Exploring Potential of Freshwater Microalgae in Northern Ontario for Bioremediation and Bioenergy Production

A thesis presented to

The faculty of Graduate Studies

of

Lakehead University

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy (PhD)

in Biotechnology

Submitted By

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August 15, 2024

Abstract

Native freshwater microalgae play a crucial role in biofuel production due to their unique characteristics and environmental suitability. This study investigated the diversity of microalgae species in various freshwater ecosystems across Northwestern Ontario, Canada and evaluated the nutrient removal, bioremediation and subsequent biomolecule production. Microalgae are known for their biochemical diversity, synthesizing an extensive range of compounds including proteins, lipids, carbohydrates, vitamins, pigments, and bioactive molecules In Chapter One, we provided a comprehensive review on the microalgae cultivation and harvesting techniques along with pretreatment methods for the extraction of biomolecules.

In Chapter Two, we isolated 75 potential microalgal isolates and characterized these stains to identify those with high potential for lipid and biomolecule production capacity through a combination of microscopy, molecular techniques, and biochemical analysis and identified the optimal light condition for the biomass and lipid production. Among these isolated strains, a five microalgae strains with higher lipid production and growth were identified using molecular and morphological approaches. Among four different photoperiods, 16L:8D light/dark cycle produced significantly higher biomass and lipids for *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Chlamydomonas* sp.

In Chapter Three, we optimized wastewater conditions for the cultivation of *Chlorella vulgaris* to enhance biomass and lipid production in three types of wastewaters: malting effluent, municipal wastewater, and paper mill wastewater. Microalgae have demonstrated the ability to efficiently remove pollutants like nitrogen and phosphorus from wastewater, offering a cost-effective and environmentally friendly alternative to traditional treatment methods. The optimal wastewater conditions were determined to be 50 times diluted malting effluent (ME-50), undiluted municipal wastewater (MC-undiluted), and undiluted paper mill wastewater (PM-undiluted). Among them, ME-50 demonstrated the maximum cell density, leading to maximum biomass growth and lipid accumulation.

Chapter Four focused on the use of microalgae for the effective management and bioremediation of microplastic by using microalgae. Our study evaluated the effect of various concentrations of polyethylene terephthalate (PET) microplastic (25–200 mg/L) on the growth, chlorophyll content, and toxicity of *Scenedesmus* sp. from aquatic and terrestrial habitats for 24 days. Our results showed that microplastics with higher concentrations (200 mg/L) have a significantly higher inhibitory effect on microalgae. Also, a higher concentration of extracellular hydrogen peroxide (H_2O_2) extracellular polymeric substance (EPS) was found on microalgae exposed to the microplastic and assisted in the adsorption of microplastic particles and formation of hetero-aggregation and facilitated the sedimentation.

Chapter Five aimed to optimize the reducing sugar production from microalgae by chemical (sulphuric acid), physical (ultrasonication) and enzymatic pretreatments using response surface methodology (RSM). The pretreatment process helps to release intracellular polysaccharides and other biomolecules, which can be used for the biofuel and value-added products production. Our results showed that microalgal biomass hydrolyzed with sulfuric acid led to a higher yield of reducing sugar (247.55 mg/g) compared to physical (ultrasonication) (184.6 mg/g) and enzymatic treatments (216.36 mg/g). In enzymatic pretreatments, crude enzyme was produced by fermenting the lignocellulosic biomass (wheat straw) by using *Bacillus* sp. which was employed for microalgal cell wall degradation. It reveals that the cocktail enzymes weakened the cell wall and facilitated in lipid and other extracellular molecules extraction process.

Chapter Six explored an integrated approach of bioconversion and bioelectricity production, coupled with bioproducts recovery from multifunctional microalgae microbial fuel cells (MMFCs). Microalgal biomass was cultivated in an MMFC and pretreated with ultrasonication followed by xylanase-producing bacterial hydrolysis to maximize xylanase production. The maximum current generation was 875 mA and xylanase activity of 4.537 u/ml was achieved. This approach not only offers the potential to reduce the environmental impact of industrial effluent, conserve resources, contribute to climate change mitigation, and safeguard the environment, but also enables the generation of renewable energy and biomaterials, marking a substantial advancement toward a more sustainable and environmentally responsible society.

In summary, this study integrated ecological, biotechnological, and engineering perspectives to explore the potential of freshwater microalgae for their multifaceted capabilities for wastewater treatment, biofuel production, and biomolecule synthesis.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Dr. Wensheng Qin, for his invaluable guidance, unwavering support, and insightful feedback throughout this journey. His expertise and encouragement have been instrumental in shaping this thesis.

I am immensely thankful to my thesis committee members, Dr. Brian Ross and Dr. Robert Mackereth, for their constructive critiques and suggestions that have significantly enriched the quality of this work. My sincere thanks to external examiner Dr. Wen Chen, Department of Biology, University of Ottawa for the invaluable comments and suggestions which improved the quality of the thesis.

I am thankful to Dr. Brenda Magajna for the constructive suggestions through out my PhD journey. My special thanks to Dr. Guosheng Wu of his help for the SEM imaging. I extend my sincere thanks to Dr. Susanne Walford, Michael Moore, and all technical staffs of Biology Department, Lakehead University for the untiring support on autoclave, gel-electrophoresis and Nanodrop. My sincere thanks to Albi Sojan from Department of Electrical Engineering, for proving necessary equipment for microbial fuel cell study. I extend my appreciation to NSERC and Ontario Graduate Scholarship for their financial support, which made this research possible.

Heartfelt thanks to my colleagues and friends in Dr. Qin's lab who have offered their encouragement, shared their expertise, and provided moral support during this journey.

I am indebted to my family for their unconditional love, patience, and belief in my abilities throughout this pursuit.

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Chapter 1

This chapter has been published as:

Deng, Z.*, J.R Khatiwada*, L.F. Fan & Wensheng Qin. 2022. Algae for Wastewater Treatment and Production of Biofuels and Bioproducts. International Journal of Environmental Research. 16:34.

https://doi.org/10.1007/s41742-022-00412-x

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1.1 Algae for wastewater treatment and production of biofuels and bioproducts

Abstract

The third-generation biofuel production from algae may be the cheapest and most promising alternative compared to other renewable energy sources. Algae can be effectively grown in a nutrient-rich environment and have the potential to accumulate nutrients and heavy metals from the wastewater, which makes them an extremely attractive means for a more extensive remediation role. Moreover, the algal biomass contains carbohydrates, proteins, vitamins, and other high-value elements and can be used as food and animal feedstock. They have the potential to produce renewable biofuels like biohydrogen and provide a future source of clean energy. The key technical aspects of algal cultivation and harvesting techniques are the major bottleneck for the industrial-scale production of algal biomass and biofuel. This review discussed the current state of large-scale algae cultivation utilizing wastewater as a nutrient source to produce biofuels. Lastly, the research progress, the challenges, and the future development trends in this field are summarized.

Keywords: Microalgae, cultivation, harvest, bioremediation, bioproducts

1.1.1 Water crisis and biological wastewater treatment process

The crisis of water resources might be one of the biggest challenges humans face in the 21st century. With the rapid development of human economic activities, numerous nutrients, mainly

dissolved nitrogen and phosphorus, have been discharged into natural water bodies, leading to eutrophication, which stimulated the growth of unwanted plants such as algae and aquatic macrophytes and deteriorated the water quality (Friberg et al. 2010, Molinuevo-Salces et al. 2019). At the same time, almost 40 percent of the countries and regions worldwide encountered water shortages problems. Therefore, it is urgent to develop sustainable usage of water resources (Liu et al. 2017). One of the important measures to solve this problem is to exploit highly effective, low-cost technology for purifying water deeply.

Based on the origin, wastewater significantly varies in water quality and contaminants. Domestic wastewater generally contains sediment, fecal materials, grease, soaps/detergents, fruit, paper, food scraps, pathogenic organisms, and other debris (Mara 2013). It is released from daily activities such as discharge from residency, restrooms, bathrooms, laundry rooms, and kitchen, respectively. Organic pollutants are the major pollutants in domestic wastewater. Similarly, municipal wastewater includes organic materials, nutrients, metals, inorganic materials, and pathogenic microorganisms (Henze et al. 2008, Zhou et al. 2014). Likewise, the wastewater from industries contains variable contaminants such as oils, pharmaceuticals, chemicals, and other industrial byproducts (Ranade and Bhandari 2014). Depending on the type of industry, the composition of industrial wastewater varies. Industrial wastewater contains organic compounds, inorganic compounds, and heavy metals such as Cd, Cr, and Zn

(Guldhe et al. 2017, Salama et al. 2017). Wastewater discharged from manufacturing or other industrial plants is usually more difficult to treat than domestic wastewater (Lin et al. 2012).

1.1.2 Algae for wastewater treatment and production of biofuels and bioproducts

Energy is the backbone of modern industry and the driving force of the national economy. Since the first industrial revolution in the 18th century, various mineral resources (also known as fossil fuels) have been largely applied. With the rapid increase of human population and industrial development in recent decades, humans are confronted with the dilemma of excessive consumption of resources and energy, even exhaustion of these resources. It is estimated that, at current consumption levels, worldwide reserves will only be sustainable for 40 years of utilization of fossil fuels (Vasudevan and Briggs 2008). Meanwhile, the consumption of fossil fuels leads to more environmental pollution, specifically, an increment of global greenhouse gas (GHG) emissions in the atmosphere and a severe impact on the earth's ecosystem. Therefore, finding renewable and environmentally friendly alternative energy sources is becoming one of the most challenging problems faced by humankind (Pittman et al. 2011).

Microalgae are photosynthetic organisms widely used to produce biofuels and other valuable products because of their higher growth rate, shorter harvesting period, and significantly higher average photosynthetic efficiency compared to other plants (Ghirardi et al. 2000, Khan et al. 2018). In addition, due to simplified unicellular cell structure and rapid cell division rate,

microalgae perform around 10–50 times higher carbon dioxide fixation through photosynthesis compared to terrestrial plants (Li et al. 2008, Williams and Laurens 2010). Algae capture atmospheric carbon dioxide and store as a form of biomass. Green plants capture atmospheric CO_2 by C_3 and C_4 pathways. Most algal species utilized the C_3 pathways (Calvin Cycle) for carbon sequestration. In this pathway, CO_2 is converted to two 3-carbon compounds (3-phosphoglycerate) from 5-carbon compound (Zhang and Liu 2021).

Microalgae can be employed to produce biofuels economically and environmentally sustainably and have a high potential to replace the current consumption rate of fossil fuels (Parmar et al. 2011, Madadi et al. 2021). In addition, microalgae can be effectively grown in nutrient-rich environments and have the potential to accumulate nutrients and metals from the wastewater, making them an extremely attractive means for a more extensive remediation role, particularly during the tertiary treatment phase of sewage (Christenson and Sims 2011). The resulting high-value algal biomass can be harvested as a feedstock for producing biofuels. Thus, the wastewater treatment system can be converted from a single process for treating sewage to a dual-use process to produce more value-added products (Pittman et al. 2011, Mathimani and Pugazhendhi 2019).

Compared to other liquid fuels such as diesel and alcohol, microalgal biodiesel has several promising aspects. These include 1) no ceresin wax; 2) the higher flash point of biodiesel makes it a safer fuel to use, handle, and store; 3) renewable biofuels can be obtained by cultivating

microalgae all year round and not restricted by season; and 4) lower sulfur and nitrogen content which will reduce the SO_2 and NO emissions in the atmosphere (De-Bashan and Bashan 2010, de Godos et al. 2010). Considering these advantages, there have been a growing number of studies in wastewater treatment and sustainable biofuel production by microalgae. This review highlights the recent developments and uses of algae for wastewater treatments and the production of value-added products.

Presently, chemical and physical technologies are widely used in wastewater treatment; however, these methods consume significant amounts of energy and chemicals, making implementing these processes costly (Ahmad et al. 2022). Meanwhile, chemical treatment often leads to secondary pollution, creating additional problems for safe disposal. Therefore, researchers focus on biological-based technology, especially algal-based wastewater treatment (Molinuevo-Salces et al. 2019). Specifically, researchers are dealing with difficulties on further denitrifying and removing phosphorus in the effluent of secondary treatment (Ansari et al. 2017). In the traditional wastewater treatment method, phosphorus was removed using chemical technology. Still, large amounts of phosphorus remain in wastewater after chemical treatment and ultimately reach into the aquatic ecosystem. Due to the ability to use inorganic nitrogen and phosphorus for their growth, microalgae are particularly useful to reduce the concentration of inorganic nitrogen and phosphorus in wastewater (Rasoul-Amini et al. 2014). Besides, microalgae are also efficient absorbers and could accumulate heavy metal (for

example, Zn, Hg, Cd, Cu, and Pb) pollutants from industrial wastewater (Christenson and Sims 2011, Karman et al. 2015). Therefore, purifying the wastewater using microalgae has been one of the most important and promising strategies in wastewater treatment (Table 1).

The following sections present the treatment of wastewater by microalgae:

1.1.2.1 Removal of nitrogen and phosphorus

The discharge and accumulation of nutrient loads into natural water bodies have increased in recent decades due to anthropogenic activities, such as urbanization, industrialization, and modern agricultural practices. These nutrients largely contain nitrogen and phosphorus and are key to eutrophication in marine and freshwater bodies (Lewis et al. 2011). Several studies have demonstrated that conventional sewage treatment systems using aerobic or anaerobic biological degradation could effectively remove most organic and inorganic compounds in the wastewater (Joss et al. 2004, Huang et al. 2005). However, the treated water still contained a significant amount of inorganic compounds such as nitrate, ammonium, and phosphate ions, leading to eutrophication in natural water bodies (Urrutia et al. 1995, Lewis et al. 2011). Although the efficiency of phosphorus removal by chemical treatment is high, adding chemical reagents to the system increases the total cost, and the chemicals left in the sludge might lead to secondary pollution, causing new environmental damage (Khan and Yoshida 2008). The concept of bio-treatment of wastewater with algae to remove nutrients such as nitrogen and

phosphorus was proposed over 60 years ago by Oswald et al. (1957). Since then, there have been numerous laboratories and pilot studies of this process; and several sewage treatment plants using various versions of this system have been constructed (El Hamouri et al. 1995, Mallick 2002, Salama et al. 2017). A series of algal bioreactors, such as High-Rate Algal Pond (HRAP) and Algal Photobioreactor (APBR), have been designed for commercial applications. (García et al. 2000, Soni-Bains et al. 2017, Pereira et al. 2018, Galès et al. 2019).

Algae remove nitrogen from wastewater generally by four possible mechanisms: ammonia volatilization, nitrification, denitrification, and anabolism (Su et al. 2016). Algae can grow autotrophically utilizing nitrogen from inorganic and organic nitrogenous compounds and carbon from carbon dioxide and carbonate compounds. Nitrate, nitrite, and ammonium uptake by algal cells could be utilized to synthesize amino acids and proteins (Garbisu et al. 1991, Vílchez and Vega 1995, Vílchez et al. 1997, Gonçalves et al. 2017, Salama et al. 2017, Wang et al. 2017).

Table 1.1 Removal of various nutrients from wastewater using microalgae.

Algae species	**Wastewater types**	**Phosphate or orthophosphate ion PO^{-3}_4–P (%)**	**Nitrate nitrogen (NO3-N) or N–NH_4 (%)**	**SO^{-2}_4**	**Chemical Oxygen Demand (COD)**	**Turbidity**	**References**
Chlorella sp.	Raw institutional wastewater from the primary septic tank	70.5	98.2	100	84.86	93.73	(Ansari et al. 2017)
Scenedesmus sp.	Raw institutional wastewater from the primary septic tank	80.5	99.7	100	95	94.93	(Ansari et al. 2017)
C. vulgaris	Artificial wastewater and urban wastewater	70	74.3	-	-	-	(Ruiz-Marin et al. 2010)
S. obliquus	artificial wastewater and urban wastewater	60	100	-	-	-	(Ruiz-Marin et al. 2010)
Chlorella vulgaris	Swine wastewater	-	91	-	70	-	(Wang et al. 2015)
Chlorella pyrenoidosa	Swine wastewater	75	92	-	58	-	(Wang et al. 2012)

Chlorella sp.	Wastewater treatment plants	95	96	-	75	-	(Wang et al. 2014)
Micractinium sp.	Wastewater treatment plants	95	94	-	75	-	(Wang et al. 2014)
Chlamydomonas incerta	Palm oil sludge	-	-	-	67	-	(Kamyab et al. 2015)
Scenedesmus sp.	Raw tannery wastewater	97	86	-	80	-	(da Fontoura et al. 2017)
Chlorella vulgaris	Tannery wastewater	99	97-100	60	99	-	(Das et al. 2017)
Chlorella vulgaris-Bacillus licheniformis systems	Synthetic wastewater	80	89	-	86	-	(Ji et al. 2018)
Microcystis aeruginosa-Bacillus licheniformis	Synthetic wastewater	79	69	-	66	-	(Ji et al. 2018)

Phosphorus in wastewater could be removed through different pathways. In the phosphorylation pathway, microalgae directly absorb the phosphorus from the wastewater and transform it into ATP and phospholipids (Ravi et al. 2018). The photosynthesis of microalgae causes an increase in the pH level of water, which results in the removal of orthophosphate and $NH_3 \cdot H_2O$ through precipitation and volatilization, respectively (Delgadillo-Mirquez et al. 2016). Meanwhile, a high pH value also functions as a disinfection agent to some extent (Muñoz and Guieysse 2006). In brief, depending on photosynthesis, algae can remove high concentrations of nutrients, particularly nitrogen and phosphorus, in the wastewater, and can store these nutrients in the forms of organic substances in the algal cells (Delgadillo-Mirquez et al. 2016, Ravi et al. 2018).

There are several advantages in utilizing microalgae to remove nitrogen and phosphorus in the sewage: 1) utilize solar energy for the photosynthesis; 2) the algae grown in the wastewater can help to treat the wastewater by removing nutrients, and bio-fix CO_2 at the same time; 3) there is no exogenous carbon source added in the wastewater by removing nutrients 4) plentiful dissolved oxygen in the effluent; 5) no generation of sludge and no secondary pollution; 6) the algae biomass harvested after wastewater cultivation have a number of usages, such as low-cost fertilizer, animal feed, high-value P-rich products such as polyphosphate, biogas, and biodiesel; and 7) the wastewater after microalgal treatment could be discharged into the water bodies (Jämsä et al. 2017, Khan et al. 2018, Molinuevo-Salces et al. 2019). Presently, the

methods of secondary treatment of wastewater have been greatly modified; however, the total N or total P of the effluents is still too high. If adopting the general biological process to remove nitrogen and phosphorus in the sewage, there will be a problem of shortage in organic carbon sources to sustain microorganisms (Molinuevo-Salces et al. 2019). While under this low N/P ratio circumstance, microalgae can efficiently cost-effectively remove nitrogen and phosphorus. Numerous studies showed that various microalgae species could provide very high removal efficiency. Wang et al. (2010) reported that green algae *Chlorella* sp. has approximately 60-99% of N and 80-100% of phosphate removal efficiency, respectively.

1.1.2.2 Removal of heavy metals

The cell wall of algae consists of exterior and interior layers. The exterior layer is a porous structure composed of multi-microfibrils, including cellulose, pectin, alginate fucoidan, and polygalacturonic acid (Domozych 2016). The main structure of the interior layer is cellulose. On the outside of the cell wall, there are some extracellular products released by algae cells such as peptides and polysaccharides. For example, *Chlorella vulgaris* consists of an average of 32.99 mg g^{-1} protein, 495.44 mg g^{-1} total carbohydrate content, 210.65 mg g^{-1} sulfate content, and 171.97 mg g^{-1} uronic acid (El-Naggar et al. 2020). These multimeric complexes provide a large number of diverse functional groups: carboxyl, amino, aldehyde, hydroxy, thiol, phosphoryl, and carbonyl, in combination with metal ions, and play an important role in the adsorption of heavy metal ions (Sargın et al. 2016, Vetrivel et al. 2017). The multilayer cell

walls and presence of many diverse functional groups on algal biomass, integrated with the trait of easy collection, make algae a highly suitable candidate for binding metal ions and removing heavy metals in the wastewater (Bilal et al. 2018). It has been verified that utilizing algae to bioremediate the water polluted by heavy metals is a highly effective, low-energy-consumption, environmentally friendly method to remove the heavy metals in wastewater (Bilal et al. 2018, Yin et al. 2019).

Generally, the removal of heavy metals via microalgae can be described as a process in which heavy metals are initially absorbed on the surface of the cells, followed by bioaccumulation to the inside of the cells due to the metal uptake and metabolism (Cheng et al. 2019). Adsorption is a passive process that is composed of physical adsorption and bio-adsorption. Heavy metals may bind to algal biomass via negatively charged reactive sites (typically polysaccharides) on the cell surface of algae (Kanamarlapudi et al. 2018). Bioaccumulation is an active process involving metabolic activity and energy consumption (Cheng et al. 2019). In removing heavy metals from wastewater, algae can bioaccumulate 10%-20% of the total heavy metals and bio-adsorb 80-90% of heavy metals; this demonstrated that bio-adsorption is the main approach to removing heavy metals (Lesmana et al. 2009). Furthermore, many types of non-living algae show a high capacity for heavy metal uptake (Pavasant et al. 2006). In living algal cells, due to the given surface/volume ratio, the algal cells' membrane is highly selective only allowing the neutral molecules to pass through. While in the non-living algal cells, the death of cells

disintegrates the cell wall and exposes more functional groups, which enhance the opportunities to bind with heavy metals (Pavasant et al. 2006). On the other hand, if the membrane has lost the function of selective permeability, metal ions can easily pass through the membrane of algal cells. Considering the above reasons, the non-living algae always showed a higher capacity to adsorb heavy metals from the wastewater (Barakat 2011). There is an equilibrium between adsorption and desorption in the process of bio-adsorption. The entire biosorption process of metal removal includes sorption, followed by desorption; i.e., the heavy metals from the algae are diluted and reused by the algae (Kumar et al. 2018). Biotechnological exploitation of biosorption technology for the removal of heavy metal(s) depends on the efficiency of the regeneration of bio-absorbent after metal desorption. It is found that plenty of algae, including marine macroalgal species and freshwater microalgal species, are excellent bio-sorbents for heavy metal ions not only in lab research but also in industrial applications (Mokhtar et al. 2017, Cheng et al. 2019).

Arsenic (Ar), Cadmium (Cd), lead (Pb), Nickel (Ni), chromium (Cr), mercury (Hg), Zinc (Zn), and copper (Cu) are widely available heavy metals in wastewater. Wilke et al. (2006) reported that *Lyngbya taylorii* had the highest biosorption abilities of heavy metals (Cd, Pb, Ni, and Zn) among thirty strains of algae used. The heavy metal absorption ability by *L. taylorii* varies with the highest biosorption of Pb followed by Ni, Cd and Zn respectively. In the study of Inthorn

et al. (2002) who exhibited the highest Hg removal by *Scenedesmus* sp. (97%), Cd removal by *Chlorococcum* sp. (94%) and Pb removal by *Scenedesmus acutu* (89%).

