Effect of controlled pH on the biochemical characteristics of estuarine phototrophic biofilms

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Effect of controlled pH on the biochemical characteristics of estuarine phototrophic biofilms

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by

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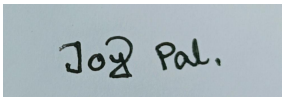
DECLARATION

I hereby declare that the work presented in this thesis report titled "**Effect of controlled pH on the biochemical characteristics of estuarine phototrophic biofilms**," submitted to Jadavpur University, Kolkata, in partial fulfillment of the requirements for the award of the degree of M.Tech, is a bona fide record of the research work carried out under the supervision of Prof. Joydeep Mukherjee and co-supervision of Dr. Reshmi Das. The contents of this thesis report, in whole or in part, have not been submitted to and will not be submitted by me to any other institute or university in India or abroad for the award of any degree or diploma.



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CERTIFICATE

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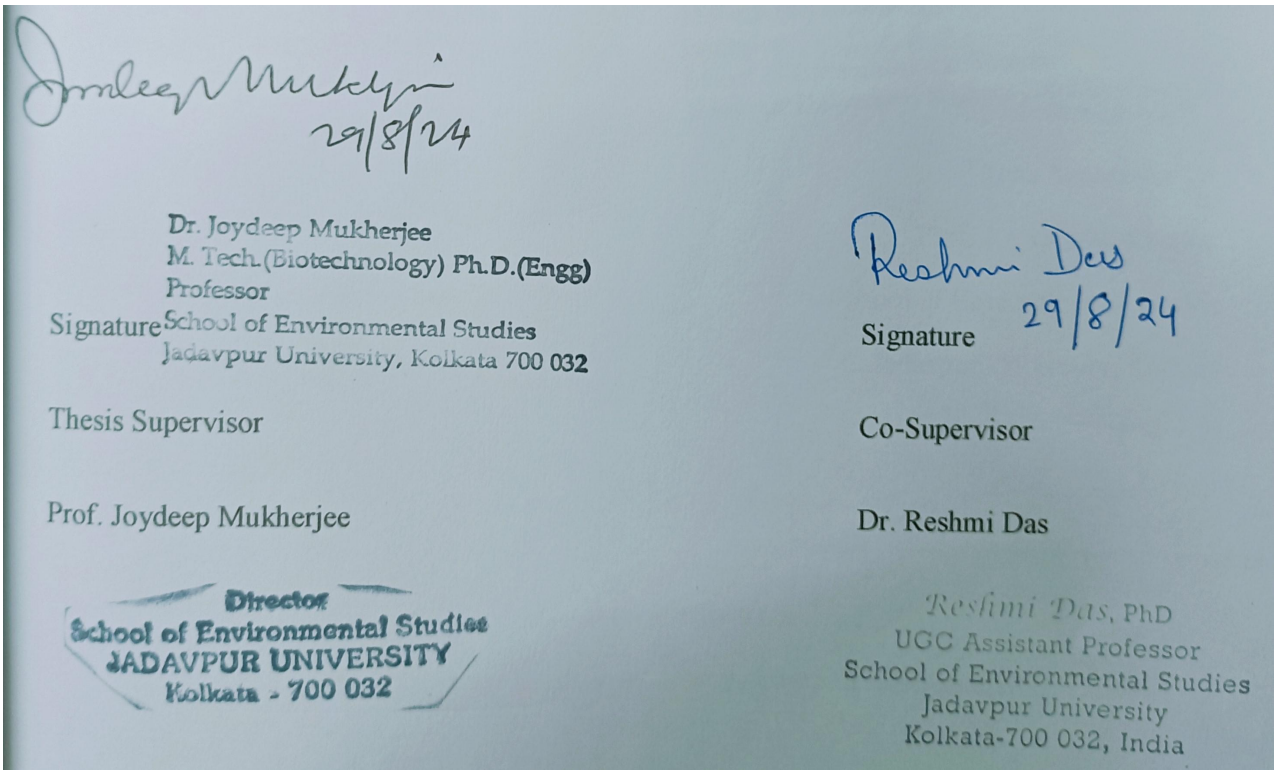

TO WHOM IT MAY CONCERN

It is hereby notified that this thesis titled "**Effect of controlled pH on the biochemical characteristics of estuarine phototrophic biofilms**", is prepared and submitted for the partial fulfillment of the continuous assessment of the Master of Technology in Environmental Biotechnology course of Jadavpur University by Joy Pal (002230904004), a student of the said course for session 2022-2024. It is also declared that no part of this thesis has been presented or published elsewhere.

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This foregoing thesis is hereby approved as a credible study of an engineering subject carried out and presented in a manner satisfactorily to warranty its acceptance as a prerequisite to the degree for which it has been submitted. It is understood that by this approval the undersigned do not endorse or approve any statement made or opinion expressed or conclusion drawn therein but approve the thesis only for the purpose for which it has been submitted.

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Joy Pal

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List of Abbreviations:

PMMA	Polymethyl methacrylate	g	Gram
EPS	Extracellular polymeric substances	mg	Milligram
ASNIII	Artificial Sea water Natural media (III)	µmol	Micromole
BG11	Blue Green 11	°C	
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	%	Percentage
AMD	Acid Mine Derange	nm	Nanometer
CPS	Capsular Polysaccharide	ml	Milliliter
RPS	Released polysaccharides	µl	Microliter
CCF	Conico Cylindrical Flasks	mM	Millimole
EDTA	Ethylenediaminetetraacetic acid	µg/mg	Microgram/Milligram
BSA	Bovine serum albumin	m/z	Mass-to-charge ratio
Chl	Chlorophyll	Da	Dalton
MS	Mass Spectroscopy		
UV	Ultra-Violate		
DNA	Deoxyribonucleic Acid		

Abbreviation	Signify	Denoted by color
HC(PMMA)	Non-buffered sample cultured in a PMMA vessel (Initial medium pH-7.2)	**Blue**
H(PMMA)	Sample buffed with HEPES cultured in a PMMA vessel (Initial medium pH-7.2)	**Orange**
TC(PMMA)	Non-buffered sample cultured in a PMMA vessel (Initial medium pH-7.8)	**Red**
T(PMMA)	Sample buffed with Tricin cultured in a PMMA vessel (Initial medium pH-7.8)	**Yellow**
HC(GLASS)	Non-buffered sample cultured in a glass vessel (Initial medium pH-7.2)	**Purple**
H(GLASS)	Sample buffed with HEPES cultured in a glass vessel (Initial medium pH-7.2)	**Green**
TC(GLASS)	Non-buffered sample cultured in a glass vessel (Initial medium pH-7.8)	**Sky-blue**
T(GLASS)	Sample buffed with Tricin cultured in a glass vessel (Initial medium pH-7.8)	**Brown**

ABSTRACT

The intertidal zone is a sensitive indicator of climate change, with wide-ranging human activity and related impacts on the surrounding ecosystems. The changed pH of estuarine water by modifying factors such as oil spills, AMD, or pollution may alter the solubility and bioavailability of some nutrients and contaminants, potentially perturbing marine ecosystems and the organisms that inhabit them. As primary producers, Cyanobacteria form one of the bases for aquatic ecosystems. Seawater pH is crucial in impacting biochemical reactions, nutrient availability, and the health of marine organisms. Changes in its pH could alter the stability of the ecosystems and biodiversity. Shifts in pH will affect photosynthetic efficiency, nutrient uptake, and community composition, impacting the entire food web and biogeochemical cycles in marine environments. This underlines the importance of pH stability during cyanobacterial cultivation and emphasizes optimizing growth conditions to enhance biomass and extracellular polymeric substances (EPS) production. The discovered results bear tremendous importance for studying biotechnological applications of cyanobacteria with a focus on biofilm-based systems. The present thesis focuses on studying the effects of different pH regimes on growth, photosynthetic pigment concentrations, and EPS production in cyanobacterial biofilms isolated from the Sundarbans.

Key Words: Intertidal, Biofilm, Heavy metal, Bioavailable, Buffer, Pigment.

1. INTRODUCTION

Life on Earth originated in the oceans, which are home to unique ecosystems and serve as critical sources of biodiversity. The oceans are large masses of water bordering the continents. Covering 71 % of the earth's surface, oceans contain approximately 97.5% of its water. However, point and non-point source pollution significantly degrade marine waters. Pollution in the coastal system is visible because of different anthropogenic activities carried out on the coast and land adjoining the ocean environment (Malan and Sharma, 2017). Marine pollution drastically impacts ocean pH, causing harmful acidification that threatens the delicate balance of aquatic ecosystems. This shift endangers countless species, from tiny plankton to majestic coral reefs, disrupting the entire oceanic food web.

Ocean pH is a critical measure of the acidity or alkalinity of seawater and plays a vital role in marine ecosystems. The pH of seawater influences a wide range of chemical and biological processes, including chemical reactions, biological toxicity, and equilibrium conditions. Oceanic pH, an important indicator of the acidity or alkalinity of seawater, plays a key role in marine ecosystems. The physiology, development, and survival of aquatic creatures, like phytoplankton, zooplankton, corals, molluscs, and fish, are all directly affected by the pH of saltwater.

Since the preindustrial era, the pH of ocean surface waters has decreased by approximately 0.1 units. Projections suggest an additional reduction of 0.3 units by the end of this century (Orr et al., 2005). Due to the narrow pH tolerance ranges present in numerous oceanic creatures, such fluctuating pH levels could harm their abilities to calcify, reproduce, and maintain biological functions. The habitat also provides significant ecological services comprising nutrient cycling, water filtration, coastal protection, and contributions to fisheries and recreational uses. Variations in pH could disturb the functionality of these

services, with a possible consequence on human well-being and socioeconomic activities depending on the estuaries.

In the current scenario, a decrease in pH is something to take into consideration since the oceans are sucking up more CO_2 from the air. It absorbs about one-quarter of the CO_2 emitted into the atmosphere (Marion et al., 2011). Acids and heavy metals are only a few instances of pollutants that may be discharged into the environment by industrial activities like mining, manufacturing, and chemical production. These contaminants have the potential to directly acidify marine ecosystems and cause localized pH drops, especially in regions with significant industrial activity or insufficient waste management procedures.

Most of the world's rivers carry different waste material from the land, which ultimately ends up in the oceans. The majority of the contaminants found in rivers are discharged by industrial and urban sewage systems. These runoffs contain high amounts of phosphorous and nitrogen from agricultural and industrial sources. The issue of sulfide oxidation and the associated acid mine drainage (AMD) or acid rock drainage (ARD) has been a primary area of study in the past few years (Kumar, 2010). AMD-contaminated water from abandoned mines can flow into nearby rivers and streams. Runoff from these water bodies, if they empty into the ocean, can contaminate the coastal watercourses with acid and metals. As a consequence, this phenomenon can locally decrease the pH of the receiving marine environment(Simate and Ndlovu, 2014). It is possible for suspended sediment and particles from AMD-contaminated rivers to travel downstream and deposit themselves in coastal regions. Acidic substances and heavy metals that may be present in these sediments have the potential to slowly seep into the nearby seawater and cause pH drops in certain areas (Gray, 1997).

Oil slicks on the water's surface can lead to decreased dissolved oxygen in seawater. These slicks have the potential to block atmospheric oxygen from reaching the ocean, which can cause hypoxic or anoxic conditions in the impacted areas. The anaerobic breakdown of organic materials in these oxygen-depleted

conditions produces acidic by-products, further lowering seawater pH (Aislabie et al., 2001). Numerous organic substances, such as hydrocarbons and volatile organic compounds (VOCs), are found in crude oil. When oil is spilled into saltwater chemical interactions between these substances can result in the creation of organic acids and other acidic chemicals. As a result, the pH of seawater drops.

Land-based inputs are the main cause of significant pH fluctuations in coastal ecosystems. These include freshwater inputs that typically dilute the alkalinity of seawater, resulting in reduced buffering capacity, nutrients that enhance productivity and pH, as well as organic matter that supports excess respiration driving acidification. In some coastal ecosystems, upwelling of nutrient-rich and corrosive water may also contribute to pH variability (Carstensen and Duarte, 2019).

Low pH negatively impacts microalgal consortia by inhibiting growth, causing molecular changes, and disrupting their capacity to construct habitats and cycle nutrients in aquatic environments. Many species of microalgae have different levels of sensitivity to pH change. For some organisms, a low pH could be ideal for their growth while it could be a threat to others by providing competitive advantages to more tolerant species.

Cyanobacteria form an important group of organisms in the marine environment and play a potentially crucial role in primary production, and nutrient cycling. They are very tolerant of many environmental stressors, such as variations in pH and temperature, making them perfect models for studying pH change under simulated future conditions. Cyanobacterial biofilms, which are composed of eukaryotic and prokaryotic cells encased in an extracellular matrix, assist in primary production by forming organic compounds consumed as food by other organisms in the food chain (Egan et al., 2008). Many organisms, such as herbivorous zooplankton, aquatic insect larvae, and some fish species, rely on biofilms as a direct source of food. Numerous species, such as insects, and small fishes, find a home and protection in

cyanobacterial biofilms. These biofilms form intricate microenvironments with an array of niches, providing resources and an oasis for many species in the food chain (Faria et al., 2020).

