Electrochemical characterization of extracellular polymeric substances and biosurfactants produced by Gram-positive and Gram-negative bacteria

Aisholpan Kaziullayeva, B. Eng in Chemical Engineering

Submitted in fulfillment of the requirements for the degree of Master of Science in Chemical and Materials Engineering

School of Engineering and Digital Sciences
Department of Chemical and Materials Engineering
Nazarbayev University

53 Kabanbay Batyr Avenue,
Astana, Kazakhstan, 010000

Supervisors: Almagul Mentbayeva
Tri Pham

May 2023

DECLARATION

I hereby, declare that this manuscript, entitled “Electrochemical characterization of extracellular polymeric substances and biosurfactants produced by Gram-positive and Gram-negative bacteria”, is the result of my own work except for quotations and citations which have been duly acknowledged. I also declare that, to the best of my knowledge and belief, it has not been previously or concurrently submitted, in whole or in part, for any other degree or diploma at Nazarbayev University or any other national or international institution.

Name: Aisholpan Kaziullayeva
Date: 03.05.2023

Abstract

A biofilm can be defined as a community of microorganisms enfolded in a matrix of peptides, nucleic acids, and poly-saccharides that the bacteria release. Planktonic or free-floating microorganisms are produced as a result of these species growing and eventually sloughing off from this matrix, which provides them with a protected environment. The matrix is a sophisticated, three-dimensional structure with attributes that facilitate the effective distribution of waste and nutrients throughout the matrix. Biofilm growth and formation might also be linked with the generation of intermediate bioproducts such as biosurfactants and biopolymers as a result of response to environmental stress. For instance, the biofilm matrix consists of extracellular polymeric substances (or shortly known as EPS), polymers present in nature, typically responsible for structural integrity of the biofilm. The EPS components are secreted by microorganisms to facilitate biofilm growth and adherence to surfaces for further preservation of microbial aggregates. Certain microorganisms, particularly Gram-negative bacteria are able to secrete biosurfactants like rhamnolipids within the biofilm matrix to resist external pathogenic attacks and develop antimicrobial counteractions in the matrix. These so-called 'intermediate' bioproducts secreted within the biofilm play a significant part in its growth and are applicable in a variety of industries. Standard test procedures biofilm analysis include mostly chemical and biological methods. However, the characterization of biopolymers can be carried out also with physical methods borrowed from soft matter analysis, such as Electrochemical Impedance Spectroscopy (or EIS). It is already offered in inexpensive equipment and has the potential to become a widespread technique for regular characterization of biomaterials of microbial origin. This study aims to showcase the potential of EIS method to successfully characterize the biopolymers (polyglutamic acid) and biosurfactants (rhamnolipids) secreted by Gram-positive and Gram-negative microorganisms, respectively, and enable to understand how their electrochemical activity behaves.

Acknowledgements

I want to start by expressing my sincere gratitude to my supervisor, Prof. Enrico Marsili, for his leadership and unending support during this process. I appreciate you mentoring me and imparting your scientific expertise and experiences, which will serve as a constant source of motivation for the rest of my life. Being a member of your group has always been a privilege for me.

In addition, I want to thank my supervisor Prof. Tri Pham for his assistance and advice throughout my studies. Another person who truly deserves to be acknowledged is Prof. Almagul Mentbayeva, who never ceased to support me from the very start.

Thank you so much to everyone in the Biofilm lab. There are simply no better or more amiable colleagues one could ask for. I'll never forget your assistance, encouragement, and enjoyable conversations.

My sincere gratitude to Obinna Ajunwa for all of his assistance, conversations, and friendship. Special thanks go to my best friend Kayode Olaifa. There are no words to describe how am I grateful for having you in this period of my life. Your technical assistance along with emotional support and true friendship made it possible.

Table of Contents

List of Abbreviations

AHLs	N-Acyl homoserine lactones
ANOVA	Analysis of variance
CLSM	Confocal laser scanning microscopy
CPE	Constant phase element
CV	Crystal Violet staining
C_{dl}	Double layer capacitance
C_{eff}	Effective capacitance
DSF	Diffusible signal factor
EDTA	Ethylenediaminetetraacetic acid
EEC	Equivalent electric circuit
EF	Electrofermentation
EIS	Electrochemical Impedance Spectroscopy
EPS	Extracellular polymeric substances
E_t	Sinusoidal voltage
FCS	Fluorescence correlation spectroscopy
FLBA	Fluorescence lectin binding analysis
HA	Natural humectant
HAAs	3-(3-hydroxyalkanoyloxy)alkanoic acids
IMS	Imaging mass spectrometry
I_t	Current response
IPTG	Isopropyl-D-1-thiogalactopyranoside
MIC	Microbially induced corrosion
PGA	Polyglutamic acid
γ-PGA	Poly-γ-glutamic acid
PIA	Polysaccharide intracellular adhesions
R_{ct}	Charge-transfer resistance
R_s	Intrinsic resistance
RL	Rhamnolipids
SM	Secondary metabolites
SPE	Screen printed carbon electrodes
SPEIS	Staircase potential electrochemical impedance spectroscopy
SSF	Solid-state fermentation
TGY	Tryptone glucose yeast
QS	Quorum sensing
qPCR	Quantitative polymerase chain reaction
Z_w	Warburg resistance

List of Figures

List of Tables

Chapter 1 – Introduction

1.1. General background information: biofilm and bioproducts generation

Microbiologists have classified bacteria as having two types of life in nature over the past years. The microorganisms appear as single, independent, free-floating cells in one of their forms (planktonic). The bacteria are arranged in surface-attached sessile aggregation in the alternative form (biofilms). The term "biofilm" comes from a biomaterial on a surface, and aggregated bacteria have also been separated as surface and non-surface attached (1, 2). The non-surface attached aggregates are termed granules and are commonly observed in wastewater treatment and the ocean.

The microbial species that make up biofilms are encased in an exopolymeric matrix and are connected to one another. These organisms are the main form of microbial biomass on our planet, and they provide a number of ecological benefits include nutrition availability, metabolic cooperation, environmental protection, and the acquisition of emerging properties (2). The majority of them are extremely tough to get rid of and are the cause of numerous stubborn illnesses.

The creation of an extracellular matrix which encases the connected cells is typical of many biofilms. This usually contains water and microbial macromolecules, and it offers the connected cells with a broad range of microenvironments (1, 2). Additionally, the matrix structure and integrity may be thought of as a dynamic environment because they are strongly affected by changes in the macro-environment around them. Exopolysaccharide overproduction is hypothesized to be a result of a series of physiological changes in cells that are brought on by attachment to a surface (3).

These exopolysaccharides aid in both the spatial organization of various species within a biofilm as well as the attachment of cells to the surface that has been colonized. The metabolic and physiological abilities of the biofilm community are provided by these interactions, which are not feasible for the individual, unattached cells. The scope of the effects that biofilms can have on society, which are typically seen as harmful, is sometimes ignored. In fact, the surrounding matrix is often blamed for the persistent and troublesome nature of biofilms. Therefore, it is remarkable that knowledge of the characteristics, production, and morphology of microbial cells and their constituent parts is still insufficient for such a common and significant occurrence, despite 60 years of research (4).

It is important to define some matrix nomenclature at this point. The existence of bioactive extracellular microbial polymers is a crucial characteristic of the matrix. Exopolysaccharides, nucleic acids, peptides, glycoproteins, and phospholipids are some of these extracellular biopolymers. Polysaccharides are among the main components, leading to the coining of the name "glycocalyx" to characterize the gelatinous substance encasing connected cells. The glycocalyx results in a specific structure in eukaryotic cells. This is inaccurate for microbial biofilms, since both the residing cells and the external biopolymers are dynamically changing. Therefore, the term "biofilm matrix" is preferable because it suggests a multicomponent, variance decomposition system (1, 4).

In biofilms, the technique of quorum sensing (QS) enables intracellular communication. The chemical species responsible for this process are species-specific. Fungi and bacteria release a variety of secondary metabolites (SM) (2). These secreted molecules typically have a low molecular weight and numerous biological functions. Although these substances are not essential for the main metabolic processes (such as the production of energy and growth), they are involved in biological processes that are crucial for bacteria' survival in a crowded ecological setting. These secondary metabolites, which play an essential role in biofilm development and growth, can be secreted by various microorganisms into their living environment (3, 5).

Environmental strains of Gram-positive bacteria *Bacillus subtilis* that produce strong floating biofilms, generate an extracellular matrix that was isolated and found to be primarily made of γ-polyglutamate (γ-PGA), a molecule with a molecular mass of approximately 1000 kDa (6, 7). Numerous applications for the highly anionic polymer poly(glutamic acid) (PGA) have been proposed. It is possible to employ the polymer itself, as well as the form of the polymer in hydrogels or nanoparticles. Its molecular mass distribution, the proportion of D- to L-glutamic acids monomers, and, most crucially, the carboxy-group (or) involved in the peptide bond can all be used to define it (7).

Gram-negative microorganisms *Pseudomonas aeruginosa* produce rhamnolipids in a coordinated manner with various virulence-related characteristics. The rhamnolipids that *P. aeruginosa* secretes are biosurfactants or glycolipidic surface-active compounds with potential biotechnological uses (8). As they are involved in various phases of the process of biofilm development, from the first cell-to-surface interplay to maintenance and propagation of the biofilm architecture, it has been increasingly demonstrated that *P. aeruginosa's* production of rhamnolipids actually has an essential role in the founding of the biofilm lifestyle (7, 8).

1.2. Problem statement

Since Costerton's first definition of biofilms was made public in the late 1970s, biofilm technology and science have been an active area of study (9). Today, it is widely accepted that most microorganisms in nature are attached to surfaces and part of an organized biofilm ecosystem, rather than existing as free-floating creatures. The growth of technology and its adaptation to biofilm research, notably innovative imaging systems, biochemical techniques, and molecular environmental biology tools, has significantly altered how biofilms are perceived during the past 40 years. The 3-D microbiological structure can now be seen in its entirety, and it can be studied in greater depth all the way down to the nanoscale (10). The development of effective methods to control harmful biofilms or to enhance and modulate valuable ones (bioproducts like biopolymers and biosurfactants) will be aided by a deeper understanding of the biofilm as a whole and at the level of separate intermediate products secreted within biofilm, as well as how they interact with the surrounding environment. This necessitates a multidisciplinary strategy supported by appropriate techniques.

The platform chosen for biofilm research will define the type of data that can be extracted; thus, it is important to make sure that it will meet the needs of the experiments. Each platform has benefits and drawbacks, however it can be easily noticed that majority of modern techniques used for biofilm and bioproducts characterization are based on chemical and biological testing. On the other hand, there is an arising interest towards the implementation of electrochemical methods in analysis of biofilm and bioproducts since most of them provide non-destructive and real time measurement approaches. Although it may provide valuable insights, one of the most promising electrochemical technique, the advanced Electrochemical Impedance Spectroscopy (EIS) has not been used much in the research of biofilm growth and development. Despite its importance, most research on EIS for microbial electrodes has concentrated on an incorrect or too simplistic use of the technology, and mainly very simple spectra analysis has been successful in determining the internal resistances of an MFC (9, 10). Later, efforts to use it to describe electrode attributes were put forth, but still there are very few attempts made to demonstrate its full potential in characterizing target materials' properties.

With the goal of illuminating the physical significance of some of the characteristics that can be collected, efforts can be undertaken to strengthen the analytical framework for data produced for some microbially produced bioproducts through the EIS technique. It is hoped that the studies on electrochemical analysis of biofilm metabolites via EIS will aid in understanding the composition and characteristics of microbial electrodes. They may also

provide useful methods for analyzing the EIS spectra obtained for the microbial samples like biopolymers and biosurfactants and provide instructions for deriving unique, valuable parameters from the EIS results. It is anticipated a wider and better application and interpretation of EIS in the near future, not just for polyglutamic acid and rhamnolipids, but also for many other types of bioproducts.

1.3. Thesis objectives

The general objective of the given study is to conduct electrochemical analysis on bioproducts generated from the Gram-positive and Gram-negative bacteria via electrochemical spectroscopy technique and validate its practicality in characterization of biofilms and biofilm processes. The study is limited to characterization of extracellular polymeric substance – polyglutamic acid (PGA) secreted from Gram-positive bacteria *Bacillus subtilis* and biosurfactants – rhamnolipids secreted by Gram-negative bacteria *Pseudomonas aeruginosa.*

1.4. Thesis outline

The six chapters that make up this project are briefly detailed below:

In Chapter 2, the essential theoretical background on biofilm formation and development is presented followed by sub-chapters dedicated to bioproducts secretion process within biofilm matrix and more detailed review on polyglutamic acid and rhamnolipids. Chapter 2 is finalized with a brief summary of current methods applied for analysis of bioproducts and an independent sub-chapter that reviews EIS method.

In Chapter 3, the methodology employed for the experimental portion of the investigations is thoroughly described with a closer inspection on EIS technique and its setting properties. The novel approach on estimation of essential electrochemical parameter, effective capacitance, is introduced in the chapter's last section.

In Chapter 4, polyglutamic acid and rhamnolipid samples were dropcast on screen-printed electrode and analysed through electrochemical impedance spectroscopy (EIS), and the outcomes are presented in forms of *Origin-generated* graphical diagrams with the broad description given for each graph. The conclusions drawn from the results obtained using the EIS method are discussed and main results are compared and verified against findings from the similar studies.

In Chapter 6, the possibility of future research based on experimental findings made possible by electrochemical characterization technique is discussed. Main findings of the analysis and conclusions are provided.

Chapter 2 - Literature review

2.1. Introduction to biofilm

A biofilm can be defined as a bacterial community framed in a matrix made up of polysaccharides, proteins, and nucleic acids that the bacteria release. Planktonic or loose bacteria are produced as a result of these organisms growing and eventually sloughing off from this matrix, which provides them with a protected environment. The matrix is a sophisticated, three-dimensional structure with attributes that facilitate the effective distribution of waste and nutrients throughout the biofilm.

The fact that bacteria cells are typically found near surfaces and inter-faces in natural settings (with the relevant exception of oceanic biomass), in the form of multilayered aggregates entrenched in matrices known as biofilms, is now generally acknowledged. The most crucial benefit of biofilms for the cells that make them up is resistance against toxins and antimicrobial invasions. Some characteristics, such as inadequate antibiotic penetration, sluggish or no expansion of biofilm cells, and the presence of small colony variants and persister cells unique to biofilms are hypothesized to contribute to the biofilm defense. Many harmful bacteria in the form of biofilm can cause persistent infections, often fatal for the patients. In addition to a number of other infections and ailments, they are to blame for dental caries, nosocomial infections, and more. In many situations, biofilms are harmful to the industrial sector. Natural biofilms, for instance, can impair heat transmission in cooling towers and heat exchangers, deteriorate reverse osmosis membrane, corrode ferromagnetic materials, and contaminate food processing facilities. Biofilms, which contain cells embedded in a polymeric matrix, exhibit higher frequencies of genetic transformation than planktonic cells and are extremely resistant to antibiotics. Despite the fact that planktonic cells are undetected following antibiotic or chemical treatment, biofilm cells endure and are frequently to blame for recurrent symptoms and treatment failure (11, 12).

2.1.1. Mechanism of biofilm formation

Biofilm goes through about five different phases. Reversible adhesion, irreversible adhesion, biofilm growth, biofilm development, and dispersion are the names for these processes. Initially, a planktonic cell binds to and attaches to a surface. Flagella are used by motile bacteria to approach and grasp surfaces. Following attachment, the bacterium employs adhesins that are strain-specific to stay anchored to the surface and forge a more durable attachment. The bacterium reacts when it encounters the surface, which encourages the

secretion of the biofilm matrix's constituent parts. The bacteria then continue to grow, advancing the biofilm matrix. To protect the cells in this initial stage of biofilm formation, the microorganisms will also release toxins and immunoavoidance substances (1, 2).

In order to foster a suitable environment for the whole colony, the colony actively recruit nutrient and maintains the matrix. The biofilm will expand to the greatest extent that the environment will allow, indicating the colony's maturity. When the biofilm is fully developed, the bacteria within it will disperse and go into a planktonic condition, where they can form new colonies. These bacteria can spread in a variety of ways, including passively as a result of shear force, where they are torn off the biofilm. Because of environmental pressures, bacteria will disperse because of the changes in their environment, such as the presence of toxins or a lack of nutrients. Some bacteria that create biofilms will secrete enzymes that break down the polymers in their biofilm to encourage separation (12). Others create biofilms that degrade over time naturally. All biofilms encourage growth of normally unicellular organisms by establishing an environment in which they can communicate with one another similarly to multicellular organisms. They also protect the colony from of environmental factors, enabling the bacteria to survive even in hostile environments that would normally wipe out planktonic bacteria (13, 14).

2.1.2. The quorum sensing phenomenon in biofilm development

The stages of the biofilm production include initiation, establishment, maintenance, and dissemination. Free-living bacterial cells have the capacity to exist as pseudo-multicellular organisms through QS mechanisms if they reach a certain threshold density, when cell-to-cell communication is established by the release of signaling molecules. The following signals have a variety of consequences on the genetics of bacteria. Changes in gene expression profiles and the resulting phenotypes are the main results of QS, which are known to influence the establishment of biofilms, three-dimansional structure, antimicrobials production, cell migration, and pathogenicity in pathogenic organisms. The QS-mediated functioning of biofilms is influenced by a number of environmental parameters, including pH, nutrition, and concentration of the chemical signals (13, 14).

By tracking the buildup of a particular signaling chemical that community members secrete, QS gives bacteria the ability to detect population density. The buildup of the stimulus in the microenvironment is only adequate to trigger the reaction whenever the density of population is large. AHLs, furanosyl-borate diesters (AI2), unsaturated essential fats (DSF family signals), and amino acids are just a few of the chemical families that QS signal molecules

can be found in. QS signal molecules also have a low molecular weight, to facilitate secretion and uptake by cells (13).