1.1.2.3 Bioaccumulation and degradation of organic compounds/organic pollutants

Organic compounds in wastewater could be important carbon sources for algal growth (Cuellar-Bermudez et al. 2017). Algae play an effective role in accumulating and degrading numerous types of organic compounds, such as hydrocarbons, organic chlorine compounds, organic nitrogen compounds, and metal-organic compounds (Baghour 2019). Bernal et al. (2008) demonstrated that microalgae including Cyanophyceae and Chlorophyceae are very effective to reduce the biochemical oxygen demand (BOD), chemical oxygen demand (COD), and total suspended solids (TSS), observing that 97.3% of BOD, 88% of COD and 88.6% of TSS were removed, respectively. Coogan et al. (2007) reported that algae could accumulate and degrade organic chlorine compounds with high efficiency. Triclosan (TCS), methyl-triclosan (M-TCS), and triclocarban (TCC) in water samples downstream from the wastewater treatment plant were at low ppt concentrations of 50-200 ng l^{-1} and were elevated to ppb concentrations of 50-400 ng l^{-1} in algae collected from same stations (Coogan et al. 2007). Kurashvili et al. (2018) also found that Blue-green alga *Spirulina* could obtain the maximum enrichment capacity removal of 70% of DDT after 15 days of treatment. Megharaj et al. (2000) demonstrated that unicellular green alga and dinitrogen-fixing cyanobacteria metabolized DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) to DDE (1,1-dichloro-2,2-bis(p-

chlorophenyl)ethylene) and DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane), the transformation to DDD was more significant in the case of nitrogen-fixing cyanobacteria. Moreover, seaweed also enhances the remediation of DDT from contaminated soil (Sudharshan et al. 2013).

Algae could also effectively remove metals and organic compounds. Tributyltin (TBT), as a component of antifouling paints, can be toxic to many organisms at a concentration of 1 μg/L or less (Amara et al. 2018). Algae have shown to play an important role in removal of TBT from wastewater (Tam et al. 2002). Jin et al. (2011) demonstrated *Chlorella vulgaris* can have the potential to remove 75 -55 % of TBT. Two major mechanisms are involved during the removal of TBT; at first physico-chemical adsorption of TBT occurs in the algal cell wall and then is gradually absorbed into the cell (Jin et al. 2011). *Ankistrodesmus falcatus* could biodegrade tributyltin through progressive removal of organic groups from the tin, yielding dibutyltin (DBT), monobutyltin (MBT), and inorganic tin, which generally resulted in a reduction of toxicity, and approximately 50% of the accumulated TBT was degraded to DBT over a 4-week period (Gadd 2000). Bioaccumulation and removal of organic solvent is largely determined by the light and photosynthetic aeration by microalgae (Holmes et al. 2020). Finkel et al. (2010) revealed that higher light intensity increases the cell size of phytoplankton (algal and cyanobacteria). Larger cell size signifies an increase in the cellular uptake of pollutants from water sources.

The biodegradation capacity of TBT differed largely between the algal groups. For example, *Chlorella vulgaris* and *Chlorella* sp. both showed a high tolerant ability for TBT. They degraded TBT to DBT, while only *C. vulgaris* is capable of further metabolizing to MBT with a different metabolizing pathway (Tsang et al. 1999, Tang et al. 2018). In the metabolic activities of *Ankistrodesmus falcatus*, a dealkylation sequence normally trimethyl lead was also degraded and formed dimethyl lead and lead (II) compounds (Wong et al. 1987).

Besides organic chlorine, farm chemicals, and metal-organic compounds, algae can remove other organic contaminants in the wastewater. Ren et al. (2017) showed that the algae (*Microcystis aeruginosa*, *Chlorella pyrenoidosa*, and *Pseudokirchneriella subcapitata*) can utilize both organic and inorganic phosphonate for their growth. Similarly, *Ankistrondesmus braunii* and *Scenedesmus quadricauda* degraded over 50% of the low molecular weight phenols contained in olive oil mill wastewater after 5 days of treatment (Pinto et al. 2003). More than 30 azo compounds can be degraded and decolored by the alga *Chlorella pyrenoidosa*, *Chlorella vulgaris,* and *Oscillateria tenuis* with the azo dyes decomposed into simpler aromatic amines due to an induced form of an azoreductase (Jinqi and Houtian 1992, Vijayaraghavan and Yun 2007, Saratale et al. 2011), and further, metabolize the aromatic amines to simpler organic compounds or CO_2 (Acuner and Dilek 2004). There are three intrinsically different mechanisms of assimilative utilization of chromophores for producing algal biomass and the transformation of coloured molecules to non-coloured ones (Sarayu and Sandhya 2012, El-

Kassas and Mohamed 2014). In addition, the interactions of algae and bacteria can also be used to degrade synthetic organic compounds such as phenols, acetonitrile, and p-cresol (Papazi et al. 2012, Sellami et al. 2020).

1.1.3 Cultivation systems of algae using wastewater

Presently, various species of *Scenedesmus* (Ansari et al. 2017, Oliveira et al. 2018), *Chlorella* (Gao et al. 2018, Gao et al. 2019), and *Spirulina* (Sankaran and Premalatha 2018, Bhuvaneshwari et al. 2019) have been successfully utilized for the treatment of primary and secondary sewage (Cuellar-Bermudez et al. 2017). The following are the most used methods for the treatment of wastewater:

1.1.3.1 High-rate algal ponds (HRAP)

High-rate algal ponds are shallow raceway reactors where microalgae and bacteria grow in symbiosis, forming microalgal–bacterial systems, which function to remove organic compounds, nitrogen, and phosphorus (Arashiro et al. 2019, Pham et al. 2020). In these systems, photosynthetic microalgae generate oxygen, which is consumed by the metabolic activities of aerobic heterotrophic bacteria, which decompose organic compounds, generating carbon dioxide, inorganic nitrogen, and phosphorus compounds (Arashiro et al. 2019, Ranjan et al. 2019). These compounds could be assimilated as carbon, nitrogen, and phosphorus sources for algal growth (Ranjan et al. 2019). The higher efficiency of nitrogen and phosphorus removal

requires much less land area for cultivation (Sutherland et al. 2020a). Also, it allows for high-value biological derivatives to be produced with many possible commercial applications, such as fertilizers, animal feed, pharmaceuticals, colouring substances, and raw materials to ferment for producing ethanol or methane (Hussein et al. 2019). Thus, there are growing scientific studies on the economic feasibility of high-rate algal ponds. Presently, the parameters which could effectively increase biomass productivity and remove the nutrients in wastewater have been studied (Sutherland et al. 2020b). Those parameters include environmental factors such as light, photoperiod, light penetration, and temperature; operational factors such as CO_2 availability, hydraulic retention time (HRT), mixing, cultivation mode, algal recycling, nutrients; and biological factors such as invertebrates (Cuellar-Bermudez et al. 2017, Arashiro et al. 2019, Sutherland et al. 2020b, Vassalle et al. 2020). Compared to conventional stabilization ponds, high-rate algal ponds have several advantages, such as: 1) can be utilized in non-productive land, 2) low operating cost, and 3) easy maintenance and management (Ranjan et al. 2019, Sutherland et al. 2020a, Sutherland et al. 2020b). The major disadvantage of HRAP is the lack of economical harvesting methods to collect algal biomass for producing microalgae-based biofuels. Hydrothermal liquefaction (HTL) has been extensively used by researchers to produce microalgae-based biofuels. It offers striking advantages such as the conversion of wet biomass to biofuel without drying and higher energy efficiency (Makut et al. 2020).

1.1.3.2 Activated algae system

An activated algae system is a system in which highly concentrated algae are artificially cultivated, and then algae are further cultured in photoreactors by partial or whole wastewater (Chen et al. 2019). This system effectively removes nitrogen, phosphorus, COD, and BOD and is suitable for the degradation of the organic compounds from artificial wastewater (Chen et al. 2019, Sepehri et al. 2020).

1.1.3.3 Stabilization ponds

Oxidation ponds are designed to treat a range of wastewater, such as industrial and municipal wastewater (Butler et al. 2017). These ponds are effectively used for pathogen, pharmaceuticals, and pesticide removal. There are three major stabilization ponds: anaerobic, facultative, and aerobic maturation ponds, which help maximize the overall pollutant removal (Butler et al. 2017).

1.1.3.4 Immobilized algae

In this process, the algal cells are prevented from moving freely in an aqueous system (Johnson and Wen 2010). Presently, two immobilization types have been defined, active and passive (Gonçalves et al. 2017). In natural or passive immobilization, microalgae are attached to surfaces and grow on them, resulting in the formation of biofilm (Kube et al. 2018). Artificial or active are the most commonly used immobilization methods, such as adsorption, covalent

coupling, and entrapment (Gonçalves et al. 2017). Adsorption is the most common process in filamentous algae, which naturally tend to attach to surfaces and grow on them, resulting in biofilm formation (Kube et al. 2018). Entrapment is another common immobilization method in which microbial cells are entrapped in the network space formed by water-insoluble polymers. Due to the easy manipulation, no restriction between the cells and the carrier, less effect on the activities of microorganisms, and high particle strength, the entrapment in polymers is suitable for most algae to immobilize (Liu et al. 2020). Thus, it is the most widely used technique for algal immobilization. These immobilization methods have some major advantages for treating wastewater: 1) high density of algal cells, especially for small sized microalgal cells; 2) fast reaction speed; 3) high removal efficiency; 4) easy to harvest the algal cells; 5) the water diffused out of the polymers could be collected and reused. Therefore, as an important bioengineering technology, immobilized algae show wide potential applications in wastewater treatment (Efremenko et al. 2012, Liu et al. 2020).

1.1.3.5 Dialysis culture and algal mats

A dialysis culture system uses a semipermeable membrane as a diffusion barrier in culturing microalgae (Bilad et al. 2014). The microalgal population is separated from the wastewater by the diffusion barrier. The solutes containing wastewater (high concentration) as a nutrient source for microalgae growth could pass through the diffusion barrier into the culture compartment (low concentration) under the concentration gradient. Dialysis culture works

under the principle of osmosis, in which osmotic pressure between different culture and wastewater compartments (Klaysom et al. 2013). In the dialysis culture system, the algae growing in the culture compartment should keep a high algal cell density to increase the consumption efficiency of nutrients. The most promising advantage of this system is that the membrane blocks high-molecular-weight substances, and it allows the harvesting of pure algae biomass free of pathogens and microbes (Abdel-Raouf et al. 2012). Although dialysis techniques have not been further exploited for microalgae cultivation in wastewater treatment, it is necessary to carry out further research and explore this area.

Algal mats are an alternative system that utilizes attached macroalgae or other aquatic plants to remove nutrients from wastewater. In this system, the algae (a range of turf-forming species such as *Spirulina*, *Sphacelaria*, *Ectocarpus*, *Ceramium*, *Polysiphonia*, *Herposiphonia*) are grown on a net or mesh and the nutrient-rich wastewater is passed over them (Kiran et al. 2017, Zhou et al. 2017). The algae containing the nutrients are regularly removed mechanically from the mats.

1.1.4 Harvesting algae cells

Due to the small size of microalgal cells (< 20 μm) with a density similar to water, intensive energy consumption is required for harvesting microalgae (Tiron et al. 2017). In the microalgae cultivation system, biomass harvesting can potentially contribute to 20–30% of the total

production costs. Additionally, the microalgal cells exist in aqueous suspension with a large volume of water in broth (0.5-5 kg m^{-3} dry weight). The algae needs to be condensed at least 100-1000 times for industrial applications (Christenson and Sims 2011, Rawat et al. 2013). Traditional solid-liquid separation technology can not directly be applied to algal harvesting. Therefore, searching for suitable and cost-effective microalgal biomass recovery techniques is an ongoing exercise. The harvested biomass is either extracted to obtain its oil or converted into biodiesel through biochemical conversion. The available techniques to recover algal biomass from the algal culture system are natural gravity sedimentation, flocculation, flotation, centrifugation, membrane filtration, immobilization, and electrocoagulation (Xu et al. 2020). Some examples of harvesting methods of microalgal biomass are listed (Table 2).

1.1.4.1 Natural gravity sedimentation

Natural gravity sedimentation is the most common and rapid method used for harvesting and separating microalgal biomass from larger volumes of water and wastewater. Sedimentation efficiency is influenced by the density of flocculants and the concentration of algal cells (Roselet et al. 2019). Although, this method can be operated at a low cost, the harvesting process is slow, unreliable, and the biomass may deteriorate during the settling time (Christenson and Sims 2011).

Table 1.2 Advantages and disadvantages of microalgae harvesting techniques.

Harvesting Technique	Advantages	Disadvantages	References
Natural gravity sedimentation	• Easy and efficient to use • Low cost	• Only useful for larger or heavier microalgae • Long sedimentation periods	(Christenson and Sims 2011, Roselet et al. 2019)
Flocculation	• Low energy consumption • Low cost	• Higher chemical and microbial contamination • Difficult to separate coagulants from algal cells	(Vandamme et al. 2013, Roselet et al. 2019)
Flotation	• Fast and easy technique • Applicable for large scale algae harvest • Low energy input	• Chemical contamination • Harmful to algal cells • Costly	(Zhang et al. 2016, Leite et al. 2019)
Immobilization	• Rapid and reliable method • Cell grows in a high density	• Microbial infestation • Rupture of microalgae beads • Less nutrient transfer because of the formation of biofilm • Costly	(Difusa et al. 2015, Gonçalves et al. 2017)
Centrifugation	• Highly efficient for cell harvesting. • Reliable for small scale harvesting	• Highly energy inefficient • Costly	(Singh and Patidar 2018)
Filtration	• Low cost and high recovery efficiency • No chemical required. • Low energy input	• Time consuming • Applicable for larger, longer, or easily colony-forming microalgae • Membrane fouling and replacement	(Singh and Patidar 2018)

1.1.4.2 Flocculation

Flocculation is the most promising method of dewatering microalgae. There are two types of flocculation methods: chemical and electrical for the separation of microalgae. In this process, microalgae cells are aggregated and can be separated by a simple gravitational method (Vandamme et al. 2013).

1.1.4.3 Flotation

Flotation can be described as a physio-chemical type of gravity separation in which chemical or biological flocculants are often added to the liquid as a pretreatment to increase the particle size and make suspended algae cells flocculate (Branyikova et al. 2018). Then, air or gas is bubbled at the bottom, and the gaseous molecules are attached to the algal cells, reducing the densities, and are carried to the surface of the liquid. Finally, the foam and liquid layers are separated, and the algal cells are collected (Van Haver and Nayar 2017). There are several flotation techniques to generate bubbles/foams, such as dispersed air flotation, dissolved air flotation (DAF), and electrolytic flotation. Dispersed air flotation is a method to form bubbles with a high-speed mechanical agitator and an air injection system. Gas mixing with liquid is introduced to the top and allowed to pass through a disperser (Alhattab and Brooks 2020). The dissolved air flotation (DAF) is a multi-step process. Firstly, a stream of water is pre-saturated with high-pressure air (higher than atmospheric air). Then, the pressure of the stream of water

is reduced to atmospheric pressure. Thirdly, by going through nozzles or needle valves, the liquid containing the dissolved air is injected into a flotation tank and generates bubbles, which rise through the liquid and carry the suspended solids to the surface; the solids can then be skimmed off (Leite et al. 2019). The flotation method is more beneficial and effective than sedimentation in removing microalgae (Zhang et al. 2016).

1.1.4.4 Immobilization

In this process, the algal cell is attached to a neighboring cell by the natural or artificial method. These neighbouring cells help to prevent the algal cell from moving independently in the bioreactor (Gonçalves et al. 2017, Srinuanpan et al. 2018). The filamentous fungi have been widely used for harvesting microalgae. The fungi and microalgae grow together and resulting in the formation of algal biofilms (Gonçalves et al. 2017). Various immobilization methods have been used for microalgae harvest; the alginate gel entrapment process is the most desirable. There are some disadvantages of immobilization, such as microbial infestation, rupture of microalgae beads, and less nutrient transfer due to the formation of biofilm (Difusa et al. 2015). Taking the requirements of effluent quality in algal wastewater treatment systems and high-density culture for algal cells in photobioreactors into account, biofilm separation is a potentially promising harvesting method. Through a biofilm attached to photobioreactors, algal cells are restricted in movement and remain in the photobioreactors. Therefore, algal cells re-

circulate and continue to grow in a high density. As a result, clean effluent with a low concentration of nitrogen and phosphorus is obtained.

1.1.4.5 Centrifugation

This method is the most widely used and preferred process of concentrating algal biomass of smaller cell sizes. The main advantage of this technique is that microalgal cell separation is achieved more rapidly by increasing the gravitational field subjected to the microalgae suspension, thereby concentrating the biomass into a cake with >95% cell harvesting efficiency at 13000 g. The harvest recovery is related to the microalgae sedimentation characteristics, the duration time of broth, and sedimentation rate. This method requires more electricity and is relatively more expensive, is a rapid and reliable method; however higher in cost (Singh and Patidar 2018).

1.1.4.6 Filtration

Filtration is a conceptually simple process to separate liquids and solids. This process is carried out commonly on membranes of various kinds with the aid of a suction pump. The size of the algal cells is the main factor influencing filtration efficiency. In general, larger, longer, or easily colony-forming microalgae display good filtration efficiency, not causing fouling and clogging of the membrane. This method is suitable for harvesting filamentous algae; there are significant costs for membrane fouling and replacement (Singh and Patidar 2018).

Considering all of the harvesting techniques, decisions on choosing the most suitable methods depend on microalgal species and the final product desired (Show et al. 2017). Normally, algae with large cell sizes and high specific gravity are easy to harvest compared to the medium cell size and are reliable to auto-flocculation. For harvesting a particular microalgal species, minimum energy requirements and economic costs should be considered.

1.1.5 Bioproducts production using algae

The harvested algal cells can be used in various sectors, such as pharmaceuticals, chemicals, aquaculture/animal feed, fertilizer, biofuel, and bioremediation (Khatoon and Pal 2015). Biodiesel is a mixture of fatty acid alkyl esters produced through a transesterification process followed by oil extraction (Brennan and Owende 2010, Chowdhury et al. 2019). The process involves mechanical crushing followed by squeezing or supercritical CO_2 extraction technology. On the other hand, algal cells can also be converted into multiple biofuels, primarily methane (CH_4), biomass oil, coke, syngas, hydrogen, etc., by anaerobic digestion (Guldhe et al. 2017). Chemical solvent extraction is the most common method used to extract lipids from microalgae biomass. Chemical solvents such as n-hexane (Saengsawang et al. 2020), mixed methanol-chloroform (2:1 v/v) (Bligh and Dyer 1959), and mixed ether-petroleum (Hidalgo et al. 2019) are effective solvents to extract microalgae lipids.

Bio-oil from algae can be extracted from both dry and wet extraction methods (Ranjith Kumar et al. 2015). Presently, there are some controversies about methods of oil extraction from microalgae. Up until now, it is not conclusive which methods of extraction are most effective (Shin et al. 2018, de Jesus et al. 2019). de Jesus et al. (2019) compared the economics and efficiency of five methods of lipid extraction from wet and dry microalgae using green solvents and reported that dry biomass yields a higher percentage of lipids than using wet biomass. Similarly, lipid extraction from traditional methods such as Folch et al. (1957) and Bligh and Dyer (1959) methods provided higher lipid yields. However, Lardon et al. (2009) demonstrated lipids extracted through the wet extraction method would decrease the amount of energy consumption, and it would greatly facilitate the overall process chain toward positive energy balance. It is estimated that 90% of the processed energy consumption is dedicated to dry lipid extraction (70% when considering wet extraction) (Lardon et al. 2009, Shi et al. 2018). Therefore, optimization of oil extraction techniques would have a significantly positive impact on sustainable and cost-efficient biofuel production processes.

Another important aspect is dealing with the residual biomass of algae after the transesterification process in biodiesel production (Taher et al. 2011). In addition to oils, microalgal biomass contains large quantities of proteins, carbohydrates, and other nutrients, which store 35 to 73% of the accumulated energy, even more than the energy generated by biodiesel produced from oil in algal biomass (Lardon et al. 2009, Tibbetts et al. 2015a).

Therefore, it is pivotal to dispose of and manage the residual biomass from biodiesel production processes; otherwise, huge quantities of nitrogen and phosphate in microalgal residual biomass will lead to serious negative impacts on the environment (Sialve et al. 2009). The residual biomass from biodiesel production processes can be used potentially as animal feed and organic fertilizers. Furthermore, the residual biomass that can be used to produce methane, hydrogen, and ethanol would contribute to a significant reduction in overall energy consumption (Lardon et al. 2009, Guldhe et al. 2017).

1.1.5.1 Food and other high-value products

Algae have been consumed as food and animal feedstock for thousands of years and is one of the most popular in East Asian cuisine. They are reported to contain carbohydrates, proteins, oils, and other essential elements (Tibbetts et al. 2015a, Tibbetts et al. 2015b). The most commonly used algae species for food products are *Chlorella*, *Arthrospira* (spirulina) and *Aphanizomenon* (Niccolai et al. 2019). Likewise, high-value natural products such as medicines and cosmetics have been widely produced from algae (Mehariya et al. 2021, Ahmad et al. 2022).

1.1.5.2 Fertilizer

Dry algal biomass is rich in nutrients and minerals that promote plant growth as a form of organic fertilizer. Saadaoui et al. (2019) highlighted the potential of *Tetraselmis* sp. based

biofertilizer, exhibiting its ability to enhance the growth of date palm (*Phoenix dactylifera*) cultivation. Algal biofertilizers can be a very promising alternative to chemical fertilizers because they: (i) can be cultivated in verities of climates including arid zones (Saadaoui et al. 2019), (ii) have various nutrients and minerals essential for plant growth (Ansari et al. 2021), (iii) enhance beneficial microbial activities in soil (Kang et al. 2021), (iv) helps in CO_2 fixation (Saini et al. 2021).

1.1.5.3 Biohydrogen

Biohydrogen (H_2) is considered a promising alternative clean energy source that produces water after combustion (Hosseini and Wahid 2016). There has been increasing demand for hydrogen in different sectors; for example, in the production of chemicals, electronic devices, food industries, and desulfurization of crude oil in oil refineries and steel industries (Glenk and Reichelstein 2019, Nicita et al. 2020). It is reported that about 95% of current hydrogen production is based on fossil fuels (IRENA 2018, Thomas et al. 2018). Available techniques for hydrogen production are highly cost-ineffective and require sophisticated technology. The bioconversion process of plant biomass (algal biomass) and solid waste is the most common process of renewable and environmentally friendly sources of hydrogen production in recent decades (Kim et al. 2021). In the initial stage of the conversion process, algal biomass and organic wastes are converted into methane by applying chemical reactions and bacteria. Then, organic matter is hydrolyzed and fermented into fatty acids, which are converted into hydrogen.

Biohydrogen has several positive impacts on sustainable energy production, global energy use, and environmental protection. There are several advantages of biohydrogen as an energy source because the combustion of hydrogen is about 50% more efficient than gasoline (Kim et al. 2018), a higher energy yield (122 kJ/g) compared to other hydrocarbon fuels (Sharma et al. 2020), hydrogen batteries can be used as future power for automobiles (Xiong et al. 2019) , and it can be easily stored as metal hydrides such as magnesium hydride, sodium aluminum hydride, lithium aluminum hydride, and palladium hydride (Kojima 2019).

Prospective

Using microalgae to treat wastewater and improve water quality is a potentially promising and ongoing technology and has shown widely applied prospects. However, significant obstacles still need to be overcome before microalgae-based wastewater treatment becomes effective in application. For example, how to harvest the algal biomass more efficiently as well as to reduce the costs in this process; how to effectively separate and collect the liquid products in the algal biorefinery process; how to effectively recover and recirculate the solid and gaseous products in the algal biorefinery process to reduce the energetic input, etc. The following points need to be considered to overcome the current technological constraints:

There should be an effective screening process for the selection of algal strains or species that can efficiently remove different kinds of pollutants on one hand and produce biofuel on the

other. The locally grown microalgae can survive in extreme temperatures and are suitable for biofuel production and bioremediation (Park et al. 2012, Schmidt et al. 2016). Therefore, the selection of indigenous microalgae, which could be remarkably used for wastewater treatment and biofuel production, is another choice. It is testified that local microalgae could adapt to wastewater more easily and remove more nutrients than commercial species (Ansari et al. 2017). Moreover, native microalgae have higher nutritional value, greater tolerance to wastewater toxicity, effectively grow in the sewage, and simplified harvesting method (Schmidt et al. 2016). Using microalgal strains genetically engineered to increase the tolerance level to wastewater is another potential way.