Phototrophic biofilms can be most accurately described as microbial communities that are adhered to a surface and powered by light energy, allowing them to perform photosynthesis. These types of biofilms are made up of oxygenic phototrophic microorganisms such as benthic diatoms (which can be both unicellular and filamentous, as well as centric and pennate), unicellular and filamentous cyanobacteria, and benthic green algae. These microorganisms are responsible for producing organic substrates and oxygen, while also reducing carbon dioxide. The biofilms are held together by extracellular polymeric substances (EPS) that are produced by the microorganisms (Roeselers et al., 2008)(Flemming, 1993). Microorganisms have significant and well-established functions in intertidal systems that have the potential to cause significant alterations. A "microbial biofilm" is created when bacteria and microalgae attach themselves to surfaces, such as plant surfaces or the soil particles of intertidal sandflats, by secreting a matrix of mucilaginous extracellular polymers (EPS).

These biofilms are considered pivotal elements of the coastal ecosystem because they are the base of the ecological pyramid, protect the intertidal beds from erosional stress, and have bio-stabilizing capabilities along with remarkable potential in the bioremediation of organic pollutants and heavy metals (Gerbersdorf and Wieprecht, 2015). Biofilms and their mucilaginous matrix are essential in heavy metal in-situ bioremediation. This metal sequestering ability is being studied for its potential to protect intertidal and coastal ecosystems from heavy metal toxicity and lower the likelihood of this toxoid entering the food chain.

Under the RAMSAR convention, the largest delta in the world, Sundarbans, located in both Bangladesh and India, is recognized as a UNESCO World Heritage Site. This biome plays a crucial role in climate regulation, acting as a carbon sink by sequestering large amounts of carbon dioxide from the atmosphere.

Considering its importance as one of the Earth's most significant coastal ecosystems, pollution, climate change, and over-exploitation of natural resources pose major risks to it. Thus it is receiving a lot of attention on a global scale for preservation and protection (Chakraborty, 2011).

This study focused on the intertidal biofilms collected from a few heavily contaminated locations in the Sundarbans, specifically industrial areas, ferry terminals, or fishing harbors where motorized launch and trawler operations are regularly observed. Samples S14 and K16 were collected from the Patharpratima (21°43′37.00″N, 88°20′41.99″E) and the Kakdwip location (21°51'34.488"N, 88°10'32.3976"E) of the Indian Sundarbans. The selection of these two specific areas was made because, compared to Patharpratima, Kakdwip is closer to urban centers or industrial zones, leading to elevated levels of contamination from industrial activities, urban runoff, and sewage discharge. Consequently, this area hosts more industrial activities, such as manufacturing or processing plants, which can release pollutants into the environment, including chemicals, heavy metals, and organic compounds.

As cyanobacterial biofilm grows in a culture medium, the pH of the medium often tends to increase over time. This phenomenon is influenced by various biological and chemical processes associated with cyanobacterial metabolism and the composition of the growth medium(Touloupakis et al., 2016). To maintain the pH level within the appropriate range, a buffer solution was utilized. Buffering agents react with acids or bases by releasing or absorbing hydrogen ions (H+), which prevents pH levels from fluctuating. To maintain pH stability and provide the ideal growing environment for cyanobacterial biofilm, buffering agents are added to the growth medium. During the cultivation process, the pH of the growth medium was regularly monitored using a pH meter.

Due to the use of buffering agents, the cyanobacterial biofilms likely underwent several biochemical changes to adapt to these stressed conditions. Therefore, this study aimed to estimate the biochemical parameters of the biofilm samples, including chlorophyll content and exopolysaccharides (EPS). Total

chlorophyll content and primary components of total EPS (both capsular polysaccharide and released polysaccharide) including carbohydrate, protein, and uronic acid contents, were measured to determine the impact of these environmental stressors on the biofilms.

2. AIMS & OBJECTIVES

General Objective:

The primary objective is to assess the impact of controlled pH levels (7.2–7.8) on the development and biochemical characteristics of cyanobacterial biofilms, as well as the polysaccharides they produce.

Specific Objectives:

The primary objectives of this study are to employ a buffer system to create and sustain a controlled pH environment throughout the cyanobacterial biofilm growth period, ensuring precise pH stabilization. This methodology will facilitate the accurate measurement of biomass production in biofilms exposed to different pH levels, enabling a comprehensive analysis of pH-dependent growth dynamics. Additionally, the study aims to quantify and compare the pigment content, specifically carotenoids, and chlorophyll, in biofilms from both sites under various pH conditions. Furthermore, the research will involve detailed characterization of the extracellular polymeric substances (EPS), with a focus on their biochemical composition, including carbohydrates, proteins, and uronic acids, under varying pH environments. This analysis will elucidate the structural and functional adaptations of EPS in response to pH fluctuations, thereby contributing to our understanding of biofilm resilience and pollutant bioremediation potential in the Sundarbans' intertidal zones.

3. LITERATURE REVIEW

Biofilms and their formation:

Intended mudflats under intertidal regions contribute significantly to coastal habitats. These ecosystems sustain a vast diversity of organisms and provide numerous ecological services. The principal constituting material is slimy clay and silt, with charged surfaces, leading to the development of thin organic films derived from the single centration or multiple microbial aggregations known as biofilms. Biofilms are thought to be amongst the most widely distributed life forms on Earth (Yin et al., 2019). When prokaryotic and eukaryotic bacteria come into contact with soil particles and a mucilaginous matrix that they have generated on their own, they form biofilms. These biofilms are composed of many different microorganisms, such as bacteria, diatoms, algae, fungi, protozoa, etc. They can be either phototrophic or heterotrophic, depending on the type of microbes that make them up. The cohesive interaction between the mucilaginous extra polymeric components released from the biofilm and the sedimentary particles allows the biofilms to attach to the surface (Decho, 2000). Multi-species biofilms are complex assemblages of micro-organisms that may remain embedded in an exopolysaccharide (EPS) matrix. EPS facilitates close, mutually beneficial interactions within the microbial communities and protects the microbial cells against stress conditions. Biofilm is made up of 90% of EPS and 10% of algal cells. Biofilms can be used as metal pollution bioindicators. Biofilm formation can be divided into five stages: Initial reversible attachment (1), irreversible attachment (2-3), maturation (4) and dispersion (5). The initial contact of the moving planktonic bacteria with the surface is the starting point, which is still reversible at this stage. The bacteria will then start to form a monolayer and will produce an extracellular matrix or “slime” for protection. The matrix consists of extracellular polysaccharides, structural proteins, cell debris, and nucleic acids; referred to as extracellular polymeric substances (EPS) (Rather et al., 2021).

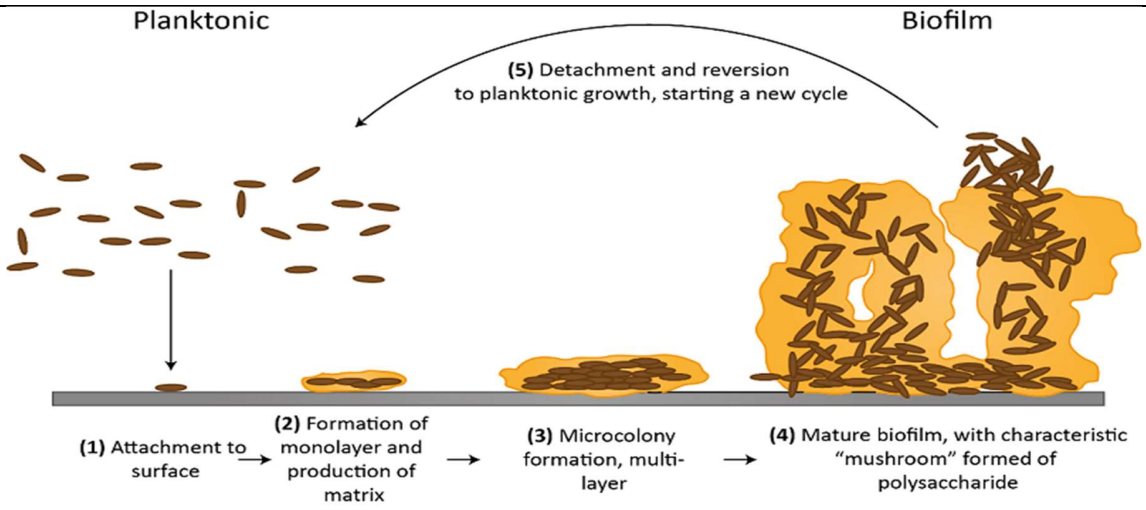


Figure 1: Formation and development of biofilm in different steps (Vasudevan, 2014).

Role of cyanobacterial exopolysaccharides in biofilm formation:

A crucial stage in the cyanobacterial cells adherence to solid surfaces is the excretion of extracellular polymers (EPSs), which is necessary to form phototrophic biofilms. Microbial adherence to solid substrates and the aggregation of coarse substrates appear to be enhanced through capsular polysaccharides (CPSs). The adhesion of cyanobacterial cells to solid surfaces is enhanced by the hydrophobic characteristics of the EPS (Neu and Marshall, 1990). Nearby cells in microbial partnerships also benefit from cyanobacterial extracellular polymers. Preferential fluxes of nutrients and water are created by the structuring of the biofilms, which is allowed by EPSs. A source of carbon for heterotrophs, EPM (Extracellular polymeric matrix) also produces moist microenvironments that shield the cells from physical damage and damaging sun radiation (Rossi and De Philippis, 2015).

EPS is composed of multiple biomolecules such as proteins, lipids, glycoproteins, and mono- and polysaccharides. Together with phospholipids, nucleic acids, and other macromolecules, these mucilaginous substances can occasionally impose restrictions on a startling amount of extracellular DNA.

The bulk of EPS is polyanionic because they include either ketal-linked pyruvate or uronic acids (glucuronic acid, mannuronic, and galacturonic), while others consist of neutral macromolecules. Inorganic residues and environmental biofilms often contain polysaccharides as their primary component, with phosphate being the most common and occasionally less frequently encountered. Due to this composition, they are sometimes referred to as extracellular polysaccharides (Karunaratne, 2012).

EPS can be classified into two primary categories: those that are released into the environment (also known as released polysaccharides or RPS) and those that are bound to the cell surface (also known as CPS). Based on the characteristics of the EPS bounded to the cell surface, these can be referred to as sheaths, capsules, or slimes, depending on their thickness, consistency, and appearance (ten Cate et al., 2009). The soluble parts of polysaccharide material known as RPS have been released into the medium, either from the outer layer or layers or as a result of a biosynthetic process that is separate from the CPS synthesis process. There can be large differences in the sugar contents of CPS and RPS, indicating different biosynthetic pathways that rely on different environmental factors (Ibrahim et al., 2022).

Adhesion to surfaces, bacterial cell aggregation into flocs and biofilms, stabilization of the biofilm's structural integrity along the process of stabilization of the adhering substratum of the same, formation of a protective barrier that offers resistance to biocides and other harmful effects, retention of water, sorption of exogenous organic compounds for the absorption of nutrients from the environment, and accumulation of enzymatic activities, such as the digestion of exogenous macromolecules for nutrient absorption, among other functions carried out by the EPS matrix (Laspidou and Rittmann, 2002).

According to Sutherland (Sutherland, 1999) and Davies (Di Martino, 2018)genes generating polysaccharide lyases, responsible for breaking down EPS, are linked to the process of exopolysaccharide production. During the polysaccharide synthesis, cells appear to continuously manufacture these lyases; however, they are shielded from the developing polysaccharide. But when these exopolysaccharide-

producing bacteria lyse, these enzymes passively secrete, which causes the EPS matrix to dissolve slowly but continuously. But when these exopolysaccharide-producing bacteria lyse, these enzymes passively secrete, which causes the EPS matrix to dissolve gradually but steadily.

Natural and anthropogenic influence on ocean pH:

One of the major reasons for health and sustainability in marine ecosystems is the ocean pH, a basic component of marine chemistry. There are cumulative problems for the multiple factors that decrease pH in marine ecosystems and biodiversity. Although most agree that the primary driver of ocean acidification is the absorption of atmospheric CO2, a wealth of research suggests natural and man-made impacts on the ocean's pH.

During volcanic eruptions, sulfur dioxide gas and other gases are emitted into the atmosphere. This gas reacts with seawater to form sulfuric acid, thereby forming localized acidification of marine environments. This outgassing of volcanic aerosols can perturb the chemistry of the oceans and, in this way, carbonate ion distribution and pH levels (Harvey and Huang, 1995).

Natural weathering processes, for example, the disintegration of rocks and minerals, produce ions and alkaline materials in the ocean. The weathering of silicate minerals is a source of bicarbonate ions that act as a buffer against any variation in pH. However, how much the buffering would depend on the constitution of rock and its rate of weathering (Caldeira and Wickett, 2003).

Coastal waters are overflowed with nutrients due to human activities including wastewater discharge and agricultural runoff. Because of the nutrient enrichment, algal blooms are fuelled, and when they break down, dissolved oxygen is reduced and carbon dioxide is released, which causes localized acidification and hypoxia. Changes in ecosystem dynamics that follow may have a domino effect on marine biodiversity and ecological services (Doney, 2010).