Microorganisms that belong to the Gram-positive and Gram-negative types are known to use a variety of QS chemical messengers. The main elements of these signals include N-acyl homoserine lactone, oligos, furanoses borate, hydroxyl-palmitic acid methyl-esters, and methyl-dodecanoic acids. Diffusible AHLs are used by Gram-negative bacteria to pass through cell membranes and attach to transcription factors in recipient cells. In contrast, peptide-based sensing systems used by Gram-positive bacteria necessitate membrane-bound receptor kinases (14).

2.1.3. Biofilm architecture

Figure 2.1a depicts a biofilm in a picture on a conductive polymer to which it is adhered. A polymeric matrix shields bacterial cells from drugs and prevents some host immunological responses, such as phagocytosis by macrophages. It is crucial to remember that the layer is more than just a slime covering and has a sophisticated architecture with channels that enable the distribution of nutrition to the overall population. The matrix can make up more than 90% of a biofilm's dry matter, whereas the bacteria typically make up less than 10%. The extracellular substance known as the matrix, which the biofilm colonies are embedded in, is primarily created by the organisms themselves (13).

Extracellular polymeric substances (EPS) are a collection of various biopolymers that serve as the scaffolding for the biofilm's three-dimensional pattern and are in charge of the cohesiveness of the biofilm as well as its ability to adhere to surfaces (Figure 2.1b). The development of a biofilm enables a way of life completely distinct from the planktonic condition. The creation of synergistic microconsortia and strong contacts, including cell-cell communication, are made possible by the immobilization and closeness of biofilm cells by EPS. The preservation of extracellular enzymes results in the generation of a flexible external digestive system that sequesters absorbed and granular nutrients from the aqueous phase so they can be used as food and energy sources. By retaining all of the lysed cell's components readily available, the matrix also serves as a recycling facility. Included in this is DNA, which might serve as a gene repository for horizontal gene transfer . It is unknown if the biofilm's cells, especially those that are located the farthest from the surface, receive any ecological advantages from the matrix. A considerable evolutionary advantage for polymer producers over non-producers was seen in simulations of competitiveness in a biofilm, probably due to the fact that polymers force the new cells nearer to environments with high oxygen concentrations (11, 13).

Because there is such a wide variety of matrix biopolymers and it is so challenging to analyze them, EPS has been referred to as "the dark matter of biofilms." Based mostly on microorganisms that are present, the temperature, the extent of nutrient elements, the shearing pressures, EPS can differ significantly between biofilms. The term "extracellular polysaccharides," which had been used to describe EPS, was changed after it was realized that grid additionally includes lipids, fatty acids, proteins, and other biopolymers like sulfates compounds. The matrix can also be stabilized by extracellular microbial structures such flagella, pili, and fimbriae.

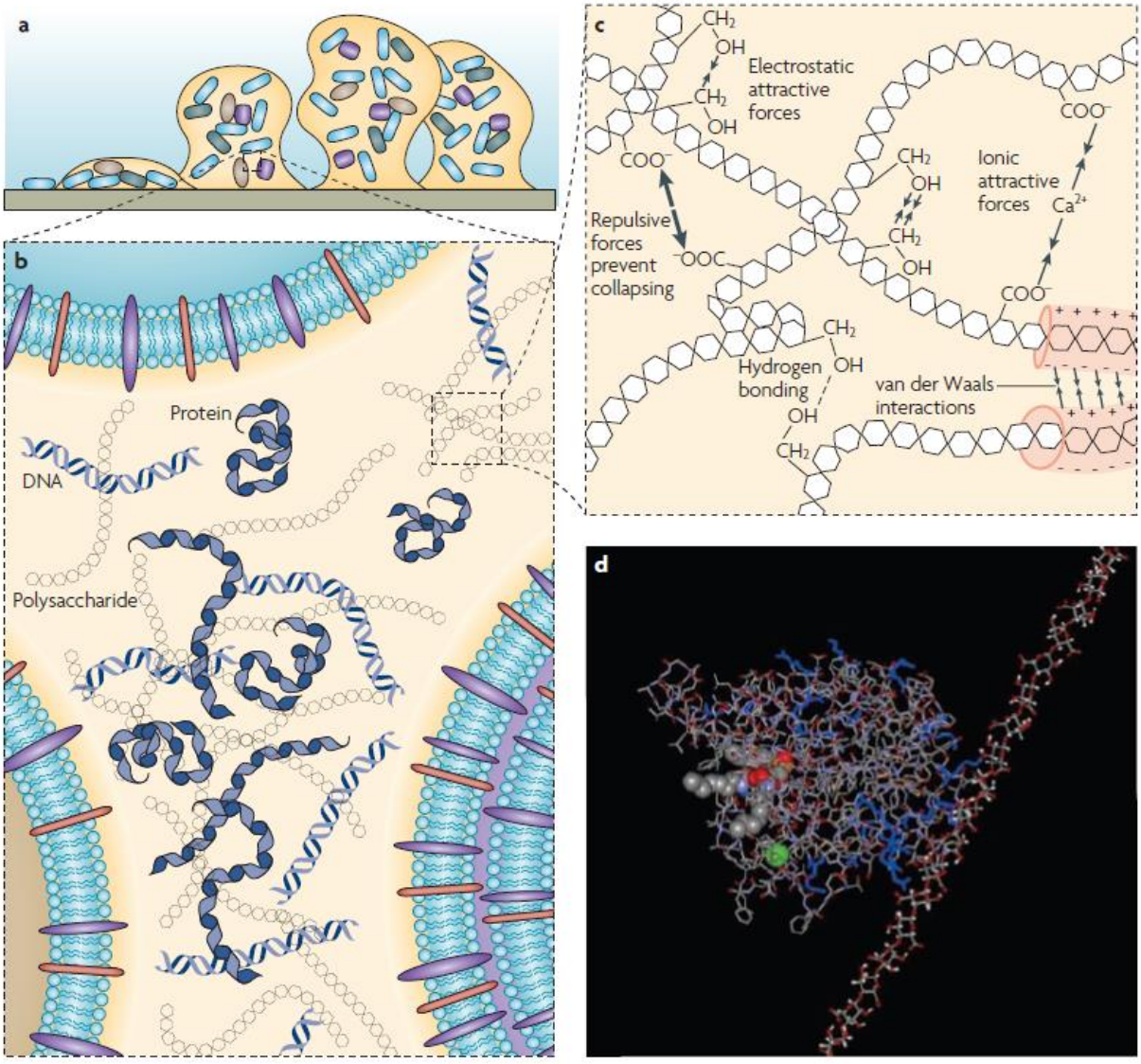

Figure 2. 1. Schematic illustration of a biofilm being developed on a surface: a - A biofilm of bacteria adhered to a solid substrate; b - Exopolysaccharides, proteins, and DNA, which make up the bulk of the matrix; c - The biopolymer entanglement and weak physicochemical interactions; d - A molecular modeling simulation (13).

A number of enzymes and proteins can be transported by virions created from the external membrane of Gram-negative bacteria., modify the properties of the matrix, and occasionally serve as "killer vesicles" meant to eliminate rival bacterial and fungal species (13, 14).

2.2. EPS and its structure

A biofilm's cells are surrounded with EPS, which serves as their immediate environment. Some EPS, especially those that form capsules, are more closely related to surface of cells than others. The generation and amount of EPS are essential in development and upkeep of organized multicellular microbial consortia. The configuration of survival in a specific biofilm is affected by the rate, cohesiveness, charge, adsorption capacities, selectivity, and type of the distinct EPS materials with respect to the three-dimensional arrangement of the composites (the dense areas, crevices, and channels). As a result, biofilm can have a variety of morphologies, including flat and smooth rough, fluffy, or filamentous, as well as different levels of porosity, including macrocolonies that resemble mushrooms that are encircled by water-filled holes. Every one of these morphological configurations have about the similar result: they temporarily keep biofilm cells immobilized and enable the development of long-lasting combined micro consortia with their interconnections, which creates extremely disparate ecosystems on a considerably low scale and promotes biodiversity (11, 13).

Weak biophysical interactions and EPS entanglement play a major role in the biofilm matrix's stability (Figure 2.1 c). Hydrogen bonds, Van der Waals, ionic electrostatic interaction, and electrostatic repulsion are only a few of the cohesive forces that are related to these interactions (preventing collapse) (14).

2.2.1. EPS structure: Exopolysaccharides

The majority of EPS matrix is made up of polysaccharides. The majority have molecular mass of 1-2 × 10^6 Da, and are long, linear or branching molecules (13). Different poly-saccharides have been visualized by electron microscopy as complicated nets made up of small strands connected to the surface of cells. Microscopical methods coupled with carbohydrate staining utilizing fluorescently labeled lectins or immune cells, and also biochemical evaluations for independent confirmation, have shown the occurrence of composite polysaccharides not merely in biofilms from organic sea, freshwater, and soil habitats and even from human-made waterways, as well as in biofilms connected to persistent human infections and also in pure-culture novel biofilms. Exopolysaccharides have recently been identified and described from a wide range of bacterial organisms from various habitats (13, 15).

Exopolysaccharides produced by streptococci in oral biofilms, as well as those made by cellulose-forming bacteria including *Gluconacetobacteria xylinus*, *Agrobacterium tumefacienns,* and several species from the *Enterobacteriaceae* and *Pseudomonadacea*, are all homopolysaccharides. The majority of exopolysaccharides, on the other hand, are heteropolysaccharides made up of a combination of neutral and charged glucose molecules. They may have organic or inorganic substituents, which significantly alter their biological and physical characteristics. Numerous exopolysaccharides are known to include uronic acids, which make them polyanionic. These exopolysaccharides include alginate, xanthan, and colanic acid (15).

2.2.2. Extracellular proteins

On a bulk basis, the protein composition of the biofilm matrix may be significantly higher than the polysaccharide content. This has been mentioned in relation to environmental biofilms, activated sludge, and sewage biofilms. Biofilms have been found to include a variety of extracellular enzymes, most of which are engaged in the breakdown of biopolymers. The substrates for these extracellular enzymes include polymers, such as numerous polysaccharides, peptides, and nucleic acids, as well as water-insoluble materials, such as celluloid, chitosan, and lipids, as well as particles that become enmeshed in biofilms (13).

The matrix functions as an exterior digestive tract that breaks down biopolymers into compounds with a low molar size that can be used as energy sources because it contains enzymes that break down the components of EPS. Additionally, some enzymes may contribute to the breakdown of structural exopolysaccharides in order to facilitate the separation of microorganisms from the biofilms. In the course of infectious processes, several enzymes function as virulence determinants in medical biofilms. Commercially valuable extrinsic proteins from bacteria are manufactured on a huge scale in industrial settings. Extracellular enzymes also engage in similar activities in soils, sediments, and water; similar processes have been employed for the phytoremediation of consumable effluents, with the aid of biofilms and flocs to break down organic compounds (16).

2.2.3. Surfactants and lipids

Proteins and extracellular polysaccharides are all highly hydrophilic molecules, however certain EPS are hydrophobic. One *Rhodococcus sp.* strain, for instance, may cling to Teflon and colonize wax leaf edges utilizing EPS with hydrophobic characteristics. This strain lacks fimbriae but has a capsule. The EPS's hydrophobic nature was related to substituents like methyl and acetyl combination to polysaccharides. The matrix also contains lipids. *Thiobacillus*

ferrooxidans needs lipopolysaccharides to cling to pyrite substrates, and *Serratia marcescens* makes external lipids with surface-active characteristics. Surfactin and emulsan are other surface-active EPS that can distribute repellent substances and making them accessible, hence these compounds might be helpful in oil spill bioremediation and microbially enhanced oil recovery (15-17).

Biosurfactants are crucial for the adhesion and detachment of bacteria from oil droplets and can have antifungal and antibacterial properties. Further research on this kind of molecule may be aided by the search for "green" compounds. Naturally, microorganisms at the air-water interface of rivers and streams produce biosurfactants, which have a significant impact on surface tension and, in turn, the oxygen supply between the seas and the atmosphere. It's interesting to note that the EPS structure of *P. aeruginosa* contains rhamnolipids, which can function as surfactants. They have been hypothesized to have a role in the initial development of microcolonies, enable bacterial motility and the production of spore structures, restrict channel colonization, and contribute to biofilm dispersal (17).

2.2.4. Bioproducts generated in the biofilm matrix

The biofilm growth and development process tend be one of the most intriguing yet not extensively discovered phenomena in microbiology. This process cannot be only limited to generation of a sludgy biofilm matrix responsible for the protection of microbial colony, but also secretion of various bioproducts of a great significance within this matrix (13). For instance, biopolymer, poly-glutamic acid is released into the fermentation environment in *B. subtilis, B. licheniforemis,* and *Natrialba aegyptiaca* to promote the survival of generating strains when subjected to environmental stressors. In the same manner, biosurfactants rhamnolipids are also typically generated by *Pseudomonas aeruginosa* due to the resistive nature of the matrix itself.

2.3. Biopolymer Poly-glutamic acid (PGA) formation

2.3.1. *Bacillus subtilis* PGA

Exploring effective methods to prevent the production of hazardous biofilms and encourage the generation of good biofilm requires a solid perception of the principles behind biofilm development process. Most research studies have been done using bacteria that are clinically relevant since there are stringent regulations to prevent the production of biofilm by harmful bacteria. *Escherichiae coli, P. aeruginosa, Vibrio cholearae, Streptococcus sp., Staphylococcus sp.,* and *Candida spp*. are some of these microorganisms. The basic concerns of how or why bacteria create biofilms may be addressed by great reviews focused on what can

be learned about these clinical pathogens. It has long been known that *B. subtilis* and similar Gram-positive microorganisms are used in industry to produce a variety of secretory enzymes, including amylase, protease, pullulanase, chitinase, xylanase, and lipase, among others that are manufactured on a commercial basis, and they account for around 60% of all industrial enzymes produced commercially. *Bacillus* species have evolved methods of surviving under difficult conditions. Although it has mostly been investigated in a single cell, seed production by *B. subtilis* has traditionally been researched as a framework for the cell distinction of bacteria (18).

B. subtilis, a seed shaped Gram-positive organism, had been developed into a model organism for the investigation of biofilm formation in recent years. Some studies were able to demonstrate that strains of *B. subtilis* descended from the domestic laboratory strain are less likely to generate robust floating biofilms than wild-type strains. The sporulation of the wild strain *B. subtilis* forms highly organized floating biofilms and colonies, with the majority of sporulation taking place at the tip of aerial projections. This demonstrates that the creation of biofilms can play a crucial role in this organism's developmental activities (19). The exopolysaccharide (EPS) generated by the eps locus is a substantial constituent of the extracellular pattern in clusters and floating biofilms for *B. subtilis*. Several wild-type strains of *B. subtilis* may generate colonies and drifting biofilms with remarkably different macroscopic characteristics. The production of floating biofilms in *B. subtilis* is thought to be triggered by oxygen deficiency, and gamma-polyglutamate (γ-PGA) seems to be a significant extracellular polymeric material (18, 19).

Spore production showed hitherto undetected spatial structure when it was examined in the setting of biofilms. An extracellular layout that is primarily made of exo-polysaccharides mediates the secretion and preservation of multicellular clusters in organized microbial communities like biofilms. The exopolymer-producing genes were studies by Stanley and Lazazzera from a natural, exo-polymer positive bacterium to cultivated, exo-polymer negative strains (20). γ -poly-DL-glutamic acid has been identified as an exopolymer which acts as a promoter of biofilm development, potentially via improving cell surface contacts, as a result of mapping these genetic determinants. γ-poly-DL-glutamic acid seems to promote cell-surface contacts, which in turn promotes *B. subtilis* biofilm development. The majority of the γ-PGA produced by *B. subtilis* is released into the media . It is well known that salt ions like Mg^{2+} and Ca^{2+}, can serve as a bridge connecting two negatively charged surfaces. Between both the negative charge γ-PGA and abiotic surfaces as well as between the γ-PGA and the cell surfaces, salt-bridges may develop. When γ-PGA has covered an abiotic interface, there are several locations for the cells to build salt-bridges because the concentration of negative charges on the

γ -PGA may be larger than on the given surface (18, 20).

It is thought that ribosomes are not required for the synthesis of PGA. The majority of multienzyme systems work on the basis of the thiotemplate mechanism, which typically provides strong stereoselectivity for amino acid precursor and productivity of relatively short peptides with tightly regulated amino acid configurations. In bacteria, PGA is produced in two phases during biosynthesis. L- and D-glutamic acids are created in the first stage, and then these two amino acid units are combined to generate PGA in the second (21, 22).

2.3.2. Introduction to γ-PGA

Multiple *Bacillus* species naturally create poly-γ-glutamic acid, which can be defined as an extracellular type of polymers consisting of D and L type glutamate elements. Polyglutamic acid is usually not toxic to people and the environment, edible, and biodegradable. As a result, it has been recommended as a strong contender for a number of industrial usages: thickener, medicine carrier, disposable fibers, water-absorbent hydrogel compounds, and heavy metal absorber. The following microorganisms can be defined as a one of the most famous bacteria generating PGA: *B. subtilis* and *B. licheniformis*. PGA is typically released by the abovementioned bacteria to improve survival when subjected to environmental stress (21, 23).

Ivanovic and his coworkers first recognized PGA as a capsule-like substance secreted by *Bacillus anthraceis*, Gram-positive microbe that activates bacteria and releases its contents into the medium when autoclaved, in the 1970s. It has been recorded that the main ingredient in the gluey Natto, one of the well-known food in Japan, is PGA. For more than a thousand of years, people have used and consumed natto, which is made by heating tiny beans and culturing them with a bacterial strain of *B.subtilis* (22).

Figure 2.2 demonstrates the structure of each glutamine unit of PGA, a biopolymer that is present in significant amounts. Because glutamate is quickly produced by a number of microbial organisms and is polymerised via an amide bond, this anionic amino has an unusual feature. The homopolymers of D- and L-glutamate (D-PGA and L-PGA, respectively), as well as the co-polymers of D- and L-glutamate (D-L-PGA), were found to be active stereo-chemicals (23). The environment under which PGA is produced, including the pH and solution temperature, can affect the structure of the compound. In accordance with the level of polymerization and the availability of other ions and molecules in the solution, PGA can exist in a variety of shapes, along with a linear chain, a branching arrangement, and a cross-linked network. (21, 22).

