

**MICROALGAL BIOREMEDIATION OF ORGANIC
POLLUTANTS AND HEAVY METALS FROM
PETROLEUM DERIVED PRODUCED WATER**

BY

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DHAHRAN, SAUDI ARABIA

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Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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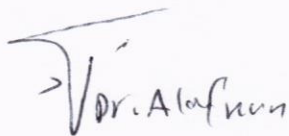
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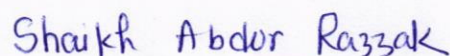
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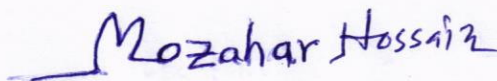
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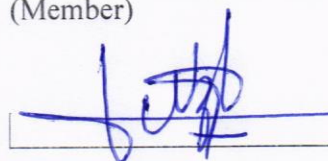
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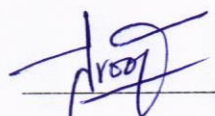
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Dedicated to my parents and my family

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LIST OF ABBREVIATIONS

ATP	: Adenosine Triphosphate
BBM	: Bold's Basal Medium
BOD	: Biochemical Oxygen Demand
CA	: Carbonic Anhydrase
CO ₂	: Carbon Dioxide
COD	: Chemical Oxygen Demand
CHNS	: Carbon, Hydrogen, Nitrogen, Sulfur
DOPs	: Dissolved Organic Pollutants
FTIR	: Fourier Transform Infrared
HHV	: Higher Heating Value
HM	: Heavy Metal
MC	: Moisture Content
NP	: Nitrogen to Phosphorus
P _b	: Biomass Productivity (mg L ⁻¹ d ⁻¹)
ROS	: Reactive Oxygen Species
SPV	: Sulfo-Phospho-Vanillin
SMW	: Synthetic Municipal Wastewater
SPW	: Synthetic Produced Water
TDS	: Total Dissolved Solid
TN	: Total Nitrogen (mg/L)
TOC	: Total Organic Carbon
TP	: Total Phosphorus (mg/L)
TGA	: Thermogravimetric Analysis
μ _g	: Specific Growth Rate (d ⁻¹)
X	: Biomass dry weight (mg/L)

ABSTRACT

Full Name : [MOHAMMED OMAR FARUQUE]

Thesis Title : [MICROALGAL BIOREMEDIATION OF ORGANIC
POLLUTANTS AND HEAVY METALS FROM PETROLEUM
DERIVED PRODUCED WATER]

Major Field : [CHEMICAL ENGINEERING]

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Produced water (PW), a byproduct of oil and gas extraction, contains hazardous dissolved organic pollutants (DOPs) and heavy metals (HM), posing significant environmental risks. This study introduces the first comprehensive investigation of *Chlorella sorokiniana* (*C. sorokiniana*) for its feasibility in terms of growth, organic pollutant degradation, and HM removal in petroleum-derived PW. The research was conducted in five phases, assessing the microalga's adaptability in hypersaline condition, bioremediation potential in different gradients of synthetic PW (SPW), and biomass valorization for bioenergy applications as well as for potential bioadsorbent to remove HM.

Initially, *C. sorokiniana* was cultivated in photobioreactor successively having hypersaline brine (HSB) supplemented with synthetic municipal wastewater (SMW) as a nutrient source, demonstrating its adaptability to extreme salinity while attaining high biomass concentration (up to 1373 mg/L) and substantial nitrogen (89.1%) and phosphorus (98.48%) elimination. Further studies explored the microalga's capability to bioremediate dissolved organic pollutants distinctly in acetate based SPW and phenol based SPW supplemented with SMW. The results uncovered effective biodegradation of organic pollutants, with total organic carbon (TOC) elimination reaching 85% in 50% PW. Notably, phenol removal (96%) was mainly attributed to biodegradation rather than bioadsorption, while lipid accumulation ranged from 12–30%, emphasizing the bioenergy potential of the biomass.

Subsequent examinations assessed the effect of lead (Pb^{2+}) stress on *C. sorokiniana* during the cultivation in phenol-acetate based SPW supplemented with SMW, proving its

resilience and ability to eliminate Pb^{2+} through biosorption, bioaccumulation, and pH-induced precipitation. The highest biomass productivity (124.63 mg/L/d) was monitored at moderate Pb^{2+} exposure, while TOC removal efficiency peaked at 95% at 50% PW. Lastly, the study considered the biosorption potential of *C. sorokiniana* dry biomass for Cd(II) removal, achieving a maximum sorption capacity of 92 mg/g at pH 7. Adsorption kinetics followed a pseudo-second-order model, while equilibrium data fitted well with the Freundlich isotherm, emphasizing the heterogeneous nature of Cd(II) adsorption.

Overall, this research provides the first detailed investigation of *C. sorokiniana* for bioremediation and bioenergy applications in PW treatment. The findings exhibit its dual role as an efficient bioremediation agent for removing dissolved organic pollutants and HM, while also serving as a suggesting bioresource for sustainable energy production. This study offers an eco-friendly approach to integrating PW bioremediation with biomass valorization, contributing to environmental sustainability and circular bioeconomy strategies.

Keywords: *Chlorella sorokiniana*; Produced water; Microalgal bioremediation; Total organic carbon; Heavy metal removal; Lipid accumulation

ملخص الرسالة

الاسم الكامل: محمد عمر فاروق

عنوان الرسالة: **تقنيات معالجة مياه الصرف الصحي باستخدام الأغشية في معالجة مياه الصرف الصحي**

التخصص: الهندسة الكيميائية

تاريخ الدرجة العلمية: رياربف 2025

مما، ثقيلة ومعادن خطرة مذبذبة عضوية ملوثات على تحتوي، والغاز النفط لاستخراج ثانوي ناتج وهي، المنتج المياة سوروكينيانا كلوريل الدقيق الطحلب استخدام حول شامل تحقيق أول الدراسة هذه تقدم. كبيرة بيئية مخاطر يشكل تم. البترول من المشتقة المنتج المياة من الثقيلة المعادن وإزالة، العضوية الملوثات تحلل على وقدرته، نموه لإمكانية الحيوية المعالجة في وإمكاناته، الملوحة شديدة الظروف مع الدقيق الطحلب تكيف لتقييم مراحل خمس على البحث إجراء الطاقة إنتاج تطبيقات في الحيوية الكتلة استغلال إلى بالإضافة، الاصطناعية المنتج المياة من مختلفة درجات ضمن الثقيلة المعادن لإزالة حيوية ماصة كمادة واستخدامها الحيوية

بمياه مدعم الملوحة عالي ملحي محلول على يحتوي ضوئي مفاعل في سوروكينيانا كلوريل زراعة تم، البداية في مع الشديدة الملوحة مع التكيف على قدرتها أظهر مما، للمغذيات كمصدر الاصطناعية الصناعية الصحي الصرف والفسفور 89.1% بنسبة للنيتروجين كبيرة وإزالة لتر/ملغ 1373 إلى يصل الحيوية الكتلة من عالٍ تركيز تحقيق العضوية للملوثات الحيوية المعالجة على الدقيق الطحلب قدرة لاستكشاف إضافية دراسات إجراء تم. 98.48% بنسبة إضافة مع، الفينول على والآخر الأسيتات على قائم أحدهما، الاصطناعية المنتج المياة من مختلفين نوعين في المذبذبة الكربون إزالة نسبة وصلت حيث، العضوية للملوثات فعال تحلل عن النتائج كشفت. الاصطناعية الصناعي الصرف مياه بشكل تعود 96% بنسبة الفينول إزالة أن بالذكر الجدير ومن. المنتج المياة من 50% في 85% إلى الكلي العضوي الإمكانيات يؤكد مما، 12-30% بين الدهون تراكم تراوح بينما، الحيوي الامتزاز من بدلاً الحيوي التحلل إلى أساسي الحيوية الطاقة إنتاج في الحيوية للكتلة العالية

اصطناعية منتج مياه في زراعته أثناء سوروكينيانا كلوريل الدقيق الطحلب على الرصاص إجهاد تأثير دراسة تمت وإزالة التحمل على قدرته أثبت مما، الاصطناعية الصناعي الصرف بمياه معززة، والأسيتات الفينول على تعتمد تسجيل تم. الهيدروجيني الرقم تغيرات عن الناتج والترسيب، الحيوي والتراكم، الحيوي الامتزاز خلال من الرصاص الكربون إزالة كفاءة بلغت بينما، للرصاص معتدل تعرض عند يوم/لتر/ملغ 124.63 بمقدار الحيوية للكتلة إنتاجية أعلى الحيوية للكتلة الحيوي الامتزاز قدرة تقييم تم، ذلك إلى بالإضافة. المنتج المياة من 50% عند 95% الكلي العضوي اتبعت 7. الهيدروجيني الرقم عند غرام/ملغ 92 بلغت قصوى امتزاز قدرة حققت حيث، الكاديوم لإزالة للطحلب الجافة الطبيعة إلى يشير مما، فروندليش نموذج مع التوازن بيانات تطابقت بينما، الكاذبة الثانية المرتبة نموذج الامتزاز حركية الكاديوم امتزاز لعملية المتجانسة غير

الحيوية المعالجة في سوروكينيانا كلوريل الدقيق الطحلب استخدام حول مفصل تحقيق أول الدراسة هذه تقدم، عام بشكل الحيوية المعالجة في فعال كعامل المزدوج دوره النتائج تظهر. المنتج المياة معالجة ضمن الحيوية الطاقة وتطبيقات توفر. المستدامة الطاقة لإنتاج واعدًا حيويًا مصدرًا كونه إلى إضافة، الثقيلة والمعادن المذبذبة العضوية الملوثات لإزالة تعزيز في يساهم مما، الحيوية الكتلة واستغلال المنتج المياة الحيوية المعالجة بين يدمج للبيئة صديقًا نهجًا الدراسة هذه الدائري الحيوي الاقتصاد استراتيجيات ودعم البيئية الاستدامة

إزالة؛ الكلي العضوي الكربون؛ الدقيقة بالطحالب الحيوية المعالجة؛ المنتج المياة؛ سوروكينيانا كلوريل؛ **المفتاح كلمات** الدهون تراكم؛ الثقيلة المعادن

CHAPTER 1

INTRODUCTION

1.1 Background

Due to the ever-increasing population, rapid industrialization, and urbanization, large amounts of wastewater are generated that must be treated before being safely disposed of or reused [1]. The wastewater created during the extraction of oil or gas from subsurface reservoirs is known as petroleum-derived produced water (PW) [2]. PW is a major waste stream by-product, representing up to 80% of all waste produced by the oil and gas sector. Every day, the world produces almost 250 million barrels of PW compared to 80 million barrels of oil [3]. Therefore, it is clear that the relationship between PW generation and the extraction of oil from reservoirs expressed as a ratio of roughly 3:1, and that due to the aging of oil wells, it will approach 12:1 by 2025 [4], [5]. In particular, Saudi Arabia, one of OPEC's top producers of crude oil, reported an average daily production of over 11.5 million barrels in 2022 [6], which is ultimately associated with the generation of vast amount of petroleum derived PW. Practically, 40% of PW are reportedly discharged into the environment, which poses a serious hazard to the ecology [7], [8]. Because PW is inappropriate for surface discharge, disposal in groundwater resources, or reuse for other uses due to the presence of hazardous hydrocarbons, dissolved salts, heavy metals, and other pollutants [9]. Even so, the well frequently must be shut down because the cost of controlling PW is frequently greater than the well's income. Therefore, an environmentally

responsible PW treatment is essential for the oil and gas business to be ecological and lucrative as well as to comply with environmental standards and allow for the reuse and recycling of PW.

Each reservoir's geological properties and the content and features of naturally appearing chemical compounds in PW are tightly related [10]. The PW is made up of hazardous organic pollutants like phenol, organic acids, inorganic pollutants like total dissolved solids (TDS), HMs, chemical additives utilized in the production of oil and gas, polycyclic aromatic hydrocarbons (PAHs), and BTEX (benzene, toluene, ethylbenzene, and xylenes), as well as other pollutants that are harmful to the environment [2]. Untreated or insufficiently treated PW discharged into bodies of water can seriously destabilize ecosystems and destroy marine life. In recent years, PW discharges have been the focus of the most attention and research due to their high overall discharge volumes, complicated chemical composition, and unknown potential long-term ecological effects [8]. Conservative treatment procedures have been employed to decontaminate PW for a while, but they have never been able to comply with environmental laws, especially when the goal is to reuse the water.

However, due to their efficiency, environmental friendliness, and potential for cost savings, biological technologies for PW treatment provide a number of benefits. Numerous contaminants found in PW, such as nutrients, suspended particles, organic compounds, hydrocarbons, and specific HMs, can be handled using these techniques. Due to their adaptability, biological treatments can be used to treat many types of PW [11]. Utilizing microalgae as a foundation for Phycoremediation for the treatment of various sorts of wastewaters has been increasingly popular in recent years. Because these microorganisms

can grow on hydrocarbons and inorganic salts found in wastewater as well as atmospheric CO₂, using microalgae as bioremediation instruments is a viable choice [12]. Microalgae are favored over conventional biological wastewater treatment methods owing to their numerous returns, like enhanced elimination of pollutants and pathogens, reduced energy consumption, cost-efficiency, decreased sludge formation, nutrient recycling, mitigation of greenhouse gas emissions, and valuable biomass production through nutrient recovery [13]. Additionally, microalgae not only purify wastewater but also provide a foundation for other bio-based products like bioenergy [14]. PW treatment with microalgae growing is more difficult than another bioremediation of nutrient-rich wastewater. Additionally, there are two significant difficulties that must be considered: Given PW's complex makeup, a mix of actions may be necessary to improve growth of algal biomass [5]. Additionally, some researchers have proposed that PW might exhibit a deficiency in nitrogen and phosphorus, which could bring the C/N ratio falling below the optimal range for fostering microalgal growth [15]. Due to these factors, pretreatment prior to bioremediation and the adding together of these two nutrients may be necessary for growing microalgae in PW, particularly throughout effluent mixing [5].

Microalgae are unicellular, freshwater or saltwater photosynthetic microorganisms that use light, water, and carbon dioxide to create biomass that is rich in carbohydrates, lipids, proteins, and other precious organic molecules [16]. Microalgal cultivation approaches encompass both open and closed reactors, which can be operated in various modes including continuous or semi-continuous, batch, and, fed-batch utilize heterotrophic, phototrophic or mixotrophic metabolic approaches [17]. Because microalgae can use some contaminants as nutrition sources while growing, they can bioremediate PW effluents

during culture [15]. Generally speaking, PW's complex elemental makeup makes it challenging to cultivate algae and frequently calls for extra treatment to make it suitable for algal development. According to a number of authors, the salinity of PW and the lack of suitable nutrients are what prevent algal growth in PW [5]. Depending on where the PW came from, its salinity could range from a few milligrams per liter to approximately 300 grams per liter [18], which can be significantly higher than the salinity of seawater, which is typically around 35 grams per liter [5]. Some of the problems of PW, like the presence of harmful organic compounds and excessive salinity, can be alleviated by supplementing it with synthetic media or nutrient-rich wastewater [19]. Some scientists opted to add a synthetic medium to PW and progressively acclimatize the microalgae to the effluent. Hakim (2016) conducted a study comparing the biomass yields achieved through cultivation of microalgae in PW with those in a traditional artificial culture medium known as BG-11, and he hypothesized that the addition of macronutrients could increase the biomass density of microalgae cultivated in PW [20]. Similarly, Das et al. (2019) hypothesized that adding nitrogen and phosphorus to PW can increase the production process' overall economics [21].

As it was already established, the petroleum derived PW contains an intricate blend of HMs, hydrocarbons, and other contaminants. The deadliest organic components among them are phenol, and organic acids which are within the category of dissolved organic carbon . It is extremely poisonous to aquatic, human, and plant life and is profoundly destructive even at low concentrations. Therefore, managing PW that is phenol and other organically polluted poses significant environmental issues. To remove dissolved organic carbon from industrial wastewater, including phenol and its derivatives, organic acids, and

other organic carbons, numerous conventional wastewater treatment procedures have been developed over the years. High intensity organic pollutant physicochemical degradation is a challenging process because it produces secondary harmful byproducts such as toxic hydrocarbons and chlorinated phenols [22]. When it is not feasible to completely degrade a specific pollutant, converting the harmful contaminant into a less lethal or non-noxious chemical is an essential bioremediation method. In light of this, phycoremediation of organic pollutants appears to be a suitable option, and a number of microorganisms were recommended for the remediation of organic pollutants using microalgae from industrial effluent. According to the most recent research, microalgae may be able to digest and breakdown organic acids, phenol, and its derivatives. The type of microalgae or substrate, the pace of degradation, and the initial pH all affect a microalgae's capacity to absorb organic molecules like phenol and organic acids. However, when pollutant concentration increased, the pace at which microalgae degraded phenol also reduced. This is because microalgae have a particular tolerance range for phenol, and the elevated the concentration of phenol, the more hazardous it is to cells[23]. Eukaryotic microalgae and certain cyanobacteria have demonstrated the capability to eliminate phenolic compounds. This is noteworthy because most algae typically exhibit limited tolerance to the acute toxicity of phenols [24]. It is proven that microalgae have the capability to bioremediate the organic compounds from different industrial wastewater [25] but few research have been conducted yet to remove the dissolved organic compounds from petroleum derived produced water due to the complex nature of the PW compositions.

PW often contains HMs in addition to dissolved organic carbon. Some HMs can be toxic to microalgae, and their presence may affect the growth and vitality of the microorganisms,

further complicating the remediation process. Many times, the presence of HMs in PW is blamed as a contaminant for the limited production of biomass and the decline of microalgae. Hakim (2016) showed that the presence of HMs in PW prevented several microalgae species from growing [20]. Based on the specific species, the type of metal involved, and the environmental conditions for growth, HMs can prevent microalgae from growing in various ways [26]. Rahman et al. (2021) recently observed that *Chlorella vulgaris* and *Galdieria sulfuraria* development at low PW dilutions was likely hampered by the existence of HMs, reflected by the excessive content of TDS [27]. HMs in PW have not been thoroughly explored to comprehend the bioremediation and inhibitory process, despite being touted as an issue in the growth of microalgae. Therefore, investigations on the HMs facilitated reduction of microalgal development in PW are required, taking into account the species of microalgae, the concentration of metal, and the growing conditions. Preparing PW by pretreatment before it is mixed with other substances or media may be required since free oil, volatile organic compounds (VOCs), and scale-forming mineral precipitation have the potential to hinder the growth of algae. Various physicochemical and biological methods can be utilized alone or in sequence to meet the needs, depending on the type of contaminants in PW and the final reuse. Single technologies haven't been able to render PW in a reusable form or fulfill disposal criteria due to its complicated composition. In order to meet the regulatory limit as well as create another resource of water in the event of a freshwater shortage, a combination of physicochemical processes followed by microalgal bioremediation would present a potential method.

The proposed research aimed at finding suitable algae species and investigating their growth kinetics in petroleum derived PW medium using a closed photobioreactor with the

object of microalgal bioremediation of toxic organic pollutants (organic acids, phenol) and HMs. While there are challenges associated with using microalgae for the elimination of organic pollutants and HMs from PW, there are significant opportunities to address these challenges through research, technology development, and process optimization. Selecting appropriate microalgal species, microalgal adaptation process in PW, integration with existing systems, biorefinery approach, sustainability and energy efficiency can make microalgae-based systems a promising and sustainable solution for the bioremediation of organic contaminants (organic acids, phenol) and HMs in PW.

Researching microalgal bioremediation of organic contaminants and HMs in petroleum-derived PW is motivated by the pressing need to address environmental challenges, comply with regulations, recover valuable resources, and advance scientific knowledge. This research has the potential to provide innovative and sustainable solutions for a critical issue in the oil and gas industry and contribute to broader efforts in environmental remediation. Microalgal bioremediation not only cleanses the petroleum derived PW but also presents an opportunity for its beneficial reuse. In a region like KSA where freshwater resources are scarce, the treated water from microalgal bioremediation can be reclaimed for various non-potable purposes, such as irrigation in agriculture or cooling in industrial processes. This approach reduces the burden on the KSA's limited freshwater sources, making it an essential component of the nation's water management strategy. By turning PW into a valuable resource, microalgal bioremediation aligns with the KSA's vision 2030 for sustainable development and responsible resource utilization.

1.2 Scope of this work

The scope of this research encompasses the comprehensive study of microalgal bioremediation as a sustainable approach for treating petroleum-derived PW by targeting both organic pollutants and heavy metals. Initially, selected microalgal species were evaluated and best suited halotolerant microalgal species are utilized for its ability to degrade organic compounds, including dissolved organic carbon (DOC) and phenol, and to adsorb heavy metals such as lead (Pb) from aqueous solutions. The study also investigates the valorization of microalgal biomass obtained post-bioremediation to enhance the overall cost-effectiveness of the process. Biomass characterization was performed using advanced instrumental techniques, including Fourier-transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), and CHNS elemental analysis, to assess its bioenergy potential. Additionally, the dried microalgal biomass was utilized as a bioadsorbent for the removal of cadmium from aqueous solutions, further contributing to the circular utilization of resources. The research includes optimizing culture conditions for effective bioremediation, exploring the mechanisms of pollutant removal, and assessing the feasibility of integrating this process into existing wastewater treatment systems. The findings aim to advance the practical application of microalgae in environmental remediation of PW while demonstrating its potential for economic sustainability through bioresource recovery.

1.3 Structure of the Thesis

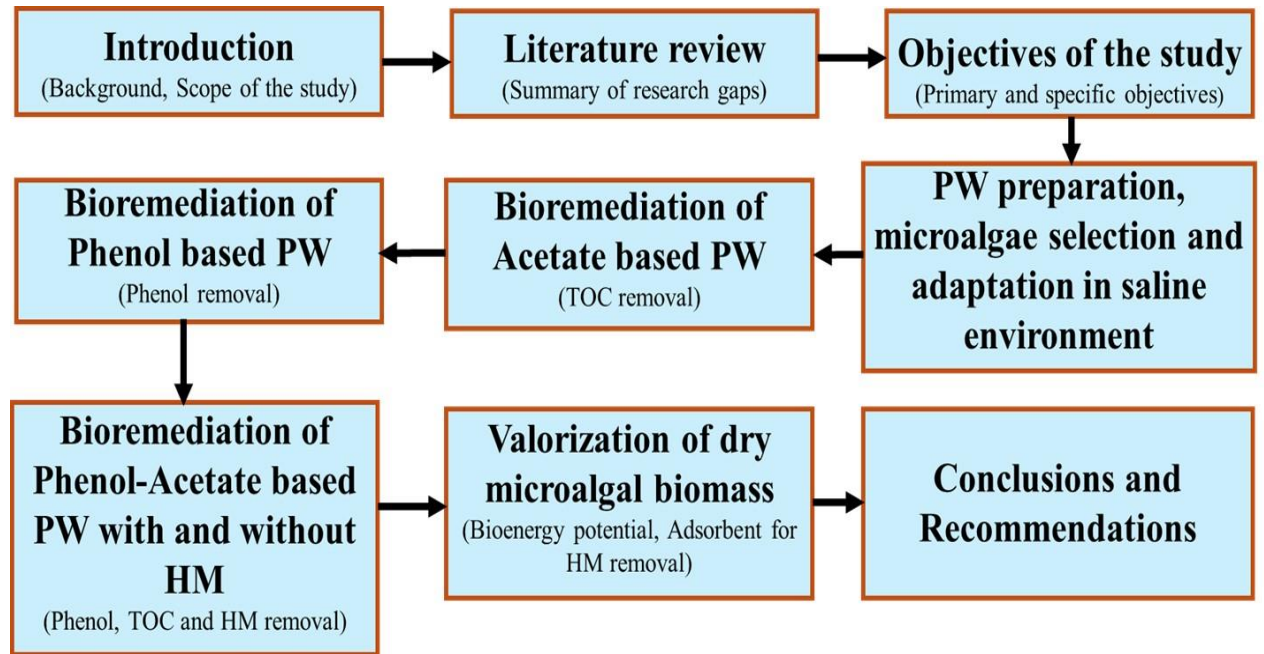


Fig. 1.1 Organization of the Thesis

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The literature review chapter critically examines existing research pertinent to microalgal bioremediation of organic pollutants and HMs, aiming to identify prevailing theories, methodologies, and findings. This analysis not only highlights the current state of knowledge but also uncovers gaps that this study seeks to address. By situating the present research within the broader academic discourse, this chapter establishes the foundation for the subsequent investigation.

2.2 Volume and characteristics of produced water

The amount of PW from a certain reservoir fluctuates. Initial water production is quite low, but as the reservoir gets older, it starts to increase [28]. As was previously indicated, the world produces almost 250 million barrels of PW every day compared to 80 million barrels of oil[3]. Given that the majority of the countries produce a large amount of water [29], hence, it is now essential to control this type of effluent. PW's intricate makeup changes with time. Its physicochemical characteristics are controlled by the field's geographic site, extraction technique, geological formation, and the hydrocarbon product being generated [30]. The key components of produced water derived from petroleum are listed in **Table**

2.1.

Table 2.1 Typical composition of produced water

Parameter	Concentration (mg/L)	References
TSS (Total suspended solids)	1.2-1000	[28]
TDS (Total dissolved solids)	100-400000	[31]
TOC (Total organic carbon)	0-1500	[4]
Total oil & grease	2-560	[10]
COD (Chemical oxygen demand)	1220-2600	[32]
Total organic acids	0.001–10000	[30]
Phenol	11.5-1000	[33]
Total BTEX	0.73–24.1	[4]
PAH (Polycyclic aromatic hydrocarbons)	0.04–3	[30]
Aliphatic hydrocarbons	17-30	[30]
Sodium	0–150000	[2]
Chloride	0–250000	[30]
Calcium	0–74000	[2]
Potassium	24–4300	[10]
Magnesium	8–6000	[2]
Bicarbonate	0–15000	[2]
Sulfate	0–15000	[30]
Lead	0.01–10	[4]
Cadmium	0.01–1.2	[4]
Manganese	0.004–175	[10]
Acetate	347	[33]

A variety of hydrocarbons, including phenol, organic acids, BTEX, and PAHs (polyaromatic hydrocarbons), are among the features of dispersed and dissolved oil components. In PW, typically find fewer soluble PAHs and heavy alkyl phenols in the form of dispersed oil, whereas substances like phenols, carboxylic acids, BTEX, low-molecular-weight aromatic compounds, and aliphatic hydrocarbons are categorized as dissolved oil. The quantity of dissolved and dispersed oil present in PW poses a threat to the environment, and in some oil fields, their concentration can be extremely high [34].

2.3 Pollution impact of produced water

The dissolved organic matter and HMs in petroleum-derived PW can have a considerable influence on the environment. This impact varies depending on the PW's composition, its treatment, and the methods used for discharging or disposing of it. The physical, chemical, and biological characteristics of receiving water bodies can be upset by the release of PW. Increased concentrations of dissolved organic compounds and HMs can affect fish, plants, and other aquatic life, changing the composition and structure of aquatic ecosystems [35]. Since the long-term impacts of dissolved organic elements, HMs, and manufacturing chemicals on the environment are not well-documented and understood, current study is focusing more on the effects of these substances on living things. Reports indicate that fish that come into contact with alkyl phenols display abnormalities in their organs and experience reduced fertility. Metals and hydrocarbons from oil platforms have also been shown to be extremely hazardous to the ecology [36]. Untreated PW can be harmful to people's health if it gets into drinking water supplies or is utilized for irrigation. Exposure to HMs and certain chemical compounds through food or water can effect in a number of health concerns, including cancer and neurological diseases [37].

2.4 Conventional produced water treatment

PW has traditionally been treated using chemical, physical, and mechanical means. Physical treatments (adsorption, cyclone, enhanced flotation), methods involving heat-based technologies, including evaporation, multi-stage flash, multi-effect distillation, vapor compression distillation, and freeze-thaw evaporation, chemical treatments (chemical precipitation, electrochemical processes, ion exchange, advanced oxidation processes, macro porous polymer extraction technology), and membrane treatments

(polymeric and inorganic membranes, microfiltration, ultrafiltration) are among the principal physicochemical methods [2]. These methods have several disadvantages, such as being expensive with substantial energy requirements (in the case of thermal processes), issues related to scale build-up (in membrane and thermal methods), demanding prerequisites for both pre-treatment and post-treatment (especially evident in thermal processes, membrane techniques, and adsorption), challenges concerning the management of residual sludge (notably in chemical precipitation and electrochemical systems), and limitations in their overall effectiveness (particularly observed in membrane and electrochemical processes) [38]. Additionally, these techniques are frequently difficult since they heavily rely on specific effluent properties [34].

Although conventional wastewater treatment systems have been used with some success, it is clear that these systems can produce waste byproducts like sludge that may need to be disposed of or treated further [39]. Therefore, it is required to create and adapt technologies with a significant reduction in energy consumption and sludge formation in order to lessen the environmental impact of wastewater treatment. Reducing operating complexity without compromising performance in terms of maintaining required effluent standards is a crucial factor in obtaining lower energy consumption. For the treatment of many types of wastewaters, microalgae-based Phycoremediation has been gaining increasing popularity in recent years [12], [40].

2.5 Treatment of produced water by microalgae cultivation

Eukaryotic algae and cyanobacteria, both belonging to the category of microalgae, have demonstrated their potential as environmentally sustainable and durable alternatives to the conventional, energy-intensive methods used in biological wastewater treatment [40].

Microalgae-based bioremediation presents a viable and eco-friendly alternative to conventional PW treatment methods. Because they have a variety of metabolic routes, microalgae may absorb different contaminants from wastewater, including PW, and use them as sources of carbon and energy [41]. Since they can photosynthesize, they are particularly effective at reducing greenhouse gas emissions and providing integrated wastewater treatment. They can absorb solar energy and turn carbon dioxide into biomass [12]. In addition, microalgae have the capacity to store large amounts of lipids, proteins, and carbohydrates that can then be transformed into beneficial goods including biofuels, animal feed, and nutraceuticals [42]. The combination of PW treatment and microalgae farming presents a special possibility for resource recovery and the development of a sustainable circular economy within the oil and gas sector, similar to other wastewater treatments. Microalgae are efficient in removing organic substances from PW, such as organic acid and phenol. They can also eliminate certain HMs and nutrients like phosphorus and nitrogen to some extent [5]. A schematic illustration of the use of microalgae photobioreactors for the production of biofuels, CO₂ capture, and nutrient uptake is shown in **Fig. 2.1**.

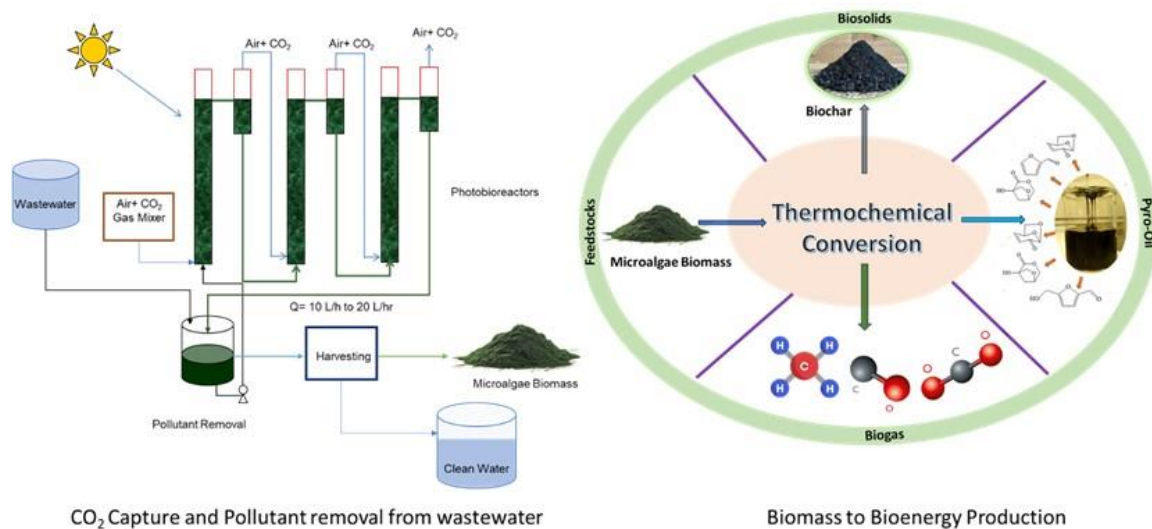


Fig. 2.1 Schematic diagram of microalgae photobioreactors applications

The large variety of values shown in Table 1 clearly illustrates the enormous variance in the chemical and physical characteristics of PW, and no two regions' PW can be the same. Therefore, to successfully develop algae using PW, a number of actions are frequently taken, including pre-treating PW, choosing an algal species based on PW-specific traits, adapting to PW, and their combinations. A revolutionary strategy known as eco-technology has been introduced for the PW treatment procedures in order to achieve a higher removal rate of contaminants. The use of microalgae-based treatment is thus identified by these eco-technology techniques as a viable alternative to the current treatment methods. Microalgae are frequently employed for the bioremediation of PW effluents because of their capability to utilize particular pollutants as a source of nutrients. There are four essential measures that must be taken in order to boost the growth of algae in PW. **Fig. 2.2** introduces these actions. Pretreatments that concentrate on eliminating salt rather than dispersed organic components ought to frequently be the most economical. As a result, preparing algal media by matching the PW's available salinity with the ideal salinity for a specific farmed alga

can be very cost-effective. Nevertheless, achieving cultivation objectives in the context of PW necessitates a comprehensive comprehension of its chemical composition [43].



Fig. 2.2 Major steps to promote the growth of algae in produced water

2.5.1 Pre-treatment of produced water

Effective pretreatment maximizes the removal of pollutants while ensuring that the resulting water is acceptable for microalgae culture. By enabling the combination of PW treatment and microalgae biomass production for future value-added applications like bioremediation or biofuel production, it also contributes to the process' overall sustainability. The characteristics of the PW and the objectives of the microalgae-based biological treatment system will determine the pretreatment processes that should be used. Solid removal, oil/water separation, chemical flocculation, desalination, pH correction, and nutrient addition are some frequent pretreatment procedures related to microalgae-based PW bioremediation. The water can be fed into the microalgae cultivation system after pretreatment. Microalgae will help further purify the water by using pollutants like fertilizers and carbon sources as substrates for their growth. In the majority of prospective investigations, pretreatment of PW is a typical step that primarily entails filtration and sterilization procedures. For instance, PW pretreatment is required, according to Ghouti et al. (2019), to promote improved microalgal development. They utilized a 0.45 μm millipore filter to filter PW from a natural gas field in Qatar in order to get rid of the majority of the suspended particles and other harmful impurities [29]. Then, five

chlorophytes were successfully grown using the PW to remove many HMs, including Al, Zn, and Fe.

2.5.2 Choice of right algae strain based on salinity of produced water

The high salinity of PW makes growing algae one of the difficulties. In PW with high salinity, freshwater algae don't typically appear to thrive effectively. However, due to their ability to tolerate osmotic pressure, algae naturally develop at a variety of salinities [44]. Freshwater and marine algae that are halotolerant may both endure high salinities. *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Chlorella sorokiniana* are the most prevalent halotolerant freshwater algae and can reportedly withstand salinities of up to 11 gL⁻¹ [45]. Gani et al. (2021) examined how *Botryococcus sp.* responded to changes in temperature, photoperiod, light intensity, and salinity [46]. They discovered that the freshwater green algae grew best at temperatures of 33°C and salinities of up to 35 gL⁻¹. This study demonstrates that freshwater algae that are halotolerant may endure salt stress.

As an alternative, marine algal strains have a competitive edge over their freshwater counterparts since they can endure even higher salinity. In culture media containing 50% PW, *Nannochloropsis oculata*, for instance, had excellent potential for growing and yielded considerable biomass at rates comparable to those seen in conventional culture systems [47]. The researchers claimed that, despite the low biomass production, it was also feasible to cultivate algae in 100% PW. According to a prior study, *Nannochloropsis sp.* can withstand a high salinity of about 40 g TDS L⁻¹ [48]. Hopkins et al. found that biomass productivity remained consistent (with no significant differences in biomass accumulation rates) in salinity ranges spanning from 30 to 120 grams of Total Dissolved Solids (TDS) per liter however, they observed growth inhibition in response to higher salinity levels at

180 g TDS L⁻¹. In another study, *Dunaliella tertiolecta*, a marine algal species, demonstrated successful growth across a broad spectrum of salinities, ranging from 30 to 210 g TDS L⁻¹ [49]. The propensity of the genus *Dunaliella* to flourish in settings ranging from mild hypersaline conditions to high salinity is generally well recognized. Additionally, it has been noted that this species thrives in salty hydraulic fracturing flowback water and brackish coal gas seams [50], [51].

2.5.3 Stepwise adaptation of algae in produced water

Allowing algae to acclimatize in PW is just as important for attaining optimal development as pre-treating PW and selecting a halotolerant species. In contrast to microalgae that weren't pre-adapted, Arriada and Abreu (2014) reached favorable outcomes by pre-conditioning *Nannochloropsis oculata* in PW, directing to a notable increase in growth rates. Specifically, they observed an 83% improvement in growth rates when using 50% PW and a 50% enhancement when using 100% PW [47]. In a separate study conducted by Ammar et al., they cultivated two marine microalgal species, *Nannochloropsis oculata* and *Isochrysis galbana*, in a stepwise adaptation process using PW media at three different concentrations: 10%, 25%, and 50% PW media. The research team continued the microalgae cultivation experiments two times at each stage to accept the microalgae sufficient time to acclimate to the new PW conditions. Their findings revealed that both *N. oculata* and *I. galbana* exhibited a prolonged lag phase when the concentration of PW in the media was increased. These findings suggest that as the concentration of PW in the growth media was incrementally increased, the microalgae required more time to adapt to the altered conditions. Researchers noted a noteworthy improvement in the growth of both microalgal strains during the second experiment of each adaptation stage when compared to their growth in PW, as opposed to the control condition with 0% PW [52]. Godfrey

successfully cultivated *Synechocystis sp.* in PW for a comparable investigation by routinely adding copious sodium nitrate to the culture. If given enough nitrate, *Synechocystis sp.* was found to grow quickly in diluted PW. These studies consequently suggest that preadapted cells in PW may have an adaptive advantage [53].

2.5.4 Bioremediation evaluation of produced water by microalgae

For the purpose of removing biodegradable contaminants, HMs, and nutrients that are still present after the initial process, biological treatment is often characterized as secondary or tertiary treatment. There have been reports of numerous microalgae genera, including *Chlorella*, *Scenedesmus*, *Phormidium*, *Botryococcus*, *Chlamydomonas*, and *Limnospira* as exceptional bioremediation agents. *Scenedesmus*, *Ankistrodesmus*, *Euglena*, *Chlorella*, *Chlamydomonas*, *Oscillatoria*, and *Chlorella* have all demonstrated exceptional growth and resistance to toxins [54]. The major three methods that microalgae can use to remove the organics from wastewater are biodegradation, consumption, and biosorption. The polymer groups in the cell walls of microalgae may provide potential sorption sites for organic contaminants. Despite the fact that most microalgae are photosynthetic in nature, a particular set of microalgae may be able to use diverse organics in either a mixotrophic or heterotrophic mode, allowing the mixing of organic-rich streams from various industrial wastewaters [41].

Organic pollutant removal

Through the metabolically inactive process of biosorption, microalgae can eliminate organic contaminants [55]. The microalgae's cell wall has a number of binding sites and functional groups that aid in chelation, electrostatic contact, surface precipitation, adsorption, and ion exchange during biosorption[56]. Microalgae participate in the biodegradation process by converting organic contaminants into low molecular weight

molecules, which are then used as a source of nutrients for microalgal growth. Furthermore, microalgae have a propensity to remove pollutants by bioaccumulating inorganic and organic contaminants from wastewater. Bioaccumulation is a procedure that depends on growth and is metabolically active. The chemicals and nutrients are absorbed by biomass through the surface. The organic and inorganic contaminants are transported inside the microalgal cell and eliminated via aqueous solutions, as shown in **Fig. 2.3**.

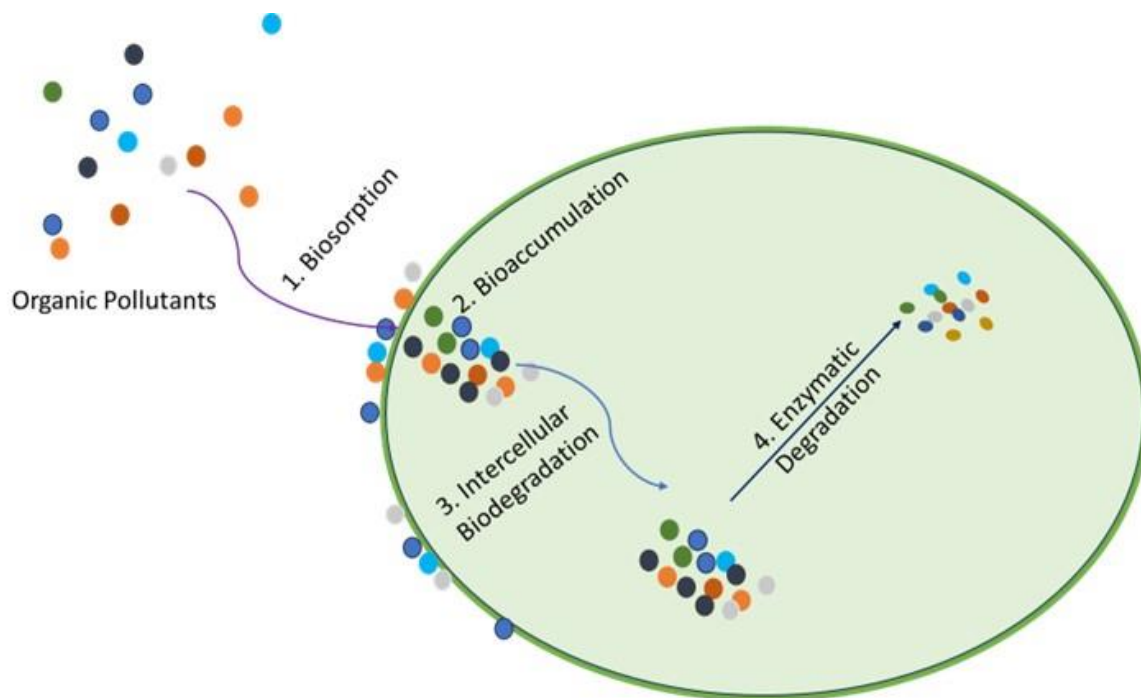


Fig. 2.3 Mechanism involved for industrial wastewater treatment using microalgae [56].

The documented information regarding various microalgae strains' effectiveness and their capacity to eliminate organic substances and nutrients from diverse wastewater sources is now comprehensive [41] but there are a few studies have been conducted yet for the bioremediation of PW with the object of dissolve organic pollutants and HMs removal. The utilization of algae for bioremediation in the context of wastewater treatment is increasingly regarded as a promising approach due to its effectiveness and cost-effectiveness in terms of both capital and operational expenses [57]. As an example, in a study by Das et al. conducted in 2019, it was noted that *Chlorella sp.*, following a 15-day growth period, demonstrated the capability to eliminate 73% of the TOC and 92% of the total nitrogen (TN) from pre-treated PW. They did this to evaluate the contaminant removal efficiency. Due to the low microbial nitrogen need during the lag phase, the TN elimination

rate was low during the first three days. But for the following eight days, the rate was higher, and this continued until *Chlorella sp.* entered the stationary phase [21].

Moreover, in research accomplished by Ojagh et al. in 2020, it was discovered that *Rhodococcus erythropolis* PTCC 1767 exhibited significant removal efficiencies. Specifically, it achieved a removal rate of 97% for COD, 85% for TOC, and 93% for hydrocarbon compounds (C14-C26). These efficiencies were observed in both synthetic and real PW samples, with a synthetic PW concentration of 600 mg/L and a NaCl concentration of 60 g/L. Furthermore, the leftover cells from the synthetic PW remediation were added, resulting in a COD removal effectiveness of 52% from PW [58]. Parsy et al. (2020) conducted research that primarily focused on cultivating the marine alga *Nannochloropsis oculata* in a blended medium containing seawater. The wastewater (WW) from anaerobic digestions was combined with the PW, which was acquired during oil extraction. Additionally, *Nannochloropsis oculata* demonstrated exceptional ammonium removal efficiency of around 100% and restricted COD removal of about 40% while growing swiftly in a mixed medium[59].

‘Halotolerant microalgae, particularly, have gained attention for their ability to adapt and proliferate in saline environments, making them prime candidates for the treatment of PW, which is often characterized by elevated salinity levels (TDS: 7744 to 38,000 mg/L) [43], [45], [60]. In recent years, several studies have demonstrated the efficacy of various halotolerant microalgae in removing organic pollutants in PW. Khairuddin et al. (2024) examined the potential of the microalga *Scenedesmus obliquus* for bioremediating pretreated produced water (PW) in a photobioreactor. In their study, varying concentrations of PW were combined with the microalgal culture to assess its growth and pollutant

removal capabilities. After 40 days of cultivation, the results showed removal efficiencies of 64% for total organic carbon (TOC), 49.8% for total dissolved solids (TDS), and 49.1% for electrical conductivity (EC)[61]. Rahmani et al. (2022) investigated the effectiveness of *Chlorella pyrenoidosa* in treating oil field PW. According to their findings, *C. pyrenoidosa* was found to be highly effective in removing organic pollutants and nutrients throughout the 21-day outdoor cultivation period [62]. Abdul Hakim et al. (2018), for example, evaluated nine strains of microalgae in PW and found that five species of green algae (*Chlorella* sp., *Dictyosphaerium* sp., *Monoraphidium* sp., *Scenedesmus* sp., and *Neochloris* sp.) could thrive in them. Despite the limited biomass generation, *Neochloris* sp. was able to remove 41.61% of the total organic carbon from the various concentrations of PW, as per their findings. Additionally, *Scenedesmus* sp. was successful in eliminating 63.76% of PW's total nitrogen. Finally, the authors suggested that petroleum-derived PW effluents may be cleaned up using halotolerant microalgae strains [63]. This strategy differs from conventional bioremediation strategies in that it makes use of microalgae strains that are specifically suited for high-salinity conditions, such those found in PW. Thus, there is an opportunity to explore the potential of freshwater halotolerant microalgae for the bioremediation of dissolved organic compounds as well as nutrient removal in PW.

According to literature research, microalgae *C. sorokiniana*, have a proven record to remove pollutants from different types of wastewaters under mixotrophic conditions. For instance, Lliopoulou et al. (2022) cultivated *C. sorokiniana* in different types of dairy wastewater with the objects of pollutants removal and biomass characterization. Their investigation found that for all types of dairy effluents, the removal of $\text{NH}_4\text{-N}$ and chemical oxygen demand was greater than 91% and 80%, respectively, whereas the removal of total

phosphorus varied between 20% and 84% [64]. Moreover, Gupta et al. (2016) examined the dual function of *C. sorokiniana* during the microalgal treatment of raw sewage for removing organic carbon and valuable biomass production for biofuels. They found that *C. sorokiniana* demonstrated better adaptability to physiological stresses and was able to remove $69.38 \pm 1.81\%$ COD, $86.93 \pm 3.49\%$ nitrogen, $68.24 \pm 11.69\%$ phosphorus [65]. To the best of our knowledge, no study has been conducted yet to remove the dissolved organic carbon from PW using microalgae *C. sorokiniana*. However, some researchers have proposed that PW might exhibit a deficiency in nitrogen and phosphorus, which could bring the C/N ratio below the optimal range for fostering microalgal growth [15]. Due to these factors, pretreatment prior to bioremediation and the addition of these two nutrients together may be necessary for growing microalgae in PW, particularly throughout effluent mixing [5]. Thus, it is anticipated that *C. sorokiniana* can be a potential halotolerant mixotrophic microalgae to bioremediate the dissolved organic carbon in PW if it is supplemented properly with nutrient rich tertiary municipal wastewater. Beyond organic pollutant removal, microalgae such as *C. sorokiniana* can offer the potential for biomass recovery and resource utilization[66].’ Additional details regarding algae species capable of thriving in PW are condensed in **Table 2.2**.

Table 2.2 Pollutant removal from produced water

Algae Species	Cultivation System	Medium Culture	PW Concentration	Biodegraded Materials	References
<i>Chlorella vulgaris</i>	3 L glass flask	F/2 Guillard	0%, 25%, 75%, 100% and crude oil	Hydrocarbons and total phenols, PAHs	[67]
<i>Nannochloropsis oculata</i>	500 mL glass test bottles	Mix of seawater and saline produced water	0–50%	Nitrogen (ammonium) and COD	[59]
<i>Dunaliella salina</i>	--	PW and sea water	W and sea water (1:1, 1:2 and 1:3, respectively)	Nitrogen, phosphorus, Ni and Zn	[57]
<i>Rhodococcus erythropolis</i> PTCC 1767	250 mL Erlenmeyer flasks	synthetic and real PW	600 mg/L	Total organic carbon (TOC), COD,	[58]
<i>Nannochloropsis oculata</i> and <i>Isochrysis galbana</i>	250 mL Erlenmeyer flask	BG11 medium	10–50%	COD and oil content	[52]
<i>Scenedesmus</i> sp	Open pond-600 L	Wuxal (W), BG-11	50%, 60%, 75%, 100%	Al, Zn, Fe	[20]

Heavy metals removal

Heavy metal accumulation in wastewater may prevent microalgal photosynthesis [43]. However, microalgae can be used to remove metal from wastewater by effectively concentrating the metal contaminants both internally and outside. Multiple metals are required by microalgal cells in trace levels for growth. Microalgae can, however, focus on different HMs through a variety of ways [26]. Biomolecules like lipids, carbohydrates, and proteins present on the surface of algal biomass portray a significant task in interacting with HMs ions [68]. Functional groups such as Hydroxyl, carboxyl, phosphate, sulfate, imine, sulfhydryl (thiol), phenol, amino, imidazole, amide, carbonyl (ketone), thioether,

phosphodiester, and phosphonate (as shown in **Fig. 2.4**) are appear on the surface of algal biomass and are involved in metal binding processes [69], [70]. Also, the surface of the algal biomass, with its diverse functional groups, is often referred to as a mosaic form owing to the existence of several connecting sites for metal ions [69], [70]. Amino acids are the monomer of protein and provide the amino functional group, while polysaccharides supply functional groups such as carboxyl groups, amino, and sulfate.

The functional groups available on the surface of algal biomasses contribute significantly to the elimination of HMs from aqueous solutions. At the binding locations on the functional groups on the algal cell wall's exterior tier, negatively charged groups on the cell wall surface interact with positively charged metal ions. When attempting to gain an understanding of complex systems like biosorption, it is necessary to have a solid grasp of the features of these functional groups. The term "biosorption" describes the process by which ions from aqueous solutions are bound rapidly and dynamically to functional groups on the surface of biomass. Algal cell walls have anionic and cationic sites, which contribute to their amphoteric properties. Microalgae, both living and non-living (inanimate), can absorb HM owing to the existence of functional groups. Furthermore, environmental pH affects whether or not these functional groups are deprotonated or protonated [26].

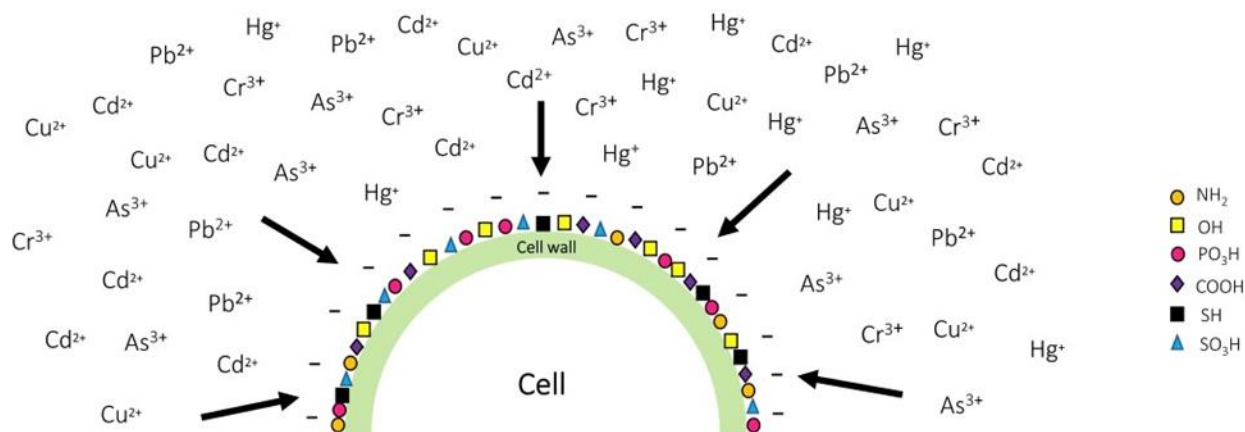


Fig. 2.4 Biosorption of metal ions to the functional groups on the microalgal cell wall [69].

In current research, conducted by Al-Ghouti et al., they cultivated five separate *chlorophytes*, namely *Dictyosphaerium* sp., *Scenedesmus* sp., *Neochloris* sp., *Chlorella* sp., and *Monoraphidium* sp. They assessed the capacity of these algae species to eliminate HMs using filtered PW sourced from a natural gas field in Qatar. The team claimed to have completely removed all Zn, Al, and Fe. *Dictyosphaerium* sp. outgrew the other species in terms of growth and was able to recover more components [29]. Additionally, the final biosorption capacity of algae can be affected by the initial metal ion concentration in the PW. Up to a certain point, increasing the metal concentration can increase metal biosorption initially, but going much higher may cause the adsorption capacity to degrade [57]. Furthermore, Talebi et al. achieved a reduction of over 84% in nickel ions (ranging from 5 to 100 mg L⁻¹) from PW within a remarkably short span of just six hours, employing *D. salina* algae [57]. Nonetheless, when the researchers increased the concentration of nickel ions from 100 mg L⁻¹ to 200 mg L⁻¹, they observed a decrease in *D. salina*'s bioremediation capacity. The active cells of *D. salina* absorbed free ions through their available binding sites, but these sites became saturated as the ion concentration

augmented. According to the same research, during the six-hour cultivation period, *D. salina* managed to eliminate 78% and 91% of free zinc ions at concentrations varying from 2 to 5 mg L⁻¹, respectively [57]. **Table 2.3** reveals the phycoremediation of HMs from different types of wastewater media including PW using potential microalgae species along with removal efficiency and other parameters.

Table 2.3 Phycoremediation of heavy metals

Wastewater media/media	Microalgae species	Metal	Temp.	Bioremediation	Removal efficiency	Ref.
Steel industry wastewater	<i>Spirulina sp.</i>	Iron	25°C	10 d	97.9%	[71]
Textile Wastewater	<i>Chlorella vulgaris</i>	cadmium	25°C	5 d	93%	[72]
Piggery wastewater	<i>Scenedesmus obliquus</i>	Zinc	25°C	10 d	41.33%	[73]
Jaworski's Medium	<i>Desmodesmus sp. AARLG074</i>	Copper	25°C	7 d	80%	[74]
Synthetic media	<i>Scenedesmus almeriensis</i>	Arsenic	23°C	3 h	40.7%	[75]
Synthetic media	<i>Chlamydomonas reinhardtii</i>	Arsenic	23°C	180 h	38.6%	[75]
Metal mine tailings water	<i>Chlorella vulgaris</i>	Molybdenum	23°C	72 h	99.9%	[76]
Bold's basal medium supplemented with Cu ²⁺ and Zn ²⁺	<i>Chlorella sorokiniana</i>	zinc	30°C	7	98.1%	[77]
Synthetic media	<i>Spirulina platensis</i>	Chromium	25°C	10 h	59.5%	[78]
Acid mine drainage	<i>Nannochloropsis oculata</i>		25°C	21	99.92%	[79]
Industrial effluents	<i>Chlorella vulgaris</i>	Chromium (VI)	25°C	10 d	43%	[80]
BG11 medium supplemented with metal	<i>Desmodesmus sp.</i>	Nickel	22°C	12 d	39%	[81]
Artificial wastewater	<i>Chlorella minutissima UTEX 2341</i>	Manganese	28°C	7 d	83.68%	[82]
Tannery effluent without dilution	<i>Chlorella vulgaris</i>	Chromium	27°C	15 d	42.87%	[83]
Tannery effluent without dilution	<i>Pseudochlorella pringsheimii</i>	Chromium	27°C	15 d	46.6%	[83]
Bold's basal medium	<i>Pseudochlorococcum typicum</i>	Mercury (II)	25°C	30 h	97%	[84]
Produced water	<i>D. salina</i>	Nickel	25°C	6h	84%	[57]
Produced water	<i>Dictyosphaerium sp.</i> , <i>Scenedesmus sp.</i> , <i>Neochloris sp.</i> , <i>Chlorella sp.</i> and <i>Monoraphidium sp.</i>	Aluminum, Iron and Zinc	-	-	100%	[29]

2.6 Factors affecting microalgal growth in produced water

The effectiveness of the treatment method can be determined by microalgal growth. Understanding and managing the critical elements that directly affect microalgae growth is crucial for optimum growth. Several environmental factors, including inoculation density, extracellular pH, initial nutrient content, temperature, and light intensity, have an impact on the rate of nutrient intake for different species of microalgae. Additionally, enough levels of CO₂ are needed to accelerate development in large-scale photobioreactors (PBRs) for an efficient microalgae production system [85]. Additionally, operational elements like reactor design, mixing, and aeration have an impact on the development of microalgae. The many elements influencing the growth of microalgae are shown in **Fig. 2.5**.