The designing of suitable photobioreactors plays an important role in sustainable biofuel production. It is necessary to exploit highly efficient, large-scale bioreactors which combine microalgal growth in photobioreactors with wastewater treatment, achieving the combination of pollution control and sustainability of resource utilization (Rosli et al. 2020).

The development of effective polymers is essential for the immobilization of algal cells. Presently, several natural polymer derivatives of algal polysaccharides (agar, alginate) and synthetic polymer derivatives (polyacrylamide, polyvinyl) have been experimentally used in the entrapment of polymers method (Vasilieva et al. 2018). Natural polymer derivatives are non-toxic to organisms, have higher diffusivity, are more vulnerable to environmental degradation by microbes, and are less hazardous to produce. Whereas synthetic polymer

derivatives have a higher intensity of immobilization but are toxic to the algal (Emami Moghaddam et al. 2018). Therefore, exploiting compound immobilization polymers and improving their performance is important for future research on immobilization algae technology. Besides, it is necessary to clarify the physiological and biochemical characteristics of immobilized algae and the mechanism of treating wastewater. Also, it is important to explore suitable methods to preserve and activate the immobilization of algae for future industrial mass production.

Moreover, wastewater treatment using algae is cost-ineffective and produces several inhibitors of algal growth. At the same time, it is worth considering finding the optimal algal growth conditions with lower costs. The cost of biofuel production from algae using available techniques is unaffordable. Harvesting, extraction, and separation are the most important energy-consuming processes. Optimization of the biofuel production process and reduction in cost is a major hurdle faced by researchers.

Conclusions

Currently, the third-generation biofuel production from microalgae has drawn the attention of many researchers. Microalgae perform around 10 to 50 times higher carbon dioxide fixation through photosynthesis compared to terrestrial plants and can be effectively grown in nutrient-rich environment and have the potential to accumulate nutrients and heavy metals from the

water, making them an extremely attractive means for a more extensive remediation role. It has the potential to produce renewable biofuels like methane, biodiesel, gasoline, biohydrogen, jet fuel, and provides a future source of clean energy. Microalgae have received more and more interest as a renewable energy and bioproducts source. Current technologies are still not met the human needs for the economic production of algae and the further production of biofuels and bioproducts. The integration of microalgae cultivation for wastewater treatment and biofuel generation is an attractive strategy for reducing energy cost, nutrients input, and CO_2 emissions. The high biomass productivity of wastewater-grown microalgae demonstrates that this cultivation method offers great potential as a viable means for the biofuels industry.

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1.2 Pretreatment Process and Bioethanol Production from Microalgae

Abstract

Bioethanol is gaining attention as a renewable alternative fuel with the potential to reduce greenhouse gas emissions. Traditional feedstocks like corn and sugarcane have limitations in terms of land use and competition with food production, making the search for sustainable alternatives imperative. Microalgae have emerged as a promising feedstock for bioethanol production due to their high growth rates, ability to thrive in non-arable land, and its significant carbohydrate content. Environmental and economic assessments indicate that microalgal bioethanol could offer a sustainable and competitive alternative to fossil fuels. Several techno-economic hurdles, including efficient biomass harvesting and effective pretreatment, must be overcome to make large-scale production viable. Bioethanol production involves fermenting reducing sugar with microorganisms like *Saccharomyces cerevisiae* and *Zymomonas mobilis*, followed by critical steps of distillation and purification for recovering high quality bioethanol. Despite the promising potential of microalgal-based bioethanol production, several techno-economic hurdles, including efficient biomass harvesting and effective pretreatment, must be overcome to make large-scale production viable. Advancements in genetic engineering, optimization of microalgae cultivation and pretreatment of biomass show potential for improving yields and reducing costs. Further research on biomass-specific pretreatment and optimization of the fermentation process is necessary to understand its full potential.

Keywords: Microalgae, Pretreatment, Reducing sugar, Bioethanol

1.2.1 Introduction

1.2.1.1 Biofuel and importance

Biofuels are renewable biological resources and have the potential to serve as a sustainable alternative to fossil fuels. Unlike fossil fuels, which have limited reserve and contribute to greenhouse gas emissions. In contrast, biofuels offer a renewable energy solution that can help mitigate climate change (Bölük and Mert 2014). Biofuels are produced from various feedstocks, including food crops, non-food biomass, and microorganisms (Padder et al. 2024). These feedstocks undergo various biochemical processes to produce biofuels, such as bioethanol, biodiesel, and biogas (Bhushan et al. 2023). An advantage of biofuel is its ability to be seamlessly integrated into existing energy infrastructure, particularly in the transportation sector, which accounts for a substantial portion of global energy consumption and emissions (Verma and Goel 2023). The importance of biofuels extends beyond environmental benefits. Economically, biofuels contribute to energy security by reducing dependence on imported fossil fuels and enhancing national energy independence. The biofuel industry also fosters rural development by creating agriculture, processing, and distribution jobs, thereby supporting local economies (Renzaho et al. 2017). Furthermore, the application of biofuels can support to improved waste management practices by converting agricultural residues and organic waste into valuable energy sources (Koul et al. 2022). This dual role in energy production and waste reduction highlights the multifaceted benefits of biofuels. However, the transition to biofuels

must be managed carefully to avoid potential drawbacks, such as competition with food production in the case of first-generation biofuels, and to ensure that the environmental benefits outweigh the associated costs (Jeswani et al. 2020). Through continued innovation and supportive policies, biofuels can be crucial in achieving a sustainable and resilient energy future. Biofuels can be broadly categorized into four generations based on the feedstock used and the production processes involved (Singh et al. 2023) (Table 1.3).

First-generation biofuels

First-generation biofuels are derived from food crops such as corn, sugarcane, soybeans, and wheat. The primary examples include bioethanol and biodiesel. Bioethanol is produced by fermenting sugars from crops like corn and sugarcane, while biodiesel is made from vegetable oils or animal fats through a process known as transesterification. These biofuels are well-established, with mature technologies and significant production volumes. However, their production raises concerns about food security and environmental sustainability as they compete with food crops for land, water, and other resources (Haji Esmaeili et al. 2020).

Second-generation biofuels

Second-generation biofuels are produced from non-food biomass, including agricultural residues, wood, and lignocellulosic biomass (Haji Esmaeili et al. 2020). Examples of these biofuels include cellulosic ethanol and biobutanol. Cellulosic ethanol is derived from the

breakdown of lignocellulosic biomass into sugars, followed by its fermentation, while biobutanol is produced from the fermentation of biomass by specific microorganisms. These biofuels address the food vs. fuel dilemma by utilizing waste materials and non-food crops, reducing pressure on food resources (Das and Gundimeda 2022). However, their production processes are more complex and costly, requiring advanced technologies and infrastructure.

Third-generation biofuels

Third-generation biofuels are derived from microalgae and other microorganisms (Khatiwada et al. 2022). Algal biofuels, such as biodiesel, bioethanol, and biogas, are produced from microalgal biomass's high lipid and carbohydrate content. Microalgae can grow in non-arable land and saline or wastewater, making them highly sustainable. They also have rapid growth rates and can yield higher biomass per hectare than traditional crops. Despite their potential, third-generation biofuels face high initial production costs and technological challenges that must be addressed through continued research and development (Ananthi et al. 2021).

Fourth-generation biofuels

Fourth-generation biofuels involve advanced technologies such as genetic modification, synthetic biology, and biochemical engineering to produce biofuels with higher efficiency and yield (Aamer Mehmood et al. 2021). With the latest advancements in renewable energy, using cutting-edge technologies and innovative approaches, the fourth-generation biofuels such as

engineered biofuels, biohydrogen, and electrofuels can overcome many limitations of earlier biofuel generations by enhancing production efficiency and sustainability. Fourth-generation biofuels often involve the genetic engineering of microorganisms and plants to enhance their biofuel production capabilities. For example, genetically modified algae can be engineered to grow more rapidly under specific conditions or to produce higher amounts of lipids for biodiesel, thereby increasing the overall biofuel output. Scientists manipulate the genetic makeup of these organisms to increase their efficiency in converting biomass into biofuels. Biohydrogen, produced during the metabolic processes of microorganisms such as bacteria and algae, can be enhanced through genetic engineering and optimization of cultivation conditions, offering a clean fuel alternative with water as its only byproduct. Electrofuels, on the other hand, use renewable electricity to convert CO_2 and water into liquid fuels, providing a carbon-neutral or even carbon-negative energy solution. While these biofuels present numerous advantages, such as improved yields, enhanced waste management, reduced reliance on arable land and minimized competition with food crops, they also faces challenges like high costs and requirement of sophisticated technology, substantial investment in research and development, and ethical and regulatory issues (Shokravi et al. 2021). These biofuels can be tailored for specific applications and have the potential to reduce carbon footprints by integrating renewable energy sources. Continued innovation and supportive policies are essential for these advanced biofuels to contribute effectively to a sustainable future energy.

Table 1.3 Types of biofuels with examples along with their advantages and disadvantages.

Generation	Feedstock	Examples	Advantages	Disadvantages	References
First Generation	Food crops (corn, sugarcane, soybeans, wheat)	− Bioethanol (corn ethanol, sugarcane ethanol) − Biodiesel (from vegetable oils, animal fats)	− Established technology. − Well-developed infrastructure − Significant production volumes	− Competes with food production − Can lead to food price increases − Extensive land and water use	Haji Esmaeili et al. (2020)
Second Generation	Non-food biomass (agricultural residues, wood, forest biomass)	− Ethanol − Biobutanol	− Reduces competition with food crops − Utilizes waste materials − Can be grown on marginal lands	− More complex and expensive production processes − Requires advanced technologies and infrastructure	Das and Gundimeda (2022)
Third Generation	Microalgae and other microorganisms	− Algal Biofuel (biodiesel, bioethanol, biogas) − Biogas − Biohydrogen	− High biomass yield − Can be cultivated in non-arable land and wastewater − Rapid growth rates − Potential for high lipid and carbohydrate content	− High initial costs − Technological challenges − Need for further research and development to achieve commercial viability	Ananthi et al. (2021)
Fourth Generation	Genetically modified microalgae and microorganism	− Genetically engineered biofuels (advanced bioethanol, advanced biodiesel) − Electrofuels (produced using renewable energy)	− High efficiency and yield − Can be tailored for specific applications. − Potential to significantly reduce carbon footprint − Integrates renewable energy sources	− Ethical and regulatory concerns − High research and development costs − Requires sophisticated technology and infrastructure − Limited current production scale	Shokravi et al. (2021)

1.2.1.2 Bioethanol and its importance as a renewable energy source

Bioethanol is a cleaner form of renewable fuel which can be derived from biomass such as corn, sugarcane, and lignocellulosic materials. This biofuel plays a pivotal role as a sustainable energy solution because of its zero greenhouse gas emissions and serves as a renewable alternative to gasoline (Rezania et al. 2020). Bioethanol can be blended with gasoline to power vehicles, thereby decreasing the carbon footprint of the transportation sector, which is a major contributor to global emissions (Duarte Souza Alvarenga Santos et al. 2021). Additionally, bioethanol production supports agricultural economies by providing an alternative market for crops and agricultural residues (Renzaho et al. 2017). It also promotes energy independence by reducing reliance on imported fossil fuels. However, the sustainability of bioethanol depends on the feedstock and production methods used, necessitating advancements in technology and practices to maximize its environmental and economic benefits.

Bioethanol production is one of the major components of the global biofuel industry, with production levels varying by region and driven by factors such as government policies, technological advancements, and market demand (Ebadian et al. 2020). In 2022, the global production of bioethanol was approximately 106 billion liters (Fig. 1.1). The United States, Brazil, and the European Union are the largest bioethanol producers, accounting for a substantial share of global production. The United States is the largest producer of bioethanol (53%), with production reaching over 60 billion litres in 2020.

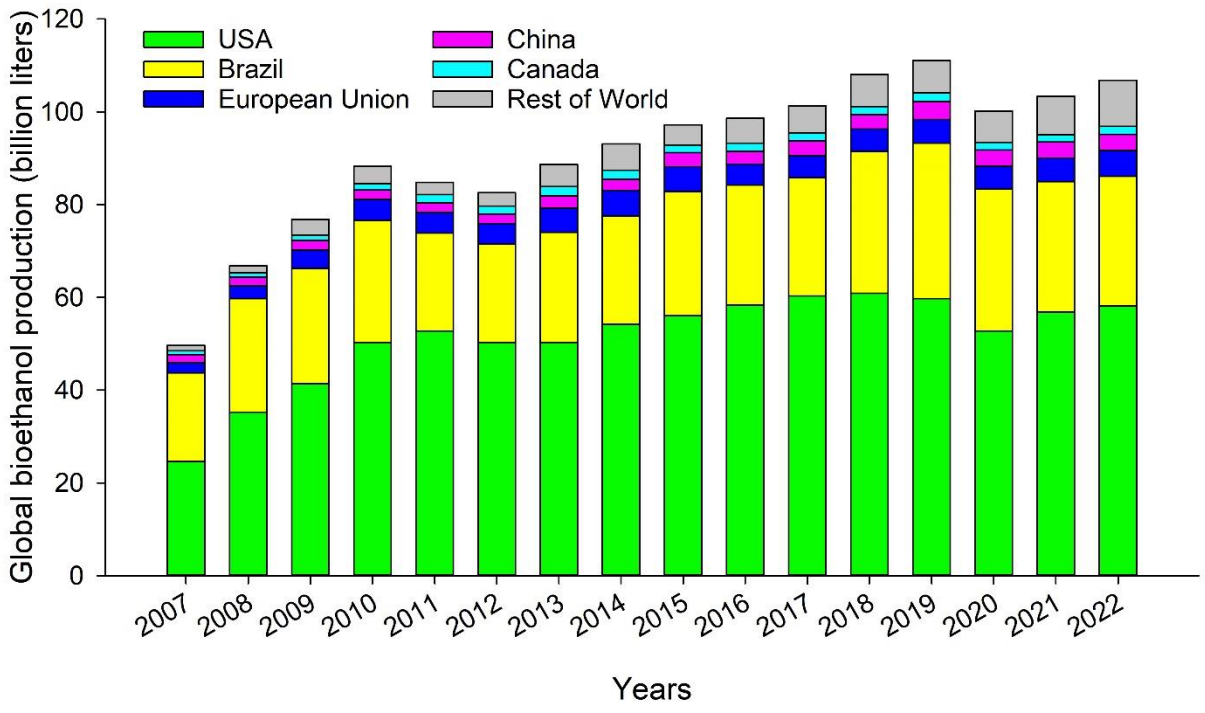

Figure 1. 1 Global Ethanol Production by Country or Region (billion liters) from 2007 to 2022 (Data Source: Renewable Fuels Association (https://ethanolrfa.org/markets-and-statistics/annual-ethanol-production, date of access: May 16, 2024).

Brazil is the second-largest producer, primarily utilizing sugarcane as feedstock for ethanol production (Santos et al. 2023). In 2022, Brazil produced over 30 billion liters of bioethanol (Fig. 1.1). The EU countries collectively produced over 6 billion liters of bioethanol in 2020 (Fig. 1.1). The projected statistics for 2050 suggest a massive increase in global bioethanol production, driven by advancements in technology, policy support, and increasing demand for renewable energy sources. While the USA and Brazil continue to lead in bioethanol production contributing 89% and 68% respectively, significant growth in China (113%) indicates a major shift towards renewable energy (Fig. 1.2).

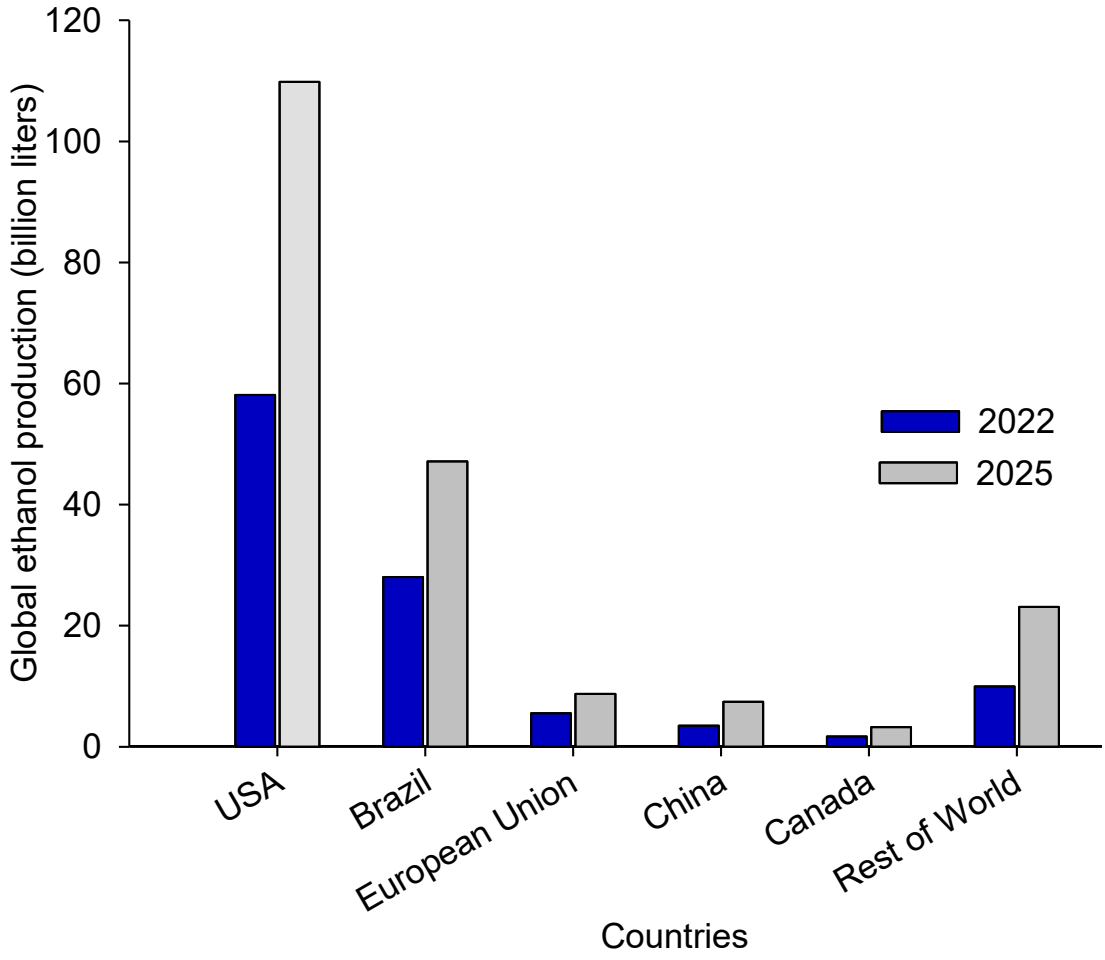

Figure 1. 2 Precited trends of global bioethanol production by 2050 by different countries (Data Source: Renewable The bars refer to global ethanol production in billions liter. The data derived from Fuels Association (https://ethanolrfa.org/markets-and-statistics/annual-ethanol-production, date of access: May 16, 2024).

1.2.1.3 Major feedstocks used for bioethanol production

Corn

In the United States, corn is the predominant feedstock for bioethanol production, with a significant portion of the country's corn crop dedicated to this purpose (Lin et al. 2023). In 2022,

approximately 40% of the U.S. corn harvest was used for ethanol production, equating to about 5.2 billion bushels (RFA. 2024). This utilization not only supports the biofuel industry but also contributes to reducing greenhouse gas emissions, as corn-based ethanol can lower carbon emissions by up to 52% compared to conventional gasoline (Desta et al. 2021). The extensive use of corn for bioethanol highlights its critical role in the U.S. renewable energy landscape, driving economic benefits and advancing environmental sustainability efforts.

Sugarcane

Sugarcane is a highly efficient and prominent feedstock for ethanol production in Brazil, where it constitutes a significant part of the biofuel industry (Karp et al. 2021). In 2022, Brazil produced approximately 28.01 million litres of ethanol from sugarcane, accounting for a substantial portion of the country's total ethanol output (Fig. 1.1). Sugarcane has a higher ethanol yield, which can reach about 7,000 litres per hectare, compared to corn's 3,800 liters per hectare (Colussi et al. 2023). Moreover, the energy balance of sugarcane ethanol is favorable, with an energy return on investment (EROI = 8) (Shelar et al. 2023). These statistics suggested that it produces eight times more energy than it consumes. This high yield and energy efficiency, coupled with the ability to use byproducts like bagasse for electricity generation, make sugarcane a leading and sustainable feedstock for ethanol production.

Lignocellulosic biomass

Lignocellulosic biomass, composed of cellulose, hemicellulose, and lignin, represents a promising feedstock for bioethanol production due to its abundance and sustainability. Globally,

lignocellulosic biomass production is estimated to be over 220 billion tons annually, with sources including agricultural residues (such as corn stover and wheat straw), forestry waste, and dedicated energy crops like switchgrass and miscanthus (Bhatt et al. 2018). Despite its potential, the conversion of lignocellulosic biomass to bioethanol is challenged due to its complex structure, which requires advanced pretreatment and enzymatic hydrolysis technologies. Recent advancements have improved yields, with some pilot plants achieving conversion efficiencies of up to 50 to 60%, though commercial viability still necessitates further technological and economic optimization (Cherubini and Stromman 2010).

Bioethanol production traditionally relies on agricultural feedstocks like corn, sugarcane, and wheat. These crops are rich in fermentable sugars or starches that can be converted into ethanol through biochemical processes involving fermentation. However, the use of such feedstocks raises significant sustainability concerns. The diversion of food crops to biofuel production can exacerbate food security issues and increase the food prices (Ananthi et al. 2021). Additionally, the cultivation of these crops requires substantial land, water, and agrochemical inputs, which can lead to environmental degradation. To address these challenges, researchers have been exploring alternative feedstocks that do not compete with food production and have a lower environmental footprint. Among the various resources, microalgae hold the most promising alternative. This review explores the potential of microalgae as a feedstock for bioethanol production, highlighting their unique characteristics and advantages.

1.2.1.4 Microalgae as a feedstock for ethanol production

Microalgae are diverse photosynthetic microorganisms in various aquatic environments, including freshwater and marine ecosystems. They are known for their rapid growth rates and high biomass productivity, attributes that make them attractive for biofuel production (Ahmed et al. 2022). Unlike terrestrial plants, microalgae do not require arable land and can grow in saline or brackish water, reducing the competition for freshwater resources. One of the key advantages of microalgae is its ability to accumulate significant amounts of carbohydrates, which are essential for bioethanol production (Ibrahim et al. 2023). Microalgal biomass can contain up to 50% carbohydrates by dry weight, depending on the species and cultivation conditions. These carbohydrates can be broken down into monomeric sugars through enzymatic hydrolysis, followed by fermentation of sugars to produce ethanol. Microalgae also offer additional environmental benefits. They have the ability to sequester carbon dioxide (CO_2) during photosynthesis, helping to mitigate greenhouse gas emissions (Dahai et al. 2024). Furthermore, microalgae can be cultivated in wastewater, where they can absorb nutrients and contaminants, thus contributing to water purification (Khatiwada et al. 2022, Khatiwada et al. 2023).

Selecting microalgae strains for bioethanol production involves several critical factors to ensure efficiency, sustainability, and economic viability. Key criteria include high biomass productivity, with strains exhibiting rapid growth rates and efficient photosynthesis alongside high carbohydrate content (Maity and Mallick 2022). Strains should be easy to cultivate, require minimal and inexpensive nutrients, and tolerate various environmental conditions (Khatiwada et al. 2022). Genetic stability and favourable metabolic pathways that synthesize higher carbohydrates over

lipid accumulation are essential, so the different pretreatment processes can convert the carbohydrate into fermentable sugars. Here, larger cell sizes and less rigid cell walls would facilitate in efficient harvesting and pretreatment process (Buchmann et al. 2019). Resistance to contaminants and pathogens is vital to maintain high yields. Studies showed that microalgae species which are compatible with co-cultivation with other microorganisms enhance yields (Naseema Rasheed et al. 2023). Sustainability considerations include efficient water and land use, effective carbon sequestration, and low production costs, while economic viability can be improved by producing valuable by-products. Finally, strains must be non-toxic, environmentally safe, and compliant with regulatory standards (Su et al. 2023, Cruz and Vasconcelos 2024). By assessing these criteria, suitable microalgae strains can be selected for effective bioethanol production.