Nitrogen oxides (NO_x), sulfur dioxide (SO_2), and other pollutants are released into the environment during industrial processes. Rainfall and atmospheric deposition cause these pollutants to undergo chemical reactions in the atmosphere, generating acidic chemicals that end up in the ocean (Bencs et al., 2008). The resilience of marine ecosystems may be compromised by this acidification process, which could also make the problems caused by ocean acidification worse.

During mining operations, sulphide minerals in rocks and ores come into contact with air and water and can result in acid mine drainage or AMD. The process of oxidation of sulphide minerals produces sulfuric acid and metal ions that contaminate surface and groundwater. This can consequently result in the local acidification of the affected areas and the pollution of the marine environment because AMD is released as extremely acidic, metal-rich fluids into nearby water bodies such as rivers and coastal zones. Specifically, near mining areas, AMD can reduce pH, increase metal toxicity, and disrupt the function of marine ecosystems (Simate and Ndlovu, 2014).

Acid rain is a result of acidic pollutants such as nitric and sulfuric acids. Acid rain directly decreases the pH of seawater when it hits the ocean's surface, which has an effect on marine life and ecological processes. Both offshore and coastal habitats may be impacted by acid rain-induced acidification, which can happen across large spatial dimensions (Driscoll et al., 2007).

Ocean acidity and carbon cycling are influenced by climate-driven variables such as sea surface temperature, precipitation patterns, and circulation patterns. pH gradients in various maritime regions and the distribution of carbon-rich waters can both be impacted by climate-induced changes in ocean circulation and upwelling dynamics (Gruber, 2011).

The principle and selections of the buffering system:

The term "buffering" originates from the discovery that weak acid or weak base partially neutralized solutions are resistant to pH shifts upon the addition of tiny volumes of strong acid or strong base. An acid and its conjugate bases, such as acetate and acetic acid or carbonate and bicarbonate, make up a buffer. A buffer's buffering capacity, or its ability to withstand pH changes caused by the addition of strong acids or bases, determines its quality (Perrin and Dempsey, 1974).

An effective buffer operates via the common ion effect. When a given ion is added to a mixture that already contains the given ion at equilibrium, the common ion effect happens. The balance moves away from producing more of that ion at that point. The Henderson-Hasselbalch equation (pH = pka + log(acid/base) if working with a weak acid and conjugate base) makes it possible to determine buffer pH without first determining the hydrogen ion concentration (Tallarida and Murray, 1987).

The amount of acid or base that can be added to a buffer without significantly changing its pH is known as its buffer capacity. More acid and base can be added to a substance with a higher capacity before the pH noticeably shifts. $B = n/\Delta pH$, where B is buffer capacity (which is unitless), n is the number of moles of acid or base that was added to the buffer per liter of the buffer, and ΔpH is the pH difference between the initial buffer's pH and the pH after the addition of acid or base to the buffer (Moran, 2018).

Researchers have historically had limited access to compounds that may be used as hydrogen ion buffers between pH 6 and 8. Consequently, biological research has employed several highly unsuitable buffers. The buffers proposed by Good et al(Good et al., 1966) were: MES, ADA, PIPES, ACES, cholamine, BES, TES, HEPES, N-(2- acetamido)glycine, tricine, glycinamide hydrochloride, and bicine. Here, twelve pH buffers are proposed for use in biological studies as alternatives to traditional buffers.

The purpose of using these buffers is that Good's buffers possess strong buffering capabilities within the physiological pH range (typically between pH 6 and 8), along with well-defined pKa values. They are made to interact with biological molecules and cellular functions as little as possible. They are created or chosen to be non-toxic, chemically inert, and compatible with biological materials so as not to disrupt the course of experiments or impair the survival of cells. Good's buffers come in a variety of chemical compositions, concentrations, and pH ranges, the choice of buffers can be focused on specific experimental needs. They find application in a lot of biological procedures, from molecular biology methods through cell culture and protein purification to enzyme tests (Ferreira et al., 2015).

4. MATERIALS AND METHOD

4.1 Site of Study:

Our senior research teams conducted systematic collections of intertidal biofilm samples during low tides in November 2018 and January 2023 from strategically selected sites in the Sundarbans. These samples were meticulously sub-cultured under controlled laboratory conditions to maintain their integrity and viability. Two of these sub-cultured biofilms were used in this research, which allowed for a thorough analysis of their physiological and biochemical reactions to different experimental conditions. The study focused on two distinct riverine intertidal sites within the South 24 Parganas district of the Indian Sundarbans: Patharpratima (21°43′37.00″N, 88°20′41.99″E) and Kakdwip (21°51'34.488"N, 88°10'32.3976"E). These sites were deliberately chosen for their differing levels of anthropogenic influence and environmental conditions, allowing for a comprehensive comparative analysis of biofilm responses under varying pH regimes. The selection criteria ensured a robust evaluation of how biofilms adapt to diverse stressors, providing valuable insights into the ecological dynamics of these critical coastal ecosystems.

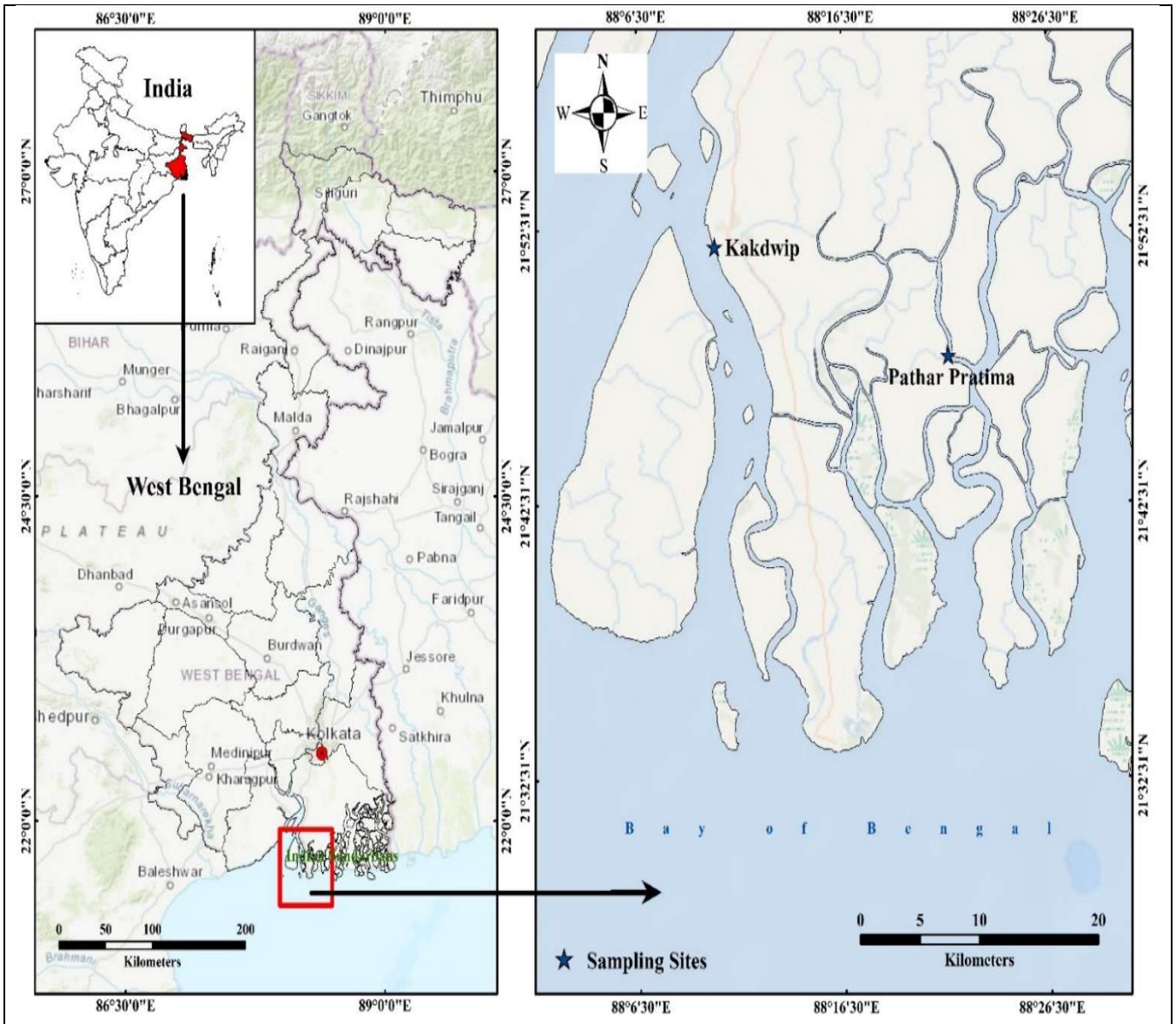


Figure 2: Two main sampling sites Patharpratima and Kakdwip.

4.2 Experimental culturing of Biofilm in a controlled environment:

Biofilm samples were cultured in Conico Cylindrical Flasks (CCF) containing a 1:1 mixture of BG11 and ASN III media. To stabilize the pH of the media, two different types of buffers were used separately (according to their favorable conditions). Additionally, non-buffered samples were grown as controls for each experimental condition. These samples were grown in two sets of CCFs: one made of polymethyl methacrylate (PMMA) and the other made of glass. Approximately 150 mg of each sample was inoculated into the sterile mixed media within these flasks. The cultures were grown for 35 days, with continuous pH monitoring of each medium. After the growth period, the samples were subjected to biochemical assays.

Figure 3: Biofilm culture in two conico cylindrical flasks (CCF) made of PMMA and glass.

4.2.1 Media preparation:

The appropriate amounts of salts were weighed according to the desired composition of ASN III and BG11 mixed medium(Witthohn et al., 2023). The salts were dissolved in sterilized water, excluding potassium phosphate (K_2HPO_2) and sodium carbonate (Na_2CO_3). The medium was autoclaved to sterilize it, ensuring thorough mixing before autoclaving to achieve a uniform concentration. After autoclaving, the medium was allowed to cool to around room temperature. Then, the two remaining salts (phosphate and carbonate) were added, ensuring thorough mixing. The pH of the medium was adjusted to 7.5-8.0 by adding the desired amount of acid (e.g., hydrochloric acid, HCl) or base (e.g., sodium hydroxide, NaOH), and a pH meter was used for accurate measurement.

Table 1. Chemical composition of the media:

ASN-III medium composition		BG-11 medium composition		*Trace metal mix A5	
Ingredients	Amount	Ingredients	Amount	Ingredient	Amount
NaCl	25 g	$NaNO_3$	1.5g	H_3BO_3	2.860g
$MgSO_4$ *$7H_2O$	3.5g	K_2HPO_4	0.04g	$MnCl_2$* $4H_2O$	1.810g
$MgCl_2$ *$6H_2O$	2g	$MgSO_4$ *$7H_2O$	0.075g	$ZnSO_4$ *$7H_2O$	0.222g
$NaNO_3$	0.75g	$CaCl_2$ *$2H_2O$	0.036g	Na_2MoO_4 *$2H_2O$	0.390g
K_2HPO_4 *$3H_2O$	0.02g	Citric acid	0.006g	$CuSO_4$ *$5H_2O$	0.079g
KCl	0.5g	Ferric ammonium citrate	0.006g	$Co(NO_3)_2$ *$6H_2O$	0.0494g
$CaCl_2$ *$2H_2O$	0.5g	EDTA (disodium salt)	0.001g	Distilled water	1 l
Citric acid ($C_6H_8O_7$)	0.003g	Na_2CO_3	0.02g		
Ammonium ferric citrate ($C_6H_8O_7$ * nFe *nNH_3)	0.003g	Trace metal mix A5*	1.0 ml		
$EDTANa_2Mg$	0.0005g	Distilled water	1 l		
Na_2Co_3	0.02g				
Trace metal mix A5*	1 ml				
Distilled water to	1 l				

4.2.2 Selection of buffer:

In a general way, the control of the pH was achieved by adding an appropriate buffer to the system, according to the desired pH range. There were certain drawbacks to using conventional buffers in biological or complex systems, such as phosphate, citrate, borate, and succinate. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and Tricine (N-[Tris(hydroxymethyl)methyl]glycine) had good buffering capacity, low toxicity, chemical stability, and compatibility with specific experimental conditions(Ferreira et al., 2015). The following buffers (25 mM) were used for the measurement of the effect of pH on the activity of cyanobacteria during the growth period: HEPES for pH 6.8 to 7.5, Tricine for pH 7.5 to 8.5. Buffering cyanobacterial growth media with Tricine and HEPES buffer ensured the maintenance of pH homeostasis during cultivation. Tricine and HEPES were commonly used buffering agents in cyanobacterial culture due to their effectiveness within the physiological pH range of most cyanobacterial species. The appropriate amount of Tricine and HEPES was dissolved separately in distilled or deionized water to achieve the desired concentration (25 mM) (Cohen et al., 1986). The appropriate volume of the prepared buffer solution (Tricine or HEPES) was added to achieve the desired buffering capacity. The pH was adjusted to 7.2 for HEPES and 7.8 for Tris using HCl(1N) or NaOH(1N) and a pH meter.