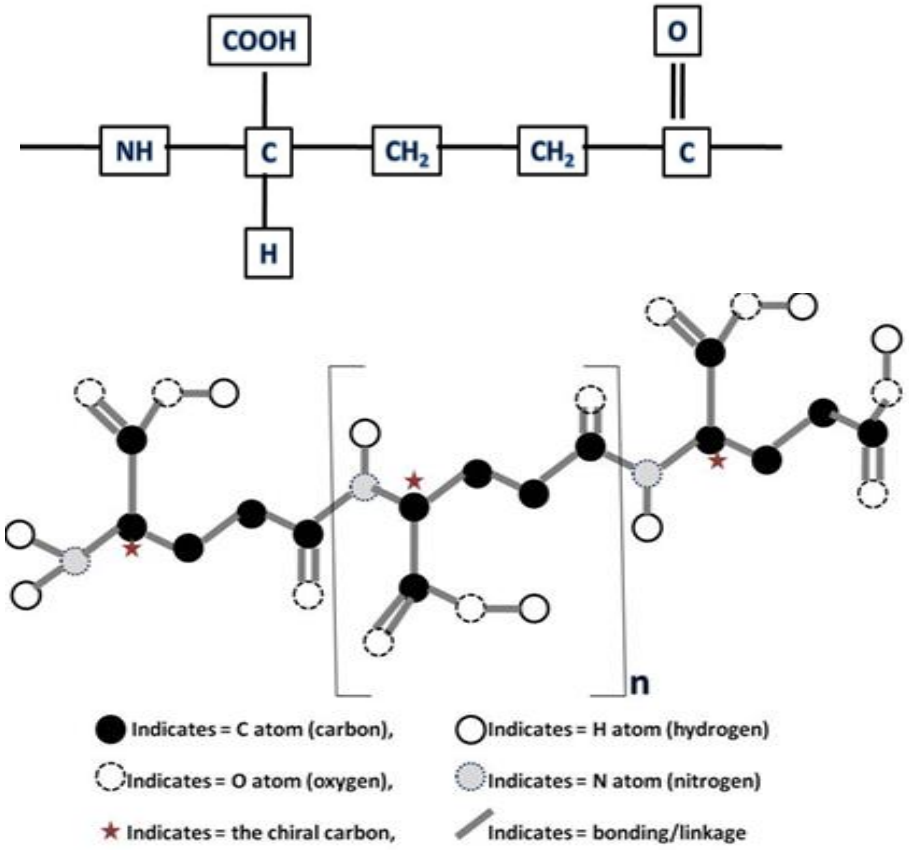

Figure 2. 2. Representation of poly-glutamic acid structure (6).

2.3.3. PGA: Physical and chemical properties

The anionic polymer γ-PGA is water-soluble, but its un-ionized form (H+) is not. In contrast, γ-polyglutamates' salt forms, such as K^+, NH_4^+, Ca^{2+} are fully soluble in water. It can be utilized as biological glues because it is fully biodegradable and harmless to people. It has similar hygroscopic and moisturizing qualities like hyaluronic acid and functions as a natural humectant (HA). It is employed as an absorbent molecule because of its extensive adsorbing capabilities. The final molecular mass of PGA depends on a variety of variables, such as the creation of enzyme that catalyzing the hydrolytic degradation of PGA, which will result in a drop in molecular weight with longer fermentation times. The additional elements that influence the fermentation-produced PGA's molecular weight are pH, oxygenation, agitation, ionic strength, and others. The PGA's dynamic molecular size allows for a multitude of modifications, which expands the spectrum of possible applications. Sizes of B. Subtilis PGA filaments have been seen to range from 160 to 1500 KDa (23).

Polyglutamate can take on a wide variety of patterns. By triggering the peptide of 10 to 20 glutamate molecules, the architecture of PGA has also been anticipated and postulated. It

has been reported that the conformational model in an aqueous solution is a left-handed complex that is supported by intra-molecular hydrogen bonds. Additionally, other investigation into PGA, which was isolated from *B. licheniformis*, revealed that the conformation of the compound is flexible and is related to PGA concentration along with the pH of the solution. When the pH is below 7.0 and the concentration is low (0.1% w/v), PGA adopts a shape that is mostly based on -helices, whereas at pH levels over 7.0, a -sheet-based conformation predominates (21-23).

2.3.4. PGA functions

PGA is produced by a large number of microorganisms for a variety of purposes. Several of the features include one of the most frequent pathogens responsible for infectious diseases throughout the past few decades, *S. epidermidis*, which is frequently resistant to medication. Poly-glutamic acid aids in host defense and contributes to the inoculation of PGA generating Bacteria. Recent research has shown that the given organism circumvents the human immune system by producing a unique exopolysaccharide known as PIA, or polysaccharide intracellular adhesions. It should be noticed that only a small portion of the microorganism is subject to PIA. The resistance feature of these bacteria should therefore be supported by a larger mechanism (20).

It has been established that *S. epidermidis'* cap locus produces the PGA, which gives the bacteria resistance to the host's immunological defense system. It was demonstrated that cap mutant strains tend to be more susceptible to antibacterial oligopeptides: LL-37 and defensing molecules. Additionally, these compounds demonstrated enhanced neutrophil phagocytosis in people, indicating that PGA contributes to the pathogenicity of this organism. It has also been discovered that PGA makes it easier for *S. epidermidis* to survive in the host (23).

The definition of a biofilm is an organized community of bacteria population contained in a matrix material either on biotic or abiotic substrates. PGA aids in the biofilm manufacturing in *Bacillus subtilis*. Exopolysaccharide (EPS) is a crucial part of the extracellular environment in *B. subtilis* 3610 wild types. However, in certain *B. subtilis* strains, PGA is detected to earn a substantial role in the growth and further biofilm development. Several studies showed that in the case of no PGA formation the strain could not create biofilm but produced very little of it in the best-case scenario (20, 23).

2.3.5. PGA production: fermentation and culture conditions

There are two methods for producing glutamic acid, namely solid-state and submerged type fermentation. At most a small number of strains are employed in solid-state fermentation

(SSF) to manufacture PGA, including *Bacillus s*. CCTCT-202048, *Bacillus s.* B6-L, *Bacillus s.* ME-714, and *Bacillus l.* NCLM-2324. Soybean cake mixture, soy protein, wheat, dairy, and swine manure have all been used either separately or in combination for the production of PGA via solid substances fermentation (21, 23).

The majority of reports on the manufacture of PGA use submerged fermentation. Submerged fermentation (SmF) has been used to produce PGA in a number of ways, However, because of barriers on quantitative oxygen molecular diffusion that hinder proper cell growth, a considerable rise in media viscosity that results in uncontrollable foaming, and a loss in PGA yield, the technology behind it is still difficult to implement. Additionally, the relatively greater cost of SmF- medium prevents PGA from being considered a widely used and lucrative commercial product. On the other hand, the Solid-State Fermentation is a method in which bacteria develop on or inside of substrates or supports without or almost without free water, efficiently excreting desired products (19-21).

This conventional method has become widely known and is the researchers' preferred method because of the fact that the less energy is needed, ease of cultivating equipment along with the considerably high productivity. This fermentation process is used to produce a wide range of industrially produced goods, such as biofuel, antibiotics, proteins, alkaloids, amino acids, regulators of crop growth, pathogenic microbes for the decontamination of industrial residues, and biopharma products. Out of the many generic products available, these are only a few. SSF is a stable, and energy-efficient process that only needs small fermenters, can be used in lower quantities, and produces less harmful effluent (23).

When comparing Submerged Liquid and Solid-State Fermentation methods, the latter technology offers higher productivity and has a wide range of other benefits. While other microorganisms may also be used, the SSF approach primarily uses fungi to grow on solid surfaces that contain moisture but lack water. Cerea-based substrates are still number one selection for fermentation by SSF, along with unconventional substrates such waste from agricultural sources, woodlands, and food processing establishments (19, 22).

PGA synthesis media demands and host factors vary depending on the microorganism strains utilized. The PGA-producing bacterium might be divided into two distinct groups based on the nutrient needs: those that depend on glutamic acid and those that do not. L-glutamic acid is utilized in the production of -Poly-glutamic acid as a precursor and transcription factor; it also interacts with other medium components. The concentration of this component must be optimized because it is a relatively expensive component and significantly influences PGA production (21-23).

In most cases, around 20 g/L of the acid was used to produce PGA. Additionally, a number of additional elements like carbon and nitrogen supplies, medium pH, oxygenation, vibration, and ionic strength, in addition to glutamic acid, had an impact on the production and quality of PGA. Citric acid is also used in the majority of fermentation processes for producing PGA together with glutamic acid. According to a report, the supplied acid is converted by the citric acid cycle into ketoglutarates and isocitrates, which are then converted into glutamic acid and PGA (23).

The decision of raw material for PGA formation lies on the strain. Glucose is consumed more promptly and provides a better carbon supply for cell growth when opposed to glycerol, citric, or glutamine. Yet, in most strains, increased glucose concentrations can lead to the synthesis of polysaccharide rather than PGAs. The efficiency of γ-PGA synthesis was improved by combining glycerol and glucose or even supplementary complex carbohydrates in moderate mixtures. Among the saccharides such as glucose, fructose, maltose and especially glucose can be considered the preferred choice (23).

2.4. Biosurfactant rhamnolipids production

2.4.1. *Pseudomonas aeruginosa* rhamnolipids

The petroleum, pharmaceutical, cosmetic, and food sectors all utilize surfactants and emulsifiers extensively. Only recently have surface-active molecules with biological origins been described; the majority of these substances are chemically produced. Considering that biosurfactants are currently easily biodegradable and may be made from less expensive, renewable sources, they may be able to take the place of chemically created counterparts. A rhamnose compound and a chain of fatty acids make up the two primary parts of rhamnolipids. The fats chain is a substantial hydrocarbon chain, while the rhamnose unit is a particular kind of sugar. Rhamnolipids have special characteristics that result from the interaction of these two elements. Rhamnolipids' capacity to reduce liquid surface tension is among its most significant qualities.

The *Pseudomonas* rhamnose-containing glycolipids are a varied group of biosurfactants. Rhamnolipid is a biosurfactant that *P. aeruginosa* produces during the fermentation process (24-26). *P. aeruginosa* can be defined as a human pathogen with a great potential that can cause significant nosocomial infections. It is first and foremost a creature that lives in the environment and may be recovered from a range of environments, including freshwater, sediment, and plants.

In 1949 Jarvis and Johnson first identified the biosurfactant rhamnolipids, which are

amphiphilic biomolecules with a hydrophobic fatty acid moiety and a hydrophilic portion comprised of one or two rhamnose, to be produced by this bacterium. Additionally, the bacteria release 3-(3-hydroxy-alkanoyloxy) alkanoic acids, which are tensio-active precursors of rhamnolipid anabolism but lack the sugar moiety.

While *P. aeruginosa* is known for producing rhamnolipids, several isolates of the pathogen *Burkholderia pseudomallei* and the non-pathogenic pseudomonades *P. putida* and *P. chlororaphis* have also recently been found to produce a range of rhamnolipids. Rhamnolipid has been made using a variety of carbon sources, including ethanol, glucose, vegetable oil, and hydrocarbons. In comparison to synthetic surfactants, microbial surfactants are typically less harmful and more biodegradable. *Pseudomonas aeruginosa*-produced rhamnolipid biosurfactants have a lot of potential for industrial use and bioremediation (26, 27).

As it can be seen from Figure 2.3, rhamnolipids can be usually made up of a complex of 3-hydroxy-fatty acids connected to mono- or di-rhamnose molecule by a β -glycosidical linkage. On the other hand, cells that are dormant or that use naphthalene as a carbon source contain biosurfactant with a singular chain of lipids. It is unclear if RhlB can metabolize 3-hydroxyfatty acid monomers or if the smaller rhamnolipids are a byproduct of the breakdown of rhamnolipids with two fatty acids (26).

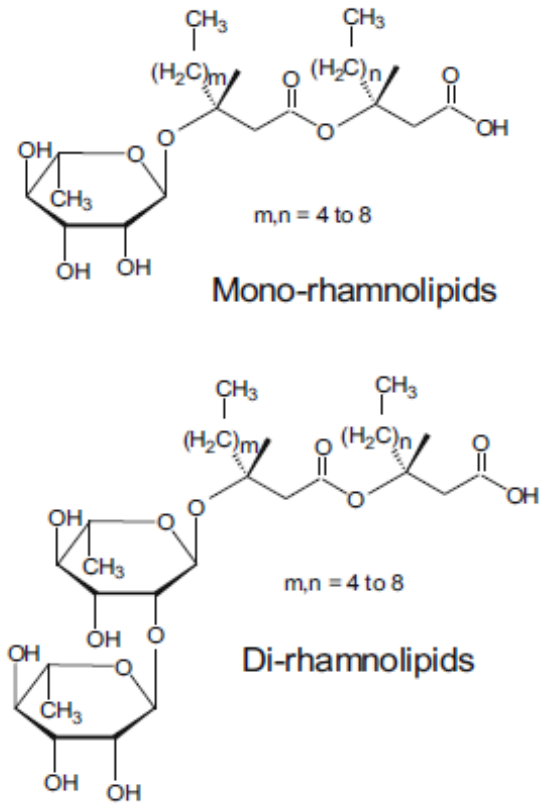

Figure 2. 3. Representation of chemical structure of rhamnolipids (7).

2.4.2. Rhamnolipids production

Rhamnolipids are usually secreted by *P. aeruginosa* by three successive processes. RhlA appears to be loosely binded to the inside membrane and is engaged in the formation of the fatty acids di component of rhamnolipids and unbound HAAs, as explained further below. The subsequent reaction, which takes dTDP-L-rhamnoses and HAAs as precursors and is catalyzed by the lattice RhlB rhamnosyltransferase, results in mono-rhamnolipids. RhlC uses these substances as substrates to create di-rhamnolipids in the presence of dTDP-L-rhamnoses. Although Rhl-C is specialized in the di-rhamnolipid synthesis, it shares sequence similarity with rhamnosyltransferases involved in LPS synthesis (24).

Rhamnolipids are referred to as "secondary metabolites," and as such, the start of the solid phase is when they are produced. This is consistent with the notion that translation from the rhlAB promoters is predominantly controlled by QS in a cell intensity manner, as was previously established. Rhamnolipid production, however, also needs ideal development circumstances. These culture variables have been thoroughly studied, mostly because to their economic and biotechnological relevance as potential substitutes for synthetic surfactants (25). Rhamnolipids have undergone substantial study, however it is still unclear exactly how they work in nature. In reality, they appear to play several parts. First, these compounds are regarded surfactants due to their strong surface tension-reducing and emulsification properties. Consequently, they can be generally researched for the capacity to solubilise and encourage the absorption of hydrophobic compounds, particularly hydrocarbons like alkanes. Rhamnolipids also increase the microbial degradation of weakly soluble compounds by increasing the hydrophobicity of the cell surface. Rhamnolipids can also be synthesized effectively when grown on soluble substrates, hence it seems unclear that their primary purpose is to aid in the uptake of insoluble substrates (26, 27). With *P. aeruginosa* being a highly effective and widespread bacteria, another ecological function for these active at the surface chemicals pertains to their toxic modification against a range of microbes that may offer an edge in the race to colonize niches. Rhamnolipids have some Gram-negative and primarily Gram-positive bacterial antibacterial action (26, 27). Rhamnolipids are thought of as virulence-associated exoproducts since QS that can be considered as a mechanism responsible for the secretion of most virulence components in *P. aeruginosa*, regulates rhamnolipid synthesis. Rhamnolipids, on the other hand, are undoubtedly one of the virulence factors produced by this bacterium that are least understood. They engage in a variety of biological and chemical processes, most of which are related to their abrasive qualities. Rhamnolipids were initially thought to play a role

in pathogenesis because they were shown to be the heat-stable hemolysins of *P. aeruginosa* (27).

2.4.3. Functions of rhamnolipids

Rhamnolipids have undergone substantial study, however it is still unclear exactly how they work in nature. In reality, they appear to play several parts. First, these compounds are regarded surfactants due to their strong surface tension-reducing and emulsification properties. A, they have mostly been researched for their capacity to solubilize and encourage the absorption of hydrophobic substrates, particularly hydrocarbons like n-alkanes. Rhamnolipids also increase the bioremediation of poorly soluble compounds by increasing the hydrophobicity of the cell surface. Rhamnolipids can also be synthesized effectively when grown on soluble substrates, hence it seems unclear that their primary purpose is to aid in the incorporation of insoluble substrates (24).

In subsequent studies, it was discovered that rhamnolipids had a range of impacts on mammalian cells, including disturbance of mononuclear cells leukocyte chemotaxis responses, downregulation of regular macrophage characteristic, stimulation of the secretion of cytokines from epithelia, interference with normal ciliary function, inhibition of the feasible fimbriae of tracheal epithelium, decelerating of the biological ciliary beat rate, and mucu (26, 27).

PQS is required for the controlling the expression of genetic loci in *P. aeruginosa* pathogenicity. Rhamnolipids were also discovered in the saliva of patients with cystic fibrosis who seemed to have *P. aeruginosa* as just a colonizer, despite the fact that all of these findings were predicated on in vitro studies. However, there hasn't yet been any in vivo proof of the significance of rhamnolipids' role in pathogenesis (25). Swarming motility has recently been clearly shown to require rhamnolipids and HAAs, and it has been suggested that this multicellular activity is connected to the formation of biofilms. In fact, there is growing evidence that rhamnolipids play a crucial part in how biofilm architecture normally develops (24).