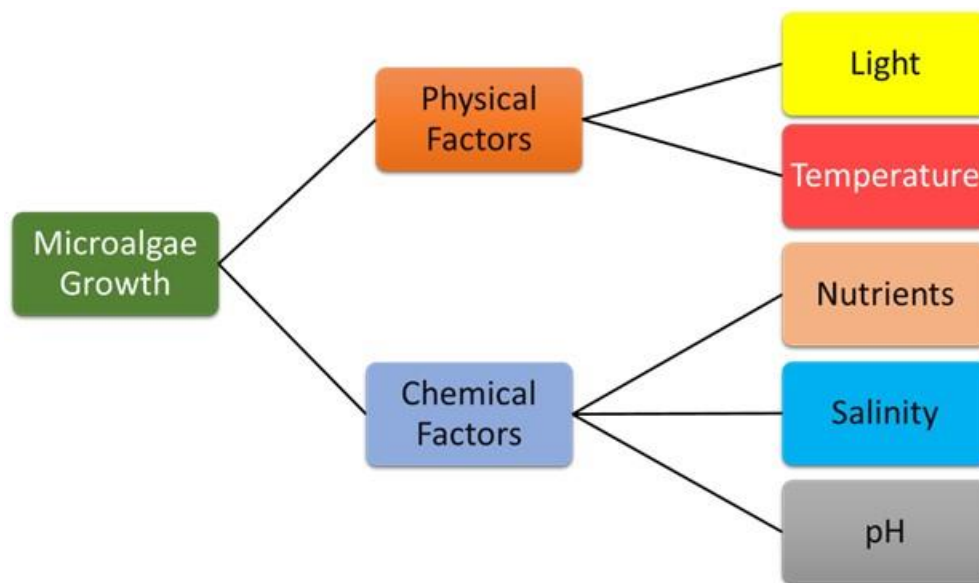


Fig. 2.5 Major parameters affecting microalgae growth

2.6.1 Temperature

The rate of microalgae growth is greatly influenced by temperature. In order for microalgae to grow well, the proper temperatures must be provided. Extremely low or high temperatures could have an adverse effect on the growth of microalgae and possibly slow it down. Algal growth can be accelerated by raising the temperature until it reaches the ideal level. Algal culture typically requires a temperature between 25°C and 30°C [86]. When the temperature exceeds the ideal level by more than 4°C, potential culture damage becomes apparent [87]. In terms of cultivation mode, open ponds lack temperature control mechanisms to maintain the ideal development rate of the culture media. This implies that only robust organisms can thrive in an environment with unregulated temperature. In contrast, the temperature is manageable in closed systems. So for algae development that is sensitive to temperature change, closed systems are acceptable [88].

2.6.2 pH

The metabolic rate of the organisms that make up microalgae can be impacted by the pH of the wastewater. pH needs to be in the right range for microalgal development to function. Most microalgae species can thrive best in a pH range of neutral. A few organisms can thrive in acidic environments. Due to increased photosynthetic activity, pH can rise. The addition of CO₂ can be used to prevent pH increases. Although it's best to have a pH of 7, some species can survive with a pH as low as 4 and as high as 10 [87]. Additionally, the pH can have a substantial effect on the biological makeup of the effluent. As an example, in a research accompanied by Hodaifa et al. in 2009, it was uncovered that the pH level plays a decisive role in influencing the growth of *Scenedesmus obliquus* microalgae and the composition of its biomass [89].

2.6.3 Light

Light serves as the primary energy source for microalgae when they undergo phototrophic growth, making it a fundamental factor that directly impacts their development. Algae are capable of engaging in photosynthesis within the range of wavelengths spanning 400 to 700 nm, which is stated to as photosynthetically active radiation (PAR) [90]. Maximum algal growth yield is attained in environments with saturated light. The efficiency of converting light into energy is a critical factor in determining the efficiency of food synthesis within algal cells. It's worth noting that only about 10% of the incident light is transformed into chemical energy, while the remaining energy is released as heat. This process involves the conversion of light energy into the necessary chemical energy for the algal cell [91]. Moreover, research indicates that just a mere 2% of the light energy is actually transformed into chemical energy [92]. Adenosine triphosphate (ATP), which is employed as an energy source for cell amalgamation and maintenance, can only be produced by microalgae through photosynthesis in the presence of light. Strong illumination, however, can impede the photosynthetic process [93].

2.6.4 CO₂ and O₂ loading

Algal cells require carbon dioxide (CO₂) to develop and make oxygen during the photosynthesis process. Because about 45–50% of the biomass of algae is made up of carbon, a low atmospheric CO₂ level (0.033%) can prevent the growth of algae [94]. Given such, it makes sense to supplement the extra CO₂. On the other hand, photo-oxidative can harm the chlorophyll reaction centers when oxygen concentrations in the algal medium exceed saturation, which restricts photosynthesis and reduces output [95]. However, microalgae can develop in a variety of CO₂ levels [96].

2.6.5 Nutrients

Apart from macronutrients and organic substances, which are required in relatively small quantities, algal cultures rely on numerous inorganic elements that constitute the essential components of algal cells. Nitrogen (N) and phosphorus (P) are the two macronutrients that are most important, with minor amounts of sulfur, iron, magnesium, and silicon [97]. Lack of nitrogen inhibits growth because it makes it more difficult to capture photosynthetic energy, which reduces the effectiveness of photosynthesis. Microalgae cells that are N-starved or N-limited must shift resources to support other crucial cellular functions rather than photosynthesizing. The microalgae then undergoes changes in pigment composition, such as losing chlorophyll and developing a rapid ingestion of inorganic nitrogen[10]. Phosphorus plays a crucial role in microalgae as it is indispensable for the synthesis of organic compounds like nucleic acids, adenosine triphosphate (ATP), and phospholipids. These compounds are vital for cell growth and metabolic processes in microalgae [98]. As the supplementary nutrients have different ratios and not all of them are accessible, extra nutrients are typically given to avoid nutritional limitation during culture [10].

2.7 Major obstacles of microalgae cultivation in produced water

Several challenges related to PW hinder the growth of microalgal biomass. These obstacles include the unavailability of essential micronutrients, the replenishment of utilized freshwater, and the requirement for an alternate carbon source [5]. The five main parameters that are frequently cited in the literature as being obstacles to microalgal growth in PW are shown in **Fig. 2.6** such as heavy metals, soluble and distributed organic components such phenol, organic acid, and BTEX, low concentrations of nitrogen and phosphorus, salinity, and turbidity.

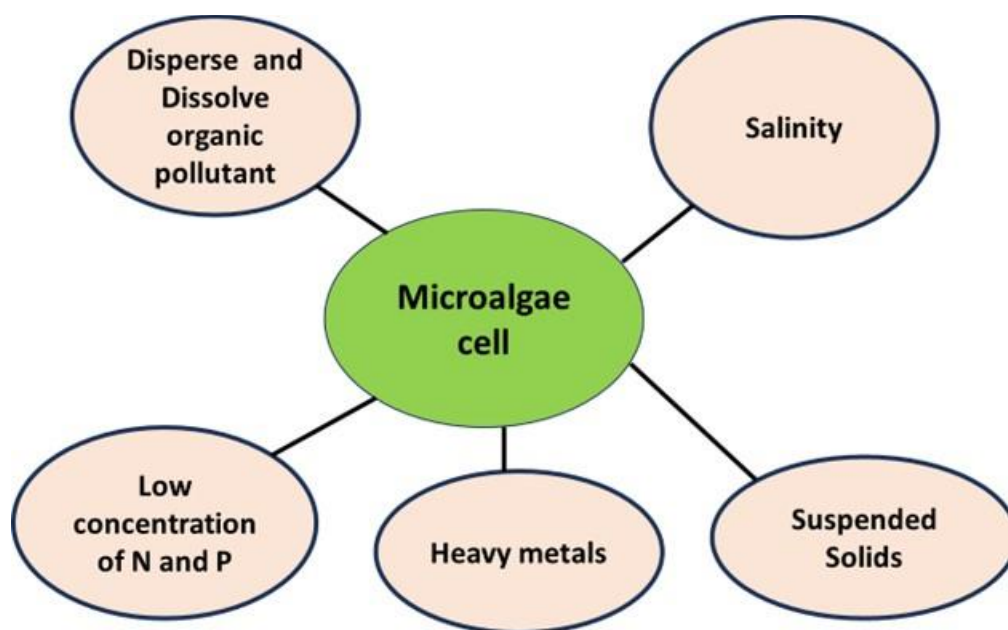


Fig. 2.6 Common obstacles to microalgal growth in produced water

In 50%, 60%, 75%, and 100% PW, Hakim (2016) cultured five chosen microalgae and saw superior growth as the effluent concentration dropped. According to the author, the primary impediments to growth are the elevated salinity levels of PW and the shortage of nutrients [20]. As seen in their work, Parsy et al. (2020) established a somewhat close figure (60 g/L) as the salinity threshold beyond which *Nannochloropsis oculata* growth can be inhibited at PW concentrations above 40% [59]. When the concentration of dissolved salts in the medium exceeds that of the interior of the cell, it is known that this causes osmotic pressure to be applied on cell membranes [5]. Selecting PW with the appropriate salinity levels is of paramount importance for fostering microalgae growth while also inducing the necessary osmotic stress to encourage the development of desired fatty acid profiles. HMs found in PW are often implicated as toxins that can account for both low biomass concentrations and microalgal mortality. Despite being acknowledged as a concern in microalgae growth, the mechanisms behind HM-mediated restraint in PW have not been

thoroughly investigated. Hence, there is a pressing need for research focusing on the impact of HMs on microalgal growth in PW, taking into consideration factors such as microalgal species, metal concentrations, and growth provisions. It can be inferred from the limitations mentioned above that the usage of PW effluent in large quantities may not be possible. Other scientists, besides Hakim, have also noted that microalgal growth is inhibited by high PW concentrations. The growth of *Nannochloropsis oculata* was shown to be inhibited by PW concentration by Marques et al. (2021) [15].

2.8 Summary of the literature review and research gap

- ❑ A few research have been conducted yet to cultivate the microalgae in PW due to its complex characterization. Required more exploration in this area.
- ❑ Higher salinity, toxic organic pollutants, presence of heavy metals, lower nitrogen and phosphorus are the major challenges during the cultivation of microalgae in PW. Appropriate selection of microalgae species as well as supplementation of PW with nutrient rich wastewater media could be a potential choice.
- ❑ While there is substantial research on microalgal bioremediation of organic pollutants and HMs from different industrial wastewater other than petroleum derived PW, there is a pressing need for more comprehensive studies that address the removal of both types of contaminants from petroleum-derived PW. Based on the above literature review, it is apparent that microalgal bioremediation of organic contaminants and HMs from petroleum derived PW is substantive but associated with some challenges due to above mentioned barrier to the cultivation of microalgae in PW. Selecting appropriate microalgal species, microalgal adaptation process in PW, integration with existing systems, biorefinery approach,

sustainability and energy efficiency can make microalgae-based systems a promising and sustainable solution for the bioremediation of dissolved organic carbon and heavy metals in PW.

CHAPTER 3

OBJECTIVES

Overall Objective

The primary aim of this study is to investigate and develop an environmentally sustainable and gainful bioremediation approach employing halotolerant microalgae to treat and bioremediate petroleum-derived PW contaminated with organic pollutants and HMs. In this context, three main objectives comprising six specific objectives were set to attain the overall objectives.

Main objectives of this research – to investigate

- i. Treatment of produced water (PW) using microalgae specie(s)
- ii. Bioenergy production using the produced microalgae biomass
- iii. Microalgae biomass as an adsorbent for heavy metal removal

Specific objectives:

- a) Preparation and characterization of synthetic PW
- b) Identification of halotolerant microalgae species
- c) Evaluate the bioremediation (TOC) of supplemented PW
- d) To remove organic pollutants in the presence of HMs and vice versa
- e) Energy content analysis of the produced biomass
- f) Adsorption kinetics and isotherm studies using produced biomass

CHAPTER 4

METHODS AND MATERIALS

4.1 Introduction

This chapter provides a comprehensive overview of the experimental procedures and methodologies employed in this research. In addition, this section provides a detailed account of the specific materials utilized and the systematic approaches undertaken to address the research objectives, ensuring the study's reproducibility and validity. By meticulously outlining each step, this chapter aims to offer a clear and precise account of the processes involved, facilitating a thorough understanding and potential replication of the study by future researchers. A block diagram outlining the overall methodology is presented in **Fig. 4.1** to provide a clear and concise overview of the study.

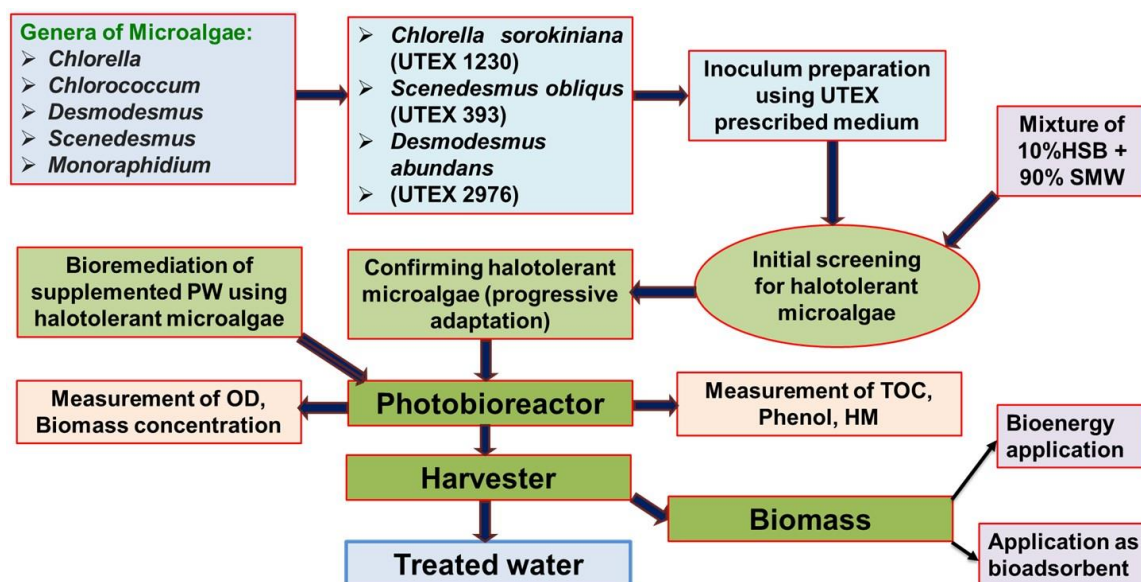


Fig. 4.1 Methodology of overall study at a glance.

4. 2 Microalgae strains and their initial screening

After a thorough review of the literature, three freshwater potential halotolerant microalgae strains—*Chlorella sorokiniana* (UTEX 1230), *Scenedesmus obliquus* (UTEX 393), and *Desmodesmus abundans* (UTEX 2976)—were obtained from the University of Texas at Austin, USA.

The inoculum was prepared separately for each of the three strains using the UTEX-prescribed medium. Once the microalgae cultures reached a sufficient concentration, with an optical density (OD₆₈₀) of 4.2, a 50 mL sample was taken from each flask to serve as the inoculum for the initial screening of microalgae in a mildly saline environment (10% HSB loadings) (as shown in Fig. 4.3). Based on the findings of the initial screening (**Fig. 4.2**), *Chlorella sorokiniana* was identified as relatively better prospective halotolerant strain among the three, demonstrating superior growth in the 10% HSB medium. Consequently, *Chlorella sorokiniana* was selected and used throughout the study.

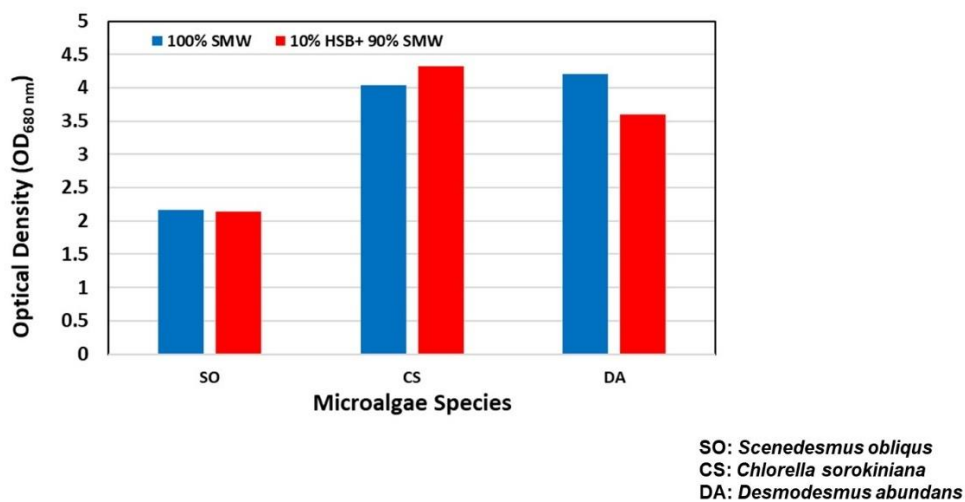


Fig. 4.2 Initial screening of microalgae in 10% HSB medium

4.3 Inoculum preparation for progressive adaptation strategy

A colony of *C. sorokiniana* (UTEX 1230) cells was transferred to a 500 mL conical flask containing 200 mL of the UTEX-recommended proteose medium for initial-stage cultivation. The microalgae were incubated under continuous white fluorescent light at an intensity of 1800–1900 LUX, maintained at a temperature of 22 ± 1 °C, without aeration. Once microalgae culture went sufficient concentration, optical density (OD_{680}) of 4.2, 50 mL of microalgae sample was taken from the flask as inoculum to start the initial cultivation with the object of attaining progressive adaptation strategy of microalgae in HSB media (**Fig. 4.3**).

4.4 Synthetic oil field hypersaline brine

Synthetic oilfield hypersaline brine (HSB) is precisely designed in controlled laboratory settings to replicate the composition and properties of actual HSB produced during petroleum extraction. Formulating HSB involves a thorough analysis of the chemical makeup of authentic HSB from specific oil reservoirs. In this study, HSB was prepared by accurately mixing various salts in specific proportions to closely match the desired composition. The choice of salts and their concentrations was carefully tailored to reflect the characteristics of real HSB. A synthetic oil field HSB that has been modified from previously published HSB formulations [99] was employed as the base of actual oil field HSB during these experiments. The composition of HSB that was employed during this study was as follows: 16.75 g.L⁻¹ NaCl, 28.39 g.L⁻¹ CaCl₂.2H₂O, 4.18 g.L⁻¹ MgCl₂.6H₂O, 0.45 g.L⁻¹ KCl, 1.14 g.L⁻¹ Na₂SO₄, 0.34 g.L⁻¹ SrCl₂.6H₂O.

4.5 Synthetic municipal wastewater

Treated wastewater from secondary municipal wastewater treatment, often referred to as tertiary municipal wastewater, contains essential nutrients such as nitrogen and phosphorus.. This resource can be effectively utilized for microalgal growth, promoting sustainability and reusability [100]. Based on this scrutiny, synthetic municipal wastewater (SMW) was utilized to simulate a tertiary municipal wastewater system. Its formulation was designed to replicate the concentration and composition of trace elements present in Bold's Basal Medium (BBM) [101]. The composition of BBM is as follows (in mg.L^{-1}): 250 mg NaNO_3 , 25 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 75 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 75 mg K_2HPO_4 , 25 mg NaCl , 175 mg KH_2PO_4 , 50 mg alkaline EDTA, 11.45 mg H_3BO_3 , and 4.98 mg acidified $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 milliliter per liter of trace elements. The trace element solution is prepared by dissolving 8.82 grams of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.49 grams of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 1.44 grams of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.57 grams of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.71 grams of MoO_3 in 1 liter of distilled water.

4.6 Progressive adaptation strategy

Initial cultivation and culture conditions

As an initial cultivation, 50 mL of prepared inoculum was added in 800 mL of SMW for a total volume of 850 mL working volume in a 1-L Erlenmeyer flask. Batch photobioreactors made of borosilicate glass Erlenmeyer flasks, featuring a conical shape with a wide flat base and tapered neck, were employed. These flasks contained a 0% HSB mixture, composed solely of SMW medium (low salinity) and microalgae strains. At the start of the experimental trial, the initial optical density (OD_{680}) of the microalgae sample in the photobioreactor fell within the range of 0.375 to 0.4 due to the addition of prepared

inoculum. The SMW medium was prepared for inoculation by adjusting its initial pH to 6.8 using 1 N NaOH, followed by sterilization at 120 °C for 15 minutes in an autoclave. To minimize the risk of contamination, foam stoppers were used to securely seal the photobioreactors. Fluorescent tubes, arranged horizontally and emitting continuous white light, were placed over most of the photobioreactors' surface under a fume hood. The light intensity at the reactor surface was measured between 56 and 57 $\mu\text{mol m}^{-2}\text{s}^{-1}$ using a Fisher Scientific™ Traceable™ Dual-Display Light Meter. Cultivation proceeded until the stationary phase at an ambient temperature of 22 ± 1 °C.

The system's hydrodynamics were aerated using the air-CO₂ mixing device FC-SH (Live Cell Instruments, South Korea). A glass rotameter integrated into the mixing system regulated the airflow, maintaining it at a rate of 1500 cc/min. Gas bubbles formed within the liquid culture medium were released through the top of the reactor, effectively preventing cell sedimentation. To further inhibit microalgae settling, the photobioreactor was manually agitated three times a day. *C. sorokiniana* was cultivated under phototrophic conditions, with the culture medium receiving inorganic carbon in the form of atmospheric air.

Cultivation in hypersaline brine supplemented with synthetic municipal wastewater

In HSB with high salinity, freshwater microalgae do not typically appear to thrive effectively. However, with stepwise adaptation, freshwater halotolerant microalgae strains can resist even higher salinities. Thus, in this work freshwater microalgae, *C. sorokiniana* was investigated successively for saline adaptation and simultaneously evaluating the growth performance. In this regard, a set of photobioreactors was utilized, each containing varying concentrations of HSB. The photobioreactors were accompanied with SMW to

create six different concentration gradients: 10%, 20%, 30%, 40%, 50%, and 60% of HSB. Here, HSB was supplemented with tertiary level SMW with the aim of reducing salinity as well as providing the necessary nutrients for microalgal growth.

The characterization of different formulated microalgal growth media is presented in **Table 4.1**. To start the progressive saline adaptation, firstly, 50 mL of microalgae sample from the exponential growth phase of initial cultivation (after 10 days) was taken and transferred to photobioreactor having 10% HSB and incubated for 24 days. After that, 20% HSB photobioreactor was inoculated with 50 ml of microalgae sample that was collected from the exponential growth phase (after 10 days) of 10% HSB photobioreactor. The same strategy of consecutive cultivation was maintained for 30%, 40%, 50% and 60% HSB containing photobioreactors.

Table 4.1 Characterization of HSB media supplemented with SMW.

HSB loading (%)	pH	EC (mS/cm)	TDS (g.L ⁻¹)	Salinity (g.L ⁻¹)	Total Nitrogen (TN) (mg. L ⁻¹)	Total Phosphorus (TP) (mg. L ⁻¹)
0	6.65	0.962	0.553	0.42	41.18	53.25
10	5.88	11.47	5.75	6.50	35.33	36.24
20	5.75	20.81	10.39	12.37	27.6	30.55
30	5.61	29.60	14.81	18.27	25.0	27.82
40	5.54	37.94	18.98	24.04	20.0	26.4
50	5.46	46.74	23.32	30.29	18.0	25.0
60	5.46	55.31	27.59	34.46	14.0	22.3

Fig. 4.3 Illustrates the progressive adaptation strategy of *C. sorokiniana* in HSB media with tertiary SMW. The initial microalgae sample in a small cultivation volume of 50 mL from the inoculum flasks. This sample is then transferred and cultivated in a larger working volume of 850 mL, comprising supplemented HSB media with a composition of 10% HSB and 90% SMW. As the cultivation progresses, subsequent stages involve variations in the composition of the supplemented HSB media. For instance, the cultivation volume of 850

mL is maintained, but the composition of the media changes to 20% HSB and 80% SMW. This change reflects an increase in the HSB concentration. Continuing further, the cultivation volume remains constant at 850 mL, while the composition of the supplemented HSB media shifts to 30% HSB and 70% SMW. This stage repeated to a further increase in HSB concentration, indicating the progressive adaptation of microalgae to higher salinity levels up to 60% HSB. Throughout these stages, microalgae samples are periodically taken to assess their growth kinetics, biochemical composition, and other relevant parameters. The figure provides a visual representation of the experimental setup and the gradual adaptation of microalgae to the challenging conditions of oilfield HSB media supplemented with SMW.'

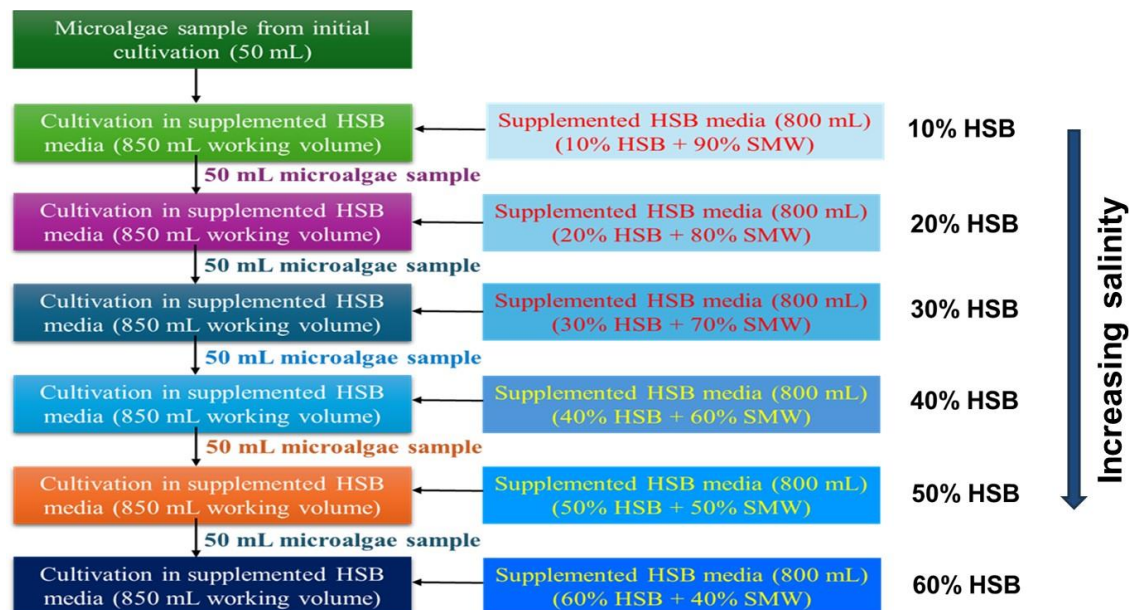


Fig. 4.3 Progressive adaptation strategy of microalgae in HSB media

4.7 Preparation and characterization of synthetic produced water

PW, generated in vast amounts by the oil and gas industry globally, contains high levels of dissolved salts, organic pollutants, and other contaminants, posing environmental risks if improperly treated. Examining various concentration gradients of PW, as done in this study, is essential to mimic real-world conditions, given that PW compositions vary widely. Effective bioremediation solutions, such as using *C. sorokiniana*, offer sustainable approaches to treat this globally prevalent PW, addressing its complex pollutant load and reducing its environmental impact. Synthetic produced water (SPW) is meticulously engineered within controlled laboratory environments to closely emulate the composition and characteristics of actual PW generated during petroleum extraction processes. The creation of SPW entails a detailed analysis of the composition of genuine PW derived from specific gas or oil reservoirs. In this study, SPW is formulated by carefully blending chemical reagents, salts, and contaminants to mirror the intended composition as closely as possible. The selection of reagents and their concentrations should align with the characteristics of real PW. A hypersaline SPW that has been modified from previously published PW formulations [99] was utilized as the basis of real PW during these experiments.

In this context, acetate was used as model dissolved organic carbon source to create an organic load in the hypersaline medium to obtain the acetate based SPW (**Fig. S1** in supplementary materials) [99].

Similarly, the required volume of phenol from stock solution (1000 ppm) was used as dissolved organic carbon source to create an organic load in the 1-L hypersaline medium to obtain the phenol based SPW (**Fig. S2** in supplementary materials)

In the same manner, phenol and acetate were employed as model dissolved organic pollutants to establish an organic load in the 1-L hypersaline medium. This was done in order to obtain the phenol-acetate based SPW, which was used for the entire bioremediation experiment without HM stress (**Fig. S3** in supplementary materials) [99]. The use of both phenol and acetate provides a realistic representation of the organic composition typically found in produced water (PW), which often includes a combination of recalcitrant compounds (e.g., phenol) and biodegradable compounds (e.g., acetate). To simulate conditions with heavy metal (HM) stress, phenol-acetate-HM-based synthetic produced water (SPW) was prepared by incorporating phenol, acetate, and lead (Pb) into a 1-L hypersaline medium, thereby creating a controlled organic load and HM stress (**Fig. S4** in supplementary materials). This prepared phenol-acetate-HM-based SPW was employed throughout the bioremediation experiments conducted under HM stress conditions.

The pH of the samples was determined using a pH meter (OAKTON, pH 700), while the salinity, total dissolved solids (TDS), and electrical conductivity (EC) were measured using a conductivity meter (Fisherbrand, accumet AB330). The analysis of total organic carbon (TOC) was performed using a TOC analyzer (Model: TOC-V_{CSN}, SHIMADZU, Japan). A key characterization of the different types of SPW used in this study, including its composition and salinity are listed in **Table 4.2, Table 4.3, Table 4.4, Table 4.5.**

Table 4.2 Characterization of acetate based SPW media supplemented with SMW (after autoclave).

PW loading (%)	pH	EC (mS/cm)	TDS (g/L)	Salinity (g/L)	Total Nitrogen (TN) (mg/L)	Total Phosphorus (TP) (mg/L)	TOC (mg/L)
0	6.65	0.96	0.55	0.43	41.17	53.24	21.77
10	5.11	11.98	5.14	6.80	35.34	36.22	61.48
20	5.11	21.93	9.43	13.15	27.5	30.54	94.71
30	5.11	31.51	13.65	19.79	25.0	27.8	124.7
40	5.08	40.99	17.58	26.19	20.0	26.4	157.5
50	5.39	49.91	21.36	32.61	18.0	25.0	191.3
100	7.42	89.6	38.32	63.46	0.0	0.0	358.4

Table 4.3 Main compositions of petroleum derived phenol based 100% SPW.

Parameter	Values
TDS (Total dissolved solids) (mg/L)	43010
Salinity (mg/L)	60760
EC (Electrical conductivity) (mS/cm)	86.14
TOC (Total organic carbon) (mg/L)	78.46
Phenol (mg/L)	100
pH	5.58

Table 4.4 Characterization of phenol-acetate based SPW media supplemented with SMW (without heavy metal) (after autoclave).

PW loading (%)	pH	EC (mS/cm)	TDS (g/L)	Salinity (g/L)	Total Nitrogen (TN) (mg/L)	Total Phosphorus (TP) (mg/L)	Phenol (mg/L)	TOC (mg/L)
10	5.05	10.55	5.284	5.90	35.30	36.20	8.5	61.48
20	5.20	19.33	9.628	11.34	27.45	30.50	17.67	94.71
30	5.24	28.67	14.38	17.52	25	27.80	25.36	124.7
40	5.43	38.04	19.04	23.95	19.5	26.40	37.41	157.5
50	5.45	44.01	21.95	28.05	17.5	25.00	45.74	191.3
100	6.82	78.25	39.03	53.91	0.0	0.0	88.2	358.4

Table 4.5 Characterization of phenol-acetate based SPW media supplemented with SMW (with heavy metal) (after autoclave).

PW loading (%)	pH	EC (mS/cm)	TDS (g/L)	Salinity (g/L)	Total Nitrogen (TN) (mg/L)	Total Phosphorus (TP) (mg/L)	Phenol (mg/L)	TOC (mg/L)	Lead (Pb) (mg/L)
10	5.64	11.79	5.879	6.68	35.30	36.20	10.08	40.39	1.97
20	5.82	21.53	10.77	12.90	27.45	30.50	19.55	84	3.77
30	6.89	31.28	15.61	19.42	25	27.80	29.18	127.8	5.61
40	7.37	40.13	20.10	25.67	19.5	26.40	37.88	172.4	7.02
50	7.22	48.17	23.99	31.36	17.5	25.00	46.83	219.5	7.93
100	7.11	86.80	43.36	61.32	0.0	0.0	93.157	426.2	10.72

4.8 *Chlorella sorokiniana* (*C. sorokiniana*) inoculum for bioremediation of PW

The microalgae strain *C. sorokiniana* (UTEX 1230) was transferred to a 500 mL conical flask having 200 mL of UTEX prescribed protease medium for first stage culture. The microalgal cells were incubated under continuous, white, fluorescent light illumination at 50–54 $\mu\text{mol photons/m}^2/\text{s}$ maintaining 22 ± 1 °C temperature without any aeration until reached at enough concentration, at least optical density (OD_{680}) of 4.2. Because a sufficiently high cell density at inoculation, providing a robust starting population for adaptation and growth in hypersaline condition is necessary for second stage cultivation. For the purpose of second stage cultivation, 50 ml of microalgae sample was transferred to 1-L conical flask consisting of 800 mL medium of a mixture of 20% hypersaline brine (HSB) solution (**Fig. S1**) and 80% SMW by volume and incubated under aeration.

The use of a 20% hypersaline brine and 80% SMW mixture for acclimatization was selected to allow *C. sorokiniana* to be gradually introduced to elevated salinity levels while ensuring adequate nutrient availability. This balanced mixture enabled the microalgae to adapt to saline conditions similar to those found in PW. By combining hypersaline brine with SMW, a controlled environment was provided that supported initial growth and metabolic activity, facilitating cellular adaptation mechanisms required for halotolerance. This approach minimized osmotic stress, promoting resilience and preparing the cells for effective bioremediation in higher-salinity conditions. Finally, 50 mL of microalgae sample from the end of growth phase of second stage cultivation (after 10 days) was collected and utilized as inoculum to conduct the bioremediation experiment in different types of SPW (Acetate based PW, Phenol based PW, Phenol-Acetate based PW without HM and with HM). 50 mL of microalgae sample was utilized as inoculum size to maintain sufficient initial optical density of microalgae sample at different photobioreactors having different PW loadings with the object of optimum growth and bioremediation of organic pollutant. It has been fixed based on some previous experiments (data not recorded here). In addition, this inoculum size of 50 mL was selected to ensure a uniform distribution of cells in the medium, facilitating even exposure to light and nutrients throughout the growth period. This volume allowed for a reliable assessment of growth dynamics and pollutant removal over time.'

4.9 Experimental set-up

4.9.1 Acetate based SPW treatment

‘To assess how effectively microalgae can remove dissolved organic pollutants from acetate based SPW, *C. sorokiniana* inoculum was cultivated in seven distinct experimental media formulations. For this purpose, a series of photobioreactors were used, each containing a different concentration of SPW. The photobioreactors were supplemented with SMW to create seven different concentration gradients: 0%, 10%, 20%, 30%, 40%, 50%, and 100% PW. The photobioreactor contained 0% of PW consisting solely of SMW medium and microalgae strains. Borosilicate glass Erlenmeyer flasks, characterized by their conical form, wide flat bottom, and tapered neck, were utilized as batch photobioreactors. The flasks or photobioreactors were filled with a total volume of 850 mL of the working liquid medium, consisting of inoculum and 800 mL of supplemented SPW. At the start of the experimental trial, the photobioreactors were filled with 50 mL of prepared inoculum to ensure that the initial optical density (OD_{680}) fell within the range of 0.375 to 0.4, allowing for accurate comparison. Prior to the operation, the SPW media in all photobioreactors were supplemented and adjusted to an initial pH value of 6.8 by adding 1 N NaOH. The media were then sterilized by autoclaving at a temperature of 120 °C for a duration of 15 minutes. In order to protect the contents from being contaminated, the photobioreactors were tightly sealed using foam stoppers. Under a fume hood, a large percentage of the surface area of the photobioreactors was covered by four fluorescent tubes that were horizontally altered, uninterrupted, and white in color. Since microalgae relies on light as their primary energy source for photosynthesis, maintaining continuous illumination ensures a consistent supply of photons, which support sustained metabolic and

growth processes. Additionally, PW contains organic and inorganic contaminants that may stress the microalgae, so providing uninterrupted light helps the cells maintain energy production and resilience under these conditions. The light intensity at the surface of the reactor was measured to be in the range of 50–54 $\mu\text{mol photons/m}^2/\text{s}$ using a Fisher ScientificTM TraceableTM Dual-Display Light Meter. The tests were conducted for a duration of 16 days under ambient temperature conditions, namely at a temperature of 22 ± 1 °C.

The air-CO₂ mixing system FC-SH (Live Cell Instruments, South Korea) was used to aerate the system's hydrodynamics. The air flow rate was controlled by a glass rotameter on the mixing device, and it was maintained at 1500 cc/min as needed. Gas bubbles are generated inside the liquid cultivation medium and are released through the upper part of the reactor, thereby avoiding the settling of the cells. To avoid the settling of microalgae, the photobioreactor was agitated manually three times daily. *C. sorokiniana* was grown in a mixotrophic environment, where the culture medium was supplied with both inorganic (atmospheric air) and organic (produced water) carbon. A 16-day growth period was established based on preliminary studies indicating that this timeframe was sufficient to observe significant biomass accumulation and TOC removal. This duration allowed for the assessment of both initial adaptation and sustained growth under continuous illumination, providing a comprehensive picture of *C. sorokiniana*'s halotolerance and bioremediation performance in PW. The elimination of the organic pollutants selected for this study was to be monitored at eight-day distinct time intervals: day 0, 8, and 16. The choice of 8-day intervals for measuring organic pollutant removal was based on preliminary experiments, which indicated that significant changes in total organic carbon (TOC) levels occurred

approximately within this timeframe. This interval allowed for observable and meaningful trends in pollutant removal efficiency without excessive sample disturbance, while also providing sufficient time for *C. sorokiniana* to adapt to the conditions and exhibit measurable bioremediation effects.'

4.9.2 Phenol based SPW treatment

'To assess how effectively microalgae can biodegrade and remove phenol pollutant from phenol based SPW, *C. sorokiniana* inoculum was cultivated in seven distinct experimental media formulations. For this purpose, a series of photobioreactors were used, each containing a different concentration of SPW. The photobioreactors were supplemented with SMW to create seven different concentration gradients: 0%, 10%, 20%, 30%, 40%, 50%, and 100% PW. The photobioreactor contained 0% of PW consisting solely of SMW medium and microalgae strains (control). Borosilicate glass Erlenmeyer flasks, characterized by their conical form, wide flat bottom, and tapered neck, were utilized as batch photobioreactors. The flasks or photobioreactors were filled with a total volume of 860 mL of the working liquid medium, consisting of inoculum and 800 mL of supplemented SPW. At the start of the experimental trial, the photobioreactors were filled with 60 mL of prepared inoculum to ensure that the initial salt free biomass concentration was 160 mg/L, allowing for accurate comparison. Prior to the operation, the SPW media in all photobioreactors were supplemented and adjusted to an initial pH value of 6.8 to 6.9 by adding 1 N NaOH. The media were then sterilized by autoclaving at a temperature of 120 °C for a duration of 15 minutes. In order to protect the contents from being contaminated, the photobioreactors were tightly sealed using foam stoppers. Under a fume hood, a large percentage of the surface area of the photobioreactors was covered by four

fluorescent tubes that were horizontally altered, uninterrupted, and white in color. The light intensity at the surface of the reactor was measured to be in the range of 1800-1900 LUX using a Fisher Scientific™ Traceable™ Dual-Display Light Meter. The tests were conducted for a duration of 10 days under ambient temperature conditions, namely at a temperature of 24 ± 1 °C.

The air-CO₂ mixing system FC-SH (Live Cell Instruments, South Korea) was used to aerate the system's hydrodynamics. The air flow rate was controlled by a glass rotameter on the mixing device, and it was maintained at 1500 cc/min as needed. Air bubbles are generated inside the liquid cultivation medium and are released through the upper part of the reactor, thereby avoiding the settling of the cells. To avoid the settling of microalgae, the photobioreactor was agitated manually three times daily. *C. sorokiniana* was grown in a mixotrophic environment, where the culture medium was supplied with both inorganic (atmospheric air) and organic (phenol containing produced water) carbon. The elimination of the phenol pollutant selected for this study was to be monitored at two-day distinct time intervals until day 10.'

4.9.3 Phenol-Acetate based SPW treatment without HM and with HM

To assess the capability of *C. sorokiniana* in removing dissolved organic pollutants (DOPs), two successive experiments were conducted: one using phenol-acetate-based synthetic produced water (SPW) without metal stress and the other using phenol-acetate-HM based SPW with heavy metal (HM) stress. Each set consisted of six distinct experimental media formulations corresponding to different SPW concentration gradients. For this purpose, a series of photobioreactors were employed, with each photobioreactor

containing SPW at one of the following concentrations: 10%, 20%, 30%, 40%, 50%, and 100% PW. In both cases (without HM and with HM), synthetic municipal wastewater (SMW) was added to supplement nutrients and support microalgal growth under the respective stress conditions. Erlenmeyer flasks made of borosilicate glass were employed as batch photobioreactors due to their conical shape, wide flat bottom, and tapered neck. The flasks or photobioreactors were filled with a total volume of 850 mL of the working liquid medium, which included 800 mL of supplemented SPW and inoculum. The photobioreactors were initially filled with 50 mL of the prepared inoculum to guarantee that the initial optical density (OD_{680}) was within the range of approximately 0.4 to 0.5, which would facilitate a precise comparison at the commencement of the experimental trial. All photobioreactors were supplemented and adjusted to an initial pH value of 6.8 by adding 1 N NaOH to the SPW media prior to operation. The media were subsequently sterilized by autoclaving at 120 °C for 15 minutes. The photobioreactors were securely sealed with foam stoppers to prevent contamination of the contents. Four fluorescent tubes that were horizontally altered, uninterrupted, and white in color were covering a significant portion of the surface area of the photobioreactors under a fume shroud. The experiments were conducted at a temperature of 22 ± 1 °C for a duration of 8 days. The system's hydrodynamics were aerated using the air-CO₂ mixing device FC-SH (Live Cell Instruments, South Korea). A glass rotameter integrated into the mixing system regulated the airflow, maintaining it at a rate of 1500 cc/min. Gas bubbles formed within the liquid culture medium were released through the top of the reactor, effectively preventing cell sedimentation. To further prevent microalgae colonization, the photobioreactor was manually stirred thrice daily. *C. sorokiniana* was cultivated in a mixotrophic environment,

utilizing both inorganic (atmospheric air) and organic (synthetically produced water) carbon in the growth medium. The removal of the organic pollutants chosen for this study was observed every two days, specifically on days 0, 2, 4, 6, and 8.

4.10 Analytical methods

4.10.1 Microalgal growth and biomass concentration

‘Sterile water was introduced into the photobioreactor before sampling to replenish the volume lost due to evaporation during cultivation. A 15 mL sample was taken every 24 hours to evaluate the optical density (OD), dry biomass concentration, and pH of the culture medium. To measure the OD, the absorbance of the microalgae sample was recorded at 680 nm against a blank sample [59] using a UV–VIS spectrophotometer (Evolution 260 Bio-Thermo Scientific). To ensure accurate OD measurements when values exceeded one, the culture medium was diluted as required. For estimating the dry biomass content, 15 mL of the culture medium was centrifuged and washed twice with distilled water to eliminate residual surface salts. The washed biomass with distilled water was vacuum-filtered using pre-weighed and pre-dried glass microfiber filter paper (Whatman GF/C, 0.45 μm pore size, 0.47 mm diameter). The filter paper containing the biomass was dried at 60 °C for 24 hours in an oven, after which it was reweighed. The difference in weight before and after drying was used to calculate the microalgae’s dry cell content, which was then expressed as biomass concentration in milligrams per liter (mg.L^{-1}) [102].

4.10.2 Growth kinetics parameters

Specific growth rate

The specific growth rate, symbolized as μ_g , was computed using equation (4.1) to quantify the daily rise in dry biomass weight [52].

$$\text{Specific growth rate, } \mu_g = \frac{\ln\left(\frac{OD_t}{OD_0}\right)}{t-t_0} \quad (4.1)$$

where, OD_t and OD_0 are the optical densities measured at the initial (t_0) and final (t) duration of the exponential growth phase.

Biomass productivity

The following equation (4.2) was employed to ascertain the biomass productivity, which is frequently referred to as the biomass production rate [103]:

$$\text{Biomass productivity, } P_b = \frac{X_t - X_0}{t_t - t_0} \quad (4.2)$$

where X_0 is the initial dry biomass weight, which is measured at the start of the culture or growth phase at time t_0 , and X_t is the dry biomass weight at the end of the cultivation phase, which is observed at time t_t .

4.10.3 Nutrient removal analysis

To evaluate nutrient elimination, 10 mL samples of microalgae culture were taken from the photobioreactors, and the subsequent techniques were implemented.

Sample preparation: The samples were centrifuged at 4500 rpm for 10 minutes to separate the supernatant, which was then kept for further analysis. Nitrogen and phosphorus concentrations were measured using a spectrophotometer (DR 3900-HACH, USA) and a digital reactor (DRB 200-HACH, USA). These instruments allowed for the

determination of total nitrogen (TN) as N-NO_3^- and total phosphorus (TP) as PO_4^{3-} in the cultivation medium

Calculating removal rates: The removal rates of TN and TP were estimated based on batch kinetics analysis. The calculation was performed utilizing the specified equation

$$(4.3): \quad \% \text{ removal} = \frac{S_0 - S_t}{S_0} \times 100 \quad (4.3)$$

S_0 and S_t denote the substrate concentration at two distinct time points: S_0 indicates the concentration at the initiation of the cultivation, corresponding to time t_0 , whereas S_t reflects the concentration at the end of the cultivation, recorded at time t_t .

4.10.4 Phenol and Total organic carbon (TOC) removal analysis

The sample from photobioreactors was collected after 2-day interval and was filtered properly before analyzed for residual phenol concentration using standard protocol[104]. In this context, UV–vis spectra of phenol containing solutions were evaluated by utilizing UV–vis spectrophotometer (SHIMADZU, UV-1601PC, Japan) from 200 to 800 nm. The maximum absorbance wavelength (λ_{max}) of phenol was found at 270 nm. Phenol concentration in aqueous solution was measured at that wavelength. The UV–Vis Spectrophotometer was routinely calibrated using specific phenol standards.

‘TOC measurement: As part of the intended experiment, 20 mL samples were taken from each photobioreactor. The samples that have been collected are subjected to centrifugation at 4500 rpm for a period of 10 minutes. This technique separated the supernatants, which were kept and utilized for TOC analysis using a TOC analyzer (Model: TOC-V_{CSN} , SHIMADZU, Japan).’ TOC was measured as an aggregate parameter to quantify the

removal of dissolved organic pollutants (DOPs), with phenol and acetate serving as representative components of the pollutant load.

4.10.5 Heavy metal removal analysis

Atomic absorption spectroscopy (AAS) method was employed to assess the concentration of Pb(II) ions in the solution (Model: Perkin Elmer AAnalyst 700). All measurements were performed in accordance with standard protocols for AAS analysis. Pb(II) ion concentrations were expressed in mg/L.

Moreover, the Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) method was employed to assess the concentration of Cd(II) ions in the solution (Model: Thermo Scientific iCAP 6300 Duo). All measurements were performed in accordance with standard protocols for ICP-OES analysis. Cd(II) ion concentrations were expressed in mg/L.

4.10.6 Evaluation of lipid content in microalgae biomass

The lipid composition was estimated using the sulfo-phospho-vanillin (SPV) technique [105]. The mechanism of lipid learning in the SPV-based colorimetric assay is illustrated in **Fig. S5** of the supplementary materials. Canola oil was employed to establish a calibration curve in accordance with the procedures specified in the SPV method. To determine the intracellular lipid content, five milliliter samples of microalgae were collected from the photobioreactor at the end of cultivation period. Following centrifugation of the samples for a duration of 10 minutes at a speed of 4500 rpm to eliminate any salts adhering to the cell surface, the samples were subsequently diluted as required and washed three times using distilled water. The biomass underwent exposure to

one milliliter of 98% sulfuric acid, followed by heating at 100 °C for a duration of ten minutes. The sample underwent cooling in an ice bath for a duration of five minutes prior to the application of five milliliters of freshly prepared SPV reagent. Subsequently, samples underwent shaking at a rate of 200 rpm for a duration of 15 minutes at a temperature of 37°C within an incubator. Following the incubation period, the absorbance of the samples was measured at a wavelength of 530 nm utilizing a UV spectrophotometer. The lipid content of the samples was calculated using the equation $y_{OD530} = 0.0333X_{lipid} + 0.1316$, resulting in an R^2 value of 0.9878 from the measured absorbance (refer to **Fig. S6** in supplementary materials). The total lipid content was subsequently determined using the provided equation (4.4), expressed as a proportion of the dry biomass weight.

$$\text{Lipid content (\%)} = \frac{X_L}{X_B} \times 100 \quad (4.4)$$

In this equation, X_B stands for the dry weight of the biomass that is present in the sample, and X_L is the amount of lipid that is present in the sample.'

4.11 Analysis of dry biomass

4.11.1 Fourier Transform Infrared (FTIR) Spectroscopy analysis

'The application of Fourier transform infrared (FTIR) spectroscopy enabled the identification of the structural and chemical bonding patterns, specifically pursuing functional groups, within several biomolecules present in the microalgal biomass. Molecular vibrational spectroscopy, such as FTIR, analyzes chemical functional groups by studying their different absorption regions, which typically range from 4000 to 500 cm^{-1} . The identification of cellular macromolecules (lipids, carbohydrates, and proteins) in dried

algal biomass was performed using Fourier Transform Infrared (FTIR) spectroscopy (Model: Thermo Scientific Nicolet Is5).

4.11.2 Thermogravimetric analysis (TGA)

Thermogravimetric examination of microalgae biomass yields valuable information regarding the thermal behavior of the sample. The TGA data were collected using a TGA instrument of the model STDG 600, manufactured by TA Instruments in the United States. Mass samples that were required were taken on the sample holder and heated at a rate of 10 °C per minute from ambient temperature to 800 °C in an inert environment (nitrogen). The TGA curve makes it easy to assess the moisture content (MC), fixed carbon (FC), volatile matter (VM), and ash content of biomass samples.

4.11.3 CHNS analysis and Higher heating value (HHV) quantification

The PerkinElmer 2400 Series II CHNS Elemental Analyzer was utilized to determine and assess the weight proportion of hydrogen, carbon, sulfur, and nitrogen in the dried algal biomass. Using the following two equations, the HHV of the dried algal biomass sample was calculated based on the proximate and ultimate analysis of the biomass [106].

$$HHV(MJ/Kg) = 0.3383C + 1.422 \left(H - \frac{O}{8} \right)$$

$$\% O = 100 - \% of (C + H + N + S + Ash)$$

The weight percentage of carbon, hydrogen, nitrogen, sulfur, oxygen, and ash content in the algal biomass is represented by C, H, N, S, O, and Ash, respectively.

4.11.4 Protein and carbohydrate content

Assuming that algal protein comprises 16% of nitrogen, total protein content was computed by multiplying total nitrogen content by 6.25 ($N \times 6.25$) [107]. The carbohydrate content

in the microalgal biomass was calculated utilizing the formula provided by the work of Silva et al. (2022) [108]. Total carbohydrate content was computed by differences applying the following equation:

$$\text{Carb. (\%wt)} = 100 - \% \text{ of (moisture + Ash + Total Lipids + Protein)'}$$

4.12 Dry biomass preparation for adsorbent

After the cultivation time, the microalgal biomass was collected by centrifuging the culture at 6000 rpm for 10 minutes utilizing a centrifuge (HITACHI-CR22GIII) and washed the biomass three times with distilled water to remove surface salts. After that, the wet biomass was collected and it was dried in an oven at a temperature of 60 °C for 48 hours in order to acquire the dry biomass of *C. sorokiniana*. The dried microalgae biomass was subsequently ground into a fine powder by means of a mortar and stored in sealed container for further use as bioadsorbent.

4.13 Cadmium solution preparation

Merck, Germany, provided all of the chemicals and reagents utilized in the investigation. About 2.7435 grams of $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ was dissolved in 1.0 L of Milli-Q deionized water to create a stock solution of Cd(II) (1000 ppm). This stock solution was then used to create aqueous solutions with different concentrations to act as the Cd(II) source. After being diluted to the appropriate concentrations, these solutions were examined.

4.14 Biosorption experiments

Using 250-mL Erlenmeyer flasks, batch biosorption—equilibrium experiments were applied to examine the biosorption of Cd(II) ions on the dry biomass of *C. sorokiniana*. A number of variables, such as the pH of the solution, the dosage of biomass, the duration of contact, and the initial amount of metal ions, influence the biosorption of Cd(II) in aqueous

solution. The consequence of pH on the adsorption of cadmium was investigated using 100 mL Cd(II) solution under different pH values (3, 4, 5, 6, 7 and 8) maintaining the constant experimental conditions (biomass dosage 50 mg/100 mL solution, initial Cd ion concentration 25 mg/L, 24 hours contact time and temperature 25 °C). The influence of the dry weight of *C. sorokiniana* biomass on cadmium bioadsorption was investigated utilizing different biomass dry weights (25, 50, 75, 100, and 125 mg) in a 100 mL Cd(II) solution under controlled conditions (pH 7, initial Cd concentration 25 mg/L, 24 hours contact duration, and temperature 25 °C). The impact of initial metal ion concentration of cadmium on biosorption was evaluated using 100 mL Cd(II) solution by varying the initial concentration of cadmium (10, 25, 40, 55 and 70 mg/l) under constant experimental conditions (pH 7, biomass dosage 125mg/100 mL solution , contact time 24 hours and temperature 25 °C). The impact of contact time on the removal of Cd(II) by *C. sorokiniana* dry biomass was studied for different periods of time (5-1452 min) maintaining experimental conditions such as pH = 7, initial Cd concentration = 25 mg/L, biomass dosage 250mg/500 mL solution, and temperature 25 °C. All biosorption experiments were conducted using a rotary orbital shaker fixing the stirring speed at 250 rpm. Following the completion of the biosorption trials, the cadmium solution underwent filtration using syringe filters that had a specific pore size of 0.25 µm. The procedure outlined in section 2.5 was employed to determine the concentration of cadmium ions in the aqueous solution prior to and following adsorption.

Biosorption experiments were carried out in triplicate, and mean results were utilized for data analysis. The elimination of metal by dry biomass was ascertained in accordance with Eq. (4.5):

$$R(\%) = \frac{C_0 - C_t}{C_0} \times 100 \dots \dots \dots (4.5)$$

where R is the percentage removal of metal from aqueous solution, C_0 is the preliminary metal ion concentration in mg/L, and C_t is the leftover concentration of metal ion at time t in mg/L.