Several microalgae species have been employed for bioethanol production. Under optimal conditions, *Chlorella* spp. can accumulate up to 70% of their dry weight as carbohydrate content. Studies have shown that *Chlorella zofingiensis* can achieve a carbohydrate content of approximately 66.7% under nitrogen-deprived conditions (Zhu et al. 2014). Acebu et al. (2022) reported that the carbohydrate content reached 3.6 gL^{-1} of the dry weight of *Chlorella vulgaris* ESP-31 cultivated in unsterilized swine wastewater, with a bioethanol yield of about 4.2 gL^{-1}. Genetic modifications have been employed to increase the starch content in microalgae species. Qu et al. (2020) reported a maximum bioethanol yield of 61 g/L from mutant *Chlamydomonas* sp. QWY37 cultivated in swine wastewater. In contrast, a genetically modified strain of *Synechococcus* sp. PCC 7002 can produce an ethanol yield of 0.1 g of ethanol per day under laboratory conditions (Kopka et al. 2017).

1.2.1.5 Pretreatment of microalgal biomass for bioethanol production

Microalgal cell wall composition

Microalgae have diverse cell wall compositions which vary significantly among species. Generally, the cell wall consists of polysaccharides (cellulose, hemicellulose, and pectin), glycoproteins, and sometimes, silica or calcium carbonate (Md Nadzir et al. 2023). For instance, the cell walls of green algae (Chlorophyta) are mainly composed of cellulose and hemicellulose (Baudelet et al. 2017), while diatoms (Bacillariophyta) have silica-based cell walls (Domozych et al. 2012). The complexity and rigidity of these components necessitate effective pretreatment methods to break down the cell wall structure.

Physical pretreatment methods

The robust nature of microalgal cell walls poses a significant challenge to the efficient extraction of intracellular components such as lipids, proteins, and carbohydrates. To enhance the accessibility of these valuable compounds, physical pretreatment methods are often employed to disrupt the cell walls. Various physical pretreatment techniques, including mechanical disruption, thermal treatment, ultrasonic disruption, and hydrodynamic cavitation, have been used for microalgal biomass processing.

Mechanical Disruption

Mechanical disruption methods apply physical forces to break down cell walls. These techniques include bead milling, high-pressure homogenization, and grinding.

Bead milling

Bead milling involves the agitation of microalgal cells with small beads inside a milling chamber (Liu et al. 2021b). The beads collide with the cells, causing mechanical shear forces that break the cell walls. This method effectively disrupts hard-to-break cell walls and can be scaled up for industrial applications. However, the high energy consumption and potential for heat generation are significant drawbacks. Studies have shown that bead milling can achieve high disruption efficiencies for various microalgal species, including *Chlorella* and *Parachlorella* (Liu et al. 2021b, Liu et al. 2022).

High-pressure homogenization

In high-pressure homogenization, microalgal suspensions are forced through a narrow valve at high pressure, generating intense shear and cavitation forces that disrupt cell walls. This method is highly effective and can be applied continuously, making it suitable for large-scale operations (Günerken et al. 2015). However, it requires significant energy input and can generate heat, which may require cooling mechanisms to prevent thermal degradation of sensitive compounds (Magpusao et al. 2021).

Grinding

Grinding involves the physical abrasion of cells using mills or grinders. While it is a straightforward and low-cost method, it may not be as effective as bead milling or homogenization for certain robust microalgal species. Additionally, grinding can be labor-intensive and may require multiple passes to achieve sufficient disruption (Safi et al. 2014).

Thermal treatment

Thermal pretreatment methods utilize heat to weaken or break down microalgal cell walls. These methods include autoclaving, microwave heating, and freeze-thaw cycles.

Autoclaving

Autoclaving involves subjecting microalgal biomass to high temperatures (typically 121°C) and pressures (15 psi) for a specified duration. The heat and pressure cause thermal and mechanical stresses that disrupt cell walls. Autoclaving is effective for cell disruption and sterilization, making it suitable for laboratory-scale studies. However, it is energy-intensive and may not be practical for large-scale applications (Lee et al. 2010).

Microwave

Microwaves use electromagnetic radiation to generate heat within microalgal cells. The rapid heating causes water inside the cells to vaporize, creating pressure that disrupts the cell walls. This method is efficient and can achieve uniform heating but requires specialized equipment and may have high energy costs. Studies have shown that microwave treatment can enhance lipid extraction efficiency from microalgae such as *Auxenochlorella protothecoides* (Zhang et al. 2022) and also useful for reducing sugar extraction from *Chlorella pyrenoidosa* (Rana and Prajapati 2021).

Freeze-thaw cycles

Freeze-thaw cycles involve repeatedly freezing and thawing microalgal suspensions. The formation of ice crystals during freezing and their subsequent melting during thawing cause

physical damage to the cell walls. This method is simple and low-cost, but it is generally less effective than other thermal treatments and may require multiple cycles to achieve significant disruption (Loureiro et al. 2023).

Ultrasonic disruption

Ultrasonic disruption uses high-frequency sound waves to create cavitation bubbles in microalgal suspensions. The collapse of these bubbles generates intense shear forces that disrupt cell walls (Chisti 2003). Ultrasonic disruption is a versatile method that can be scaled up for industrial applications. It is effective for freshwater and marine microalgae and can be combined with other pretreatment methods to enhance overall efficiency. However, the process can be energy-intensive and may generate heat, requiring cooling systems to prevent thermal degradation of sensitive compounds. Additionally, the high initial cost of ultrasonic equipment can be a barrier for some applications (Günerken et al. 2015). This method has been successfully used to enhance the extraction of lipids, proteins, and carbohydrates from various microalgal species. For example, using ultrasonic pretreatment, studies have demonstrated improved lipid extraction from Chlorella and Nannochloropsis sp. (Krishnamoorthy et al. 2023).

Hydrodynamic cavitation

Hydrodynamic cavitation involves the generation of cavitation bubbles due to rapid flow of liquid through a constriction, such as a venturi or an orifice plate. The collapse of these bubbles generates intense shear forces that disrupt microalgal cell walls. The subsequent increase in pressure causes the bubbles to collapse, generating localized high temperatures and pressures that disrupt cell walls

(Lee and Han 2021). This method is a scalable and energy-efficient method for cell disruption. It can be integrated into continuous processing systems, making it suitable for large-scale applications (Günerken et al. 2015). However, the effectiveness of this method depends on the specific design of the cavitation device and the properties of the microalgal suspension. Additionally, it may require optimization of operating parameters, such as flow rate and pressure, to achieve maximum disruption efficiency (Lee and Han 2021). Hydrodynamic cavitation has been used to enhance the extraction of intracellular components from various microalgal species. For instance, research has shown that this method can improve lipid extraction from *Nannochloropsis salina* (Lee and Han 2021).

Chemical methods

Chemical pretreatment methods are essential in breaking down microalgae's rigid cell walls to enhance biofuel production's efficiency, particularly bioethanol. The cell walls of microalgae are complex structures composed of polysaccharides, proteins, and lipids, which act as barriers to the extraction of intracellular components. Effective chemical pretreatment can significantly improve the accessibility of these components, thereby increasing the yield of fermentable sugars.

Acid hydrolysis

Acid hydrolysis involves using strong acids to break down the cell wall polysaccharides into simpler sugars (Hernández et al. 2015). The acidic environment hydrolyzes the glycosidic bonds in the polysaccharides, converting them into monosaccharides and oligosaccharides (Liu et al. 2021a). This method is particularly effective for microalgae with high carbohydrate content.

Concentrated sulfuric acid (H_2SO_4) and hydrochloric acid (HCL) are commonly used acids for hydrolysis due to their effectiveness in breaking down complex carbohydrates (Hong and Wu 2020). It can achieve high yields of fermentable sugars but requires careful handling due to its corrosive nature and the need for neutralization post-treatment. The efficiency of acid hydrolysis depends on several parameters, including acid concentration, temperature, and reaction time. Higher acid concentrations, elevated temperatures, and longer reaction times generally enhance the breakdown of cell wall components but can also lead to the formation of inhibitory by-products, such as furfural and hydroxymethylfurfural (HMF), which can hinder subsequent fermentation processes (Harun and Danquah 2011).

Alkaline hydrolysis

Alkaline pretreatment uses bases like sodium hydroxide (NaOH) or potassium hydroxide (KOH) to solubilize cell wall components, particularly lignin, hemicellulose, and other complex carbohydrates (Loow et al. 2016). This method effectively breaks down the ester bonds within the cell wall matrix (Xu et al. 2016). Key factors influencing the efficiency of alkaline hydrolysis include the concentration of the alkaline agent, temperature, and reaction time. Higher concentrations and temperatures increase the disruption of cell walls but also raise the risk of degradation of desirable intracellular components. Optimal conditions must be carefully determined to balance cell wall disruption and preservation of valuable metabolites (Chen et al. 2013).

Oxidative treatments

Oxidative treatments involve the use of oxidizing agents to break down the organic components of the cell wall (Qiu et al. 2020). These treatments lead to the oxidative cleavage of cell wall polysaccharides and proteins, enhancing the release of intracellular components. Hydrogen peroxide (H_2O_2) and Ozone (O_3) are commonly used due to their strong oxidizing properties and ability to break down into water and oxygen, leaving no harmful residues (Qiu et al. 2020). It is often used in combination with other treatments to enhance cell wall disruption. Ozone is a powerful oxidizing agent that can effectively degrade cell wall components (Barone et al. 2021). It is used in aqueous or gaseous forms and can enhance the permeability of the cell wall. The effectiveness of oxidative treatments depends on the concentration of the oxidizing agent, exposure time, and temperature. Higher concentrations and longer exposure times generally improve cell wall disruption but can also lead to excessive oxidation and degradation of intracellular components. The process parameters must be optimized to maximize cell wall disruption while minimizing damage to valuable metabolites (Lee et al., 2017).

Organic solvents

Organic solvents are used to dissolve cell wall components and enhance the permeability of the cell wall (Yap et al. 2014). These solvents can disrupt the lipid bilayers and protein networks, facilitating the release of intracellular components. Alcohols like methanol (CH_3OH) and ethanol (C_2H_5OH) are effective in disrupting cell walls due to their ability to solubilize lipids and proteins (Kusmiyati et al. 2023). They are often used in combination with other treatments to enhance cell wall disruption. Chloroform ($CHCl_3$) and acetone (CH_3COCH_3) are used for their strong solubilizing properties and ability to disrupt cell wall structures (Shankar et al. 2019). They are

particularly effective for microalgae with high lipid content. The effectiveness of organic solvent treatments depends on the type of solvent, concentration, and exposure time. The choice of solvent and its concentration must be carefully considered to balance cell wall disruption and the preservation of intracellular components. Additionally, the potential toxicity and environmental impact of solvents must be taken into account (Kim et al. 2013).

Enzymatic hydrolysis

Crude enzymes secreted by microorganisms offer a potential solution for breaking down the complex microalgal cell walls, facilitating the release of intracellular components for subsequent biofuel production (Guo et al. 2017). Crude enzymes secreted by microorganisms typically contain a mixture of hydrolytic enzymes, each targeting different components of the microalgal cell wall (Monjed et al. 2021). The major enzyme classes involved include cellulases, hemicellulases, pectinases, and proteases (Guo et al. 2017).

Cellulase

Cellulose is a primary component of many microalgal cell walls, and cellulase enzymes hydrolyze it into glucose units (Maffei et al. 2018). Cellulases are classified into endoglucanases, which cleave internal bonds within cellulose chains; exoglucanases, which remove cellobiose units from the chain ends; and β-glucosidases, which hydrolyze cellobiose into glucose (Juturu and Wu 2014). Cellulases bind to cellulose fibers and initiate hydrolysis by cleaving internal bonds (endoglucanases), releasing cellobiose units (exoglucanases), and ultimately producing glucose

(β-glucosidases). The combined action of these enzymes results in the depolymerization of cellulose into fermentable sugars (Juturu and Wu 2014).

Hemicellulases

These enzymes target hemicellulose, a heterogeneous polysaccharide consisting of various sugars such as xylose, mannose, and arabinose (Qaseem et al. 2021). Hemicellulases include xylanases, which degrade xylan into xylose, and mannanases, which break down mannan into mannose (Qaseem et al. 2021). Hemicellulases target the various sugar linkages within hemicellulose, breaking it down into its constituent monosaccharides. Xylanases cleave the β-1,4-glycosidic bonds in xylan, while mannanases hydrolyze the β-1,4-mannosidic bonds in mannan (Saini et al. 2022). This enzymatic action releases sugars such as xylose and mannose, which can be further utilized in biofuel production (Qaseem et al. 2021).

Pectinases

Pectinases degrade pectin, a complex polysaccharide found in some microalgal cell walls. These enzymes include polygalacturonases, which cleave the α-1,4-glycosidic bonds in pectin, and pectin lyases, which break down pectin by β-elimination (Li et al. 2024). Pectinases hydrolyze the complex pectin network by cleaving α-1,4-glycosidic bonds and breaking down the pectin structure. Polygalacturonases and pectin lyases work together to depolymerize pectin into galacturonic acid and other smaller molecules, aiding in cell wall disintegration (Satapathy et al. 2020).

Proteases

Proteases hydrolyze proteins in the cell wall, facilitating the breakdown of proteinaceous components and potentially enhancing the accessibility of polysaccharides to other enzymes. The degradation of microalgal cell walls by crude enzymes involves a synergistic action of various enzymes, each contributing to the overall hydrolytic process. Proteases hydrolyze peptide bonds within proteins in the cell wall matrix, breaking down protein structures and facilitating the action of polysaccharide-degrading enzymes (Esfandi et al. 2019). The proteolytic activity helps to weaken the cell wall and enhance the accessibility of other enzymes to their respective substrates (Udatha et al. 2015).

1.2.1.6 Importance of crude enzyme for cell wall degradation and biofuel and value-added products production

The application of crude enzymes for microalgal cell wall degradation has implications for biofuel production. By breaking down the cell wall, these enzymes facilitate the release of intracellular lipids, carbohydrates, and proteins, which can be converted into biofuels and other valuable products (Guo et al. 2017). Enzymatic degradation of the cell wall releases glucose, xylose, and other sugars, which can be fermented by microorganisms such as *Saccharomyces cerevisiae* or *Zymomonas mobilis* to produce bioethanol (Xiros et al. 2016). Lipids, primarily stored in microalgal cells as triacylglycerols (TAGs), are crucial feedstocks for biodiesel production. Enzymatic cell wall degradation enhances lipid extraction efficiency by disrupting the cell wall and releasing intracellular lipids, making them more accessible for downstream processing (Guo et al. 2017). In addition to biofuels, the enzymatic breakdown of microalgal cell walls can yield valuable by-products such as amino acids, pigments, and polysaccharides. These by-products can

be utilized in various industries, including food, pharmaceuticals, and cosmetics, enhancing the overall economic viability of microalgal bioproduct production (Mehariya et al. 2021).

Several studies have demonstrated the effectiveness of crude enzyme preparations for microalgal cell wall degradation. For example, a study Bader et al. (2020) reported using a crude enzyme cocktail from *Trichoderma harzianum* to degrade the cell walls of *Chlamydomonas reinhardtii*, resulting in a significant increase in reducing sugar (22.4 $g.L^{-1}$) efficiency. Another study by Kim et al. (2015) utilized crude enzymes from *Trichoderma reesei*. to hydrolyze the cell walls of *Nannochloropsis* sp., achieving high yields of fermentable sugars (1.70 $g.L^{-1}$) for bioethanol and lipids (77% of biomass) for biodiesel production. Guo et al. (2017) used *Cellumonas xylanilytica* and *Escherichia coli* JM109 in crude enzyme production for the microalgal biomass hydrolysis and reported the higher production of reducing sugar and facilitated in lipid productivity. Prajapati et al. (2015) reported the efficient pretreatment of microalgae species using the crude enzyme (20%: v/v) produced by *Aspergillus lentulus*. Enzymatic pretreatment induces significant changes in the structure and composition of biomass (Prajapati et al. 2015), affecting its susceptibility to subsequent conversion processes.

Despite the promising potential of crude enzymes for microalgal cell wall degradation, several challenges remain. These include optimizing enzyme production and activity, cost-effective enzyme recovery and reuse, and the development of enzyme formulations targeted to specific microalgal strains. Future research should focus on the genetic engineering of microorganisms to enhance enzyme production, the discovery of novel enzymes with superior activity and stability, and the integration of enzymatic processes with other bioconversion technologies for improved

efficiency and sustainability (Zhang et al. 2012). Crude enzymes secreted by microorganisms offer a viable solution to overcome the challenge of microalgal cell wall degradation in biofuel production. An enzyme cocktail comprising a mixture of cellulases, hemicellulases, pectinases, and proteases can work synergistically to break down complex cell wall structures, facilitating the release of valuable intracellular components, thereby enhancing lipid extraction and fermentable sugar production, contributing to the efficient conversion of microalgal biomass into biofuels (Guo et al. 2017).

1.2.1.7 Bioethanol production processes

Fermentation

Fermentation is a metabolic process that converts fermentable sugars (glucose, xylose, mannose, and other monosaccharides) into bioethanol and other by-products in anaerobic conditions by microorganisms (Karimi et al. 2021). The general reaction represents the conversion of glucose into ethanol and carbon dioxide by fermentative microorganisms (Eq. 1)

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$ ……...…… (Eq. 1)

Several microorganisms can ferment sugars into bioethanol, each with unique characteristics and efficiencies. The most used microorganisms include:

Saccharomyces cerevisiae (Baker's Yeast)

S. cerevisiae is the most widely used yeast for industrial bioethanol production due to its high ethanol tolerance, rapid fermentation rates, and ability to ferment hexose sugars like glucose and

mannose. However, it has limited ability to ferment pentose sugars such as xylose (Ochoa-Chacón et al. 2022).

Pichia stipites

This yeast can ferment hexose and pentose sugars, making it suitable for lignocellulosic biomass hydrolysates containing a mixture of these sugars. However, *P. stipitis* has lower ethanol tolerance and slower fermentation rates than *S. cerevisiae* (Phaiboonsilpa et al. 2020).

Zymomonas mobilis

This bacterium is known for its high ethanol yield and productivity, and it can ferment glucose and fructose effectively. It operates via the Entner-Doudoroff pathway, which has a higher theoretical ethanol yield compared to the glycolytic pathway used by *S. cerevisiae* (Todhanakasem et al. 2020).

Escherichia coli

Genetically engineered strains of *E. coli* can ferment a broad range of sugars, including hexoses and pentoses. These strains are often modified to enhance ethanol production and reduce by-product formation (Bañares et al. 2021).

***Clostridium* spp.**

Clostridium species, particularly *Clostridium thermocellum* and *Clostridium acetobutylicum*, are anaerobic bacteria capable of fermenting cellulose and hemicellulose directly into ethanol and other biofuels (Hon et al., 2017). *C. thermocellum* is known for its robust cellulolytic capabilities,

which enable the direct conversion of lignocellulosic biomass into ethanol without the need for separate enzyme production (Hon et al., 2017). *C. acetobutylicum*, on the other hand, is more commonly associated with acetone-butanol-ethanol fermentation but has been engineered for enhanced ethanol production, making it a versatile microorganism in biofuel research (Xue et al., 2017).

Fermentation conditions

The optimal fermentation conditions for maximizing ethanol production vary significantly among microorganisms, primarily influenced by temperature and pH levels (Table 1.4). For *Saccharomyces cerevisiae*, moderate temperatures of around 30°C and slightly acidic pH levels of approximately 5.0 are highly effective, yielding up to 410 g kg^{-1} h^{-1} of suspended solids (Lin et al. 2012). At higher temperatures of 37°C, *Saccharomyces cerevisiae* still performs well with a 90% ethanol yield (Liu and Shen 2008). Co-culture fermentation of *Saccharomyces cerevisiae* and *Pichia stipitis* also benefit from elevated temperatures and slightly acidic conditions, achieving notable ethanol yields (Table 1). *Zymomonas mobilis*, another efficient ethanol producer, performs optimally at 30°C with a pH of 5.5, producing up to 23.3 $g.L^{-1}$ of ethanol, while more neutral pH conditions result in moderate yields (Table 1.4). Genetically modified *Escherichia coli* demonstrates the best ethanol yield of 25 $g.L^{-1}$ at 37°C with a near-neutral pH of 6.8. Slightly alkaline conditions (pH 8.2) at lower temperatures (31°C) (Table 1.4).

Distillation and Purification

Distillation is the primary method for ethanol recovery and purification in industrial settings. This process involves heating the fermented mixture to separate ethanol from water and other components based on differences in boiling points (Zentou et al. 2019). The most common setup is a fractional distillation column, where repeated condensation and vaporization cycles increase the ethanol concentration (Bessa et al. 2012). Azeotropic distillation techniques are employed to achieve high-purity ethanol (over 95% purity), often using additional agents like benzene or cyclohexane to break the ethanol-water azeotrope (Kunnakorn et al. 2013). Membrane separation and molecular sieves are also used as alternative or supplementary methods to distillation. These methods can enhance ethanol purity by selectively allowing ethanol molecules to pass through while retaining water and other impurities (Górak and Sorensen 2014).

Table 1.4 Bioethanol production by different microorganisms under different temperature and pH conditions.

Species	**Temperature (°C)**	**pH**	**Ethanol yield**	**References**
Saccharomyces cerevisiae	30	5.0	410 g kg^{-1} h^{-1} of suspended solids	Lin et al. (2012)
Saccharomyces cerevisiae	37	5	90 %	Liu and Shen (2008)
Saccharomyces cerevisiae	30	5	9.64 $g.L^{-1}$	El-Dalatony et al. (2016)
Zymomonas mobilis	30	7	9.35 $g.L^{-1}$	Condor et al. (2022)
Zymomonas mobilis	30	5.5	23.3 $g.L^{-1}$	Mazaheri and Pirouzi (2022)
Zymomonas mobilis	33	4	7.9 % (v/v) ethanol	Khoja et al. (2018)
Saccharomyces cerevisiae + *Pichia stipitis*	40	5.5	12 $g.L^{-1}$	Srilekha Yadav et al. (2011)
Trichoderma reesei + *Pichia stipitis*	35	4.8	0.411 $g.g^{-1}$	Pothiraj et al. (2014)
Escherichia coli	31	8.2	8.9 g ethanol dm^{-3}	Lopez-Hidalgo et al. (2017)
Escherichia coli	37	6.8	25 $g.L^{-1}$	Munjal et al. (2012)

Efficiency and energy considerations are crucial in the distillation and purification process. Traditional distillation is highly energy-intensive, accounting for a significant portion of the total energy consumption in ethanol production. Advanced techniques such as vacuum distillation and pressure-swing distillation have been developed to reduce energy requirements by lowering the boiling points of ethanol and water, thus requiring less heat (Kiss 2014). Additionally, integrating heat recovery systems can capture and reuse the energy from the distillation process, further improving efficiency. The use of membrane technologies and molecular sieves, while initially costly, can provide significant energy savings and higher ethanol recovery rates in the long term (Saw et al. 2019, Saini et al. 2020). The optimization of these processes is essential to minimize operational costs and environmental impact, making bioethanol production more sustainable and economically viable.