2.5. Analysis of EPS components – generated bioproducts

Polysaccharidic compounds, e-DNA, and proteins released by the cells inside the biofilm during the formation and maintenance of the biofilm make up the majority of EPS. However, it's not always easy to tell which released molecules belong to the cellular membrane and which ones make up the biofilm matrix. In actuality, deposited polysaccharides and complex enzymes cohere between lattice molecules as well as the biofilm matrix, making it difficult to separate and purify EPS from cellular components. The basis of the examination of

the biofilm is their interaction. Ex-situ and in-situ approaches can be used to characterize and quantify the proteins and carbohydrates that make up EPS (28).

Ex situ analyses heavily rely on the extraction techniques. In EPS extraction procedures, physical methods like ultrasonic, blending or high-speed centrifugation, heating, heating, cation exchange resins, or irradiation are used, as well as chemical agents like ethanol, methanol, esters, NaOH, EDTA, or glyoxal. Since it relies on the species makeup of the biofilm and the complexity of the EPS, there is no agreement on the optimum methodology to employ. In comparison to only physical approaches, chemical agents are generally used in extractions to boost EPS yields. For instance, EPS per g of dry biofilm was 7.2 and 12.7 mg when extracted with centrifugation or ultrasonic, whereas 164.5 and 114.7 mg were obtained when extracted with ethylenediaminetetraacetic acid (EDTA) or EDTA-formaldehyde (28, 29).

In most cases, depending on the biofilms, the extraction process needs to be optimized. To guarantee extraction of enriched proportions of EPS having a minimal contamination from intracellular content, testing a mixture of mechanical procedures linked to active ingredients in conjunction with tests at various ionic strengths and exposure duration is necessary. The scientific question that needs to be answered will also influence the best extraction process. For instance, extraction by chemical agents or chelating agent resin may have an impact on the attachment of ion by the EPS because the ion strength may change the creation of metal ion complexes. In this situation, it is not advised to extract EPS using chemical reagents (28, 29).

The carbohydrate fraction of extracted EPS is typically purified and reduced by ethanol precipitation, while the protein fraction is typically generated by trichloroacetic acids (TCA) precipitation. Then, protein diversification could be investigated using proteomics techniques, and carbohydrates could be defined using a variety of analytical methods from hydrolysed polysaccharides to produce a carbohydrates fingerprint of biofilm-based EPS. In situ analyses of the EPS biofilm have evolved with the development of microscopy equipment. The imaging of EPS using 3d digital microscopy disclosed the fundamental structure of the biofilm, as well as the dispersion of the EPS within the biofilm and the activities of the EPS during adhesion, development, maturation, dispersal, and hypercolonization of the surface. EPS aid in the three-dimensional organization of biofilms. CLSM is the method of preference for EPS dispersion and in situ characterization assessments since it is non-intrusive (29).

Fluorescence lectin binding analysis or FLBA, is another method that determines the biofilm's glycoconjugate spread, could be used to identify the EPS carbohydrates. The sensitivity of the lectins utilized determines how to characterize the EPS carbohydrate using FLBA (28, 29).

Using typical fluorescent dye sensors that were detected using fluorescence correlation spectroscopy (FCS) for the structural system of the biofilm, the fluidity and permeability of the biofilm could be assessed. FLBA and fluorescence labeled probe studies must be integrated with CLSM analyses and specific cell fluorescent probes to create a 3D model of the dispersion of the EPS in line with cell localization (28, 29).

Imaging mass spectrometry (IMS) is lately becoming a novel method for examining the components of the biofilm matrix. These imaging approaches allow 2-D monitoring of the distribution of several components (such as metabolite, interfacial lipids, and enzymes) directly from biological matter, such as biofilms, without the requirement for chemical identification or antibodies (30).

There are also numerous standard test procedures widely applied for characterization of biofilm and its components, and majority of them use chemical and biological testing (Crystal Violet (CV) staining, colorimetric techniques, quantitative polymerase chain reaction (qPCR), etc.) (31). However, none of the abovementioned methods provide information about electrochemical parameters of biofilm components and bioproducts. With the knowledge of electrochemical parameters of the bioproducts, one could get a better understanding of bioproducts formation and further development processes and many more, since electron transfer and electrical charge are the essential parts of the biofilm development, especially, in the quorum sensing phenomena. The most significant area of analytical chemistry is electroanalytical methods, which identify the kind and quantity of a particular analyte in an electrolytic system. At the electrode interface, electrochemical characteristics are measured, and the link between the intensity of the feature being evaluated and the amount of a certain chemical species is shown by this measurement. Electroanalytical techniques are more suitable for quick and precise detection since they are cheaper and easier to miniaturize than other analytical techniques, such as chromatography or spectroscopy.

One of the most promising electrochemical methods that can be applied in microbiology is Electrochemical Impedance Spectroscopy (EIS) that measures a circuit's impedance in Ohms. EIS brings many benefits due to its consistent nature, utilization limited signal analysis, and capability to investigate transmission relaxations across an extremely broad range of resonant load, with less than 1 mHz to even more than 1 MHz, utilizing readily available electrolytic working terminals (potentiostat) (31).

2.6. Implementation of EIS in bioproducts characterization

2.6.1. Working principle of EIS

A method for examining the electrical properties of substances and systems in electrochemical processes is electrochemical impedance spectroscopy (EIS). Impedance, or the resistance of a substance or system towards the transmission of an electrical current, is measured in this process. EIS operates by providing a small amplitude AC signal to a system and measuring the system's response in terms of current flow over a frequency range, generally from mHz to mHz. A frequency response spectrum with a real (resistive) and an imaginary (reactive) component is then displayed from the measured impedance (31, 32).

The real element of the impedance spectra reflects the resistance to flow of current resulting from the system's physical characteristics, such as the electrodes' resistance or the electrolyte solution's conductivity. The capacitance or conductance of the unit, which is associated with the energy storing and transferring capabilities of the system, is represented by the fictitious portion of the impedance spectrum (31, 32). The function of electrode, galvanic cells, and corrosive processes are all commonly studied using EIS in electrochemistry. The kinetics and principles of electrochemical processes, the characteristics of particle surface, and the influence of environmental conditions on electrochemical processes can all be revealed.

In a typical electrochemical cell, interactions between substance (redox species) and the electrodes also entail the concentrations of ionic species, energy transfer, and mass-transfer out from bulk fluid to the electrode surface, in addition to the resistance of the electrolytes. An electrical circuit that consists of capacitors, constant potential components, or resistances placed in series or parallel to form a circuit model can be used to represent each of these qualities. Consequently, processes like mass transport, charge carrier, and diffusion process might be studied using the EIS. As a result, the EIS provides the ability to investigate inherent material properties or specific processes that may have an impact on the conductivity, resistivity, or capacitive of an electrochemical system. Resistance in Electric circuits directly adheres to Ohm's Law, which distinguishes it from impedance. The electrochemical cell response is pseudo-linear in where a transition is acquired, although the response to a sinusoidal waveform is a sinewave at the operating frequency (31-33). As a result, Equation 1 illustrates the excitation signal with relation to time:

$$E_t = E_0 \cdot \sin(wt) \qquad \text{(Eq.1)}$$

, where E_t – potential at the given time (t), E_0 – signal amplitude, ω – frequency of the radian.

The relation between the frequency of the radial and applied frequency can be evaluated as follows:

$$w = 2 \cdot \pi \cdot f \quad \text{(Eq.2)}$$

The signal in the linear system is different in amplitude and has a phase shift (Φ):

$$I_t = I_0 \sin(wt + \Phi) \quad \text{(Eq.3)}$$

Consequently, Equation 4 can be used to determine the impedance of an entire system:

$$Z = \frac{E}{I} = Z_0 \exp(i\Phi) = Z_0(cos\Phi + isin\Phi) \quad \text{(Eq.4)}$$

The versatile technique of electrochemical impedance spectroscopy (EIS) is used to investigate bio-electrochemical processes and biofilm electrodes. Biofilm impedance and biofilm capacitance, which cannot be measured directly using current, are examples of features of biofilm electrodes that can be examined using EIS. Because of misunderstandings or a lack of data validation, EIS in microbiological electrochemistry is occasionally employed superficially or examination of supplied data is not comprehensive. This prevents a wider implementation of this technology, both practically and for the measurement of particular biofilm electrode characteristics (30, 31). Capturing the current response (I_t) to a sinusoidal voltage perturbation stimulus (E_t) is the foundation of EIS. Scanning E_t at steady ΔE under a frequency band, typically in the mHz to MHz span for biofilm electrodes, yields the impedance spectrum. To visualize and analyze EIS data, two distinct types of charts are frequently employed. The Bode plot displays the absolute magnitude of the impedance *Z*, also known as modulus, and the phase transition f at two separate Yaxes over the log10 of the applied frequencies. The Nyquist Plot displays the imaginary part $(Z_{lm} = i|Z|sin\varphi)$ over the real part $(Z_{Re} = i|Z|cos\varphi)$ of the impedance (Figure 2.4) (31).

An electric circuit (or equivalent circuit) comprising electrical components is used to modelling and computation of the electro-chemical activities linked to the electrolyte/interface and reduction-oxidation processes (resistors, capacitors, inductors). To comprehend and assess the distinct units of the EIS system, this equivalent circuit has been created and put into use. The Randles equivalent circuits, simplify the Warburg resistance (Z_w), charge transfer resistance (R_{ct}), resistance of the solution (R_s), and double-layer capacitance at the electrode surface (C_{dl}). Warburg resistance is created by a diffusion process at the electrode-electrolyte interface. Therefore, a constant phase component (CPE) is used to model or reproduce this non-ideal capacitive activity as the ideal capacitor is often not present in studies (31-33). Following the Nyquist shape, the components of the respective circuit are identified and connected using Nyquist plots. Because of this, obtaining the EIS curve is the most crucial first step, after which

surface properties are assessed by fitting the electric circuits simulations (Figure 2.4). The composition of the electrode material, or electrode matrix, as well as the chemical oxidation occurring at the working electrode's surface or in the bulk of the solution, all affect the form of a Nyquist plot. As a result, several Nyquist plot curves may be produced; for example, one, two, or two half-semicircles might be formed for a particular electrochemical procedure (32).

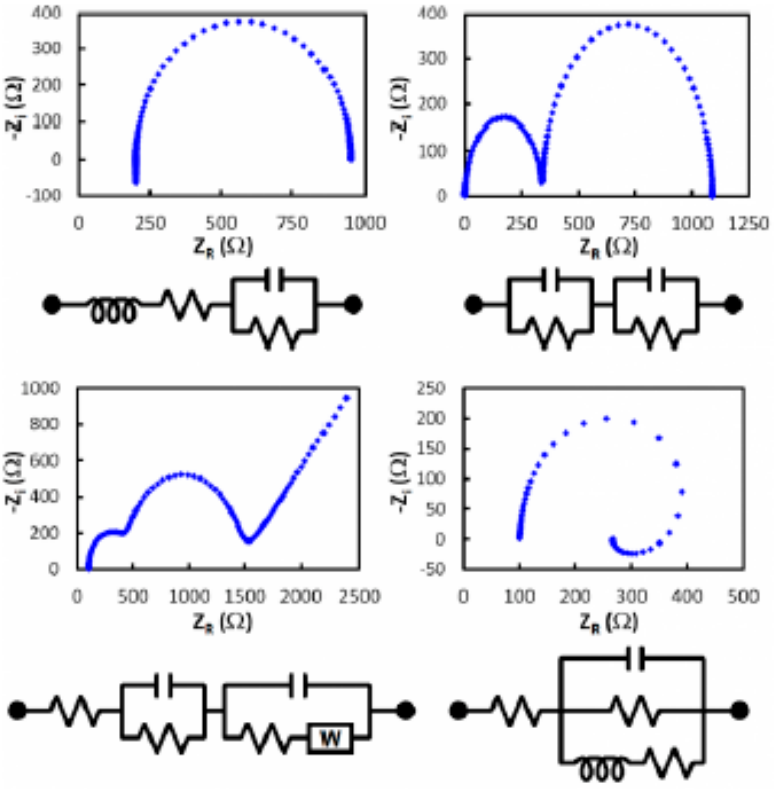

Figure 2. 4. Nyquist plot examples and their corresponding equivalent circuits (31).

2.6.2. EIS for microbial electrodes characterization

When examining the electrical characteristics of metals exposed to aquatic environments, electrochemical impedance spectroscopy (EIS) is thought to be a potent technique. This method can be used to study surface processes that take place at the interface in greater detail, like charge transfer process via a conducting electrode, sorption, dissolving, and the dispersion of charged species. EIS can be used to investigate and measure layers of oxides and other corrosion products that form at the boundary between a metal and a solution and have strong, non-ideal capacitive characteristics as well as faradaic resistance. Localized corrosion, changes in corrosion rate, corrosion inhibition (MICI), and alterations in the nature and composition of corrosion products can all be caused by microbially-induced electrochemical changes. Due to the metabolism of microorganisms, the corrosion mechanism will also be more complicated in their presence. Understanding the involvement of microbes

can be aided by electrochemical factors identified by EIS and approximated through an equivalent electric circuit (EEC). However, explaining impedance curves is not simple since they are the product of various physical processes that might each contribute in a different way to an identical electric output. Therefore, knowledge of a system's physical properties and competence can help prevent drawing assumptions about material behavior that are hypothetical (33).

The involvement of biologically produced substances, particularly enzymes, will complicate the interface between the solid and the solution in microbial environments. These metabolites can change the surface's electrochemical performance in a number of ways, including its propensity to accept or release electrons and the rate at which corrosion processes occur (34). Extracellular polymeric substances (EPS) are made up of organic polymers released by microorganisms and give biofilms their structural and functional integrity. Based on the progression of the microbial communities and the durability of the substrate, the creation of biofilms will lead to a development of the interface and mass transport phenomena, fostering novel equilibria that may survive for a while. This evolution may lead to modification of the impedance signaling (32).

Additionally, due to deficiencies and non-ideal activity, bioproducts within the EPS matrix frequently display various reactance and pseudo-capacitive qualities. In order to describe their response, constant phase elements (CPEs) instead of pure capacitance might be used (35). Knowing the characteristics of time-constant dispersion, which correspond to a given variable in the system under study, is necessary for the understanding of CPE variables in terms of physically feasible features. For instance, the impedance of a constant phase element can be written as follows (35):

$$Z(CPE) = Y_0^{-1}(j\omega)^{-n} \quad \text{(Eq.5)}$$

, where Y_0 can be considered a capacitance of a CPE. When experimental capacitance measurements are to be used to identify system parameters quantitatively, conversion of this CPE parameter data into capacitance is crucial. The conversion of CPE into a qualitative parameter is widely practiced when analyzing heterogenous systems such as EPS matrix components, and it can also be attempted to characterize microbial samples of biopolymers and biosurfactants using the abovementioned CPE conversion practices.

Chapter 3 – Methodology

3.1. Bacterial strains growth

The two microbial species were cultivated using sterile, nutrient-rich broth and kept in agar cambers at a constant temperature of about 5 °C are *P. aeruginosa* and *B. subtilis*. The culture medium contained necessary nutrients like glucose, yeast extract, glutamic acid (for production of PGA): 0.17 M sucrose, 0.3 M L-glutamic acid, 0.1 M $(NH_4)_2SO_4$, 3 mM K_2HPO_4, 2 mM $MgSO_4 \cdot 7H_2O$, 0.15 mM $FeSO_4 \cdot 6H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 0.7 mM $MnCl_2 \cdot H_2O$, 9 mM NaCl, 1% skimmed milk (and 1mM IPTG when applicable). An orbital mixer was utilized to maintain the cultured strains at a specific temperature of roughly 35-37°C while the strain's growth was carried out inside the flasks by using inoculation method.

3.2. Sample extraction

3.2.1. PGA samples extraction

By cultivating a bacterial culture by applying a Tryptone glucose yeast extracts (TGY) medium, the growth and development of the biofilm was achieved. For the accumulation of biomass and/or bioproducts as well as subsequent management, TGY medium is frequently used in microbiology. The EPS was removed from the biofilm by first centrifuging the biomass under certain circumstances, and afterwards suspending the condensed biofilm in a solution with 80% sodium chloride and around 20% formalin (w/w). Centrifuging the dispersed EPS for extraction represented the final and last step. The list of PGA samples extracted for the EIS analysis are given in Table 3.2 with their growth conditions.

3.2.2. Rhamnolipids extraction

Similar to EPS, biosurfactant extraction involved first isolating the *Pseudomonas aeruginosa* strain, which was then cultivated for around ten days at a temperature of 35-37°C in the nutrient-rich medium. After the medium had been sterilized, biomass was produced by using inoculation technique, and it proceeded through a fermentation process based on carbon sources. Finally, centrifugation for 20 to 30 minutes was used to extract biosurfactants. The list of rhamnolipid samples extracted for the EIS analysis are given in Table 3.3 with their growth conditions.

3.3. Electrochemical Impedance Spectroscopy

On *Bacillus subtilis* PGA samples and *Pseudomonas aeruginosa* rhamnolipids grown under various conditions, the electrochemical measurements (EIS) were carried out utilizing

three electrode-based screen-printed carbon electrodes (Metrohm) at a room temperature through the use of a VMP3 multi-channel potentiostat (Bio-logic, France) (pH, incubation time, temperature, medium). With an efficient surface area of 3.67 mm^2, screen printed carbon electrodes (SPE) were applied for impedance analysis. The impedance spectra were measured with a sinusoidal potential of 10 mV intensity throughout a frequency range of 100 kHz to 100 mHz. The potential range between -0.2 V and 0.6 V was used for staircase potential EIS (SPEIS). All tests were carried out in 0.1 M KCl with 0.1 mM potassium ferricyanide ($K_4[Fe(CN)_6]\cdot 3H_2O$) acting as a redox mediator.

Following was the experimental settlement process:

1) The working electrode (SPE) was covered with 15 µL of the extracted material, which was then dried for 20–30 minutes.

2) A second layer of 15 µL of the extracted material was spread out and kept in the fume hood for an additional 20 to 30 minutes to dry completely.

3) The working electrode was plugged into the biopotentiostat via channels, and 80 µL of KCl containing 0.1 mM potassium ferricyanide was spread on top of the specimen, totally covering it.

EIS analysis is conducted based on the parameters listed in the Table 3.1 below.