At equilibrium, the determination of metal uptake by biomass was conducted in accordance with Eq. (4.6):

$$Q_e = \frac{C_i - C_e}{M} \times V \dots \dots \dots (4.6)$$

In this equation, Q_e represents the metal adsorbed measured in mg/gm biomass. The variable V denotes the volume of the metal-containing solution that interacts with the biosorbent, expressed in liters (L). C_i and C_e indicate the initial and equilibrium concentrations of metal in the solution, respectively, measured in mg/L. M refers to the quantity of biomass added, calculated on a dry basis in grams [109].

4.15 Adsorbent characterization

4.15.1 SEM-EDX

SEM was conducted to observe the surface morphology of the microalgae biomass before and after cadmium bioadsorption. SEM analysis was accomplished utilizing a scanning electron microscope (Model: Quanta FEG 250) at an accelerating voltage of 10 kV. Simultaneously, EDX (Model: Oxford Instruments, X-Max^N) assessment was used to determine the elemental composition and confirm cadmium adsorption on the biomass. EDX spectra were recorded, focusing on identifying cadmium and other key elements. The results before and after bioadsorption were compared to assess the presence and distribution of cadmium on the biomass surface.

4.15.2 XRD analysis

To investigate the structural changes in the *C. sorokiniana* biomass before and after cadmium bioadsorption, X-ray Diffraction (XRD) analysis was conducted. Prior to cadmium exposure, dried microalgae biomass subjected to XRD analysis using a XRD equipment (Model: Rigaku MiniFlex II). The data was collected in the 2θ range of 0° to 90° at a scanning rate of $2^\circ/\text{min}$. The post-bioadsorption samples were collected, dried, ground and analyzed under the same XRD parameters to ensure consistent comparison. The diffraction patterns obtained were used to assess the crystalline structure, phase changes, and any shifts in peak positions or intensities that indicated the interaction between cadmium ions and the microalgae cell wall components. This comparative analysis allowed for the identification of structural modifications attributable to cadmium binding and provided insights into the bioadsorption mechanism at the microstructural level.

4.16 Kinetics study

To study the kinetics of Cd(II) biosorption onto *C. sorokiniana* dry biomass, batch kinetic experiments were performed. A known mass of 0.250 gm of dried biomass was added to 500 mL of Cd(II) solution with an initial concentration of 25 mg/L in Erlenmeyer flasks. At a temperature of 25°C , the flasks were stirred at a speed of 250 rpm. The kinetic studies were carried out under experimental conditions of pH 7, for different time interval (5-1452 min). Samples were acquired at specified time intervals and promptly filtered to isolate the biomass from the solution. The concentration of cadmium in the solution at each time point was quantified following the methodology outlined in section 4.10.5.

The amount of cadmium adsorbed at time t was estimated utilizing the following Eq. (4.7).

$$Q = \frac{C_i - C_t}{M} \times V \dots \dots \dots (4.7)$$

In this equation, Q represents the quantity of cadmium adsorbed (mg/g) at time t . V denotes the volume of the metal-containing solution in contact with the biosorbent, measured in liters (L). C_i indicates the initial concentration of Cd(II), while C_t signifies the concentration of Cd (II) at time t . M represents the quantity of added biomass expressed on a dry basis in grams (gm). The kinetic data were analyzed using common kinetic models such as the pseudo-first-order (PFO) and pseudo-second-order (PSO) models to determine the rate constants and the potential rate-limiting steps in the biosorption process. Moreover, the Elovich model was also applied to describe the adsorption process that can be involved in a chemical reaction between the adsorbate and the adsorbent surface. This is relevant for HMs, as they often bind strongly to functional groups on the biomass (e.g., carboxyl, hydroxyl, and amino groups). The nonlinear form of the PFO rate equation is given as following Eq. (4.8).

$$Q(t) = Q_e(1 - e^{-k_1 t}) \dots \dots \dots (4.8)$$

In this context, Q_e and $Q(t)$ (mg/g) represent the quantities of metal ions that have been bioadsorbed at equilibrium and at time t (min), correspondingly. Additionally, k_1 denotes the rate constant of the equation, expressed in min^{-1} . The trial data were also evaluated utilizing the PSO kinetic model, represented by Eq. (4.9).

$$Q(t) = \frac{k_2 Q_e^2 t}{1 + k_2 Q_e t} \dots \dots \dots (4.9)$$

The variable k_2 (g/mg min) represents the rate constant in the second-order equation. $Q(t)$ (mg/g) denotes the amount of biosorption at time t (min), while Q_e indicates the amount of biosorption at equilibrium (mg/g). Additionally, the experimental data were analyzed using the Elovich kinetic model, represented by Eq. (4.10).

$$Q(t) = \left(\frac{1}{\beta}\right) (\ln(1 + \alpha\beta t)) \dots \dots \dots (4.10)$$

In this context, $Q(t)$ (mg/g) represents the quantity of biosorption at time t (min). The parameter α denotes the initial adsorption rate (mg/g·min), while β signifies the desorption constant, which is associated with the degree of surface coverage and activation energy (g/mg). The parameters α and β provide valuable information about the adsorption rate and the binding strength, helping in the optimization of the adsorption process.

4.17 Adsorption equilibrium isotherms

Adsorption isotherm models are vital for interpretation the adsorption process, characterizing the efficiency and capacity of adsorbents, and aiding in the design and application of adsorption systems. For each isotherm experiment, 0.125 gm of dry microalgae biomass was added to 100 mL of Cd(II) solution in Erlenmeyer flasks. The mixtures were stirred at 250 rpm at room temperature (25 °C) for 24 hours to ensure the equilibrium was reached. The equilibrium isotherms studies were conducted under experimental situations of pH 7, with different initial metal concentrations (20-350 mg/L). After 24 hours, the left over concentration of Cd(II) in solution was evaluated corresponding to the protocol explained in section 2.5. For data analysis, biosorption data were calculated using the formula in Eq. (2). Further, data were interpreted with different adsorption isotherm models such as Langmuir, Freundlich and Temkin adsorption isotherms. The Langmuir adsorption isotherm is specified by the following Eq. (4.11).

$$\frac{1}{Q_e} = \frac{1}{Q_{max}} + \frac{1}{bQ_{max}C_e} \dots \dots \dots (4.11)$$

In this context, Q_e represents the quantity of cadmium ion that has been adsorbed at equilibrium, measured in milligrams per gram (mg/g). Q_{max} and b (L/mg) are the Langmuir constants that pertain to the maximum adsorption capacity (mg/g) and the energy of adsorption. C_e represents the equilibrium concentration of cadmium ions in the solution, measured in milligrams per liter (mg/L).

The Freundlich isotherm is utilized to evaluate the biosorption processes occurring on heterogeneous surfaces. The isotherm model was utilized to define the surface heterogeneity, active sites, and their corresponding energies. The Freundlich adsorption isotherm is represented by the equation shown in Eq. (4.12).

$$\ln Q_e = \ln K_F + \frac{1}{n} \times \ln C_e \dots \dots \dots (4.12)$$

In this context, Q_e represents the quantity of cadmium ion that is adsorbed at equilibrium, measured in milligrams per gram of biomass adsorbent. C_e represents the equilibrium concentration of cadmium ion in the solution, measured in mg/L. K_F and n represent the constants of the Freundlich model. The ratio of $\frac{1}{n}$ is a critical parameter for assessing the appropriateness of adsorption processes.

The Temkin isotherm indicates that an increase in surface coverage leads to a decrease in the adsorption heat for all molecules present in the layer. This model is applicable solely within moderate concentration ranges.

The equation of this isotherm is presented by the following Eq. (4.13).

$$Q_e = B \ln K_t + B \ln C_e \dots \dots \dots (4.13)$$

Where, K_t represents the Temkin isotherm equilibrium binding constant (L/mg), which corresponds to the maximum binding energy, while B is associated with the adsorption heat (J/mol).

4.18 Statistical analysis

‘The analysis was carried out using the mean of the data from the three replicate sets of tests. To assess the level of variation across the various treatment groups, a one-way analysis of variance (ANOVA) was carried out. When the p-value was less than 0.05, a difference was considered to be statistically significant.’

CHAPTER 5

Progressive Adaptation of *Chlorella sorokiniana*

5.1. Introduction

The chapter intends to examine the feasibility of growing *C. sorokiniana* in synthetic HSB supplemented with SMW, utilizing stepwise saline adaptation to enhance the alga's productivity and halotolerance. This approach provides insights into the physiological responses of *C. sorokiniana* under saline and nutrient-variable conditions while evaluating the potential of this innovative cultivation strategy for bioenergy production, bioremediation, and environmental sustainability.

5.2 Growth characteristics and final biomass concentration

Microalgae *C. sorokiniana* (UTEX 1230) was incubated in successive manner (**Fig. 4.3**) in different levels of HSB medium supplemented with SMW as a nutrient source to evaluate the growth parameters and bioenergy potential of biomass. **Table 4.1** represents the major characterization of different growth mediums. *C. sorokiniana* was grown in SMW (0% HSB) as an initial culture and was considered as control. **Fig. 5.1** illustrates time profiles of optical densities (OD₆₈₀) indicating the growth feasibility of *C. sorokiniana* under varying HSB loadings. According to **Fig. 5.1**, microalgae was cultured about 24 days for medium with lower HSB loadings (up to 30%). However, for medium with higher HSB loadings (up to 60%), incubation lasted for about 14 days. This resultant variation of cultivation period may be due to the associated salinity stress prevailing in different concentration of HSB medium [110]. Generally, cultivation medium with higher HSB

loadings consist of higher salinities as reported in **Table 4.1**. Due to the utilization of preadapted microalgae cells in each stage culture, lag phase was escaped in the culture of all photobioreactors except 40% and 50% HSB loadings photobioreactors. The exponential phase was initiated in these two photobioreactors with increased HSB loadings after a single day of incubation. The common endpoint of the exponential growth phase was monitored on day 17 in the photobioreactors containing culture media with 0%, 10%, and 20% HSB loadings. However, photobioreactor with 30% HSB grew well until day 23. Furthermore, photobioreactors with 50% HSB and 60% HSB grew exponentially until day 7, whereas it was day 9 for photobioreactors with 40% HSB loadings.

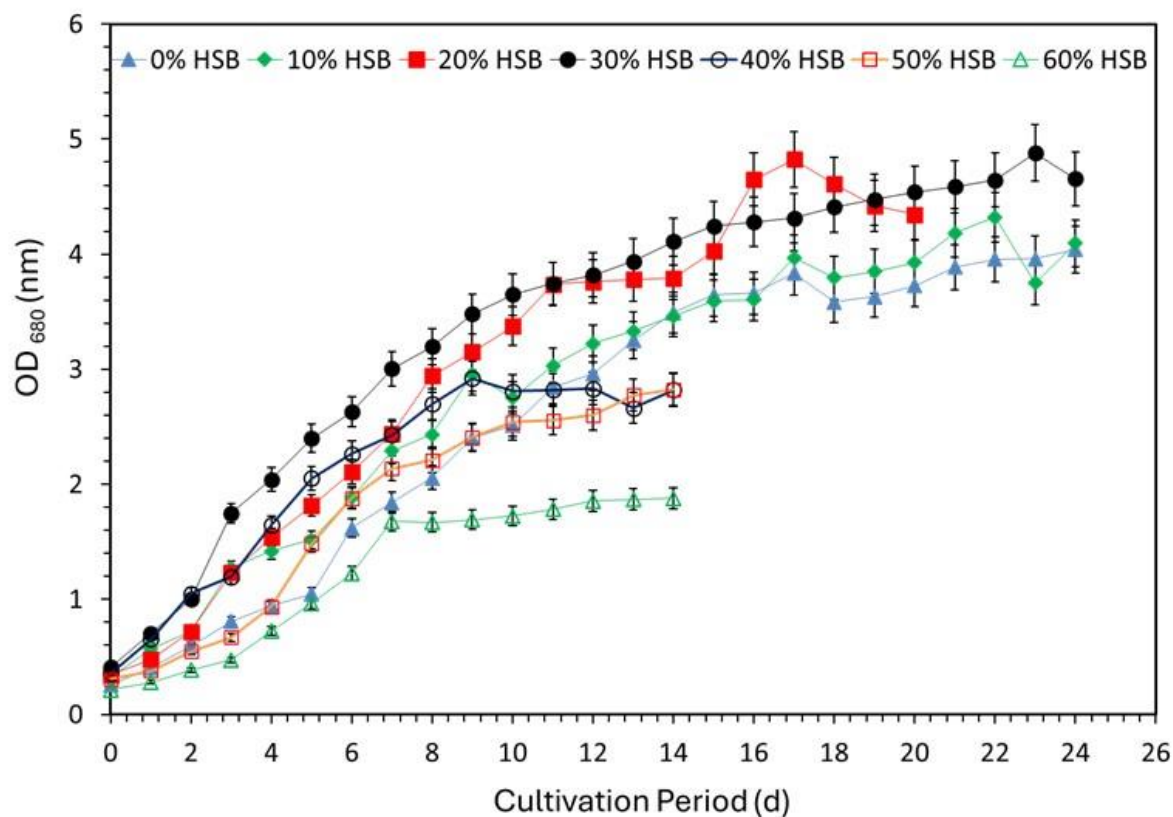


Fig. 5.1 Time profiles of optical densities (OD_{680}) as a growth feasibility of *C. sorokiniana* in different HSB loadings. Data are means $\pm$ SD ($n=3$).

As can be seen in **Fig. 5.1**, microalgae culture in SMW (0% HSB) showed the fair OD values during the cultivation period and reached the maximum OD values ($OD_{680} = 4.04$) on day 24. However, there was a slight drop in OD values on day 18 and maintained a slow growth pattern for the rest of the cultivation period. During the 10% HSB loadings, experiment was performed with a mix including microalgae sample from the initial culture (50 mL) and a media composed of 10 % HSB (v/v) and 90% SMW (v/v) to complete the total volume. It is seen that *C. sorokiniana* adapted well in relatively higher saline medium and maintained a better growth profile during the whole cultivation period than that of control (0% HSB). The maximum OD values ($OD_{680} = 4.32$) achieved on day 22 at the end of stationary phase. It indicates that enrichment of HSB medium with SMW increased the OD values by 12.62% and found overall growth pattern statistically significant ($p < 0.05$). Considering 20% HSB loadings, experiments were conducted in a similar manner. It is seen that microalgae *C. sorokiniana* revealed better adaptation in incremental saline medium and showed relatively better growth pattern than that of control and medium with 10% HSB loadings. Maximum OD values ($OD_{680} = 4.825$) attained on day 17 before declining the growth curve. There is a noticeable increase in maximum OD values by 25.78% for 20% HSB loadings than that of control and found overall growth pattern statistically significant ($p < 0.05$). It is noteworthy that microalgae in 30% HSB medium displayed the highest OD values during the cultivation period compared to the control and other tested cultivation medium. Maximum OD values ($OD_{680} = 4.88$) reached on day 23 before dropping the growth curve. The maximum OD values recorded for 30% HSB loadings is statistically significantly higher than those of others HSB loadings including

control ($p < 0.05$). As observed in **Fig. 5.1**, microalgae *C. sorokiniana* revealed the better growth pattern successively until 30% HSB loadings. It indicates that progressive adaptation helps the *C. sorokiniana* to grow well in incremental saline environment [59]. After that, relatively decreased growth profile was seen for 40%, 50% and 60% HSB loadings medium due to the hypersaline environment.

Considering 40% HSB loadings, microalgae grew well compared to that of the control until day 9 and its highest growth was showed on that day with OD values ($OD_{680} = 2.92$). Subsequently its growth began to decay slowly for the rest of the cultivation period. Thinking of the successive culture with 50% HSB loadings, microalgae displayed very slow growth until day 3. Later, growth rose rapidly until day 7 before starting a slow growth phase until the rest of the cultivation period and reached the highest OD values ($OD_{680} = 2.824$) on day 14. Lastly, the 60% HSB loadings presented lower growth of *C. Sorokiniana*, where microalgae grew very slowly until day 4. After that, microalgae grew gradually until day 7 and remained almost constant during the rest of the period attaining the highest OD value ($OD_{680} = 1.88$) on day 14. It is seen that there is a 53.46% reduction in maximum OD values obtained for 60% HSB loadings compared to the control and found overall growth pattern statistically significant ($p < 0.05$). As anticipated, a significant decrease in growth was observed for HSB loadings of 40%, 50%, and 60% (v/v) consecutively. This is likely due to excessively high salinity levels, specifically 24.04 g.L^{-1} , 30.29 g.L^{-1} , and 34.46 g.L^{-1} for the respective HSB loadings. These salinity values are too high for the growth of the freshwater microalgae *C. Sorokiniana*. Microalgae have significant challenges due to environmental stress caused by high salinity. Unicellular photosynthetic microalgae, such as *C. Sorokiniana*, are particularly susceptible to ionic imbalance, osmotic stress, and

interference from reactive oxygen species (ROS) that disrupt their photosynthesis [7]. Due to the successive reduced growth pattern observed cultivation with 40% HSB loadings and so on, experiment was not conducted beyond 60% HSB loadings.

Table 5.1 primarily delineates the growth kinetics characteristics, including the maximum specific growth rate ($\mu_{\max}$) and maximum biomass productivity ($P_{\max}$) of *C. sorokiniana* cultivated under varying HSB loadings. As showed in **Table 5.1**, the value of maximum specific growth rates at HSB loadings of 0%, 10%, 20%, 30%, 40%, 50% and 60% were 0.16, 0.15, 0.16, 0.14, 0.23, 0.21, 0.29 day⁻¹ (d⁻¹) respectively. According to the obtained results, maximum specific growth rate for 10%, 20%, and 30%, HSB loadings were closer to each other and found closer to control. The specific growth rate that was gained in this investigation was comparable to the specific growth rate that was discovered by Ammar et al. (2018) during their work on the treatment of PW by two different species of marine microalgae [52]. In addition, **Table 5.1** appears the biomass productivity of microalgae culturing at different HSB loadings. Maximum biomass productivity for 0%, 10%, 20% , 30%, 40%, 50% and 60% HSB was found to be about 35.3 mg. L⁻¹d⁻¹, 39.5 mg. L⁻¹d⁻¹, 48.0 mg. L⁻¹d⁻¹, 53.9 mg. L⁻¹d⁻¹, 47.2 mg. L⁻¹d⁻¹, 44.2 mg. L⁻¹d⁻¹, and 11.2 mg. L⁻¹d⁻¹ respectively. The obtained results of biomass productivity for different HSB loadings except 60% HSB are 1.5 to 1.1 times higher than that of 0% HSB (control). The range of biomass productivity that was established in this study is validated by the findings of other researchers who have obtained equivalent results. For example, in contrast to the results of Ishika et al. (2018), which indicated biomass productivity of 25 mg/L/day and 25.8 mg/L/day for halotolerant *Amphora* and *Navicula* species respectively under elevated salinity conditions[111], *C. sorokiniana* in this investigation demonstrated enhanced

performance with a maximum biomass productivity of 53.9 mg/L/day at 30% HSB loading. The notable disparity can be ascribed to the cultivation strategy utilized, particularly the incremental adaptation to increasing salinity, which presumably allowed *C. sorokiniana* to acclimatize to osmotic stress and ionic composition of the hypersaline environment more proficiently than other halotolerant species. Furthermore, the supplementation of SMW supplied vital nutrients that may have synergistically improved growth and metabolic activity in *C. sorokiniana*. Moreover, the comparison of biomass productivity between this study and the work of Kobayashi et al. (2013) highlights the significant influence of cultivation media composition and adaptation strategies on the growth performance of *C. sorokiniana*. Kobayashi et al. (2013) assessed the growth of three *C. sorokiniana* strains, including UTEX 1230, in an unconventional medium of 10% anaerobic digester effluent (ADE) from cattle manure, documenting a biomass productivity of 12.77 mg/L/day for UTEX 1230[112]. The present investigation revealed a significantly elevated biomass production of 53.9 mg/L/day for the same strain when cultivated in synthetic HSB supplemented with SMW and exposed to stepwise saline adaptation. The results demonstrate the versatility and resilience of *C. sorokiniana* for applications in biomass production and wastewater treatment, as well as its superior growth potential under saline conditions when supported by strategic cultivation techniques.

Table 5.1 Conditions and growth parameters of *C. sorokiniana* cultivated in supplemented HSB.

HSB loading (%)	Salinity (g/L)	Maximal (OD ₆₈₀)	Max. specific growth rate μ_m (d ⁻¹)	Cultivation Period (d)	Max. Biomass productivity (mg. L ⁻¹ d ⁻¹)
0	0.42	4.04 ± 0.08	0.16 ± 0.004	24	35.3 ± 0.7
10	6.50	4.32 ± 0.09	0.15 ± 0.003	24	39.5 ± 0.8
20	12.37	4.82 ± 0.10	0.16 ± 0.004	20	48.0 ± 0.9
30	18.27	4.88 ± 0.10	0.14 ± 0.002	24	53.9 ± 1.1
40	24.04	2.92 ± 0.06	0.23 ± 0.005	14	47.2 ± 0.9
50	30.29	2.82 ± 0.05	0.21 ± 0.004	14	44.2 ± 0.9
60	34.46	1.88 ± 0.04	0.29 ± 0.006	14	11.2 ± 0.2

It is noted that the highest maximum biomass productivity (53.9 mg. L⁻¹d⁻¹) was measured for 30% HSB, which was linked to the lowest maximum specific growth rate (0.14 d⁻¹) for same percentage of HSB loadings. Under saline conditions, microalgae may face several challenges such as osmotic stress, ionic imbalance and toxicity that can affect their specific growth rate [113]. However, despite these limitations on growth rate, microalgae cultures can still exhibit high biomass productivity under moderate saline conditions like 30% HSB loadings due to their ability to adapt to such environments and continue producing biomass, even though at a slower rate. On the other hand, highest maximum specific growth rate (0.29 d⁻¹) was evaluated for 60% HSB, which was linked to the lowest maximum biomass productivity (11.2 mg. L⁻¹d⁻¹) for equal percentage of HSB loadings. Although, the relationship between specific growth rate and biomass productivity is generally positive, however, it is possible for a microalgae culture to exhibit a high specific growth rate while still having low biomass productivity under saline conditions. This may be due to the presence of high salinity level at 60% HSB loadings. Because, salinity stress can divert energy away from biomass production and towards mechanisms for osmoregulation or defense against oxidative stress [114]. As a result, even though the cells are growing

rapidly, they may not accumulate biomass as efficiently as they would under optimal conditions. Moreover, in a saline environment, light penetration may be reduced due to factors such as increased scattering or absorption by dissolved salts. This can limit the amount of light available to the microalgae for photosynthesis, thereby reducing their overall biomass productivity despite a high specific growth rate [115]. The growth kinetics parameters recorded in this study for freshwater *C. sorokiniana* grown under varying HSB loadings validates its ability to tolerate high salinity.

Fig. 5.2 illustrates the maximum biomass yields achieved by *C. sorokiniana* cultures under different HSB loadings ranging from 0% to 60%. Across all HSB concentrations, the average highest biomass production varied between 237 mg. L⁻¹ and 1373 mg. L⁻¹. Microalgae culture with 30% HSB established the highest biomass concentration (1373 mg. L⁻¹) whereas, as expected, lowest biomass concentration (237 mg. L⁻¹) was obtained for 60% HSB loadings. It is seen that biomass attained for 10%, 20% and 30% HSB loadings is higher than that of control (0% HSB), whereas biomass achieved for 40%, 50% and 60% HSB loadings is lower than that of control (0% HSB). Thus, it is obvious that supplementation of HSB with SMW up to certain levels (30% HSB) enhances the microalgal growth to accumulate more biomass concentration. Because moderate salinity levels can provide an optimal environment for microalgal growth by helping to maintain osmotic balance and providing essential nutrients [110]. It has been observed that after a certain degree of salinity, the concentration of biomass dropped with increasing salinity, which indicates that the HSB loadings in the culture medium increased. This findings is corroborated with the other stated work [49], [59]. The maximum biomass yields achieved in this investigation for various HSB loadings surpassed those reported in a comparable

study by Eladel et al. (2019) in certain instances. Eladel et al. (2019) cultured *C. sorokiniana* in municipal wastewater and BBM-enriched wastewater for concurrent nitrogen elimination and biodiesel production. Their conclusions indicated that the maximum biomass concentration ranged from 747 mg.L⁻¹ to 833 mg.L⁻¹, which was lower in certain instances than in this investigation [116]. Moreover, Hamidian et al. (2022) examined the bioenergy and biodiesel production potential of *C. sorokiniana* EAK1 grown in untreated dairy wastewater (DWW). Their research indicated a highest biomass concentration of 1500 mg/L when cultivated in undiluted DWW [117]. These findings are closely aligned with the results of this study, where *C. sorokiniana* (UTEX 1230) achieved a biomass concentration of 1373 mg/L in 30% HSB supplemented with SMW. In addition, Salama et al. (2013) cultivated two freshwater halotolerant microalgae, *Chlamydomonas mexicana* and *Scenedesmus obliquus*, in Bold's Basal Medium (BBM) with salinity levels up to 100 mM NaCl, achieving maximum biomass concentrations of 800 mg/L and 650 mg/L, respectively [113]. In contrast, the current study recorded a significantly higher biomass concentration of 1373 mg/L for *C. sorokiniana* cultured in 30% HSB supplemented with SMW. These findings highlight *C. sorokiniana*'s exceptional ability to adapt and survive in high-salinity conditions, making it a suitable candidate for large-scale biomass production and sustainable applications in saline wastewater treatment and bioenergy generation.

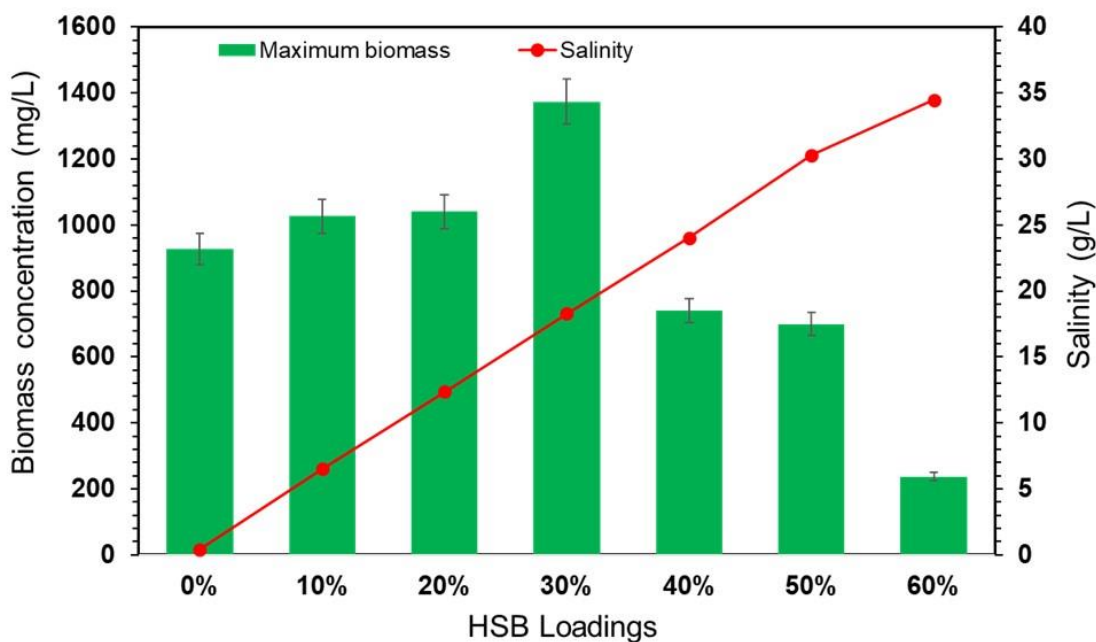


Fig. 5.2 Maximum biomass concentration obtained from the cultivation of *C. sorokiniana* under different HSB loadings. Data are means $\pm$ SD (n=3).

5.3 Nutrient removal

Microalgae can uptake nitrogen (N) and phosphorus (P) from wastewater as nutrients for their growth and metabolic interest [118]. In this particular investigation, the HSB that was imitated does not have any nitrogen or phosphorus in its composition (section 2.2). As a result, additional SMW was added to HSB in order to provide a significant source of nutrients for the growth of microalgae. Due to the supplementation with SMW, the photobioreactor having the dilutions of 0%, 10%, 20%, 30%, 40%, 50%, and 60% of HSB showed that the initial average nitrogen values for each dilution were 41.17 mg. L⁻¹, 35.34 mg. L⁻¹, 27.5 mg. L⁻¹, 25 mg. L⁻¹, 20 mg. L⁻¹, 18 mg. L⁻¹, and 14 mg. L⁻¹ respectively. To investigate the nutrient uptake capacity of *C. sorokiniana* under HSB environment, two primary nutrients (N and P) were monitored after the respective cultivation period. **Fig.**

5.3a reveals the total nitrogen (TN) content measured in the culture medium of different HSB loadings at the beginning and at the end of cultivation periods. As can be noticed in **Fig. 5.3a**, initial nitrogen concentration decreased with an increase in HSB loadings. In general, it is seen that the concentration of nitrogen reduced dramatically with an increase in the growth of microalgae, taking into consideration all of the HSB loadings. At the end of cultivation period, nitrogen concentration reduced to around 4.5 mg. L⁻¹, 4.6 mg. L⁻¹, 5.0 mg. L⁻¹, 4.0 mg. L⁻¹, 4.7 mg. L⁻¹, 4.2 mg. L⁻¹, and 6.5 mg. L⁻¹ in the photobioreactor having the HSB loadings of 0%, 10%, 20%, 30%, 40%, 50%, and 60% respectively, representing 89.1%, 86.98%, 81.81%, 84%, 76.5%, 76.7%, and 53.57% removal of nitrogen. Nitrogen uptake generally promotes microalgal growth (Fig. 2) and is influenced by the initial concentration of nitrogen [62], [119]. The percentage elimination of nitrogen (in the form of nitrate) achieved in this study aligns well with the findings from other reported research [116], [120]. Moreover, Gao et al. (2021) employed a dilution strategy to improve nutrient removal and biomass production of *C. sorokiniana* in saline frigon wastewater, achieving total nitrogen (TN) removal efficiencies ranging from 38.9% to 60.4%[121]. In comparison, this study demonstrated a notably higher TN removal efficiency of 53.57% to 89.1% when *C. sorokiniana* (UTEX 1230) was cultivated HSB supplemented SMW. In addition, Von Alvensleben et al. (2013) investigated the nitrogen removal efficiency of the halotolerant microalga *Picochlorum atomus* under high salinity conditions and reported a nitrogen removal efficiency of 50% [122]. However, this study showed better nitrogen removal efficiencies of 89.1% by *C. sorokiniana* (UTEX 1230) cultivated in HSB supplemented with SMW. The superior performance observed in this study can be attributed to the optimized nutrient composition of the HSB-SMW medium

and the stepwise saline adaptation method, which enhanced the alga's nutrient uptake capabilities under saline conditions. When exposed to reduced salt levels, microalgae *C. sorokiniana* have been shown to be capable of absorbing a greater amount of nitrate [123]. As a result, considerable quantities of this nutrient were diminished from the supplemented HSB medium owing to the growth of microalgae by the end of the culture period. While the microalgae present in the supplemented HSB medium can not entirely eliminate the nitrogen, the remaining nitrogen concentrations generally reside within the parameters of 4 to 6.5 mg. L⁻¹. This can be regarded as a effective nitrogen absorption, in alignment with the European Commission Directive 98/15/EC, which establishes a maximum threshold of 10 mg. L for Total Nitrogen discharge [90]

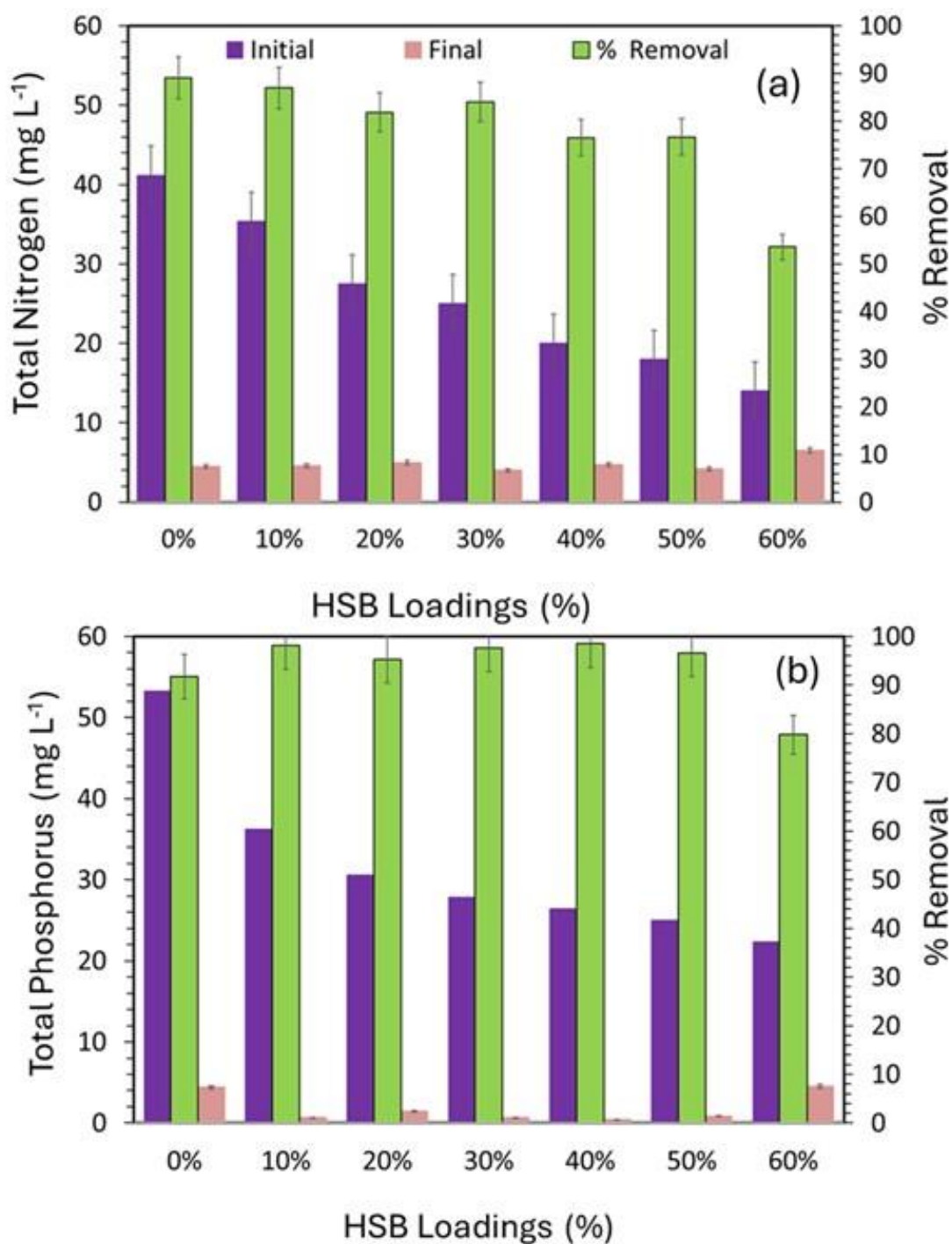


Fig. 5.3 Initial and Final concentration and percentage removal of **(a)** Total Nitrogen (TN) **(b)** Total phosphorus (TP) in the culture medium under different HSB loadings. Data are means $\pm$ SD (n=3).

Fig. 5.3b portrays the total phosphorus (TP) elimination by *C. sorokiniana* grown under different levels of HSB loadings. As showed in **Fig. 5.3b**, the photobioreactor having 0%, 10%, 20%, 30%, 40%, 50%, and 60% HSB loadings presented that the initial average values for each dilution were 53.25 mg. L⁻¹, 36.24 mg. L⁻¹, 30.55 mg. L⁻¹, 27.82 mg. L⁻¹, 26.4 mg. L⁻¹, 25 mg. L⁻¹, and 22.3 mg. L⁻¹ respectively and were reduced to around 4.4 mg. L⁻¹, 0.67 mg. L⁻¹, 1.45 mg. L⁻¹, 0.65 mg. L⁻¹, 0.4 mg. L⁻¹, 0.84 mg. L⁻¹, and 4.5 mg. L⁻¹ after the end of cultivation, correspond to the average removal of 91.74%, 98.15%, 95.25%, 97.66%, 98.48%, 96.64%, and 79.82% of phosphorus correspondingly. It is important to note that the average percentage of phosphorus elimination for all HSB loadings was higher than that of 0% HSB (control), with the exception of 60% HSB loading.

The phosphorus elimination achieved in this study for varying HSB loadings is consistent with the results of comparable research conducted by other researchers. For instance, Rahmani et al. (2022) evaluated the potential of *Chlorella pyrenoidosa* for the treatment of oil field PW. They discovered that over the course of the cultivation period, TN and TP were removed at rates of 57.14% and 75.51%, respectively [62]. Furthermore, Talebi et al. (2016) conducted a study examining the application of microalgae culture within indoor photobioreactors for the treatment of petroleum-derived produced water. The researchers found that microalgae exhibited an impressive ability to reduce nitrogen levels by 65% and phosphorus levels by 40% [57]. Moreover, Iliopoulou et al. (2022) examined the culture of *C. sorokiniana* in several dairy effluent streams, emphasizing pollutant removal and biomass characterization, and reported total phosphorus removal efficiencies between 20% and 84% [64]. The present investigation attained markedly superior phosphorus removal efficiencies, varying from 79.82% to 98.48%, when *C. sorokiniana* (UTEX 1230) was

cultivated in synthetic HSB augmented SMW. The results obtained in **Fig. 5.1** and **Fig. 5.3b** clearly indicate that the removal of phosphorus from the photobioreactors of HSB loadings medium is mostly influenced by the growth of microalgae as well as the imposition of salt stress compelled the microalgae to use the nutrients, even in the presence of an imbalanced ratio [118]. The microalgal cell wall has the ability to adsorb phosphate, which has the potential to then undergo phosphorylation [100]. *C. sorokiniana* exhibited successful phosphorus elimination and effective nitrogen absorption, despite the imbalanced supplemental HSB (N/P) ratio in this investigation. In conclusion, *C. sorokiniana* consumed P from all photobioreactors in full, in contrast to N uptake, thereby meeting the Total P requirement, Directive 98/15/EC of the European Commission, [90] limits of 1 mg. L⁻¹ except 20% and 60% HSB loadings as an exception. It is noted that 60% HSB loadings comparatively showed the lower percentage of TN and TP removal, which may be caused by ions causing downregulation and blocking the transport system, which alters the microalgae's cellular structure by making it more salinized and preventing the uptake of nutrients (N and P) [45].

5.4 Lipid content and productivity

The lipid content of *C. sorokiniana* cultured in seven distinct HSB loadings medium was measured at the end of corresponding cultivation period and is revealed in **Fig. 5.4**. In summary, the findings suggest that the lipid accumulation in microalgae biomass is influenced by the compositions of the culture medium. Considering all HSB loadings, the lipid content widely varied within the range of 3.7 % to 30.94%. These lipid content results were comparable to those of other researchers [124], where *C. sorokiniana* was cultured mixotrophically on waste dark fermentation effluents having acetate. In addition, similar

observations regarding total lipid content have been reported in previous studies. For instance, the total lipid content in three *C. sorokiniana* strains was found to range between 25% and 35% of ash-free dry weight (DW) under both Bold's Basal Medium (BBM) and 10% anaerobic digester effluent (ADE) conditions[112]. Furthermore, the lipid levels of *C. sorokiniana* (UTEX 1230) in this study align closely with those of *Chlorella* species cultivated in fermented swine manure, where lipid content reached approximately 30% of DW[125]. These consistent findings further validate the lipid production potential of *C. sorokiniana* under varying cultivation conditions, highlighting its suitability for bioenergy applications. Moreover, As appeared in **Fig. 5.4**, lipid content increased steadily with an increase in HSB loadings up to 50% HSB loadings and then it experienced a boost for 60% HSB loadings. The increasing trend of lipid content with increasing HSB loadings indicates that salinity as well as nutrients in optimum level have an influence to accumulate lipid in *C. sorokiniana* biomass.

These findings are corroborated with the similar findings of other reported work [126]. The highest lipid content of 30.94% of dry weight of biomass was acquired with 60% HSB loadings, whereas it was only 3.7% of dry weight of biomass link to the 0% HSB loadings. The highest lipid content with 60% HSB loadings may be due to the prevailing salinity stress. Because excessively high salinity levels can still stress the cells, diverting energy towards lipid production for cellular protection rather than biomass growth [127]. The lipid productivities of *C. sorokiniana* in seven different cultures were computed as depicted in **Fig. 5.4**. Lipid productivities ranged from 1.31 to 9.03 mg. L⁻¹ d⁻¹, according to the data. When 10% to 50% HSB loadings were considered, the lipid productivity of *C. sorokiniana* across various treatments was mostly caused by variations in their biomass, while the lipid

contents of the species within those various treatments did not differ significantly. Microalgae *C. sorokiniana* cultured in 0% HSB loadings showed the lowest lipid productivities compared to other experiment groups associated with the lower lipid content in microalgal biomass. However, *C. sorokiniana* grown in 60% HSB loadings had significant lipid levels, but its biomass productivity was constrained, leading to comparatively low lipid productivity. This findings are corroborated with similar findings of other researcher [128]. Considering 50% of HSB loadings having lipid content of 20.45% dry weight of biomass revealed the highest lipid productivity link to the moderate level of biomass productivity.

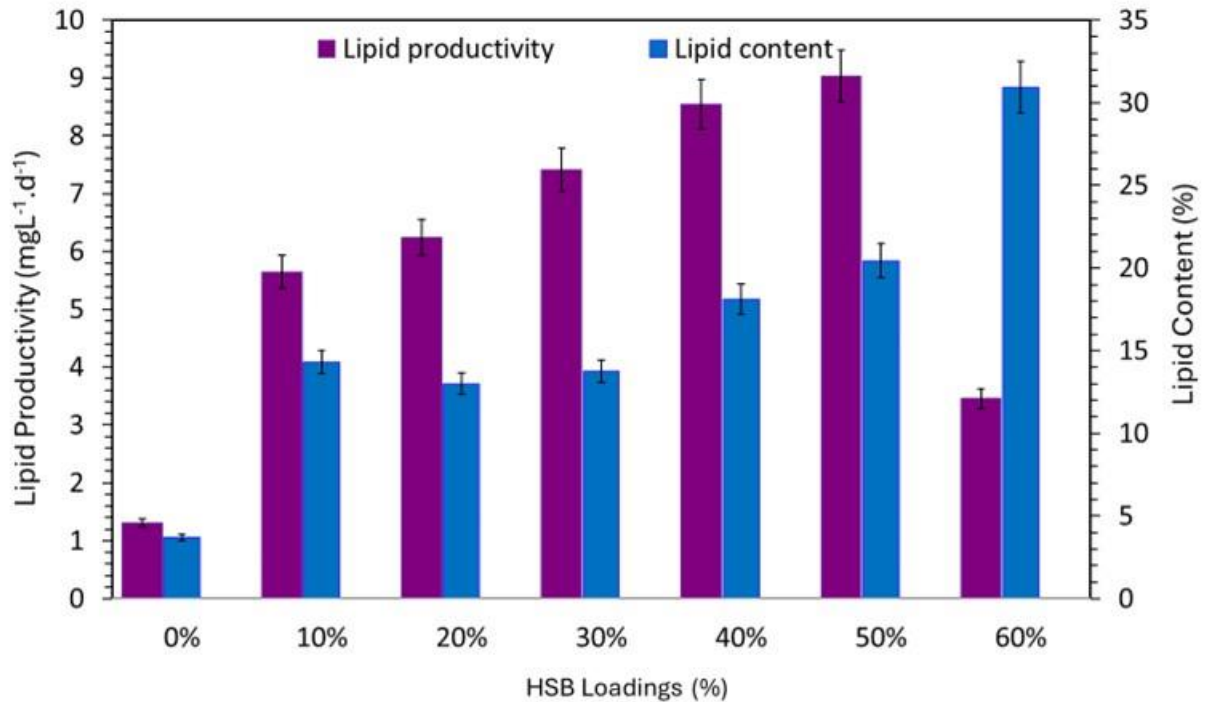


Fig. 5.4 Lipid content and lipid productivity of *C. sorokiniana* measured for different HSB loadings at the end of cultivation period. Data are means $\pm$ SD (n=3).

5.5 Dry biomass characterization

Although the rapid SPV method was used to evaluate the total lipid amount of biomass at the end of the microalgae *C. sorokiniana* cultivation and the results were reported in the previous section, a deeper comprehension of the biochemical, chemical, physical, and HHV of the dried microalgae biomass is required to establish a suitable method to produce commercial biofuel. Therefore, to categorize microalgal species according to their applications, a comprehensive bio-composition analysis is needed. The objective of the subsequent section is to determine the bio-compounds (proteins, carbohydrates, and lipids) present in dried algal biomass through a successive method of FTIR/TGA/CHNS instrumental analysis. This is done to conduct a systematic characterization of the biomass.

Structural characterization by FTIR spectroscopy

By analyzing the entire FTIR spectrum, including peaks associated with various biomolecules (lipids, proteins, carbohydrates, etc.), it is possible to assess the overall composition of microalgae biomass obtained under different HSB loadings. The FTIR transmittance of the *C. sorokiniana* algal biomass indicates the occurrence of -COOH, -OH, -CO -, and NH₂ organic chemical groups under different HSB loadings (0%, 30%, and 60%) corresponding to low, medium, and high salinity levels (**Fig. 5.5**). According to the obtained FTIR spectra, the *C. sorokiniana* biomass FTIR characterization has shown the shift in infrared (IR) peaks in some positions due to the different salinity level in the culture medium. As appeared in **Fig. 5.5**, the IR peaks from 0% HSB biomass such as 3283 cm⁻¹, 2933 cm⁻¹, 2853 cm⁻¹, 2365 cm⁻¹, were shifted to 3284 cm⁻¹, 2922 cm⁻¹, 2852 cm⁻¹, 2359 cm⁻¹, respectively associated with both 30% and 60% HSB biomass. Moreover, IR peak 2161 cm⁻¹ of 0% HSB biomass was sifted to 2164 cm⁻¹ (30% HSB biomass) and 2160

cm⁻¹ (60% HSB biomass). IR peak 1735 cm⁻¹ of 0% HSB biomass was sifted to 1742 cm⁻¹ for both 30% and 60% HSB biomass also. Similarly, shifting of other IR peaks of 0% HSB biomass was seen when compared to 30% and 60% HSB biomass. Changes in peak intensities, positions, and shapes can provide valuable information about the biochemical responses of microalgae to salinity stress [129]. Cellular macromolecules (lipids, proteins, and carbohydrates) can be identified in various study locations based on their distinct transmission bands by analyzing the acquired FTIR spectra. Two sites are frequently used in the literature to determine the lipid content by FTIR: the vibrational stretching of ester bonds at 1742 cm⁻¹ and the methylene and methyl groups at 2800–3000 cm⁻¹ [130].

The presence of "lipid band spectra" linked with the symmetric stretching of methylene's C-H bonds is responsible for the FTIR bands observed at 2922 cm⁻¹ and 2852 cm⁻¹ for 30% and 60% HSB loadings respectively (**Fig. 5.5**). These lipid-associated bands are stronger in comparison to similar findings by other studies [131]. The band observed at 3284 cm⁻¹ (30% and 60% HSB loadings) and 3283 cm⁻¹ (0% HSB loadings) is a result of the stretching of the O-H bonds. The presence of a prominent peak at 1742 cm⁻¹ (30% and 60% HSB loadings) can be attributed to the stretching of C=O bonds in esters (inserted table in Fig. 6). This peak is believed to be mainly associated with lipid and fatty acid ester bonds [132].

Another significant band observed at a wavenumber of 1646 cm⁻¹ is attributed to the stretching of the C=O bond in the amide I region, which is connected with proteins [133].

The relative lipid content was determined by calculating the ratio of the intensity of the lipid band at 1742 cm⁻¹ to the intensity of the amide I band at 1646 cm⁻¹ [134]. The ratio of the lipid: amide band was higher in the saline medium containing 30% and 60% HSB

loadings compared to the medium with 0% HSB loadings. This indicates that HSB enhances the lipid content in microalgal biomass [120].

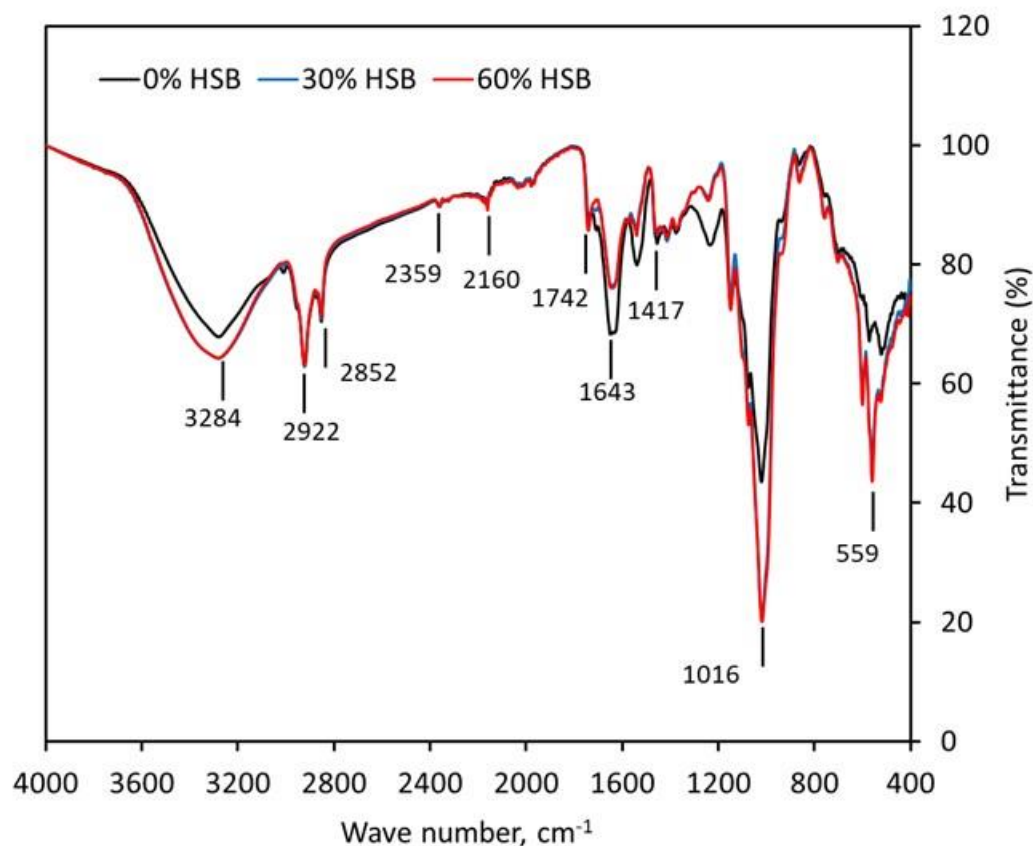


Table: Tentative assignments of bands obtained in the FTIR spectra of *Chlorella sorokiniana* biomass

Wave number range (cm ⁻¹)	Band assignment	Comments
3512-3003	Water, (O-H) stretching; Protein, (N-H) stretching (amide A)	Water, protein
2940-2897	(C-H) from methylene groups of lipids	Primary lipids
2860-2835	(C-H) from methylene groups of lipids	Primary lipids
1811-1720	(C=O) stretching of esters	Esters of lipids and fatty acids
1692-1599	Amide I band mainly (C=O) stretching	Protein (Amide I)
1545-1460	Amide II band mainly (C-H) and (N-H) associated with protein	Protein (Amide II)
1455-1416	(CH ₂) and (CH ₃) bending of methyl	Proteins
1398-1350	(CH ₃) and (CH ₂) of proteins, and C-O of COO ⁻ group	Proteins and carboxylic acids
1312-1204	(P=O) of phosphodiester groups	Nucleic acids (DNA and RNA)
1200-900	(C-O-C) of polysaccharides	Carbohydrates
1020-957	(C-O) stretching, (C-O-C) of polysaccharides	Carbohydrate
880-570	Polysaccharides of cell wall	--

Fig. 5.5 FTIR spectra of *C. sorokiniana* biomass cultured with 0%, 30%, and 60% HSB loadings. Embedded table indicates the interpretation of the associated functional groups and corresponding biomolecules obtained in biomass.

The spectra of the amide II band, which mostly consists of stretching vibrations of C-H and N-H bonds associated with proteins, were observed at around 1541 cm^{-1} (30% and 60% HSB loadings) and 1539 cm^{-1} (0% HSB loadings). The spectra at 1455 cm^{-1} (0% HSB loadings) and 1414 cm^{-1} (30% and 60% HSB loadings) correspond to the bending of methyl groups (CH_2 and CH_3) and the stretching of carboxylic groups (C-O) associated with the presence of proteins in *C. sorokiniana* biomass (inserted table in **Fig. 5.5**). Due to the asymmetric stretching of the phosphodiester P=O, the protein band spectra exhibited a moderately significant band at around 1238 cm^{-1} . The 'carbohydrate band spectra' exhibited strong and medium features in the range of approximately $1149\text{--}522\text{ cm}^{-1}$ due to C-O-C and C-O stretching. The presence of the main constituents of the *C. sorokiniana* species, specifically lipid, carbohydrate, and protein, was detected by the spectral bands. These outcomes are consistent with several others [131], [133].

TGA of biomass

Thermogravimetric Analysis (TGA) plays a pivotal role in assessing the bioenergy potential of microalgae biomass, offering valuable insights into its thermal behavior and decomposition characteristics. This analytical technique helps in understanding the intricate interplay between temperature, biomass composition, and energy release, laying the foundation for informed decisions in bioenergy research and development. TGA can be used to measure the proximate composition of biomass samples, including moisture content (MC), volatile matter (VM), ash content, and fixed carbon (FC) [47]. In this study, TGA analysis has detected three stages of degradation of microalgae biomass, similar to what other authors have shown [135]. It was noticed that samples cultured in HSB loadings.

The TGA analysis of the pyrolytic characteristics of *C. sorokiniana* biomass in microalgae grown in low salinity conditions (0% HSB loadings), moderate salinity conditions (30% HSB loadings), and high salinity conditions (60% HSB loadings) are presented in **Fig. 5.6**. The predominant source of the initial stage of mass loss, which comprised the loss of free water and water loosely bound to biomolecules before 160°C (0% HSB biomass), 180°C (30% HSB biomass), and 185°C (60% HSB biomass), was the moisture content (MC) of the microalgae biomass sample, as shown in **Fig. 5.6** [136]. It is seen that *C. sorokiniana* biomass had a moisture level in the range of 4.18% to 8.05% considering all HSB loadings in this study (**Table 5.2**), suggesting it a suitable option for direct combustion. Due to the endothermic nature of evaporation, a combustible material utilized in thermochemical systems requires a low moisture content (less than 10%) in order to enhance the amount of usable energy [137]. According to **Table 5.2**, it is seen that salinity in culture medium had an influence on moisture content in biomass and almost revealed a positive correlation. Because salinity can affect water uptake and retention in cells, potentially leading to variations in moisture content.

During the devolatilization stage, the *C. sorokiniana* biomass underwent vaporization of its main organic constituents within specific temperature ranges. For biomass with 0% HSB, this vaporization occurred between 185°C and 510°C. For biomass with 30% and 60% HSB loadings, the vaporization took place in the temperature range of 195°C to 435°C and 190°C to 385°C respectively. These vaporization were a result of the thermal breakdown of proteins, carbohydrates, and lipids [138]. The volatile matter content of *C. sorokiniana* biomass was in the range of 47.81% to 80.31% considering all HSB loadings (**Table 5.2**). Particularly, it was 80.31%, 59.51%, and 49.71% for 0%, 30% and 60% HSB

loadings biomass respectively. The variation in obtained volatile matter content in microalgal biomass may be due to the incremental salinity level at different HSB loadings. Because salinity influences the cellular metabolism of microalgae. High salinity levels can stress microalgae, altering their metabolic pathways and affecting the accumulation of lipid, protein, and carbohydrate in microalgal biomass, which in turn affect the volatile matter content. The percentage of volatile matter achieved in this study is consistent with the similar findings of other researcher [139], [140].