Challenges and limitations of bioethanol production

Bioethanol production faces several technical and economic challenges, especially in large-scale operations. One of the primary technical challenges is the efficient conversion of lignocellulosic biomass into fermentable sugars, which requires advanced pretreatment methods and enzymatic hydrolysis (Khan et al. 2021, Alawad and Ibrahim 2024). These processes are often energy-intensive and costly, impacting the overall economic viability of bioethanol. Additionally, the fermentation process itself can be limited by the tolerance of microbial strains to inhibitors present in the hydrolysate, which can reduce ethanol yields (Baral and Shah 2014). Economically, the high initial capital investment required for biorefineries, coupled with fluctuating feedstock prices, can make large-scale bioethanol production less competitive compared to fossil fuels (Raj et al. 2022).

Furthermore, market volatility and policy changes can affect the stability and predictability of bioethanol production economics (Tanaka et al. 2023).

Issues with microalgal biomass harvesting and pretreatment present significant hurdles in the bioethanol production process. Efficiently collecting and transporting large volumes of biomass is logistically challenging and can be cost-inefficient. Pretreatment methods, necessary to break down the complex cellular structure, often involve harsh chemicals or extreme conditions, which can generate byproducts that inhibit fermentation (Jönsson and Martín 2016). These methods also require substantial energy inputs, further complicating the sustainability and economic feasibility of bioethanol production. Improvements in pretreatment technologies, such as the development of more efficient processes, are critical to overcoming these barriers.

Conclusion

The fermentation of pretreated microalgal biomass to produce bioethanol is a complex, yet feasible process involving various microorganisms, optimal conditions, and innovative strategies to maximize yields. Preparation of the fermentation medium, selection and optimization of microbial strains, control of fermentation conditions, and effective downstream processing are all critical aspects of this process. Moreover, advancements in genetic engineering, optimization of fermentation process, and the design of efficient bioreactor are crucial for enhancing bioethanol production from microalgae. These efforts contribute greatly to the development of sustainable biofuels. Furthermore, integrating novel biotechnological methods and using cutting-edge research in metabolic engineering can further boost the efficiency and viability of microalgae-based bioethanol production.

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1.3 Study Area, Sample Collection, and Objectives

1.3.1 Study area

The study area lies around Thunder Bay, Ontario, Canada and is characterized by ancient Precambrian rock formations, including granite and gneiss (Brzozowski et al. 2023). Glacial activity during the last ice age left behind rugged landscapes, numerous lakes, and dense forests (Ellis et al. 2011). Thunder Bay experiences a humid continental climate, characterized by warm summers and cold winters. Lake Superior helps in moderating temperatures, leading to milder conditions compared to inland areas (Blanken et al. 2011). The summers have longer daylight hours while the winters are cold. The area is rich in biodiversity, with a variety of terrestrial and aquatic habitats supporting diverse flora and fauna. Thunder Bay is situated along the shores of Lake Superior, the largest of the Great Lakes, which harbors unique freshwater ecosystems. The surrounding boreal forests are home to a range of plant species, contributing to the organic matter and nutrients that sustain aquatic life, including microalgae.

1.3.2 Sample collection and microalgae isolation

Water samples were collected from Thunder Bay and nearby areas, Ontario, Canada (Table 1.5 and Fig. 1.3). We collected water samples into the depth of 10 cm and placed into 50 ml autoclaved tube samples from the depth of 10 cm and were place and kept into icebox and transferred to the laboratory. Coarse materials and plants parts were removed by filtration using

cheese cotton cloth and samples were cultivated in BG-11 medium for 2-3 weeks. Then, samples were spread onto BG-11 agar plates and were placed in continuous illumination at room temperature (22 ± 2 °C) for a period of two to three weeks. Individual algal colonies were again replated, to obtain the single microalgal strain (Fig. 1.4). Purely isolated microalgal strains were cultured in BG-11 medium in a one L flask with a working volume of 500 ml under 16 hours light and 8 hours dark (16L:8D) cycles with continuous aeration

Figure 1.3 Map showing the sampling locations.

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Table 1.5 Water sampling locations with number of species found in each location.

S.N.	Place	Number of isolates	Coordinates		Site
			Y	X	
1	Boulevard Lake	3	48.4335	89.2166	Lake
2	Burk Lake	2	48.6545	89.4444	Lake
3	Cascades	3	48.5036	89.2341	River
4	Cloud lake	3	48.1361	89.5391	Lake
5	Current river	2	48.4691	89.2011	River
6	Dog Lake	5	48.6820	89.5861	Lake
7	Dorion	7	48.7855	88.5310	Lake
8	Hazelwood Lake	3	48.5961	89.3202	Lake
9	Kakabeka Falls	6	48.4166	89.6317	River
10	Lake Tamlyn, Lakehead University	3	48.4202	89.2631	Lake
11	Loch Lomond	3	48.2856	89.2998	Lake
12	Mapleward creek	6	48.6820	89.5861	Creek
13	Marina park, Lake superior	5	48.4335	89.2166	Lake
14	Murillo creek	3	48.4138	89.4975	Creek
15	Nipigon	5	49.0244	88.2531	River
16	Oliver Lake	3	48.2649	89.5826	Lake
17	Saganaga Lake	4	48.2412	92.8330	Lake
18	Silver Falls	5	48.6912	89.6420	River
19	Walkinshaw Lake	3	48.6322	89.0732	Lake
20	Whitefish Lake	2	48.2221	89.9837	Lake

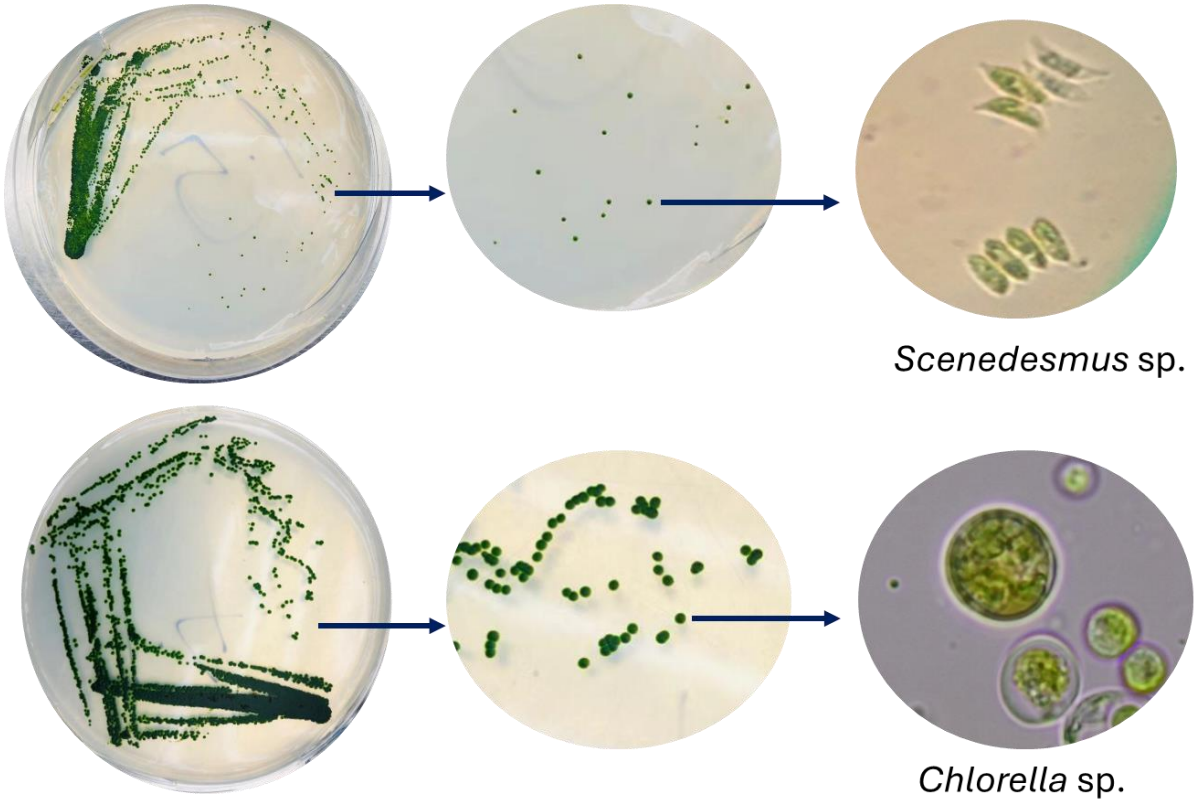

Figure 1.4 Process of microalgae isolation

1.3.3 Microalgae identification

1.3.3.1 DNA extraction and PCR

The DNA extraction of selected microalgal strains was carried out using the CTAB (cetyltrimethylammonium bromide) method. This procedure was chosen for its efficacy in isolating high-quality DNA from various plant materials, including microalgae, due to its ability to remove polysaccharides and secondary metabolites (Schenk et al. 2023). Nucleotide sequences of the 18S gene were downloaded from the NCBI GenBank database and aligned with BioEdit Version 7.0.5 (Hall 1999). Maximum likelihood (ML) analysis was carried out in

MEGA7 (Kumar et al. 2016) to show the phylogenetic relationships among the species based on the 18S gene sequences.

Initially, the microalgal culture was harvested by centrifugation at 12,000 rpm for 10 minutes, and the supernatant was discarded. The cell pellet was then resuspended in 500 µL of pre-warmed (65°C) CTAB extraction buffer, which contained 2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 1.4 M NaCl. The mixture was transferred to a 2 mL microcentrifuge tube, to which 20 µL of proteinase K (20 mg/mL) was added. The tube was incubated at 65°C for 30 minutes with occasional gentle mixing to ensure cell lysis and the release of DNA. Following incubation, 500 µL of chloroform:isoamyl alcohol (24:1) was added to the lysate, and the mixture was gently inverted. This step was performed to denature proteins and separate them from the nucleic acids. The mixture was then centrifuged at 10,000 rpm for 10 minutes at room temperature to separate the phases. The upper aqueous phase, containing the DNA, was carefully transferred to a new microcentrifuge tube. To precipitate the DNA, 0.6 volumes of ice-cold isopropanol were added to the aqueous phase, and the tube was gently mixed by inversion. The sample was left at -20°C for 30 minutes to enhance DNA precipitation. After the incubation period, the DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the DNA pellet was washed with 1 mL of 70% ethanol to remove residual salts and CTAB. The ethanol wash was followed by centrifugation at 10,000 rpm for 5 minutes at room temperature. The ethanol was carefully removed, and the DNA pellet

was air-dried at room temperature to ensure complete evaporation of the ethanol. The dried DNA pellet was then resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). To ensure complete dissolution of the DNA, the tube was incubated at 37°C for 10 minutes with occasional gentle mixing. The purity and concentration of the extracted DNA were assessed using a NanoDrop spectrophotometer by measuring absorbance at 260 nm and 280 nm. The size and quality of the DNA was further confirmed by agarose gel electrophoresis.

The PCR amplification of the 18S rRNA gene was amplified using universal primers (Khaw et al. 2020, Song et al. 2023):

18ScomF1: 5'- GCTTGTCTCAAAGATTAAGCCATGC - 3'

18ScomR1: 5'- CACCTACGGAAACCTTGGTTACGAC- 3'

ss3: 5'- GATCCTTCCGCAGGTTCACCTACGGAAACC - 3'

ss5: 5'- GGTGATCCTGCCAGTAGTCATATGCTTG- 3'

The PCR reaction was carried out in 50 µL working volume with 1 µL of template DNA, 1 µL each of forward and reverse primers, 25 µL of 2x Taq PCR Master Mix (containing Taq DNA polymerase, dNTPs, and $MgCl_2$), and 22 µL of nuclease-free water. The PCR amplification was carried out in a thermal cycler with the following cycling conditions: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. A final extension was performed at 72°C for 5 minutes to ensure complete amplification of the target gene.

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. A 1 kb DNA ladder had been used as a molecular weight marker. The gel was visualized under UV light to confirm the presence and size of the amplified 18S rRNA gene. The amplified PCR products were purified using a PCR Purification Kit (BioBasic) according to the manufacturer's instructions. The quality of purified PCR products was further verified by measuring ratio of the absorbance 260/280 nm from NanoDrop spectrophotometer. The purified amplicons were sequenced using Sangar sequencer. Reference sequences of the 18S gene were downloaded from the NCBI GenBank database. The combined query and reference sequences aligned using BioEdit (version 7.0.5) (Hall 1999). A maximum likelihood (ML) tree was reconstructed using MEGA7 (Kumar et al. 2016) to determine the phylogenetic relationships among the species based on the 18S gene sequences.

1.3.3.2 Morphology

Microalgal strains were identified morphologically based on characteristics such as cell shape, cell size, cell wall, chloroplasts number and shape, flagella, colony structure, eyespots (stigmata), pyrenoids, mucilage envelopes, and motility (Bellinger and Sigee 2015, Wehr et al. 2015).

1.3.4 Rationale for the work

Canada is rich with its diverse geography, encompassing various climatic zones and habitat types, and provides a wide range of niches for life. From the boreal forests of the north to the temperate lakes of the south, organisms exhibit remarkable adaptability to different environmental conditions. Due to the vast expanse of freshwater ecosystems, Canada harbors a rich and diverse array of microalgae species (Park et al. 2012). These microscopic organisms play crucial roles in aquatic ecosystems, contributing to nutrient cycling, primary productivity, and food webs (Carrasco Navas-Parejo et al. 2020). Despite their significance, the diversity and distribution of freshwater microalgae in Canada remain relatively understudied (Wehr et al. 2015). The exploration of freshwater microalgae biodiversity in the Northwestern Ontario region holds significant potential for various applications, including bioremediation, biofuel production, biomolecule extraction, and bioelectricity generation.

Rationale 1

The exploration of biodiversity in freshwater microalgae within Northwestern Ontario is crucial for understanding the health and stability of aquatic ecosystems in this region. The locally adapted microalgae with higher growth rate with higher lipid and biomolecule production capabilities can be used for bioremediation and biofuel production.

Rationale 2

Freshwater microalgae have demonstrated significant potential for bioremediation (Khatiwada et al. 2023), the process of using biological organisms to remove or neutralize contaminants from the wastewater. They can absorb and accumulate heavy metals, nutrients, and other pollutants from the aquatic environment, making them effective agents for bioremediation (Khatiwada et al. 2022). Selection of specific species with high bioremediation capabilities is needed. This could lead to the development of targeted bioremediation strategies to address pollution from wastewater, thereby improving water quality and protecting public health and the environment.

Rationale 3

Microalgae are a promising source of bioethanol and other valuable biomolecules due to their high photosynthetic efficiency and ability to accumulate lipids and carbohydrates. Bioethanol, a renewable biofuel, can be produced from the fermentation of microalgal biomass, offering a sustainable alternative to fossil fuels (Kaur et al. 2022, Yirgu et al. 2023). Additionally, microalgae can produce a wide range of biomolecules, including proteins, lipids, and pigments, which have applications in the pharmaceutical, cosmetic, and food industries (Deng et al. 2022). It is necessary to identify strains with high biomass productivity and desirable biochemical profiles. Therefore, this study optimized the efficient bioethanol production processes and extracted valuable biomolecules. This study therefore will contribute to the bioeconomy and reduce dependency on non-renewable resources.

Rationale 4

Microalgae Microbial Fuel Cells (MMFC) have emerged as a promising solution with versatile applications, encompassing wastewater treatment, microalgae cultivation for commercial enzyme production, and the generation of sustainable bioelectricity (Nayak and Ghosh 2019, Hadiyanto et al. 2022, Ribeiro et al. 2022). Such integrated approach not only offers the potential to reduce the environmental impact of industrial effluent, conserve resources, contribute to climate change mitigation, and safeguard the environment, but also enables the generation of renewable energy. The optimal fuel cell and dilution of wastewater is necessary to produce higher microalgal biomass and bioelectricity.

1.3.5 Research objectives

The overall objective of the research was to isolate and identify the microalgal species with higher biomass productivity along with lipid and other value-added products production utilizing wastewater. The specific objectives of this study were to:

1. Explore the diversity of freshwater microalgae using both molecular and morphological approaches and determine the growth rate, biomass yield, nutrients removal efficiency, and lipid productivity.
2. Evaluate the bioremediation potential of native freshwater microalgae.

3. Enhance the microalgae pretreatment process for higher reducing sugar extraction and bioethanol and biomolecule production.
4. Optimize bioelectricity and xylanase enzyme production employing microalgae microbial fuel cells (MMFCs).

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Chapter 2

2.1 Isolation and Identification of Freshwater Microalgae in Northwestern Ontario, Canada for Sustainable Lipid and Biomass Production

Abstract

Native freshwater microalgae play a crucial role in biofuel production due to their unique characteristics and environmental suitability. This study isolated and identified 75 microalgae isolates with higher biomass and lipid production capacity from various freshwater ecosystems across Northwestern Ontario, Canada. Of these isolated strains, five with higher lipid production and growth were identified using molecular and morphological approaches. Among four different photoperiods, the 16L:8D light/dark cycle produced significantly higher biomass and lipids in *Chlorella sorokiniana, Chlorella vulgaris*, and *Chlamydomonas* sp. (JRK10). The results indicated a rich diversity of microalgae species from the region of Northern Ontario, with promising lipid accumulation isolates with high biomass productivity. This study highlighted the potential for utilizing native microalgae strains as the source of renewable lipids and biomass for biofuel and biomolecule production.

Keywords: Native microalgae; Northern Ontario; Molecular phylogenetics; Morphology; Cell density; Biomass production; Lipid content

2.1.1 Introduction

Microalgae are diverse groups of organisms and their global population is declining alarmingly, with habitat loss being a primary factor contributing to the biodiversity crisis among microalgal communities (Ahmed et al. 2022). Eutrophication, climate change, pollution, and acidification have further exacerbated threats to microalgae diversity and distribution (Salles and Mercado 2020). The taxonomic status of many microalgae species remains unknown in various geographic regions, leading to frequent new species discoveries compared to other organisms (Jagielski et al. 2022). Canada is prosperous with its diverse geography, encompassing various climatic zones and habitat types, and provides a wide range of niches for life. From the boreal forests of the north to the temperate lakes of the south, organisms exhibit remarkable adaptability to different environmental conditions. Due to the vast expanse of freshwater ecosystems, Canada harbors a rich and diverse array of microalgae species (Park et al. 2012). These microscopic organisms play crucial roles in aquatic ecosystems, contributing to nutrient cycling, primary productivity, and food webs (Carrasco Navas-Parejo et al. 2020). Despite their significance, the diversity and distribution of freshwater microalgae in Canada remain relatively understudied (Wehr et al. 2015). Northwestern Ontario, with its pristine lakes and diverse ecosystems, offers a unique environment for the study of microalgae diversity (Estepp and Reavie 2015). Northwestern Ontario harbors the world's largest freshwater lake, such as Lake Superior, alongside small ponds, rivers, and streams, each

nurturing a rich array of microalgal species (Ivanikova et al. 2007). Microalgae, comprising various microscopic organisms such as Cyanophyceae (blue-green algae), Chlorophyceae (green algae), Porphyridiophyceae (red algae), and Bacillariophyceae (diatoms) and exhibiting remarkable adaptability and ecological versatility (Guiry and Guiry 2024).

Cyanophyceae, also known as cyanobacteria or blue-green algae, are characterized by their blue-green pigmentation due to the presence of phycocyanin and chlorophyll α (Gupta et al. 2013). They are often found in freshwater, marine, and terrestrial habitats. Some cyanobacteria are capable of nitrogen fixation, converting atmospheric nitrogen into a form usable by plants and other organisms (Jiménez-Ríos et al. 2024). While many cyanobacteria are beneficial, some species can produce toxins under certain conditions, leading to harmful algal blooms that can have detrimental effects on aquatic ecosystems and human health (Brooks et al. 2016).

Chlorophyceae are a diverse group of green algae that are most found in freshwater habitats, although they can also inhabit marine and terrestrial environments. They are unicellular, colonial, or multicellular organisms with a wide range of morphologies, including filamentous, coenocytic, and parenchymatous forms (Guiry and Guiry 2024). Green algae serve as important primary producers in aquatic ecosystems, contributing to oxygen production and serving as food for various aquatic organisms.

Porphyridiophyceae, or red algae, are primarily marine organisms, although some species are found in freshwater habitats. They are characterized by their red pigmentation, which is due to the presence of phycoerythrin and phycocyanin pigments, masking the green color of chlorophyll (Guiry and Guiry 2024). Red algae are typically multicellular, with complex morphologies ranging from filamentous to sheet-like structures. They are important components of marine ecosystems, forming extensive underwater habitats such as coral reefs and providing food and shelter for many marine organisms. Diatoms are a major group of microalgae found in both freshwater and marine environments, as well as in damp soil. They are characterized by their unique silica cell walls, which form intricate and often ornate patterns (Guiry and Guiry 2024).

Diatoms are unicellular organisms, but they can form chains or colonies. They are significant primary producers in aquatic ecosystems, contributing a large portion of the world's oxygen production through photosynthesis (Naselli-Flores and Padisák 2023). Diatoms play crucial roles in nutrient cycling and are a vital food source for various aquatic organisms, including zooplankton and filter-feeding invertebrates.

Microalgae are known for their biochemical diversity, synthesizing an extensive range of compounds including proteins, lipids, carbohydrates, vitamins, pigments, and bioactive molecules (Ibrahim et al. 2023). These organisms have evolved unique metabolic pathways to survive in diverse ecological niches, leading to the synthesis of specialized biomolecules with

commercial and industrial significance (Abu-Ghosh et al. 2021). They serve as a vital source of nutraceuticals and functional food ingredients. Species such as *Spirulina* and *Chlorella* are rich in high-quality proteins, essential amino acids, vitamins (e.g., B12), and omega-3 fatty acids. These biomolecules serve as antioxidant, anti-inflammatory, and immune-modulating properties, making them valuable in enhancing human health and combating nutritional deficiencies (Abidizadegan et al. 2021). Microalgae can produce bioactive compounds with therapeutic potential. For instance, compounds derived from microalgae, like polyunsaturated fatty acids (PUFAs) and carotenoids, exhibit anti-cancer, anti-inflammatory, and neuroprotective properties (Talero et al. 2015).

Due to high lipid content and rapid growth rate, microalgae are recognized as the sustainable source for the production of biofuels such as biodiesel, biohydrogen and bioethanol (Srivastava et al. 2020). Lipids extracted from microalgae can be converted into biodiesel, offering a renewable alternative to fossil fuels (Kumar et al. 2021). Microalgae-based biofuels have the potential to reduce greenhouse gas emissions and mitigate the environmental impacts associated with conventional fuels (Dahai et al. 2024). Moreover, some of the microalgae species play a crucial role in bioremediation by absorbing heavy metals, nutrients, and organic pollutants from wastewater and industrial effluents (Khatiwada et al. 2022, Khatiwada et al. 2023). This capability not only helps in cleaning up polluted water bodies but also contributes to nutrient recycling and sustainable waste management.

Despite the importance of freshwater microalgae, several challenges hinder their comprehensive assessment in Northern Ontario, Canada. Limited taxonomic expertise, inadequate sampling protocols, and lack of standardized molecular databases pose obstacles to accurately cataloging and identifying microalgal diversity (Prasanthi et al. 2020). Furthermore, anthropogenic impacts such as pollution, habitat alteration, and climate change threaten the stability of freshwater ecosystems, potentially altering the composition and dynamics of microalgal community. Therefore, this study involved extensive sampling of various freshwater bodies including lakes, rivers, and streams across the Northwestern Ontario region, and aimed to isolate and characterize these microalgae strains to identify potential candidates with higher capacity for lipid and biomolecule production through a combination of microscopical, molecular and biochemical techniques.

2.1.2 Materials and methods

2.1.2.1 Isolation and identification of microalgae strains

Sampling location, isolation and detailed identification processes have been described in section 1.3.1 – 1.3.3. Purely isolated microalgal strains were cultured in BG–11 medium and 1 ml of culture (OD680 = 1.52 nm) was inoculated in a 4-L glass jar with a 3-L working volume under four different light cycles: 0-hour light and 24-hours (0 L:24 D) dark, 12-hour light and

12-hour dark (12L:12D), 16-hour light and 8-hour dark (16L:8D), and 24-hour light and 0-hour dark (24L:0D) cycles along with continuous aeration.