4.2.3 Growth condition and continuous monitoring of pH:

Each strain of cyanobacteria was cultured under fluorescent irradiance (50 μmol photon /m^2 s) with a 14:10 h light: dark photoperiod at 27 ± 1 °C. The pH of each culture was regularly monitored using a calibrated pH meter.

4.3 Estimation of chlorophyll and carotenoids:

Chlorophyll and carotenoids of cultured samples were determined on the 35th day. 20 mg of biofilm samples were homogenized in 5 ml of 100% methanol. The solution was then transferred to a falcon tube and centrifuged for 5 minutes at 10,000 RPM. After centrifugation, the supernatant was collected, and absorbance was measured spectrophotometrically at five different wavelengths (480nm, 632nm, 652nm, 665nm, 696nm, and 750nm) and different types of chlorophylls were measured, and the total chlorophyll content was determined using the procedure described in (Osório et al., 2020), as outlined below:

Chl a (µg/ml) = − 2·0780×(A632-A750) − 6·5079×(A652-A750) +16·2127× (A665-A750) − 2·1372×(A696-A750) (±0·0070)

Chl b (µg/ml) = − 2·9450 × (A632-A750) +32·1228×(A652-A750) − 13·8255× (A665- A750) − 3·0097× (A696 -A750) (±0·0212)

Chl c (µg/ml) = 34·0115 × (A632-A750) − 12·7873×(A652-A75) +1·4489× (A665-A750) − 2·5812×(A696-A750) (±0·0120)

Chl d (µg/ml) = − 0·3411 × (A632-A750) +0·1129×(A652-A750) − 0·2538× (A665-A750) +12·9508×(A696-A750) (±0·0031)

Total Chl (µg/ml) = Chl a+ Chl b+ Chl c+ Chl d

Carotenoids (µg/ml) = 4 × (A480 − A750)

4.4 Estimation of Extracellular Polymeric Substances (EPS):

The primary components of EPS include proteins, lipids, exopolysaccharides, and nucleic acids, combining to form a three-dimensional polymer network that binds the biofilm to surfaces and keeps cells near to one another, creating a community that works well together.

Two fractions of exopolysaccharides, CPS and RPS, were extracted from the biomass obtained from strains grown in culture using the method outlined in (Barranguet et al., 2005, 2004). Biomass harvested from both flasks was centrifuged at 5000 rpm (revolutions per minute) for 10 minutes, resulting in the separation of RPS in the supernatant and CPS and other cells as the pellet.

4.4.1 CPS Extraction:

The pellet containing the cell-bound portion of the extracellular polymeric substances was incubated with 0.1 M sulfuric acid at 95°C for 1 hour. After the incubation period, samples were centrifuged at 5000 rpm for 5 minutes. The resultant supernatant, containing the cell-bound exopolysaccharides, was separated from the pellet (cells) and precipitated in a 1:3 ratio of 96% ethanol. The ethanol-containing CPS was then centrifuged at 10,000 rpm for 10 minutes, and the pellets were washed with 65% ethanol to remove any contamination. After washing, the pellets were freeze-dried and stored at -20°C.

4.4.2 RPS Extraction:

To precipitate the EPS fraction from the supernatant containing RPS, 99% chilled ethanol in a ratio of 1:3 (v/v) was used. The ethanol solution containing RPS was centrifuged at 10000 rpm for 10 minutes. Subsequently, the pellets were washed with 65% ethanol to remove any contamination. After washing, the pellets were freeze-dried and then stored at -20℃.

4.5 Dubois's method:

The phenol–sulfuric acid method (Dubois's method) (DuBois et al., 1956) demonstrated high sensitivity with stable color development. Glucose was used as the standard for creating the calibration curve to determine the carbohydrate concentration. 1 ml of standard or extracted sample was added to 1 ml of 5% phenol and 5 ml of 96% sulfuric acid. After 10 minutes of reaction, the samples were vortexed and left for 30 minutes at room temperature. Subsequently, the samples were measured spectrophotometrically at 490 nm.

Figure 4: Different concentrations of glucose can be visually distinguished by changes in color intensity.

4.6 Bradford protein assay:

Coomassie Brilliant Blue G-250 is a dye that has a λ_{max} of 470 nm when not associated with protein, but the λ_{max} shifts to 590 nm when bound to protein. This reaction occurred relatively rapidly, and the resulting complex remained stable for approximately 1 hour. Bovine serum albumin (BSA) from Sigma was used as the standard. 3 ml of Bradford reagent was added to 1 ml of standard or extracted sample. After that, each sample was incubated for 30 minutes at 37°C. When the reagent was added to protein standard

solutions (ranging from 100–1000 µg/ml), the color changed to blue. Samples were measured spectrophotometrically at 590 nm (Richards et al., 2020).

Figure 5: Visualization of protein concentration via Bradford assay colorimetric change.

4.7 Estimation of uronic acid:

The presence of uronic acid in the EPS was checked by obtaining the absorbance of the reaction mixture at 530 nm, using galacturonic acid as a standard, and employing the sulfuric acid-carbazole method(Haug et al., 1962). 1 ml of standard or extracted sample was added to 3 ml of 96% sulfuric acid (for samples, acid borate was used instead of sulfuric acid) and 0.1 ml of 0.1% carbazole. Each sample was then incubated in a boiling water bath for 60 minutes at 90°C.

Figure 6: Colorimetric detection (purple color) of Uronic Acids via sulfuric acid-carbazole Method.

4.8 Resazurin assay:

The resazurin assay protocol is based on the reduction of oxidized non-fluorescent blue resazurin to a pink fluorescent dye (resorufin) by the mitochondrial respiratory chain in live cells. The amount of resorufin produced is correlated with the amount of fluorescence produced. 180 µl of phosphate buffer solution (pH-7.2) was added to a 96-well microtiter plate containing 10mg of fresh biofilm, followed by the addition of 20 µl of 0.2 mg/ml resazurin. The plate was then immediately incubated in the dark for 12 hours. Absorbance measurement values at 570 nm were obtained using a microplate reader(Van den Driessche et al., 2014).

Figure 7: Fluorescence detection of live cell activity in biofilm using the Resazurin Assay.

4.9 Mass-Spectroscopy-

Mass spectrometers are typically not standalone instruments. Most often, they are connected physically and electronically to a chromatograph and a computer. The chromatograph separates mixtures and introduces the sample into the mass spectrometer. The mass spectrometer ionizes analyte molecules, and then separates and detects the resulting ions. The computer system controls the operation of the

chromatograph and the MS, and provides data manipulation and storage during and after data collection (Smith, 2004). Figure 7 shows a typical arrangement of a chromatograph/mass spectrometer/computer system.

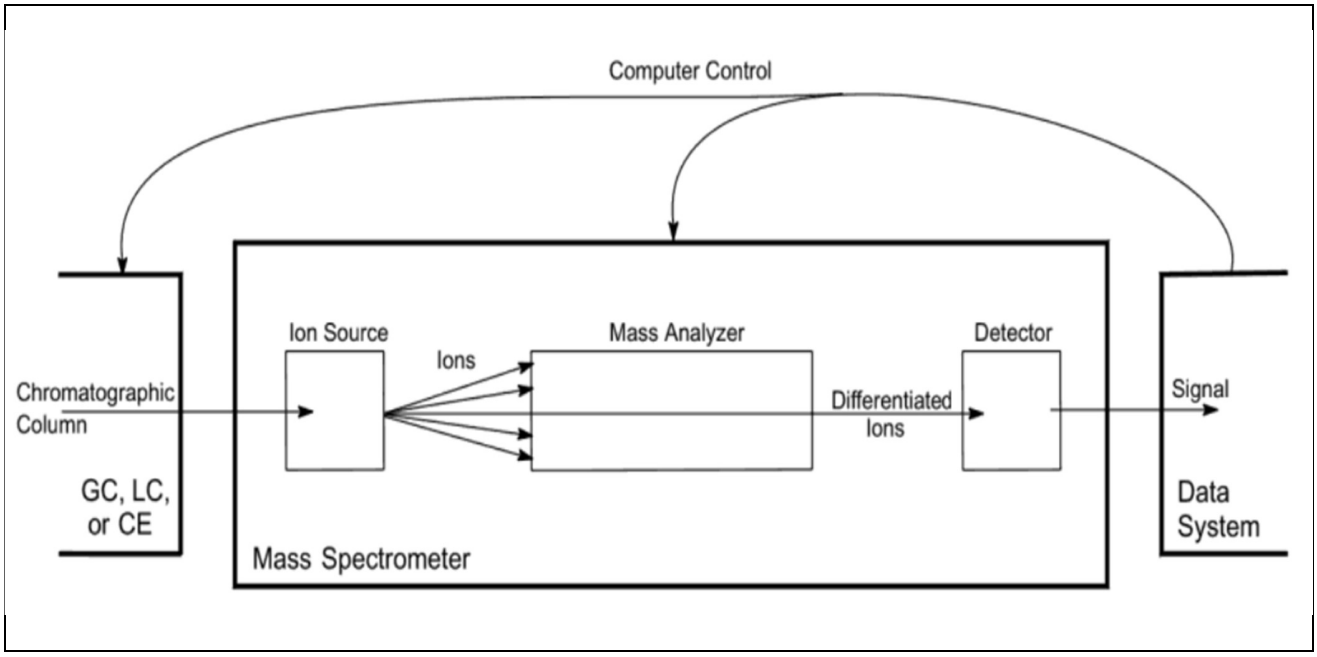


Figure 8: Block diagram of a chromatograph/mass spectrometer/computer system.

Due to the low pH conditions, the sample designated K16 did not exhibit optimal growth even after 35 days. Consequently, the sample's growth media was then examined using mass spectrometry to look for any potential metabolites or compounds to determine what might be affecting the growth. Mass spectrometry analysis of the biofilm culture medium provided different m/z values for different ions. As a way of making sure that proper identification of the compounds present is done, provisions were made to factor in the contributions of hydrogen and potassium. The observed m/z values were initially put into consideration against the potential protonation. Hydrogen ions, that contribute approximately 1.00784 Da, were subtracted from the observed m/z to obtain the actual mass of the compounds. Adducts of potassium, contributing approximately 39.0983 Da to the observed m/z values, were also taken into consideration. In

this respect, the mass of the compound without potassium adducts could be deducted when the mass of potassium was subtracted from the observed m/z values. These values were then used to query mass spectral databases and software tools for identification of the compounds in the biofilm culture medium. These corrected values gave more correct molecular masses that enabled the identification of exact compounds (De Vijlder et al., 2018).

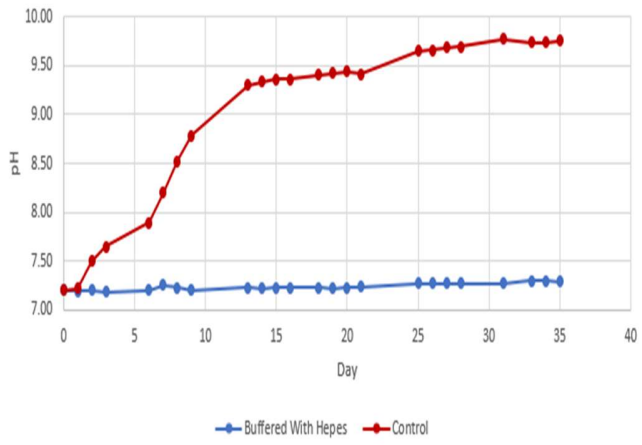
5. RESULTS & DISCUSSION

5.1 Growth of biomass and pH evolution:

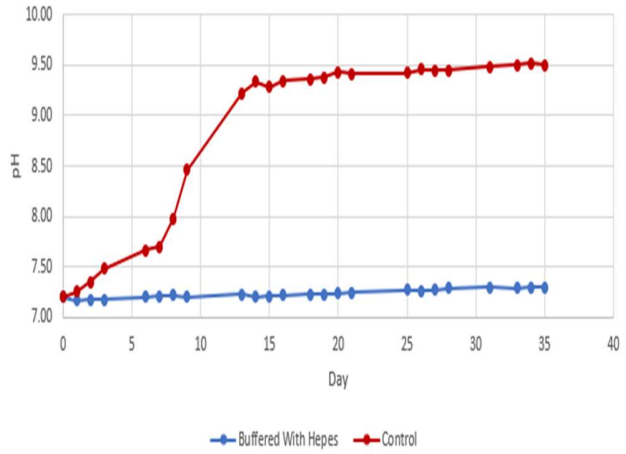
Two growth experiments were carried out in triplicate with cultures of K16 and S14. The medium had an initial pH of 7.2 and was either non-buffered or buffered by HEPES (25 mM). In another set of experiments, Tricine (25 mM) was used as a buffer, and a non-buffered medium with an initial pH of 7.8 was tested. Both experiments were carried out in two type of culture vessel types: Polymethyl methacrylate (PMMA) and glass.

For the S14 samples inoculated with the non-buffered medium, the pH increased within seven days from 7.2 to approximately 8.5 (in both PMMA and glass flasks). Then, the pH increased slowly during cell growth to reach 9.5-9.8 after 35 days at the time of EPS harvest. In the buffered medium, the pH remained near to contestant at 7.2±0.2(in both PMMA and glass flasks) for HEPES and 7.8±0.3(in both PMMA and glass flasks) for Tricine. The growth in the non-buffered medium was faster than in the buffered medium.