Table 3. 1. Setting parameters for EIS analysis.

E_i (V)	-0.2	**E range min (V)**	-10
dt (s)	5	**E range max (V)**	10
f_i (kHz)	100	**E_f (V)**	0.6
f_f (mHz)	100	**V_a (mV)**	10

Table 3. 2. List of the PGA samples used for the EIS analysis with their growth conditions.

#	Sample	Conditions
1	*B. subtilis* PB5760 mutant strain (diluted in water)	incubation at 35°C for 72 h conventional method
2	*B. subtilis* PB5760 mutant strain (concentrated)	incubation at 35°C for 72 h conventional method

3	*B. subtilis* wild strain	incubation at 35°C for 48 h conventional method
4	*B. subtilis* PB5760 mutant strain	incubation at 35°C for 48 h conventional method
5	*B. subtilis* wild strain	incubation at 35°C for 48 h conventional method in the presence of IPTG
6	*B. subtilis* PB5760 mutant strain	incubation at 35°C for 48 h conventional method in the presence of IPTG

Table 3. 3. List of the rhamnolipid samples used for the EIS analysis with their growth conditions.

#	**Sample**	**Conditions**
1	*P. aeruginosa* Duo2_nadE	incubation at 37°C for 48 h via conventional method
2	*P. aeruginosa* Duo2_rhlBA (1.7 kb)	
3	*P. aeruginosa* Duo2_rhlBA (5 kb)	
4	*P. aeruginosa* Duo2	
5	*P. aeruginosa* wild type strain	
6	*P. aeruginosa* Duo2_nadE	incubation at 37°C for 48 h via electrofermentation method
7	*P. aeruginosa* Duo2_rhlBA (1.7 kb)	
8	*P. aeruginosa* Duo2_rhlBA (5 kb)	
9	*P. aeruginosa* Duo2	
10	*P. aeruginosa* wild type strain	

3.4. PGA concentration measurements

The concentration of *Bacillus subtilis* PGA samples was measures using NanoDrop™ 8000 Spectrophotometer at a wavelength of 216 nm.

3.5. Data analysis

EC-Lab software was used to get analysis results, and data collected was fitted using the software's Z-fit function based on the equivalent circuit shown below:

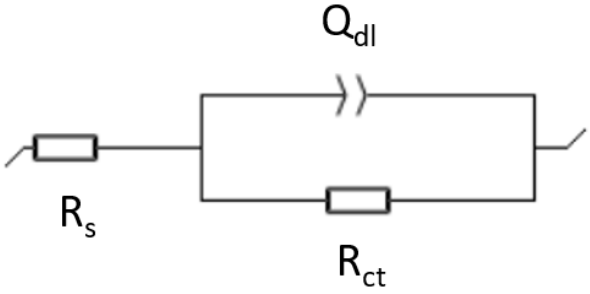

Figure 3. 1. The equivalent circuit diagram used for fitting the data obtained from EIS analysis.

Calculating crucial electrochemical properties such as double-capacitance and charge transfer resistance was achievable using the provided circuit. Additionally, the collected data might be valuable for drawing the Nyquist diagram, which might reveal important details about the studied sample. In addition, the Hsu-Manfield relation was intended to be applied in order to achieve an effective capacitance value:

$$C_{eff} = \frac{(Q_{dl}R_{ct})^{1/\alpha}}{R_{ct}}$$

(Eq.6)

, where C_{dl} - Constant Phase Element (CPE) (F $s^{\alpha-1}$), R_{ct} - charge transfer resistance and α is the fitting coefficient (36).

3.6. Statistical analysis

The level of significance of retrieved electrochemical parameters was evaluated using analysis of variance (ANOVA) at a 95% confidence level. The results of ANOVA are presented in Appendix.

Chapter 4 – Impedance results and discussion

4.1. *Bacillus subtilis* PGA samples

B. subtilis PGA samples listed in the Table 3.2 were analyzed by EIS technique, and the obtained raw data was fitted via fitting tool in the EC-lab software to retrieve electrochemical parameters.

4.1.1. Nyquist plot

Nyquist plots for electrodes at an open circuit are shown in Figure 4.1 in both the absence and presence of various PGA concentrations. The charge-transfer process is shown by the semicircle at high frequencies, which is followed by a linear section of the Nyquist plot at low frequencies, up to 0.15 Hz. With a slope of the Nyquist plot close to unity, the mass transfer of redox couple to and from the electrode surface dominates the impedance response in this frequency range. The equivalent circuit depicted in Figure 3.1 can provide an excellent description of the observed impedance findings for the bare electrode. According to this circuit, R_s can be referred to as electrolyte or intrinsic resistance and R_{ct} is a charge-transfer resistance based on the redox species' oxidation and reduction. Meanwhile, the double layer capacitance or the overall capacitance in series of the covered electrode/solution junction is represented by C_{dl}, a constant phase element, which later can be converted to the effective capacitance C_{eff} (Eq. 6).

Nyquist plots for SPE electrodes with PGA samples exhibit semi-circles in higher frequencies that might attribute to interfacial capacitance along with charge transfer resistance (Figure 4.1). Another capacitive loop that exhibits constant slope from its startup follows this semicircle, which can be assumed to be linked with mass transfer of redox species. In case of the slope followed by capacitive loop not approaching unity one could speculate that equivalent circuit in Figure 3.1 cannot be applied for impedance analysis due to the presence of disproportional diffusion pattern, which would require additional parameter for describing this anomality (37).

Compared to the electrodes with PGA samples, the impedance response for the bare SPE had a significantly larger capacitive loop. In fact, the suppression of charge transfer resistance is what is responsible for the given discrepancy. These findings suggested that the dielectric character of the bare electrode is associated with the shift in capacity. The Nyquist

figure, which shows erratic semicircles due to frequencies dispersion, demonstrates that charge transfer regulates the process (Figure 4.1).

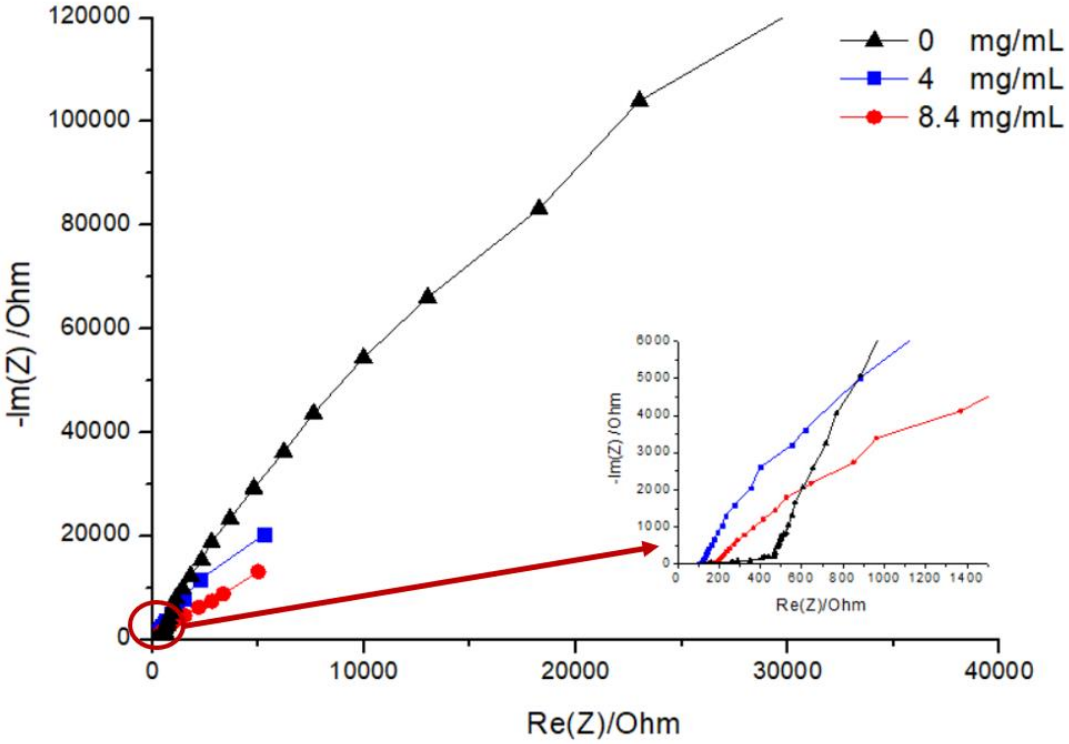

Figure 4. 1. Nyquist plot of different concentration PGA samples secreted by Bacillus subtilis PB5760 mutant strain incubated at 35°C for 72 h via conventional method.

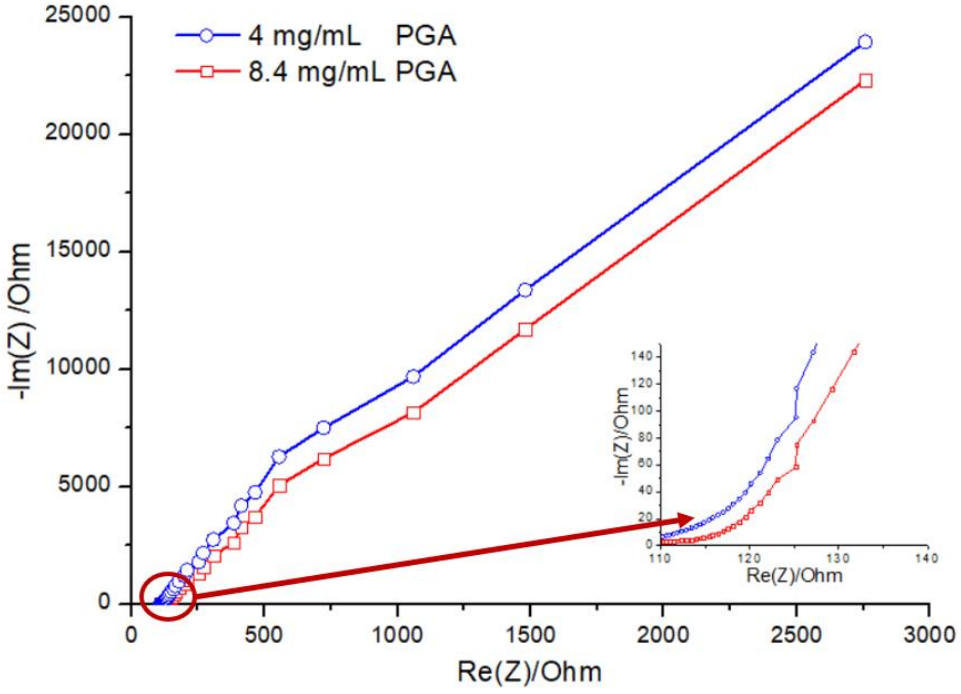

Figure 4. 2. Nyquist plot of different concentration PGA samples secreted by Bacillus subtilis PB5760 mutant strain incubated at 35°C for 72 h via conventional method.

When comparing diluted and concentrated PGA samples, it might be recognized that there is a slight variance in diameter of capacitive loop with the higher concentration PGA exhibiting greater size semicircular portion (Figure 4.2). The semicircular diameter in Nyquist plots, as illustrated in this image, directly reflects the resistance to redox process at the working electrode surface (38). It may be argued that the semicircular section of the lower concentration PGA is substantially larger than the higher concentration PGA because of the chemical structure of the acid itself. Polyglutamic acid usually has a negative charge at pH values of 7, which may account for why impedance rises with acid concentration. These have carboxylic acid rings on their side chains that have sufficiently low pKa readings to deprotonate, producing a negative charge. At the SPE/KCl solution interface, these negatively charged side chains may prevent chloride ions from passing through (38). The findings show that adding polyglutamic acid, which has a negative charge, to the electrode's surface significantly inhibits chloride ion exchange, leading to an increase in impedance.

These simulation results demonstrate that a variety of impedance diagram behaviors, which indicate variations in capacitive effects, might be summed up by the effective capacitance obtained by combining the electrical properties of the circuit via Equation 6.

4.1.2. Effective capacitance

Figure 4.3 demonstrates the effective capacitance of various concentration PGA samples calculated using the Equation 6. As it is mentioned earlier, the comparative capacitance of a complicated structure or circuit that may be described by a unified capacitance value is referred to as effective capacitance. It is the capacitance, in other words, that acts just like the circuitry or unit is a separate capacitor.

It was anticipated earlier that effective capacitance for bare SPE electrode is close to zero verifying its dielectric nature. It is shown that effective capacitance of the concentrated PGA is higher than that of the diluted sample. Given that effective capacitance can be linked to surface conditioning, it can be suggested that concentrated sample indicating a greater value of effective capacitance may correlate to a higher production of *Bacillus subtilis* PGA. A higher percentage of possible active sites for charge transfer actions can result in improved surface conditioning, which can be indicated by an increase in effective capacitance (10). In other words, these results made it possible to claim that *Bacillus subtilis* PGA with a higher yield has a greater dispersion of charge-transfer active sites, resulting in increased quantities of effective capacitance.

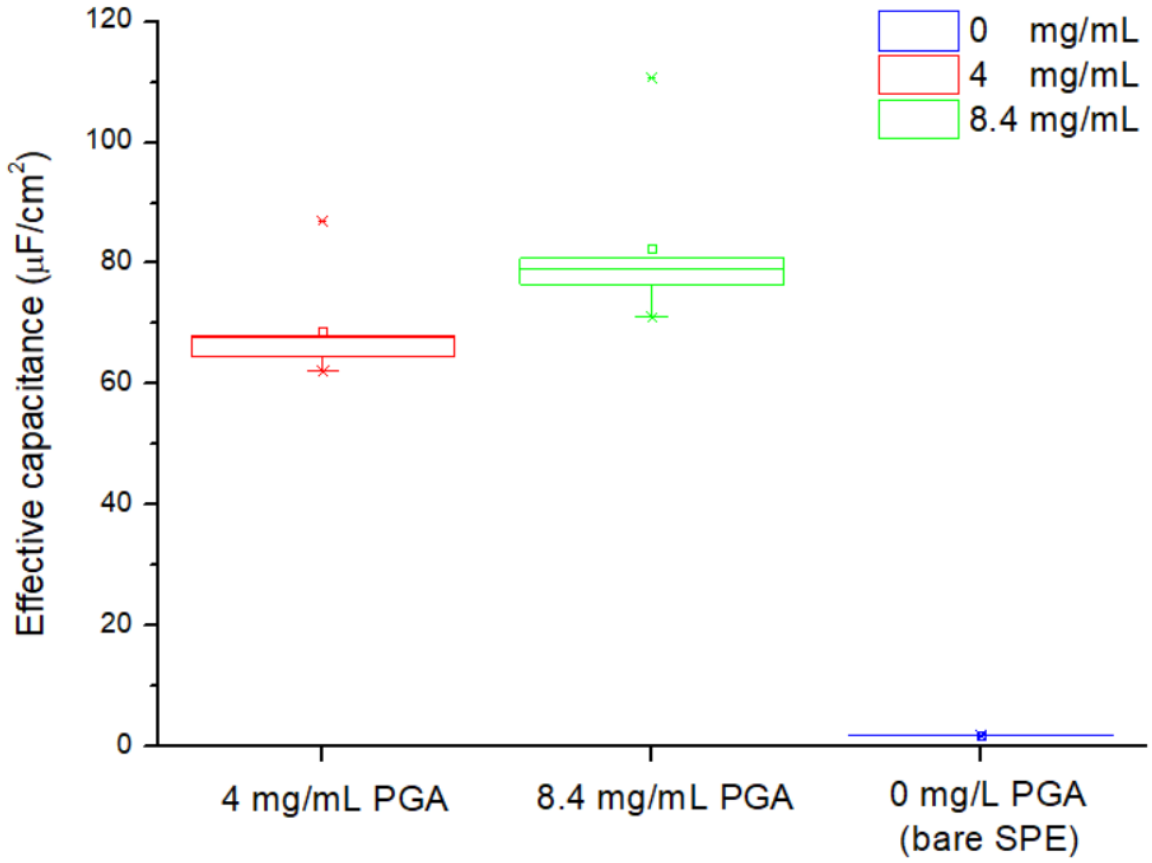

Figure 4. 3. Effective capacitance of different concentration PGA secreted by Bacillus subtilis PB5760 mutant strain incubated at 35°C for 72 h via conventional method.

Table 4. 1. Mean effective capacitance of PGA samples with various dilution extents.