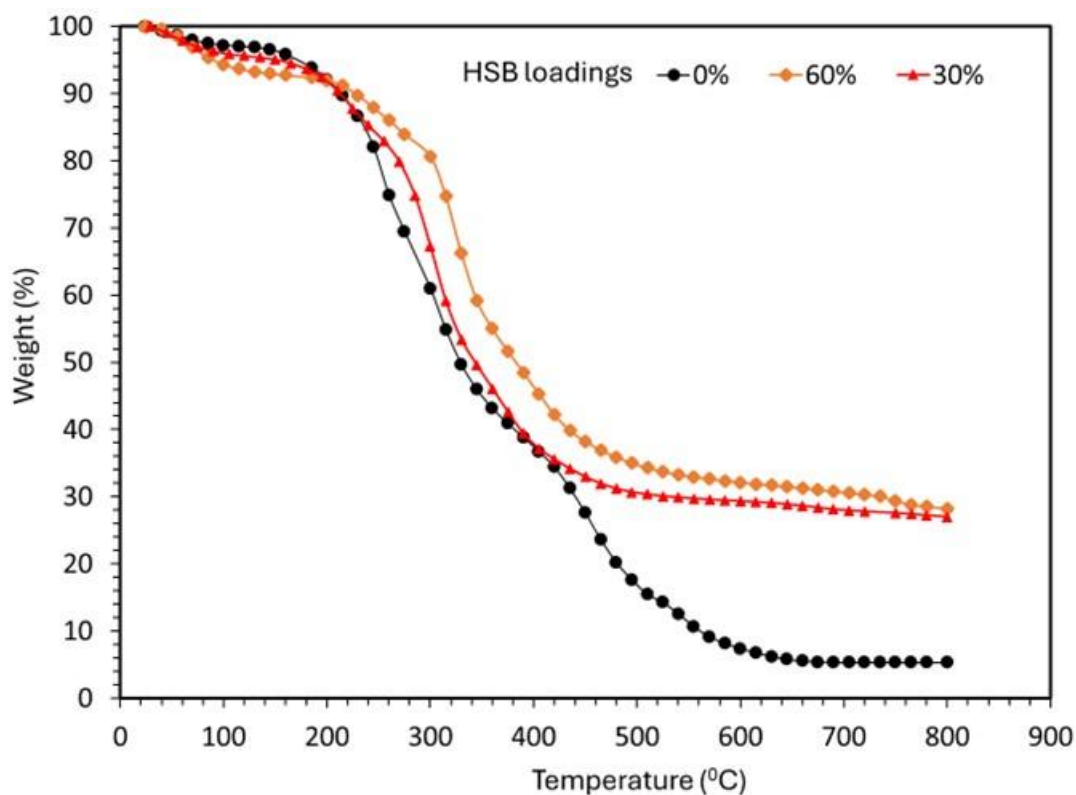


Fig. 5.6 TGA curve of *C. sorokiniana* biomass cultured with 0%, 30%, and 60% HSB loadings.

Different HSB loadings have similar thermal characteristics except for a few differences in 0%

As appeared in **Fig. 5.6**, the left over mass loss in the third stage after 510 °C to until 800 °C (0% HSB biomass), after 435 °C to until 800 °C (30% HSB biomass) and after 385 °C to until 800 °C (60% HSB biomass) was due to both ash content and fixed carbon (FC) [141].

According to the reports expressed in **Table 5.2**, the percentage of fixed carbon and ash content in biomass were in the range of 6.19% to 17.06% and 5.31% to 32.80% respectively considering all HSB loadings in this study. In general, *C. sorokiniana* biomass burns more quickly during the devolatilization phase because to its high volatile matter and low fixed carbon contents [139]. The study's proximate analysis, together with comparable data from other sources are presented in **Table 5.2**.

Table 5.2 Proximate analysis of *C. sorokiniana* biomass in different HSB loadings compared with similar findings of previously reported

Microalgae biomass	Moisture content (MC) (dry wt.%)	Volatile matter (VM) (dry wt.%)	Fixed carbon (FC) (dry wt.%)	Ash content (dry wt.%)	Reference
<i>Chlorella vulgaris</i>	9.10	37.3	5.0	48.6	[142]
<i>Spirulina sp.</i>	6.50	59.5	15.5	18.5	[135]
<i>Scenedesmus sp.</i>	5.72	67.94	6.8	19.64	[131]
<i>Nannochloropsis oculata</i>	3.99	67.45	8.08	24.47	[139]
<i>Tetraselmis suecica</i>	4.27	58.31	15.77	21.65	[143]
<i>Scenedesmus almeriensis</i>	2.90	67.90	9.70	19.40	[144]
<i>Scenedesmus dimorphus</i>	3.6	62.79	6.51	27.1	[133]
<i>Chlorella sorokiniana</i> (0% HSB or SMW)	4.18	80.31	10.2	5.31	This study
<i>Chlorella sorokiniana</i> (10% HSB)	6.75	48.88	13.95	30.42	This study
<i>Chlorella sorokiniana</i> (20% HSB)	6.71	54.3	6.19	32.80	This study
<i>Chlorella sorokiniana</i> (30% HSB)	6.31	59.51	7.17	27.01	This study
<i>Chlorella sorokiniana</i> (40% HSB)	6.66	47.81	15.47	30.06	This study
<i>Chlorella sorokiniana</i> (50% HSB)	7.75	49.11	17.06	26.08	This study
<i>Chlorella sorokiniana</i> (60% HSB)	8.05	49.71	13.99	28.25	This study

CHNS analysis, protein, carbohydrate and HHV in biomass

CHNS analysis provides insights into the elemental composition of microalgae biomass, specifically carbon (C), hydrogen (H), nitrogen (N), and sulfur (S). These elements are fundamental in determining the energy content and combustion properties of biomass. Ultimate analysis is another word for the elemental determination of hydrogen, carbon, sulfur, and nitrogen [145]. Before proceeding with additional processing, it is necessary to do an ultimate analysis on a sample of microalgae biomass in order to examine the beneficial benefits of microalgae as a feedstock for biofuel [146]. As indicated in **Table 5.3**, the C, H, N, and S contents of the *C. sorokiniana* algal biomass were measured at various HSB loadings. The outcomes were compared to related observations from other published studies. The elemental composition of 0% HSB biomass was found different as compared to others, representing highest carbon content (49%) in biomass than those of others considering all HSB loadings. It is true that salinity had a direct effect on carbon content in biomass. Considering all HSB loadings, relatively reduced amount of carbon content was achieved, ranged from 36.12% to 40.27% with increasing salinity. Because high salinity can lead to cellular changes such as increased production of osmolytes (compounds that help maintain osmotic balance), which may indirectly affect carbon metabolism and storage. It is seen that sulfur content in biomass decreased with increase in salinity in the culture medium. Even though, zero sulfur content was measured for the biomass obtained from 20% to 60% HSB loadings. High carbon and hydrogen content relative to nitrogen and sulfur is desirable for efficient biofuel production [147] and are in good accord with the obtained outcome in this study. Protein in microalgae biomass is a

valuable resource with diverse applications. Microalgae are rich sources of high-quality protein, encompassing essential amino acids vital for human and animal health.

As reported in **Table 5.3**, protein content of *C. sorokiniana* was relatively higher in 0% HSB with 28.12% of dry weight. Total protein content for other HSB loadings ranged from 15.37% to 19.81%, showing a small inclination to decrease as salinity increases. Comparatively, higher protein content with 0% HSB may be due to the nitrogen rich condition of the culture medium. In their study, Mtaki et al. (2021) found that increasing the nitrogen content in the growth medium can stimulate protein synthesis in microalgae [148]. Haris et al. (2022) claim that salinity instability may cause microalgae to produce less total protein content. Elevated levels of salt may limit the activation of ATP-synthase, hence inhibiting the synthesis of proteins. This may help to explain why *C. vulgaris*'s protein content decreases as salinity rises [110].

Carbohydrate is a significant constituent of *C. sorokiniana* biomass, and its synthesis can be influenced by variations in growth conditions, such as the properties of the nutritional media, including the levels of nitrogen, phosphates, and other micronutrients. In this study, the carbohydrate content of *C. sorokiniana* varied between 25% and 62.87% of the dry weight of biomass, surpassing the values reported in some existing literature [107]. Moreover, Ishika et al. (2018) examined biochemical composition of two halotolerant microalgae such as *Amphora* and *Navicula* species cultivated under high salinity conditions and obtained average carbohydrate contents of 28.2% and 20.5% (as a percentage of ash-free dry weight), respectively[111]. In contrast, the current study demonstrated that *C. sorokiniana* (UTEX 1230), cultivated in synthetic HSB supplemented SMW, exhibited a significantly broader range of carbohydrate content, varying between 25% and 62.87% of

the dry weight. These results suggest that *C. sorokiniana* not only adapts effectively to hypersaline environments but also accumulates carbohydrates at levels that surpass or are comparable to other halotolerant species. This highlights its potential as a robust candidate for applications in bioenergy production and other biotechnological processes requiring carbohydrate-rich biomass. Carbohydrate content was comparatively higher in 0% HSB and 30% HSB with 62.87% and 43.87% of dry weight of biomass respectively. It is seen that carbohydrate content in all HSB loadings except 60% HSB (**Table 5.3**) was higher as compared to total lipid content obtained in this study (**Fig.5.4**). According to Simas-Rodrigues et al. (2015), *Chlorella* and *Tetraselmis* are microalgae genera that have a high carbohydrate content and are well-suited for bioethanol synthesis [149]. Because the synthetic pathway is tightly related, competitions arise in the generation of lipid and carbohydrate content in microalgae under stress conditions [150]. This statement likely elucidates the findings of the current study about the carbohydrate composition of *C. sorokiniana*, which exhibited a lower lipid content when compared to its carbohydrate content. During nutrient limitation in the stationary phase, which is the phase characterized by growth conditions that are restricted, the majority of carbon is typically transformed into carbohydrate content [110].

The HHV of microalgae biomass is a crucial parameter in determining its bioenergy potential. HHV represents the maximum amount of heat released when a fuel is completely combusted in an environment where the products are allowed to return to their original temperature, typically expressed in units of energy per unit mass (e.g., MJ/kg or kcal/kg). **Table 5.3** also presents a comparison of the higher heating value of *C. sorokiniana* biomass with similar studies conducted in the past. the HHV of *C. sorokiniana* biomass in the

present study ranged from 16.15 MJ/kg to 22.84 MJ/kg , which is like the other works for different species including *C. sorokiniana* reported in the literature [138]. Maximum amount of HHV (22.84 MJ/kg) was achieved in this study for microalgae culturing with 10% HSB loadings. It may be due to the presence of relatively higher carbon as well as lower oxygen content in 10% HSB loadings biomass. The study found that *C. sorokiniana* had a higher HHV compared to lignocellulosic biomass sources like rice husks (16.7 MJ/kg) and corn stalks (15.81 MJ/kg) [151], bagasse sugarcane (16.4 MJ/kg) [152], and pistachio shells (17.47 MJ/kg) [153]. Microalgal biomass samples that have a high calorific value are characterized by low nitrogen levels and almost little sulfur. Microalgal biomass possesses a diminished nitrogen and sulfur composition, rendering it a favorable option for the production of environmentally sustainable biofuels [141]. In addition, solid fuels commonly utilized in bioenergy production, such as wood sawdust with a HHV of 18 to 19 MJ/kg, can be used as a reference to assess the energy potential of *C. sorokiniana* in thermal conversion processes [106].

Table 5.3 Ultimate analysis, protein, carbohydrate and HHV of *C. sorokiniana* biomass in different HSB loadings compared with similar findings of previously reported

Microalgae biomass	Carbon (C) (dry wt.%)	Hydrogen (H) (dry wt.%)	Nitrogen (N) (dry wt.%)	Sulfur (S) (dry wt.%)	Oxygen (O) by difference	Protein (dry wt.%)	Carbohydrate (dry wt.%)	HHV (MJ/Kg)	Reference
<i>Chlorella sorokiniana</i>	49.74	7.60	8.25	0.38	34.03	37.7	27.51	20.96	[107]
<i>Chlorella sp.</i>	46.10	6.10	6.70	0.62	39.1	29.6	16.5	17.30	[154]
<i>Spirulina platensis</i>	51.81	7.11	3.95	1.27	32.90	65.64	7.20	20.97	[155]
<i>Nannochloris oculuta</i>	43.88	6.95	5.74	1.32	42.11	n/a	n/a	17.25	[156]
<i>Tetraselmis suecica</i>	27.72	4.59	4.70	1.11	40.23	29.37	n/a	9.38	[143]
<i>Scenedesmus almeriensis</i>	41.90	6.70	5.90	0.80	44.70	44.2	25.2	15.76	[144]
<i>Scenedesmus sp.</i>	24.45	7.68	5.42	n/a	22.45	n/a	n/a	15.20	[131]
<i>Scenedesmus dimorphus</i>	44.59	8.28	4.02	0.16	15.85	51.75	n/a	24.04	[133]
<i>Chlorella sorokiniana</i> (0% HSB or SMW)	49	8	4.5	0.51	32.68	28.12	62.87	22.14	This study
<i>Chlorella sorokiniana</i> (10% HSB)	39.56	8.88	3.17	0.17	17.8	19.81	35.46	22.84	This study
<i>Chlorella sorokiniana</i> (20% HSB)	36.12	6.97	2.59	0	21.52	16.18	38.02	18.30	This study
<i>Chlorella sorokiniana</i> (30% HSB)	40.20	6.67	2.46	0	23.66	15.37	43.87	18.87	This study
<i>Chlorella sorokiniana</i> (40% HSB)	38.72	5.87	3.03	0	22.32	18.93	32.91	17.47	This study
<i>Chlorella sorokiniana</i> (50% HSB)	38.40	5.64	2.57	0	27.31	16.06	37.41	16.15	This study
<i>Chlorella sorokiniana</i> (60% HSB)	40.27	6.35	2.53	0	22.6	15.81	25	18.64	This study

n/a = not availabl

5.6 Conclusions

This study demonstrates the feasibility and effectiveness of cultivating *C. sorokiniana* in synthetic oilfield HSB supplemented with SMW as a nutrient source. Through progressive adaptation in different HSB loadings, *C. sorokiniana* exhibited a better growth pattern and showed maximum biomass productivity ($53.9 \text{ mg. L}^{-1}\text{d}^{-1}$) with 30% HSB loadings. However, cultivation with higher HSB loadings could limit the growth of *C. sorokiniana*. At the end of cultivation, average nitrogen removal was in range of 53.57% to 89.1% considering all HSB loadings. In comparison to nitrogen uptake, phosphorus uptake was higher by *C. sorokiniana*, representing 79.82% to 98.48% phosphorus removal thinking of all HSB loadings. The suitability of *C. sorokiniana* biomass as a biofuel feedstock was determined by analyzing its physicochemical parameters, including proximate analysis, ultimate analysis, and determining HHV. *C. sorokiniana* biomass under different HSB loadings exhibited desirable biochemical composition, including higher carbohydrate content compared to protein and lipid, indicating its suitability for various biotechnological applications beyond biofuel production. The stepwise saline adaptation strategy employed demonstrates the feasibility of cultivating *C. sorokiniana* in high-salinity environments, positioning it as a viable candidate for large-scale operations in regions with limited freshwater resources.

However, specific limitations of the study must be recognized. The application of synthetic HSB and SMW created a regulated environment that fails to entirely emulate the intricacy and variety of actual oilfield brine and municipal wastewater. Actual systems exhibit a broader spectrum of chemical components, biological contaminants, and variable salinity levels, which may influence the physiological adaption, growth, and bioenergy production of *C. sorokiniana*. Moreover, variables such as temperature fluctuations, microbial competition, and nutrient discrepancies inherent in actual wastewater may affect the results observed in this investigation. To mitigate these limitations and improve the relevance of the findings, subsequent research should concentrate on evaluating the cultivation method with actual oilfield brine and municipal wastewater to gain a deeper insight into the effects of varied and dynamic environments. Addressing these problems will more completely confirm the potential of *C. sorokiniana* for bioenergy production and wastewater bioremediation in hypersaline environments, facilitating its effective implementation in industrial applications.'

CHAPTER 6

Bioremediation of Acetate Based Produced Water

6.1 Introduction

The primary objective of this chapter is to assess the efficiency of microalgae *C. sorokiniana* in reducing the concentration of dissolved organic carbon in PW while analyzing its growth and survival in such a complex saline environment. To address this aim, *C. sorokiniana* was cultivated in SPW supplemented with tertiary synthetic SMW as a novel growth medium to simultaneously remove dissolved organic carbon and produce valuable biomass. The evaluation of this microalga's growth and its ability to bioremediate pollutants included measuring its growth rate, the reduction of organic pollutants through total organic carbon (TOC) analysis, lipid content, and the efficiency of nutrient removal across different concentrations of synthetic produced water (SPW) effluent.

6.2 Microalgal growth, pH profiles and biomass concentration

Preadapted microalgae *C. sorokiniana* inoculum was cultivated in SPW supplemented with SMW to evaluate the growth parameters and bioremediation capacity. **Fig. 6.1a** demonstrates the time profiles of optical densities (OD₆₈₀) as a growth feasibility of *C. sorokiniana* in different PW loadings. As observed in this figure, microalgae successfully adapted at PW loadings of 0% (control), 10%, 20%, 30%, 40% and 50%. However, microalgae did not grow well in 100% PW due to the lack of appropriate nutrients and high salinity. Due to the utilization of preadapted microalgae cells, lag phase was avoided in the

culture of all photobioreactors except 100% PW. It is seen that the common end of exponential growth phase was observed on day 10 in all the photobioreactors having the culture media of 10%, 20%, 30%, 40% and 50% PW loadings. Briefly, it is apparent that microalgae grew well for the 16-day cultivation period maintaining higher OD values for all PW loadings than that of 0% PW (control). But, as expected, OD values of 100% PW were always lower than that of 0% PW (control) during the cultivation period. The obtained growth patterns of *C. sorokiniana* under different PW loadings are corroborated by the findings of another researcher for different species. For instance, Hopkins et al. (2019) grew *Dunaliella tertiolecta* in a medium based on produced water, varying the salinity levels with the object of biomass and lipid productivity and found similar growth behavior like this study [49].

As can be observed in **Fig. 6.1a**, microalgae in 10% PW showed the highest OD values during the cultivation period compared to the medium composed of 20%, 30%, 40%, and 50% PW respectively. This may be due to the increasing salinity with increasing PW loadings in the culture medium [59]. Microalgae experience osmotic stress due to the high quantities of salts (NaCl and other ions) found in hypersaline conditions linked to greater percentages of PW loadings[114]. Algal cells lose water due to the high concentration of salt outside, which causes dehydration and cell shrinkage. This osmotic imbalance can lower photosynthetic efficiency and impair cell activity. There was a little variation in the observed values of OD of microalgae cultures until day 6 among the 10%, 20%, 30%, 40%, and 50% PW loadings. After that, microalgae culture in 10% PW revealed significantly higher OD values for the rest of cultivation period than those of other PW loadings.

It is seen that at 10% PW concentration, the microalgal growth on day 6 was nearly identical to that observed on day 4. However, following day 6, a significant increase in optical density (OD) was recorded, indicating a rapid enhancement in growth. This phenomenon may be attributed to the synchronization of cell division and self-shading of microalgae during the cultivation phase[157]. During this period, cell biomass or size may see a minor rise without substantial alterations in cell count, resulting in minimal or no variation in optical density (OD). Furthermore, as the culture density escalates, light may fail to penetrate deeply into the culture, especially under mixotrophic conditions where photosynthesis remains operational. Self-shading can diminish the photosynthetic activity of deeper strata, momentarily constraining growth. Following cellular adaptation or redistribution, OD may subsequently rise once more. Moreover, from day 6 to day 16, a similar trend of growth was observed among 20%, 30%, 40%, and 50% PW loadings and found higher OD values in the order of lower PW loadings after end of the cultivation. The drop in microalgal growth seen on day 12, followed by a successive increase on days 14 and 16, can be described by a combination of environmental and physiological factors that led to temporary growth inhibition, followed by recovery. These fluctuations may have been prompted by accumulation of toxic byproducts, pH fluctuations, or oxygen-related stress, which provisionally inhibited microalgal metabolism. Recovery on days 14 and 16 could be ascribed to the stabilization of these factors, including nutrient redistribution, detoxification, improved gas exchange, or adaptation to light conditions. Moreover, the metabolic activity of microalgae, particularly CO₂ uptake and organic acid creation, can lead to alterations in the culture's pH. A quick drop in pH around day 12 by 0, 30, 40 and 50% PW could have caused adverse conditions for growth by inhibiting nutrient

accessibility or enzyme activity. The succeeding increase in microalgal growth on day 14 could be due to a stabilization or correction of the pH, allowing the cells to resume growth under more favorable conditions. According to the analysis of variance (ANOVA), it is proven that the mean differences of OD values between the treatment groups (PW loadings) are statistically significant ($p\text{-value} = 0.000128 < 0.05$).

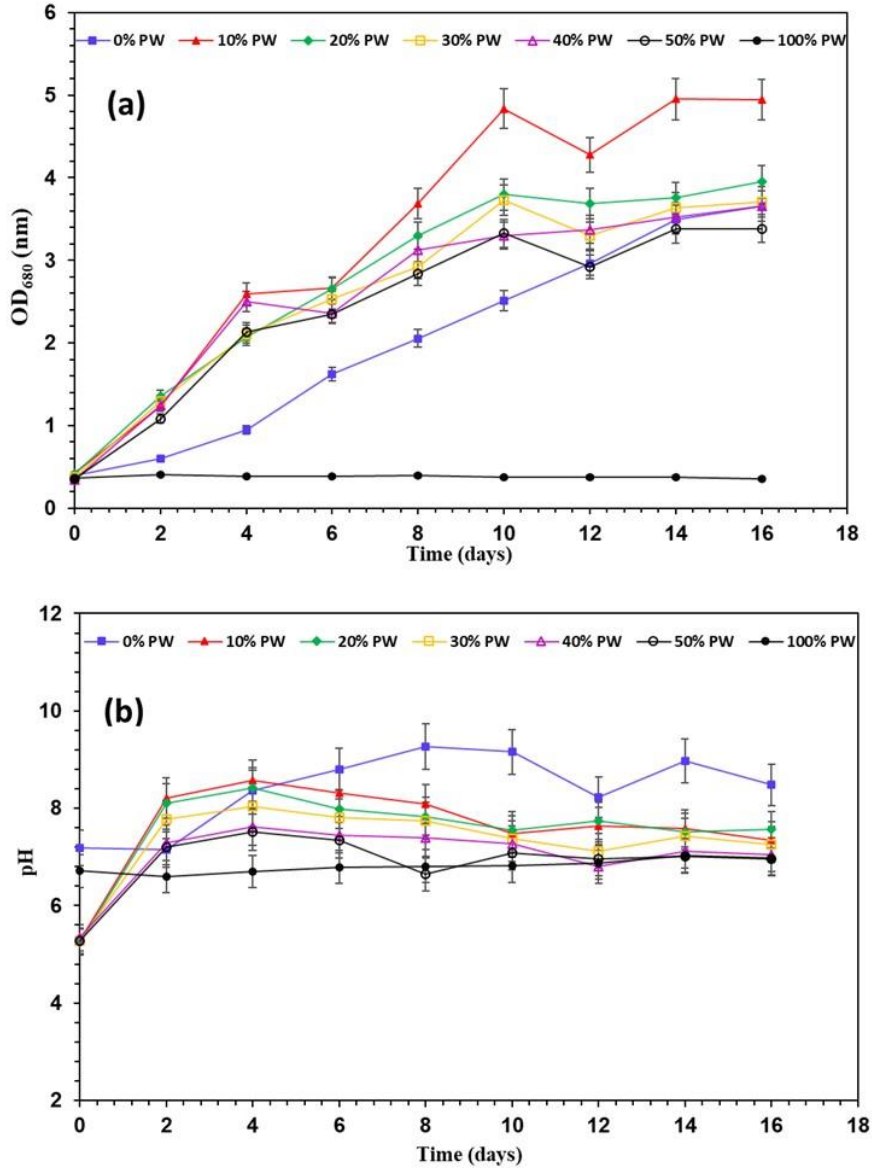


Fig. 6.1 (a) Time profiles of optical densities (OD₆₈₀) as a growth feasibility of *C. sorokiniana* in different PW loadings. **(b)** pH profiles over time during the cultivation of *C. sorokiniana* under different percentage of PW loadings. Data are means $\pm$ SD (n=3).

One of the most significant environmental variables influencing the development and metabolism of microalgae is pH [158]. **Fig. 6.1b** demonstrates the pH profiles over time during the cultivation of *C. sorokiniana* under different percentage of PW loadings. As observed in **Fig. 6.1b**, the initial values of pH for 10%, 20%, 30%, 40% and 50% PW loadings were in the range of 5.25-5.34 indicating very close values with respect to each other. However, initial pH values for 0% PW and 100% PW were 7.19 and 6.71 respectively. It is noted that pH adjustment of the culture medium prior to autoclave has a significant influence on the initial values of pH in the photobioreactors having different PW loadings. On day 2, pH values of culture medium drastically increased for photobioreactors containing 10%, 20%, 30%, 40% and 50% PW loadings and was in the range of 7.20 to 8.21. It is noteworthy that these pH rises were positively correlated with the microalgal growth on day 2 shown in **Fig. 6.1a**. On the contrary, there was a slight drop in the pH on day 2 of cultivation for 0% PW and 100% PW loadings associated with insignificant microalgal growth. After day 2, pH values maintained the same level with minor fluctuations until day 10 for 10%, 20%, 30%, 40% and 50% PW loadings and was in the range of 7.09 to 8.57. Similar pH variation was observed with microalgae *Dunaliella tertiolecta* when tested in similar PW-based medium over a range of salinities [49]. Generally, the trend to neutral and more basic conditions of the culture medium correlates the exponential growth of microalgae within this duration of culture medium (**Fig.6.1a**). In comparison to other PW loadings, the pH rise in the comparatively slower growing 0% PW medium (SMW) was considerably higher after day 2. It might be caused by the 0% PW medium's low TOC content, the intake of nitrogen for algal cell growth, and the microalgae's subsequent nitrate adsorption [100], [159]. The pH value for 100% PW was

almost leveled off throughout the cultivation period due to the no growth of microalgae. According to **Fig. 6.1b**, it is seen that salinity of culture medium has a direct influence on pH variation during the cultivation period. Considering all PW loadings, the trend of higher pH value was observed for lower salinity and vice versa. Overall, growth curves observed in **Fig. 6.1a** for 10%, 20%, 30%, 40% and 50% PW loadings were corroborated with the trend of higher pH value accordingly (**Fig. 6.1b**).

Table 6.1 mainly describes the growth kinetics parameters such as maximum specific growth rate ($\mu_{\max}$), and maximum biomass productivity ($P_{\max}$) of *C. sorokiniana* cultivated under the different PW loadings. As appeared in **Table 6.1**, the average specific growth rates at PW loadings of 0%, 10%, 20%, 30%, 40% and 50% were 0.15, 0.24, 0.22, 0.23, 0.15, 0.22 day⁻¹ respectively. The variations in specific growth rates at different PW concentrations reflect a dynamic balance between nutrient availability and salinity stress. At lower PW concentrations (10%–30%), the nutrients from the supplemented PW enhance growth, while moderate salinity provides a slight stress that stimulates metabolic activity. However, at higher PW concentrations (40%–50%), the salinity stress becomes more pronounced, reducing growth rates[160]. Despite this, at 50% PW, *C. sorokiniana* adapts to the salinity stress, allowing the specific growth rate to increase compared to 40% PW. These fluctuations highlight the importance of optimizing PW concentrations for balancing nutrient supply and osmotic stress to achieve optimal growth. According to the results obtained, the highest specific growth rate was accounted for 10% PW loadings. In addition, the specific growth rate for others PW loadings is almost same except 0% PW and 40% PW loadings. The specific growth rate, 0.15 day⁻¹ for 40% PW is relatively lower than those of others, maybe due to the unexpected drop of growth at the end of exponential

growth phase. Particularly, at 40% PW, salinity becomes a significant stress factor for *C. sorokiniana*. The higher osmotic pressure requires the cells to expend more energy on maintaining osmotic balance, reducing the resources available for growth and division. As a result, the specific growth rate drops to 0.15 day^{-1} . A relatively lower specific growth rate observed by 0% PW (control) due to the absence of congruent amount of carbon in the culture medium compared to supplemented PW. The overall findings of the specific growth rate obtained in this study was higher compared to the specific growth rate found by Ammar et al. (2018) during their PW treatment work done by two marine microalgae species [52]. Also, **Table 6.1** shows the biomass productivity of microalgae culturing at different PW loadings. As expected, higher biomass

Table 6.1 Conditions and growth parameters of *C. sorokiniana* cultivated in supplemented SPW.

PW loading (%)	Salinity (g/L)	Maximal OD ₆₈₀	Max. specific growth rate, μ_m (day ⁻¹)	Cultivation Period (day)	Max. Biomass productivity (mg/L/d)
0	0.43	3.66 ± 0.07	0.15 ± 0.003	16	40.8 ± 0.80
10	6.80	4.95 ± 0.10	0.24 ± 0.007	16	53.1 ± 1.60
20	13.15	3.95 ± 0.07	0.22 ± 0.004	16	48.2 ± 0.96
30	19.79	3.71 ± 0.10	0.23 ± 0.006	16	50.8 ± 1.50
40	26.19	3.65 ± 0.07	0.15 ± 0.003	16	42.2 ± 0.96
50	32.61	3.39 ± 0.06	0.22 ± 0.004	16	42.4 ± 0.97
100	63.46	0.35 ± 0.007	0.013 ± 0.0003	16	0.0

Notes: Salinity recorded here after pH adjustment and sterilization of the culture media

productivity was achieved at the average figure of 53.1 mg/L/d for 10% PW loadings. This may be happened due to presence of sufficient amount of nitrogen and phosphorus as well as the fair amount TOC in the culture medium [119]. Average biomass productivity for 0% PW, 20% PW, 30%PW, 40% PW and 50% PW was found 40.8 mg/L/d, 48.2 mg/L/d, 50.8 mg/L/d, 42.2 mg/L/d, and 42.4 mg/L/d respectively. The shifting equilibrium between salt stress and the microalgae's capacity for adaptation and nutrient use accounts for the pattern of biomass productivity, which shows decreases at 20% and 40% PW and increases at 30% and 50% PW. Cells' osmotic pressure rises with salinity, while modest salinity might trigger advantageous stress reactions. Growth is inhibited by excessive salt (at 40% PW), but at even higher salinity levels (at 50% PW), adaptive mechanisms enable the cells to regain some production[161] . This variation emphasizes how crucial it is to strike the ideal balance between stress and nutrient availability in order to maximize biomass yield in microalgal cultures. Microalgal adaptation to fluctuating salinity levels entails physiological modifications and biochemical mechanisms that facilitate homeostasis, safeguard cellular integrity, and promote survival under stress conditions. The amalgamation of osmoregulatory mechanisms, ion transport regulation, antioxidant

defenses, and metabolic reprogramming enables microalgae to not only endure but also flourish in high-salinity settings[114]. The results obtained of biomass productivity for different PW loadings are 1.3 to 1.2 times higher than that of 0% PW (control). The range of biomass productivity found in this study is supported by comparable results of other researchers [49], [133]. The comparable growth kinetics parameters obtained in this study for freshwater microalgae *C. sorokiniana* cultivated under hypersaline PW confirms its halotolerant capacity.

The maximum biomass yields obtained from *C. sorokiniana* culture with varying PW loadings (0% to 100%) are shown in **Fig. 6.2**. Considering all PW loadings, the average maximal biomass output was found to vary from 733 mg/L to 1077 mg/L, with the exception of 100% PW, which was only 107 mg/L and essentially indicated no microalgae development in that culture medium. Microalgae culture with 10% PW showed the highest biomass concentration (1077 mg/L) followed by 30% PW (924mg/L), 20% PW (851mg/L), 40% PW (788mg/L), 50% PW (785mg/L), and 0% PW (733mg/L). It is obvious that the dry biomass concentration achieved for different PW loadings is higher than that of 0% PW (control). It is almost true that biomass concentration decreased with increase in salinity that means increasing PW loadings in the culture medium. This findings is corroborated with the other reported work [49], [59]. However, the biomass yield of 30% PW is higher than that of 20% PW. Although, the salinity level at 30% PW may create a slightly higher osmotic pressure compared to 20% PW, but not high enough to inhibit growth significantly. At 30% PW, the concentration of essential nutrients from the supplemented SMW is likely more optimal for supporting microalgal growth than at 20% PW. The 30% PW concentration might provide a better balance of nutrients such as

nitrogen, phosphorus, and trace elements, which are necessary for growth and biomass accumulation. In contrast, the lower biomass yield at 50% PW compared to 40% PW is likely due to increased salinity stress, toxic compound accumulation, and nutrient imbalances that inhibit growth. Maximum biomass yields obtained in this study for different PW loadings were higher than those of similar study conducted by Eladel et al. (2019). For example, Eladel et al. (2019) cultivated the *C. sorkokiniana* under municipal wastewater medium and BBM enriched wastewater medium for simultaneous nutrient removal and biodiesel production. According to their findings maximum biomass concentration was in the range of 747 mg/L to 833 mg/L which was lower than compared to this study [116].

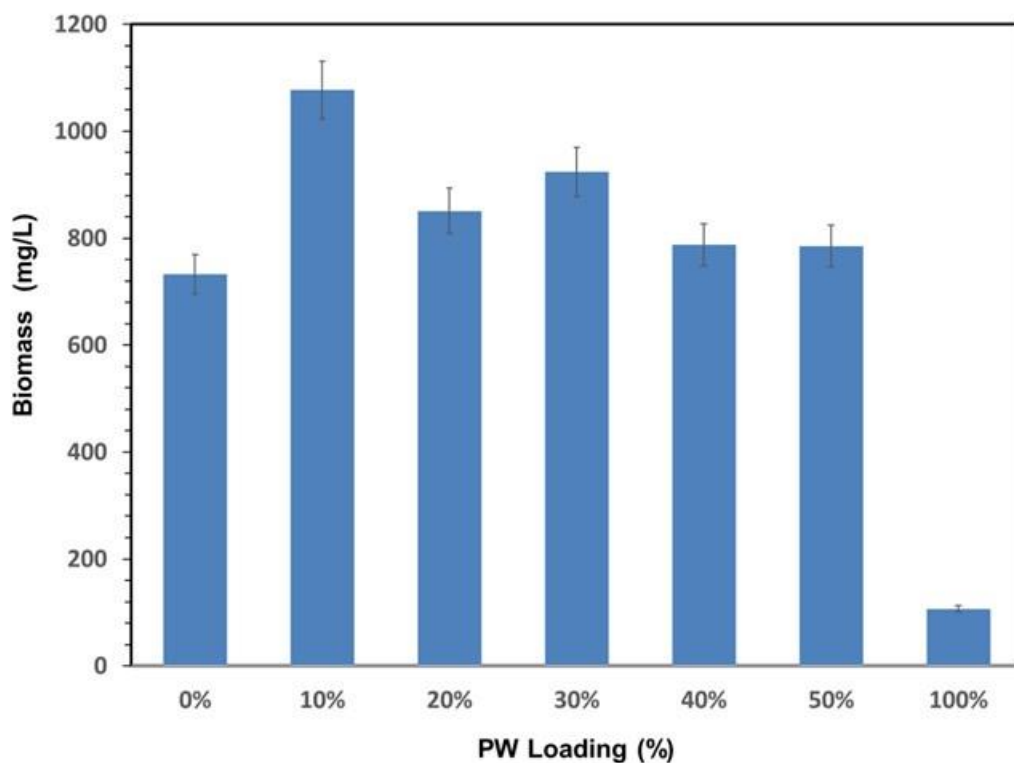


Fig. 6.2 Maximum biomass yields achieved from the cultivation of *C. sorokiniana* under different PW loadings. Data are means $\pm$ SD (n=3).

6.3 Bioremediation of PW- TOC removal

To assess the bioremediation efficiency of PW by microalgae, TOC of culture medium of different photobioreactors was monitored on day 0, 8 and 16 according to the experimental plan. **Fig. 6.3a** illustrates the TOC content in different PW loadings on day 0, 8 and 16. According to **Fig. 6.3a**, higher PW loadings showed the expected incremental initial TOC value in the culture medium. As a good trend, it was observed that the highest amount of carbon uptake by microalgae happened on day 8 showing lowest TOC value for all photobioreactors except photobioreactors having 100%PW. In addition, as appeared in **Fig. 6.3b**, TOC removal efficiency increased gradually with increasing PW loadings except

100% PW. The initial TOC value of 50% PW was 191.3 mg/L and after bioremediation by microalgae it reached to around 28.84 mg/L on day 8 indicating the maximum average removal efficiency (85 %) than those of other PW loadings. Maximum average TOC removal efficiency on day 8 for other PW loadings was in the average figure of 41.66%, 40.79%, 61.54%, 72.8%, and 80.38% for 0%, 10%, 20%, 30%, and 40% respectively. It is seen that the variation of TOC removal efficiency among the treatment groups is statistically significant ($p\text{-value} = 0.015904 < 0.05$). According to **Fig. 6.3a and 6.3b**, higher TOC removal efficiency accounted for higher initial TOC value considering all PW loadings except 100% PW. The TOC removal efficiency for 100% PW was 6.05% only (initial TOC = 358.4 mg/L) on day 16. As 100% PW did not show any growth (section 3.1), the minor percentage of TOC removal may be due to biodegradation only. The findings of this investigation demonstrate that microalgae *C. sorokiniana* can effectively decompose and utilize the organic compounds in the supplemented PW to support their growth. Moreover, as observed in **Fig. 6.3a**, TOC of culture medium in all photobioreactors except 100% PW was slightly higher on day 16 than those of day 8. These phenomena perhaps occurred due to the starting of stationary phase of microalgae culture after day 10 of cultivation for almost all photobioreactors. At that stage of cultivation, some of the dead cells can release organic carbon to the culture medium which ultimately can increase the TOC value slightly [162].

As appeared in **Fig. 6.3a**, carbon uptake by microalgae is strongly influenced by nutrient concentration in the culture medium. Reducing the levels of nitrogen (N) and phosphorus (P) while increasing the amount of carbon (C) in SPW-SMW media, specifically by increasing the PW loading, results in an enhanced uptake of carbon by microalgae

throughout the cultivation period. Therefore, the introduction of higher PW effluent loadings into the growth medium is anticipated to enhance the absorption of carbon by microalgae. These results are consistent with those obtained during PW treatment by different microalgae species [52], [163], [164]. A suitable nutritional energy balance among C, N and P may be a pivotal factor to remove organic pollutant from PW under mixotrophic conditions [119], [165]. The photobioreactor having the dilutions of 10%, 20%, 30%, 40%, and 50% of PW confirmed that the initial average TOC values for each dilution were 61.48 mg/L , 94.71 mg/L, 124.7 mg/L, 157.5 mg/L and 191.3 mg/L respectively. As a result, C:N:P ratios (mass) were found 1.15:0.7:1, 3.1:0.9:1, 4.48:0.89:1, 5.96:0.75:1, and 7.65:0.72:1 for 10%, 20%, 30%, 40%, and 50% of PW loadings respectively. Considering C:N:P ratios for photobioreactor having the dilutions of 10%, 20%, 30%, 40%, and 50% of PW confirmed that the initial average TOC values for each dilution were 61.48 mg/L , 94.71 mg/L, 124.7 mg/L, 157.5 mg/L and 191.3 mg/L respectively. As a result, C:N:P ratios (mass) were found 1.15:0.7:1, 3.1:0.9:1, 4.48:0.89:1, 5.96:0.75:1, and 7.65:0.72:1 for 10%, 20%, 30%, 40%, and 50% of PW loadings respectively. Considering C:N:P ratios for incremental PW loadings, it is noteworthy that decreasing N and P concentrations by maintaining the almost constant N:P ratios and concurrently increasing organic carbon leads to maximum removal of TOC from the culture medium. At lower C/N ratio, microalgae often grow well, but the ability to remove organic carbon (TOC) may decrease, as they rely on available carbon sources for energy and biomass synthesis. However, at higher C/N ratios can promote TOC removal but may lead to limited growth if nitrogen is insufficient for protein synthesis and other cellular functions.

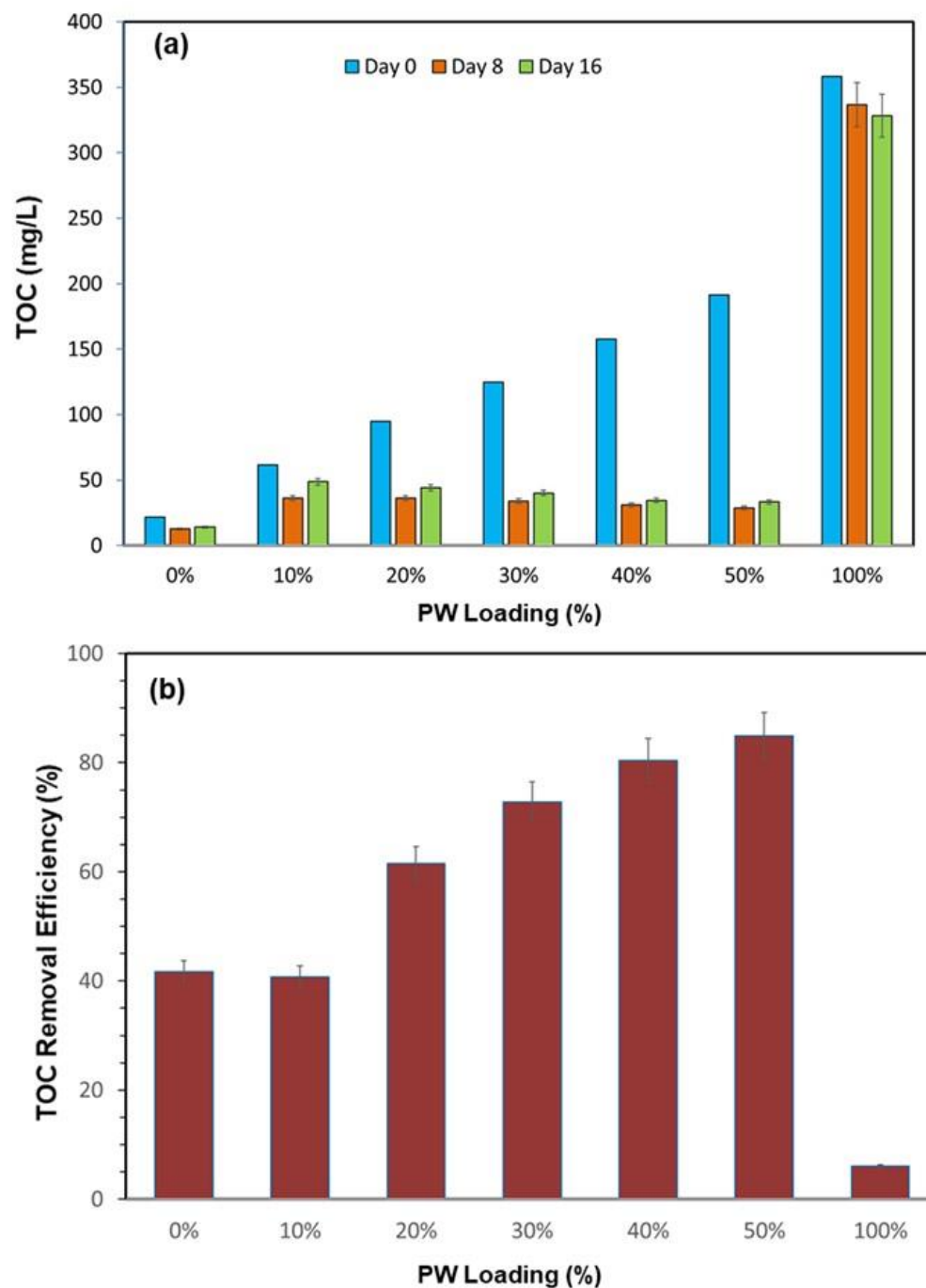


Fig. 6.3 (a) TOC content in culture medium of different PW loadings at day 0, 8 and 16. **(b)** Maximum TOC removal efficiency on day 8. Data are means $\pm$ SD (n=3).

Despite the imbalanced cultivation conditions regarding macronutrients, the halotolerant freshwater microalgae *C. sorokiniana* demonstrated adaptation by incorporating supplemented SPW. Additionally, it achieved a remarkable efficiency in removing TOC, reaching 85% (for 50% PW). The findings are consistent with the findings reported by Marques et al. (2021) for several species [15]. Moreover, Abdul Hakim et al. (2018) evaluated the pollutant removal efficiency of five local microalgae species from the PW of a petroleum industry, focusing on TOC removal. The study found that, despite low biomass production, *Neochloris* sp. achieved 41.61% TOC removal across various PW concentrations. However, these TOC removal efficiency were notably lower than those observed in the present study, where *C. sorokiniana* achieved higher removal efficiencies at 50% PW[63]. Das et al. (2019) investigated the bioremediation of low-salinity, low-pH PW utilizing six microalgal strains, both with and without nitrogen and phosphorus supplementation. *Chlorella* sp. and *Scenedesmus* sp. were the sole strains to grow under both circumstances, with *Chlorella* sp. attaining the maximum biomass density of 1.2 g/L in nutrient-enriched water. In a 1 L photobioreactor, *Chlorella* sp. eliminated 73% of total organic carbon (TOC) after 15 days. Their findings align with the outcomes of this investigation[21]. Overall, it is evident that freshwater halotolerant microalgae *C. sorokiniana* materialized to be a possible candidate to bioremediate supplemented PW under mixotrophic conditions.

Mixotrophy refers to the capacity of specific microalgae to utilize dissolved organic carbon as a source of carbon when light is available [7]. The three major methods that microalgae can use to remove organics from wastewater are biodegradation, bioaccumulation, and biosorption. The polymer groups in the cell walls of microalgae may provide potential

sorption sites for organic contaminants. The microalga *C. sorokiniana* exhibited mixotrophic behavior, enabling effective TOC removal from the PW through a combination of biosorption, bioaccumulation, and biodegradation processes (**Fig. 6.4**). These findings are consistent with results reported in other studies [56]. Through the metabolically inactive process of biosorption, microalgae can eliminate organic contaminants [55]. The microalgae cell wall has numerous binding sites and functional groups that aid in electrostatic contact, adsorption, surface precipitation, chelation, and ion exchange during biosorption [56]. The study found that 85% of TOC was removed in the photobioreactor with 50% PW. However, the microalgae showed less growth compared to other PW loadings. These findings indicate that the microalgae mostly employed biosorption of the compounds present in the biomass [15]. The higher TOC removal efficiency in the 50% PW, despite lower biomass, can be attributed to *C. sorokiniana*'s metabolic adaptations under elevated salinity and nutrient-limited conditions. In such environments, microalgae can activate mechanisms such as mixotrophic metabolism and increased biosorption, focusing energy on breaking down organic compounds rather than biomass production. This metabolic flexibility allows for effective TOC removal without directly correlating with biomass increase, adhering to conservation principles while prioritizing pollutant uptake and biodegradation over growth. A percentage of TOC may be removed from microalgae culture medium due to the consumption or bioaccumulation of organic components inside the cell during microalgal growth. Bioaccumulation is a procedure that depends on growth and is metabolically active.

As appeared in **Fig. 6.4**, during the process of total organic carbon (TOC) consumption, acetate is first converted into acetyl coenzyme A (acetyl-CoA) and then incorporated into

algal carbohydrates through the glyoxylate cycle, in collaboration with the tricarboxylic acid cycle (TCA cycle) [119]. The glyoxylate cycle and the TCA cycle are essential junctions in metabolism that connect acetyl-CoA to oxidative catabolism and synthesis pathways. This global overview of acetate metabolism indicates that microalgae can employ acetate as the exclusive carbon and energy source for cellular proliferation and the accumulation of biomolecules of industrial significance, including starch, proteins, and triacylglycerols (TAGs) [166].

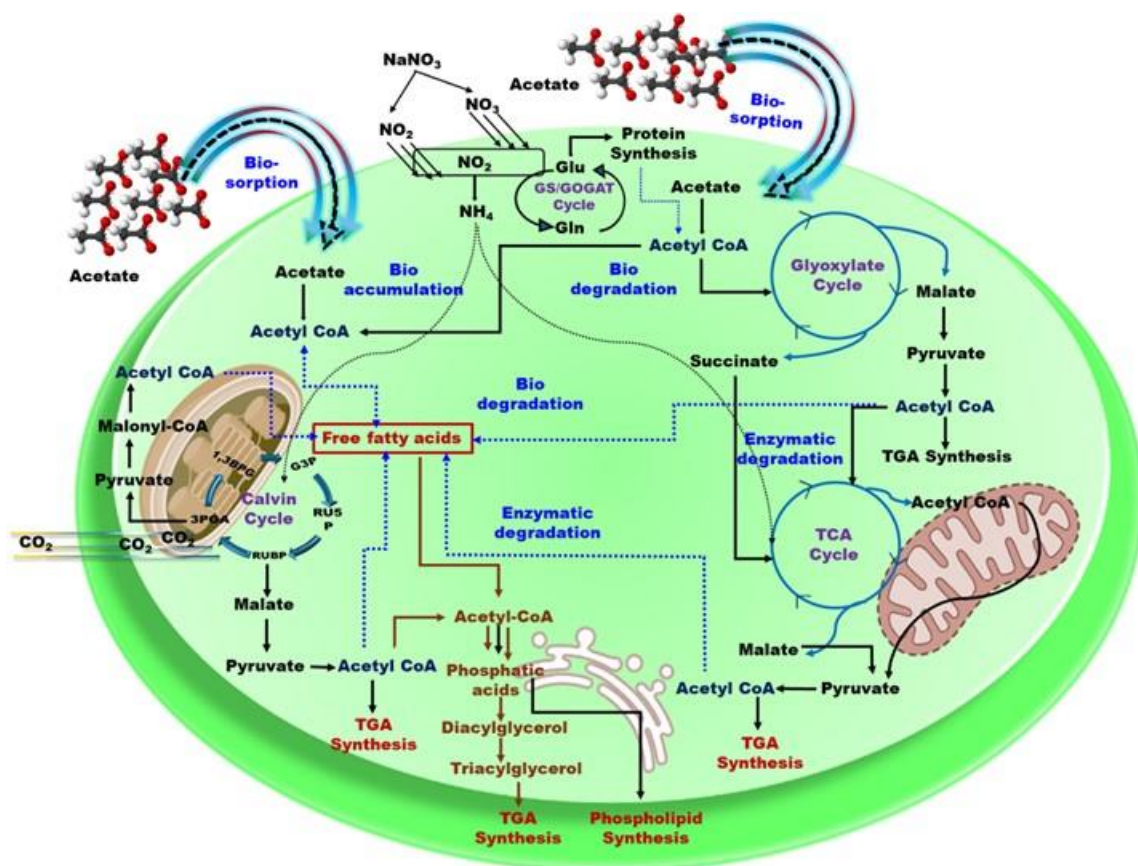


Fig. 6.4 The potential mechanism of organic pollutant removal from acetate based produced water by *C. sorokiniana* owing to the combination of biosorption, bioaccumulation and biodegradation.

According to earlier mentioned, the TOC removal efficiency for 100% PW was 6.05% only (initial TOC = 358.4 mg/L) on day 16. As 100% PW did not show any growth, the minor percentage of TOC removal may be due to the biodegradation mechanism. While *C. sorokiniana* exhibited optimal growth at 10% PW, but its capacity to thrive and efficiently eliminate pollutants at 20%-50% PW concentrations demonstrates its resilience and appropriateness for PW remediation. The 85% reduction in TOC at 50% PW highlights the algae's ability to remediate extremely saline and organic-rich PW, even in suboptimal growing conditions. The system's dual function of pollutant removal and biomass

production renders it financially viable and environmentally sustainable, providing a scalable and cost-efficient solution for PW management. Halotolerant green microalgae (*Chlorella*) and cyanobacteria utilize intricate systems for the uptake and breakdown of organic pollutants. Cyanobacteria, due to their substantial EPS generation, photosynthetic energy use, and endurance to harsh environmental conditions, frequently possess a marginal advantage in biosorption and biodegradation within saline settings. Conversely, *Chlorella* exhibits bioaccumulation capabilities along with other mechanisms and may utilize organic pollutants as carbon sources, rendering it suitable for bioremediation across diverse environments.

6.4 Nutrient uptake

It is evident that PW might exhibit a deficiency in nitrogen and phosphorus, which could bring the C/N ratio below the optimal range for fostering microalgal growth [15]. Due to these factors, the addition of these two nutrients together may be necessary for growing microalgae in PW, particularly throughout effluent mixing [5]. SPW prepared in this study does not contain any nitrogen or phosphorus. Thus, PW was supplemented with SMW as a major source of nutrients for microalgal growth. Due to the supplementation with SMW, the photobioreactor having dilutions of 0%, 10%, 20%, 30%, 40%, and 50% of PW showed that the initial average nitrogen values for each dilution were 41.17 mg/L, 35.34 mg/L, 27.5 mg/L, 25 mg/L, 20 mg/L, and 18 mg/L respectively. 100% PW does not contain any N and P concentration as it was not supplemented with SMW.

To investigate the nutrient uptake capacity of *C. sorokiniana* under PW environment, two primary nutrients (nitrogen, and phosphorus) were monitored at day 0, 8 and 16 during the

cultivation period. **Fig. 6.5a** demonstrates the total nitrogen (TN) content remaining in the culture medium of different PW loadings at day 0, 8 and 16 of the cultivation periods. As can be observed in **Fig. 6.5a**, initial nitrogen concentration decreased with an increase in PW loadings. Generally, it was seen that nitrogen concentration decreased gradually with an increase in microalgal growth considering all PW loadings. On day 8 of cultivation period, nitrogen concentration reached around 14.7 mg/L, 11.0 mg/L, 7.0 mg/L, 12 mg/L, 14 mg/L, and 12 mg/L in the photobioreactor having the PW loadings of 0%, 10%, 20%, 30%, 40%, and 50% respectively. According to the available nitrogen on day 8, it is true that nitrogen uptake favored by microalgal growth (**Fig. 6.1a**) and depend on initial nitrogen concentration [62], [119]. At the end of cultivation on day 16, average nitrogen removal by 10% PW loadings was 87% which was slightly lower than 0% PW (89.3%) (control). The average nitrogen removal for other photobioreactors was 81.8%, 76%, 77.5%, and 77.8% for 20% PW, 30% PW, 40% PW, and 50% PW respectively and found no statistically significant difference ($p\text{-value} = 0.978467 > 0.05$). The percentage removal of nitrogen (in the form of nitrate) obtained in this study is in good agreement with the findings of the other reported work [62]. In addition, Gillard et al. (2021) documented a 92% reduction in nitrate levels using diatoms in PW, which is consistent with the nitrate removal outcomes [167]. Experimental evidence demonstrates that elevated salinity levels negatively impacted the ability of *C. sorokiniana* to remove nutrients from PW, as indicated by reduced nitrogen removal. This outcome is consistent with the results obtained from the growth rate trends and biomass production [123]. Moreover, the presence of major ion concentration in PW solution can affect the nutrient removal mechanism by microalgae. Figler et al (2019) demonstrated a strong correlation between the salt concentration in the

media and the pace of nitrate transport [168]. Additionally, it has been documented that microalgae are capable of absorbing a greater amount of nitrate when exposed to reduced levels of salt [123]. Consequently, significant amounts of these nutrients were depleted from the supplemented PW medium due to the growth of microalgae by the end of the culture period. Although the microalgae in the PW may not completely eliminate the N, the residual N levels typically fall within the range of 4 to 6 mg/L. This can be deemed as a successful N absorption, in accordance with the European Commission Directive 98/15/EC, which sets a maximum limit of 10 mg/L for Total N discharge [90].