2.1.2.2 Parameters measurements

Algal cell growth quantification via hemocytometer microscopy

Microalgal cell density was examined by counting the cell number. The cell density was measured by 0.1-mm Tiefe deep Neubauer Improved haemocytometer under a compound microscope. Ten µL of culture sample was added to counting chamber of haemocytometer. Among the 25 squares on the central part of the haemocytometer, only five squares were chosen for the cell count and the cell density was calculated as below:

$$Cell\ density\ (\frac{cells}{ml}) = \frac{Average\ number\ of\ cells\ counted \times Dilution\ factor}{Volume\ of\ the\ square\ (0.000004\ ml)}$$

Biomass measurement

The microalgal dry weight was quantified on the final day of experiment (day 18). Microalgal biomass was harvested by centrifuging 50 mL of microalgal culture at 4000 rpm for 10 minutes. To remove the salts and other impurities, the microalgal pellet was washed twice using distilled water and kept in an oven at 80 °C for 48 hours. The amount of dry biomass was measured gravitmetrically.

Lipid extraction

The total lipids from algal biomass were extracted by chloroform:methanol (1:1, v/v) method developed by Bligh and Dyer (1959) with some modifications. Briefly, 0.1 g of the dry algal biomass was mixed with a mixture of chloroform, methanol, and distilled water (1:2:0.8, v/v/v). The algal cells were broken down by ultrasonication for 10 minutes followed by centrifugation at 13000 rmp for 10 minutes. The supernatant was transferred to a pre-weighted Eppendorf tube (W_1 g), and the cell pellet was re-extracted with the mixture of chloroform and methanol (1:2, v/v). The mixture was centrifuged again, and then the supernatant was collected in the same pre-weighed Eppendorf tube. Chloroform and water were then added to the supernatant to form a final ratio of 1:1:0.9 (chloroform:methanol:distilled water, v/v/v). The mixture was homogenized thoroughly by vertexing followed by centrifugation at 13000 rpm for 5 min. The top layer was then removed, and the bottom layer was evaporated and dried at 80 °C until reaching a constant weight (W_2 g). The total lipid content was calculated by subtracting W_1 from W_2 and presented as a percentage of the dry weight as follows:

Percentage of total lipid content (% dry weight) (L_T) = $W_L/W_A \times 100$

Where, W_L is the lipid weight and W_A is the microalgae dry weight.

2.1.3 Statistical analysis

All the numerical variables were checked for normality before conducting the parametric test. One-way ANOVA was used to test for differences in the algal dry biomass and lipid contents among the photoperiods, followed by Tukey's HSD post hoc test for pairwise differences. All the experiments were performed in triplicates, and the results are shown as mean ± SD (standard deviation). Differences between treatments were evaluated at $P < 0.05$. All statistical analyses were carried out in R v. 4.4.0 (R Development Core Team 2024).

2.1.4 Results and discussion

2.4.1.1 Identification of microalgae species

Phylogenetic analysis

A total of 75 microalgal strains were isolated from 20 different water sources around Thunder Bay, Ontario, Canada. Among them, 10 promising isolates (JRK1, JRK2, JRK3, JRK4, JRK5, JRK6, JRK7, JRK8, JRK9, and JRK10) were screened based on the rapid growth and biomass characteristics. These strains were identified by the comparison of the 18S rRNA gene sequences from NCBI databases. Based on the molecular data, seven isolates were clustered into *Chlorella* spp. clade and two isolates were clustered into *Scenedesmus* spp. clade (Fig. 2.1 and 2.2). Based on the maximum likelihood (ML) tree, analysis microalgae strain JRK02 was identified as *Scenedesmus acutus.* The uncorrected genetic distance between JRK02 and

Scenedesmus acutus was lower than 4%. Similarly, the JRK09 strain was identified as *Scenedesmus dimorphus* and the uncorrected genetic distance between them was 2%.

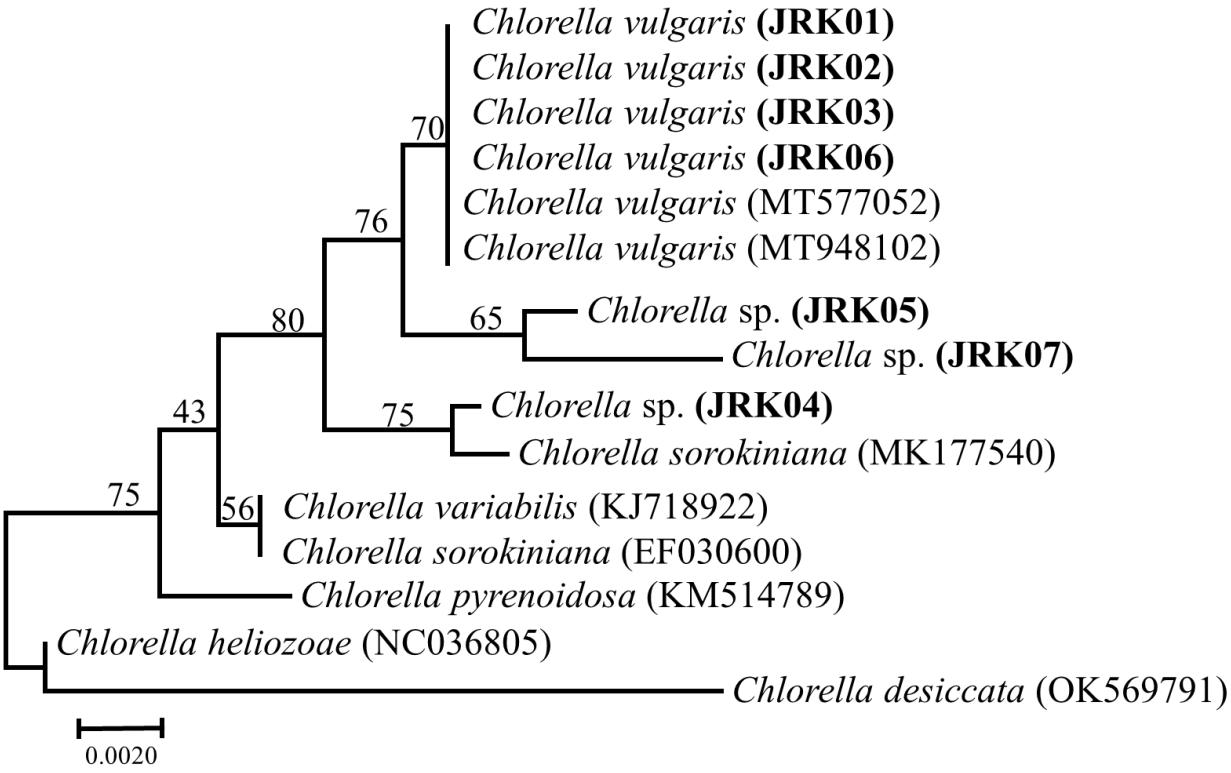

Figure 2.1 Maximum likelihood tree derived from 18S rRNA gene sequences showing the phylogenetic relationships within the genus *Chlorella*. Numbers present on branches are bootstrap support values for Maximum likelihood analysis. GenBank accession numbers are presented in parenthesis.

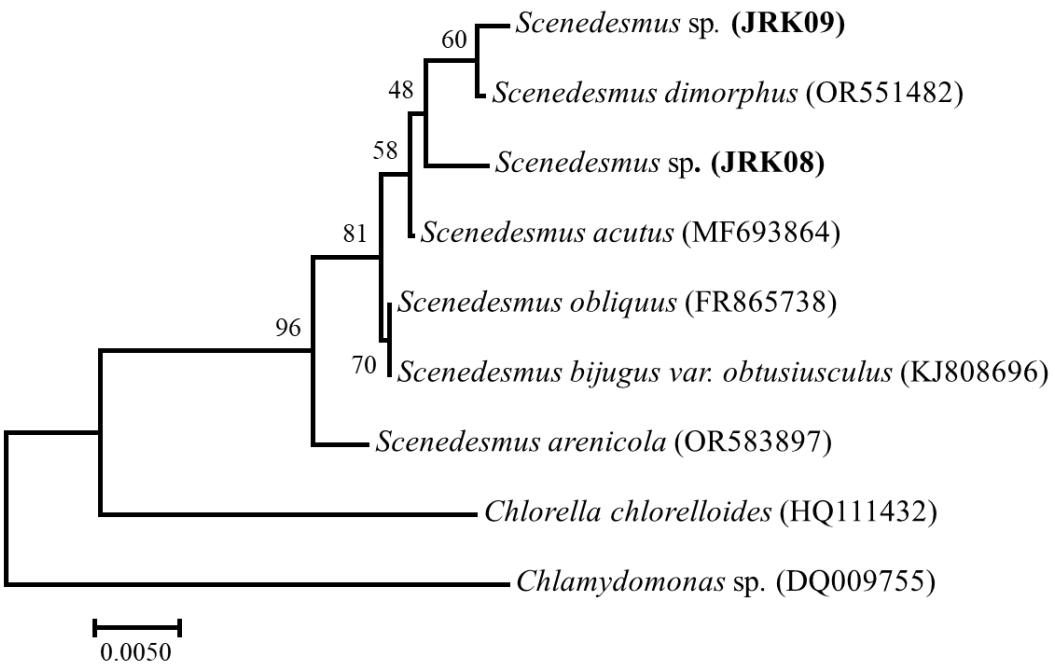

Figure 2.2 Maximum likelihood tree derived from 18S rRNA sequences showing the phylogenetic relationships within the genus *Scenedesmus*. Numbers present on branches are bootstrap support values for Maximum likelihood. Genbank accession numbers are presented in parenthesis.

Morphological analysis

Nine out of ten microalgae isolates were identified by molecular phylogenetics and *Chlamydomonas* sp. (JRK10)was identified morphologically. Among them, five species with higher biomass and lipid contents were selected for detailed morphological and biomolecular analysis.

***Chlorella sorokiniana* (JRK01)** – Unicellular, spherical, solitary cells, cell size (7.2 ± 0.14 µm, n = 10), cup-shaped chloroplasts, lack of flagella (Fig. 2.3a).

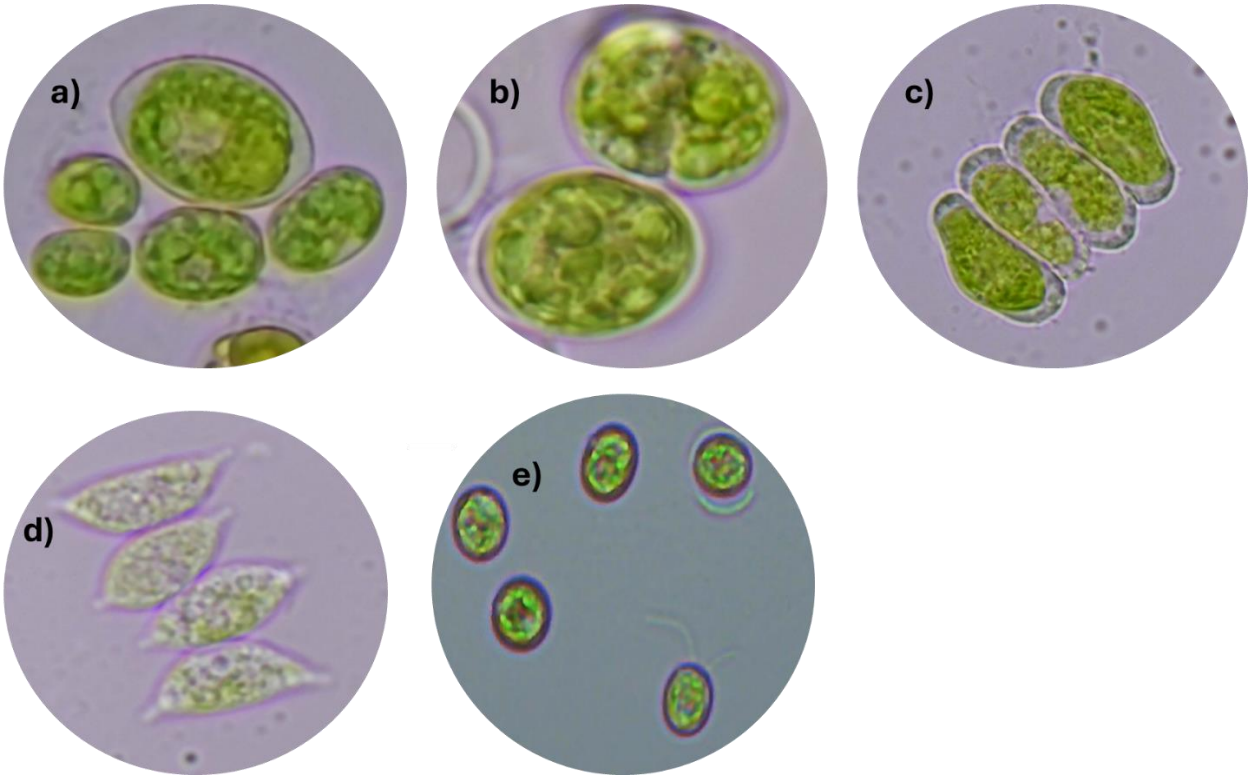

Figure 2.3 Microscopic structure of a) Chlorella sorokiniana b) Chlorella vulgaris c) Scenedesmus acutus d) Scenedesmus dimorphus and e) Chlamydomonas sp.

***Chlorella vulgaris* (JRK02)** – Unicellular, spherical to ovoid shape, solitary cells often found in colonies or clusters. cells, cell size (6.5 ± 0.25 µm, n = 10), cup-shaped chloroplasts, non-motile, and lack flagella (Fig. 2.3b).

***Scenedesmus acutus* (JRK08)** – Unicellular, cell crescent or half-moon shape, cells arranged in colonies of 2, 4, or 8 cells connected at their ends, cell width (6.2 ± 0.41 µm, n = 10) and cell length (10.1 ± 0.37 µm, n = 10), cup-shaped chloroplasts, pair of flagella per cell, contains a single nucleus, blunt-ended cells (Fig. 2.3c).

***Scenedesmus dimorphus* (JRK09)** – Unicellular, cylindrical cells are arranged in colonies or as solitary cells, cell width (6.5 ± 0.61 µm, n = 10) and cell length (10.5 ± 0.14 µm, n = 10), colonies flat or ribbon-like shape, with cells arranged side by side, pointed-ended cells (Fig. 2.3d).

***Chlamydomonas* sp. (JRK10)** – Unicellular, rounded or oval shape, single cup-shaped chloroplast and single contractile vacuole, presence of two flagella (whip-like structures) at the anterior end of the cell, cell diameter ranging from 11.5 ± 0.76 µm (n = 10) (Fig. 2.3e).

An understanding of microalgal diversity of Northern Ontario region of Canada is still vaguely known despite the historical survey efforts in this group (Park et al. 2012, Abdelaziz et al. 2014). Based on both molecular and morphological analysis several microalgae species remain to be identified from Northern Ontario region of Canada. There are more than 100 species within the genus *Chlorella* (Krivina and Temraleeva 2020). Our results identified two clades in the genus *Chlorella* from the study area. However, several unknown *Chlorella* species have also been isolated in Canada (Park et al. 2012, Abdelaziz et al. 2014) and other regions (Bock et al. 2011, Chae et al. 2023). Recent studies have highlighted the complexity of *Chlorella* taxonomy, revealing the existence of cryptic species and genetic diversity within known species complexes (Park et al. 2012, Abdelaziz et al. 2014, Krivina and Temraleeva 2020). *Chlorella* spp. exhibits a broad distribution range, with species found in freshwater, marine, and terrestrial environments (Darienko et al. 2019, Aigner et al. 2020).

Chlorella spp. is known for its unicellular structure and high nutritional value. It is widely studied for its potential applications in food and feed production, biofuel development, and environmental remediation due to its rapid growth rate and ability to fix carbon dioxide. This genus is recognized for their rich content of proteins, vitamins, minerals, and antioxidants, making them a promising candidate for sustainable nutrition and biotechnological applications (Tibbetts et al. 2015b, Tibbetts et al. 2015c).

This study identified two species within the genus *Scenedesmus*. Additional, several cryptic taxon groups within *Scenedesmus* have also been identified from Canada (Park et al. 2012, Tibbetts et al. 2015a). *Scenedesmus* comprises a diverse group of green microalgae belonging to the class Chlorophyceae, with significant ecological and biotechnological importance. In recent years, interest in *Scenedesmus* species has expanded beyond ecological studies to biotechnological applications. Several strains are being investigated for their potential in biofuel production, bioremediation, and high-value metabolite synthesis (Khatiwada et al. 2023, Condori et al. 2024). Understanding the taxonomy and distribution of microalgae species is therefore critical for selecting suitable strains with desired traits for specific biotechnological applications.

2.4.1.2 Optimization of light cycle for microalgae growth

Five different microalgae species were grown under four different light cycles: 0-hour light and 24 hour-dark, 12-hour light and12 hour-dark, 16-hour-light and 8-hour dark, and 24-hour light and 0-hour dark. There was a linear increasing trend of cell density along the cultivation days for all microalgae species with different light cycles (Fig. 2.4). The maximum cell density was reached on day 12 for all species and declined afterwards.

On day 12 (exponential growth phase), *Chlorella sorokiniana*, *Chlorella vulgaris,* and *Chlamydomonas* sp. exhibited significantly higher cell density under the 16L:8D cycle (Fig. 2.5). *Scenedesmus acutus* and *S. dimorphus* had the maximum cell density on both 12L:12D and 16L:8D cycles (Fig. 2.5). One-way ANOVA revealed that on day 12, three species (*C. sorokiniana*, *C. vulgaris,* and *Chlamydomonas* sp.) exhibited significantly higher cell density, followed by *S. acutus* and *S. dimorphus* ($F = 6.31$, $df = 3$, $P < 0.001$ and Fig. 2.5). Our study aligned with the work carried out by Holdmann et al. (2018) who reported the higher cell density *C. sorokiniana* at 16 hours light and 8 hours dark cycle.

One-way ANOVA revealed a significant difference in maximum cell growth rate during the exponential growth phase between different photoperiods ($F = 5.43$, $df = 3$, $P < 0.05$) (Table 2.1). The cell growth order different photoperiods for *Chlorella sorokiniana* was: 16L:8D ≥ 12L:12D > 24L:0D > 0L:24D, *Chlorella vulgaris* : 6L:8D ≥ 12L:12D > 24L:0D > 0L:24D,

Scenedesmus acutus: 6L:8D ≥ 12L:12D > 24L:0D > 0L:24D, *Scenedesmus dimorphus*: 6L:8D ≥ 12L:12D > 24L:0D > 0L:24D and *Chlamydomonas* sp.: 16L:8D ≥ 12L:12D > 24L:0D > 0L:24D.

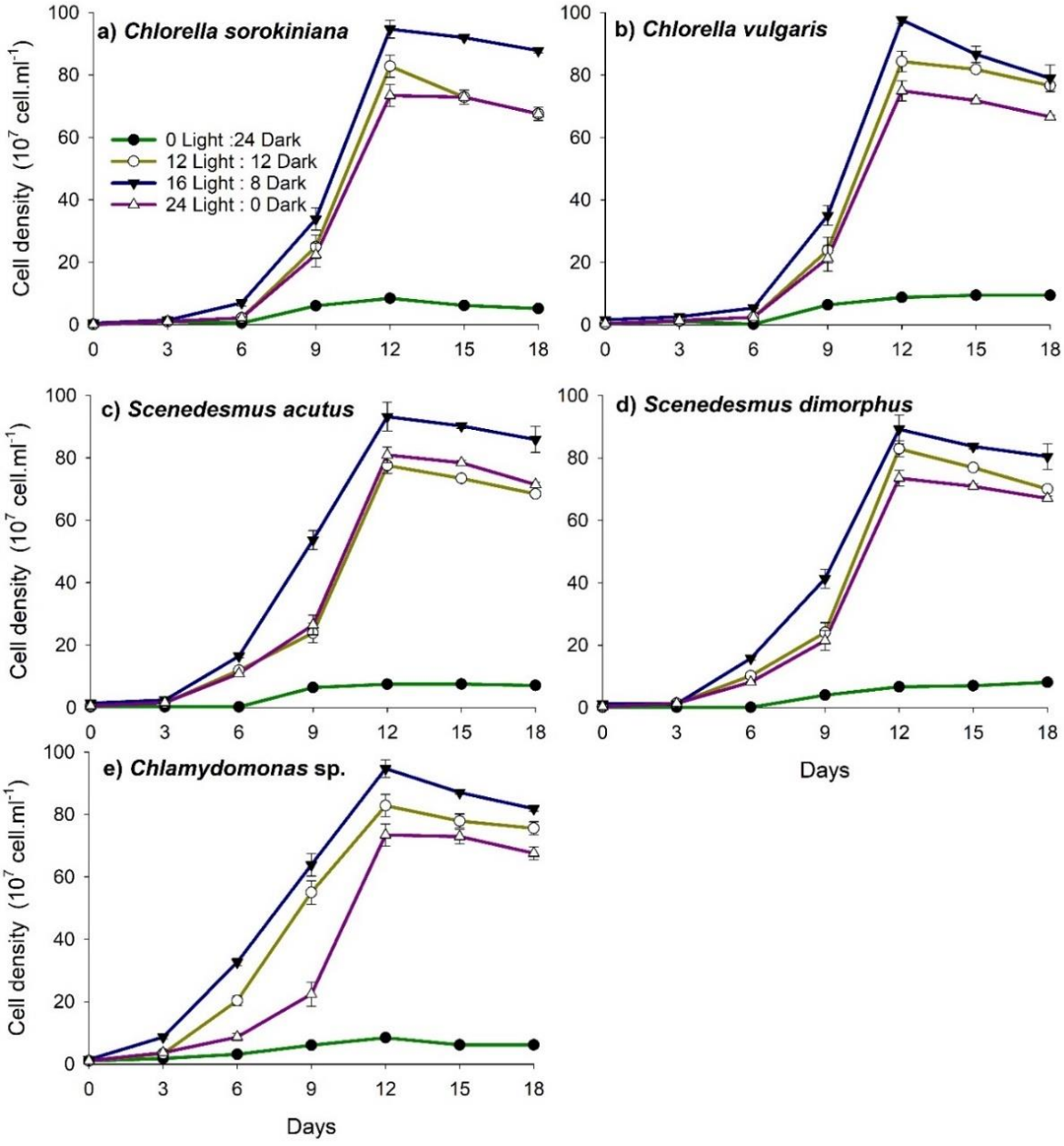

Figure 2.4 Variation in cell density along the cultivation days for different species.

For *Chlorella sorokiniana*, the maximum cell growth was in 16L:8D (1.37 ± 0.01) followed by 12L:12D (1.35 ± 0.03) and 24L:0D (1.31 ± 0.03). In contrast, the cell growth rates in *Chlorella vulgaris*, *Scenedesmus acutus*, and *Scenedesmus dimorphus* were similar in three different photoperiods (Table 2.1).

Table 2.1 Maximum growth rate of microalgae species in different photoperiods on day 12. The statistics derived from one way ANOVA with post-hoc Tukey test. The different letters in the superscript denote the significant differences at $P < 0.05$.