Sample (volume fraction)	**PGA concentration (mg/mL)**	**Mean effective capacitance (μF/cm²)**
Original	8.4	110.64
Diluted 2 times	4.2	77.10
Diluted 4 times	4	68.83
Diluted 4 times	2.1	40.83
Diluted 8 times	1.05	20.23
Diluted 16 times	0.5	9.71

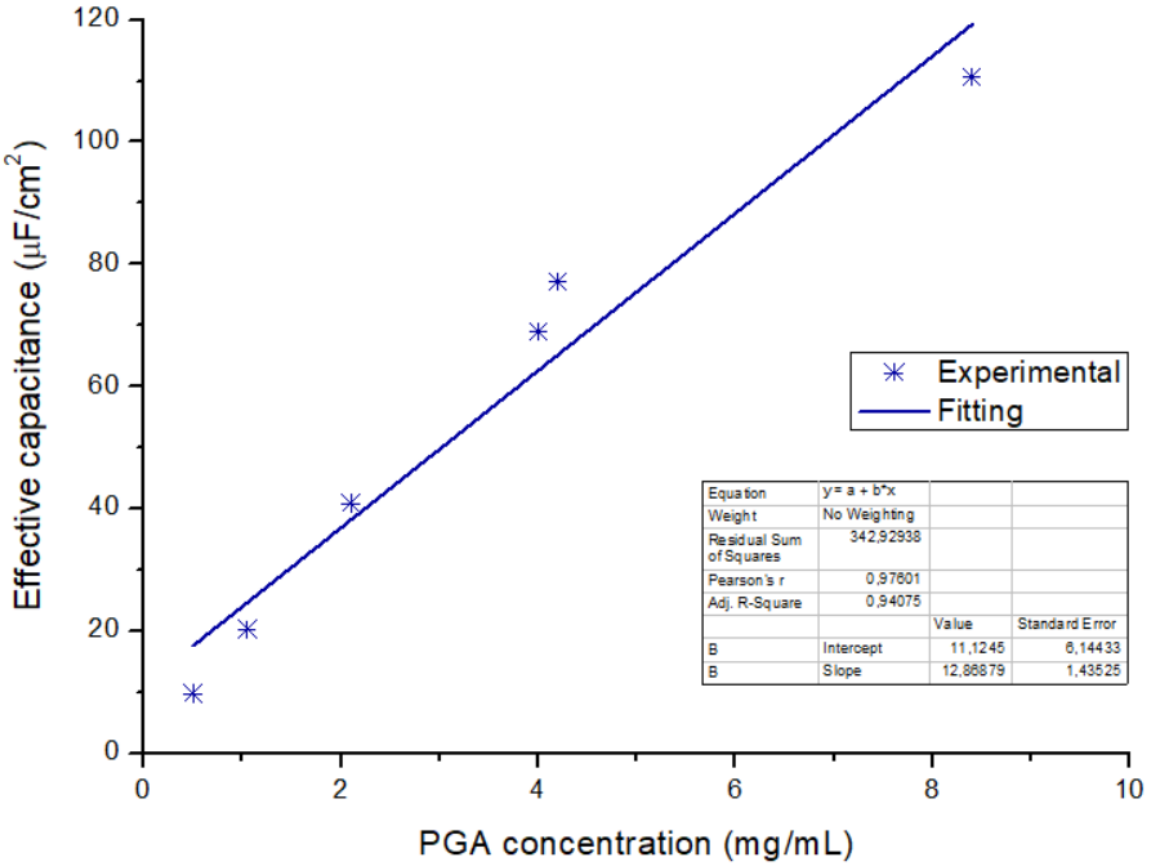

Figure 4. 4. Effective capacitance as a function of concentration of PGA secreted by Bacillus subtilis PB5760 mutant strain incubated at 35°C for 72 h via conventional method (symbol – experimental data, line – fitting).

Followed by the results from Figure 4.3, a consecutive set of six different PGA concentrations were analyzed under EIS to test whether concentration-dependent rule applies to effective capacitance or not (Table 4.1). Effective capacitance is depicted in Figure 4.4 as a function of PGA concentration, with a goodness of fit of R^2 = 0.94. When there are more entangled PGA particles, the C_{eff} increases, indicating that the PGA's effective capacitance has increased due to the rise in the net electrical double layer. Considering that R^2 value is higher than 0.9 and close to 0.95, it can be argued that C_{eff} permits a concentration-depending fitting curve for PGA samples.

The similar result was obtained from the study, in which effective capacitance retrieved from the equivalent circuit was used to monitor the suspensions of non-adherent cells. It was found out that there is a strong dependence between the effective capacitance and particle concentration of PGA samples (39). Combining it with the results from Figure 4.3, it is reasonable to argue that the number of active sites for ion insertion/charge transfer and the effective capacitance value are strongly correlated.

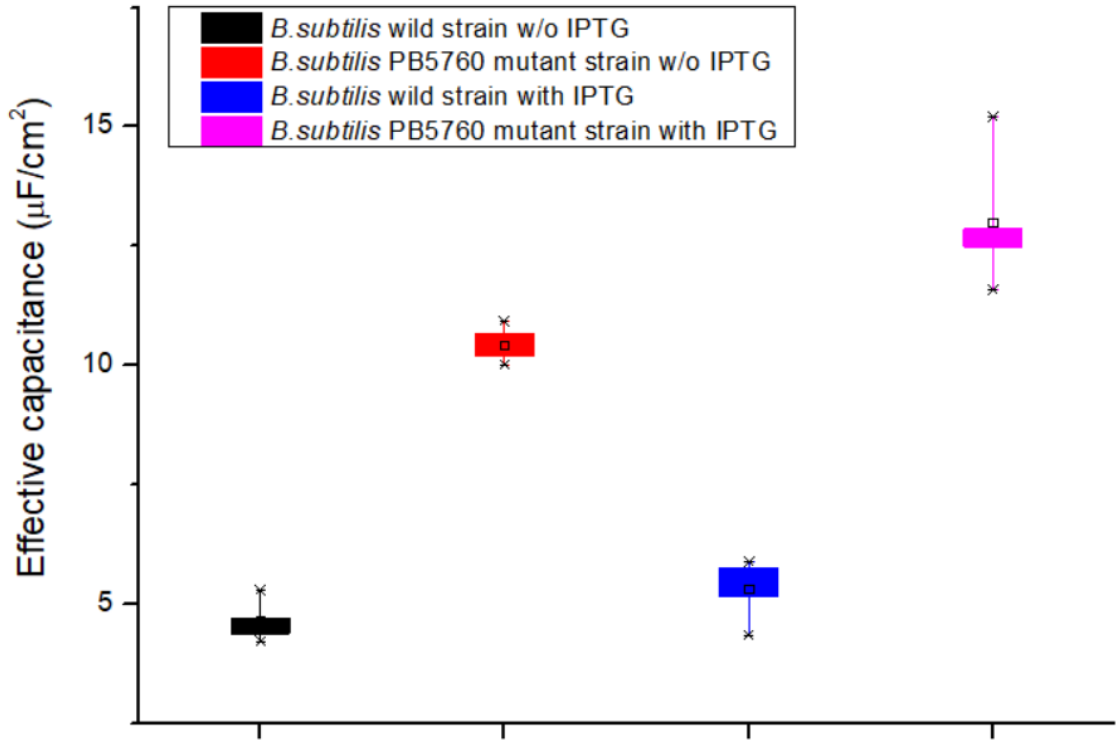

Figure 4. 5. Effective capacitance of PGA secreted by Bacillus subtilis wild and PB5760 mutant strains incubated at 35°C for 48 h via conventional method (in the presence/absence of IPTG).

Figure 4.5 illustrates the estimated effective capacitance for the PGA samples prepared by *Bacillus subtilis* wild and mutant strains cultivated both with and without IPTG. Isopropyl-D-1-thiogalactopyranoside (IPTG), also known as isopropyl-D-1-thiogalactose, is a substance that imitates allolactose and stimulates gene transcription by eliminating a blockage from the lac operator (40). In molecular biology investigations, IPTG is a frequent inducer of gene expression that is used to activate the production of genes whose promoters are controlled by its lac operon mechanism in bacterial cells. A collection of bacterial genes called the lac operon are involved in lactose metabolism. These genes' promoter regions, which are regulated by the repressor protein LacI, determine how these genes are expressed. LacI attaches towards the promoter region of the genes when lactose is not present, stopping the translation of the genes. The repressor is released from the promoter when there is lactose, causing a conformational shift that allows the genes to be expressed. Lactose binds to LacI. Since PGA-generating *B. subtilis* (wild strain) is confirmed to exhibit no genetic competence, it is generally accepted that the mutant strain *B. subtilis* is capable of receiving DNA. Hence, IPTG, artificial lactose analog that the bacteria are unable to digest is added to the medium. It attaches to LacI when it is given to the bacterial suspension, causing a shape change that relieves the repressor out from promoters and permits the transcription of the genes below of the promoter (41). The

transcription of the target gene may significantly rise as a result, which will lead to the enhanced γ-PGA production.

As it was anticipated, PGA of wild strains with and without IPTG promoter display lowest effective capacitance, which indicate their inability to be genetically competent and not allowing the promoter to control the gene expression.

It can be seen that the highest effective capacitance was recorded for the PGA sample of mutant strain grown in the presence of IPTG, which might be reasonable, since as it was discussed earlier, *Bacillus subtilis* PB5760 mutant strains are likely stimulate PGA generation only in the presence of chemical agents like IPTG that induce gene expression. Dynamic metabolic regulation can not only regulate the expression of genes but also permit the organism to exhibit an auto inducible expression profile. While the expression of γ-PGA synthetase might be activated by IPTG when the cell size reached a particular value. A significant increase in effective capacitance when growth is regulated by a promoter demonstrates IPTG's potency as a tool for regulating PGA synthesis in mutant strains.

Comparably high capacitance was observed for PGA of mutant strain without promoter, but with slightly lower values compared to the samples prepared in the presence of IPTG. Even in the lack of a promoter, mutant strains can still ferment and create a significant quantity of PGA. Considering the fact that promoters like IPTG are vital for the cell growth, stimulator-dependent growth of *B. subtilis* PB5760 mutant strains was highly anticipated. However, earlier studies have also confirmed that some mutant strains can adapt for successfully growing even in the absence of gene expression promoters (42). It turns out that genes in these mutant strains were continuously transcribed to some extent rather than rigorously being strictly repressed by LacI.

4.2. *Pseudomonas aeruginosa* rhamnolipids

4.2.1. Conventional method

Rhamnolipids, in other words, amphiphilic glycolipids synthesized by *Pseudomonas aeruginosa* are excellent biosurfactants. When received, RL molecules were a mix of mono- and di-RL types. The structures include -hydroxyl fatty acids and L-rhamnose. RL-s are attractive emulsifiers for the electrostatic dispersal of materials because of their amphiphilic nature and the electrical potential of their carboxyl group in solutions. Rhamnolipid samples extracted from *Pseudomonas aeruginosa* listed in the Table 3.2 were analyzed by EIS technique, and the obtained raw data was fitted via fitting tool in the EC-lab software to retrieve electrochemical parameters.

According to results from Electrochemical Impedance Spectroscopy (EIS) (Figure 4.6), the impedance was relatively low, and the Nyquist plot was approaching vertical line for rhamnolipids prepared in the presence of plasmid and gene expression, indicating a good capacitive performance.

At the frequency of 5 mHz, the maximum real component of capacitance reached about 8 μF. Real capacitance declined with frequency, whereas complex capacitance's component frequency dependences displayed relaxation-type dispersions (Figure 4.6B) (43).

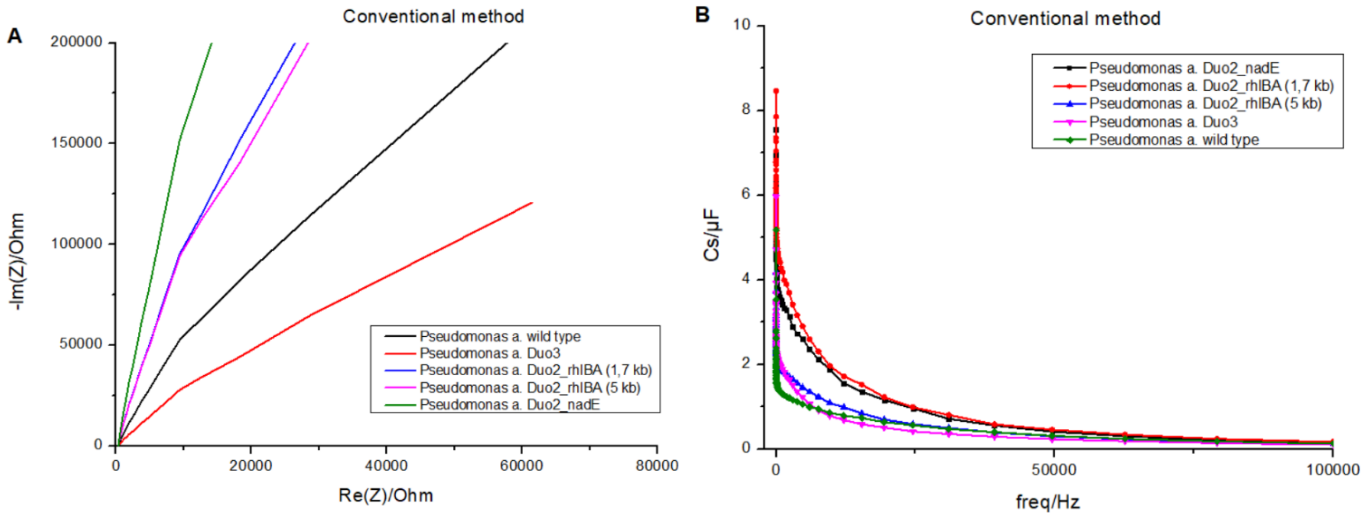

Figure 4. 6. A – Nyquist plot; B – C_s vs frequency chart for Rhamnolipids secreted by Pseudomonas aeruginosa strains incubated at 37°C for 48 h via conventional method.

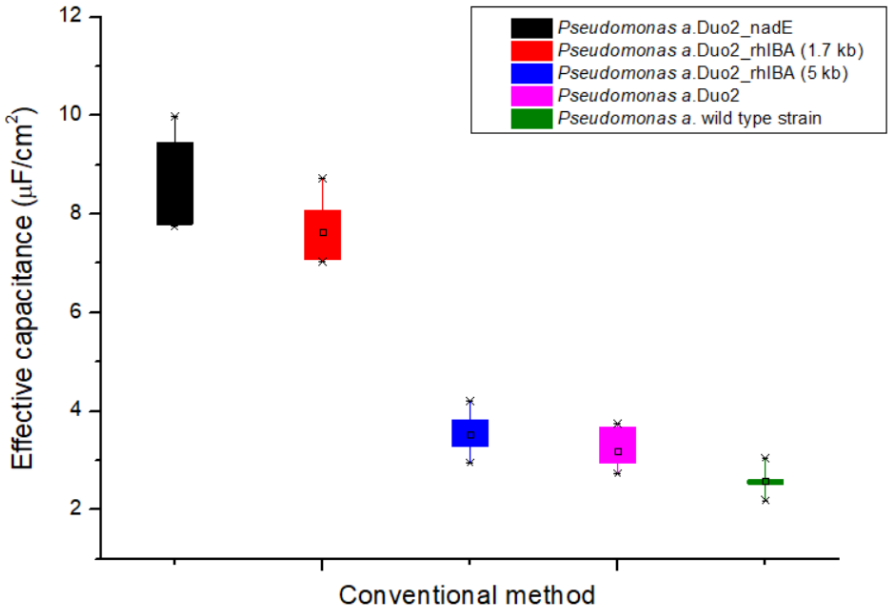

Figure 4. 7. Effective capacitance of Rhamnolipids secreted by Pseudomonas aeruginosa strains incubated at 37°C for 48 h via conventional method (in the presence/absence of plasmid and gene expression).

When nitrogen and iron concentrations are limited, *Pseudomonas aeruginosa* strains form rhamnolipids in the stationary and late-exponential growth stages. It has been determined that a regulatory locus below of the *rhlAB* domains contains the tandemly structured *rhlR* and *rhlI* alleles and is arguably necessary for their expression (44). It was intended to accomplish rhamnolipid development in recombinant hosts by trying to express the *rhlBA* rhamnosyltransferase genes, presuming that an operational rhamnosyltransferase could very well catalyze the initiation of rhamnolipid in these strains, in order to circumvent the intricate regulation of rhamnolipid syntheses. The plasmids Duo2 containing gene clusters of *nadE* (biosynthetic NAD genes) and *rhlBA* (rhamnosyltransferase) genes were applied to control gene expression and regulate rhamnolipid biosynthesis of wild and recombinant strains of *Pseudomonas aeruginosa*.

Overall, it can be shown that effective capacitance is significantly higher for Rhamnolipids secreted by strains grown in the presence of gene expression, whereas samples prepared of wild *Pseudomonas aeruginosa* strains without gene expression displayed lowest effective capacitances (Figure 4.7). Mutant strains grown in the presence of *nadE* and *rhlBA* showed the highest capacitance, presumably indicating accordingly enhanced microbial synthesis of RL-s. However, strains grown by the addition of plasmid containing gene cluster on 5-kb DNA fragment displayed substantially lower effective capacitance when compared to the same plasmid of 1.7-kb DNA fragment.

4.2.2. Electrofermentation method

The same set of RL samples but prepared via electrofermentation method demonstrated completely opposite results (Figure 4.8). Rhamnolipid sample without plasmid and gene showed the highest effective capacitance unlike in the conventional method results, which might be explained by the distinctness of both electrofermentation and conventional methods in the forthcoming sub-chapters.

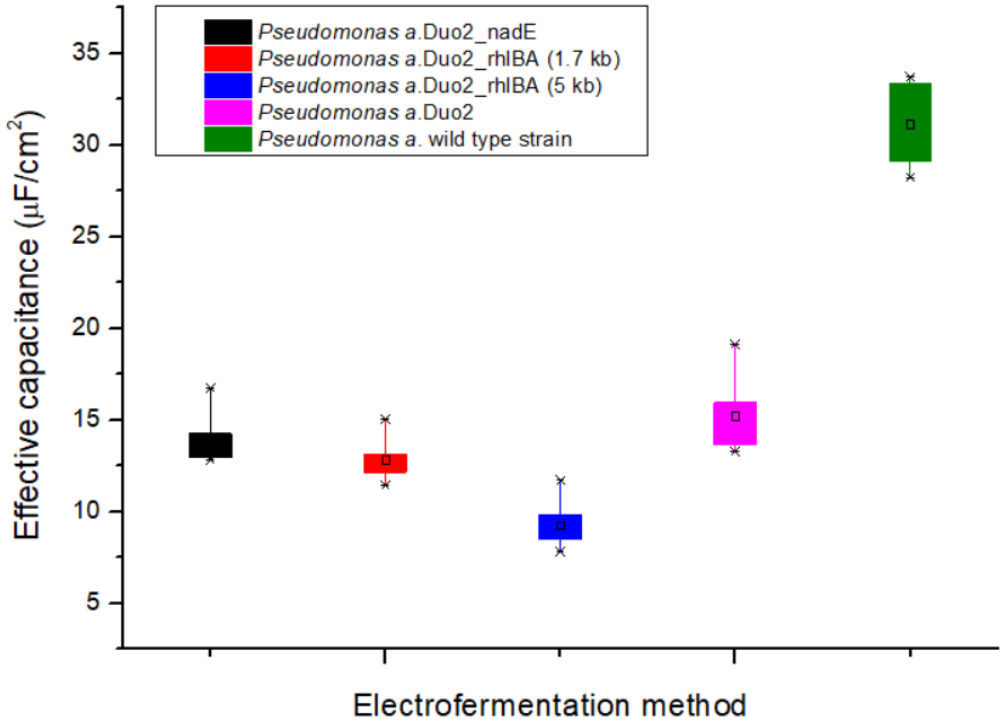

Figure 4. 8. Effective capacitance of Rhamnolipids secreted by Pseudomonas aeruginosa strains incubated at 37°C for 48 h via electrofermentation method.