Fig. 6.5b depicts the total phosphorus (TP) uptake by *C. sorokiniana* grown under varied levels of PW loadings. As appeared in **Fig. 6.5b**, the photobioreactor containing 0%, 10%, 20%, 30%, 40%, and 50% PW loadings showed that the initial average values for each dilution were 53.24 mg/L, 36.22 mg/L, 30.54 mg/L, 27.8 mg/L, 26.4 mg/L, and 25 mg/L and were reduced to around 14.7 mg/L, 11.62 mg/L, 3.52 mg/L, 11.62 mg/L, 6.85 mg/L and 0.97 mg/L respectively on day 8 of cultivation period. The effectiveness of TP removal is probably affected by a complex interaction of salinity, nutritional equilibrium, and cellular stress responses. The markedly greater TP removal at 20% PW concentration on the 8th day, in contrast to the diminished removal at 30% PW, may be ascribed to the equilibrium of nutrient availability and salt stress. At 20% PW, the moderate salinity likely establishes a more advantageous equilibrium between stress and nutrient availability, enhancing phosphorus uptake. Furthermore, the nutritional profile in 20% PW may be more conducive to facilitating the metabolic processes necessary for effective phosphorus

elimination. The findings indicate that 20% PW creates a more favorable environment for TP elimination than both 10% and 30% PW.

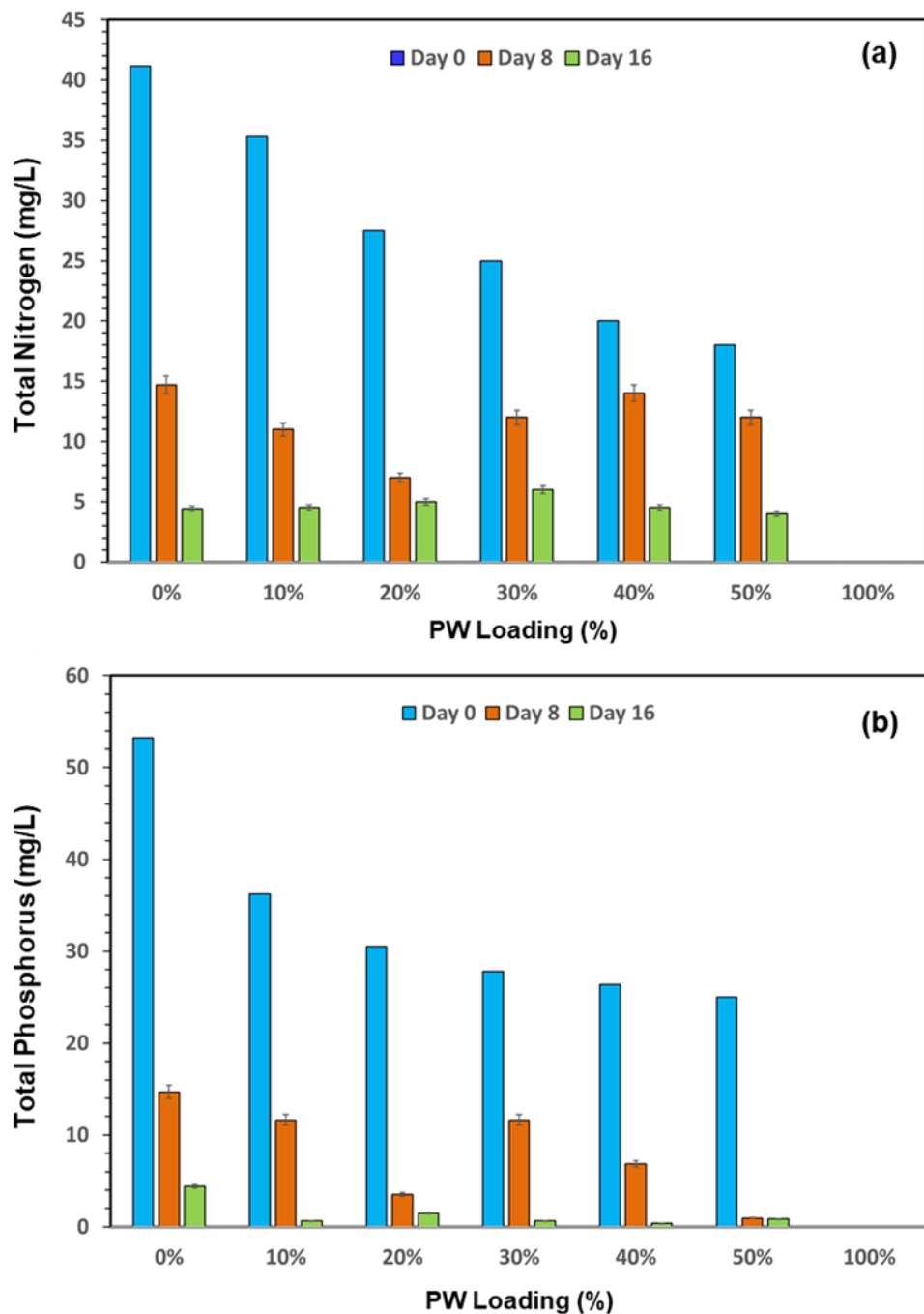


Fig. 6.5 (a) Total nitrogen (TN) content remaining in the culture medium of different PW loadings at day 0, 8 and 16. **(b)** Total phosphorus (TP) content remaining in the culture medium of different PW loadings at day 0, 8 and 16. Data are means $\pm$ SD (n=3).

At the end of 16 days of cultivation period, the concentration of phosphorus was reached around to the amount of 4.4 mg/L, 0.65 mg/L, 1.47 mg/L, 0.65 mg/L, 0.4 mg/L and 0.84 mg/L for 0%, 10%, 20%, 30%, 40%, and 50% PW loadings representing the average removal of 91.7%, 98.2%, 95.2%, 97.7%, 98.5%, and 96.6% of phosphorus respectively. It is noteworthy that the average percentage removal of phosphorus for all PW loadings was higher than that of 0% PW (control) and found no statistically significant difference among the treatment groups ($p\text{-value} = 0.98 > 0.05$). These results are in satisfactory accord with the final biomass concentration of *C. sorokiniana* at these PW dilutions, as was depicted in **Fig. 6.2**. The phosphorus removal obtained in this study for different PW loadings is consistent with the findings of similar work done by other researchers. For example, Rahmani et al. (2022) assessed the potential of *Chlorella pyrenoidosa* for oil field PW treatment and found the 57.14%, and 75.51% TN, and TP removal respectively throughout the cultivation period [62]. In addition, Talebi et al. (2016) guided a study on the use of microalgae culture in indoor photobioreactors for treating petroleum generated PW. The researchers discovered that microalgae had a remarkable capacity to eliminate nitrogen by 65% and phosphorus by 40% [57]. The results obtained in **Fig. 6.1a** and **Fig. 6.5b** clearly indicate that the removal of phosphorus from the photobioreactor growing medium is mostly influenced by the growth of microalgae. Phosphate can be adsorbed by the microalgal cell wall and then undergo phosphorylation. Additionally, at pH levels higher than 9, phosphate can be precipitated from the culture medium [100]. However, in this study, pH value was always estimated at about lower than 9 for all photobioreactors during the cultivation period (**Fig. 6.1b**).

Hence, the impact of phosphate precipitation is likely to be negligible in this case due to the low pH value (below 9). Despite the imbalanced supplemental PW (N/P) ratio in this investigation, *C. sorokiniana* demonstrated effective nitrogen absorption and successful phosphorus elimination. To summarize, in comparison to N uptake, P was completely consumed from all photobioreactors by *C. sorokiniana* thus satisfying Total P European Commission Directive 98/15/EC [90] limits of 1 mg/L except 20% PW loadings as an exception (1.47 mg/L).

6.5 Total lipid content

This work emphasized lipid analysis because of its significance in evaluating the potential of *C. sorokiniana* for biofuel applications, where lipid accumulation serves as a crucial indicator of compatibility. Lipids are essential for cellular adaptation to saline stress, offering more understanding of the microalga's response mechanisms under PW circumstances. Total lipid content as a percentage of the dry weight of biomass on different day of cultivation period considering all photobioreactors are depicted in **Fig. 6.6**. Briefly, the results indicate that culture medium compositions have an influence on lipid accumulation in microalgae biomass [90]. Seeing all PW loadings, the lipid content varied between on average 12.42% and 15.41% at the end of 16 days cultivation period and this variation was not statistically significant ($p\text{-value} = 0.972136 > 0.05$). This obtained results of lipid content were close to the findings of other researchers [124], where *C. sorokiniana* cultivated mixotrophically on waste dark fermentation effluents having acetate. Lacroux et al. (2021) examined the mixotrophic growth of *C. sorokiniana* on acetate and butyrate, focusing on the interaction between substrate, carbon-to-nitrogen ratio, and pH. They

evaluated the effect of C:N:P on biomass, lipids and carbohydrates production dynamics and finally reported the accumulation of 16.4% lipids in biomass during nutrient replete conditions. Their findings in lipid content in biomass resemble the findings of this study [169]. Moreover, Heredia-Arroyo et al. (2011) adopted mixotrophic methods to cultivate *Chlorella vulgaris* and exploring its potential for accumulating lipid from non-sugar sources. Authors found that the lipid content was lower (14-17%) in mixotrophic conditions, though it resulted in higher biomass production (1.4 g/L), compared to autotrophic (0.4 g/L) and heterotrophic (0.75 g/L) conditions. Thus, the lipid content observed in this study (12.42% - 15.41%) is similar to their reported findings [170]. Gupta et al. (2016) obtained higher lipid content (22.74%) in biomass than those of lipid content findings in this study. Authors evaluated the combined role of *C. sorokiniana* and *S. obliquus* in wastewater treatment and biomass production for biofuel generation. According to their findings, *C. sorokiniana* showed greater resilience to physiological stress and could be effective for both comprehensive wastewater treatment and substantial lipid accumulation [65].

As observed on **Fig. 6.6**, on day 8 of the cultivation period, total lipid content increased in all PW loadings. But 0% PW loading showed higher lipid content (17.98%) than those of others. This is most likely due to the lower saline condition of the culture medium and observed peak pH of the cultivation medium on that day of 0% PW loading [49]. Compared to day 8, on day 16, lipid content reduced in 0% PW (15.41%), 20% PW (12.42%) and 40% PW (13.03%), whereas it increased in 10% PW (13.23%), 30% PW (12.46%) and 50% PW (14.94%). Moreover, it was observed that lipid content increased with an increase in PW loadings at the end of cultivation for 20%, 30%, 40% and 50% PW loadings. It may

be due to the increase in C/N ratio with increase in PW loadings. The literature study indicates that lipid yield in microalgae biomass will be enhanced by raising the C/N ratio through organic carbon supplementation (PW loading) [171]. For instance, *Dunaliella tertiolecta* has demonstrated a greater lipid content in a separate investigation, particularly when there is a higher availability of carbon [172]. The potential advantages of examining additional metabolites such as carbohydrates, proteins, and pigments are recognized; nevertheless, these were deemed outside the purview of this study. Future investigations could examine a more comprehensive understanding of the biomass's worth, particularly for applications beyond biofuels, such as feed, nutraceuticals, or bioplastics.

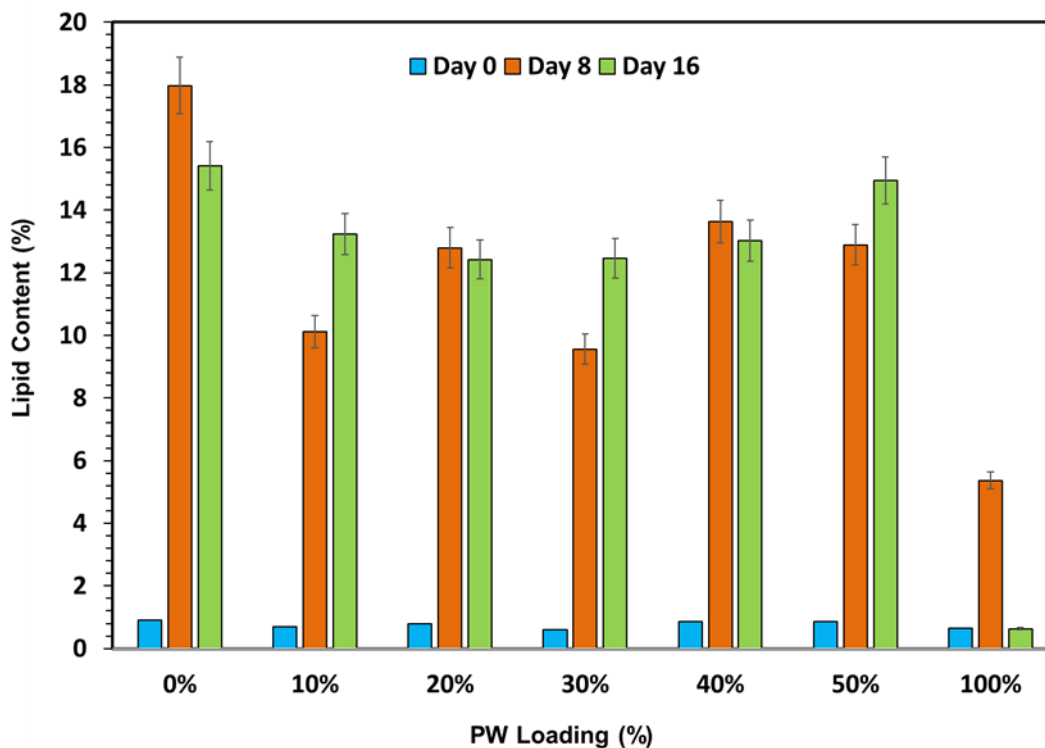


Fig. 6.6 Total lipid content as a percentage of the dry weight of biomass on different day of cultivation period considering all PW loadings. Data are means $\pm$ SD (n=3).

6.6 Conclusions

This study demonstrates the effectiveness of *C. sorokiniana* in removing dissolved organic pollutants from SPW across various concentration gradients. The results indicate that *C. sorokiniana* can adapt and perform under different PW concentrations, with specific concentration levels yielding optimal pollutant removal efficiency. Maximum TOC removal was achieved on day 8, with efficiencies increasing with SPW loadings up to 50%, reaching 85%. However, TOC removal was limited to 6.05% at 100% SPW on day 16, as no algal growth occurred at this concentration. By the end of the 16-day cultivation, nitrogen removal was highest in 0% (control) and 10% PW loadings (89.3% and 87%, respectively), while phosphorus was almost entirely consumed across all PW

concentrations, with removal efficiencies above 91%. The findings support the significance of harnessing the natural abilities of halotolerant *C. sorokiniana* microalgae, presenting a feasible avenue towards mitigating dissolved organic carbon in PW, thereby conducive to the preservation of aquatic ecosystems and human health. However, the outcomes of a particular SPW formulation may not be immediately relevant to all forms of PW due to diversity. Due to variations in its composition, PW from different sources may operate differently in microalgal-based treatment systems. To properly evaluate microalgal strains' adaptability and treatment effectiveness under various circumstances, future studies should concentrate on evaluating them in actual PW samples from various places and with various compositions.

Enhancing the phycoremediation process using *C. sorokiniana* for commercial use holds considerable promise, especially if economical production and useful byproducts can be utilized. Nonetheless, scaling issues, particularly in enhancing algae growth and managing expenses, must be resolved. Progress in automation, bioreactor design, and novel applications of algal biomass will likely be pivotal in assessing the viability and effectiveness of large-scale phycoremediation initiatives. Further research and implementation of this eco-friendly bioremediation strategy holds considerable promise for addressing the challenges associated with dissolved organic compound contamination in real PW scenarios.'

CHAPTER 7

Cultivation of *Chlorella sorokiniana* in Phenol Based Produced Water

7.1 Introduction

This chapter describes the bioremediation potential of *C. sorokiniana* by examining its effectiveness in eliminating phenol from petroleum derived PW at different levels of salinity. *C. sorokiniana* was grown in SPW with the addition of tertiary SMW as a unique growth medium. This cultivation method was used to efficiently eliminate phenol while also generating valuable biomass. Laboratory scale experiments have been conducted to investigate the kinetics of phenol removal, evaluate the influence of phenol concentration and salinity levels on microalgal growth and phenol degradation rates, and assess the potential for value-added biomass production for bioenergy applications during the bioremediation process. Moreover, the aim of this chapter was to explore the underlying mechanisms governing phenol removal and biodegradation by *C. sorokiniana* and its potential applications in the sustainable treatment of phenol-contaminated PW.

7.2 Microalgal growth, phenol bioremediation and pH profiles

The halotolerant microalgae *C. sorokiniana* inoculum was grown in a phenol-based SPW supplemented with SMW as a nutrient source. This was done to assess the growth characteristics, phenol bioremediation, and the potential for biomass to be used as a source of bioenergy. **Fig. 7.1a** illustrates the time profiles of optical densities (OD_{680}) as a measure of the growth feasibility of *C. sorokiniana* in various phenol-containing PW loadings. **Fig. 7.1b** illustrates the fluctuation of pH levels with varying PW loadings over a period of 10 days during culture. The kinetics of phenol bioremediation at varied PW loadings are illustrated in **Fig. 7.1c** and **Fig. 7.1d**, showing the residual phenol content in the growth medium and the phenol removal efficiency, respectively. According to **Fig. 7.1a**, *C. sorokiniana* were able to respond satisfactorily to different PW loadings, including 0% (control), 10%, 20%, 30%, 40%, and 50%.

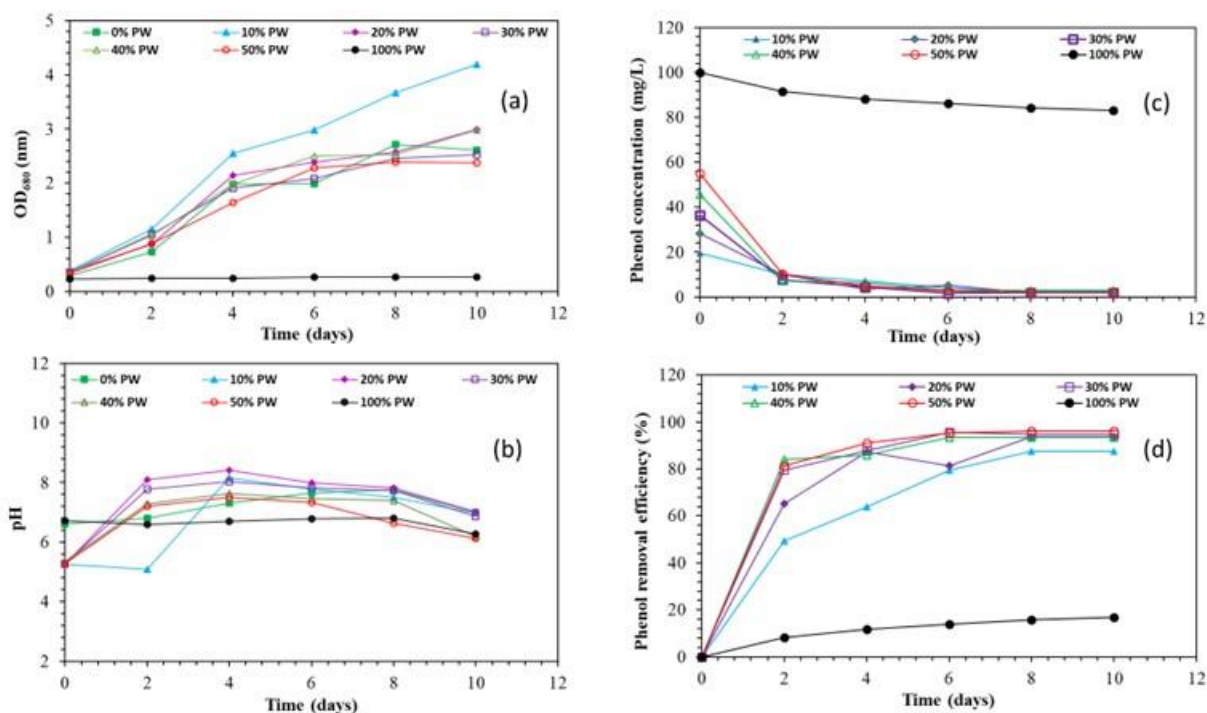


Fig. 7.1 (a) Time profiles of optical densities (OD₆₈₀) as a growth feasibility of *C. sorokiniana* in different phenol containing PW loadings (b) pH profiles over time during the cultivation (c) Phenol bioremediation profile of *C. sorokiniana* in different PW loadings (d) Phenol removal efficiency over time

However, the high salinity, high phenol concentration (100 mg/L), and inadequate nutrition caused the microalgae to not develop effectively in 100% PW. With the exception of 100% PW, lag phase was absent from the culture of all photobioreactors due to the use of preadapted microalgae cells in a saline environment. Nonetheless, until the second day of the cultivation period, a rather sluggish growth pattern of microalgae was seen in all photobioreactors (0% PW to 50% PW). It can be because the microalgae are still getting used to their new growing media[173]. Furthermore, the algae growth seen in the control group was much lower compared to the growth in the phenol-containing PW group. This suggests that the presence of phenol concentration resulted in an increase in the concentration of algae[174]. Specifically, it is seen that higher PW loadings associated with

higher phenol concentrations have a reduced effect on algal growth until day 2 of cultivation period. After that, a sudden rise in microalgal growth was noticed until day 4 of cultivation period in all photobioreactors except 100% PW during the log phase. It might be because during mixotrophic growth, algae use phenol as an organic carbon source. According to reports, phenol can be broken down into organic end products like CO₂ and pyruvate, which can help biomass growth[175]. From day 4 to day 6, there was a slight decrease in microalgae growth rate for 20%, 30% and 40% PW loadings but it was higher than that of control (0% PW). Photobioreactor with 50% PW loadings showed similar growth pattern from day 2 to day 6. But the overall growth rate of 50% PW was lower compared to other PW loadings. After day 6, 50% PW loadings entered the stationary phase and maintained the same level until the end of cultivation. Photobioreactor with 40% and 30% PW loadings started stationary phase on day 6 and on day 8 respectively, whereas 40% PW loadings showed a little rise in growth after day 8 of the cultivation period. After day 6, the photobioreactor with 20% PW loadings revealed a very slow growth pattern of microalgae until the end of cultivation period. It is noteworthy that microalgae in 10% PW showed the highest OD values during the 10 days cultivation period compared to the medium composed of 20%, 30%, 40%, and 50% phenol-based PW respectively and found statistically significant ($p\text{-value} = 0.015457 < 0.05$) with respect to control. The explanation for this may be attributed to the escalating levels of salt and phenol concentration as the PW loadings in the culture medium increase. Therefore, a photobioreactor containing 10% PW loadings, which has low salinity and low phenol concentration, generates an environment that reduces osmotic stress and harmful effects on microalgae. Microalgae are able to perform metabolic activities, maintain cellular health,

and accomplish optimal growth efficiently[114]. It is evident that photobioreactor with lower PW loadings like 10% and 20% PW did not enter stationary phase significantly but showed microalgal growth during the 10 days cultivation period. On the contrary, higher PW loadings such as 30%, 40% and 50% PW entered the stationary phase on day 8, day 6 (40% and 50% PW) respectively. In summary, the higher the PW loadings, the lower the log phase of microalgae cultivation period. Consequently, it was observed that the rate of growth reduced as the content of phenol and salt, which are linked to higher PW loadings, increased. This suggests that the presence of phenol and salinity in the PW effluent has an inhibitory effect[59].

Fig. 7.1c and **Fig. 7.1d** illustrate the phenol bioremediation kinetics in different PW loadings in terms of residual phenol concentration in the cultivation medium and phenol removal efficiency respectively. Due to the supplementation with SMW, the photobioreactor having the dilutions of 10%, 20%, 30%, 40%, and 50% of PW displayed that the initial phenol concentration for each dilution were 19.49 mg/L, 28.35 mg/L, 36.46 mg/L, 45.67 mg/L, and 55.02 mg/L respectively. 100% PW contains 100 mg/L phenol concentration as it was not supplemented with SMW. According to **Fig. 7.1c** and **Fig. 7.1d**, on day 2 of cultivation period, phenol concentration reached around 9.88 mg/L, 9.88 mg/L, 7.49 mg/L, 7.30 mg/L, and 10.22 mg/L in the photobioreactor having the PW loadings of 10%, 20%, 30%, 40%, and 50% corresponding to 50%, 65%, 80%, 84%, and 82% phenol bioremediation respectively. During the early stage of exponential phase (until day 2), the significant decrease in phenol concentration found in all PW loadings except 100% PW is likely due to the combined effect of both extracellular and intracellular degradation

mechanisms. Initially, extracellular enzymes may rapidly break down phenol outside the cells, making it easier for the microalgae to uptake and further degrade the resulting compounds intracellularly. Similar types of observations were reported in the literature by Radziff et al. (2021)[176]. Despite the slow growth pattern of the microalgae depicted in **Fig. 7.1a**, the combined effect of these two processes leads to an efficient and quick reduction of phenol levels during the early stages of microalgae production. Extracellular enzymes like laccases and peroxidases can be secreted by microalgae into their surroundings. Outside of the microalgal cells, these enzymes can catalyze the degradation of phenol into less toxic compounds. These enzymes' activity is independent of the microalgae's quick growth, enabling significant phenol breakdown even in cases where cell division is slow[177]. This phenomenon focuses on the potential of halotolerant microalgae *C. sorokiniana* for effective bioremediation in phenol-contaminated PW, comprising both extracellular and intracellular degradation pathways. Similar observation of phenol degradation by microalgae *C. pyrenoidosa* during the early stage of cultivation period (until day 2) has been reported in previous works of Das et al. (2015)[175]. Considering the photobioreactors with 40% and 50% PW loadings, there was no change of phenol removal (**Fig. 7.1d**) by *C. sorokiniana* when it entered the stationary phase of growth after day 6 (**Fig. 7.1a**) and finally found 94% and 96% maximum phenol bioremediation respectively. A similar phenomenon was observed for the case of 30% PW loadings when it entered the stationary phase after day 8 (**Fig. 7.1a**) and achieved 95% maximum phenol bioremediation. It is evident that the biodegradation of phenol depends on the exponential (or log) growth phase of microalgae. Because of their high metabolic activity and active dividing nature, microalgae are able to efficiently break down phenolic

substances during this phase[178]. After the majority of the phenol is consumed during the exponential phase, the cells enter into the stationary growth phase. The continued increase in biomass, even after most of the phenol has been depleted, can be attributed to the metabolic intermediates of phenol biotransformation. These intermediates act as growth substrates until they are mostly used[179]. Priyadharshini et al. (2017) reported the similar findings of phenol biodegradation by *C. pyrenoidosa* in the effluent of a coal gasification plant. The authors note that an increase in phenol concentration leads to the emergence of a lag phase. They also observe simultaneous phenol biotransformation during the exponential phase, followed by a stationary phase coinciding with phenol depletion. Additionally, they observe a rise in biomass even after *C. pyrenoidosa* has fully utilized the phenol[174]. Photobioreactor with 10% and 20% PW loadings did not enter the stationary growth phase during the 10 days cultivation period and was found to have 88% and 94% phenol bioremediation respectively at the end of cultivation period. Compared to higher PW loadings, at lower initial phenol concentrations (10% and 20% PW), microalgae might utilize phenol more efficiently for their growth and metabolism. However, because the initial amount of phenol is comparatively low, the percentage removal might appear lower (**Fig. 7.1d**) despite the high biomass (**Fig. 7.2**). According to **Fig. 7.1d**, phenol removal efficiency for 100% PW was found to be 16% only (initial phenol = 100 mg/L) on day 8. According to the analysis of variance (ANOVA), it is proven that the mean differences of phenol removal efficiency among the treatment groups (PW loadings) are statistically significant ($p\text{-value} = 0.013464 < 0.05$). As 100% PW did not show any growth (**Fig. 7.1a**), the percentage of phenol removal in this case may be due to non-biological (abiotic) factors such as mostly biosorption as well as volatilization[180]. In

order to counteract the volatilization loss (abiotic factor) specifically in the removal of phenol, the loss of phenol from the culture media without *C. sorokiniana* was measured. It was found that there was an 8% abiotic loss of phenol within 8 days, which is considered an acceptable level of abiotic loss when compared to the degradation of phenol by microalgae as reported in the literature[175]. The evidence demonstrates that *C. sorokiniana* is the primary contributor to the process of bioremediation of phenol in supplemented PW. This is primarily achieved by biodegradation (both external and internal), with a smaller portion attributed to bioaccumulation and biosorption. Typically, biosorption is not the primary method for removing phenol in certain research. However, it can serve as the initial stage for processes such as bioaccumulation, internal biodegradation, and other mechanisms[181]. The research on the bioremediation of phenol-contaminated wastewater using microalgae is presented in **Table 7.1**. In comparison to earlier reports, the *C. sorokiniana* culture system developed in this work demonstrates higher effectiveness in degrading phenol. This system has the potential to be applied in the treatment of phenol-containing PW.

Table 7.1 Phenolic pollutant removal by microalgae in a controlled laboratory experiment and the related mechanisms

Microalgae species	Wastewater medium/medium	Mechanism	Temp ($^{\circ}\text{C}$)	Initial concentration (mg/L)	Removal rate (%)	Time (days)	Reference
<i>Chlorella pyrenoidosa</i>	Synthetic medium	Biodegradation	25	800	97	4	[182]
<i>Lyngbya lagerlerimi</i>	Synthetic medium	Biodegradation	25	166	64.4	3	[183]
<i>Chlorella Vulgaris</i>	Modified Conway medium	Biodegradation	24	100	98	4	[184]
<i>Scenedesmus sp.</i>	Supplemented BG-11 medium	Biodegradation	26	4.88	22	11	[185]
<i>Chlorella pyrenoidosa</i>	Coal gasification wastewater	Biodegradation	30 ± 5	1475	95	7	[174]
<i>Chlorella sp.</i>	Supplemented TAP medium	Biodegradation	28 ± 1	700	86	8	[186]
<i>Scenedesmus abundans</i>	Bold's basal medium	Biosorption	25	100	87.8	0.156	[187]
<i>Chlorella sp.</i>	Synthetic medium	Biosorption	30	400	50.24	0.295	[188]
<i>Phormidium animale</i>	Pulp and paper wastewater	Bioaccumulation	26	6.45	70	7	[189]
<i>Chlorella sorokiniana</i>	Synthetic produced water	Mostly Biodegradation	24 ± 1	19.49-100	17-96	8	This study

Table 7.2 mainly describes the growth kinetics parameters such as maximum specific growth rate ($\mu_{\max}$), and maximum biomass productivity ($P_{\max}$) of *C. sorokiniana* cultivated under the different phenol-based PW loadings. As appeared in **Table 7.2**, the specific growth rates at PW loadings of 0%, 10%, 20%, 30%, 40% and 50% were 0.15, 0.16, 0.15, 0.11, 0.13, 0.12 day⁻¹ respectively. According to the obtained results, the specific growth rate accounted for 10% and 20% PW loadings was consistent with the 0% PW loading (control), indicating that growth was not substrate inhibited into these two photobioreactors. Furthermore, because phenol is used as an organic carbon source in incubated cultures, it has a suitable specific growth rate. The findings of other researchers support this conclusion[178]. But when PW loadings were increased above 30%, the growth rate was shown to decrease, indicating that phenol had a growth-inhibiting impact. The overall findings of the specific growth rate obtained in this study was consistent to the specific growth rate found by Priyadharshini et al. (2017) during the treatment of phenolic effluent of a coal gasification plant by microalgae *C. pyrenoidosa* [174]. In addition, **Table 7.2** shows the biomass productivity of *C. sorokiniana* culturing at different PW loadings. Compared to control (0% PW), higher biomass productivity was achieved in the value of 64.7 mgL⁻¹d⁻¹ for 10% PW loadings. This could be attributed to the lower PW loadings, where the presence of phenol and other potentially inhibiting factors like salinity may be at levels that the microalgae can detoxify or tolerate more efficiently. As a result, this creates optimal circumstances for growth[114]. Biomass productivity for other PW loadings such as 0% PW (control), 20% PW, 30% PW, 40% PW and 50% PW was found 27.8 mgL⁻¹d⁻¹, 24.9 mgL⁻¹d⁻¹, 25.9 mgL⁻¹d⁻¹, 30.2 mgL⁻¹d⁻¹, and 21.3 mgL⁻¹d⁻¹ respectively. It is seen that biomass productivity was found to decrease with an increase in PW loadings

where 40% PW loadings showed an exception in this regard. At higher PW loadings, the concentration of phenol increases, leading to enhanced toxicity that can inhibit various metabolic activities, reduce photosynthetic efficiency, and ultimately decrease biomass production[190]. Moreover,

Table 7.2 Conditions and growth parameters of *C. sorokiniana* cultivated in supplemented phenol based SPW.

PW loading (%)	Salinity (g/L)	Maximal OD ₆₈₀	Specific growth rate, μ (day ⁻¹)	Cultivation Period (day)	Biomass productivity (mgL ⁻¹ d ⁻¹)
0	0.43	2.61	0.15	10	27.8
10	6.66	4.2	0.16	10	64.7
20	12.59	2.99	0.15	10	24.9
30	19.22	2.53	0.11	10	25.9
40	25.62	2.98	0.13	10	30.2
50	31.90	2.38	0.12	10	21.3
100	62.97	0.265	0.0	10	0.5

Notes: Salinity recorded here after pH adjustment and sterilization of the culture media

PW frequently contains elevated concentrations of dissolved salts, which significantly contribute to the occurrence of salinity stress in microalgal cultures. As the loading of PW increases, the impact of salinity stress becomes more evident, which can potentially result in osmotic imbalance, ion toxicity, and additional hindrance of growth[191]. The range of biomass productivity found in this study is supported by comparable results reported by Das et al.[192].The comparable growth kinetics parameters obtained in this study for freshwater microalgae *C. sorokiniana* cultivated under hypersaline PW confirms its halotolerant capacity.

Fig. 7.2 illustrates the maximum biomass yields (mg/L) and specific phenol removal rate (mg phenol/g biomass) obtained from *C. sorokiniana* culture with varying phenol-based PW loadings (0% to 100%). Considering all PW loadings, maximum biomass output after

the cultivation period was found to vary from 373 mg/L to 807 mg/L, with the exception of 100% PW, which was only 165 mg/L and essentially indicated no microalgae development in that culture medium. Microalgae culture with 10% PW showed the highest biomass concentration (807 mg/L) followed by 20% PW (615 mg/L), 40% PW (462 mg/L), 30% PW (447 mg/L), 0% PW (control) (438 mg/L), and 50% PW (373mg/L). It is evident that biomass production was found to decline beyond 20% PW loadings and lowest biomass production was achieved for

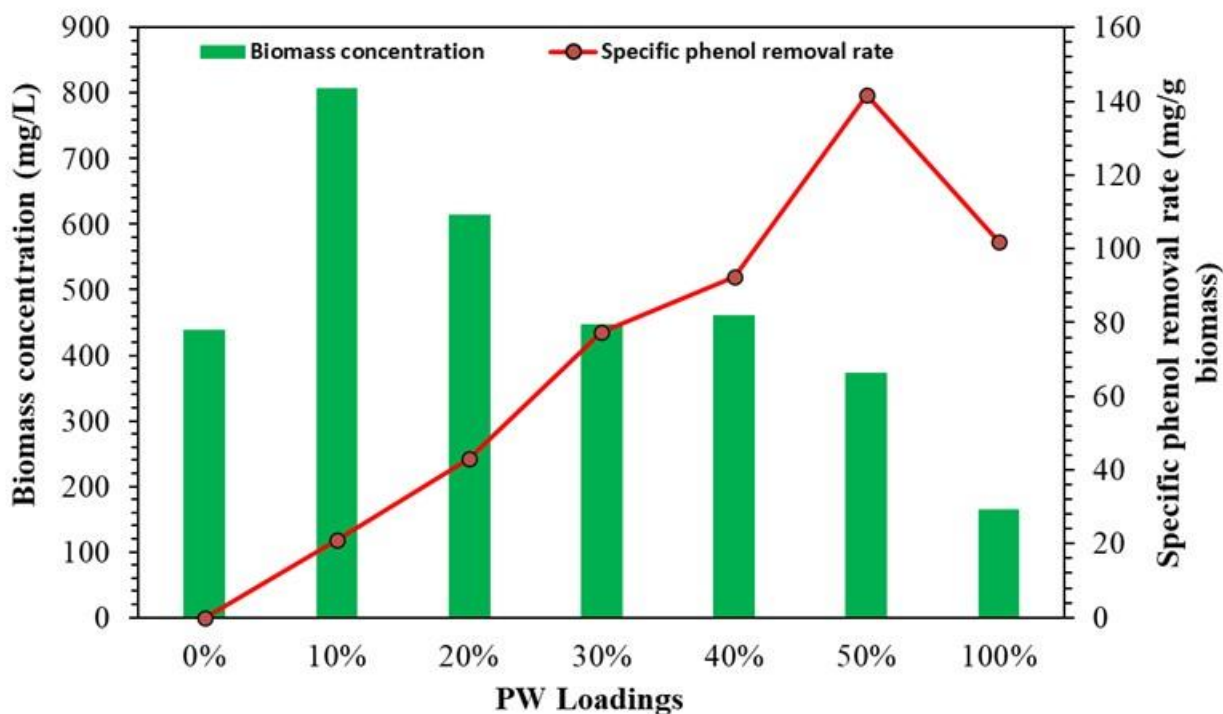


Fig. 7.2. Maximum biomass yields (mg/L) and specific phenol removal rate (mg phenol/g biomass) of *C. sorokiniana* culture with varying phenol-based PW

50% PW loadings. The results suggest that the production of microalgae biomass is maximized when exposed to low concentrations (10%) of PW, as this mild phenolic stress

promotes growth. Nevertheless, the production of biomass experiences a substantial decrease when the PW loadings above 20%, primarily because to heightened phenol toxicity and stress reactions caused by elevated salt levels. As appeared in **Fig. 7.2**, specific phenol removal rate, the amount of phenol removed per unit of microalgae biomass (mg phenol/g biomass) increased gradually with increasing PW loadings, up to 50% PW. The highest specific phenol removal rate (141.58 mg phenol/g biomass) was attained for 50% PW loadings despite lowest biomass production. The decrease in biomass concentration observed as the PW loadings increase can be attributed to the toxic effects of phenol, the activation of stress responses, metabolic shifts towards detoxification, and resource allocation strategies, which also result in increased phenol removal. Specifically, this could be attributed to metabolic trade-offs, where increased PW loadings may cause the microalgae to choose phenol breakdown over growth. The energy and metabolic intermediates that are usually utilized for growth are redirected towards the detoxification and degradation of phenol[193]. These findings highlight the complex interactions between microalgae and phenol-containing PW.

The pH of the growing medium is crucial in affecting the effectiveness of phenol removal and the growth of microalgae[190]. **Fig. 7.1b** demonstrates the pH profiles over time during the cultivation of *C. sorokiniana* under different percentage of phenol containing PW loadings. As observed in **Fig. 7.1b**, the initial values of pH for 10%, 20%, 30%, 40% and 50% PW loadings were in the range of 5.25-5.34 indicating very close values with respect to each other. However, initial pH values for 0% PW and 100% PW were 6.62 and 6.71 respectively. It is noted that pH adjustment of the culture medium prior to autoclave

had a significant influence on the initial values of pH in the photobioreactors having different PW loadings. On day 2, pH values of culture medium drastically increased for photobioreactors containing 20%, 30%, 40% and 50% PW loadings and was in the range of 7.20 to 8.10. It is noteworthy that these pH rises were positively correlated with the microalgal growth on day 2 shown in **Fig. 7.1a**. According to **Fig.7.1d**, on day 2 of cultivation period, photobioreactor having the PW loadings of 20%, 30%, 40%, and 50% showed 65%, 80%, 84%, and 82% phenol bioremediation respectively. The results of phenol bioremediation on day 2 indicate that observed pH range of 7.20 to 8.10 was favorable for phenol bioremediation and microalgal growth. Therefore, it could be suggested that the present *C. sorokiniana* favored neutral to alkaline pH for phenol bioremediation. The pH value of photobioreactors with 20%, 30%, 40% and 50% PW loadings increased slightly on day 4 and did not change well for the rest of the period. The favorable pH range observed in this study for microalgal growth and phenol degradation was consistent with the literature reported by Thakurta et al. (2018) [194]. On the contrary, photobioreactor with 10% PW loadings revealed a slight reduction in pH value of 5.09 with corresponding phenol bioremediation of 50% on day 2. This observed lower pH value may be due to the degradation of phenol by microalgae that can produce acidic by-products, which can lower the pH of the cultivation medium. After that, there was a drastic increase in pH value (8.16) on day 4 and maintained the same level with a small reduction for the rest of the cultivation period. Photobioreactor with 0% PW (control) showed the pH value in the range of 6.62-7.75 associated with a fair growth of microalgae under phototrophic mode in absence of any carbon source like phenol. The pH value for 100% PW was almost leveled off throughout the cultivation period due to the no growth of microalgae. Generally,

the trend to neutral and more basic conditions of the culture medium correlates the exponential growth of microalgae and bioremediation of phenol in PW (**Fig.7.1a and 7.1d**).

7.3 Nitrogen removal and lipid biosynthesis

In the cultivation of *C. sorokiniana* using phenol-containing PW, nitrogen concentration is a critical parameter that influences the growth and metabolic processes of microalgae, including the degradation of organic pollutants like phenol. SPW prepared in this study does not contain any nitrogen. Thus, SPW was supplemented with SMW as a major source of nitrogen for microalgal growth. Due to the supplementation with SMW, the photobioreactor having the dilutions of 0%, 10%, 20%, 30%, 40%, and 50% of PW exhibited that the initial nitrogen values for each dilution were 41.2 mg/L, 35.5 mg/L, 27.5 mg/L, 25 mg/L, 20 mg/L, and 18 mg/L respectively. 100% PW does not contain any nitrogen concentration as it was not supplemented with SMW. The phenol present in the PW causes stress on the microalgae, which may impact their nitrogen metabolism. At lower concentrations of phenol, *C. sorokiniana* demonstrates a reasonably consistent intake of nitrogen, with a gradual decline in nitrogen levels over time. Nevertheless, when phenol concentrations are elevated, the stress might impede the growth rate of the microalgae, leading to a reduced rate of nitrogen depletion. As appeared in **Fig. 7.3a**, considering all PW loadings, during the initial period of cultivation (0-2 days), there was a rapid uptake of nitrogen as the microalgae acclimated to the medium and began to multiply rapidly. This phase is marked by a rapid decrease in nitrogen content. During the mid-phase (2-6 days), when the microalgae population experiences exponential growth, nitrogen uptake persists although at a slightly slower pace compared to the early phase. The concentration of

nitrogen in the medium is gradually decreasing. During the later phases of culture, specifically between 6 to 10 days, the nitrogen concentration gradually decreases to a minimal level. The nitrogen uptake rate experiences a substantial decline as the microalgae transition into the stationary phase of growth (**Fig. 7.1a**). This decrease is mostly caused by the exhaustion of nitrogen and the buildup of metabolic byproducts, such as phenol degradation products.

Assessing the lipid accumulation in biomass can indirectly provide evidence that microalgae are breaking down phenol, as the breakdown of phenol can stimulate increased lipid production in *C. sorokiniana* microalgae. **Fig. 7.3b** displays the time course profiles of total lipid content as a proportion of dry weight of biomass at various phenol containing PW loadings. The data revealed a positive correlation between the concentration of PW and lipid accumulation in *C. sorokiniana* biomass. It was observed that higher PW loadings resulted in significantly increased lipid content in the microalgal biomass. On day 2, lipid content in biomass ranged from 4.39% to 9.8% considering all PW loadings except 0% PW loadings (control). No lipid accumulation was observed in *C. sorokiniana* biomass on day 2 when microalgae was cultured in 0% PW loadings (control). This investigation clearly indicates that a percentage of phenol or degradation products of phenol from the phenol containing PW were bioaccumulated within the microalgae cell which ultimately enhanced the lipid biosynthesis in biomass on day 2. As observed in **Fig. 7.3a**, under nitrogen-rich conditions (until day 6), *C. sorokiniana* biomass exhibited enhanced uptake and degradation of phenol (**Fig. 7.1c**) considering all PW loadings except control. The presence of nitrogen is responsible for the availability of enzymes involved in the breakdown of

phenol. Nitrogen enhances the overall metabolic activity of microalgae, hence allowing the effective degradation of phenol and its degradation byproducts[195].

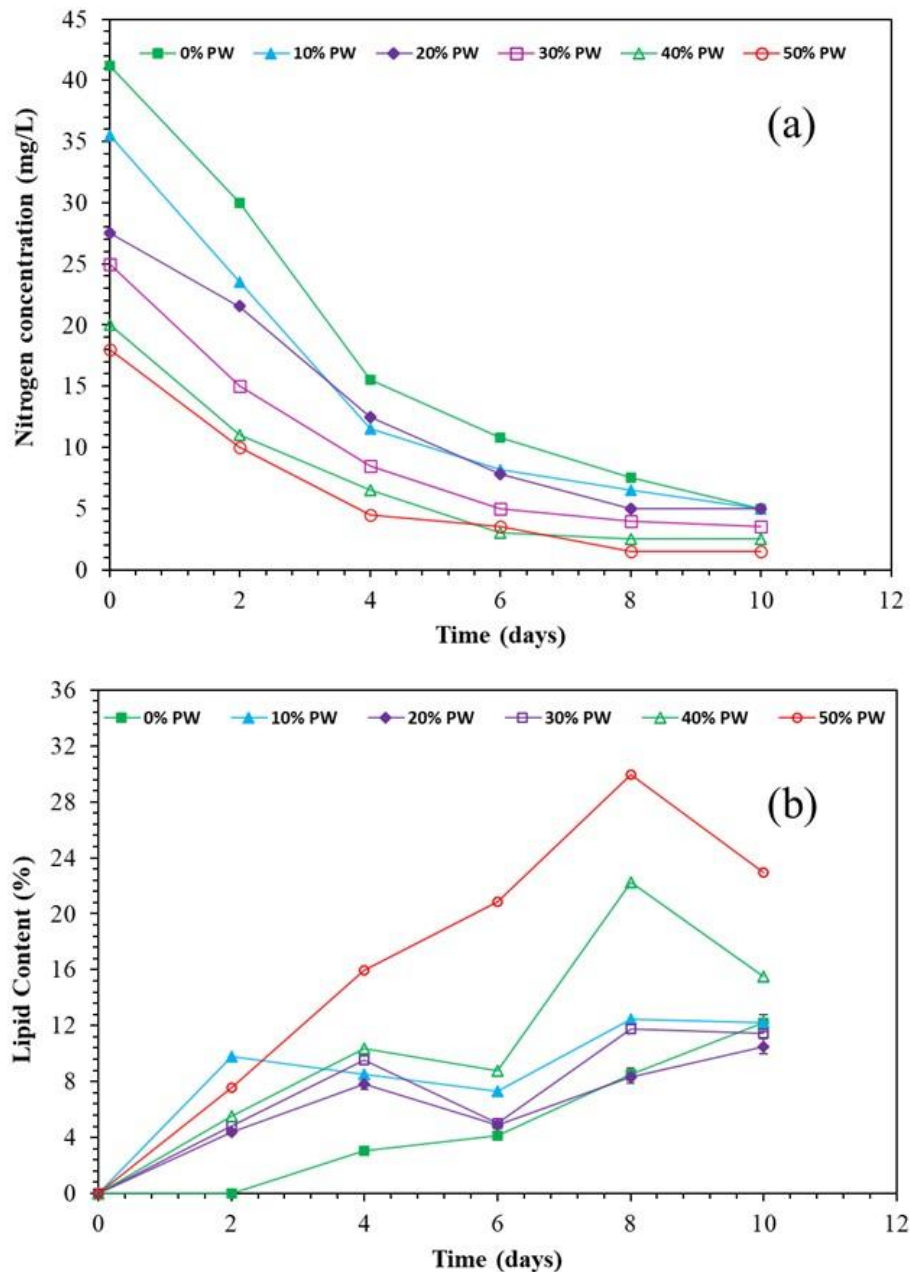


Fig. 7.3 (a) Time profiles of nitrogen uptake of *C. sorokiniana* in different phenol containing PW loadings. **(b)** Time profiles of lipid biosynthesis in *C. sorokiniana* during bioremediation of phenol in varying PW loadings

Conversely, under nitrogen-depleted conditions (6 to 10 days), the microalgae displayed a significant increase in lipid biosynthesis attaining a highest lipid content (8.3% to 30%) on day 8 considering all PW loadings compared to control. Nitrogen deficiency leads to nutritional stress, which prompts a metabolic change towards lipid storage as a means of survival. This stress response is distinguished by the redirection of carbon from phenol degradation pathways to lipid storage pathways. Microalgae use the carbon obtained from the accumulation and breakdown of phenol to produce and store high-energy lipids. These lipids act as a reserve when there is a shortage of nutrients[25]. The findings emphasize the dual function of nitrogen in controlling both the breakdown of pollutants and the synthesis of biofuels in microalgae. Phenol breakdown is facilitated in nitrogen-rich environments, which makes it advantageous for bioremediation applications. Conversely, when there is a lack of nitrogen, it promotes the buildup of lipids, which is beneficial for the creation of biofuels. As appeared in **Fig. 7.3b**, maximum lipid content in *C. sorokiniana* biomass was achieved in the amount of 30% as dry weight of biomass on day 8 when microalgae were cultured with 50% PW loadings and found statistically significant compared to control ($p\text{-value} = 0.01241 < 0.05$). The elevated lipid content seen in response to higher phenol-containing PW loadings (50% PW) is likely a result of a combination of stress-induced lipid accumulation, alternate carbon usage, nutritional constraint, osmotic stress, and changed metabolic pathways[196]. The obtained results of lipid content in this study were higher than those of the findings of other researchers, where *C. sorokiniana* cultivated mixotrophically on waste dark fermentation effluents having acetate[124].

7.4 Proposed phenol removal mechanism

As appeared in **Fig. 7.4**, microalgae *C. sorokiniana* have been shown to be effective in the bioremediation of phenol-contaminated PW through cultivation considering all PW loadings except 100% PW. *C. sorokiniana* did not grow well in 100% PW due to the lack of appropriate nutrients, high salinity as well as high phenol concentration (100 mg/L). According to **Fig. 7.1d**, phenol removal efficiency for 100% PW was found to be 16% only on day 8. As 100% PW did not show any growth (**Fig. 7.1a**), the percentage of phenol removal in this case may be due to non-biological (abiotic) factors such as mostly biosorption as well as volatilization[180]. As observed in **Fig. 7.4** (10% to 50% PW), despite the successful removal of most of the phenol

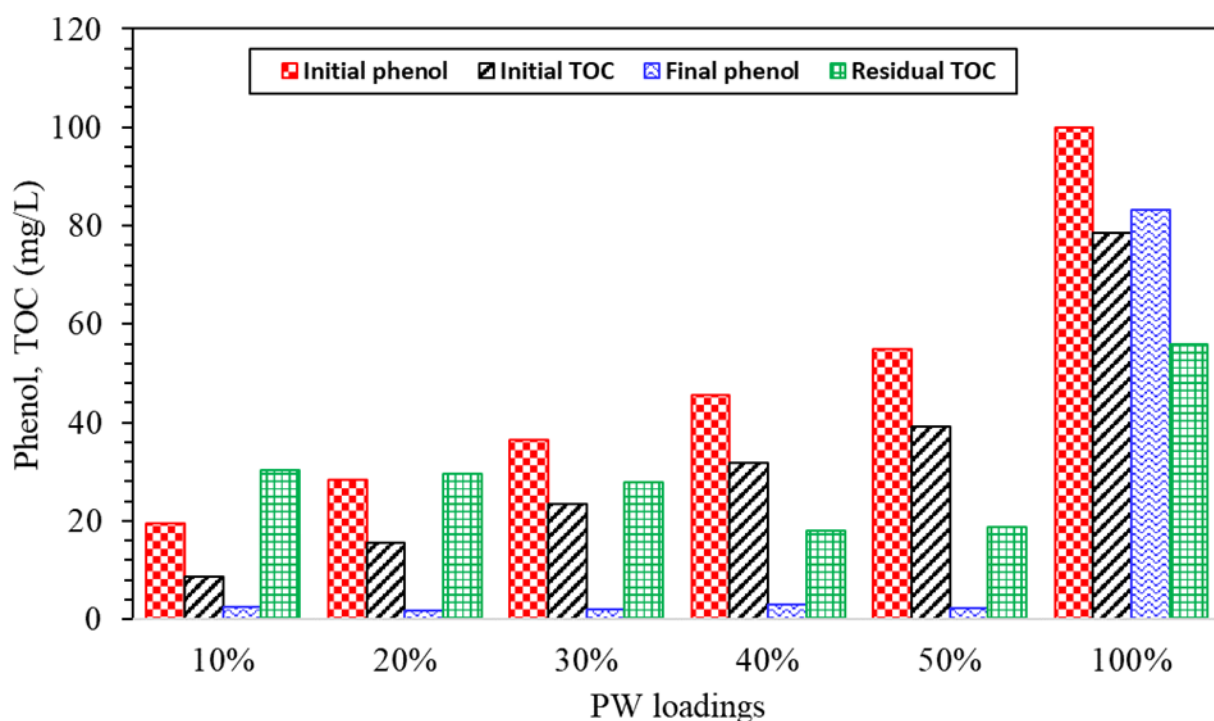


Fig. 7.4. Measured initial and final value of phenol and TOC in different phenol containing PW loadings.

the cultivation media still exhibits a residual TOC value, suggesting the presence of unassimilated organic compounds. It suggests that the *C. sorokiniana* microalgae are not fully using all of the degraded phenol or metabolic intermediates. The residual organic molecules raise the TOC content of the growing media, potentially as partly degraded phenol derivatives. According to **Fig. 7.4**, photobioreactor having 40% PW and 50% PW loadings showed lower amount of residual TOC value compared to other PW loadings. It indicates that biodegraded phenol or metabolic intermediates are mostly assimilated by the microalgae *C. sorokiniana* in these two cases. The assimilation of more organic compounds in these two cases was positively reflected in the enhancement of lipid biosynthesis in *C. sorokiniana* biomass (**Fig. 7.3b**). To clarify the phenol removal mechanism in this system, it is essential to consider the potential pathways involved, including biosorption, biodegradation as well as bioaccumulation.

Microalgae have been shown to remove phenol and other contaminants by a well-documented process called biosorption, which involves the passive adsorption of pollutants onto biomass surfaces[197]. The biomass of *C. sorokiniana* may adsorb some of the phenol, leading to its removal from the cultivation media. The observation of residual TOC (**Fig. 7.4**) indicates that biosorption may not be the exclusive mechanism accountable for the reported elimination of phenol. In this case, another possible method for phenol elimination is biodegradation—the breakdown of organic molecules by microalgae. It has been demonstrated that *C. sorokiniana* has the metabolic capacity to use phenol as a source of carbon and energy, which could result in the breakdown of some of the phenol found in

the PW[25]. Based on **Fig. 7.3b**, the lipid content in biomass varied between 4.39% and 9.8% on day 2 for all PW loadings, except for the 0% PW (control). There was no lipid buildup observed in the biomass of *C. sorokiniana* on day 2 when it was cultivated in the control medium with 0% PW loadings (without phenol). The results of this investigation indicate that phenol or its breakdown products from phenol-containing PW were bioaccumulated in the microalgae cells. Ultimately, by the second day, this accumulation caused the biomass to produce more lipids (**Fig 7.3b**). In summary, the phenol removal mechanism by *C. sorokiniana* involves a combination of biosorption, enzymatic biodegradation, and assimilation into biomass. The presence of unassimilated organic molecules and metabolic byproducts from the breakdown of phenol is probably the cause of the reported TOC value in the culture media at the end of the process. Based on the obtained result of abiotic phenol removal (16%) for 100% PW loadings, it is recommended that the removal of phenol (88% to 96%) for other PW loadings (10% PW to 50% PW) by *C. sorokiniana* cultivation is mostly owing to biodegradation rather than biosorption processes.