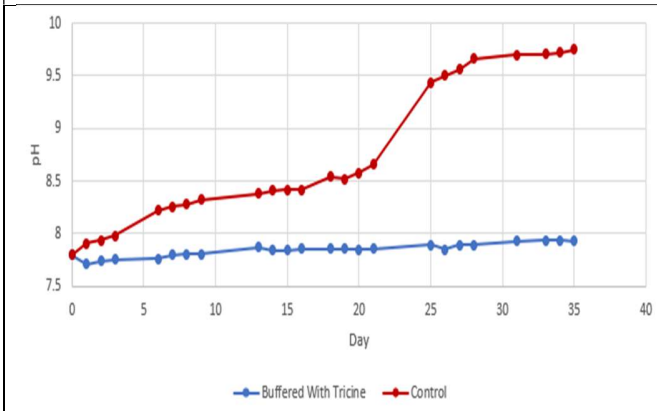
Cyanobacteria perform photosynthesis, which involves the uptake of carbon dioxide (CO_2). This process reduces hydrogen ion concentration and increases pH (Stal and Moezelaar, 1997). Cyanobacteria assimilate ammonia (NH_4^+), which can lead to the production of hydroxide ions (OH^-), raising the pH (Flores and Herrero, 2005). The natural buffering capacity of the medium might be insufficient to counterbalance the changes brought about by cyanobacterial metabolic activities (Markou and Georgakakis, 2011). These processes are the primary reasons for the increase in the pH of biofilm culture media during the growth period.



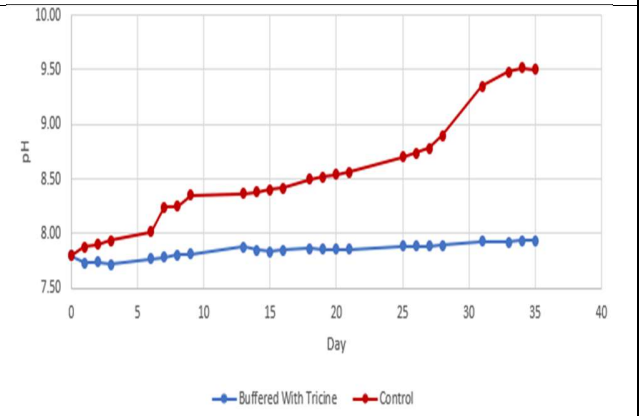
pH of medium buffered with HEPES and control for S14 sample (PMMA Flask)



pH of medium buffered with HEPES and control for S14 sample (Glass Flask)



pH of medium buffered with Tricine and control for S14 sample (PMMA Flask)



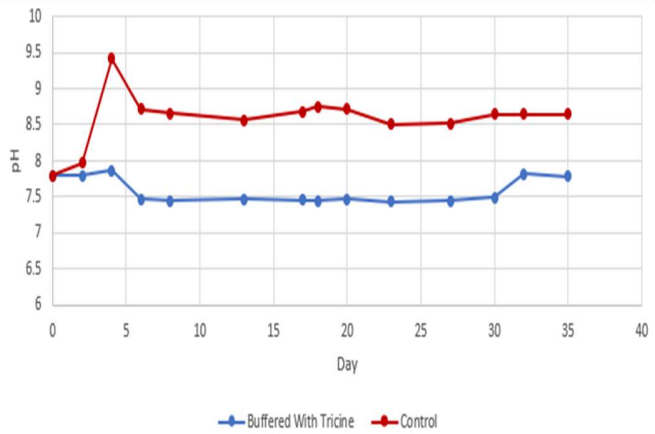
pH of medium buffered with Tricine and control for S14 sample (Glass Flask)

Figure 9: Regular pH monitoring data of S14 samples for buffered and non-buffered samples. The points represent the average pH of three replicates.

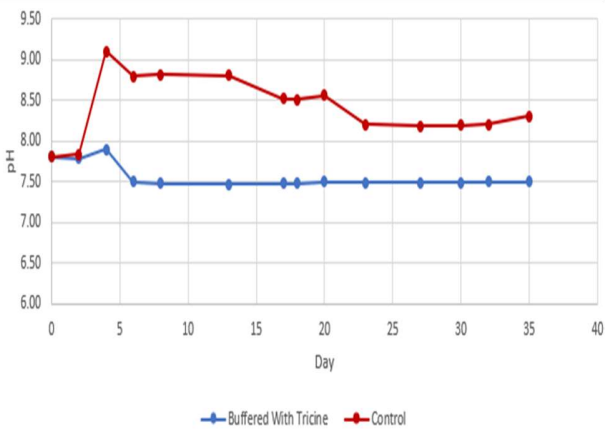
For the samples inoculated with K16, the non-buffered medium pH increased within three days from 7.2 to approximately 9.5 (in both PMMA and glass flasks). Two weeks following the inoculation date, a slightly lower pH value was recorded. Then, the pH increased slowly during cell growth to reach 8.2-9.5 (in both PMMA and glass flasks) after 35 days.

In the buffered medium, the pH remained near to contestant at 7.2±0.3 (in both PMMA and glass flasks) for HEPES and 7.8±0.3(in both PMMA and glass flasks) for Tricine.

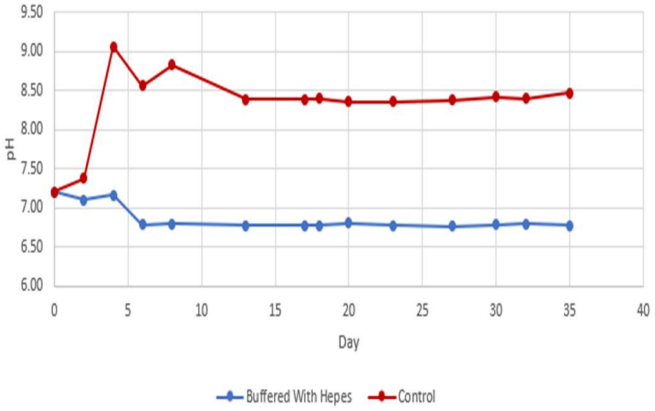
For K16 samples, even after 35 days, the biofilm culture growth was observed to be significantly low. Biomass production was quite minimal (in both PMMA and glass flasks) and not sufficient for EPS extraction.



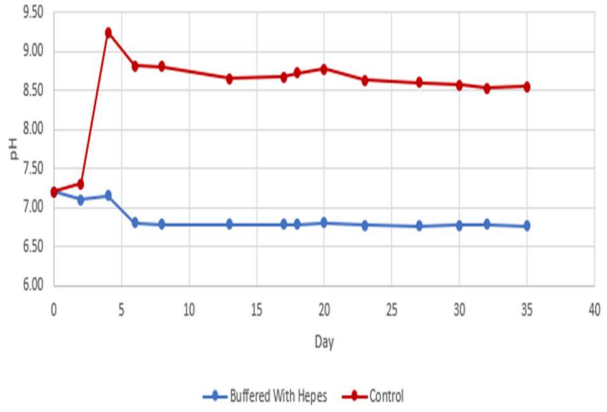
pH of medium buffered with Tricine and control for K16 sample (PMMA Flask)



pH of medium buffered with Tricine and control for K16 sample (Glass Flask)



pH of medium buffered with HEPES and control for K16 sample (PMMA Flask)



pH of medium buffered with HEPES and control for K16 sample (Glass Flask)

Figure 10: Regular pH monitoring data of K16 samples for buffered and non-buffered samples. The points represent the average pH of three replicates.

5.2 Chlorophyll Estimation:

The S14 samples buffered with tricine (initial pH of 7.8) had higher chlorophyll levels than those buffered with HEPES (initial pH of 7.2), according to the chlorophyll estimation from the samples. Chlorophyll levels in non-buffer samples were lower than those in buffer samples, with HEPES-buffered samples showing higher chlorophyll concentrations compared to non-buffered samples. The sample in the PMMA flask with tricine (initial pH of 7.8) contained the highest amount of total chlorophyll.

In comparison to the S14 samples, the total chlorophyll concentration in the K16 samples was significantly lower. All buffer samples, compared to non-buffered samples, displayed higher levels of chlorophyll, with Tricine-buffered samples showing higher chlorophyll content than HEPES-buffered samples. The sample in the PMMA flask buffered with Tricine (initial pH of 7.8) had the highest total chlorophyll concentration.

Table 2: Total Chlorophyll concentration in the S14 samples.

Sample Name	Average Chlorophyll Concentration(µg/gm)
HC(PMMA)	1557.05
H(PMMA)	1250.06
TC(PMMA)	1401.65
T(PMMA)	2233.92
HC(GLASS)	2129.10
H(GLASS)	2030.84
TC(GLASS)	2055.09
T(GLASS)	2061.80

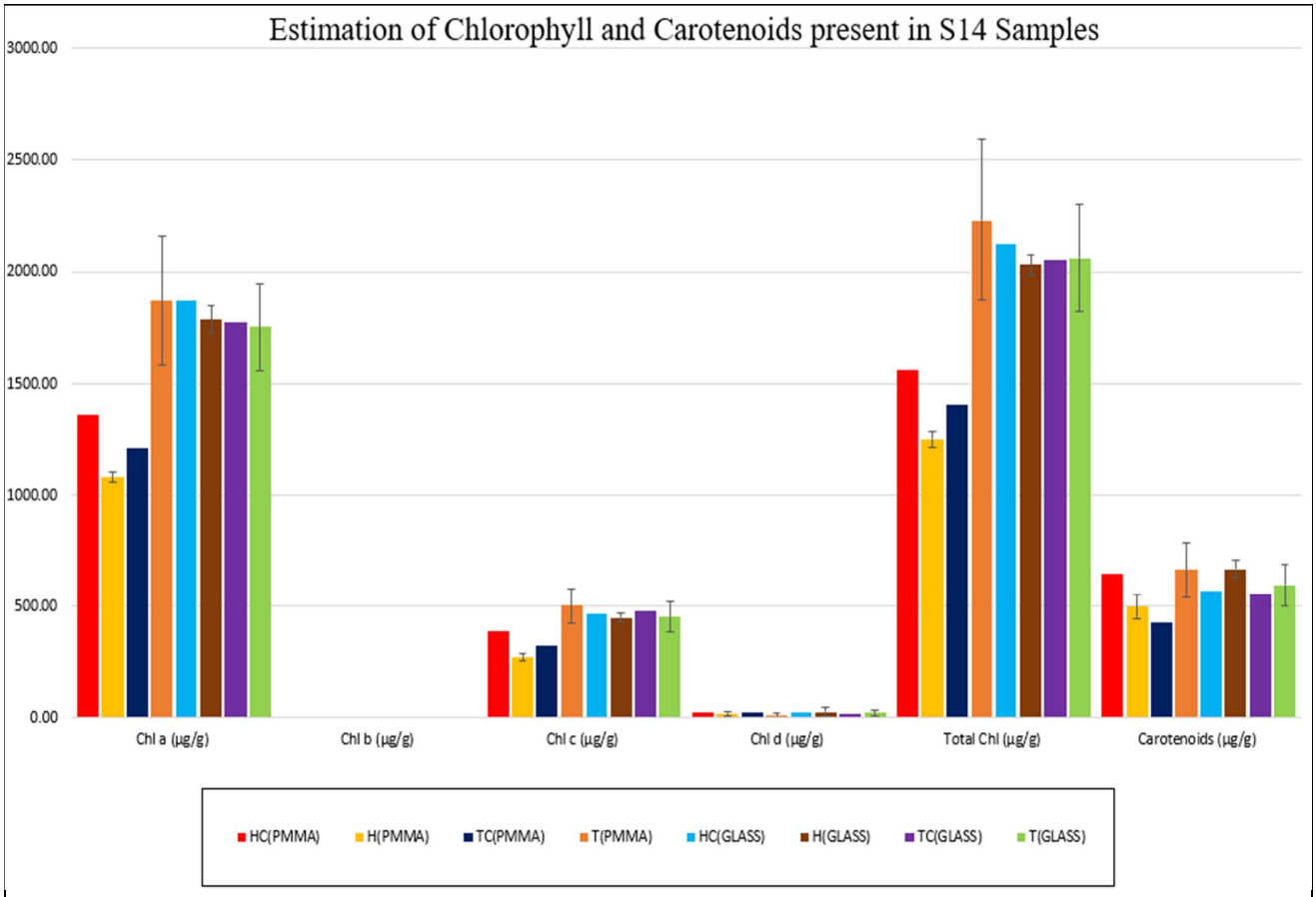


Figure 11: Bar graphs show chlorophyll and carotenoids concentration in S14 samples, with error bars representing the standard deviation of triplicate samples.

Table 3: Total Chlorophyll concentration in K16 samples.

