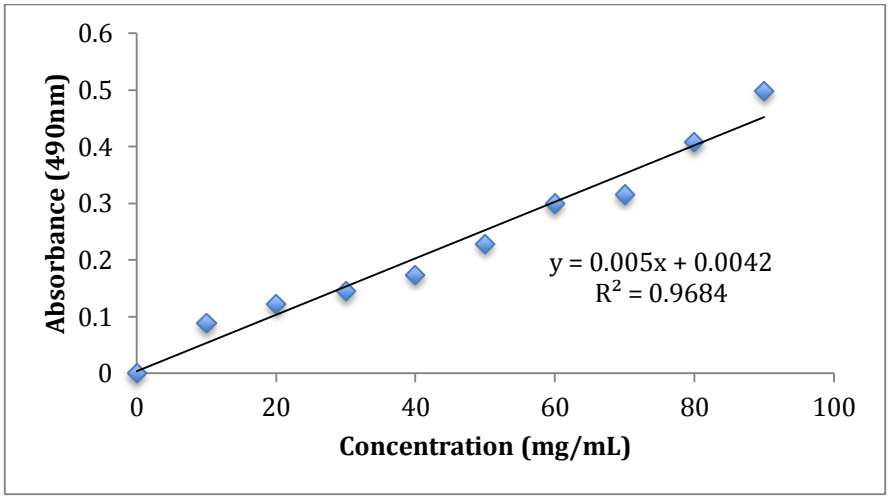
Annexure- II



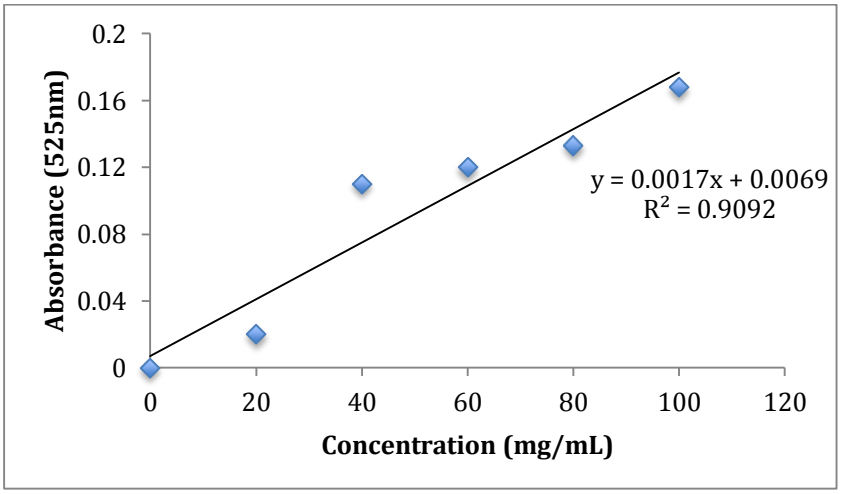
Calibration curve of EPS



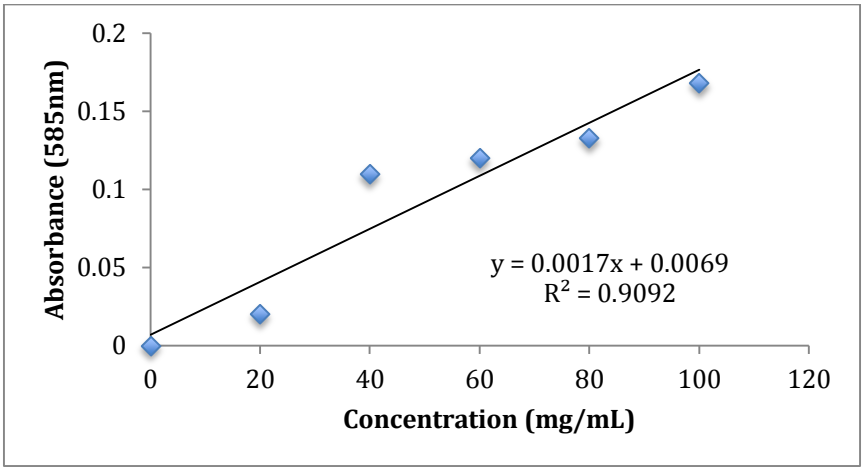
Calibration curve of protein using BSA as standard



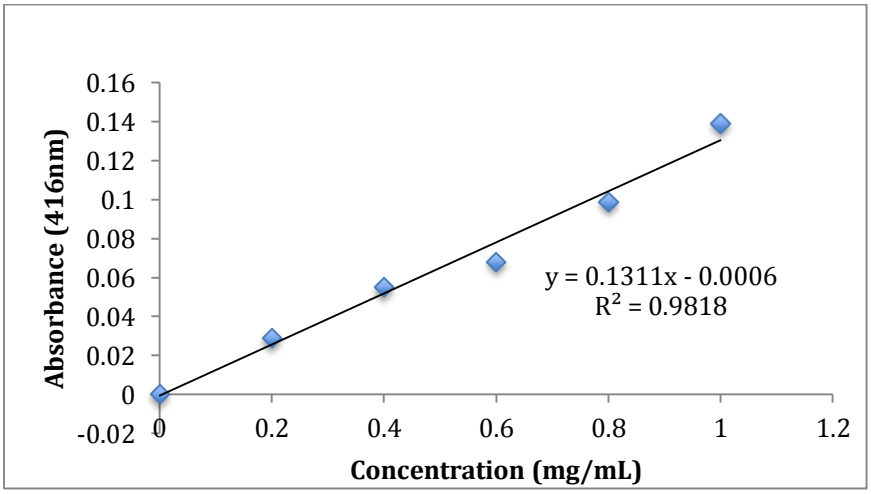
Calibration curve of sugar using glucose as standard



Calibration curve of uronic acid using D-glucuronic acid as standard



Calibration curve of amino sugars using Galactosamine as standard



Calibration curve of pyruvic acid using pyruvic acid as standard