As per the documented literature, a typical metabolic route for an aromatic compound such as phenol involves dihydroxylation of the benzene ring, resulting in the formation of a catechol derivative, and then opening of the ring via ortho- or meta-oxidation. Due to the two hydroxyl groups on catechol, enzymatic processes will inevitably oxidize this molecule right away, causing ring-fission[25]. Catechol undergoes oxidation either the ortho-cleavage pathway by the enzyme catechol 1, 2-dioxygenase, or through the meta-pathway to form 2-hydroxymuconic semialdehyde with the help of catechol 2, 3-dioxygenase. The phenol degradation route can proceed through either meta or ortho

fission, depending on the specific type of microalgae. It has been found that the majority of eukaryotic microalgae are capable of degrading phenol using the ortho route[190]. Nevertheless, *Chlorella pyrenoidosa*, a type of green algae, was shown to break down phenols via both the ortho and meta pathways. However, the ortho pathway was more efficient than the meta pathway[175]. According to the studies described above, it is proposed that the degradation of phenol in PW by the green microalgae *C. sorokiniana* may involve both the ortho and meta pathways. Pyruvate, which is a terminal product of the meta route, can enter the tricarboxylic acid (TCA) cycle and be degraded to promote lipid biosynthesis[198] in *C. sorokiniana* biomass (**Fig. 7.5**). Similar type of findings was reported by Kankılıç et al. during their phenol degradation study by microalgae *Scenedesmus regularis*. The authors mentioned that microalgae break down phenol or phenolic compounds in aerobic environments. Simple organic molecules like carbon dioxide and pyruvate, which is an essential component, are broken down during the biodegradation process via the ortho- or meta-cleavage route. Phenolic compounds have the ability to alter the functionalities of algae's cell membranes and metabolic processes during degradation. This action can alter the amount of lipid and carbohydrate synthesis in microalgae[198]. Based on the previously referenced research and related metabolic processes related to the increased lipid biosynthesis in microalgal biomass, the pathway suggested by *C. sorokiniana* for phenol bioremediation in PW is portrayed in **Fig. 7.5**. As appeared in **Fig. 7.5**, it is suggested that the biodegradation of phenol by *C. sorokiniana* can be associated with two separate metabolic pathways, resulting in the production of acetyl-CoA and pyruvate. These pathways are known as ortho-fission (acetyl-CoA) and meta-fission (pyruvate) biodegradation processes, as shown in **Fig. 7.5b**[190]. The

phenolic biodegradation products (pyruvate and acetyl-CoA) and phenol from cultivating media can be integrated into microalgal cells through the biosorption and diffusion processes, which is also referred to as the bioaccumulation process. Through the Calvin cycle process in cellular chloroplasts, acetyl-coA, pyruvate, and phenol are biodegraded into free fatty acid through microalgal cell mechanism upon activation by sunlight or energy, as shown in **Fig. 7.5a**. Lipid elongation is the process by which free fatty acid transforms into long chain fatty acid throughout the endoplasmic reticulum. The enzymatic action of Diacylglycerol acyltransferase (DGAT) and phospholipid: diacylglycerol acyltransferase (PDAT) converts elongated fatty acids into triacylglycerol (**Fig. 7.5a**). Triacylglycerol, often known as triglyceride, is the primary and simplest kind of dietary lipid found in fats and oils[199]. Triacylglycerol consists of fatty acids, which serve as the body's energy storage form. As a result, the biological or metabolic processes of bio-absorption, bioaccumulation, and biodegradation in *C. sorokiniana* play a role in enhanced lipid biosynthesis (**Fig. 7.5**).

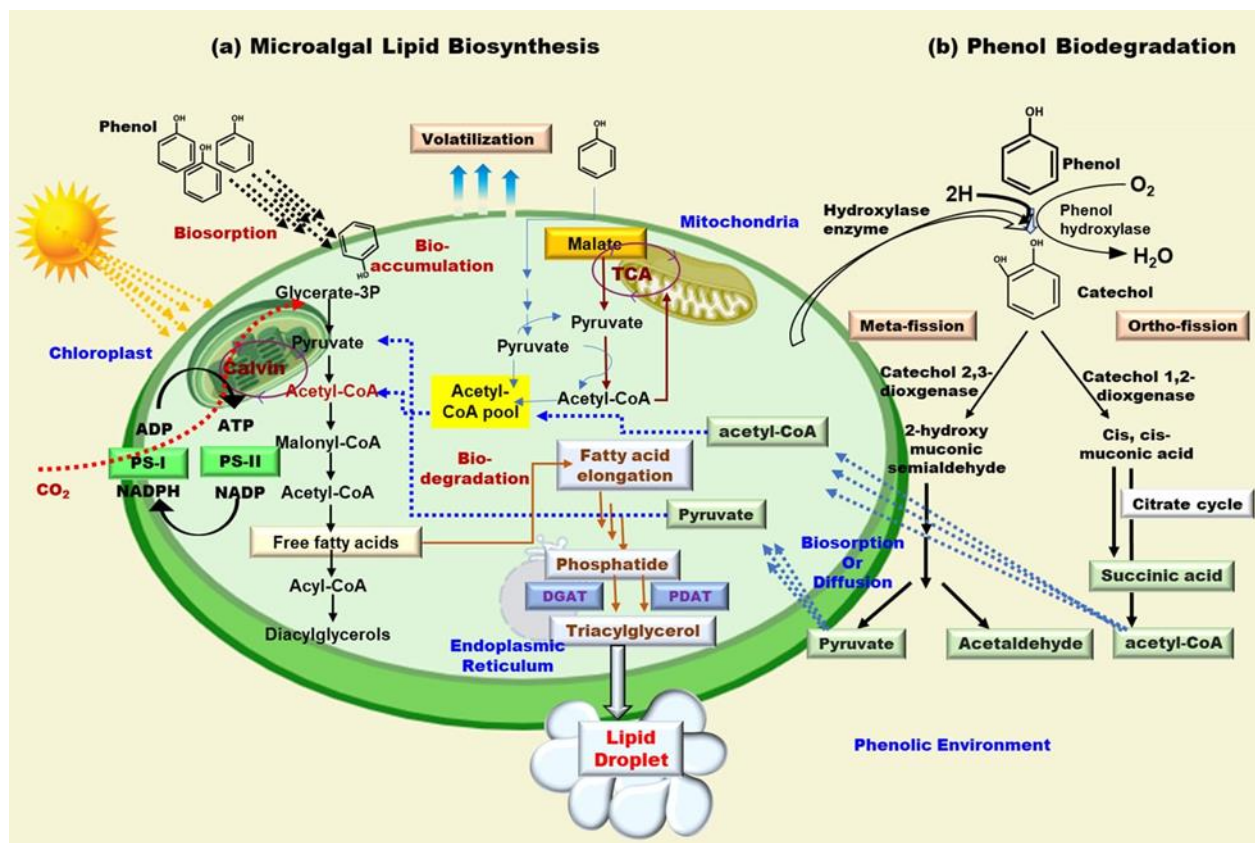


Fig. 7.5. Potential pathway proposed for phenol bioremediation in PW by *C. sorokiniana* is mainly due to biodegradation processes rather than a bioadsorption process (a) Enhancement of lipid biosynthesis obtained in *C. sorokiniana* biomass due to the assimilation of more organic compounds resulting from biodegraded phenol or metabolic intermediates (b) Enzymatic phenol biodegradation reported in the literature[190].

7.5 Valorization of phenol degraded *C. sorokiniana* biomass

Although the rapid SPV method was used to evaluate the time course profiles of total lipid content (**Fig. 7.3b**) as a proportion of dry weight of biomass at various phenol containing PW loadings. Despite, a deeper comprehension of the biochemical, chemical, physical, and HHV of the dried microalgae biomass is required to establish a suitable method to produce commercial biofuel. Therefore, to categorize microalgal species according to their applications, a comprehensive bio-composition analysis is needed. The objective of the subsequent section is to determine the bio-compounds (proteins, carbohydrates, and lipids) present in dried algal biomass through a successive method of FTIR and CHNS instrumental analysis. This is done to conduct a systematic characterization of the biomass.

7.5.1 Structural characterization by FTIR spectroscopy

To accurately assess the bioenergy potential of microalgal biomass, it is essential to determine its biochemical composition, which includes carbohydrates, proteins, and lipids. Regarding this, FTIR spectroscopy is a popular analytical method that can offer a quick and non-destructive evaluation of the macromolecular content of microalgal biomass[133]. The FTIR transmittance of the phenol degraded *C. sorokiniana* biomass indicates the occurrence of -COOH, -OH, -CO –, and NH₂ organic chemical groups under different PW loadings (0% to 50%) (**Fig. S7**). Variations in peak positions, intensities, and shapes can reveal important details regarding the metabolic reactions of microalgae to PW containing phenol. By examining the obtained FTIR spectra in **Fig. S7**, cellular macromolecules (lipids, proteins, and carbohydrates) can be distinguished in various study locations according to their unique transmission bands. Two sites are frequently used in the literature to determine the lipid content by FTIR: the vibrational stretching of ester bonds at 1743

cm^{-1} and the methylene and methyl groups at $2800\text{--}3000\text{ cm}^{-1}$ [130]. In this regard, the infrared spectrum at 1743 cm^{-1} shows that the % transmittance at this position declines with phenol incubation unlike that in control cells (0% PW) (**Fig. S7**). This peak is believed to be mainly associated with lipid and fatty acid ester bonds[132]. Decreased % transmittance or increased band area indicates increased intracellular phenol or phenol degraded products like pyruvate accumulation in biomass which have enhanced the lipid biosynthesis for 40% and 50% PW shown in **Fig. 7.3b**. The biomass of *C. sorokiniana* (after phenol biodegradation) may be used as a possible raw material for the generation of biofuel due to its higher lipid content. Mixotrophic culture has been shown to enable microalgae to acquire a greater proportion of lipids in a shorter amount of time[200]. As appeared in **Fig. S7**, the band observed at 3279 cm^{-1} to 3289 cm^{-1} considering all PW loadings is a result of the stretching of the O-H bonds. As reported in the literature, the infrared region $1610\text{--}1685\text{ cm}^{-1}$ is associated with C=O stretching of proteins[201]. Thus, significant band observed at a wavenumber of 1683 cm^{-1} is attributed to the stretching of the C=O bond in the amide I region, which is connected with proteins [133]. The reduced transmittance in this region suggests that protein production is higher in the control biomass compared to the phenol-degrading biomass (**Fig. S7**). According to **Fig. S7**, the spectra of the amide II band, which mostly consists of stretching vibrations of C-H and N-H bonds associated with proteins, were observed at around 1538 cm^{-1} (0%, 10%, 20%, and 30% PW loadings), 1539 cm^{-1} (40% PW loadings), and 1540 cm^{-1} (50% PW loadings). The spectra at 1410 cm^{-1} (0% and 20% PW loadings), 1411 cm^{-1} (10% PW loadings), 1412 cm^{-1} (30% PW loadings), 1416 cm^{-1} (40% PW loadings), and 1414 cm^{-1} (50% PW loadings) correspond to the bending of methyl groups (CH_2 and CH_3) and the stretching of carboxylic

groups (C-O) associated with the presence of proteins in *C. sorokiniana* biomass. The 'carbohydrate band spectra' exhibited strong and medium features in the range of approximately 1149-550 cm⁻¹ due to C-O-C and C-O stretching. The presence of the main constituents of the phenol degraded *C. sorokiniana* biomass, specifically lipid, carbohydrate, and protein, was detected by the spectral bands. These outcomes are consistent with several others[131], [133].

7.5.2 CHNS analysis and HHV in biomass

A key aspect of evaluating the potential of microalgae biomass for bioenergy is through its elemental composition (CHNS: Carbon, Hydrogen, Nitrogen, Sulfur) and its Higher Heating Value (HHV)[138]. The CHNS and HHV of microalgae biomass provide significant evidence for its suitability in bioenergy applications. The C, H, N, and S contents of the *C. sorokiniana* biomass were quantified at different phenol containing PW loadings, as shown in **Table 7.3**. The results were compared to analogous findings from other published investigations. Carbon is essential for bioenergy production as it is the main constituent that is transformed into energy through combustion or other bioenergy procedures. According to **Table 7.3**, elemental composition of carbon for 0% PW (control) biomass was found different as compared to others, representing highest carbon content (39.06%) in biomass than those of others considering all PW loadings. A relatively reduced amount of carbon content in biomass was achieved, ranging from 32.64% to 36.51% considering all phenol containing PW loadings. The metabolic strain of detoxifying phenol and repairing phenol-induced damage can potentially impair the overall efficiency of carbon fixation[195]. Hydrogen is another essential component for bioenergy production, contributing to the overall heating value of the biomass. As appeared in **Table 7.3**,

hydrogen content in microalgae biomass obtained ranges from 4.6 to 5.2% considering all PW loadings. The obtained percentages of hydrogen are consistent with the reported literature[202]. Seeing to all PW loadings, sulfur content in *C. sorokiniana* biomass is generally low (<1%). This is beneficial because it decreases the likelihood of sulfur dioxide (SO₂) emissions during burning, which are detrimental pollutants. Efficient biofuel production is enhanced by a high carbon and hydrogen concentration compared to nitrogen and sulfur.[147] and are in good accord with the obtained outcome in this study (**Table 7.3**). **Fig. 7.3b** shows that the phenol-degraded *C. sorokiniana* biomass has a greater lipid content, even if the overall carbon content is reduced according to **Table 7.3**. This suggests that the increased lipid content is likely due to stress-induced metabolic changes that promote lipid accumulation as a defensive mechanism. The 0% PW (control) exhibits a lower lipid content but a higher carbon content, suggesting that it is under normal growth conditions where carbon is divided more uniformly across different cellular components, such as carbohydrates and proteins. The presence of phenol triggers a stress response in the body, which in turn leads to a redistribution of metabolic resources towards lipids. This shift in metabolism is a reflection of the organism's adaptive strategy to survive in unfavorable situations.

The higher heating value (HHV) of microalgae biomass is a crucial measure for evaluating its potential as a bioenergy source. HHV is the maximum amount of heat liberated after the complete combustion of a fuel in an environment where the resulting substances are permitted to revert to their initial temperature. This value is commonly given in units of energy per unit mass, such as MJ/kg or kcal/kg. This parameter is crucial in assessing the effectiveness and viability of transforming microalgae into several types of bioenergy, such

as biofuels, biogas, or direct combustion for heat and electricity generation[203]. As reported in **Table 7.3**, the HHV of phenol degraded *C. sorokiniana* biomass in the present study ranged from 13.81 MJ/kg to 15.20 MJ/kg , which is like the other works for *Scenedesmus sp.* reported in the literature[131]. Moreover, the study found that phenol degraded *C. sorokiniana* biomass had a higher heating value (HHV) compatible to lignocellulosic biomass sources like rice husks (16.7 MJ/kg) and corn stalks (15.81 MJ/kg)[151], bagasse sugarcane (16.4 MJ/kg)[152], and pistachio shells (17.47 MJ/kg)[153]. Microalgal biomass possesses a diminished nitrogen and sulfur composition, rendering it a favorable option for the production of environmentally sustainable biofuels. In addition, solid fuels commonly utilized in bioenergy production, such as wood sawdust with a HHV of 18 to 19 MJ/kg, can be used as a reference to assess the energy potential of phenol degraded *C. sorokiniana* biomass in thermal conversion processes[203].

Table 7.3 CHNS analysis and HHV of *C. sorokiniana* biomass cultivated in phenol containing PW loadings compared with similar findings of previously reported

Microalgae biomass	Carbon (C) (% dry wt.)	Hydrogen (H) (% dry wt.)	Nitrogen (N) (% dry wt.)	Sulfur (S) (% dry wt.)	HHV (MJ/Kg)	Reference
<i>Chlorella sp.</i>	46.10	6.10	6.70	0.62	17.30	[154]
<i>Chlorella sorokiniana</i>	49.74	7.60	8.25	0.38	20.96	[107]
<i>Spirulina platensis</i>	51.81	7.11	3.95	1.27	20.97	[155]
<i>Nannochloris oculuta</i>	43.88	6.95	5.74	1.32	17.25	[156]
<i>Scenedesmus dimorphus</i>	44.59	8.28	4.02	0.16	24.04	[133]
<i>Scenedesmus almeriensis</i>	41.90	6.70	5.90	0.80	15.76	[144]
<i>Scenedesmus sp.</i>	24.45	7.68	5.42	n/a	15.20	[131]
<i>Tetraselmis suecica</i>	27.72	4.59	4.70	1.11	9.38	[143]
<i>Chlorella sorokiniana</i> (0% PW)	39.06	5.81	3.78	0.54	15.98	This study
<i>Chlorella sorokiniana</i> (10% PW)	36.51	5.16	3.73	0.90	15.20	This study
<i>Chlorella sorokiniana</i> (20% PW)	32.64	4.65	3.85	0.68	14.09	This study
<i>Chlorella sorokiniana</i> (30% PW)	33.61	4.68	3.71	0.55	14.32	This study
<i>Chlorella sorokiniana</i> (40% PW)	32.87	4.78	3.00	0.51	13.81	This study
<i>Chlorella sorokiniana</i> (50% PW)	35.65	5.26	2.72	0.50	14.54	This study

7.6 Conclusions

This study investigated the capability of *C. sorokiniana* to bioremediate phenol and its potential for bioenergy applications by cultivating it in synthetic petroleum-derived PW. The growth investigation revealed that the microalga survived in this demanding environment, attaining significant biomass accumulation. The key findings reveal a notable decrease in the content of phenol, which indicates that *C. sorokiniana* has the ability to degraded harmful organic molecules such as phenol found in PW. Maximum phenol removal efficiency on day 8 for different PW loadings was in the average figure of 88%, 94%, 95%, 94%, and 96% for 10%, 20%, 30%, 40%, and 50% PW loadings respectively. Measuring lipid accumulation in biomass can indirectly support the idea that microalgae are metabolizing phenol, as phenol biodegradation can lead to enhanced lipid synthesis in *C. sorokiniana* biomass (30% lipid content). The suitability of phenol-degraded biomass as a biofuel feedstock was assessed by examining its physicochemical properties using FTIR and CHNS analysis, as well as estimating its higher heating value (HHV). The study's results highlight the potential of utilizing microalgae *C. sorokiniana* in integrated systems for bioremediation and bioenergy generation. By utilizing PW, a waste product of the petroleum industry, this approach offers a sustainable solution for water treatment while generating valuable bioresources. In overall, the combination of environmental bioremediation and the potential for bioenergy production from phenol degraded biomass provides a strong rationale for additional research and advancement in this area.

CHAPTER 8

Microalgal Bioremediation of Phenol-Acetate Based Produced Water with and without Heavy Metal Stress

8.1 Introduction

The primary objectives of this chapter are: i) To investigate HM stress on microalgal growth, nitrogen removal, organic pollutant degradation, and lipid content in biomass across different PW gradients ii) To quantify TOC removal as an indicator of organic pollutant biodegradation across different PW concentrations, both with and without heavy metal presence, iii) To analyze the lipid accumulation in microalgal biomass as a function of stress conditions, highlighting its potential for bioenergy applications.

8.2. Growth of microalgae with and without heavy metal stress

To assess the growth characteristics and bioremediation potential, preadapted microalgae *C. sorokiniana* inoculum was cultured in SPW supplemented with SMW. **Fig. 8.1 (a-f)** shows time profiles of optical densities (OD_{680}) as a way of determining the growth feasibility of *C. sorokiniana* in different gradients of PW with and without HM stress. Due to the utilization of preadapted microalgae cells in each PW gradient, lag phase was escaped in the culture of all photobioreactors. According to **Fig. 8.1a**, the growth of *C. sorokiniana* in 10% PW shows individual trends under conditions with and without heavy metals (HM). By day 2, OD rises in both conditions, with a sharper rise in the "with HM" condition, demonstrating the microalga's quick adaptation to metal stress. OD reaches 2.08

(without HM) and 2.65 (with HM) by day 4. The exponential growth phase is evident. The change in OD between conditions widens, reflecting improved adaptation and metabolic activity under metal stress. By day 6, OD increases further to 2.73 (without HM) and 3.74 (with HM). This marks the peak of the exponential growth phase, with the "with HM" condition notably beating the "without HM" condition. By day 8, the growth rate begins to slow, showing the transition to the stationary phase as nutrients or environmental factors become limiting. Thus, the presence of lead (initial concentration = 1.97 mg/L) as HM in PW does not hinder growth; rather, it seems to stimulate a more robust response from *C. sorokiniana*, possibly due to adaptive mechanisms or a stress-related metabolic shift. Similar types of observation was reported by other researcher[204]. The analysis of variance (ANOVA) demonstrates that the mean changes in OD values between the treatment groups (10% PW loadings without HM and with HM) are statistically significant ($p\text{-value} = 0.03817 < 0.05$).

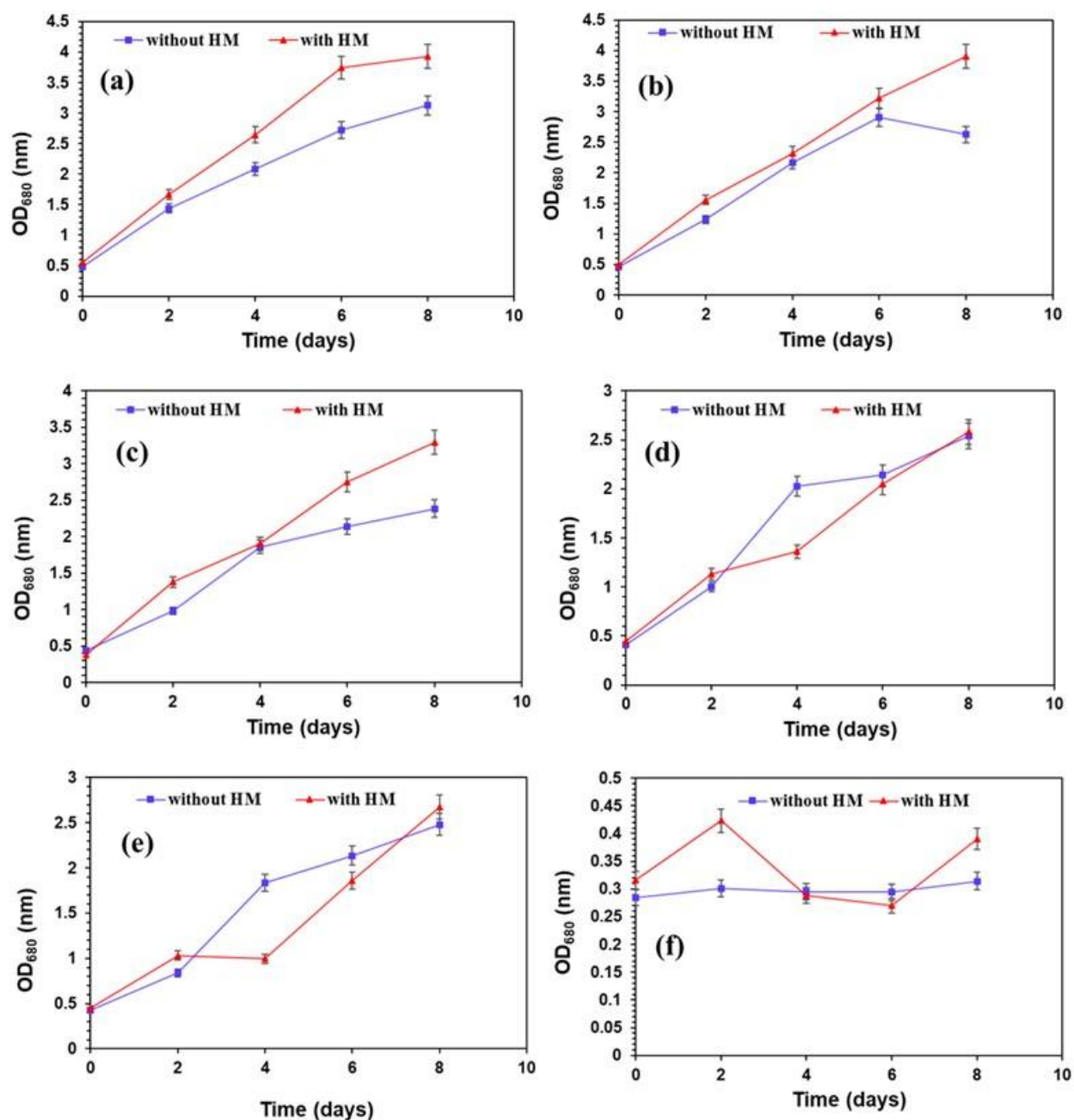


Fig. 8.1 Time profiles of optical densities (OD₆₈₀) as a growth feasibility of *C. sorokiniana* in different PW loadings without HM and with HM. **(a)** 10% PW **(b)** 20% PW **(c)** 30% PW **(d)** 40% PW **(e)** 50% PW **(f)** 100% PW. Data are means $\pm$ SD (n=3).

Considering 20% PW (**Fig. 8.1b**), both conditions show growth, but the "with HM" condition outperforms the "without HM" condition by day 2 due to its ability to adapt

quickly under metal stress. It is seen that the condition with HM demonstrated higher growth compared to the condition without HM throughout the cultivation period, achieving an OD of 3.9 after eight days of cultivation. In contrast, the condition without HM reached a maximum OD of 2.91 on day 6, after which the growth declined. This observation focuses that the existence of HM steadily supports growth advantage for the microalga, likely due to its enhanced adaptive mechanisms under HM stress[205]. This trend underlines its potential for bioremediation, as the microalga establishes remarkable adaptation capabilities in metal-contaminated environments. It is seen that the mean changes in OD values between the treatment groups (20% PW loadings without HM and with HM) are statistically not significant ($p\text{-value} = 0.129107 > 0.05$).

Considering 30% PW (**Fig. 8.1c**), by day 2, the growth trend differs significantly. The OD of the culture with HM (1.38) surpasses that of the culture without HM (0.98), proposing a possible adaptive response to HM stress, potentially due to microbial mechanisms like efflux pumps or metal sequestration[206]. By day 4, both conditions exhibit speedy growth, reaching OD values of 1.86 (without HM) and 1.90 (with HM). The slight advantage of the HM-exposed culture continues, implying enhanced metabolic activity under stress conditions. Between days 6 and 8, microbial growth further accelerates. The OD of the HM culture peaks at 3.3 on day 8, substantially higher than 2.4 in the non-HM culture, establishing a pronounced positive adaptation (not statistically significant, $p\text{-value} > 0.05$). Overall, the trend indicates that the microalgal culture adapts to HM presence and exhibits enhanced growth, potentially due to metal tolerance mechanisms or selective advantages in the stressed environment[206].

Microalgal growth in 40% PW (**Fig. 8.1d**) is induced by both heavy metal (HM) presence and higher salinity, which together initiate a dual-stress environment. By day 2, microbial growth proceeds in both conditions, with OD reaching 1.00 in the control and 1.13 in the HM-exposed culture. While HM stress alone can trigger protective responses, the higher salinity in 40% PW may cause other osmotic stress, leading to gradual adaptation. By day 4, the culture without HM continues growing (OD = 2.025), while the HM culture lags (OD = 1.36). This suggests that high salinity intensifies the toxicity of HM by disrupting cellular ion homeostasis and reducing nutrient availability[206]. By day 6, both cultures show a similar OD (~2.1), representing gradual adaptation, potentially through osmoprotectant accumulation or ion transport mechanisms. By day 8, microalgal growth reached peaks for both cases (OD $\approx$ 2.58), suggesting that microalga have developed resistance strategies to mitigate both HM toxicity and high salinity stress. The findings suggest that while high salinity initially exacerbates HM stress, microalgal adaptation mechanisms allow recovery over time. It is seen that the mean changes in OD values between the treatment groups (40% PW loadings without HM and with HM) are statistically not significant ($p\text{-value} = 0.4811 > 0.05$).

By comparing microalgal growth between 30% PW and 40% PW, it is clear that growth is only slightly affected by salinity alone. The presence of both high salinity (40% PW) and HM leads to a noteworthy drop in OD values on day 8 than that of 30% PW. This proposes that osmotic stress from high salinity elaborates the toxicity of HM, likely due to increased cellular energy demands for ion homeostasis, membrane stability, and detoxification mechanisms. Thus, microalgal adaptation is more efficient in 30% PW under HM stress compared to 40% PW, where extreme osmotic pressure may hinder survival and growth.

According to **Fig. 8.1e**, microalgal growth in 50% SPW is substantially influenced by both high salinity and HM concentrations. By day 2, remarkably, OD is slightly higher in the presence of HM (1.03 vs. 0.84). This suggests possible initial adaptation, where microalgae might be utilizing some available nutrients before toxicity effects dominate. By day 4, growth without HM increases significantly (1.835), while with HM, OD remains nearly constant (0.995). The toxicity of HM is likely inhibiting cellular processes, leading to a slowdown in growth. By day 6, OD without HM reaches 2.135, while OD with HM slightly recovers to 1.86. This suggests partial adaptation of microalgae to metal stress, but growth stays lesser than in the metal-free condition. By day 8, growth without HM reaches its peak at 2.48, while growth with HM reaches 2.68 (not statistically significant, $p\text{-value} > 0.05$). The late-stage increase in OD under HM may be due to stress-induced cellular aggregation or altered metabolic pathways.

As observed in **Fig. 8.1f**, *C. sorokiniana*'s growth in 100% PW is highly hindered owing to excessive salinity, nutrient deficiency, and high HM concentrations. Unlike lower PW gradients (10–50%), where some adaptation occurs, 100% PW lacks necessary nutrients, creating a highly stressful environment. By day 2, a slight increase is monitored, with OD rising to 0.30 (without HM) and 0.42 (with HM). The higher OD with HM might suggest an initial stress response where microalgae attempt to adapt. By day 4, growth stagnates, with OD slightly reducing to 0.29 (without HM) and 0.288 (with HM). This suggests that excessive salinity and nutrient deficiency limit cell division. By day 6, OD stays nearly constant at 0.29 (without HM) and further drops to 0.27 (with HM), proving that microalgae struggle to survive. By day 8, a minor recovery is noticed (OD 0.31 without HM and OD 0.39 with HM), maybe owing to cellular aggregation or stress-induced

metabolic shifts. High salinity disrupts osmotic balance, leading to water loss and cell shrinkage[114]. Additionally, heavy metals (Pb) at high concentrations interfere with photosynthesis and enzymatic activity, further reducing cellular energy production. The minor OD increase on day 8 might indicate the formation of cell clusters as a defense mechanism, where cells produce exopolysaccharides to bind toxic ions and reduce direct exposure. However, this does not translate into substantial biomass accumulation. Literature suggests that optimal microalgal bioremediation requires dilution of PW to reduce salinity and metal toxicity while supplementing essential nutrients to support sustained growth[101]. The inability of *C. sorokiniana* to thrive in 100% PW highlights the need of PW treatment strategies, such as pre-dilution or nutrient alterations, before considering microalgae-based bioremediation.

Table 8.1 provides critical insights into the growth kinetics of *C. sorokiniana* cultivated under different PW loadings, both with and without HM. The key parameters are maximum specific growth rate ($\mu_{\max}$), and maximum biomass productivity ($P_{\max}$). As PW loading increases, salinity and organic load also increase, which influences microalgal growth. As appeared in **Table 8.1**, at lower PW loadings (10-30%), specific growth rate ($\mu_{\max}$) ranged from 0.21 to 0.27 day⁻¹, indicating moderate tolerance to PW up to this level. The highest specific growth rate ($\mu_{\max}$) (0.27 day⁻¹) was recorded at 30% PW with HM, possibly due to stress-induced metabolic adaptations. At 40% and 50% PW, specific growth rate ($\mu_{\max}$) stayed stable (~0.22 day⁻¹) but was pointedly lower than at 10-30% PW. At 100% PW, specific growth rate ($\mu_{\max}$) dropped radically (0.01–0.02 day⁻¹), demonstrating near-total inhibition of cell division due to extreme environmental stress. The specific growth rate

achieved in this study was greater than the specific growth rate found by Ammar et al. (2018) during their work on PW treatment with two marine microalgae species[52].

Also, **Table 8.1** illustrates the biomass productivity of *C. sorokiniana* culturing at different PW loadings with HM and without HM conditions. As observed in **Table 8.1**, in the absence of HM, maximum biomass productivity ($P_{\max}$) peaked at 10% PW (67.63 mg/L/d) and then progressively turn down with raising PW loadings except 40% PW load. However, in the presence of HM, the highest $P_{\max}$ (124.63 mg/L/d) was recorded at 20% PW.

Table 8.1 Growth kinetics parameters of *C. sorokiniana* cultivated in supplemented SPW without HM and with HM

PW loading (%)	Maximal OD ₆₈₀		Max. specific growth rate, μ_m (day ⁻¹)		Cultivation Period (day)	Max. Biomass productivity (mg/L/d)	
	Without HM	With HM	Without HM	With HM		Without HM	With HM
10	3.13	3.93	0.23	0.24	8	67.63	64.5
20	2.62	3.9	0.22	0.26	8	63.38	124.63
30	2.39	3.30	0.21	0.27	8	59.00	47.63
40	2.54	2.58	0.23	0.22	8	64.38	14.75
50	2.48	2.68	0.22	0.22	8	57.38	30.00
100	0.31	0.39	0.01	0.02	8	0.0	0.0

The substantial rise in $P_{\max}$ at 20% PW with HM suggests a possible stimulatory effect of low-level HM exposure, potentially due to the activation of stress response mechanisms. Beyond 30% PW, biomass productivity dropped extensively, especially at 40% and 50% PW with the presence of HM. At 100% PW, biomass productivity was entirely inhibited (0.0 mg/L/d), validating that extreme salinity and metal toxicity inhibit growth. Overall, at higher PW levels (40-100%), HM exacerbated the negative effects of high salinity, significantly reducing biomass productivity. The results indicate that microalgal growth in

PW depends on the balance between salinity, organic load, and HM concentration. At low PW loadings (10-20%), microalgae can thrive due to adequate nutrients and manageable osmotic stress. At moderate levels (30-50%), microalgae experience increasing stress, leading to slower growth and reduced productivity. This study's range of biomass productivity is corroborated by similar findings from other studies[49], [133]. The comparable growth kinetics characteristics acquired in this study for freshwater microalgae *C. sorokiniana* cultured under hypersaline PW demonstrates its halotolerant capabilities. In summary, *C. sorokiniana* demonstrates a outstanding capacity to adapt to varying PW loadings, with optimal growth observed at lower to moderate levels. The presence of HM at certain concentrations may stimulate beneficial stress responses, enhancing growth and productivity. However, beyond a threshold, the damaging effects of high salinity and HM toxicity become predominant, slowing growth and biomass accumulation.

The maximum biomass yields acquired from *C. sorokiniana* culture with varying PW loadings (10% to 50%) under the conditions of without HM and with HM are shown in **Fig. 8.2**.

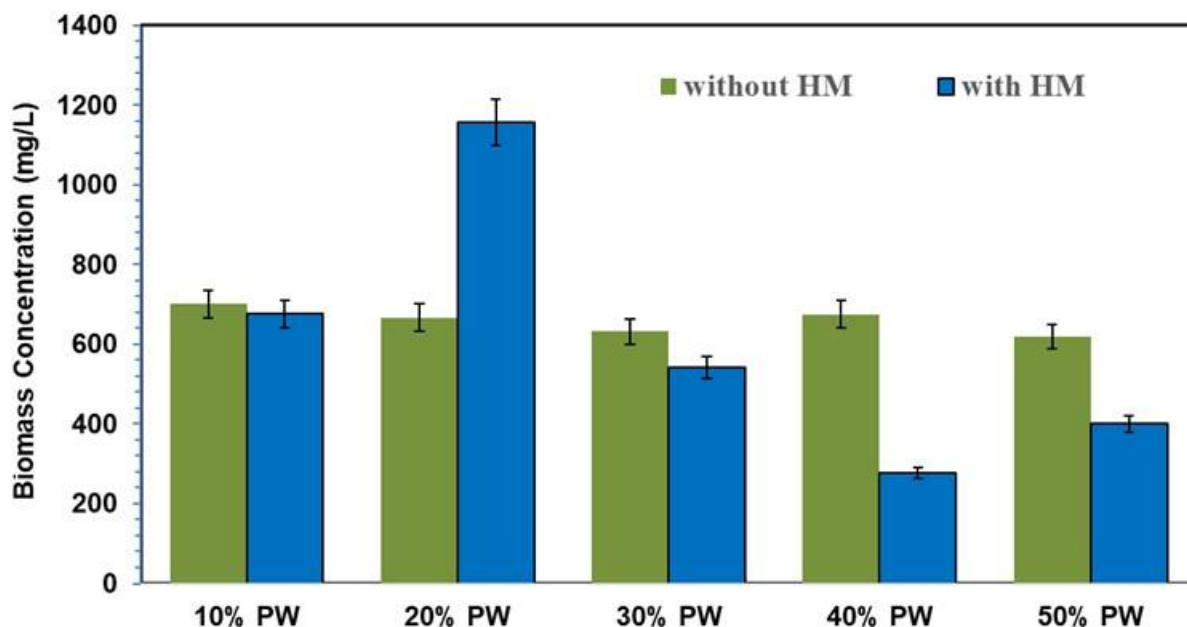


Fig. 8.2 Maximum biomass yields achieved from the cultivation of *C. sorokiniana* under different PW loadings without HM and with HM. Data are means $\pm$ SD (n=3).

The results indicate that maximum biomass production stays relatively stable without HM, ranging from 701 mg/L (10% PW) to 619 mg/L (50% PW). However, in the presence of HM, maximum biomass yield displays high variability, peaking at 1157 mg/L at 20% PW, followed by a sharp drop at higher PW loadings (e.g., 278 mg/L at 40% PW). This trend implies that while *C. sorokiniana* can tolerate moderate levels of PW, extreme concentrations lead to growth inhibition due to combined HM toxicity and increasing salinity. Studies have shown that microalgae can develop stress-adaptive mechanisms, such as increased antioxidant activity and ion regulation, when exposed to moderate salinity and HM[206]. However, beyond a critical threshold, high salinity can lead to osmotic stress, spoiling photosynthesis and nutrient uptake[207]. The severe decrease in biomass at higher PW loadings implies that extreme salinity, combined with HM toxicity, initiates an hostile growth environment. Optimizing PW dilution ratios is necessary to balance nutrient

accessibility while reducing salinity and metal-induced stress for sustainable microalgal cultivation.

8.3 Bioremediation of PW- Dissolved organic pollutants removal

TOC was evaluated as an aggregate parameter to quantify the elimination of dissolved organic pollutants (DOPs), with phenol and acetate serving as characteristic components of the pollutant load. **Fig. 8.3 (a-f)** demonstrates the phenol and TOC content in different PW loadings on day 0, 2, 4, 6 and 8 with the conditions of without HM and with HM. As observed in **Fig. 8.3a** for 10% PW loadings, TOC and phenol degradation were followed over eight days. Without HM, TOC reduced notably from 40.6 mg/L (Day 0) to 10.63 mg/L (Day 4), point out successful microalgal degradation. However, a slight surge in TOC after Day 4 suggests potential organic matter release from algal cells. With HM, TOC elimination was slower (40.39 mg/L to 15.34 mg/L by Day 4) and plateaued thereafter, indicating inhibition by HM, which likely interfered with enzymatic breakdown of organic pollutants. Phenol degradation resulted a distinct pattern. Without HM, phenol exhibited a slow initial degradation phase (8.5 mg/L to 8.059 mg/L by Day 2) before rapid reduction (~1.07 mg/L on Day 4). With HM, initial removal was faster, dropping from 10.08 mg/L to 1.98 mg/L by Day 2, likely due to early adsorption and metabolic activation. However, its slight increase after Day 4 suggests possible desorption or transformation into intermediate organic compounds.

Comparing TOC elimination with microalgae growth trends (**Fig. 8.1a**), optical density (OD) increased steadily, correlating with organic matter utilization. Without HM, OD boosted from 0.488 (Day 0) to 3.125 (Day 8), indicating robust growth. With HM, OD was

initially higher (0.556 at Day 0), reaching 3.93 at Day 8, suggesting some adaptation but potential stress responses. The slower TOC removal with HM despite higher OD suggests that HM might have led to metabolic shifts in algal utilization of organic carbon. This aligns with previous studies, which highlight that HM can hinder microalgal degradation efficiency but simultaneously trigger adaptive mechanisms in microalgae[208]. It is noted that the mean changes in TOC removal quantity between the treatment groups (10% PW loadings without HM and with HM) are statistically not significant ($p\text{-value} = 0.06707 > 0.05$).

According to **Fig. 8.3b** for 20% PW loadings, the bar chart illustrates the efficiency of *C. sorokiniana* in removing dissolved organic pollutants (DOPs) in 20% PW loading, with and without heavy metals (HM). A fast decline was seen by day 2, particularly in the presence of HM, suggesting that adsorption and metal-organic interactions contributed to pollutant removal [209]. By day 4, phenol was almost fully degraded in the absence of HM (0.72 mg/L) but slightly persisted in the HM condition (1.26 mg/L), indicating that HM inhibited microalgal enzymatic activity[210]. TOC removal sustained, reaching 8.83 mg/L (without HM) and 8.75 mg/L (with HM), establishing efficient organic matter utilization by microalgae. By day 6, TOC values slightly boosted in the HM condition (10.46 mg/L), likely due to intracellular organic matter release from algal cells under stress[208]. On Day 8, TOC further increased (9.06 mg/L without HM, 13.61 mg/L with HM), possibly due to microalgal decay and extracellular organic accumulation[162].

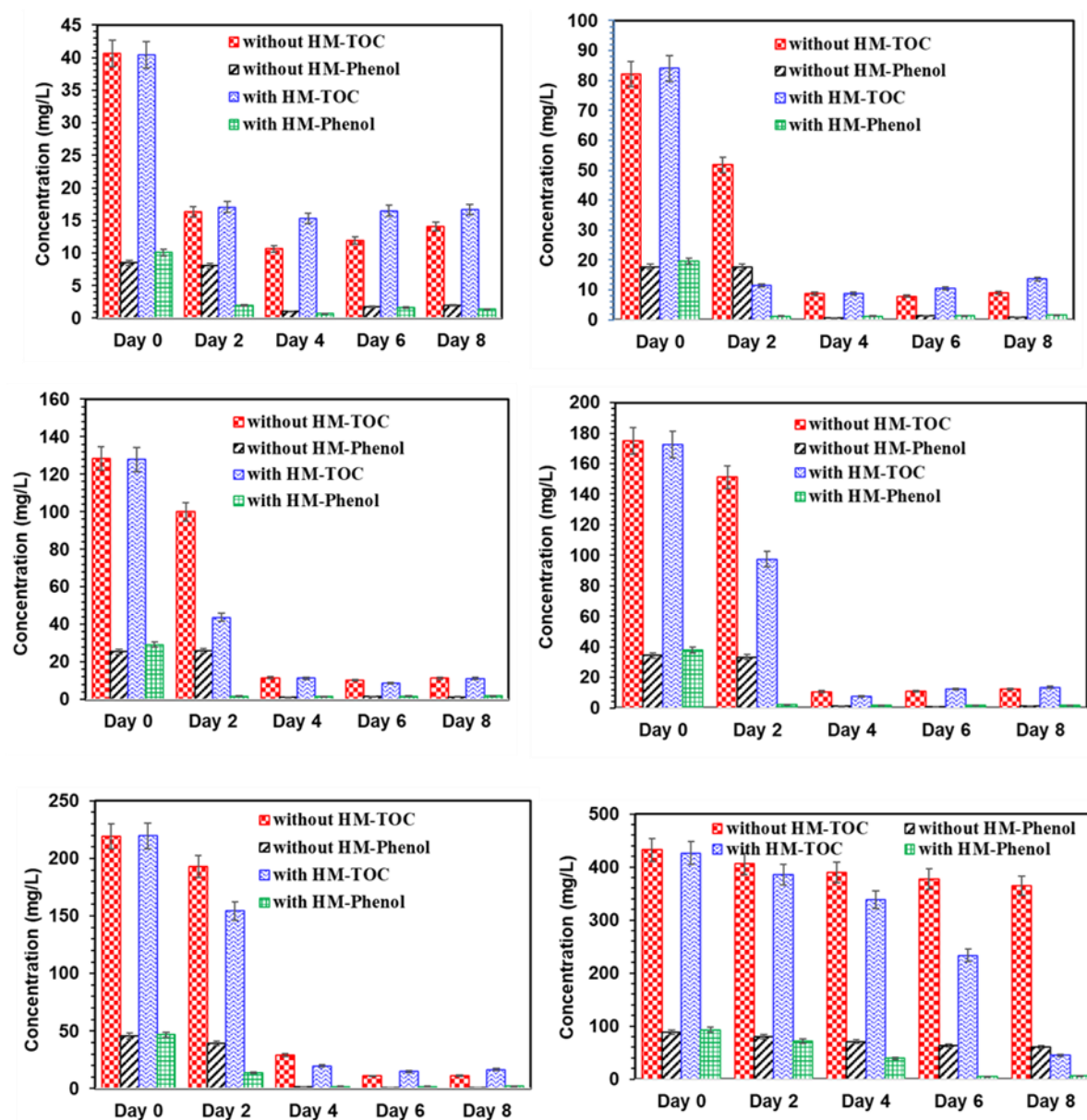


Fig. 8.3 TOC and phenol content in different PW loadings on day 0, 2, 4, 6 and 8 with the conditions of without HM and with HM (a) 10% PW (b) 20% PW (c) 30% PW (d) 40% PW (e) 50% PW (f) 100% PW. Data are means $\pm$ SD (n=3).

The experiment emphasizes that *C. sorokiniana* effectively degraded organic pollutants, with HM playing a dual role—partially hindering enzymatic degradation while promoting adsorption effects. By comparing the results with the microalgal growth curve obtained in 20% PW (**Fig. 8.1b**), it is seen that the growth curve of *C. sorokiniana* is directly linked to pollutant removal efficiency. The exponential growth phase correlates with maximum TOC and phenol removal, while later stages (day 6-8) show minor TOC fluctuations due to possible organic release from algal cells. It is also noted that the mean changes in TOC removal quantity between the treatment groups (20% PW loadings without HM and with HM) are statistically not significant ($p\text{-value} = 0.50307 > 0.05$).

As appeared in **Fig. 8.3c**, the study of 30% PW loading depicted higher initial organic loads than 20% PW, with TOC at 128.4 mg/L (without HM) and 127.8 mg/L (with HM), and phenol concentrations at 25.36 mg/L and 29.19 mg/L, respectively. By day 2, the optical density (OD) in 30% PW (**Fig. 8.1c**) remained low, however, TOC levels (**Fig. 8.3c**) declined considerably, particularly in the presence of HM, with TOC reducing to 43.6 mg/L from 127.8 mg/L. This sharp drop in TOC, despite low algal growth, suggests that mechanisms other than biological metabolism were liable for initial organic carbon removal. Microalgae have cell walls that are abundant in functional groups, including carboxyl, hydroxyl, and amino groups, enabling the binding of metal ions via biosorption. Metal ions can form complexes with dissolved organic matter, promoting their aggregation and precipitation from the solution. Furthermore, extracellular polymeric substances (EPS) produced by microalgae can augment this process by offering additional binding sites for metal-organic complexes. Such interactions may result in a rapid decrease in TOC levels, irrespective of biological metabolism[211]. By Day 2, phenol concentration remained

unchanged in the absence of HM (25.36 mg/L), whereas in the HM condition, phenol sharply degraded from 29.18 mg/L to 1.76 mg/L. This rapid decline in phenol, despite low algal growth (OD), indicates that abiotic (non-biological) mechanisms played a dominant role in phenol removal, rather than microbial degradation. The sharp drop in phenol in the HM condition by day 2 occurred independently of microalgal growth, primarily due to metal-catalyzed oxidation, adsorption onto metal hydroxides, and complex formation[211]. These mechanisms worked synergistically to eliminate phenol before *C. sorokiniana* could actively degrade it through enzymatic pathways. In contrast, without HM, phenol stayed unchanged due to the absence of these metal-driven processes, and algal enzymatic degradation had not yet started. By day 4, the phenol was almost entirely degraded in the absence of HM (1.21 mg/L) and slightly higher in HM conditions (1.40 mg/L), confirming that enzymatic activity was slightly impacted by HM. TOC values reached 11.52 mg/L (without HM) and 11.24 mg/L (with HM), implying substantial pollutant elimination. Between Days 6 and 8, TOC somewhat raised (from 10.14 to 11.28 mg/L without HM and from 8.56 to 11.04 mg/L with HM), likely due to intracellular organic matter release from algal cells under stress[162]. Despite the elevated initial organic load in 30% PW, the degradation trend was similar to 20% PW, with HM acting a dual role of inhibiting enzymatic degradation while promoting adsorption effects.

As observed in **Fig. 8.3d**, the TOC values in the absence of HM began at 175.1 mg/L on Day 0, with a gradual drop to 151.1 mg/L by day 2. A sharp reduction was followed by day 4 (10.63 mg/L), with minimal variations thereafter (11.02 mg/L at day 6 and 12.3 mg/L at day 8). The severe drop of TOC from day 2 to day 4 implies microalgal metabolism of organic pollutants. However, the superior TOC elimination despite low algal growth (OD)

at 40% PW loading (**Fig. 8.1d**) is likely due to privileged carbon metabolism for energy rather than cell division, osmotic stress from high pollutant concentrations, nutrient imbalances limiting biomass synthesis, and extracellular enzymatic activity accelerating TOC degradation[101]. These findings suggest that while *C. sorokiniana* is efficient in TOC removal, nutrient supplementation strategies or osmotic pressure modifications could be explored to increase biomass growth under high PW loadings in future PW treatment applications. Under HM stress, TOC primarily dropped from 172.4 mg/L (Day 0) to 97.48 mg/L (Day 2), but then demonstrated a more gradual drop, reaching 7.62 mg/L by Day 4. Interestingly, TOC grew slightly to 12.34 mg/L (Day 6) and 13.39 mg/L (Day 8), which suggests a probable release of intracellular organic compounds due to metal-induced stress or algal cell lysis. The mean changes in TOC removal quantity between the treatment groups (40% PW loadings without HM and with HM) are statistically not significant ($p\text{-value} = 0.3432 > 0.05$). The phenol elimination trends at 40% PW loading disclose a substantial change between HM-free (without HM) and HM-containing (with HM) conditions. In the absence of HM, phenol concentration remained almost unchanged from 34.4 mg/L on day 0 to 33.28 mg/L on day 2, followed by a quick drop to 1.04 mg/L by day 4, and become constant near 1.29 mg/L by day 8. This stagnation on day 2 proposes a lag phase in enzyme activation, as *C. sorokiniana* requires time to upregulate phenol hydroxylase and catechol pathway enzymes, which are essential for aromatic compound degradation[190]. Additionally, preference for simpler organic carbon sources from PW may have delayed phenol utilization, as microalgae often prioritize easily metabolizable compounds like acetate before breaking down complex aromatic pollutants[212]. Moreover, limited adsorption capacity in HM-free conditions might have further

contributed to the delayed decline in phenol concentration. In contrast, under HM stress, phenol levels declined significantly from 37.89 mg/L at day 0 to 2.05 mg/L by day 2, after which elimination reduced, fluctuating around 1.49–1.53 mg/L until day 8. This quick initial reduction is likely owing to metal-induced changes in cell surface charge, enhancing phenol adsorption onto algal biomass[211]. Furthermore, HM toxicity may have triggered oxidative stress responses, accelerating early detoxification pathways that facilitated faster phenol breakdown compared to the HM-free condition[206]. Additionally, nutrient uptake limitations under HM stress might have forced *C. sorokiniana* to metabolize phenol earlier as an alternative carbon source, leading to its rapid decline. However, after Day 2, enzyme inhibition by HM likely reduced further degradation. These findings suggest that HM impacts both adsorption and metabolic adaptation, substantially altering phenol removal dynamics in microalgae-based PW treatment systems.

Considering 50% PW loadings (**Fig. 8.3e**), it is seen that by day 2, TOC was reduced extensively in both conditions, but the drop is more pronounced in the presence of HM (154.2 mg/L) compared to the absence of HM (193.0 mg/L). This implies that in the initial phase, HM might have induced pollutant degradation either by direct chemical interactions (e.g., precipitation or complexation of organics) or by affecting microbial activity in a way that initially enhances TOC removal[211]. However, as the experiment progresses, HM negatively impacts microalgal metabolism, leading to slower TOC degradation in later days. Similarly, phenol concentration drops sharply in the HM-free condition (~39.4 mg/L) but experiences a more dramatic decrease in the presence of HM (~13.5 mg/L), possibly due to metal-phenol interactions affecting solubility or initial adsorption effects[211]. By day 4, a sharp drop in TOC is observed in both conditions, with levels reducing to 29 mg/L

(without HM) and 19.56 mg/L (with HM), implying improved microalgal activity and pollutant metabolism. It is seen that by day 4, the growth of *C. sorokiniana* in the with HM condition is notably lower than in the without HM condition, as indicated by the optical density (OD) values (0.995 with HM vs. 1.835 without HM) (**Fig. 8.1e**). Despite this, TOC reduction is greater in the presence of HM (19.56 mg/L with HM vs. 29.0 mg/L without HM). This visible contradiction proposes that the mechanisms driving TOC elimination are not solely dependent on microalgal metabolism but also engage abiotic processes induced by HM. The abiotic interactions (precipitation, adsorption, and oxidation reactions) portray a substantial role in organic pollutant removal in the presence of HM. However, as microalgal growth is needed for entire biodegradation, the long-term effectiveness of pollutant removal is likely to be higher in the without HM condition, where biological mechanisms remain fully active. Phenol also indicates a meaningful reduction, reaching ~1.46 mg/L (without HM) and ~1.8 mg/L (with HM), indicating effective biodegradation, though slightly inhibited by HM. On Day 6, TOC keep on to drop but at a slower rate, reaching 10.87 mg/L (without HM) and 14.74 mg/L (with HM), indicating that most readily biodegradable organic matter has been processed. Phenol levels remain low, with 0.87 mg/L (without HM) and 1.79 mg/L (with HM), viewing near-complete removal. By Day 8, TOC stabilizes around 11.32 mg/L (without HM) and 16.43 mg/L (with HM), recommending minimal further degradation due to either nutrient limitation or the presence of more recalcitrant organic matter. It is noteworthy that the mean changes in TOC removal quantity between the treatment groups (50% PW loadings without HM and with HM) are statistically not significant ($p\text{-value} = 0.39241 > 0.05$). Meanwhile, phenol concentrations are sustained at ~1 mg/L (without HM) and ~2.15 mg/L (with HM), reinforcing that HM

impede entire removal. The monitored results align with literature findings, but HM toxicity can inhibit algal metabolism, reducing the degradation process[213].

As observed in **Fig. 8.3f** for 100% PW loadings, in the absence of HM, TOC reduced steadily from 432.5 mg/L to 364.7 mg/L over eight days, while phenol fell from 88.2 mg/L to 60.52 mg/L. This slow removal rate can be ascribed to the lack of necessary nutrients such as nitrogen and phosphorus in 100% PW, which are crucial for sustaining microalgal metabolism and biomass growth[100]. Nutrient limitations likely constrained the metabolic capacity of *C. sorokiniana*, reducing its ability to biodegrade organic pollutants effectively. In contrast, when HM was present, TOC levels accompanied a much sharper drop from 426.2 mg/L to 44.7 mg/L, and phenol concentration fell radically from 93.16 mg/L to just 6.12 mg/L by day eight. This noteworthy advancement in pollutant elimination suggests that HM may have played a catalytic or synergistic role in increasing organic degradation. One possible explanation is the creation of reactive oxygen species (ROS) in the presence of elevated concentration of HM, which can facilitate the oxidation and breakdown of organic pollutants[206].

However, despite the evident advantage of HM in promoting pollutant elimination, their presence could also pose toxicity risks to *C. sorokiniana*. High concentrations of HM are known to hinder microalgal growth by disturbing cellular homeostasis, damaging photosynthetic machinery, and weakening metabolic activity[214]. This could explain why the elimination of TOC and phenol was quick despite the poor algal growth in 100% PW, as the key degradation mechanisms may have shifted from biotic (microalgal metabolism) to abiotic (oxidation via ROS or adsorption onto HM surfaces). Furthermore, the role of

biosorption—where organic pollutants bind onto the algal cell surface—cannot be ruled out, specifically in high-metal environments where exact metabolic degradation is limited.