Sample Name	Average Chlorophyll Concentration(µg/gm)
HC(PMMA)	1547.57
H(PMMA)	1768.17
TC(PMMA)	991.25
T(PMMA)	1893.63
HC(GLASS)	966.05
H(GLASS)	1475.16
TC(GLASS)	1316.11
T(GLASS)	1653.90

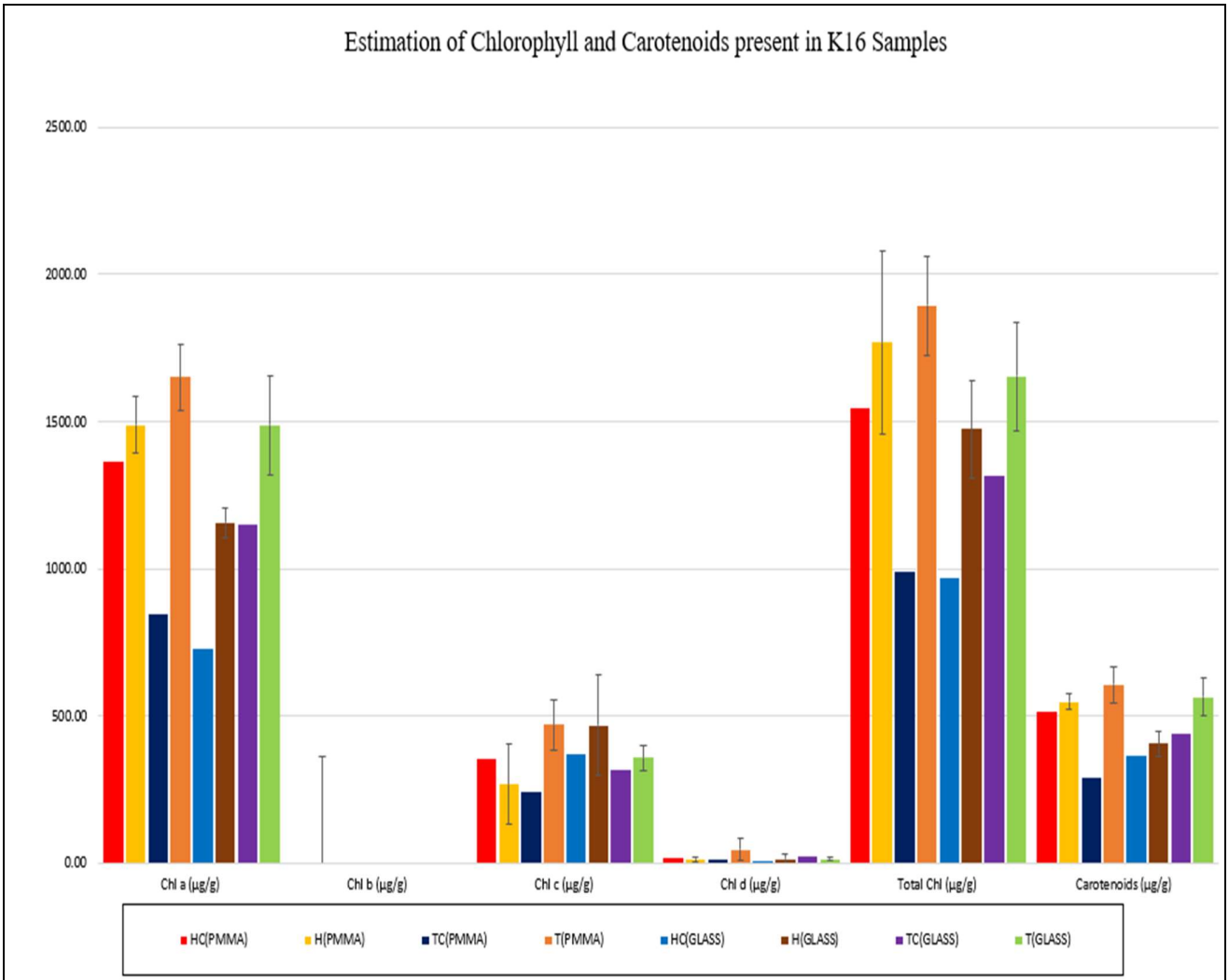


Figure 12: Bar graphs representing various chlorophyll and carotenoid concentrations in K16 samples and the error bar represents the standard deviation of triplicate samples.

The results indicate that buffering the culture media significantly enhances chlorophyll production in both S14 and K16 cyanobacterial biofilms. Tricine buffer (initial pH 7.8) is more effective in promoting chlorophyll synthesis than HEPES buffer (initial pH 7.2)(Good et al., 1966; Pratte and Thiel, 2021). Additionally, the material of the flask influences the chlorophyll concentration, with glass generally providing better conditions for chlorophyll accumulation in S14 samples, while PMMA was more effective for tricine-buffered K16 samples.

5.3 Resazurin assay:

The increased absorbance in spectrophotometry was directly correlated with the quantity of resorufin produced and the number of metabolically active cells. Compared to non-buffered samples, all buffered samples displayed higher absorbance. K16 samples had considerably lower absorbance than S14 samples. Buffered samples showed higher metabolic activity, and among them, S14 showed the most prominent activity in comparison with K16.

In buffered samples, the environment is more stable, leading to higher metabolic activity and, consequently, higher production of resorufin, which results in increased absorbance(Van den Driessche et al., 2014).

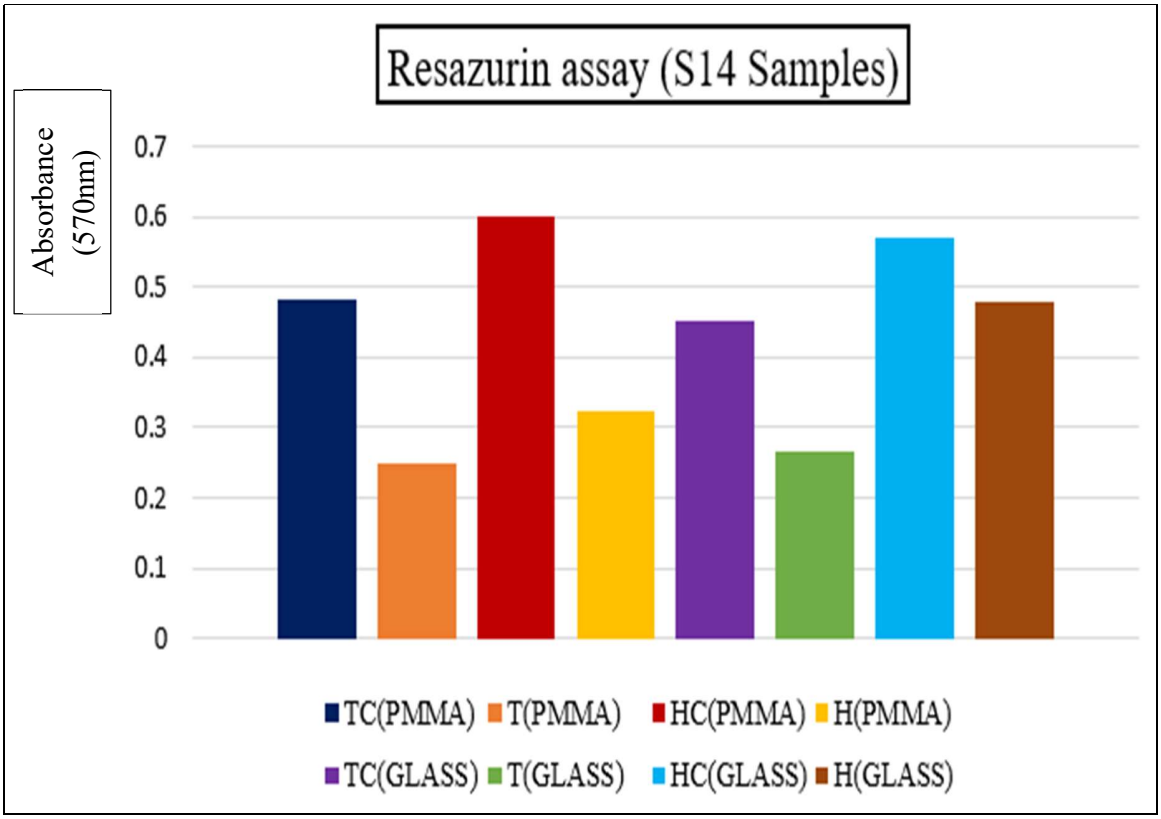


Figure 13: S14 sample's resazurin assay results. Bar charts displaying the absorbance for various samples.

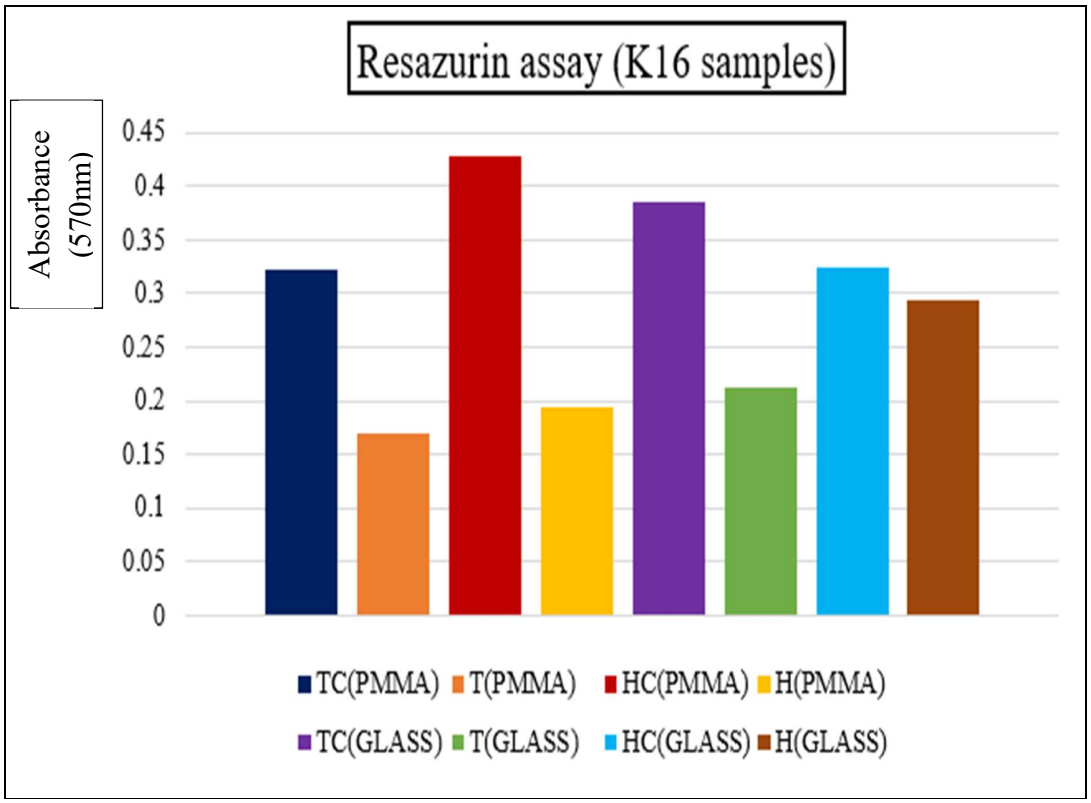


Figure 14: K16 sample's resazurin assay results. Bar charts displaying the absorbance for various samples.

5.4 EPS production estimation:

The biomass removed from the culture was measured for dry weight. The biomass produced in the S14 sample is given in Table 4 below. The PMMA flask buffered with Tricine (initial pH-7.8) produced the highest quantity of biomass. Compared to the glass flask, the PMMA flask produced more average biomass. The amount of biomass generated in the K16 samples was insufficient (>300mg) for EPS extraction. Compared to its control samples(non-buffered), all of the buffered samples (both HEPES and Tricine) consistently had higher CPS. With an initial pH of 7.8, the sample in the PMMA flask buffered with Tricine had the highest amount of CPS.

Table 4: Amount of biomass and EPS content of S14 biofilm samples.

Sample Name	Amount of biomass extracted (mg)	Capsular EPS content (mg/g)
HC(PMMA)	520	8.03
H(PMMA)	547	10.17
TC(PMMA)	574	8.18
T(PMMA)	540	12.16
HC(GLASS)	570	8.59
H(GLASS)	545	9.89
TC(GLASS)	512	6.30
T(GLASS)	536	10.52

5.5 EPS Characteristics:

The biomolecules that makeup EPS include mono and polysaccharides, proteins, glycoproteins, uronic acids (glucuronic acid, mannuronic and galacturonic), etc. EPS can be classified into two major categories, those bound to the cell surface (CPS) alongside those released into the environment (released polysaccharides, or RPS). The amounts of CPS and RPS extracted are shown in Table 5 below.

Table 5: Total amount of CPS & RPS extracted from S14 Samples.

Sample Name	Amount of CPS extracted (mg)	Amount of RPS extracted (mg)
HC(PMMA)	4.17	12.2
H(PMMA)	5.56	36.2
TC(PMMA)	4.69	13.9
T(PMMA)	6.56	24.8
HC(GLASS)	4.90	12.5
H(GLASS)	5.39	24.2
TC(GLASS)	3.22	14.7
T(GLASS)	5.63	19.2

Table 6: Concentration of CPS extracted from the biomass of S14 Samples.