Species	**Photoperiod**			
	0L:24D	**12L:12D**	**16L:8D**	**24L:0D**
Chlorella sorokiniana	0.29 ± 0.01^{c}	1.35 ± 0.03^{ab}	1.37 ± 0.01^{a}	1.31 ± 0.03^{b}
Chlorella vulgaris	0.29 ± 0.01^{c}	1.37 ± 0.04^{a}	1.38 ± 0.01^{a}	1.33 ± 0.04^{b}
Scenedesmus acutus	0.32 ± 0.04^{b}	1.37 ± 0.02^{a}	1.36 ± 0.03^{a}	1.33 ± 0.02^{a}
Scenedesmus dimorphus	0.32 ± 0.04^{b}	1.36 ± 0.02^{a}	1.35 ± 0.03^{a}	1.32 ± 0.02^{a}
Chlamydomonas sp.	0.29 ± 0.01^{c}	1.35 ± 0.03^{ab}	1.37 ± 0.01^{a}	1.31 ± 0.03^{b}

The photoperiod greatly influences the rate of photosynthesis in microalgae which potentially leads to increased growth rates due to higher energy availability and effects on cellular metabolism (Ramanna et al. 2017). During light hours, energy (in the form of ATP) is generated through photosynthesis, supporting growth processes such as cell division and synthesis of cellular components (Yang et al. 2000). As a result, longer exposure to light can enhance biomass production. On the other hand, darkness also plays a critical role in the growth and development of plants. During darkness, microalgae undergo metabolic processes like respiration and utilize the stored energy reserves in the cell. The darkness periods help balance

energy consumption and storage (Liu et al. 2022). Different species of microalgae have varying responses to photoperiod conditions. The maximum cell growth was observed under 16L:8D dark cycle for *Nannochloropsis* sp. (Wahidin et al. 2013, Matos et al. 2017) and 24L:0D photoperiod for *Tetraselmis chui* (PLY429) (Meseck et al. 2005).

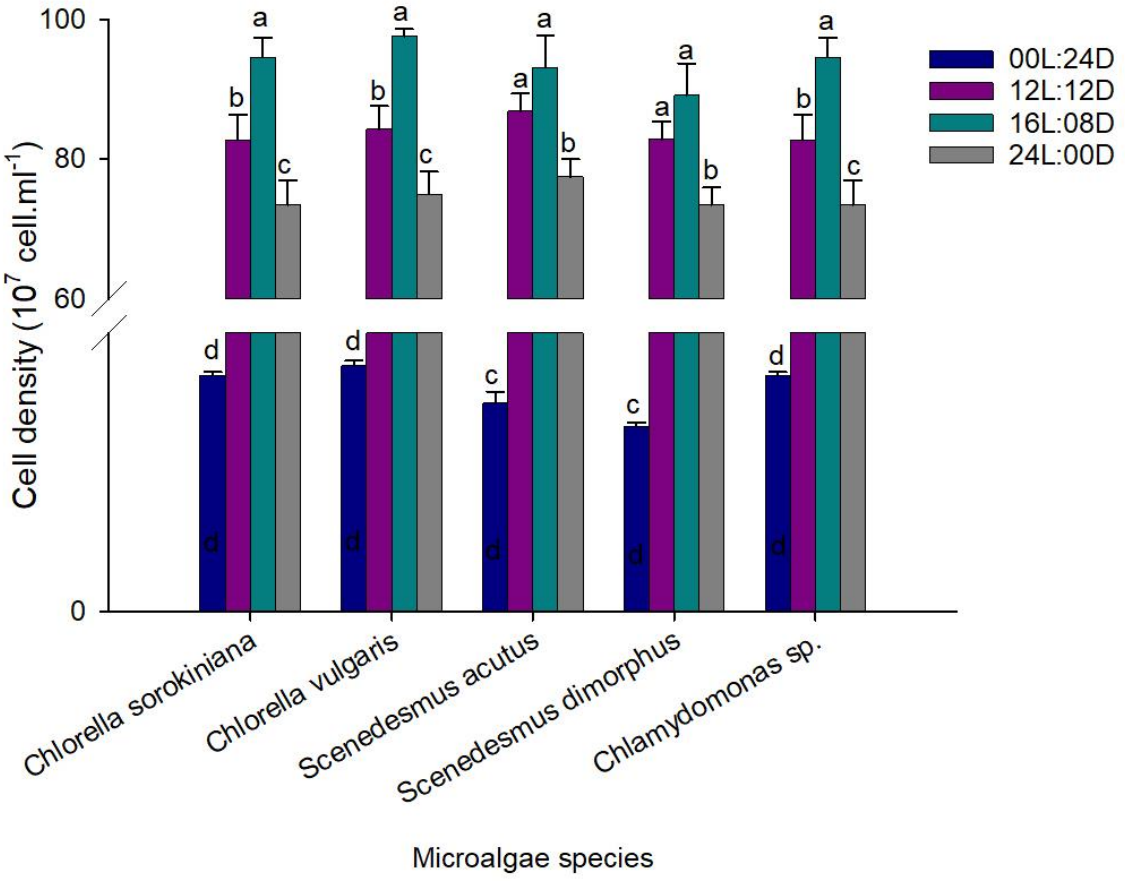

Figure 2.5 Variation in cell density during the exponential growth phase (day 12). The bar represents the mean cell density and error bar refers to standard deviation (SD). The statistics derived from one way ANOVA with post-hoc Tukey test. The different letters above the bar denote the significant pairwise differences at $P < 0.05$.

Microalgae, like other organisms, exhibit circadian rhythms that regulate biological processes in response to light-dark cycles. Disruption of these rhythms due to prolonged exposure to light or inadequate dark periods can impact growth and overall health (Holdmann et al. 2018). Optimizing photoperiods is essential for maximizing microalgae productivity for various applications.

2.4.1.3 Effect of photoperiod on biomass production

The results from one-way ANOVA showed that photoperiod had a significant effect on biomass Production (Table 2.2). *Chlorella sorokiniana* produced significantly higher biomass (2.07 ± 0.03 $g.L^{-1}$) in 16L:8D compared to 12L:12D and 24L:0D photoperiods. For *Chlorella vulgaris*, higher biomass production occurred in 16L:8D (2.01 ± 0.02 $g.L^{-1}$) and 12L:12D photoperiods followed by the 24L:0D. *Scenedesmus acutus* produced higher biomass under the photoperiod 16L:8D (1.77 ± 0.03 $g.L^{-1}$) followed by the 12L:12D and 24L:0D photoperiods. A similar trend was observed in *Scenedesmus dimorphus* with higher biomass 1.82 ± 0.01 $g.L^{-1}$ in 16L:8D photoperiods. Whereas *Chlamydomonas* sp. produced significantly higher biomass 2.03 ± 0.06 $g.L^{-1}$ in 16L:8D photoperiod compared to 12L:12D and 24L:0D photoperiods (Table 2.2).

Further, we compared the biomass production in 16L:8D photoperiods and revealed that *Chlorella sorokiniana, C. vulgaris,* and *Chlamydomonas* sp. had significantly higher biomass compared to *Scenedesmus acutus* and *S. dimorphus* ($F = 54.36$, $P = 0.001$, $df = 4$, Table 2.2). Amini Khoeyi et al. (2012) reported that *C. vulgaris* produced higher biomass 2.05 $g.L^{-1}$ under

16L:8D photoperiod and Babuskin et al. (2014) found the production of 8.35 $g.L^{-1}$ of *Isochrysis galbana* under mixotrophic culture. Light plays a significant role in growth and biomass accumulation in microalgae (Ramanna et al. 2017). Longer light periods generally enhance biomass production by providing more opportunities for cellular photosynthesis (Sun et al. 2018). However, excessive light exposure could lead to photoinhibition or cellular stress. Besides it is evident that lower biomass production is also related to nutrient availability (Liu et al. 2007).

Table 2.2 Biomass production of microalgal species under different photoperiods. The statistics derived from one way ANOVA with post-hoc Tukey test. The different letters in the superscript denote the significant differences at $P < 0.05$.

Species	**Photoperiod**		
	12L:12D	**16L:8D**	**24L:0D**
Chlorella sorokiniana	1.71 ± 0.06^{b}	2.07 ± 0.03^{a}	1.76 ± 0.01^{b}
Chlorella vulgaris	1.76 ± 0.05^{b}	2.01 ± 0.02^{a}	1.78 ± 0.03^{b}
Scenedesmus acutus	1.6 ± 0.01^{b}	1.77 ± 0.03^{a}	1.59 ± 0.02^{b}
Scenedesmus dimorphus	1.68 ± 0.01^{b}	1.82 ± 0.01^{a}	1.67 ± 0.01^{b}
Chlamydomonas sp.	1.64 ± 0.03^{b}	2.03 ± 0.06^{a}	1.69 ± 0.03^{b}

2.4.1.4 Effect of light cycle on the lipid content

The effect of photoperiod on lipid accumulation of microalgae species was measured on day 18. There was significant variation in lipid concentrations between different photoperiod among the species (Fig. 2.7). The lipid content was significantly higher in *C. sorokiniana* (38.34%), *C. vulgaris* (32.89%) and *Chlamydomonas* sp. (32.70%) under the 16L:8D

photoperiod compared to the12L:12D and 24L:0D. It is evident that higher cell density was correlated with the higher lipid content. Our study aligned with Amini Khoeyi et al. (2012) reported the higher lipid content of *Chlorella vulgaris* on the 16L:8D cycle. Photoperiod plays a critical factor in affecting growth, metabolism, and overall lipid synthesis such as triacylglycerols (TAGs) in microalgae (Wahidin et al. 2013). In contrast, *Scenedesmus acutus* and *Scenedesmus dimorphus*, the maximum lipid content was obtained in 12L:12D. Vendruscolo et al. (2019) also reported a higher lipid content in *Scenedesmus obliquus* in 12D:12L cycle. This study revealed that the effect of the light cycle on lipid productivity in microalgae is species-specific. *Verrucodesmus verrucosus* produced higher lipids in the 12D:12L cycle (Vélez-Landa et al. 2021). The effect of photoperiods on lipid synthesis in microalgae involves complex biochemical pathways that respond to changes in environmental conditions, particularly light availability.

During photosynthesis, light energy is converted into chemical energy in the form of ATP (adenosine triphosphate) and NADPH (nicotinamide adenine dinucleotide phosphate) through the light-dependent reactions (Luo et al. 2016, Grechanik and Tsygankov 2022). In this process carbon dioxide is fixed into organic molecules such as sugars (e.g., glucose). Lipid synthesis involves the conversion of acetyl-CoA (derived from the breakdown of carbohydrates) into fatty acids (Bellou et al. 2014). Acetyl-CoA is generated from pyruvate through the pyruvate dehydrogenase complex in the mitochondria during aerobic metabolism or from cytoplasmic

sources under anaerobic conditions (Krivoruchko et al. 2015). The availability of light regulates the key enzymes involved in lipid synthesis (Chen and Wang 2021). Our study also showed that extended light exposure followed by the dark periods promotes lipid accumulation.

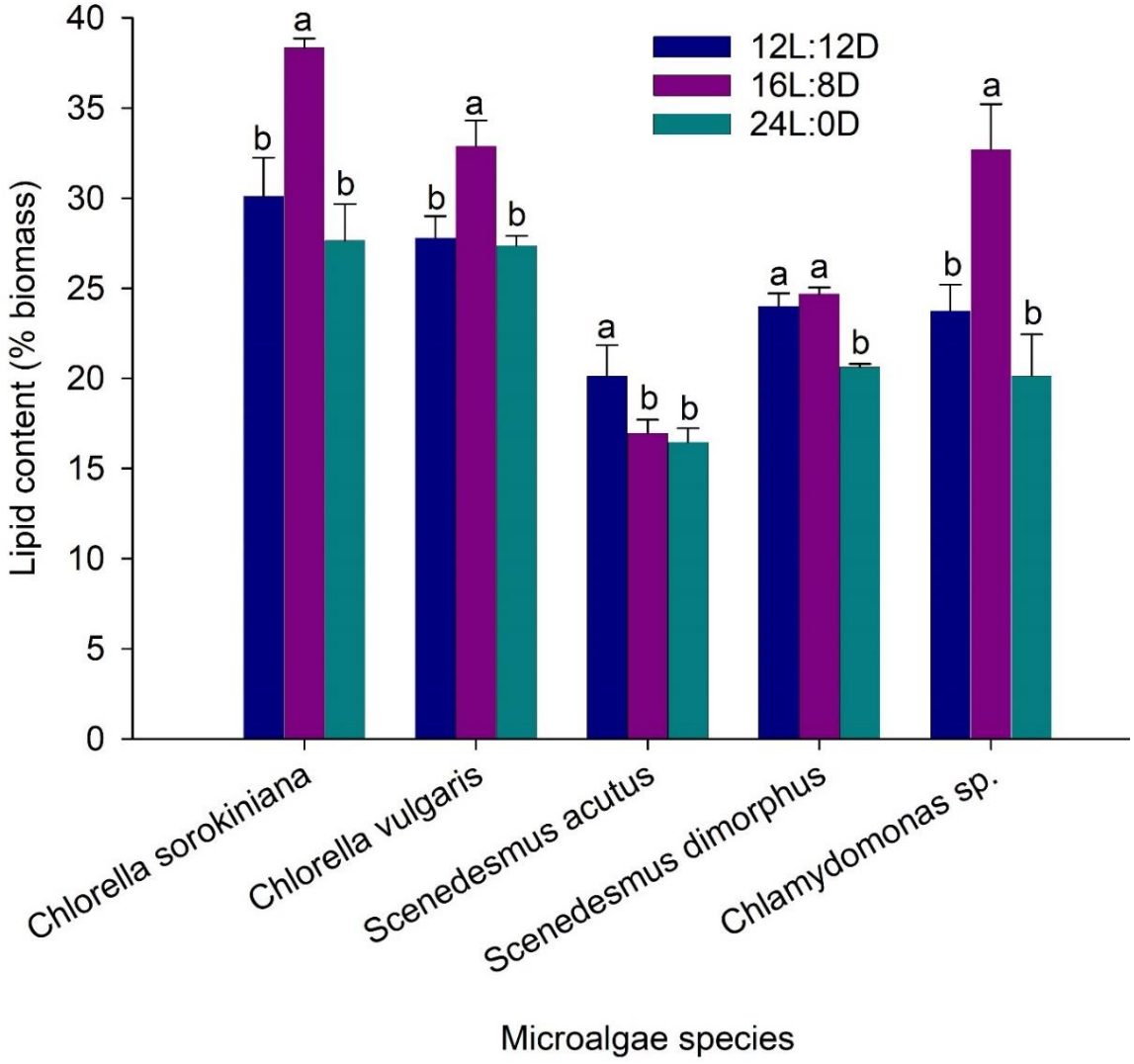

Figure 2.6 Variations in the lipid content of selected microalgae species in different photoperiods. The statistics derived from one way ANOVA with post-hoc Tukey test. The different letters in the superscript denote the significant differences at $P < 0.05$.

Conclusion

In this study, we characterized five different microalgae strains with higher levels of growth and lipid production by screening more than 75 strains of native microalgae isolated from different freshwater systems of Northern Ontario, Canada. Among four different photoperiods, 16L:8D produced significantly higher biomass and lipids for *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Chlamydomonas* sp. have on the 16L:8D photoperiods. This research contributes valuable insights into the potential of native freshwater microalgae for sustainable renewable energy production.

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Chapter 3

Part of this chapter has been published as:

Khatiwada, J. R., H. Guo, S. Shrestha, C. Chio, X. Chen, A. L. Mokale Kognou, and W. Qin. 2022. Cultivation of microalgae in unsterile malting effluent for biomass production and lipid productivity improvement. Fermentation 8:186

3.1 Comparative Assessment of Biomass and Lipid Production of *Chlorella vulgaris* Using Various Wastewater

Abstract

This study optimized wastewater concentrations for the cultivation of *Chlorella vulgaris* to enhance biomass and lipid production in three types of wastewaters: malting effluent, municipal wastewater, and paper mill wastewater. These effluents served as nutrient-rich media for promoting microalgal growth, which contributed to wastewater treatment by removing nutrients and pollutants. The optimal wastewater types were determined to be ME-50 (50 times diluted malting effluent), undiluted municipal wastewater (MC-undiluted), and undiluted paper mill wastewater (PM-undiluted). Among them, ME-50 demonstrated the maximum cell density, leading to maximum biomass growth and lipid accumulation. The nitrate and phosphate from the wastewaters significantly declined at the end of the experiment, which signified the nutrient removal capacity of microalgae. Therefore, ME-50 was selected as the optimal culture medium for further studies. These findings highlighted the dual benefits of using wastewater for cost-effective microalgal cultivation for biofuel and biomolecule production and efficient nutrient removal from polluted effluents.

Keywords: *Chlorella vulgaris*, wastewater treatment, biomass production, lipid accumulation, nutrient removal, biofuel production.

3.1.1 Introduction

Sustainable energy production has been a major environmental conundrum to fulfill the overwhelming increase in energy demands. Global energy consumption is increasing, in which fossil fuel accounted for 80% of total energy consumption (BP 2019). In contrast, alternative and renewable energy resources share only about 11% of the total energy consumed globally (REN 2021). Fossil fuel consumption leads to environmental pollution, specifically, increment of global greenhouse gas (GHG) emissions and imposed severe impact on global climatic patterns (Guo et al. 2021) which deteriorate the quality of environment and threaten various life forms (Hahladakis et al. 2018). However, the conversion of lignocellulosic biomass for energy production is a renewable, environment-friendly, cheap and promising alternative (Lay et al. 1999, Macqueen and Korhaliller 2011, Sharma et al. 2019). The third-generation biofuel production from microalgae has drawn the attention of many researchers because microalgae have a higher growth rate, shorter harvesting period, and a significantly higher average photosynthetic efficiency than other plants (Xia et al. 2016, Xu et al. 2019). Microalgae perform around 10–50 times higher carbon dioxide fixation through photosynthesis than terrestrial plants (Li et al. 2008, Williams and Laurens 2010). Microalgae can be effectively grown in a nutrient-rich environment to accumulate nutrients and heavy metals from the wastewater, making it a promising candidate for bioremediation (Christenson and Sims 2011).

Moreover, microalgae can produce renewable biofuels like biohydrogen and jet fuel as carbon-neutral clean energy (Chowdhury et al. 2019).

In Canada, wastewater is a significant source of aquatic pollution, with 139,599 tons released into the water bodies in 2019 (NPRI 2021). These effluents primarily contain nitrogen and phosphorus as major source of nutrients, causing the eutrophication of freshwater and marine ecosystems (Preisner et al. 2021). Nitrogen is the second most important constituent for plants including microalgae, absorbing it in the form of ammonium (NH_4^+) and nitrate (NO_3^-) (Farahin et al. 2021, Zhou et al. 2022). Researchers have used several methods (for example, physical, chemical, and biological) for wastewater treatment (Jhunjhunwala et al. 2021, Mao et al. 2021). However, the treated effluent still contains many inorganic compounds, such as nitrate, ammonium, and phosphate ions (Zhou et al. 2022). Although the efficiency of pollutant removal by chemical treatment is high compared to other methods, adding chemical reagents might increase the total cost and further deteriorate the environment by the leftover chemical in the effluents (Saravanan et al. 2021a). Similarly, physical treatment also requires high costs, energy, and complex technology. While the physical and chemical treatment methods are challenged by techno-economic bottlenecks, biological treatment of wastewater using microalgae can effectively remove nutrients and produce value-added products such as biomass, lipids, and biofuels (Kothari et al. 2013, Azizi et al. 2021). Thus, several studies

(Dhandayuthapani et al. 2021, Mohsenpour et al. 2021) were carried out in process optimization for optimum recovery of value-added products.

Canada is rich in natural lakes and rivers, contributing to the diversity of microalgae (Sheath and Hymes 1980, Minelgaite et al. 2020). However, studies on bioremediation, biomass, and lipid production using native microalgae are lacking in a Canadian context. Biofuel production using native microalgae could be an ideal alternative to fossil fuels in Canada. Locally grown microalgae can survive in extreme temperature conditions due to their physiological robustness allowing them to thrive in the environments with the greater fluctuations in conditions (Schmidt et al. 2016, Pankratz et al. 2017). This makes them suitable candidate for biofuel production and wastewater treatment in Canada. Therefore, the objectives of this study were to detrmine the optimal concentration of three types of wastewaters: malting effluent, papermill wastewater and municipal wastewater for microalgae growth, biomass yield, lipid productivity and nutrient removal efficiency.

3.1.2 Materials and methods

3.1.2.1 Sample collection and microalgae isolation

Water samples were collected from Lake Superior, Thunder Bay, Ontario, Canada. Filamentous plants and other coarse materials were removed by filtration using a cheese cotton cloth. Water samples were spread on the BG11 agar plate with the help of sterile disposable spreaders and

incubated at 20 ± 2 °C in continuous illumination for two to three weeks. Further, sub-cultures of the individual colony were carried out on an agar plate (BG 11) using a streak plate technique to obtain a pure single microalgal colony. Each isolated colony was grown in a one litre glass flask with 600 ml of working volume of BG 11 medium.

3.1.2.2 Cultivation conditions

Microalgae were grown in a BG-11 as a control medium at the initial pH of 7.1 (Stanier et al. 1971). The exponential growth phase algal cells (1 ml of inoculants with optical density (OD) of 1.351 at 680 nm) were used for experimental trials. Algal cultivations were carried out in 4 litre glass flask with a working volume of 3 litre and incubated in an environmentally controlled growth room at a constant temperature of 25°C (±2°C) and a photoperiod of 16-hours light and 8-hours dark cycle with continuous aeration. Two cool fluorescence tubes (200 lumens) provided light intensity on the top of the reactors (10 cm distance).

The malting effluent (ME) was obtained from Canada Malting (Thunder Bay, ON, Canada). The paper mill wastewater (PM) for this study was obtained from Resolute Forest Products (Thunder Bay, ON, Canada) and Municipal wastewater (MC) was obtained from City of Thunder Bay, Water Pollution Control Plant (WPCP), Canada. We used a clean cotton cloth to remove solids and suspended particles from wastewater and stored them at 4 °C until use in experiments. The dilution of wastewater was done based on our previous work (Khatiwada et

al. 2022). The growth characteristics of microalgae were tested into four dilution conditions of wastewater: 30% dilution (ME-30, MC-30 and PM-30) - 30 volumes of wastewater were mixed with 70 volumes of distilled water, 50% dilution (ME-50, MC-50 and PM-50) - 50 volumes of wastewater were mixed with 50 volumes of distilled water), 70% dilution (ME-70, MC-70 and PM-70) - 70 volumes of wastewater were mixed with 30 volumes of distilled water), 100% (ME- undiluted, MC-undiluted and PM- undiluted: no dilution) and control condition (BG 11 medium).

3.1.2.3 Measurement of microalgae growth characteristics

Microalgae growth was monitored by cell count in intervals of 3 days (Khatiwada et al. 2023).

3.1.2.4 Lipid extraction

The total lipids from the algal biomass were extracted using a modified version of the Bligh and Dyer method (1959), which involves a chloroform: methanol (1:1, v/v) solvent system. In this procedure, 0.1 g of dry algal biomass was combined with a mixture of chloroform, methanol, and distilled water in a 1:2:0.8 (v/v/v) ratio. The algal cells were disrupted by ultrasonication for 10 minutes, followed by centrifugation at 13 000 rpm for 10 minutes. The resulting supernatant was transferred to a pre-weighed Eppendorf tube (W_1 g), and the remaining cell pellet was subjected to a second extraction with chloroform and methanol (1:2, v/v). After another round of centrifugation, the supernatant was added to the same pre-weighed

Eppendorf tube. Chloroform and water were then added to the collected supernatant to achieve a final ratio of 1:1:0.9 (chloroform: methanol: distilled water, v/v/v). The mixture was thoroughly homogenized by vortexing and centrifuged at 13,000 rpm for 5 minutes. The upper layer was removed, and the lower layer was evaporated and dried at 80 °C until a constant weight (W_2 g) was obtained. The total lipid content was calculated by subtracting W_1 from W_2 and expressed as a percentage of the dry weight using the formula:

Percentage of total lipid content (% dry weight) (L_T) = $W_L/W_A \times 100$

Where W_L is the lipid weight and W_A is the microalgae dry weight.

3.1.2.5 Nitrate and phosphate concentrations

Nitrate concentration in wastewater was determined using UV-spectrophotometer as described by Huang et al. (2024) with some modifications. Total phosphorus concentration was determined using the ascorbic acid and molybdenum blue method as described by Dick and Tabatabai (1977) and He and Honeycutt (2005).