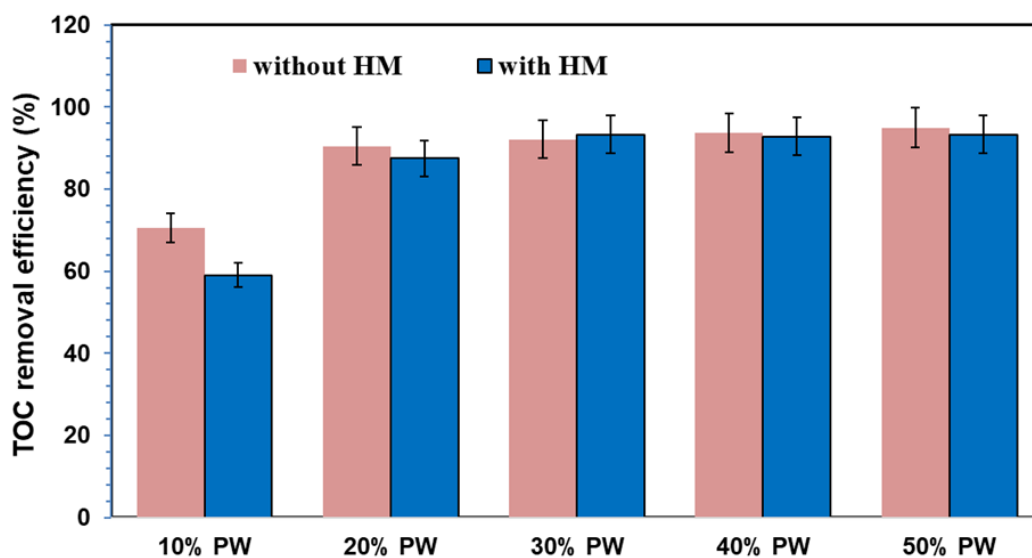


Fig. 8.4 Maximum TOC removal efficiency on day 6 under different PW loadings using *C. sorokiniana* under the conditions of without HM and with HM. Data are means $\pm$ SD (n=3).

Fig. 8.4 illustrates the analysis of TOC removal efficiency under different PW loadings using *C. sorokiniana* under the conditions of without HM and with HM. TOC elimination efficiency can be connected strongly with the biomass concentration attained for different PW loadings shown in **Fig. 8.2**. The highest TOC elimination was detected at 50% PW loading, with efficiencies reaching 95% in the absence of HM and 93.3% in their presence. Across all PW loadings, TOC elimination efficiency was lower in the presence of HM, particularly at 10% PW, where the elimination declined from 70.62% (without HM) to 59% (with HM). Biomass concentration trends show that under HM-free conditions, biomass remains reasonably constant across different PW loadings, with a slight drop at 50% PW. However, in the presence of HM, biomass production fluctuates notably, peaking at 20% PW (1157 mg/L) and dropping clearly at higher loadings (e.g., 40% PW: 278 mg/L). This proposes that while *C. sorokiniana*

thrives at moderate organic pollutant concentrations, extreme PW loading related with HM presence leads to biomass inhibition, possibly owing to enhanced toxicity or nutrient imbalances.

The monitored link between biomass concentration and TOC elimination indicates that *C. sorokiniana* degrades DOPs primarily through biomass growth and metabolic activity. Literature supports that microalgae consume organic pollutants as a carbon source, leading to augmented biodegradation through enzymatic processes and bioadsorption[215]. However, HM can damage microalgal metabolism by hindering photosynthesis, enzymatic activity, and overall biomass production, ultimately reducing pollutant degradation efficiency[214]. The sharp drop in biomass at 40% and 50% PW under HM conditions further upholds the hypothesis that extreme metal toxicity limits microalgal growth. Despite this, *C. sorokiniana* exhibits a notable degree of resilience at higher PW loadings, likely owing to adaptive detoxification mechanisms. These findings highlight the potential of *C. sorokiniana* for bioremediation applications in wastewater treatment but also highlight the requirement to optimize PW and HM concentrations to assert high pollutant elimination efficiency while preventing biomass inhibition.

8.4 Heavy metal (Pb) removal -abiotic and biotic mechanism

Since DOPs were successfully eliminated from the culture medium, Pb removal was further analyzed to verify its fate in the system. As appeared in **Fig. 8.5a**, the Pb removal profile showed that most Pb^{2+} was precipitated within the first 48 hours of cultivation, corresponding with an increase in pH (**Fig. 8.5b**) owing to photosynthetic CO_2 uptake. The pH progressively increased from ~5.6–6.9 on Day 0 to 7.0–7.5 by Day 2, stabilizing

thereafter. The elementary mechanism of Pb removal was chemical precipitation as Pb(OH)_2 and PbCO_3 at elevated pH, consistent with literature indicating Pb precipitation above pH 6.5[216]. Moreover, adsorption onto algal cell walls can be a adding factor to Pb removal, particularly in lower PW concentrations (10–30%), where the presence of carboxyl, hydroxyl, and amine functional groups enabled metal binding. In contrast, higher PW loadings (50% and 100%) demonstrated delayed Pb removal, likely owing to organic ligands forming soluble Pb complexes, slowing precipitation. The steadying of pH beyond day 2 proposed equilibrium between photosynthetic CO_2 uptake, Pb hydroxide precipitation, and bicarbonate buffering. The interplay between Pb stress and algal metabolism also impacted TOC removal, nitrogen removal, and lipid accumulation, necessitating further analysis of lipid composition and Pb speciation. These findings highlight the potential of *C. sorokiniana* for Pb bioremediation, where pH modulation plays a critical role in HM precipitation and organic pollutant degradation, with implications for optimizing microalgal treatment of PW.

While Pb elimination was primarily abiotic due to pH-induced precipitation (observed within the first two days), EDX analysis (**Fig. 8.6**) established the presence of Pb in dry *C. sorokiniana* biomass, demonstrating partial bioaccumulation. The EDX spectra uncovered detectable Pb peaks in algal biomass, supporting the hypothesis that a fraction of Pb was biologically retained. This affiliates with aforementioned studies where microalgae have been reported to uptake HM via passive adsorption and intracellular accumulation[217]. However, given that the majority of Pb was eliminated within the first two days, abiotic precipitation stays the leading mechanism, with minor impacts from biotic uptake. The

proposed mechanism for lead (Pb) removal from petroleum-derived produced water (PW) is illustrated in **Fig. 8.7**.

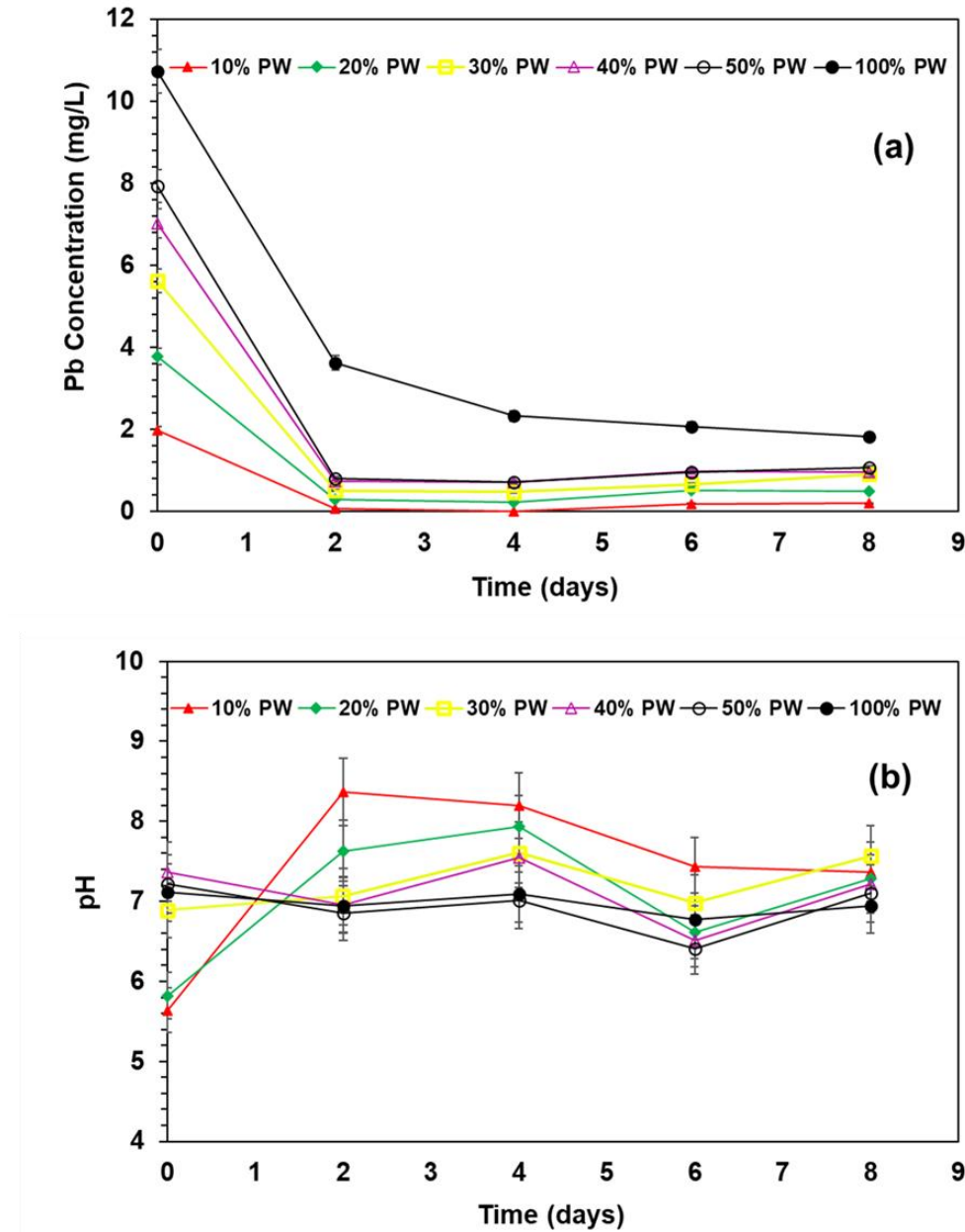


Fig. 8.5 (a) Time profiles of lead (Pb) removal in the form of both abiotic and biotic mechanism in different PW loadings **(b)** pH profiles over time during the cultured of *C. sorokiniana* under different gradient of PW loadings. Data are means $\pm$ SD (n=3).

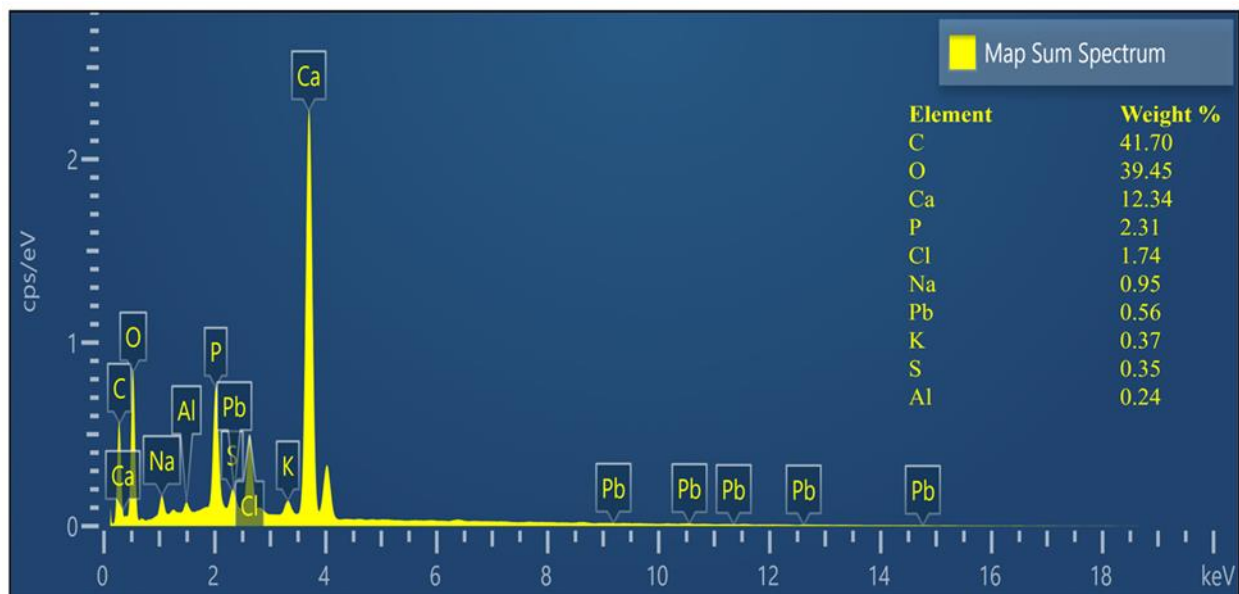


Fig. 8.6 EDX analysis of *C. sorokiniana* biomass obtained in 30% PW loadings cultured with the condition of heavy metal (Pb)

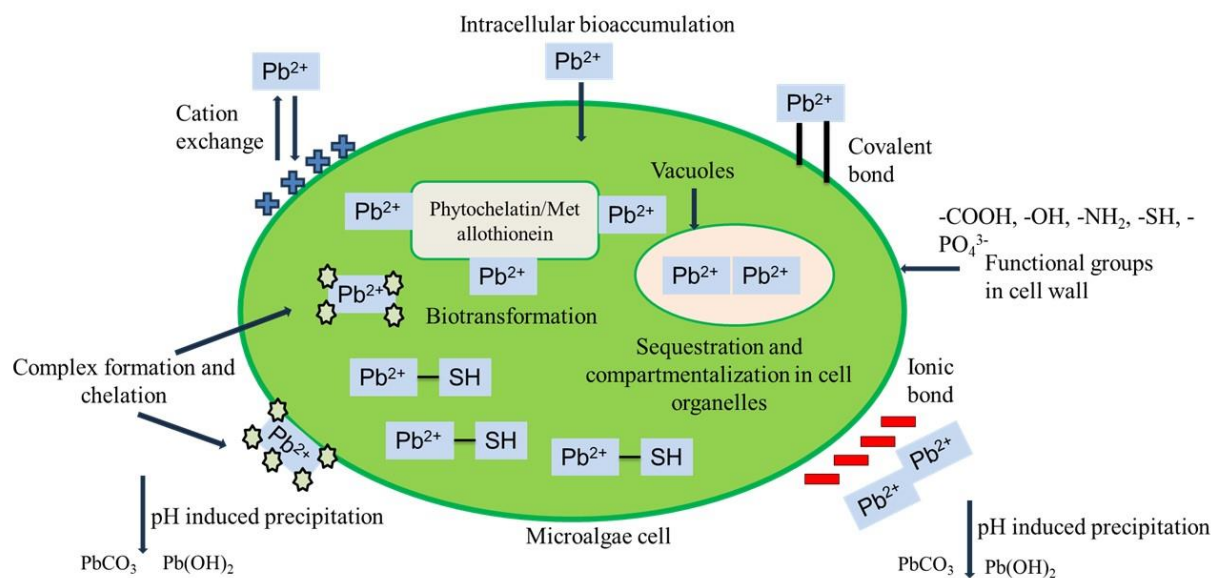


Fig. 8.7 Proposed lead (Pb) removal is a combination of pH induced-precipitation, biosorption (passive), bioaccumulation (active) and biotransformation (Modified after Faruque et al. (2024))[217].

8.5 Nitrogen removal

The nitrogen elimination efficiency of *C. sorokiniana* cultivated in different PW loadings supplemented with SMW under the conditions with and without HM exhibits a clear trend of reducing nitrogen elimination as PW concentration increases (**Fig. 8.8**). At 10% PW, nitrogen elimination efficiency is highest, with 96.8% in the presence of HM and 92.92% without HM, suggesting that lower PW concentrations allow the microalgae to efficiently assimilate nitrogen. However, as PW loading rises, nitrogen elimination efficiency decreases notably, with only 39.42% elimination at 50% PW in the presence of HM and 77% without HM. The presence of HM has a dual effect: at lower PW loadings, it looks to slightly enhance nitrogen elimination, likely owing to its role in enzyme activation. However, at higher PW

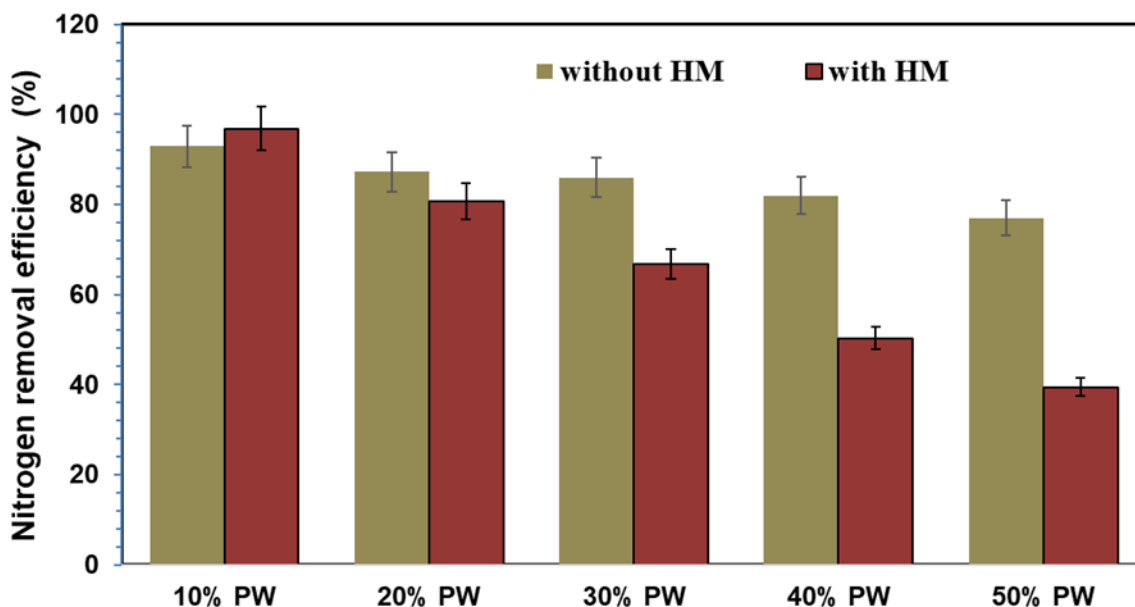


Fig. 8.8 Nitrogen removal efficiency at the end of cultivation in different PW loadings using *C. sorokiniana* under the conditions of without HM and with HM. Data are means $\pm$ SD (n=3).

concentrations (30% and above), nitrogen elimination efficiency declines more significantly in the presence of HM, proposing that HM exert a toxic effect that hinders the metabolic activity of *C. sorokiniana*. This associates with literature findings that HM disrupt enzymatic pathways and impair nitrogen metabolism, especially under stressful environmental conditions[213]. Additionally, higher PW concentrations initiate higher salinity, increased organic carbon load, all of which negatively impact algal growth and nitrogen assimilation. Since microalgae predominantly eliminate nitrogen through assimilation into biomass and denitrification, these inhibitory factors reduce overall efficiency. While some metals at lower concentrations may accelerate enzymatic activity[218], their accumulation at higher levels is harmful. The bar chart **(Fig. 8.8)** visually confirms these trends, indicating the sharp decrease in nitrogen elimination as PW concentration rises, specifically in the presence of HM.

Establishing a link between biomass concentration attained in different PW loadings **(Fig. 8.2)** and nitrogen elimination efficiency **(Fig. 8.8)**, it is seen that the nitrogen elimination efficiency was highest at 10% PW, where the biomass concentration was 701 mg/L without HM and 676 mg/L with HM, indicating that microalgal growth was optimal at this loading, supporting higher nitrogen assimilation. However, at 20% PW, an uncommon trend was detected—biomass concentration substantially increased to 1157 mg/L with HM, despite a drop in nitrogen elimination efficiency (80.7%). This implies that, at this concentration, *C. sorokiniana* may have experienced adaptive stress response mechanisms, leading to augmented biomass production while nitrogen uptake efficiency declined owing to potential metabolic shifts toward organic carbon utilization instead of nitrogen assimilation. At 30% PW and above, biomass

concentration and nitrogen elimination efficiency both reduced more suddenly under HM conditions, with biomass dropping to 541 mg/L at 30% PW, 278 mg/L at 40% PW, and 400 mg/L at 50% PW, compared to more stable biomass levels without HM. This supports with literature implying that HM at higher concentrations disrupt microalgal metabolic pathways, inhibit photosynthetic activity, and reduce nitrogen assimilation efficiency[219]. The decrease in both nitrogen elimination and biomass at higher PW concentrations indicates that organic pollutant toxicity, collective with HM stress, substantially weakened algal growth and nutrient uptake efficiency. The comparatively stable biomass values without HM, even at 50% PW, indicate that nitrogen elimination in these conditions was primarily governed by microalgal growth dynamics rather than toxicity-induced inhibition. These results focus the complex interplay between nitrogen elimination, biomass production, and environmental stress factors. While low PW loadings support efficient nitrogen assimilation and robust biomass growth, increasing PW concentrations lead to metabolic stress, lowering both nitrogen removal and biomass yield, especially in the presence of HM.

8.6 Total lipid content in biomass

The lipid content in *C. sorokiniana* biomass was examined under different PW loadings, with and without HM exposure (**Fig. 8.9**). The results imply that lipid accumulation varied substantially depending on the concentration of PW and the presence of HM. Without HM, lipid content intensified gradually from 10.51% at 10% PW to 18.00% at 50% PW, except for a noteworthy dip at 30% PW (7.95%), possibly owing to osmotic stress or toxicity effects limiting growth. In contrast, under HM exposure, lipid content fluctuated, with a substantial fall at 20% PW (4.42%), followed

by a rise at 30% PW (11.72%) and a peak at 40% PW (23.97%), before somewhat declining at 50% PW (15.32%).

The lipid content in biomass is directly correlated with the biomass concentration attained (**Fig. 8.2**) as well as nitrogen elimination efficiency by microalgae (**Fig. 8.8**). It is clear that a negative relationship was detected between biomass concentration and lipid content, meaning that as biomass growth reduced, lipid accumulation improved. This pattern is strongly induced by nutrient accessibility, particularly nitrogen, which plays a crucial role in both biomass productivity and lipid biosynthesis. Without HM exposure, biomass concentration was reasonably high at lower PW loadings (701 mg/L at 10% PW) but steadily dropped (619 mg/L at 50% PW). In contrast, lipid content boosted with PW concentration, reaching a peak of 18.00% at 50% PW, underlining the trade-off between growth and lipid accumulation[94]. Under HM stress, biomass concentration varied notably, with the highest documented at 20% PW (1157 mg/L) and the lowest at 40% PW (278 mg/L). The lipid content, however, was highest at 40% PW (23.97%), further verifying that stress conditions reduce growth but promote lipid biosynthesis. This occurrence is largely driven by nitrogen reduction, which is well-recognized to induce lipid accumulation in microalgae[133]. Nitrogen is crucial for protein synthesis, chlorophyll production, and overall biomass enhancement. When nitrogen becomes limited, microalgae alter their metabolic

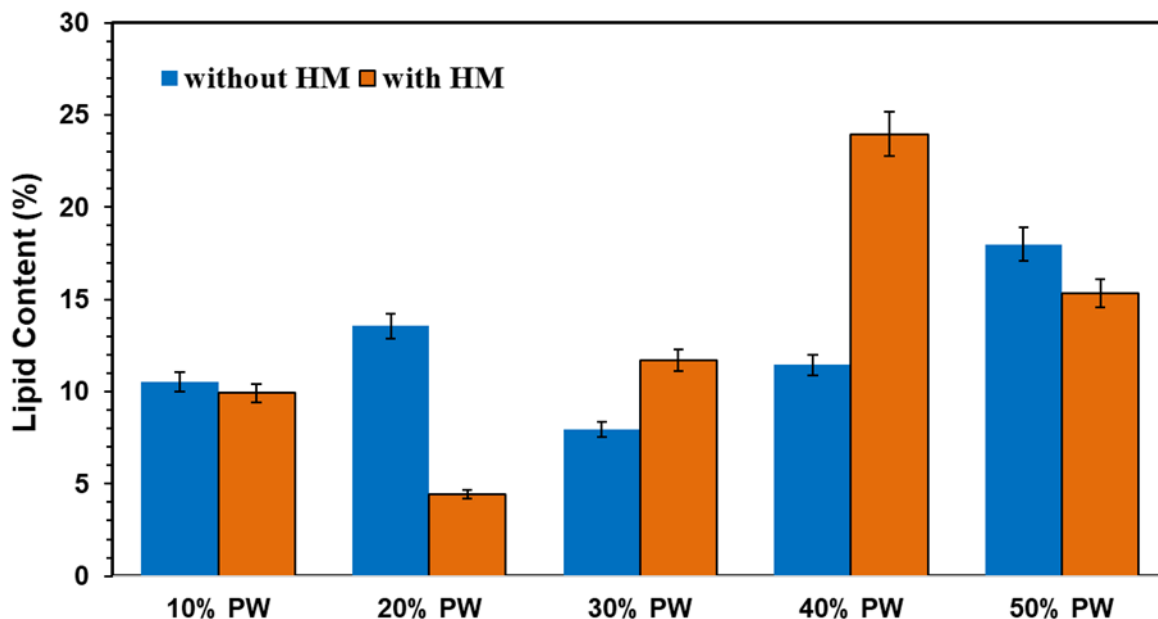


Fig. 8.9 Lipid content in *C. sorokiniana* biomass cultivated in different PW loadings under the conditions of without HM and with HM. Data are means $\pm$ SD (n=3).

pathways toward lipid production as an energy storage mechanism. This explains why lipid content was highest when biomass concentration was lowest. Additionally, the presence of HM can cause oxidative stress, further triggering lipid biosynthesis as a protective response[220].

The results recommend that PW or supplemented PW can assist as a nutrient source for microalgal cultivation, but optimal PW concentration must be affirmed to balance biomass productivity and lipid accumulation. Moreover, controlled stress conditions, such as moderate nitrogen limitation and HM exposure, can be asserted to enhance lipid yields, achieving this approach highly appropriate for biofuel applications. The findings point out the potential of *C. sorokiniana* for sustainable bioenergy production, remarkably in PW treatment systems where nutrient recycling and lipid biosynthesis can be co-optimized.

8.7 Conclusions

This study revealed the capability of *C. sorokiniana* for the bioremediation of DOPs in SPW under different salinity and HM stress conditions. The microalga demonstrated significant adaptability to varying PW concentration gradients, efficiently eliminating TOC, phenol, and nitrates while collecting lipids, emphasizing its dual utility in PW treatment and bioenergy production.

The findings indicated that *C. sorokiniana* survived best under low to moderate PW loadings (10–30%), with maximum biomass productivity and effective pollutant decomposition. Notably, at 20% PW with HM stress, the microalga demonstrated increased biomass accumulation, indicating a potential stress-induced metabolic response that facilitated higher pollutant removal. At elevated PW concentrations (40–50%), excessive salinity and HM toxicity resulted in metabolic inhibition and decreased biomass formation, but the efficiency of DOPs elimination remained comparatively high. The maximum TOC removal efficiency (95% without HM and 93.3% with HM) was recorded at 50% PW, indicating that microalgal metabolism, along with abiotic processes including adsorption and precipitation, significantly contributed to pollutant removal. Furthermore, the removal of HM was predominantly facilitated by pH-induced precipitation during the initial 48 hours of cultivation, while biosorption onto algal biomass played a role in the total sequestration of Pb^{2+} . Although pollutant degradation was successful at various produced water (PW) loadings, microalgal development was significantly hindered in 100% PW due to excessive salinity and nutrient scarcity, highlighting the necessity for dilution or nutrient enhancement to maintain efficient bioremediation.

These findings demonstrate *C. sorokiniana's* potential as a sustainable and economical alternative for PW treatment, integrating wastewater bioremediation with biomass valorization for circular bioeconomy applications. Future study must concentrate on optimizing operating parameters, scaling bioreactor designs, and investigating genetic or biochemical modifications to boost microalgal resilience and pollutant degrading efficacy in real PW systems.

CHAPTER 9

Cadmium(II) removal from aqueous solution using dry biomass

9.1. Introduction

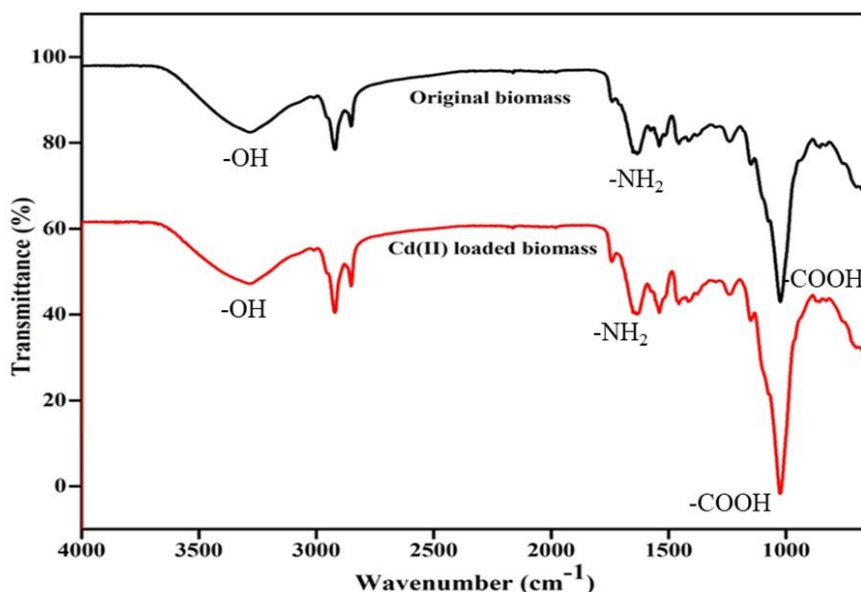
This chapter explores the potential of *C. sorokiniana* dry biomass as an effective bioadsorbent for the removal of Cd(II) ions from aqueous solutions. In this context, the specific objectives of this chapter are to (i) examine the effectiveness of cadmium removal by *C. sorokiniana* dry biomass under different environmental conditions (ii) determine the kinetics of cadmium biosorption (iii) analyze the equilibrium adsorption isotherms that describe the cadmium biosorption process (iv) characterize (FTIR, SEM-EDX, XRD) the *C. sorokiniana* dry biomass before and after Cd(II) biosorption to ensure a comprehensive understanding of the biosorption process. Moreover, by gaining insights into the mechanisms governing cadmium biosorption by *C. sorokiniana*, this study seeks to contribute to the development of sustainable, microalgae-based technologies for treating cadmium-contaminated wastewater.

9.2 Characterization of the algal biosorbent

9.2.1 FTIR analysis

The FTIR approach is employed to investigate the surface properties of the *C. sorokiniana* biomass as well as the chemical alteration that occurs following Cd(II) biosorption by microalgae biomass [221]. The technique of FTIR spectroscopy is useful in understanding the interactions of functional groups with metal ions. In other words, when this method is

employed to algal biosorbents and metal-loaded biosorbents, differences in the absorption of separate bands reveal the functional groups involved in the metal biosorption method or the formation of new bands for modified algae [222]. These functional groups are originated from macromolecules found in the algal cell wall (carbohydrate, proteins, and lipids) [221]. As seen in **Fig. 9.1**, the spectra revealed that the intensity of the absorption peaks in the Cd(II)-treated biomass was altered in some point compared to the native biomass. This alteration in peak intensity suggests that the functional groups on the biomass surface were associated with the binding of Cd(II) ions, resulting in changes to their vibrational energies [223]. Key functional groups, such as carboxyl (-COOH), hydroxyl (-OH), amine (-NH), and phosphate (-PO₄), likely played a significant role in the biosorption process[217]. These groups act as active binding sites, interacting with Cd(II) ions through mechanisms such as complexation, ion exchange, and electrostatic attraction. As shown in **Fig. 9.1 with embedded Table**, the broad and strong band from 3003 to 3512 cm⁻¹ may be due to the stretching vibration of O-H and N-H. The FTIR bands observed at 2922 cm⁻¹ and 2852 cm⁻¹ correspond to the existence of lipid band spectra, which are linked to the symmetric C-H stretching of methylene groups [133].



Wavenumber (cm ⁻¹)	Band assignment	Comments
3512-3003	Water, (O-H) stretching; Protein, (N-H) stretching (amide A)	Water, protein
2940-2897	(C-H) from methylene groups of lipids	Primary lipids
2860-2835	(C-H) from methylene groups of lipids	Primary lipids
1811-1720	(C=O) stretching of esters	Esters of lipids and fatty acids
1692-1599	Amide I band mainly (C=O) stretching	Protein (Amide I)
1545-1460	Amide II band mainly (C-H) and (N-H) associated with protein	Protein (Amide II)
1455-1416	(CH ₂) and (CH ₃) bending of methyl	Proteins
1398-1350	(CH ₃) and (CH ₂) of proteins, and C-O of COO ⁻ group	Proteins and carboxylic acids
1312-1204	(P=O) of phosphodiester groups	Nucleic acids (DNA and RNA)
1200-900	(C-O-C) of polysaccharides	Carbohydrates
1020-957	(C-O) stretching, (C-O-C) of polysaccharides	Carbohydrate

Fig. 9.1 FTIR analysis of the original biomass and Cd (II) loaded biomass of *C. sorokiniana* (inserted table indicates interpretation of bands).

A pronounced peak at 1741 cm^{-1} is attributed to the C=O stretching of esters, which is mainly associated with lipid and fatty acid ester bonds [132], which are key factors for the biosorption of heavy metal [109]. A notable band observed at 1650 cm^{-1} is attributed to the C=O stretching of Amide I. The Amide II band spectra, associated with C-H and N-H stretching in proteins, were observed at approximately 1538 cm^{-1} . The spectrum at 1454 cm^{-1} corresponds to the bending vibrations of methyl CH₂ and CH₃ groups, as well as the stretching vibrations of carboxylic C-O bonds. Due to the asymmetric stretching of the

phosphodiester P=O, the protein band spectra exhibited a moderately prominent band at approximately 1238 cm^{-1} . The 'carbohydrate band spectra' exhibited pronounced characteristics at 1022 cm^{-1} due to C-O-C stretching. It was observed that peak intensity increased at this wavenumber after Cd(II) biosorption. The increased intensity in the FTIR spectrum after Cd(II) biosorption suggests that specific functional groups (e.g., carboxyl) play an active role in metal binding [223]. This is likely owing to the formation of stable complexes or structural reorganization of the biomass surface. These changes confirm that chemical interactions are pivotal to the cadmium removal process. However, the intensity of the FTIR peaks remains unchanged at the wavenumber of 2922 cm^{-1} , 2852 cm^{-1} , and 1741 cm^{-1} before and after Cd(II) biosorption. It suggests that the functional groups at this point responsible for binding Cd(II) are not undergoing significant chemical changes. Physical adsorption is one of the potential mechanisms for Cd(II) elimination in this situation. The process of physical adsorption is affected by Van der Waals forces and the electrostatic interactions that occur between positively charged metal ions and the negatively charged surface of biomass [224]. Because of the physical structure of the biomass and the presence of functional groups on the surfaces of the algal cells, the entire process shows that the biosorption of Cd(II) onto *C. sorokiniana* biomass is probably a combination of physical and chemical adsorption.

9.2.2 SEM-EDX analysis

To examine the morphological changes prior to and following Cd(II) biosorption, *C. sorokiniana* biomass was subjected to Scanning Electron Microscopy (SEM) analysis. The SEM pictures of the raw biomass showed an uneven, rough, and somewhat porous surface structure, as shown in **Fig. 9.2(a-c)** [109].

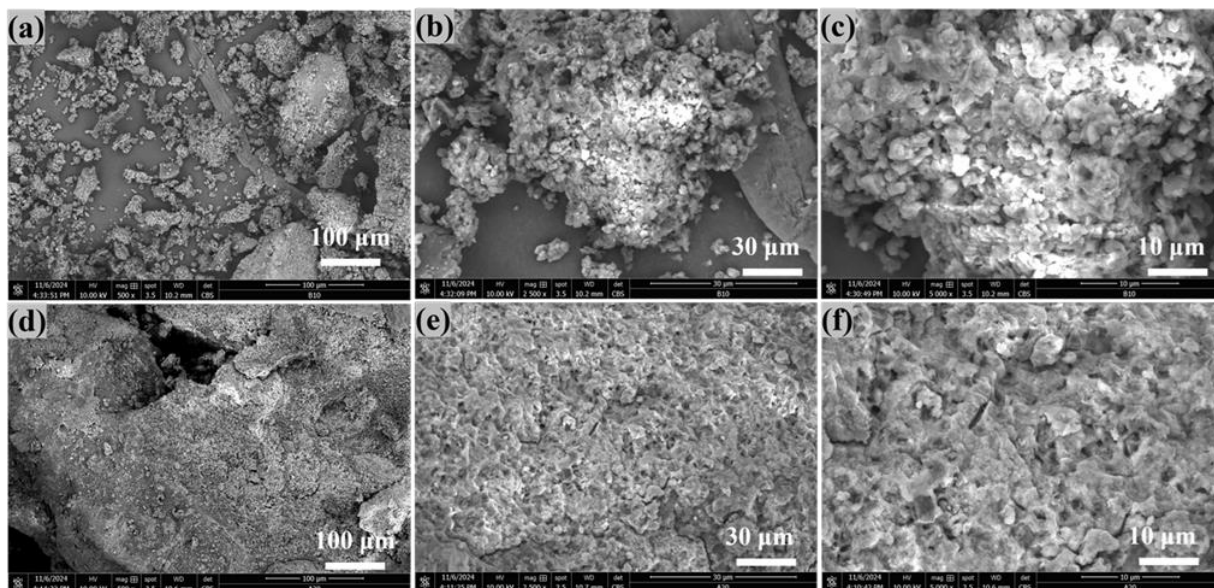


Fig. 9.2 SEM analysis of the *C. sorokiniana* biomass: **(a-c)** before biosorption; **(d-f)** after biosorption.

The biomass surface's heterogeneity enhances its biosorption capacity by enabling the adsorption of metal ions via diverse functional groups present on the surface [225]. Following biosorption illustrated in **Fig. 9.2(d-f)**, significant alterations in the surface morphology were noted. The SEM pictures indicated that the biomass surface grew more uniform and dense, with particles visibly dispersed across the surface [226]. The enhanced smoothness can be resulted from the deposition and buildup of Cd(II) ions on the biomass surface, which occupy accessible binding sites and fill surface imperfections [226]. The morphological alterations validate the effective adsorption of Cd(II) ions onto the *C. sorokiniana* biomass. The visible evidence of metal ion deposition further corroborates the physical and chemical interactions between the biomass and Cd(II). The compact structure observed after biosorption suggests that Cd(II) ions are not only adsorbed but also firmly bonded to the biomass, creating stable complexes that diminish surface porosity and roughness. These findings highlight the significance of surface morphology in the

biosorption process. This SEM investigation emphasizes the efficacy of *C. sorokiniana* biomass as a dependable biosorbent to remove the Cd(II) from aqueous solutions.

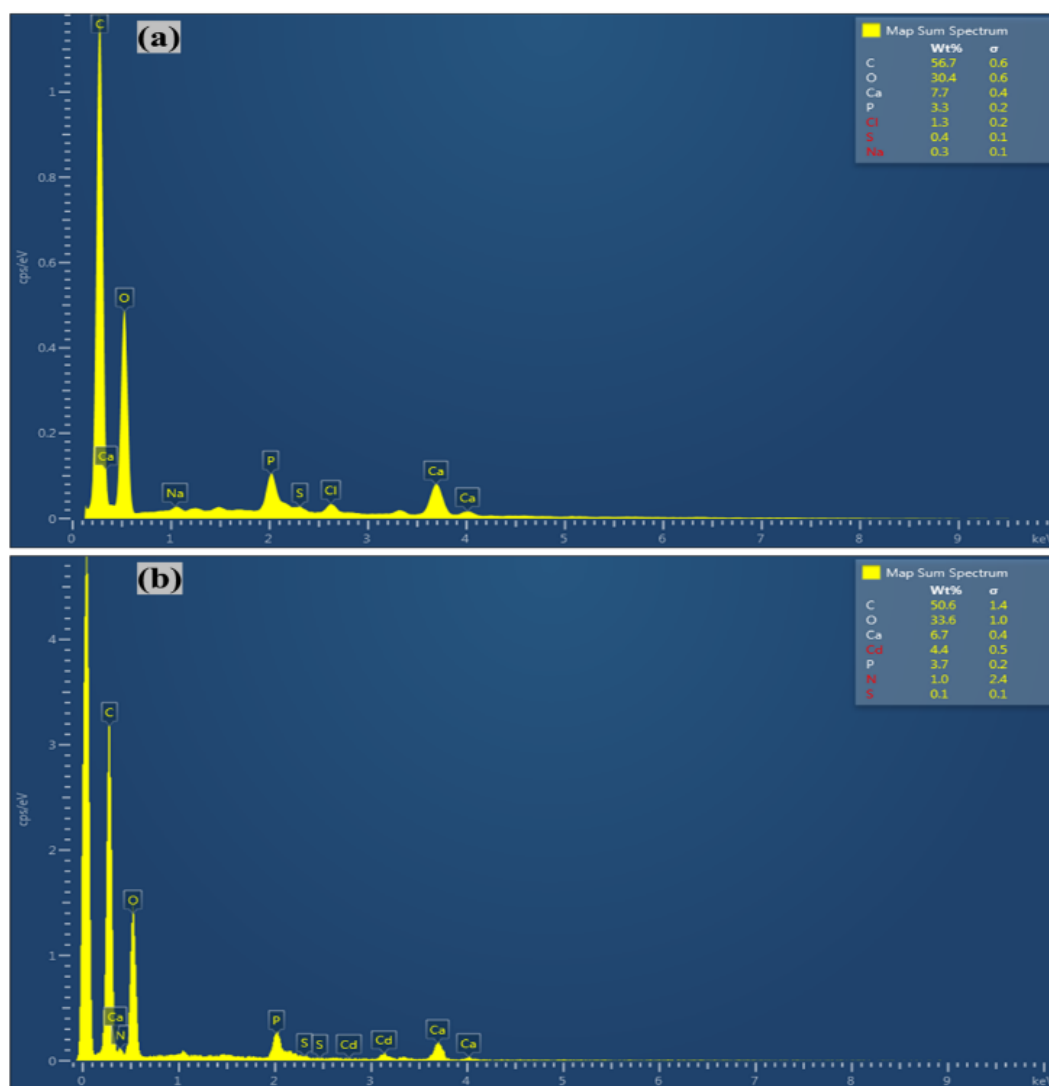


Fig. 9.3 EDX analysis of *C. sorokiniana* biomass: (a) before biosorption; (b) after biosorption

The appearance of a new peak corresponding to Cd(II), in conjunction with the peaks of virgin biomass in the EDX graph (**Fig. 9.3b**), confirms the presence of metal ions Cd(II). The EDS layered and compositional images indicated the presence of Cd(II) in conjunction

with other elements, thereby confirming the strong adsorption of metal ions (Supplementary **Fig. S8**).

Fig. 9.3b indicates that the broadened peaks for Cd are observed within the range of 3 to 3.5 keV. The concentration of Cd in the biomass was measured at 4.4 wt% following the biosorption process, as illustrated in **Fig. 9.3b**. This indicates that a substantial amount of Cd(II) was loaded onto the *C. sorokiniana* biomass. **Fig. 9.3b** illustrates that the elimination of Na⁺ and Cl⁻ ions, along with the decrease in Ca²⁺ ions following Cd(II) biosorption, provides strong evidence that ion exchange serves as a primary mechanism for the removal of Cd(II) [227]. Electrostatic attraction and complexation may also play complementary roles in stabilizing the Cd(II) ions on the biomass surface.

9.2.3 X-ray diffraction analysis

As appeared in **Fig. 9.4**, XRD analysis of *C. sorokiniana* biomass before and after Cd(II) biosorption reveals significant structural and chemical modifications. Before Cd(II) biosorption, sharp and well-defined peaks at different 2θ values indicate the presence of crystalline regions and in the same way, broad humps or diffuse patterns signify amorphous regions. The coexistence of crystalline and amorphous regions in *C. sorokiniana* biomass is advantageous for its functional properties, including biosorption of HMs. The decreased peak intensity in the treated biomass following Cd(II) biosorption suggests that crystalline areas have been disrupted by the interaction with Cd(II), most likely by ion exchange, in which Cd(II) substitutes ions like Na⁺ and Ca²⁺ at binding sites. Peak shifting also reflects changes in interatomic distances and lattice strain brought on by the addition of Cd(II) ions to the biomass structure, which modifies the electron density. Reduced and shifted broad peaks, which indicate amorphous regions, indicate that these regions have stabilized or

reorganized as a result of adsorption. These findings emphasize the dual contribution of physical and chemical interactions in the removal of Cd(II) by pointing to a combination process of ion exchange, surface complexation with functional groups (such as carboxyl and hydroxyl), and structural modification of the biomass.

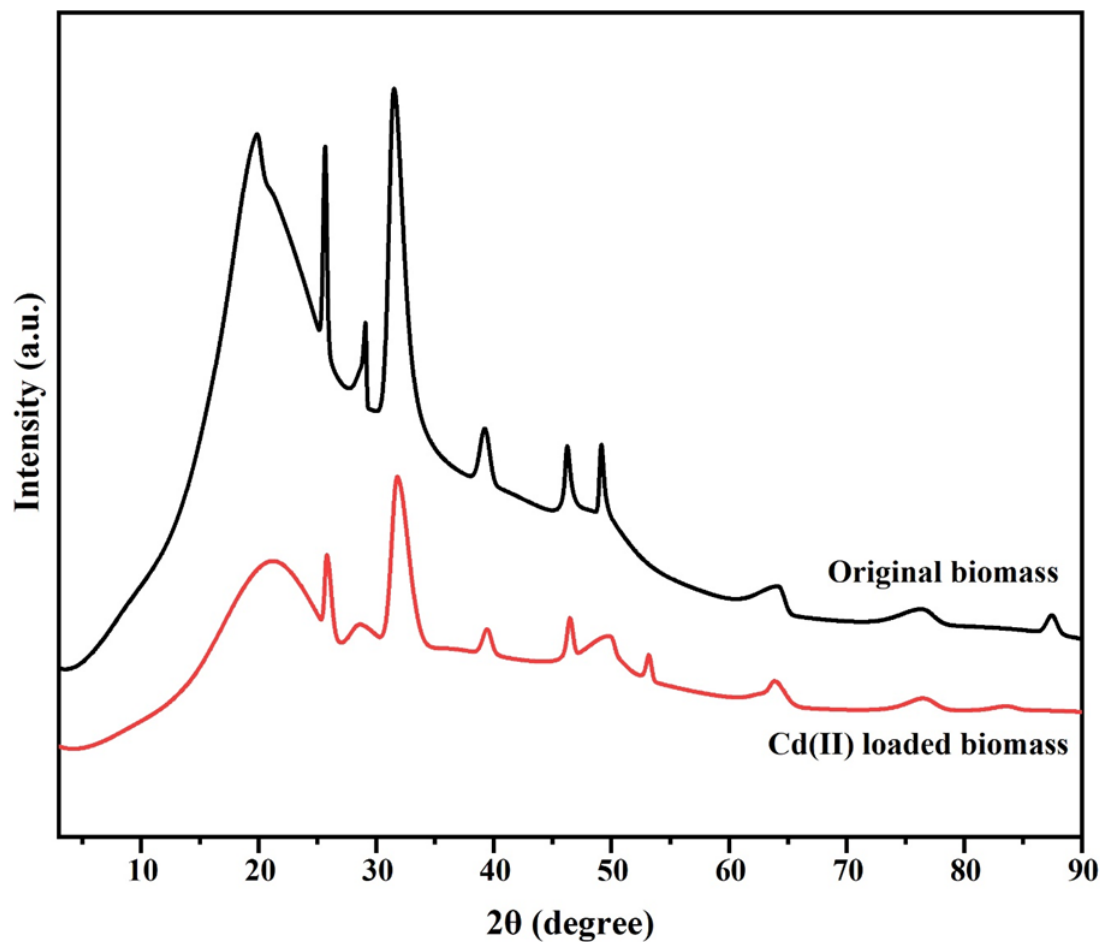


Fig. 9.4 XRD analysis of the original biomass and Cd (II) loaded biomass of *C. sorokiniana*

Similar observations were reported by others researcher. For instance, Kaparapu et al. (2018) analysed the XRD patterns of the microalgae *N. oculata* before and after Cd(II) biosorption,. The XRD model of the biosorbent after biosorption indicated a variation in

the crystallinity of the *N. oculata* biomass. They concluded that pure biomass has a poor crystallinity. Later, the change in 2θ and d spacing values in the Cd(II) loaded biomass was discovered. These results suggest the transformation in the crystallinity of the biomass after biosorption [228].

9.3 Effect of various process parameters on adsorption of Cd(II)

9.3.1 Initial pH

The pH of the solution significantly impacts heavy metal uptake by altering the surface charge of the bioadsorbent and the ionization and speciation of the adsorbate [229]. The influence of pH on Cd(II) adsorption onto the dry biomass of *C. sorokiniana* has been studied across a pH spectrum of 3 to 8, with the results pointed in **Fig. 9.5**. The biosorption capacity of Cd(II) onto *C. sorokiniana* biomass was minimal at an initial pH of 3, resulting in merely 4% removal. Nonetheless, it escalated markedly from 4% to 48% as the pH of the solution was elevated to 4. The biosorption capability rose progressively with pH levels of 5 and 6, resulting in Cd(II) removal rates of 61% and 66%, respectively. Moreover, at pH 7, the elimination of Cd(II) peaked at 76%, then decreasing to 63% at pH 8. At lower pH levels, such as 3, the biosorption effectiveness significantly diminished owing to the elevated concentration of hydrogen ions (H^+) in the solution. The protons compete with Cd(II) ions for the active binding sites on the surface of *C. sorokiniana* biomass, hence restricting the available sites for cadmium binding. This rivalry leads to diminished biosorption under very acidic environments. These findings align with the conclusions documented by other researchers. For instance, Kumar et al. (2020) performed an experiment on the biosorption of Hg(II) utilizing algal biomass and noted a diminished adsorption capacity for heavy metal removal under very acidic conditions [225].

As the pH nears neutrality (about 7), the competitive influence of H^+ ions decreases, permitting the functional groups on the biomass, including carboxyl, hydroxyl, and amino groups, to stay deprotonated and negatively charged. The negatively charged sites augment the electrostatic interaction between the biomass and the positively charged $Cd(II)$ ions, hence enhancing adsorption capacity. The optimal performance at pH 7 indicates a beneficial equilibrium between the accessibility of binding sites and the ionic condition of cadmium. A similar phenomenon regarding

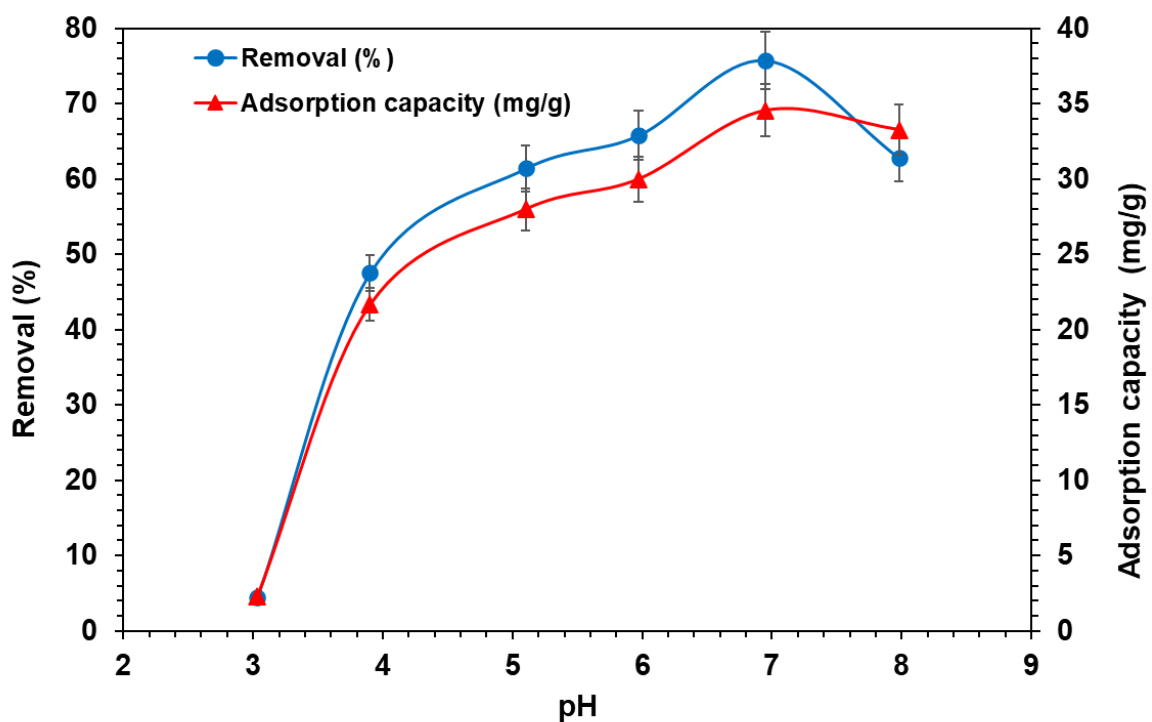


Fig. 9.5 Effect of initial pH on the Cd (II) removal (Conditions: initial Cd (II) conc., 25 mg/L; bioadsorbent dose, 50 mg/100 mL; contact time, 24 hour; Temp., 25 °C; stirring speed, 250 rpm). Data are means $\pm$ SD (n =3).

the maximum biosorption of $Cd(II)$ on microalgal biomass occurring around the neutral pH range has been reported by other researcher [230]. Beyond pH 7, the risk of cadmium

precipitation as cadmium hydroxide $\text{Cd}(\text{OH})_2$ increases. This can lead to a reduced observed biosorption capacity, as some of the metal ions form insoluble complexes and precipitate out of the solution rather than being adsorbed onto the biomass [231]. Furthermore, the chemical composition and structure of the biomass surface may change at pH levels beyond 7, potentially diminishing the affinity for $\text{Cd}(\text{II})$ binding. This result underscores the necessity of sustaining an initial pH of 7 to optimize biosorption and prevent potential precipitation and related complications. These findings emphasize the importance of controlling the pH to optimize $\text{Cd}(\text{II})$ removal, demonstrating that *C. sorokiniana* biomass is most effective as a biosorbent for cadmium at neutral pH conditions. Thus, further biosorption experiments were carried out at pH 7 in this study.

9.3.2 Biosorbent dosage

The dosage of adsorbent is important in the adsorption process, as it indicates the adsorbent's capability by providing the number of accessible binding sites for the removal of metal ions at a specified beginning concentration. In this study, the impact of adsorbent dose on the biosorption of $\text{Cd}(\text{II})$ onto *C. sorokiniana* dry biomass was assessed by varying the adsorbent doses from 25 to 125 mg per 100 mL of solution under constant experimental conditions. **Fig. 9.6** indicates that the percentage elimination of $\text{Cd}(\text{II})$ escalated significantly as the biomass dosage rose to 75 mg. This trend is attributed to the augmented number of accessible binding sites resulting from the increasing biomass, which elevates the likelihood of $\text{Cd}(\text{II})$ ions engaging with functional groups on the biomass surface. A comparable trend was seen in the removal of cadmium utilizing *Chlorella vulgaris* biomass as a bioadsorbent [232].