Sample Name	Amount of biomass extracted (mg)	Capsular EPS content (mg/g)
HC(PMMA)	520	8.03
H(PMMA)	547	10.17
TC(PMMA)	574	8.18
T(PMMA)	540	12.16
HC(GLASS)	570	8.59
H(GLASS)	545	9.89
TC(GLASS)	512	6.30
T(GLASS)	536	10.52

5.5.1 Carbohydrate content:

The carbohydrate content of EPS was higher in the buffered medium compared to the non-buffered medium (Table 7). The sample in the glass flask buffered with Tricine (with an initial pH of 7.8) had the highest carbohydrate concentration. Buffering agents such as Tricine aid in maintaining a stable pH environment, which can enhance the action of enzymes involved in the production and stabilization of carbohydrates (Journet and Douce, 1985). pH fluctuations may trigger the breakdown of carbohydrates. To maintain the carbohydrate level, buffering helps to prevent such pH shifts.

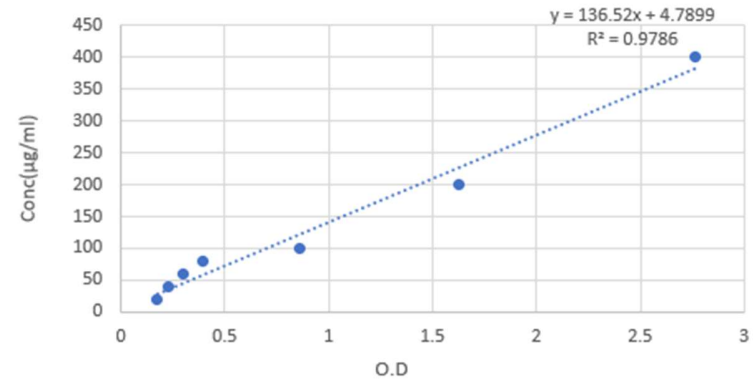


Figure 15: Standard curve of glucose

Table 7: carbohydrate content for S14 Samples in CPS and RPS.

Sample Name	CPS content (µg/mg)	RPS content (µg/mg)
HC(PMMA)	129.43	28.41
H(PMMA)	290.16	20.35
TC(PMMA)	213.12	24.18
T(PMMA)	220.58	10.43
HC(GLASS)	166.02	14.21
H(GLASS)	143.86	12.98
TC(GLASS)	67.86	17.08
T(GLASS)	351.96	13.44

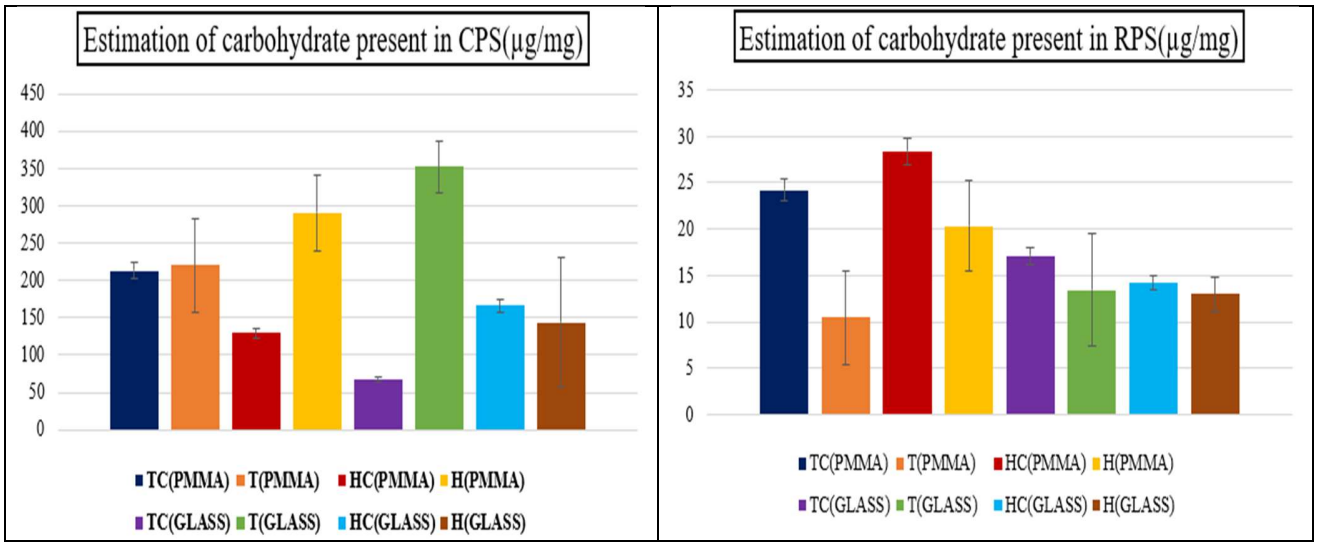


Figure 16: Quantitative comparison of Carbohydrate content in CPS and RPS fractions from samples cultured in PMMA and Glass vessels. The error bar represents the standard deviation of triplicate samples.

5.5.2 Protein content:

The protein content of EPS fraction from non-buffered cultures was higher than in buffered cultures (Table 8). Among all the buffered samples, protein concentration was highest in the sample that buffered with HEPES in the PMMA flask.

Protein content was higher in non-buffered samples, probably due to stress-induced production of EPS. When cyanobacteria are exposed to non-buffered conditions, their immediate environment can become more uncertain and stressful, leading them to generate more extra EPS as a protective mechanism. Microorganisms under stress frequently release additional EPS to aid in adhesion to surfaces, desiccation resistance, and protection against harmful agents(Brito et al., 2022).

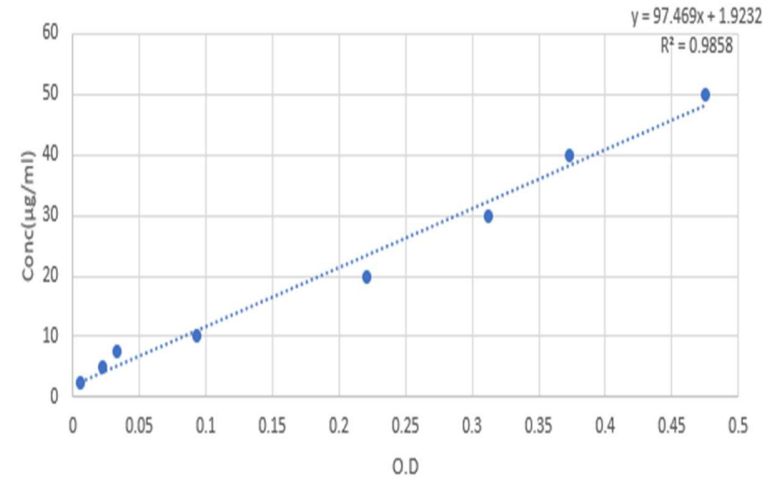


Figure 17: BSA standard curve.

Tabel 8: Protein content for S14 Samples in CPS and RPS.

Sample Name	CPS content (µg/mg)	RPS content (µg/mg)
HC(PMMA)	19.95	2.61
H(PMMA)	15.96	4.04
TC(PMMA)	36.72	2.31
T(PMMA)	13.07	3.78
HC(GLASS)	19.08	3.09
H(GLASS)	9.66	5.46
TC(GLASS)	6.70	2.22
T(GLASS)	9.14	4.04

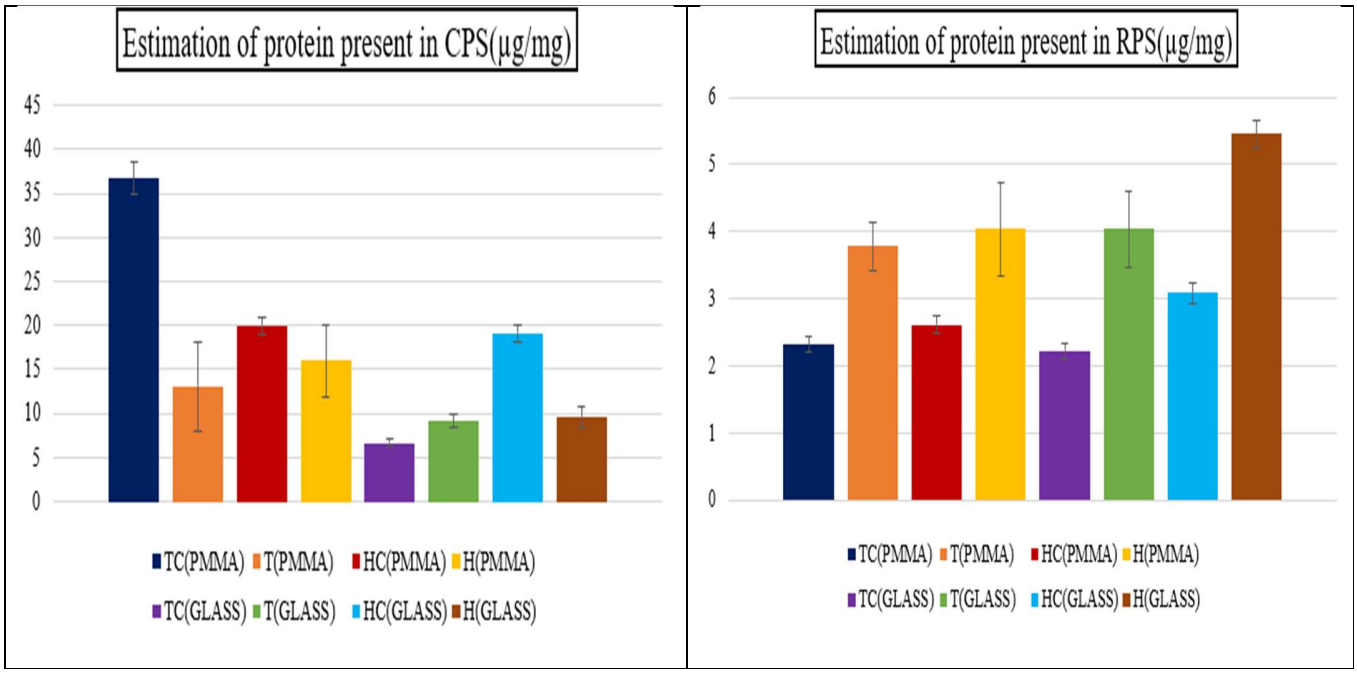


Figure 18: Quantitative comparison of Protein content in CPS and RPS fractions from samples cultured in PMMA and Glass vessels. The error bar represents the standard deviation of triplicate samples.

5.5.3 Uronic acid content:

The uronic acid content of the CPS fraction from non-buffered cultures was approximately five times higher than in buffered cultures. Compared to the CPS, the uronic acid concentration in RPS was significantly lower (Table 9).

The metabolic activities of the cyanobacteria in non-buffered cultures can cause significant pH fluctuations in the medium, which can increase the production of uronic acids as part of the CPS. Uronic acids, having a negative charge, can help neutralize pH changes and protect the cells from acidic or alkaline stress (Madsen et al., 2021).

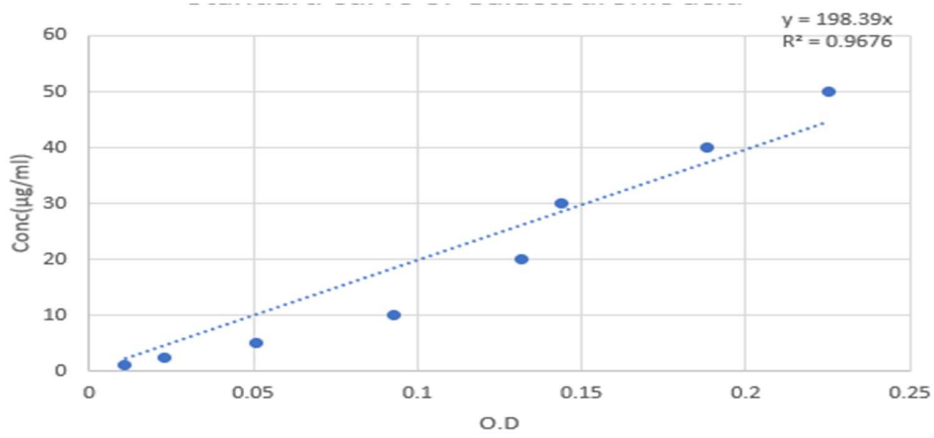


Figure 19: Standard curve of galacturonic

Tabel-9: Uronic acid content for S14 Samples in CPS and RPS.