4.2.3. Conventional vs. electrofermentation methods

Given that electrofermentation method is typically applied for outperforming conventional fermentation approaches and surpass restrictions imposed by thermodynamics, which would limit production efficiency, it is reasonable to expect explicit differences in performance of these two methods. EIS analysis results can be interpreted as a comparative study between RL-s produced via conventional and electrofermentation processes.

Figure 4.9 shows that electrofermentation method Rhamnolipid samples displayed higher effective capacitance. Since EF uses a synergistic method that combines electrochemistry and the microbial environment to accelerate the electrochemical reactions, it can be argued that electrofermentation is likely to produce a higher yield of rhamnolipids compared to conventional method. By balancing redox while controlling the electro metabolic pathways towards particular bio-based products, the synergy of biological and electrochemical reactions allows to circumvent the thermodynamic constraints of conventional fermentation. The reactions occurring at the anode and cathode are influenced by the applying voltage (positive/negative) on the electrodes, which are required to move the reaction toward a specific product synthesis. Due to electrostatic interactions, the majority of bacteria are negatively charged, hence applying a positive voltage to the anode might hasten the production of biofilms.

The negative voltage should be applied in order to aid the reduction reaction within the cathode (45). By enhancing the energy storage mechanism of the microbiome-ic interactions, the EF strategy for specialized product biosynthesis through specific metabolic pathways influences the fermentation routes.

This statement has already been tested several times, and it is claimed that the electrofermentation system functions more effectively than the traditional fermentation methods in accordance with the laboratory-scale investigations on the topic that have been done thus far. As an illustration, the fermentation of ethanol using various cultures has been the subject of numerous studies, and one of them demonstrated that electrofermentation increased production by 50% in a study using *C. thermocellum* and *S. cerevisiae* microorganisms and by 50% in a system using *Z. mobilis* (46). The findings of the other study concluded that electroactive bacteria could produce extracellular lipase at higher rates under electrochemical circumstances in comparison to via traditional (submerged) fermentation (47). This supports the idea that electro-fermentation tends to increase the output of additional metabolites with industrial importance.

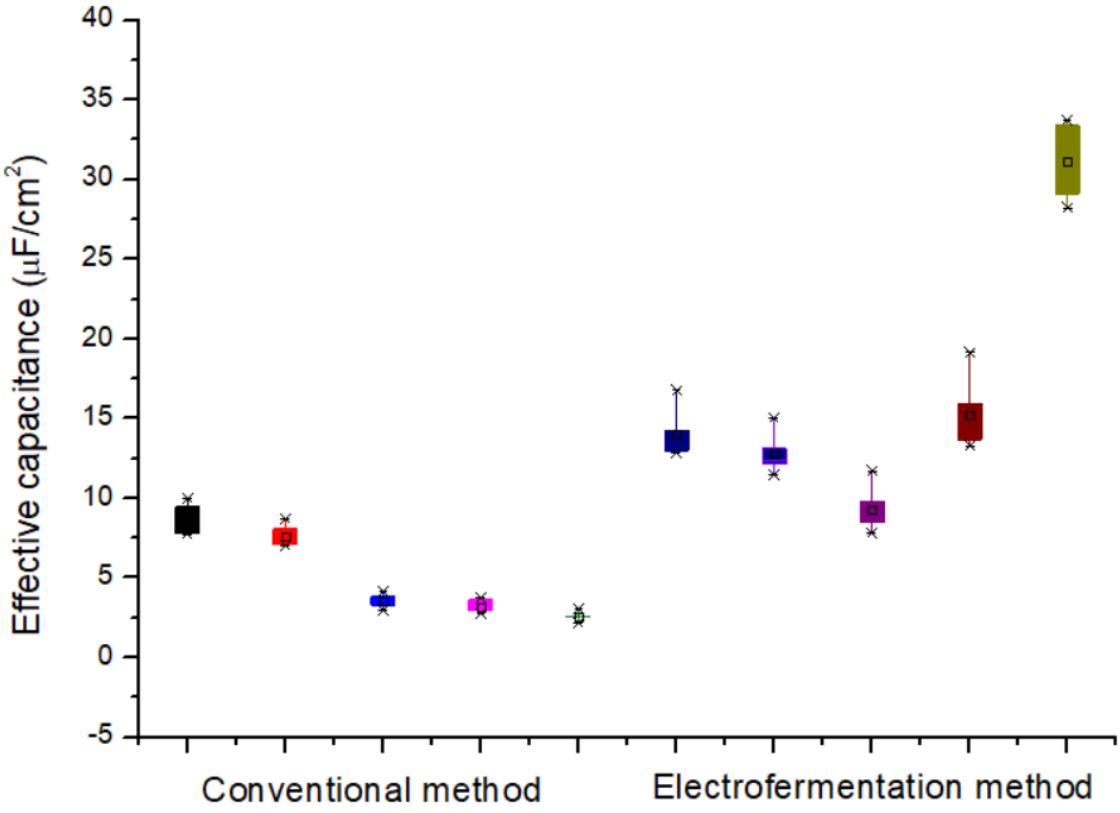

Figure 4. 9. *Effective capacitance of Rhamnolipids secreted by P. aeruginosa strains incubated at 37°C for 48 h via conventional and electrofermentation methods.*

Alternatively, electrofermentation method might lead to the generation of transient current in conjunction to faradaic activity, which in turn will add up to the accumulation of extra charge resulting in an increased capacitance compared to the conventional fermentation system (48). In other words, increased capacitance could be because of the efficient transfer of electrons within the system made possible by additionally generated electrochemical input. With respect to anodically oxidative/electro-chemical performance, the increased capacitance shows a corresponding decrease in internal and external resistance, favoring a rise in cathodic reductive ability. Together with the fermentation substrate, the electrochemical supply of electrons provides microorganisms with additional reducing power, acting as a co-reducing agent for increased electrometabolic transition to products (49).

4.3. PGA vs. RL samples: charge-transfer resistance

Tables 4.2 and 4.3 compare the intrinsic and charge-transfer resistance values obtained from fitting the EIS data for PGA and RL samples, both produced using conventional fermentation technique.

Charge transfer resistance (R_{ct}) is derived from the electrical and ionic resistance values at the electrode-electrolyte interface, whereas intrinsic resistance (R_s) is often related to the total resistance of the electrolyte and electrode material (Figure 3.1). The easiest technique to assess the electrochemical system's performance is to numerically compare the R_{ct} values because R_s is fixed for a specific medium and electrical characteristic that are independent of current density.

Significantly higher resistance for rhamnolipids compared to PGA samples might correspond to the corrosion inhibition properties of biosurfactants. Several studies have already reported that rhamnolipids have antibiofilm and anticorrosion properties, and they may potentially be a useful corrosion inhibitor for microbially induced corrosion (MIC) (50, 51). The outcomes did, in fact, support the ability of all created RL samples to inhibit the growth of microbial biofilms more effectively than PGA.

The quorum sensing (QS), which is a unique method of communication used by bacteria is connected to the biofilm mode of action. Many Gram-negative bacteria employ this biological mechanism to communicate with one another in response to changes in density of population and to produce biofilms. *P. aeruginosa* is aware of four QS systems, and each one has a distinct signaling and regulatory molecule (52, 53). The engagement of RL with phospholipid surface of cell membrane, which results in the disruption of the permeability along with consistency of the cell membrane, is directly related to the antibiofilm activity of RL (54). In the presence of

RL, the lipid content of bacterial membranes can vary. Furthermore, ion channels and holes may form, causing cell lysis and the release of intracellular chemicals. The protonation of carboxyl group on the fatty acid chain is thought to be connected to the antibacterial action of RL, which is likewise thought to be pH sensitive and favors acidic environments (55).

Biosurfactants have been shown to have antibiofilm properties in earlier investigations. For instance, when exposed to *Listeria* monocytogenes, the antibiofilm effect of rhamnolipids was demonstrated in stainless steel and polystyrene microplates (56). According to this study, interconnections between bacteria and surfaces during the early phases of biofilm development were blocked by a layer of biosurfactants on the surface. The biosurfactant's synergistic activity with some antibiotics to combat germs was demonstrated by the prevention of bacterial adhesion on surface of surgical equipment treated with biosurfactant (57). In another investigation, it was demonstrated that the prior adhesion of biosurfactants to steel metal and polypropylene surfaces achieved the necessary diminution of the bacteria colonization. Using a broth microdilution technique, Astuti and coauthors discussed the potential of biosurfactant to control biofilm (58).

As it is shown above, majority of the studies demonstrate the ability of rhamnolipids to inhibit biofilm formation of different microorganisms. However, results of the EIS analysis in this study show that it is possible for rhamnolipids to prevent *P. aeruginosa* forming its own biofilm. According to the studies that support this hypothesis, at greater quantities, rhamnolipids were observed to prevent *P. aeruginosa* W10 biofilm formation. Several functions of the rhamnolipids made by *P. aeruginosa* were hypothesized, including the preservation of biofilm matrix through the defense of diffusion pathways for gasses and nutrients. Rhamnolipids around 0.5 mg/ml were thought to be effective at rupturing *P. aeruginosa* ATCC 15442 biofilms. Rhamnolipids, however, have surface active properties that may cause them to remove extracellular polymeric substances that harm microcolonies and change the biofilm environment once the biofilm has already developed. At higher rhamnolipid concentrations, the biofilm may simply be completely disrupted (59).

Although EIS analysis allowed to evaluate the resistive properties of the samples with the help of data fitting and obtaining charge-transfer and intrinsic resistances, the exact mechanism of microbial corrosion inhibition and biofilm prevention process tends to be a subject of debate. The establishment of a complex mixture between chloride ions of the electrolyte and rhamnolipids on oxide layer sites of the carbon electrode is not fully checked out, it can be generally accepted that the pathway of corrosion inhibition is closely linked to the adsorption of the biosurfactant molecule on the SPE surface and the formation of a barrier film

(60). In a recent review of biosurfactants' mechanisms of action, Elshikh et al. noted that biosurfactants can alter the energy of absorber surface and can anchor to cell wall membranes, which increases the flowability of the cell membrane structure and causes leakage of intracellular components. Cell wall hydrophobicity may thus also be impacted as a result (61).

Table 4. 2. Intrinsic and charge-transfer resistances derived for PGA samples produced via conventional fermentation method (refer to Table 3.2 for sample characteristics).

#	R_s (Ohm/cm^2)	R_{ct} (kΩ/cm^2)
PGA Sample 1	118.92	3.67
PGA Sample 2	163.31	3.13
PGA Sample 3	133.88	3.86
PGA Sample 4	127.33	0.97
PGA Sample 5	142.29	2.05
PGA Sample 6	153.41	0.98

Table 4. 3. Intrinsic and charge-transfer resistances derived for RL samples produced via conventional fermentation method (refer to Table 3.3 for sample characteristics).

#	R_s (Ohm/cm^2)	R_{ct} (kΩ/cm^2)
RL Sample 1	313.44	6.22
RL Sample 2	273.33	4.68
RL Sample 3	334.15	5.94
RL Sample 4	309.25	7.63
RL Sample 5	317.28	7.55

Chapter 5 - Conclusion

5.1. Actuality and relevance of the EIS in microbiology

Powerful characterization methods like electrochemical impedance spectroscopy (EIS) are frequently employed in industries as diverse as energy, electrocatalysis, and medicine. EIS is particularly appealing due to two aspects. First, the electrolytic (EC) system's physical parameters, such as diffusivities and biochemical kinetics, and micro - structural traits can be determined using EIS data. Second, setting up an EIS study is not too difficult.

EIS allows the examination of time-dependent phenomena through the response in the form of current/potential of the ec process gathered at specific frequencies. Because it can explain the electrochemical mechanisms taking place at an electrified interface in just one measurement, the EIS approach is widely useful. These fundamental mechanisms include those that govern how commercial batteries function and metal corrosion, and this list can be extended to the analysis of microorganisms' growth and bacteria detection. Several studies in the microbiology already demonstrated the applicability of EIS in describing the microbial organisms, however, there has not been an extensive attempt to characterize the microbially generated bioproducts as an independent sample to our knowledge.

One of the most fascinating yet understudied phenomena in microbiology is the genesis and development of biofilms. This process also involves the production of several highly significant bioproducts within the sluggish biofilm matrix that is responsible for protecting the microbial colony. For instance, when exposed to environmental challenges, biopolymer poly-glutamic acid is released into the fermenting habitat in *B. subtilis, B. licheniforemis*, and *N. aegyptiaca* to support the survival of producing strains. Due to the resistive character of the matrix itself, *Pseudomonas aeruginosa* also frequently produces biosurfactants called rhamnolipids.

Biosurfactants are surface-active substances that are produced by a variety of microorganisms and are in high demand due to their particular environmental benefits. In the same manner, the features of biopolymers and their variants are diverse, making them excellent and crucial for a growing number of applications.

Microbiology and biotechnology are now paying more attention to the electrochemical characterization of biofilm and its constituent parts using EIS; however, it can be said that studying the secondary metabolites, such as biopolymers and biosurfactants, independently via impedance spectroscopy, is novel. Using the EIS technique to examine the biopolymer PGA

produced by Gram-positive bacteria *B. subtilis* and the biosurfactant rhamnolipids produced by different strains of Gram-negative bacteria *P. aeruginosa* is a very ambitious approach given the novel insights electrochemical analysis can offer to the evaluation of microbial systems. This work seeks to demonstrate that EIS may be regarded as one of the most promising yet efficient approaches for evaluating the bioproducts in addition to attempting to characterize the provided sample via electrochemical analysis.

5.2. Main findings

Data fitting was used to retrieve certain important electrochemical parameters, such as effective capacitance and charge-transfer resistance, and interpret the results of the EIS study. The major conclusions on the *B. subtilis* PGA samples show that the effective capacitance and PGA concentrations exhibit a strong correlation. In comparison to diluted specimens, the samples with the highest PGA concentration showed correspondingly high effective capacitance. Also, it can be inferred that EIS analysis tends to make it possible to fit a curve for effective capacitance as a function of PGA concentration. Additionally, the fitting of EIS data revealed how *B. subtilis* mutant strains in combination with inducer may be used to increase the synthesis of PGA using gene expression promoters. The results showed that samples of wild strains of *B. Subtilis* exhibited the lowest potential effective capacitance, regardless of the presence of promoter, but those samples of mutant strains demonstrated the effectiveness of greater PGA production through gene expression.

Results for the electrochemical study of rhamnolipids were consistent with that of PGA. The maximum effective capacitance was displayed by mutant strains treated on *nadE* and *rhlBA* gene expression, likely indicating improved microbiological production of RL-s. The extremely low levels of effective capacitance in *P. aeruginosa* wild strains immediately revealed their lack of genetic competence. The same set of samples as before were however generated utilizing the electrofermentation process as opposed to the usual approach, and extremely intriguing results were achieved. In both the absence and presence of gene expression, it was found that the highest effective capacitance is displayed by RL-s released by wild strains of *P. aeruginosa*, and the opposite is true for mutant strains. This intricate observation may be the result of the unique characteristics of the electrofermentation technology, which overcomes the drawbacks of traditional fermentation.

Further testing for comparing the samples from conventional and electrofermentation was motivated by these findings. The effective capacitance for the same set of samples, synthesized using both mentioned fermentation techniques, was measured, and it was

discovered that electro fermented rhamnolipids exhibit significantly stronger capacitive properties than conventional ones. It was shown that the root driver of this variation was a greater yield production when compared to conventional methods, which was achieved by balancing redox reactions and managing the electro cellular metabolism toward certain bio-based products.

The last, but certainly not least, noteworthy results came from contrasting PGA and rhamnolipid samples, which were both fermented using standard techniques under the same conditions. The result showed that RL had much higher charge transfer resistance, which can be directly related to the biosurfactants' antibiofilm and anticorrosive capabilities. It can be theorized that what inhibits the *P. aeruginosa* bacteria from forming their own biofilm is the binding of the biosurfactant molecules on the SPE surface and the development of a barrier layer, which will eventually increase charge transfer resistance of the system.

5.3. Future work and recommendations

In general, it's possible to conclude that attempts to analyze the bioproducts (biopolymer PGA and biosurfactant rhamnolipids) using the EIS method produced some intriguing results. It is challenging to label these discoveries as ground-breaking, but it can be presumed that this work paved the path for future investigations that can be equally fascinating but more crucial. It is strongly advised that additional study be conducted on the following routes:

1. To further explore the dependence of PGA concentration on effective capacitance by expanding the concentration range for PGA samples to be examined.

2. A separate, in-depth investigation focused on comparing the results of rhamnolipid samples prepared using traditional and electrofermentation methods in the absence and presence of gene expression. Several issues remain regarding why the results are in conflict. The root cause of the opposite finding in the electrofermentation samples may be due to the unique characteristics and side effects of using electric current to alter metabolic pathways, even though the results for conventional fermentation could be assumed to be reasonable.

Nonetheless, the following suggestions for further research should only be considered the tip of the iceberg. Overall, the implementation of EIS for the characterization of independent bioproducts like biopolymers and biosurfactants showed that initial steps in this direction have been taken. It is strongly believed that there are still a number of intriguing results that can be obtained with the aid of the EIS method, which is concluded to be an innovative and promising technology for characterizing microbiological products and long-term advancement of biotechnology.