Beyond the 75 mg dosage, the enhancement in biosorption efficiency became increasingly incremental and ultimately stabilized at a dosage of 125 mg. The decline in biosorption effectiveness at elevated doses is owing to the saturation of accessible metal ions in relation to the total binding sites. When biomass concentration exceeds the ideal level, the surplus binding sites do not facilitate additional substantial removal of Cd(II), as the majority of Cd(II) ions in the solution are already bound. Moreover, at elevated doses, particle aggregation may transpire, resulting in a diminished effective surface area and reduced accessibility to the binding sites [233]. This aggregation may impede the interaction between Cd(II) ions and the active areas of the biomass. Furthermore, the competition among binding sites may lead to certain sites being underutilized, thereby accounting for the little increase in biosorption efficiency as the dosage near 125 mg. These observations indicate that while increasing the dose of *C. sorokiniana* biomass initially enhances Cd(II) removal, there is an optimal dose beyond which the efficiency gains are minimal. However, as appeared in **Fig. 9.6**, it was noticed

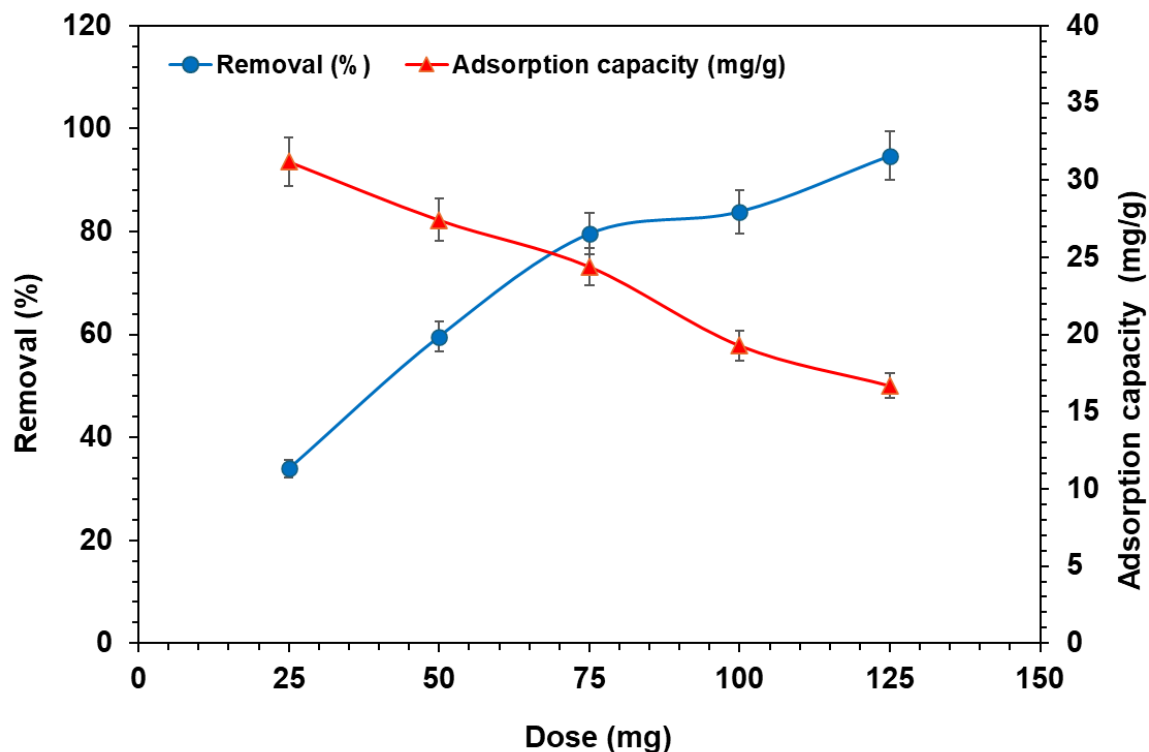


Fig. 9.6 Effect of bioadsorbent dosage on the Cd (II) removal (Conditions: initial Cd (II) conc., 25 mg/L; initial pH, 7.09; volume, 100 mL; contact time, 24 hour; Temp., 25 °C; stirring speed, 250 rpm). Data are means $\pm$ SD (n =3).

that the adsorption capacity (Q_e), defined as the amount of metal adsorbed per unit mass of the adsorbent (mg/g), reduces with an increase in adsorbent dose. This phenomenon occurs because the fixed initial concentration of metal ions is insufficient to occupy all available sites on the adsorbent. Establishing the optimum dosage is essential for enhancing biosorption efficacy and economic viability in actual implementations.

9.3.3 Initial Cd (II) concentration

The influence of initial Cd(II) ion concentration on biosorption utilizing *C. sorokiniana* dry biomass was assessed by altering the concentration from 10 to 70 mg/L, while keeping the adsorbent dosage constant at 125 mg per 100 mL of solution. As can be seen in **Fig.**

9.7, the results indicated that as the initial Cd(II) concentration augmented from 10 to 55 mg/L, the adsorption capability of the biomass also multiplied gradually from 8.5 to 34.4 mg/g biomass. At higher initial

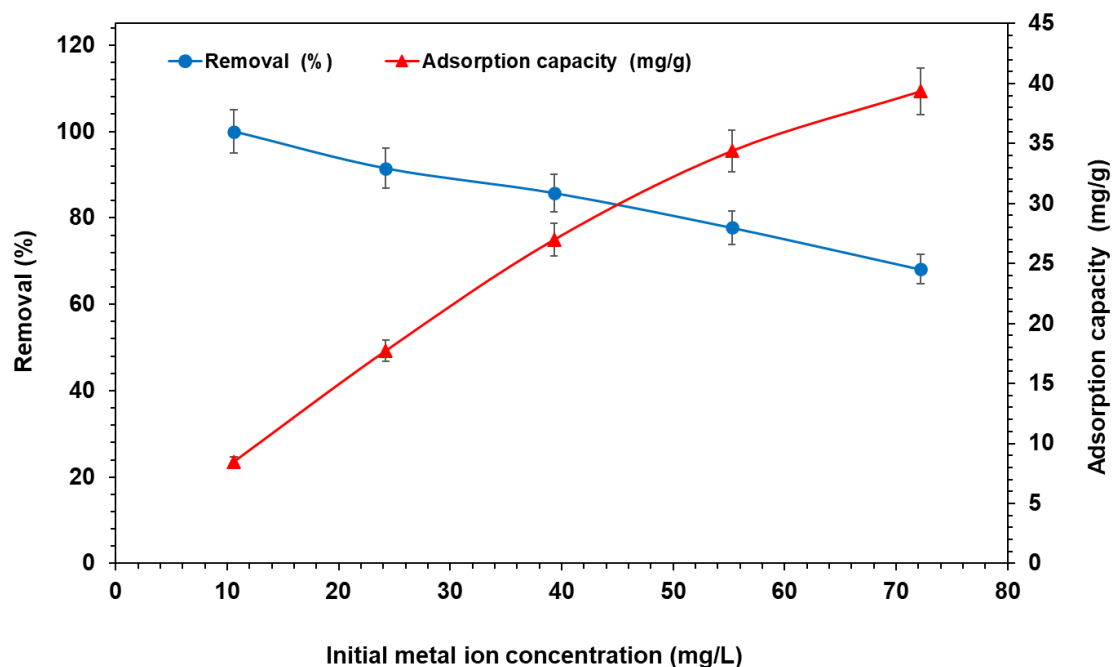


Fig. 9.7 Effect of initial Cd(II) concentration on the Cd(II) removal (Conditions: initial pH, 7.0; bioadsorbent dose, 125 mg/100 mL; contact time, 24 hour; Temp., 25 °C; stirring speed, 250 rpm). Data are means $\pm$ SD (n =3).

concentrations of Cd(II) ions, a strong driving force is created that overcomes mass transfer resistances between the liquid and solid phases, increasing the frequency of collisions between the sorbate and the sorbent surface. This is the main reason for the observed adsorption behavior [230]. Further, there was a steady increase in adsorption capacity until the initial Cd(II) concentration reached 70 mg/L. Beyond the initial Cd(II) concentration 70 mg/L, the trend of adsorption capacity approached saturation. This behavior indicates that at elevated concentrations, the quantity of Cd(II) ions exceeds the accessible binding sites, leading to the saturation of biomass functional groups and resulting in a plateau in

sorption [234]. This saturation point indicates the maximal absorption capacity of the biomass under the specified testing conditions. Nonetheless, although the adsorption capacity persisted in rising, the removal efficiency (% of Cd(II) eliminated) exhibited a declining trend as the starting concentration escalated. This unfavorable association is probably attributable to the saturation of accessible binding sites. At lower metal concentrations, the majority of binding sites on the biomass are accessible for adsorption, resulting in complete removal efficiency (**Fig. 9.7**). As concentration escalates, the finite quantity of binding sites (functional groups) gets entirely saturated, leading to a reduced percentage removal despite an increase in absolute adsorption capability [225]. These findings highlight the necessity of adjusting the initial metal ion concentration to achieve a balance between adsorption capacity and removal efficiency in effective biosorption systems.

9.3.4 Contact time

The duration of contact is crucial for optimizing biosorption effectiveness and comprehending the kinetics of the process. The impact of contact duration on the biosorption of Cd(II) onto *C. sorokiniana* biomass was investigated using various contact times, while maintaining other parameters constant. **Fig. 9.8** illustrates that the biosorption rate was initially swift, attaining an efficiency of 51% during the first 90 minutes. This speedy uptake phase is due to the many empty binding sites (functional groups) on the microalgal biomass surface, which enabled the rapid adsorption of Cd(II) ions [230]. In this first phase, the significant impetus for mass transfer from the solution to the solid phase, along with the vigorous interaction between cadmium ions and biomass, contributed to approximately 51.8% of the total Cd(II) removal. The elevated adsorption rate in the

initial phases is characteristic of biosorption processes, wherein numerous active sites (functional groups) in microalgal biomass facilitate effective ion binding [80].

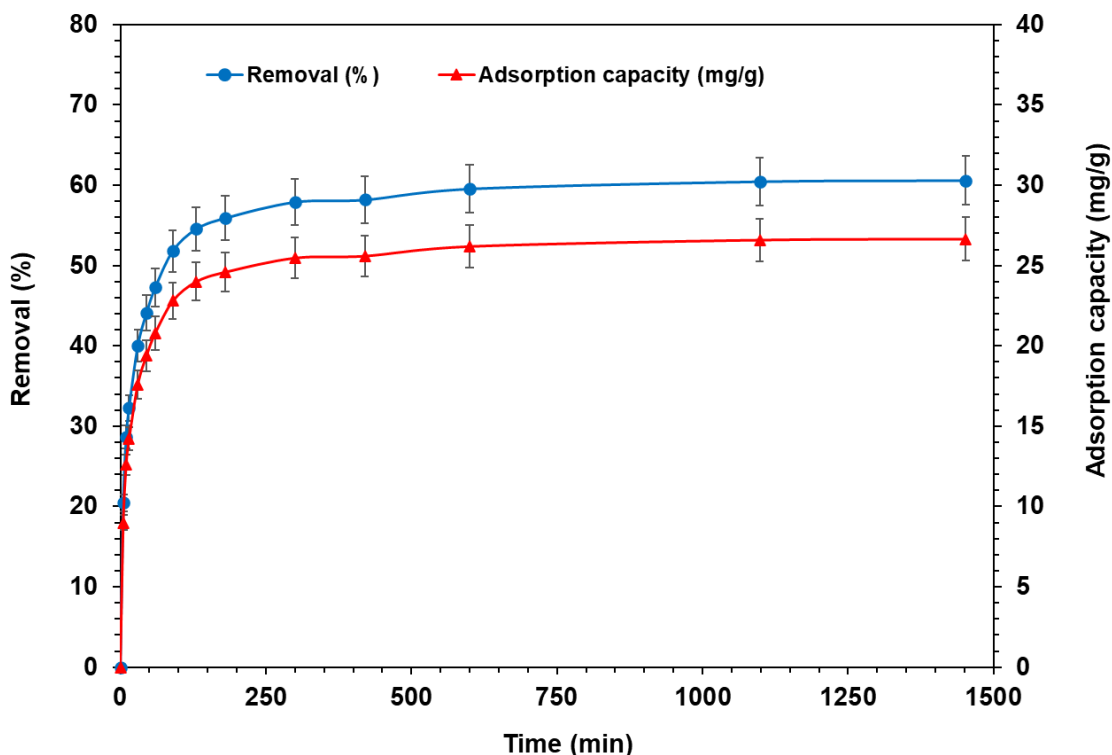


Fig. 9.8 Effect of contact time on the Cd(II) removal (Conditions: initial pH, 7.0; initial Cd (II) conc., 22 mg/L; bioadsorbent dose, 250 mg/500 mL; Temp., 25 °C; stirring speed, 250 rpm). Data are means $\pm$ SD (n =3).

As contact time increased beyond 90 minutes, the rate of biosorption slowed significantly. The elimination of Cd(II) increased by only 6% when the contact duration was increased to 300 minutes, resulting in a total biosorption efficiency of 57.9%. The gradual saturation of binding sites explains this decrease in the adsorption rate. The total rate of metal uptake decreases as these sites are occupied because there is more competition for the remaining unoccupied locations. The biosorption rate slowed after 300 minutes, and further increases in contact time resulted in only slight improvements in the elimination of Cd(II). This plateau phase indicates that equilibrium had been achieved, where the majority of the

available binding sites were fully utilized and the rates of adsorption and desorption were equal. Other researchers observed similar findings [226]. In industrial or environmental applications, it is crucial to ascertain the suitable contact time to achieve an optimal balance between high removal efficiency and effective time and resource management. The data acquired from this experiment was subsequently utilized to assess the kinetics of the adsorption process.

9.4 Kinetics studies

Kinetic studies are essential for comprehending the mechanism and efficacy of HMs removal from aqueous solutions utilizing the dry biomass of microalgae. They offer significant insights into the adsorption process, encompassing the rate-controlling mechanisms, such as chemisorption or physisorption, and the interaction dynamics between the adsorbate (heavy metals) and the biosorbent (microalgal biomass) [235]. The experimental data on Cd(II) adsorption onto *C. sorokiniana* dry biomass was investigated by fitting them to various kinetic models, including PFO, PSO, and Elovich models, to determine the best appropriate model for the adsorption process. As documented in **Table 9.1**, the experimental equilibrium adsorption capacity $Q_e(\text{exp.})$ was determined to be 26.7 mg/g, which was compared against the values anticipated by each kinetic model. The PFO model (**Fig. 9.9**) supposes that the rate of adsorption is directly proportional to the quantity of unoccupied sites available. The anticipated equilibrium adsorption capacity ($Q_e=24.69$ mg/g) was marginally inferior to the experimental result, accompanied with a rate constant (k_1) of 0.0521 min^{-1} . The correlation coefficient ($R^2 = 0.93748$) and root mean square error (RMSE = 2.01) indicate that the PFO model offers a decent fit to the experimental data.

The PSO model (**Fig. 9.9**), which assumes chemisorption as the rate-limiting phase, yielded a superior fit. The anticipated equilibrium adsorption capacity ($Q_e = 26.32 \text{ mg/g}$) closely approximated the experimental measurement, with a rate constant (k_2) of 0.00301 g/mg/min . As shown in **Table 9.1**, the PSO model had the strongest correlation coefficient ($R^2 = 0.99134$) and the lowest RMSE (0.75), suggesting that chemisorption is likely the principal mechanism controlling the adsorption of Cd(II) onto *C. sorokiniana* biomass. Waqar et al. (2023) explained similar results on the elimination of Cd(II) from aqueous solutions employing the green microalga *Scenedesmus*

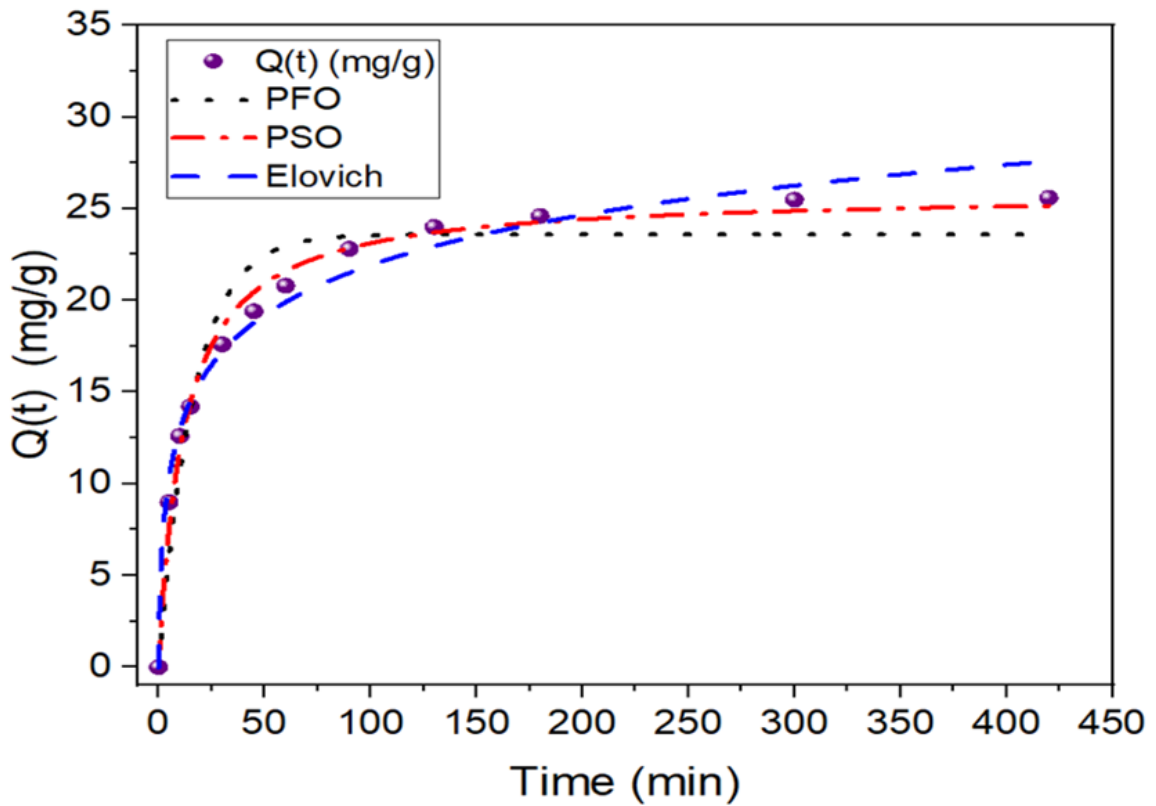


Fig. 9.9 Kinetic plot of the adsorption of Cd(II) on to *C. sorokiniana* bioadsorbent (Conditions: initial pH, 7.0; initial Cd (II) conc., 22 mg/L; bioadsorbent dose, 250 mg/500 mL; Temp., 25 °C; stirring speed, 250 rpm).

Table 9.1 Data of the kinetic coefficients for Cd(II) biosorption onto the *C. sorokiniana* biomass.

Q _e exp. (mg/g)		26.7		
Kinetic Model		Parameter Values	R ²	RMSE
PFO	$Q(t) = Q_e(1 - e^{-k_1 t})$	Q _e = 24.69 mg.g ⁻¹ k ₁ = 0.0521 min ⁻¹	0.93748	2.01
PSO	$Q(t) = \frac{k_2 Q_e^2 t}{1 + k_2 Q_e t}$	Q _e = 26.32 mg.g ⁻¹ k ₂ = 0.00301 g.mg ⁻¹ .min ⁻¹	0.99134	0.75
Elovich	$Q(t) = \left(\frac{1}{\beta}\right) (\ln(1 + \alpha\beta t))$	α = 25.34 mg.g ⁻¹ .min ⁻¹ β = 0.31869	0.95632	1.68

sp.[230]. However, Dirbaz et al. (2018) indicated that the biosorption of Cd(II) onto the dry biomass of the green microalga *Parachlorella* species was more accurately represented by the PFO kinetic model than by the PSO model when employed as a biosorbent for the elimination of Cd(II) from aqueous solutions [236].

The Elovich model (**Fig. 9.9**), commonly applied to characterize adsorption on heterogeneous surfaces, also yielded a satisfactory fit. The Elovich constants were established as α = 25.34 mg/g/min and β = 0.31869 g/mg, indicating a moderate adsorption rate and surface contact intensity. The correlation coefficient (R² = 0.95632) and RMSE (1.68) indicate that surface heterogeneity influences the adsorption process, albeit less significantly than in the PSO model. Of the three models, the PSO model exhibited the most accurate alignment with the experimental data (**Fig. 9.9 and Table 9.1**), suggesting that the adsorption of Cd(II) onto *C. sorokiniana* dry biomass is primarily governed by chemisorption, characterized by electron-sharing or exchange between the biosorbent and adsorbate. The results elucidate the adsorption kinetics and reinforce the viability of *C. sorokiniana* as an effective biosorbent for Cd(II) removal.

9.5 Adsorption isotherms

The study investigated the adsorption properties of Cd(II) onto the dry biomass of *C. sorokiniana*, employing various adsorption isotherm models to evaluate the underlying biosorption mechanisms and capacity. The linear fitting of the experimental data to the Langmuir (**Fig. 9.10a**), Freundlich (**Fig. 9.10b**) and Temkin (**Fig. 9.10c**) isotherms suggest that the biosorption of Cd(II) onto biomass can be defended by these isotherm models. According to the obtained isotherm parameters and coefficients of determination shown in **Table 9.2**, the Langmuir isotherm, which presumes monolayer adsorption on a homogeneous surface [230], produced a maximum adsorption capacity ($Q_{\max}$) of 71.42 mg/g and an adsorption affinity constant (b) of 0.063 L/mg. The R^2 value of 0.9002 indicates that the Langmuir model sufficiently represents the process, however it implies that adsorption may extend beyond homogeneous sites. The Freundlich isotherm, which characterizes adsorption on heterogeneous surfaces yielded the most accurate fit to the experimental equilibrium data of cadmium adsorption on *C. sorokiniana*, exhibiting a R^2 value of 0.9829 and the maximum experimental uptake of Cd(II) appeared at 92 mg/g at pH of 7. This observation agrees with those results obtained by Ali et al. (2016) for cadmium adsorption onto *C. vulgaris* [237]. However, Mirghaffari et al. (2015) stated that

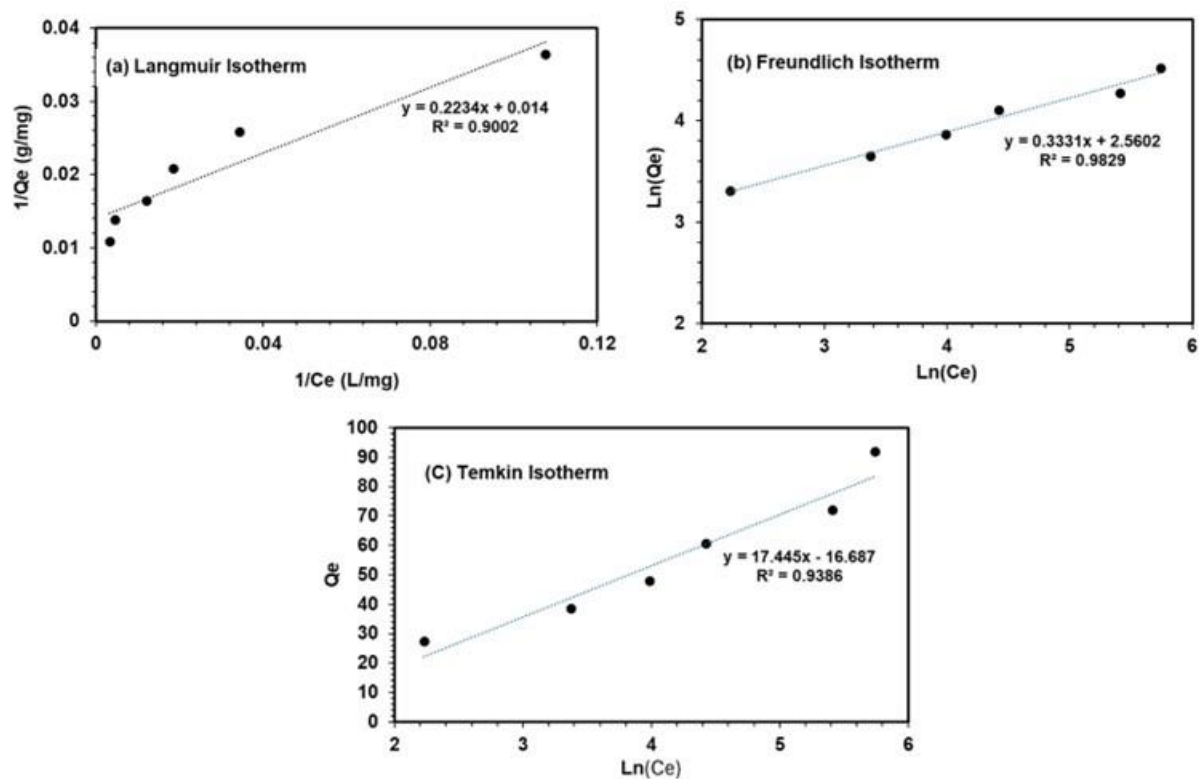


Fig. 9.10 Adsorption isotherm curves: (a) Langmuir (b) Freundlich, and (c) Temkin (Conditions: initial pH, 7.0; initial Cd (II) conc., (20 to 350) mg/L; bioadsorbent dose, 50 mg/100 mL; Temp., 25 °C; stirring speed, 250 rpm).

Table 9.2 Adsorption isotherm coefficients for the Cd(II) biosorption onto the *C. sorokiniana* biomass.

Isotherm Model		Parameter Values	R ²	RMSE
Langmuir	$\frac{1}{Q_e} = \frac{1}{Q_{max}} + \frac{1}{bQ_{max}C_e}$	$Q_{max} = 71.42 \text{ mg/g}$ $b = 0.063 \text{ L/mg}$	0.9002	10.94
Freundlich	$\ln Q_e = \ln K_F + \frac{1}{n} \times \ln C_e$	$K_F = 12.94 \text{ mg}^{0.67} \cdot \text{L}^{0.33} \cdot \text{g}^{-1}$ $n = 3.00$	0.9829	3.68
Temkin	$Q_e = B \ln K_t + B \ln C_e$	$B = 17.44 \text{ mg/g}$ $K_t = 0.384 \text{ L/mg}$	0.9386	5.31

biosorption of the Cd(II) was better fitted by Langmuir model rather than that by Freundlich model when the dry biomass of the green microalga *Scenedesmus quadricauda* was utilized as a biosorbent for the elimination of Cd from aqueous solutions [238]. This analysis yielded a Freundlich constant (K_F) of 12.94 mg/g and a heterogeneity factor (n) of 3.00, indicating preferential adsorption ($n > 1$) and the presence of diverse binding sites[228]. Furthermore, a fairly strong correlation was found with values $B = 17.44$ and $A = 3.00$ in the Temkin isotherm, which takes into consideration the influence of adsorbate-adsorbent interactions. This suggests that these interactions also contribute to the adsorption process. It is apparent that the Freundlich model most accurately depicted Cd(II) biosorption on *C. sorokiniana* biomass, emphasizing the heterogeneous characteristics of the biomass surface and the diverse energy levels of adsorption sites. Similar findings have been reported by other researchers, where metal was effectively removed from aqueous solutions using the dry biomass of microalgae *Scenedesmus* species[109]. The results of this investigation demonstrate how well *C. sorokiniana* works as a biosorbent to eliminate Cd(II) from aqueous solutions.

9.6 Comparison with other algal biosorbent

The evaluation of sorption capabilities of Cd(II) on different biosorbents underlines the superior efficacy of *C. sorokiniana* dry biomass compared to other algae species. **Table 9.3** summarizes the maximum adsorption capacity (Q_{max}) and testing conditions for various biosorbents. The highest adsorption capacity of *C. sorokiniana* dry biomass in this investigation was established at 71.42 mg/g at a pH of 7 and a temperature of 25°C. This value is competitive in comparison to other biosorbents. For instance, *Oedogonium sp.* exhibited comparable performance with an adsorption capacity of 88.2 mg/g (pH 5, 25°C).

Parachlorella sp. exhibited the greatest absorption of Cd(II) at 90.72 mg/g (pH 7, 30°C), indicating its robust biosorption capacity. Moreover, *Chlorella vulgaris* exhibited varying adsorption capacities, ranging from 45.04 to 86.6 mg/g under different conditions. When compared to marine algae, such as *Caulerpa fastigiata* (16.13 mg/g), *Hypnea valentiae* (15.78 mg/g), and *Ulva lactuca* (29.2 mg/g), *C. sorokiniana* displayed a significantly higher adsorption capacity. Additionally, biosorbents like *Spirulina platensis* (69.02 mg/g) and *Neochloris oleoabundans* (73.1 mg/g) performed similarly to *C. sorokiniana*. The results suggest that *C. sorokiniana* dry biomass is a highly effective biosorbent, particularly under neutral pH conditions, and offers a sustainable alternative to other algal biosorbents for Cd(II) removal from aqueous solutions.

Table 9.3 Comparison of adsorption capacity of Cd(II) on various algal biosorbents.

Biosorbent	pH	T (° C)	Maximum uptake (mg/g)	Ref.
<i>Chlamydomonas reinhardtii</i>	6	25	42.71	[239]
<i>Parachlorella sp.</i>	7	30	90.72	[236]
<i>Chlorella vulgaris</i>	4	25	86.6	[240]
<i>Caulerpa fastigiata</i>	5.5	25	16.13	[241]
<i>Hypnea valentiae</i>	6	30	15.78	[242]
<i>Oedogonium sp.</i>	5	25	88.2	[223]
<i>Chaetomorha linum</i>	5	25	53.76	[243]
<i>Fucus spiralis</i>	5	25	42.10	[244]
<i>Ulva lactuca</i>	5	20	29.2	[245]
<i>Neochloris oleoabundans</i>	7	25	73.1	[246]
green algae waste	5	20	34.61	[247]
<i>Chlorella vulgaris</i>	n/a	n/a	45.04	[237]
<i>Spirulina platensis</i>	5	n/a	69.02	[248]
<i>Chlorella vulgaris</i>	4	20	62.30	[26]
<i>Chlorella sorokiniana</i>	7	25	71.42	This study

9.7 Conclusions

This work successfully demonstrated for the first time the potential of *C. sorokiniana* dry biomass as an effective biosorbent to eliminate Cd(II) ions from aqueous solutions. A comprehensive evaluation was conducted of the primary process parameters that impact biosorption, including initial pH, biosorbent dosage, initial Cd(II) ion concentration, and contact time. The initial pH of the solution has a considerable impact on the biosorption process, which is best suited at a pH of about 7. The process's low cost and industrial usability were defined by its strong biosorption capacity at lower biosorbent dosage. The influence of contact duration exhibited an initial rapid rate succeeded by a gradual rate. Instrumental characterization of the biomass before and after Cd(II) adsorption revealed significant changes in surface morphology and functional groups, confirming the involvement of active sites in the biosorption process. FTIR spectra revealed that key functional groups, such as carboxyl (-COOH), hydroxyl (-OH), amine (-NH), and phosphate (-PO₄), likely played a significant role in the biosorption process. The SEM-EDX analysis revealed the binding of Cd(II) to the biomass. The coexistence of crystalline and amorphous regions in *C. sorokiniana* biomass is advantageous for its functional properties, including biosorption of Cd(II) ions. Kinetic modeling showed that the experimental data aligned closely with the PSO model with an R² value of 0.99134, suggesting that chemisorption was the primary mechanism governing Cd(II) uptake along with physical adsorption. Langmuir, Freundlich and Temkin isotherm models were fitted for Cd(II) ion removal. Among the tested isotherm models, the Freundlich isotherm model offered the best fit to the equilibrium data with an R² value of 0.9829, emphasizing the heterogeneous nature of the adsorption process and the multilayer binding of Cd(II) ions.

The monolayer biosorption capability of *C. sorokiniana* for Cd(II) was found to be 71.42 mg/g. These findings highlight the suitability of *C. sorokiniana* biomass as an eco-friendly and cost-effective alternative for Cd(II) removal, offering a sustainable solution for wastewater treatment. Further exploration of scaling and real-world applications is recommended to fully realize its potential in industrial wastewater treatment.

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CHAPTER 10

CONCLUSIONS AND RECOMMENDATIONS

10.1 Conclusions

This study conducts a thorough examination of microalga *C. sorokiniana*'s capacity for bioremediation of petroleum-derived produced water (PW) and HM removal, while evaluating its biomass valorization for bioenergy purposes. The viability of *C. sorokiniana* was assessed by a series of experimental investigations under several environmental circumstances, including hypersalinity, organic pollution gradients, and HM stress. The results collectively illustrate the dual function of *C. sorokiniana* as an effective bioremediation agent and a valuable bioresource for renewable energy generation.

The study initially verified the effective adaption and growth of *C. sorokiniana* in hypersaline brine (HSB), attaining significant biomass productivity ($53.9 \text{ mg L}^{-1}\text{d}^{-1}$) at 30% HSB concentrations. Progressive adaptation mechanisms facilitated microalgal survival in extreme salinity conditions, emphasizing its potential for extensive applications in areas with scarce freshwater resources. The microalga demonstrated substantial nitrogen (up to 89.1%) and phosphorus (up to 98.48%) removal efficiency, highlighting its efficacy in PW treatment.

Subsequent studies demonstrated that *C. sorokiniana* effectively degraded dissolved organic pollutants (DOPs), in acetate and phenol based SPW markedly. The maximum removal of TOC in acetate based SPW achieved was 85% at a 50% PW loading, while phenol degradation efficiency reaching 96%, primarily via biodegradation rather than

bioadsorption. The research indicated that lipid accumulation in biomass, reaching up to 30%, was enhanced by phenol biodegradation, highlighting its viability for biofuel production. The physicochemical characterization of biomass demonstrated its potential as a biofuel feedstock, exhibiting a higher heating value (HHV) ranging from 15.20 to 22.84 MJ/kg.

C. sorokiniana exhibited resilience and metabolic adaptation under HM stress conditions. Biomass productivity reached a maximum of 124.63 mg L⁻¹d⁻¹ in 20% PW under HM stress, with Pb²⁺ removal facilitated by biosorption, bioaccumulation, and pH-induced precipitation. Although pollutant degradation was successful at various produced water (PW) loadings, microalgal development was significantly hindered in 100% PW due to excessive salinity and nutrient scarcity, highlighting the necessity for dilution or nutrient enhancement to maintain efficient bioremediation.

Furthermore, this research demonstrates, for the first time, the potential of *C. sorokiniana* dry biomass as an effective biosorbent for the removal of Cd(II) from aqueous solutions. The biosorption process demonstrated significant efficiency, achieving a maximum uptake capacity of 92 mg/g at pH 7. Kinetic and isotherm modeling indicated that Cd(II) biosorption adhered to a pseudo-second-order mechanism, predominantly influenced by chemisorption. The Freundlich isotherm model effectively characterized the adsorption equilibrium, suggesting multilayer binding on a heterogeneous surface. Surface characterization indicated that functional groups including carboxyl (-COOH), hydroxyl (-OH), amine (-NH), and phosphate (-PO₄) were essential for the sequestration of Cd(II). The findings indicate that *C. sorokiniana* biomass may function as a cost-effective and

environmentally sustainable option for heavy metal removal in wastewater treatment processes.

To sum up, *C. sorokiniana* demonstrates potential as a microalga for mitigating environmental issues related to petroleum-derived PW. This study enhances eco-friendly, cost-effective, and sustainable strategies for PW management through its dual capabilities in organic pollutant removal and bioresource production.

10.2 Recommendations for future work

1. Optimization of Cultivation Parameters

- Further research is needed to refine and optimize the cultivation conditions of *C. sorokiniana* to enhance its efficiency in large-scale PW treatment systems.

2. Scaling Up Biosorption Experiments

- Large-scale biosorption studies should be conducted to validate the laboratory findings and determine the feasibility of *C. sorokiniana* dry biomass for industrial applications in HM removal.

3. Integration into Circular Bioeconomy Models

- The valorization of microalgal biomass should be explored further, incorporating its application in biofuel production, bioproducts, and other sustainable resource recovery strategies.

4. Techno-Economic and Life Cycle Assessment

- A comprehensive techno-economic analysis and life cycle assessment (LCA) should be conducted to evaluate the feasibility, environmental benefits, and

economic viability of implementing *C. sorokiniana* in large-scale wastewater treatment systems.

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Journal Publications:

1. Faruque, M.O., Mohammed, T., Hossain, M.M., Razzak S.A., **(2025).** Bioremediation of Dissolved Organic Compounds in Produced Water from Oil and Gas Operations using *Chlorella sorokiniana*: A Sustainable Approach. *Environmental Monitoring and Assessment*, vol. 197, no. 1.

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3. Faruque, M.O., Uddin, S., Hossain, M.M., Hossain, S. M. Z., Shafiquzzaman, M., Razzak S.A., **(2024).** A Comprehensive Review on Microalgae-Driven Heavy Metals Removal from Industrial Wastewater using Living and Nonliving Microalgae. *Journal of Hazardous Materials Advances*, pp-100492.

4. Faruque, M.O., Hossain, M.M., Razzak S.A., **(2023).** Photoautotrophic Cultivation, Lipid Enhancement, and Dry Biomass Characterization of Microalgae *Scenedesmus dimorphus* for Bioenergy Application. *Arabian Journal for Science and Engineering*, Volume-48, Issue- 12, pp. 16263-16280.

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6. Razzak S.A., Bahar. K., Islam, K.M.O., Haniffa, A. K., **Faruque, M.O.,** Hossain, S. M. Z., Hossain, M.M., **(2024).** Microalgae cultivation in photobioreactors: Sustainable

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SUPPLEMENTARY MATERIALS

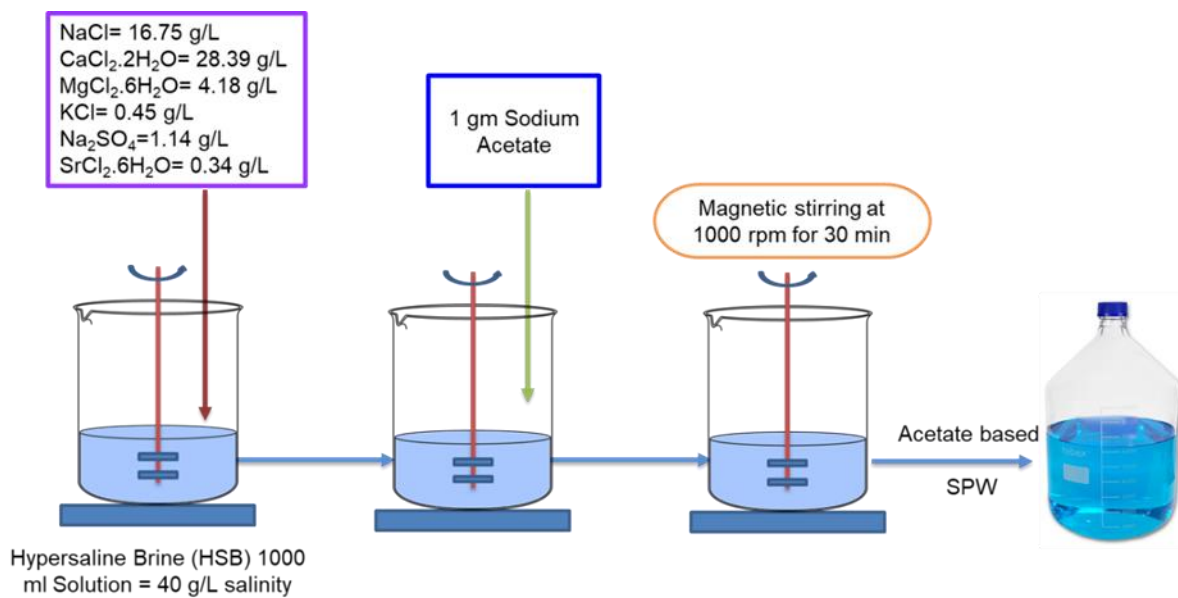


Fig. S1 Modified protocol for preparing acetate based SPW.

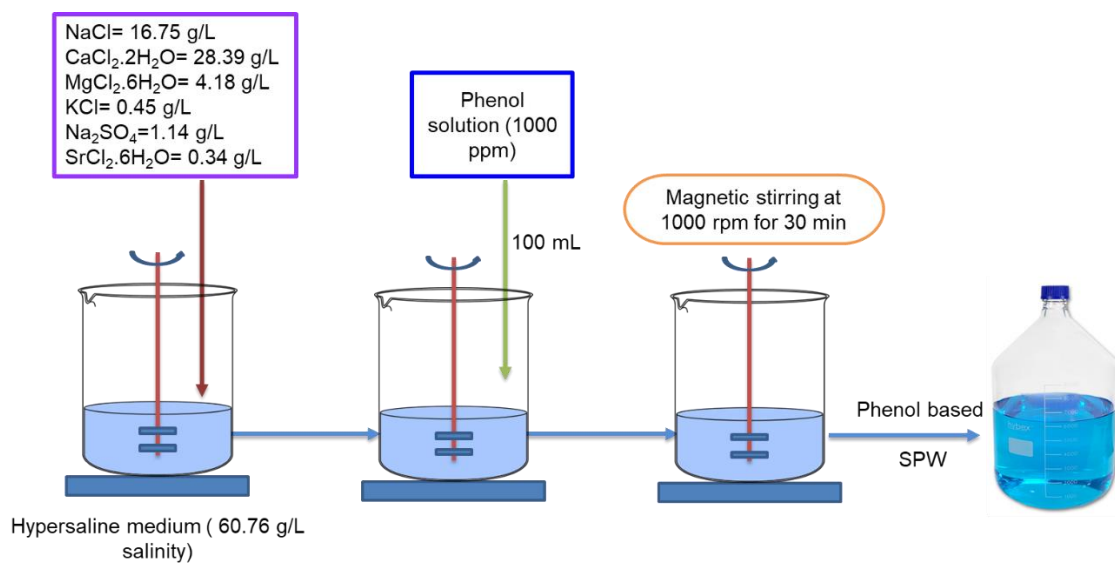


Fig. S2 Modified protocol for preparing phenol based SPW.

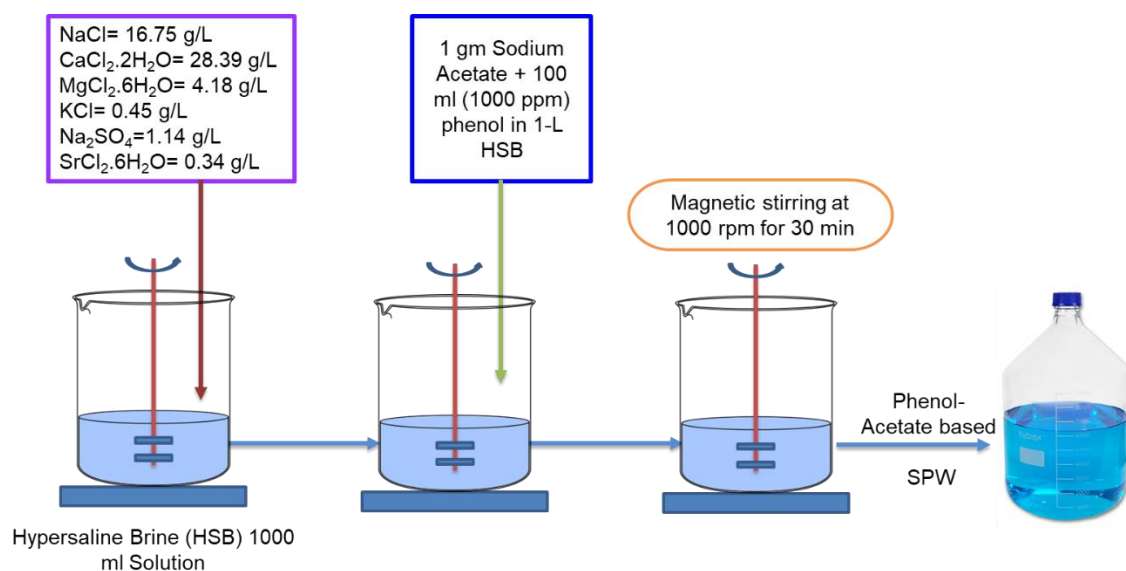


Fig. S3 Modified protocol for preparing phenol-acetate based SPW.

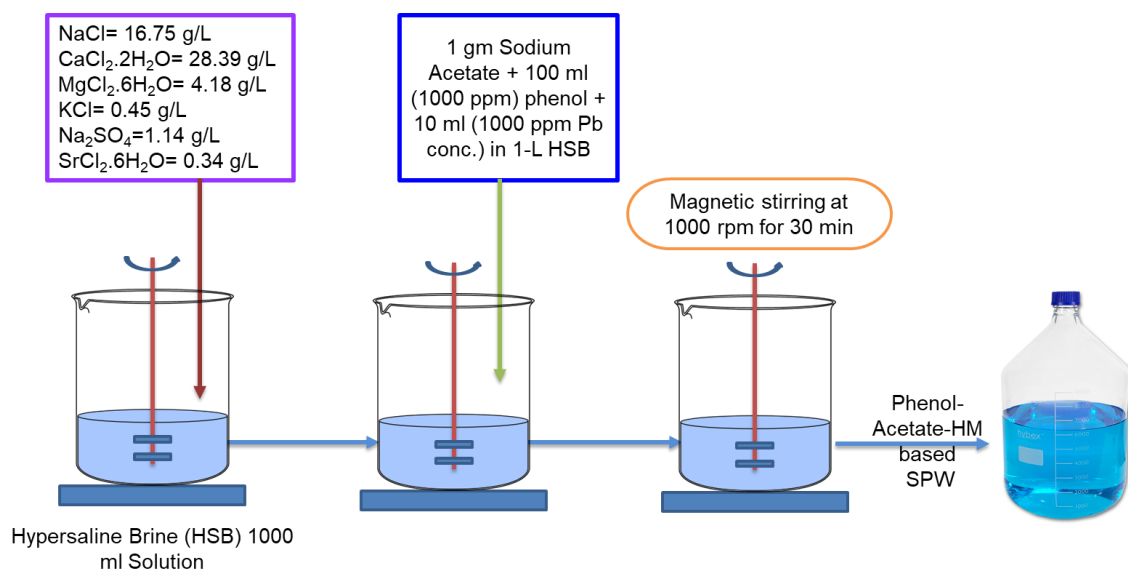


Fig. S4 Modified protocol for preparing phenol-acetate-HM based SPW.

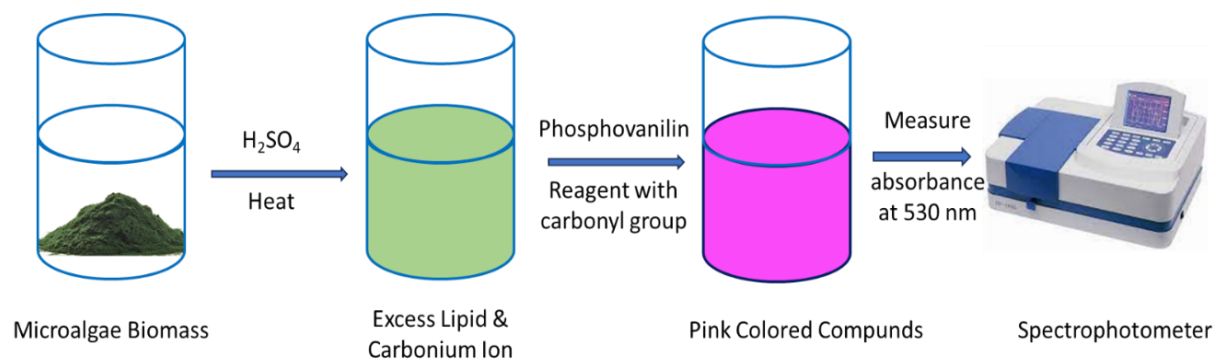


Fig. S5 Lipid detection principle in the SPV-based colorimetric assay.

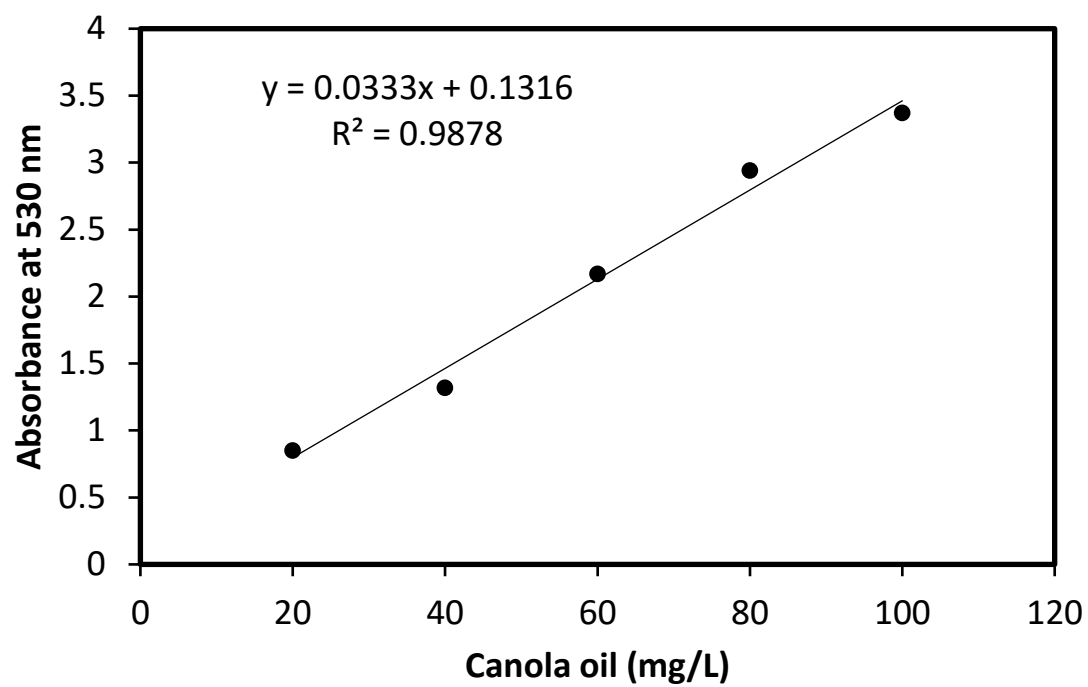


Fig. S6 Calibration curve for lipid quantification

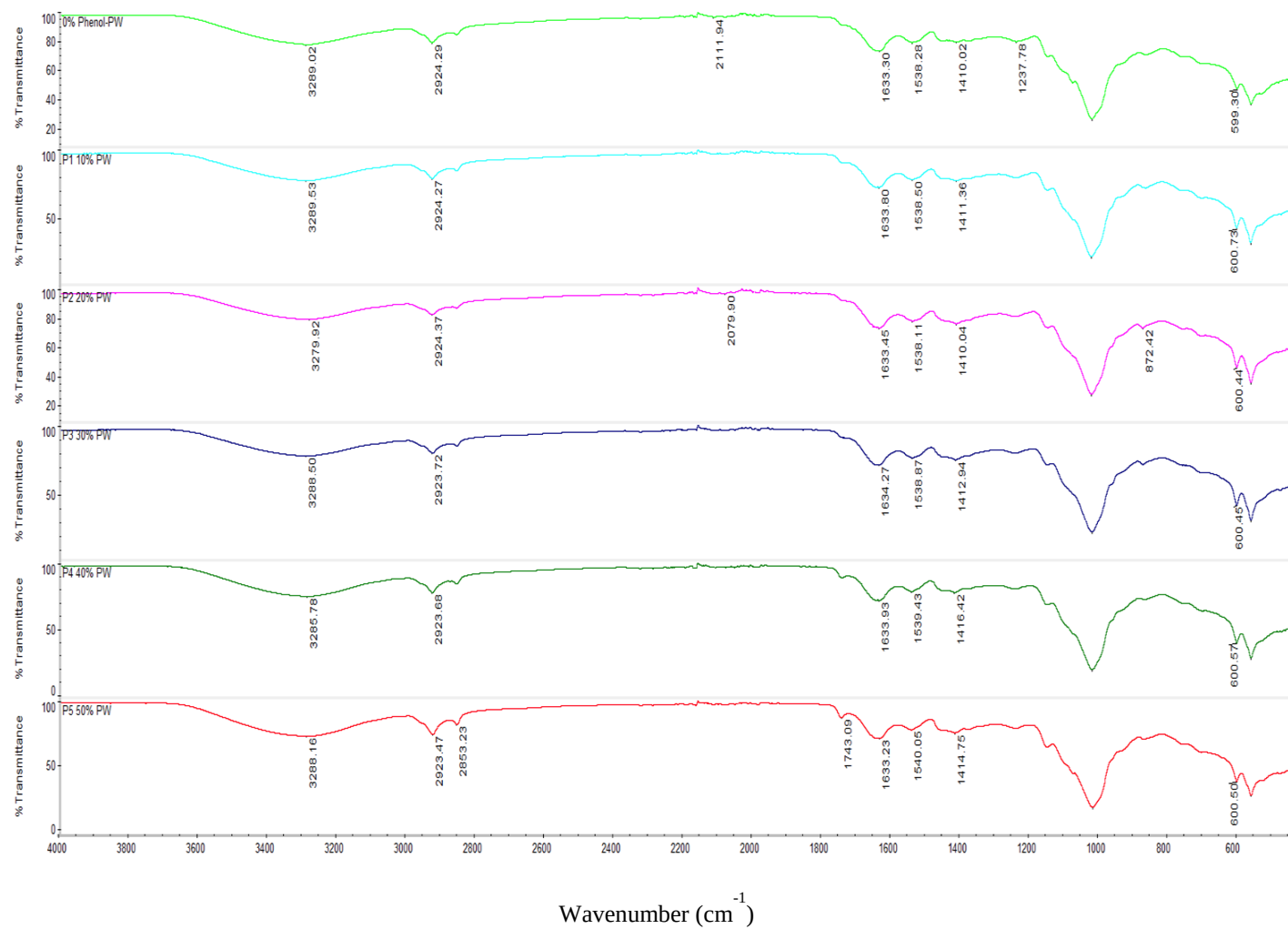
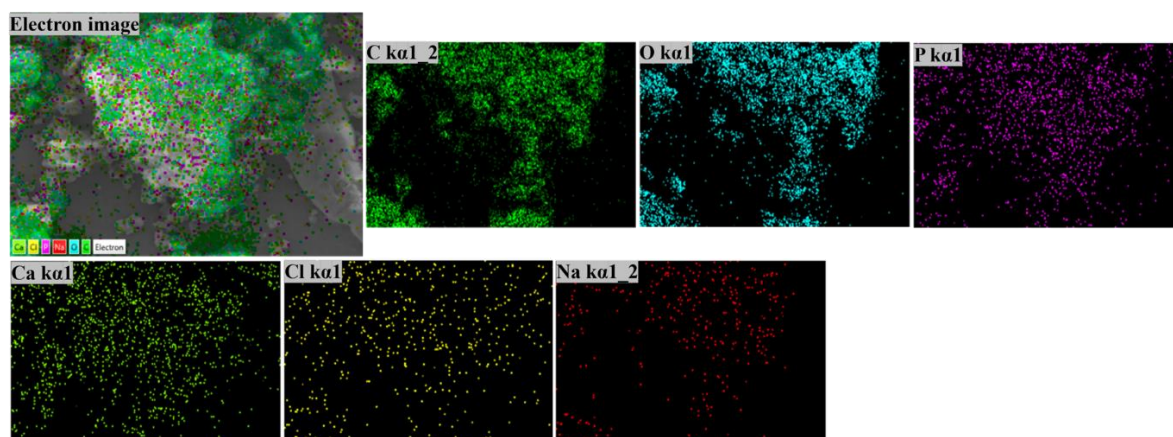
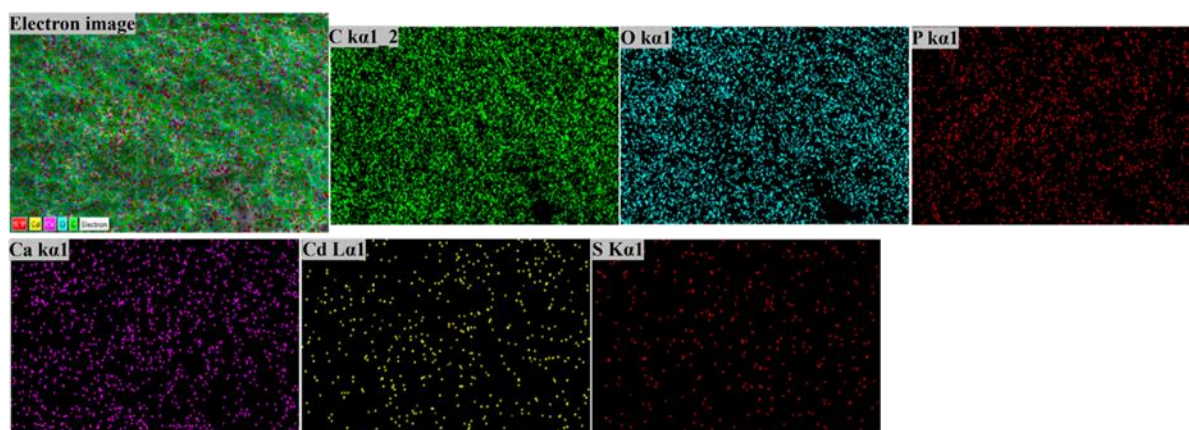


Fig. S7 The FTIR transmittance of the phenol degraded *C. sorokiniana* biomass considering different PW loadings



Before biosorption



After biosorption

Fig. S8 EDS layered and compositional images of the *C. sorokiniana* biomass before and after Cd(II) adsorption.