Sample Name	CPS content (µg/mg)	RPS content (µg/mg)
HC(PMMA)	130.94	24.01
H(PMMA)	28.63	7.54
TC(PMMA)	152.36	23.81
T(PMMA)	25.46	3.17
HC(GLASS)	155.74	12.9
H(GLASS)	23.48	2.31
TC(GLASS)	84.32	15.47
T(GLASS)	15.54	1.72

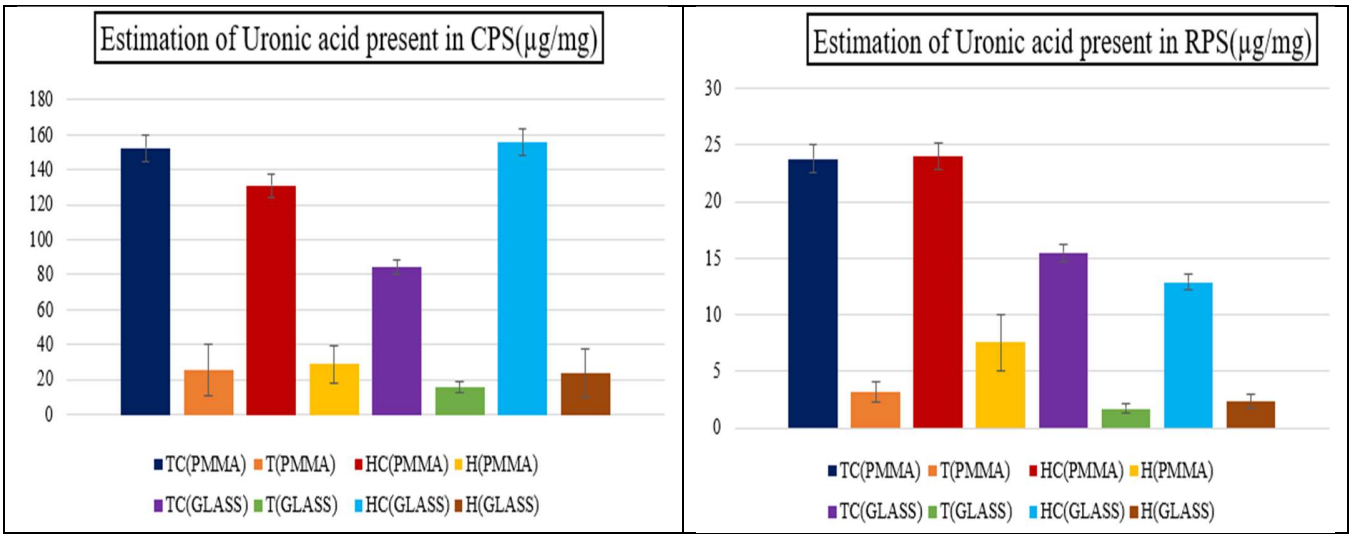


Figure 20: Quantitative comparison of Uronic acid content in CPS and RPS fractions from samples cultured in PMMA and Glass vessels for S14 samples. The error bar represents the standard deviation of triplicate samples.

5.6 Mass Spectroscopy Analysis of Biofilm Culture Media-

The biofilm culture medium (K16) underwent mass spectroscopy investigation, which produced many prominent peaks corresponding to distinct m/z (mass-to-charge) values. These peaks represent the most prevalent molecular ions found in the sample. The mass spectrometry analysis of the K16 cyanobacterial biofilm sample identified significant m/z values of 202, 261, 283, 319, 399, 401, 445, 489, 499, and 521, which were confirmed using the CyanoMet database (Jones et al., 2021). Regretfully, it is impossible to identify certain chemicals using solely m/z readings. To find possible substances connected to cyanobacterial development, each of these data underwent further analysis. Table 10 provides a summary of the findings.

Table 10: Major m/z values detected and potential corresponding compounds

m/z value	Potential Compound (Probable)	Relevance to Cyanobacterial Growth
202	4-hydroxy-7-methyl-1-indanone	Antimicrobial properties, nitrogen fixation
261	Phenazine-1-carboxylic acid (PCA)	Secondary metabolite affecting microbial interactions
283	13-O-Acetyl-12β-deoxydecarbamoylsaxitoxin, Palythine	Reduce the rate of cyanobacteria growth
319	Trichotoxin A or Phormidinine B	Known for its toxic effects and antibacterial properties.
399	Microcystin variants	Cyanotoxin affects growth and metabolism
401	Cylindrospermopsin, Taveuniamide-K	Cyanotoxin inhibits cyanobacterial growth
445	Viridisamide A, Secondary metabolites	Alkaloid with antibacterial properties
489	Bistratamides M and N, Dendroamide A	Known for cytotoxic and antibacterial activities.
499	Barbamide, 8-O-acetyl-8-epi-malyngamide C	Inhibit biofilm formation
521	Antillatoxin-A, Bistratamide C, Majusculamide A	Negative effect on biofilm growth or integrity.

It is noteworthy that similar compounds have been putatively identified in the partially purified extract of cyanobacterial from the Indian Sundarbans (Basu et al., 2021; unpublished data), demonstrating the consistency of these discoveries to our current cyanobacterial biofilm study.

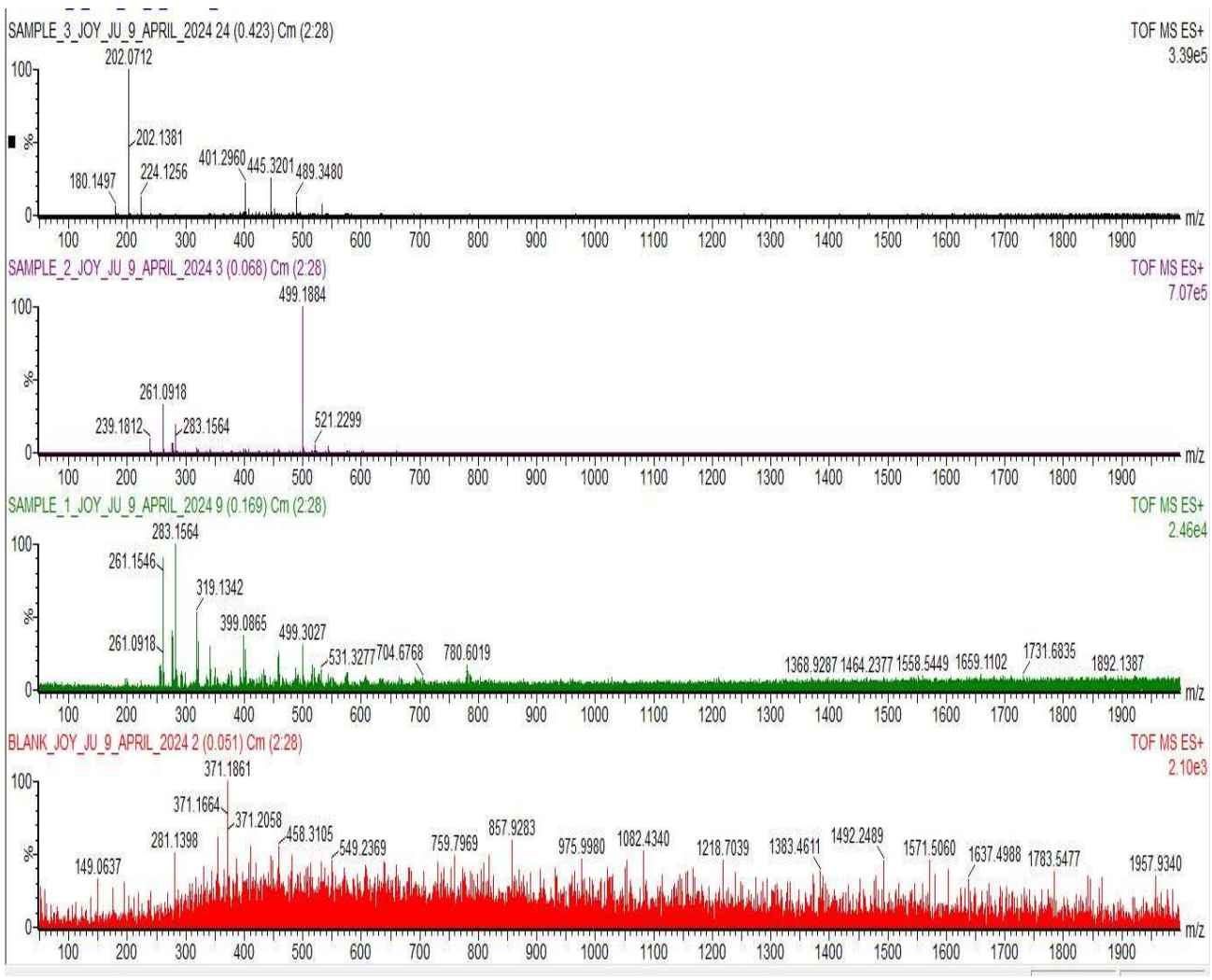


Figure 21: Sample 1 consists of the medium of K16 without any buffer, Sample 2 is the medium of K16 buffered with HEPES, and Sample 3 is the medium of K16 buffered with Tricine. The mass spectrometry data are represented in a graph where the X-axis denotes the mass-to-charge (m/z) ratio, and the Y-axis denotes the relative intensity of detected compounds, reflecting the abundance of specific proteins in each sample.

Interpretation of Major Peaks

The m/z value of 202 corresponds to 4-hydroxy-7-methyl-1-indanone, which has antimicrobial properties that can also influence the development and growth of cyanobacteria(Turek et al., 2017). The peak at m/z 261 may maybe identified as Phenazine-1-carboxylic acid (PCA), a broad-spectrum antibiotic and secondary metabolite that plays an important role in microbial interactions within the biofilm(LeTourneau et al., 2019).

A prominent peak at m/z 283 is likely 13-O-Acetyl-12β-deoxydecarbamoylsaxitoxin or Palythine. This substance, a saxitoxin derivative, is more well-known for its neurotoxic qualities than for having any anti-cyanobacterial biofilm-growth effect (Pearson et al., 2010). The m/z 319 peak indicates the possible presence of Trichotoxin A, which has a direct effect on cyanobacterial biofilms growth, and its general toxicity could potentially disrupt biofilm formation or stability.

Interestingly, the m/z 399 peak is associated with variations of Microcystin, a well-known cyanotoxin that may significantly influence the growth and metabolism of cyanobacteria(Belykh et al., 2017). Similarly, Cylindrospermopsin, another cyanotoxin affecting cyanobacterial function, was responsible for the m/z 401 peak. The m/z 445 is possibly Viridisamide A, which is more associated with natural product discovery from cyanobacteria(Ferrinho, 2023).

The m/z 499 peaks indicate probably the presence of Barbamide and 8-O-acetyl-8-epi-malyngamide C both of which have potent antimicrobial activity, including activity against cyanobacteria, potentially inhibit biofilm formation due to their general antimicrobial properties(Gerwick et al., 2013).

A prominent peak at m/z 521 is likely Antillatoxin-A or Bistratamide C. Antillatoxin-A could disrupt bacterial cell processes, potentially affecting cyanobacterial biofilm formation. Bistratamide C is known for its cytotoxic properties, which might impact biofilm integrity or formation.

6. CONCLUSION

The study has revealed deep insights into the role of environmental stressors, particularly pH, on the cyanobacteria biofilm from the Sundarbans. Among all environmental parameters, pH has emerged as one such modulator of the environment which significantly controls the growth, biochemical constitution, and resilience of biofilms in both natural and controlled environments.

Experimented analyses revealed that there are huge differences in growth dynamics, pH stability, chlorophyll concentration, and metabolic activity between cyanobacterial biofilms developed in buffered and non-buffered media. Non-buffered media displayed considerable pH fluctuations, which negatively impacted growth and biochemical synthesis. In contrast, buffered media, especially those stabilized at slightly alkaline pH levels, maintained a more stable environment that was favorable to enhanced metabolic activity, chlorophyll synthesis, and exopolysaccharide production. These findings underscore the importance of maintaining optimal pH conditions to maximize the productivity and stability of cyanobacterial biofilms.

Analysis by mass spectrometry showed important m/z values corresponding to compounds known to be inhibitory and toxic, such as phenazine-1-carboxylic acid and microcystin variants. The presence of these compounds provides a deeper understanding of the metabolic profile of cyanobacterial biofilms, suggesting that certain environmental stressors may trigger the production of bioactive or toxic metabolites. This would further prompt the initiation of the development of newer approaches toward unraveling the regulatory mechanisms for biosynthesis of such a class of compounds and also devising ways for curtailing their impacts on aquatic ecosystems.

Future studies surely need to address the optimization of pH conditions for a much larger variety of cyanobacterial strains to generalize these results. Really interesting would be the investigation of different buffering agents, concentrations, and pH ranges on metabolic and biochemical responses of various cyanobacteria to design customized cultivation protocols for specified biotechnological applications. Additionally, exploring the molecular mechanisms underlying pH-induced changes in EPS production and metabolic activity could reveal new targets for genetic or chemical modulation, thereby enhancing biofilm resilience and productivity under varying environmental conditions.

Thus, this research not only makes a significant contribution toward knowledge about the influence of pH on cyanobacteria biofilms but also sets up a strong platform for future research to optimize cyanobacteria culture for its eventual application. Therefore, knowledge acquired through these teachings could also help progress microbial ecology and harness innovative solutions in managing and utilizing cyanobacterial biofilms within natural or engineered ecosystems.

7. Future Work:

Further work should be focused on the impact of other environmental stressors in association with pH on the formation and stability of cyanobacterial biofilms, such as salinity, temperature fluctuation, and nutrient availability. To further consolidate these laboratory results into useful suggestions for the control of cyanobacterial blooms in these natural settings, these findings should be more specifically framed within field research conducted across a variety of habitats. A lot more research would also need to be done to optimize the growth characteristics and functions of these novel cyanobacterial biofilms under controlled settings, given their broad potential applications in bioremediation, carbon sequestration, and sustainable aquaculture.

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