References

1. Lee, K. W. K., Periasamy, S., Mukherjee, M., Xie, C., Kjelleberg, S., & Rice, S. A. (2014). Biofilm development and enhanced stress resistance of a model, mixed-species community biofilm. *The ISME journal*, *8*(4), 894-907.
2. Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. Nature Reviews Microbiology, 14(9), 563-575.
3. Berlanga, M., & Guerrero, R. (2016). Living together in biofilms: the microbial cell factory and its biotechnological implications. *Microbial cell factories*, *15*(1), 1-11.
4. Oliveira, N. M., Martinez-Garcia, E., Xavier, J., Durham, W. M., Kolter, R., Kim, W., & Foster, K. R. (2015). Biofilm formation as a response to ecological competition. *PLoS biology*, *13*(7), e1002191.
5. Hathroubi, S., Mekni, M. A., Domenico, P., Nguyen, D., & Jacques, M. (2017). Biofilms: microbial shelters against antibiotics. *Microbial Drug Resistance*, *23*(2), 147-156.
6. Chen, J., Shi, F., Zhang, B., Zhu, F., Cao, W., Xu, Z., ... & Cen, P. (2010). Effects of cultivation conditions on the production of γ-PGA with Bacillus subtilis ZJU-7. *Applied biochemistry and biotechnology*, *160*(2), 370-377.
7. Jujjavarapu, S. E., Dhagat, S., & Sen, R. (2019). *Thermophiles for Biotech Industry: A Bioprocess Technology Perspective*. Springer.
8. Nickzad, A., & Déziel, É. (2014). The involvement of rhamnolipids in microbial cell adhesion and biofilm development–an approach for control?. *Letters in applied microbiology*, *58*(5), 447-453.
9. Azeredo, J., Azevedo, N. F., Briandet, R., Cerca, N., Coenye, T., Costa, A. R., ... & Sternberg, C. (2017). Critical review on biofilm methods. *Critical reviews in microbiology*, *43*(3), 313-351.
10. Dominguez-Benetton, X., Sevda, S., Vanbroekhoven, K., & Pant, D. (2012). The accurate use of impedance analysis for the study of microbial electrochemical systems. *Chemical Society Reviews*, *41*(21), 7228-7246.
11. Gupta, P., Sarkar, S., Das, B., Bhattacharjee, S., & Tribedi, P. (2016). Biofilm, pathogenesis and prevention—a journey to break the wall: a review. *Archives of microbiology*, *198*(1), 1-15.

12. Tolker-Nielsen, T. (2015). Biofilm development. *Microbiology spectrum*, *3*(2), 3-2.
13. Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature reviews microbiology*, *8*(9), 623-633.
14. Horn, H., & Lackner, S. (2014). Modeling of biofilm systems: a review. *Productive Biofilms*, 53-76.
15. Marvasi, M., Visscher, P. T., & Casillas Martinez, L. (2010). Exopolymeric substances (EPS) from Bacillus subtilis: polymers and genes encoding their synthesis. *FEMS microbiology letters*, *313*(1), 1-9.
16. Maruzani, R., Sutton, G., Nocerino, P., & Marvasi, M. (2019). Exopolymeric substances (EPS) from Salmonella enterica: polymers, proteins and their interactions with plants and abiotic surfaces. *Journal of Microbiology*, *57*(1), 1-8.
17. Cruz, G. F. D., Angolini, C. F., Santos Neto, E. V. D., Loh, W., & Marsaioli, A. J. (2010). Exopolymeric substances (EPS) produced by petroleum microbial consortia. *Journal of the Brazilian Chemical Society*, *21*, 1517-1523.
18. Morikawa, M. (2006). Beneficial biofilm formation by industrial bacteria Bacillus subtilis and related species. *Journal of bioscience and bioengineering*, *101*(1), 1-8.
19. Morikawa, M., Kagihiro, S., Haruki, M., Takano, K., Branda, S., Kolter, R., & Kanaya, S. (2006). Biofilm formation by a Bacillus subtilis strain that produces γ-polyglutamate. *Microbiology*, *152*(9), 2801-2807.
20. Stanley, N. R., & Lazazzera, B. A. (2005). Defining the genetic differences between wild and domestic strains of Bacillus subtilis that affect poly-γ-DL-glutamic acid production and biofilm formation. *Molecular microbiology*, *57*(4), 1143-1158.
21. Berekaa, M. M., & Al-Otaibi, M. S. (2013). Enhanced production of poly glutamic acid by Bacillus sp. SW1-2 using statistical experimental design. *African Journal of Biotechnology*, *12*(5).
22. Bajaj, I., & Singhal, R. (2011). Poly (glutamic acid)–an emerging biopolymer of commercial interest. *Bioresource technology*, *102*(10), 5551-5561.
23. Najar, I. N., & Das, S. (2015). Poly-glutamic acid (PGA)-Structure, synthesis, genomic organization and its application: A Review. *International Journal of Pharmaceutical Sciences and Research*, *6*(6), 2258.
24. Soberón-Chávez, G., Lépine, F., & Déziel, E. (2005). Production of rhamnolipids by Pseudomonas aeruginosa. *Applied microbiology and biotechnology*, *68*(6), 718-725.
25. Maier, R. M., & Soberon-Chavez, G. (2000). Pseudomonas aeruginosa rhamnolipids: biosynthesis and potential applications. *Applied microbiology and*

biotechnology, *54*(5), 625-633.

26. Boles, B. R., Thoendel, M., & Singh, P. K. (2005). Rhamnolipids mediate detachment of Pseudomonas aeruginosa from biofilms. *Molecular microbiology*, *57*(5), 1210-1223.
27. Rashedi, H., Assadi, M. M., Jamshidi, E., & Bonakdarpour, B. (2006). Production of rhamnolipids by Pseudomonas aeruginosa growing on carbon sources. *International Journal of Environmental Science & Technology*, *3*(3), 297-303.
28. Boudarel, H., Mathias, J. D., Blaysat, B., & Grédiac, M. (2018). Towards standardized mechanical characterization of microbial biofilms: analysis and critical review. *NPJ biofilms and microbiomes*, *4*(1), 1-15.
29. Bhardwaj, C., Moore, J. F., Cui, Y., Gasper, G. L., Bernstein, H. C., Carlson, R. P., & Hanley, L. (2013). Laser desorption VUV postionization MS imaging of a cocultured biofilm. *Analytical and bioanalytical chemistry*, *405*(22), 6969-6977.
30. Magar, H. S., Hassan, R. Y., & Mulchandani, A. (2021). Electrochemical impedance spectroscopy (EIS): Principles, construction, and biosensing applications. *Sensors*, *21*(19), 6578.
31. Orazem, M. E., & Tribollet, B. (2008). Electrochemical impedance spectroscopy. *New Jersey*, 383-389.
32. Instruments, G. (2007). Basics of electrochemical impedance spectroscopy. *G. Instruments, Complex impedance in Corrosion*, 1-30.
33. Moradi, M., Ghiara, G., Spotorno, R., Xu, D., & Cristiani, P. (2022). Understanding biofilm impact on electrochemical impedance spectroscopy analyses in microbial corrosion and microbial corrosion inhibition phenomena. *Electrochimica Acta*, *426*, 140803.
34. Cabrera-Sierra, R., Pech-Canul, M. A., & González, I. (2006). The role of hydroxide in the electrochemical impedance response of passive films in corrosion environments. *Journal of the Electrochemical Society*, *153*(3), B101.
35. Hsu, C. H., & Mansfeld, F. (2001). Concerning the conversion of the constant phase element parameter Y0 into a capacitance. *Corrosion*, *57*(09).
36. Bharatula, L. D., Marsili, E., & Kwan, J. J. (2020). Impedimetric detection of Pseudomonas aeruginosa attachment on flexible ITO-coated polyethylene terephthalate substrates. *Electrochimica Acta*, *332*, 135390.
37. Martinhon, P. T., Carreño, J., Sousa, C. R., Barcia, O. E., & Mattos, O. R. (2006). Electrochemical impedance spectroscopy of lead (II) ion-selective solid-state membranes. *Electrochimica acta*, *51*(15), 3022-3028.

38. Velusamy, V., Arshak, K., Yang, C., Yu, L., Korostynska, O., Oliwa-Stasiak, K., & Adley, C. (2010, February). Label-free detection of Bacillus cereus DNA hybridization using electrochemical impedance spectroscopy for food quality monitoring application. In *2010 IEEE Sensors Applications Symposium (SAS)* (pp. 135-138). IEEE.
39. De León-Hernández, A., Romero-Ornelas, L., Ramírez-Chavarría, R. G., Ramón-Gallegos, E., & Sánchez-Pérez, C. (2022). Effective Capacitance from Equivalent Electrical Circuit as a Tool for Monitoring Non-Adherent Cell Suspensions at Low Frequencies. *Bioengineering*, *9*(11), 697.
40. Wandrey, G., Bier, C., Binder, D., Hoffmann, K., Jaeger, K. E., Pietruszka, J., ... & Büchs, J. (2016). Light-induced gene expression with photocaged IPTG for induction profiling in a high-throughput screening system. *Microbial cell factories*, *15*(1), 1-16.
41. Yeh, C. M., Wang, J. P., Lo, S. C., Chan, W. C., & Lin, M. Y. (2010). Chromosomal integration of a synthetic expression control sequence achieves poly-γ-glutamate production in a Bacillus subtilis strain. *Biotechnology progress*, *26*(4), 1001-1007.
42. Kada, S., Nanamiya, H., Kawamura, F., & Horinouchi, S. (2004). Glr, a glutamate racemase, supplies d-glutamate to both peptidoglycan synthesis and poly-γ-glutamate production in γ-PGA-producing Bacillus subtilis. *FEMS microbiology letters*, *236*(1), 13-20.
43. Milne, J., Silva, R. M., & Zhitomirsky, I. (2019). Surface modification and dispersion of ceramic particles using liquid-liquid extraction method for application in supercapacitor electrodes. *Journal of the European Ceramic Society*, *39*(12), 3450-3455.
44. Reimmann, C., Ginet, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., ... & Haas, D. (2002). Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in Pseudomonas aeruginosa PAO1. *Microbiology*, *148*(4), 923-932.
45. Stamenkovic, V. R., Strmcnik, D., Lopes, P. P., & Markovic, N. M. (2017). Energy and fuels from electrochemical interfaces. *Nature materials*, *16*(1), 57-69.
46. Yoruklu, H. C., Koroglu, E. O., Demir, A., & Ozkaya, B. (2019). The electromotive-induced regulation of anaerobic fermentation: Electrofermentation. In *Microbial electrochemical technology* (pp. 739-756). Elsevier.
47. Garuba, E. O., Ajunwa, O. M., Oguntomi, O. S., & Onilude, A. A. (2020). Electro-fermentative production of thermotolerant lipases by electrogenic bacteria isolated from palm oil mill effluents. *Novel Research in Microbiology Journal*, *4*(1), 624-640.

48. Velvizhi, G., Sarkar, O., Rovira-Alsina, L., Puig, S., & Mohan, S. V. (2022). Conversion of carbon dioxide to value added products through anaerobic fermentation and electro fermentation: A comparative approach. *International Journal of Hydrogen Energy*, *47*(34), 15442-15455.
49. Sravan, J. S., Butti, S. K., Sarkar, O., & Mohan, S. V. (2019). Electrofermentation: chemicals and fuels. In *Microbial Electrochemical Technology* (pp. 723-737). Elsevier.
50. Li, Z., Yuan, X., Sun, M., Li, Z., Zhang, D., Lei, Y., ... & Wang, F. (2022). Rhamnolipid as an eco-friendly corrosion inhibitor for microbiologically influenced corrosion. *Corrosion Science*, 110390.
51. Shubina Helbert, V., Gaillet, L., Chaussadent, T., Gaudefroy, V., & Creus, J. (2020). Rhamnolipids as an eco-friendly corrosion inhibitor of rebars in simulated concrete pore solution: evaluation of conditioning and addition methods. *Corrosion Engineering, Science and Technology*, *55*(2), 91-102.
52. Rocha, V. A. L., de Castilho, L. V. A., Castro, R. D. P. V. D., Teixeira, D. B., Magalhães, A. V., Abreu, F. D. A., ... & Freire, D. M. G. (2021). Antibiofilm effect of mono-rhamnolipids and di-rhamnolipids on carbon steel submitted to oil produced water. *Biotechnology progress*, *37*(3), e3131.
53. Wood, T. L., Gong, T., Zhu, L., Miller, J., Miller, D. S., Yin, B., & Wood, T. K. (2018). Rhamnolipids from Pseudomonas aeruginosa disperse the biofilms of sulfate-reducing bacteria. *NPJ biofilms and microbiomes*, *4*(1), 22.
54. Bharali, P., Saikia, J. P., Ray, A., & Konwar, B. K. (2013). Rhamnolipid (RL) from Pseudomonas aeruginosa OBP1: a novel chemotaxis and antibacterial agent. *Colloids and Surfaces B: Biointerfaces*, *103*, 502-509.
55. Haba, E., Pinazo, A., Jauregui, O., Espuny, M. J., Infante, M. R., & Manresa, A. (2003). Physicochemical characterization and antimicrobial properties of rhamnolipids produced by Pseudomonas aeruginosa 47T2 NCBIM 40044. *Biotechnology and bioengineering*, *81*(3), 316-322.
56. De Araujo, L. V., Abreu, F., Lins, U., Santa Anna, L. M. D. M., Nitschke, M., & Freire, D. M. G. (2011). Rhamnolipid and surfactin inhibit Listeria monocytogenes adhesion. *Food Research International*, *44*(1), 481-488.
57. Das, P., Yang, X. P., & Ma, L. Z. (2014). Analysis of biosurfactants from industrially viable Pseudomonas strain isolated from crude oil suggests how rhamnolipids congeners affect emulsification property and antimicrobial activity. *Frontiers in microbiology*, *5*, 696.

58. Astuti, D. I., Purwasena, I. A., & Putri, F. Z. (2018). Potential of biosurfactant as an alternative biocide to control biofilm associated biocorrosion. *Journal of Environmental Science and Technology*, *11*(2), 104-111.
59. Chebbi, A., Elshikh, M., Haque, F., Ahmed, S., Dobbin, S., Marchant, R., ... & Banat, I. M. (2017). Rhamnolipids from Pseudomonas aeruginosa strain W10; as antibiofilm/antibiofouling products for metal protection. *Journal of basic microbiology*, *57*(5), 364-375.
60. Zin, I. M., Pokhmurskii, V. I., Korniy, S. A., Karpenko, O. V., Lyon, S. B., Khlopyk, O. P., & Tymus, M. B. (2018). Corrosion inhibition of aluminium alloy by rhamnolipid biosurfactant derived from Pseudomonas sp. PS-17. *Anti-Corrosion Methods and Materials*, *65*(6), 517-527.
61. Elshikh, M., Marchant, R., & Banat, I. M. (2016). Biosurfactants: promising bioactive molecules for oral-related health applications. *FEMS Microbiology Letters*, *363*(18), fnw213.

Appendix

Table A1: Effective capacitance C_{eff} of different concentration PGA samples estimated from the equivalent circuit. N = number of technical replicates; a = non-ideality constant parameter of the non-ideal capacitance.

Electrode (PGA sample)	**N total**	Q_{eff} **(mF*cm^{-2})**	**a**
0 mg/mL	2	1.77±0.06	0.93±0.00
4 mg/mL	2	68.83±7.62	0.94±0.01
8.4 mg/mL	2	82.46±12.15	0.89±0.02

Table A2: ANOVA for C_{eff} of two different concentration PGA samples (4 mg/mL and 8.4 mg/mL) estimated from the equivalent circuit. Null hypothesis: no difference in the mean of the samples. Alternate hypothesis: there is difference in the mean interfacial capacitance of the two samples. As $F>F_{crit}$, we accept the alternate hypothesis; there is a significant difference between the mean effective capacitance of the 4 mg/mL and 8.4 mg/mL PGA samples.

Source of variation	**Sum of squares**	**Degrees of freedom**	**Mean square**	**Mean square ratio (F)**	**P-value**	**Fcrit**
Between groups	836.71	1	836.71	**8.14**	0.0115	**4.49**
Within groups	1645.19	16	102.82			
Total	2481.90	17				

Table A3: ANOVA for C_{eff} of PGA secreted by *Bacillus subtilis* wild and PB5760 mutant strains in the presence/absence of IPTG. Null hypothesis: no difference in the mean of the samples. Alternate hypothesis: there is difference in the mean interfacial capacitance of the two samples. As $F>F_{crit}$, we accept the alternate hypothesis; there is a significant difference between the mean effective capacitance of the samples.

Source of variation	**Sum of squares**	**Degrees of freedom**	**Mean square**	**Mean square ratio (F)**	**P-value**	**Fcrit**
Between groups	360.65	3	120.22	**123.55**	$1*10^{-17}$	**2.90**
Within groups	31.14	32	0.97			

Total	391.79	35

Table A4: ANOVA for C_{eff} of RL-s secreted by *P. aeruginosa* strains in the presence/absence of plasmid and gene expression via conventional method. Null hypothesis: no difference in the mean of the samples. Alternate hypothesis: there is difference in the mean interfacial capacitance of the two samples. As $F>F_{crit}$, we accept the alternate hypothesis; there is a significant difference between the mean effective capacitance of the samples.

Source of variation	**Sum of squares**	**Degrees of freedom**	**Mean square**	**Mean square ratio (F)**	**P-value**	**Fcrit**
Between groups	280.34	4	70.09	**194.53**	$1*10^{-25}$	**2.61**
Within groups	14.41	40	0.36			
Total	294.75	44				

Table A5: ANOVA for Q_{eff} of RL-s secreted by *P. aeruginosa* strains in the presence/absence of plasmid and gene expression via electrofermentation method. Null hypothesis: no difference in the mean of the samples. Alternate hypothesis: there is difference in the mean interfacial capacitance of the two samples. As $F>F_{crit}$, we accept the alternate hypothesis; there is a significant difference between the mean effective capacitance of the samples.

Source of variation	**Sum of squares**	**Degrees of freedom**	**Mean square**	**Mean square ratio (F)**	**P-value**	**Fcrit**
Between groups	2590.88	4	647.72	**220.29**	$1*10^{-26}$	**2.61**
Within groups	117.61	40	2.94			
Total	2708.49	44				