3.1.2.6 Statistical analysis

All the numerical variables were checked for normality before conducting the parametric test. Linear regression analysis was carried to explore the relationship between cell density and the number of cultivation days. One-way ANOVA was used to test for differences in the algal dry

biomass and lipid content among the treatments, followed by Tukey's HSD post hoc test for pairwise differences. All the experiments were performed in triplicate, and the results are shown as mean ± SD (standard deviation). Differences between treatments were evaluated at $P < 0.05$. All statistical analyses were carried out in R 4.4.1 (R Development Core Team 2024).

3.1.3 Results and Discussions

3.1.3.1 Selection of optimal concentration of wastewater for *C. vulgaris* growth

This study optimized the concentration of wastewater (malting effluent, municipal wastewater and paper mill wastewater) for the maximum growth of *C. vulgaris*. The effects of different dilutions of wastewater (30%, 50% and 70% and undiluted) on the growth of *C. vulgaris* were given in Fig. 3.1. In malting effluent, cell density showed a noticeable increase from day 3 to day 6, reaching a peak of by day 12 and then declining steadily (Fig. 3.1a). The ME-50 showed the maximum cell density of 90 x 10^7 cells/mL on day 12 compared to undiluted (30 x 10^7 cells/mL), ME-70 (70 x 10^7 cells/mL) and ME-30 (50 x 10^7 cells/mL). This could because the undiluted malting effluent is highly turbid and thereby hindering the light availability in the culture. The diluted malting effluent facilitates light penetration and thereby increases photosynthesis. The higher nutrient concentration in undiluted malting also inhibits microalgal growth.

Similarly, cell density showed a gradual increase from day 3 to day 6, reaching a peak of by day 12 and declined afterwards (Fig. 3.1b). *C. vulgaris* cultivated in undiluted municipal wastewater showed maximum cell density of 84.7 x 10^7 cells/mL on day 12 compared to MC-70 (71.1 x 10^7 cells/mL), MC-50 (63.5 x 10^7 cells/mL) and MC-30 (59 x 10^7 cells/mL).

C. vulgaris grown in paper mill wastewater showed a gradual increase of cell density from day 3 to day 6, reaching a peak of by day 12 and declined afterwards (Fig. 3.1c). The undiluted paper mill wastewater showed the maximum cell density of 77.6 x 10^7 cells/mL on day 12 compared to PM-70 (62.1 x 10^7 cells/mL), PM-50 (46.6 x 10^7 cells/mL) and PM-30 (38.8 x 10^7 cells/mL). BG-11 (control) and ME-50 (50 times diluted malting effluent) had the significantly higher cell density on day 12 followed by undiluted municipal and paper mill wastewater respectively ($F = 3.65$, $P = 0.001$, $df = 12$, and Fig. 3.2).

This study used unsterile malting effluent, paper mill, and municipal wastewater as a growth medium for *C. vulgaris* isolated from the freshwaters of Northern Ontario, Canada. We identified the optimal concentration of malting effluent, paper mill and municipal wastewater for microalgal growth. Based on our findings, microalgae grew better in diluted malting effluent (ME-50). Malting effluent, generated from the malting process in breweries, is primarily composed of organic matter such as sugars, proteins, and starches, along with minimal cleaning agents (Karlović et al. 2020). It is generally non-toxic, highly biodegradable,

and contains low levels of harmful chemicals, with pH levels that are neutral to slightly alkaline, making it environmentally benign and easy to treat (Table 3.1) (Gupta et al. 2010).

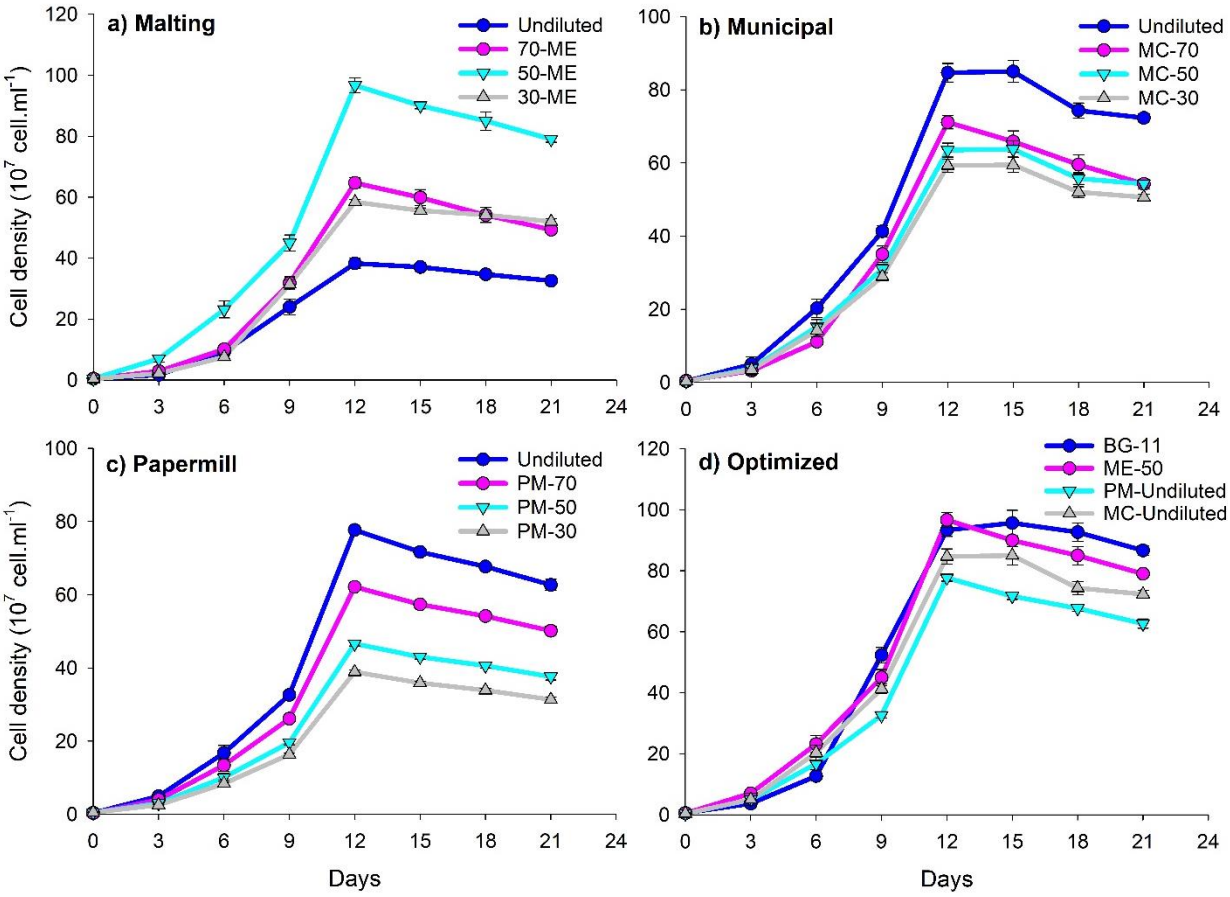

Figure 3.1 Cell density of microalgae on each cultivation day in a) Malting effluent b) Municipal wastewater c) Paper mill wastewater and d) Optimized dilution of wastewater with control medium (BG-11).

In contrast, paper mill wastewater from paper and pulp production contains a mix of organic and inorganic compounds, including lignin, chlorinated compounds, and dyes, making it highly toxic and difficult to treat due to its low biodegradability and high chemical contaminants (Pokhrel and Viraraghavan 2004, Kamali and Khodaparast 2015). Similarly, municipal

wastewater, originating from residential, commercial, and industrial sources, includes a variety of organic matter, nutrients, pathogens, and household chemicals, making it moderately toxic (Saravanan et al. 2021a).

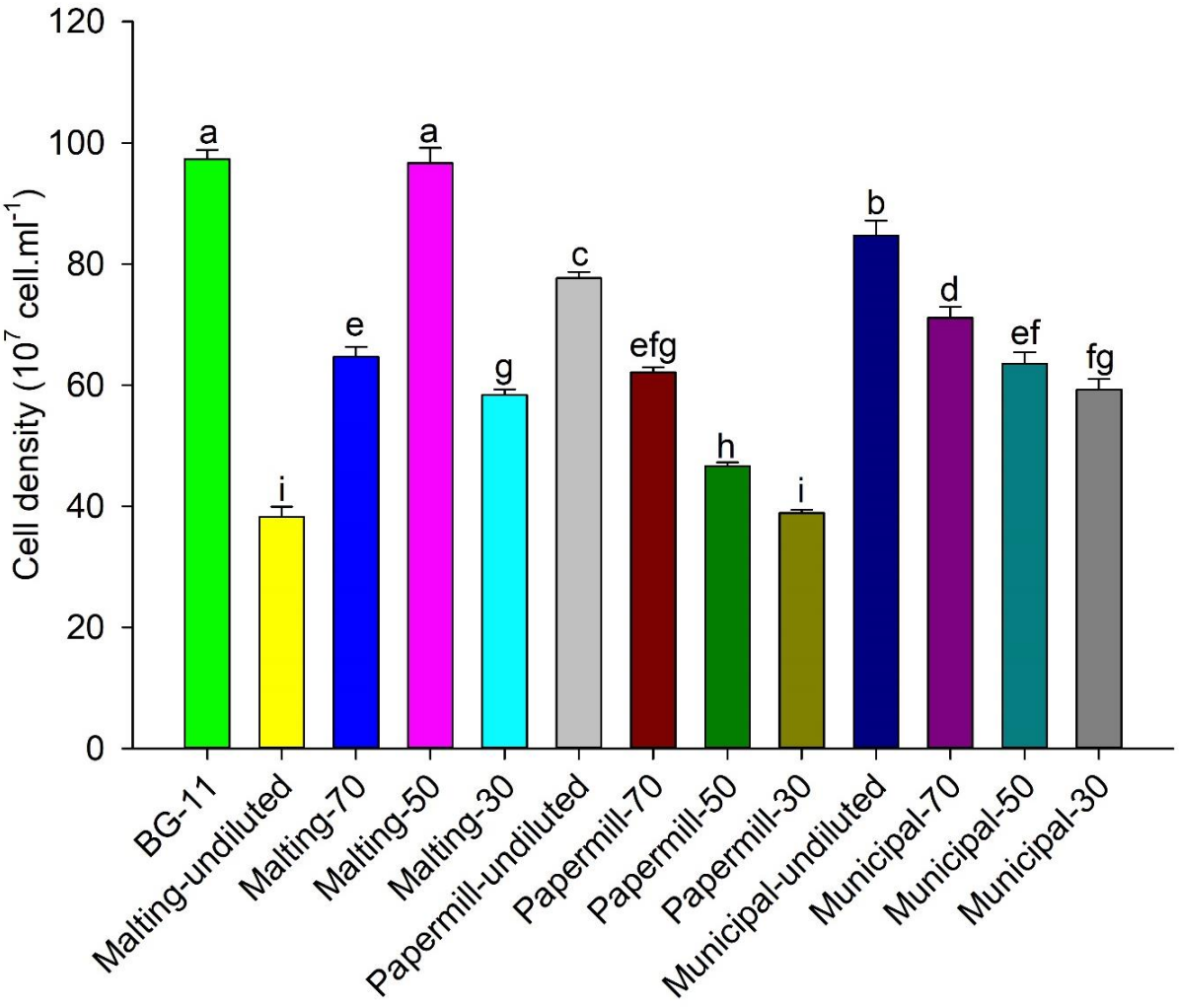

Figure 3.2 Cell density of *C. vulgaris* in different growth medium. The bar represents the mean cell density and error bar represents the SD. The letters above the bars are derived from on way ANOVA with posthoc Tukey test. The different letter above the bars signifies the significant different at $P < 0.05$.

It poses significant public health risks due to pathogens and chemical contaminants and contributes to nutrient loading, requiring comprehensive treatment to mitigate its environmental impact (Sathya et al. 2022). Compared to paper mill and municipal wastewater, malting effluent is less harmful, easier to treat, and poses lower environmental and health risks (Table 3.1).

3.1.3.2 Effect of wastewater type on microalgal biomass production

Our results showed that *C. vulgaris* grew better in BG-11 (control), ME-50 (50% diluted mating effluent), MC-undiluted (undiluted municipal wastewater) and PM-undiluted (undiluted papermill wastewater) than in other diluted concentration (Fig. 3.2). Therefore, biomass production was performed for four different media mentioned above. *C. vulgaris* cultivated in malting effluent produced higher biomass (2.08 ± 0.07 $g.L^{-1}$) than BG-11, municipal and papermill wastewater ($F = 62.53$, $P = 0.001$, $df = 3$, and Fig. 3.3). Kusmayadi et al. (2022) reported that *C. sorokiniana* species cultivated in 50% diluted dairy wastewater produced maximum biomass (3.2 $g.L^{-1}$). Similarly, Chen et al. (2020) the 50% diluted swine wastewater produced maximum biomass (6.5 $g.L^{-1}$).

3.1.3.3 Nutrient removal by microalgae

Nitrate concentrations in different wastewater decreased during the cultivation time (Table 3.2). In ME-50 treatment group, nitrate concentration significantly declined as the number of

cultivation days increased (r^2=0.972, P<0.001). The initial nitrate concentration was 302 ± 0.12 $mg.L^{-1}$ and then steadily decreased to 45.7 ± 1.24 $mg.L^{-1}$ by day 21. The removal efficiency was 84.9%, indicating a significant reduction in nitrate levels over the cultivation period. A similar trend was noticed in MC-undiluted (r^2=0.981, P<0.001) and PM-undiluted (r^2=0.982, P<0.001) treatments respectively.

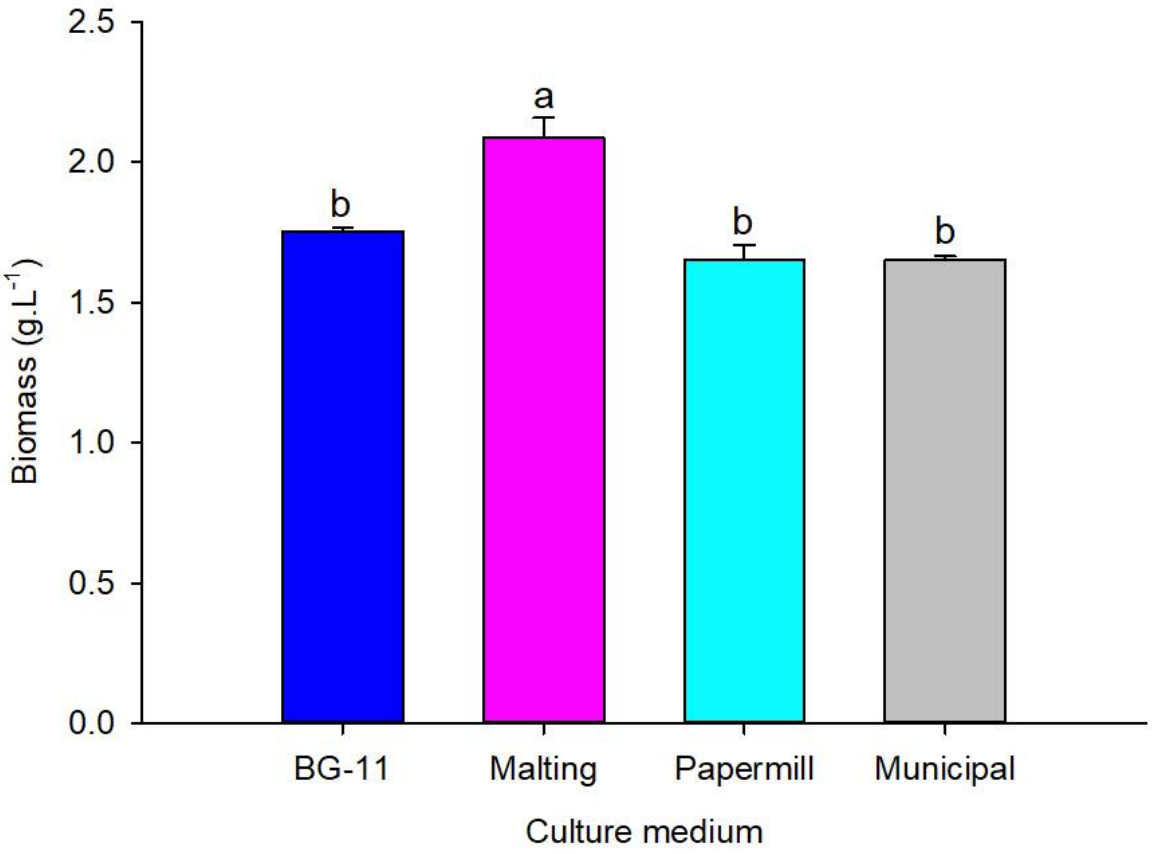

Figure 3.3 Biomass production of *C. vulgaris* in different growth medium. The bar represents the mean dry biomass and error bar refers to SD. The statistics derived from one way ANOVA with post-hoc Tukey test. The different letters above the bar denote the significant differences at P < 0.05.

The nitrate concentration significantly decreased from 125 ± 2.5 $mg.L^{-1}$ to 85.3 ± 1.1 $mg.L^{-1}$ by day 21 and the nitrate removal efficiency was 85.3%. In papermill wastewater, the nitrate concentration gradually reduced from 20.5 ± 1.6 $mg.L^{-1}$ to 4.1 $mg.L^{-1}$ by day 21 and removal efficiency was 79.8%.

In malting effluent, there was substantial reduction in phosphate concentrations from 31.8 ± 1.4 mg/L to 3.8 ± 0.9 mg/L ($r^2 = 0.972$, $P < 0.001$), with a removal efficiency of 88.1 ± 2.4%. For municipal wastewater, phosphate levels significantly dropped ($r^2 = 0.981$, $P < 0.001$) from 13.8 ± 0.7 mg/L to 2.6 ± 0.3 mg/L, achieving an 81.3 ± 1.1% removal efficiency. A similar trend was observed in paper mill wastewater with significant linear phosphate concentration decline with increasing cultivation days ($r^2=0.982$, $P<0.001$). Phosphate levels declined from 12.3 ± 0.2 mg/L to 2.2 ± 0.4 mg/L, with a removal efficiency of 82.1 ± 2.3%.

The results indicate that *C. vulgaris* can significantly reduce nitrate and phosphate levels in various wastewater types. The high removal efficiencies for nitrates and phosphates in malting, papermill, and municipal wastewater treatments suggest that microalgal cultivation is a promising method for wastewater bioremediation. These findings align with studies by Kusmayadi et al. (2022) and Chen et al. (2020), which demonstrated effective nutrient removal using microalgae in different wastewater matrices. Nitrogen and phosphorus are the primary nutrients in wastewater that contribute to eutrophication in the aquatic ecosystem (Jämsä et al. 2017, Goswami et al. 2021).

Table 3.1 Composition and toxicity of different wastewater (Pokhrel and Viraraghavan 2004, Gupta et al. 2010, Kamali and Khodaparast 2015, Karlović et al. 2020, Saravanan et al. 2021b, Sathya et al. 2022).

Aspect	Malting Effluent	Paper mill Wastewater	Municipal Wastewater
Definition	Wastewater from malting process	Wastewater from paper and pulp production	Wastewater from residential, commercial, and industrial sources
Composition	Organic matter (sugars, proteins, starches), cleaning agents	Organic and inorganic compounds (lignin, chlorinated compounds, dyes)	Organic matter (feces, urine, food waste), nutrients (nitrogen, phosphorus), pathogens, household chemicals
Toxicity	Non-toxic	Highly toxic	Moderately toxic
Biodegradability	High	Low	Moderate
Chemical Contaminants	Low	High	Moderate
Pathogens	Low	Low	High
Nutrient Loading	Low	Moderate	High
pH Levels	Neutral to slightly alkaline	Highly variable, often acidic or alkaline	Variable, often neutral to slightly alkaline
Environmental Impact	Low	High	Moderate to high
Treatment Difficulty	Easy	Difficult	Moderate

Table 3.2 Nitrate and phosphate concentration in different wastewater along the cultivation days.

Days	Malting Effluent	Paper Mill Wastewater	Municipal Wastewater
		Nitrate Concentration (mg/L)	
0	302 ± 2.5	20.5 ± 1.6	125 ± 2.5
3	255.3 ± 4.7	18.6 ± 0.4	118 ± 1.4
6	227.1 ± 3.1	16.8 ± 0.8	110.9 ± 3.1
9	196.7 ± 3.5	13.6 ± 0.6	93.2 ± 1.2
12	160.7 ± 4.2	10.5 ± 0.5	74.3 ± 1.2
15	133.3 ± 3.1	8.5 ± 0.4	55.7 ± 3.2
18	92.7 ± 4.1	5.2 ± 0.1	33.1 ± 3.6
21	45.7 ± 1.5	4.1 ± 0.4	18.3 ± 1.5
Removal efficiency	84.9 ± 0.4	79.8 ± 1.4	85.3 ± 1.1
		Phosphate Concentration (mg/L)	
0	31.8 ± 1.4	12.3 ± 0.2	13.8 ± 0.7
3	28.7 ± 0.3	11.3 ± 0.2	13.4 ± 0.3
6	25.2 ± 0.4	9.7 ± 0.2	11.3 ± 0.2
9	21 ± 0.6	7.5 ± 0.3	9.7 ± 0.4
12	15.7 ± 0.4	5.4 ± 0.3	7.5 ± 0.3
15	11.5 ± 0.3	4.3 ± 0.3	5.5 ± 0.3
18	7.9 ± 0.6	3.5 ± 0.1	3.5 ± 0.4
21	3.8 ± 0.9	2.2 ± 0.4	2.6 ± 0.3
Removal efficiency	88.1 ± 2.4	82.1 ± 2.3	81.3 ± 1.1

Therefore, proper wastewater treatment is necessary before releasing them into water bodies. The phosphate removal by microalgae species used in this study was higher than in other studies (Kothari et al. 2013, Azizi et al. 2021). Kothari et al. (2013) reported that the reduction

in nitrate and phosphate by *Chlamydomonas polypyrenoideum* grown on dairy industry wastewater were range from 48.7–90% and 3.9–70%. Azizi et al. (2021) reported 57% nitrate and 43% phosphate removal by *Chlorella Vulgaris* from pulp and paper wastewater. The study conducted by Lavrinovičs et al. (2021) reported the average reductions of nitrate and phosphate in municipal wastewater by *Chlorella vulgaris*, *Botryococcus braunii*, and *Ankistrodesmus falcatus* were 87.9% and 99.1%, respectively.

3.1.3.4 Effect of wastewater type on microalgal lipid production

There were significant differences in lipid content of *C. vulgaris* among four different cultivation media ($F = 13.10$, $P = 0.001$, $df = 3$, and Fig. 3.4). The average order of lipid production was PM-undiluted ≥ ME-50 ≥ PM-undiluted ≥ BG-11 (Fig. 4). PM-undiluted yields the highest lipid content (32.0 % ± 1.7), followed by ME-50 (29.3 % ± 1.5), MC-undiluted (28.3 % ± 1.1), and BG-11 (25.0 % ± 1.0) respectively. Biomass was collected on day 21, and the total lipid content was measured. Lipid production in microalgae is influenced by several factors, including nutrient availability, environmental conditions, and the type of wastewater used as a growth medium (Leong et al. 2022).

Nitrogen and phosphorus are critical nutrients that significantly impact lipid accumulation in microalgae (Maltsev et al. 2023). Under nitrogen-starved conditions, microalgae tend to divert carbon flux from protein synthesis to lipid synthesis, resulting in higher lipid content (Leong

et al. 2022). For example, *Chlorella vulgaris* and *Scenedesmus obliquus* enhances lipid production during nitrogen limitation (Laraib et al. 2021, Trivedi et al. 2022). Similarly, phosphorus starvation can trigger lipid accumulation by redirecting metabolic pathways towards energy storage in the form of lipids (Maltsev et al. 2023).

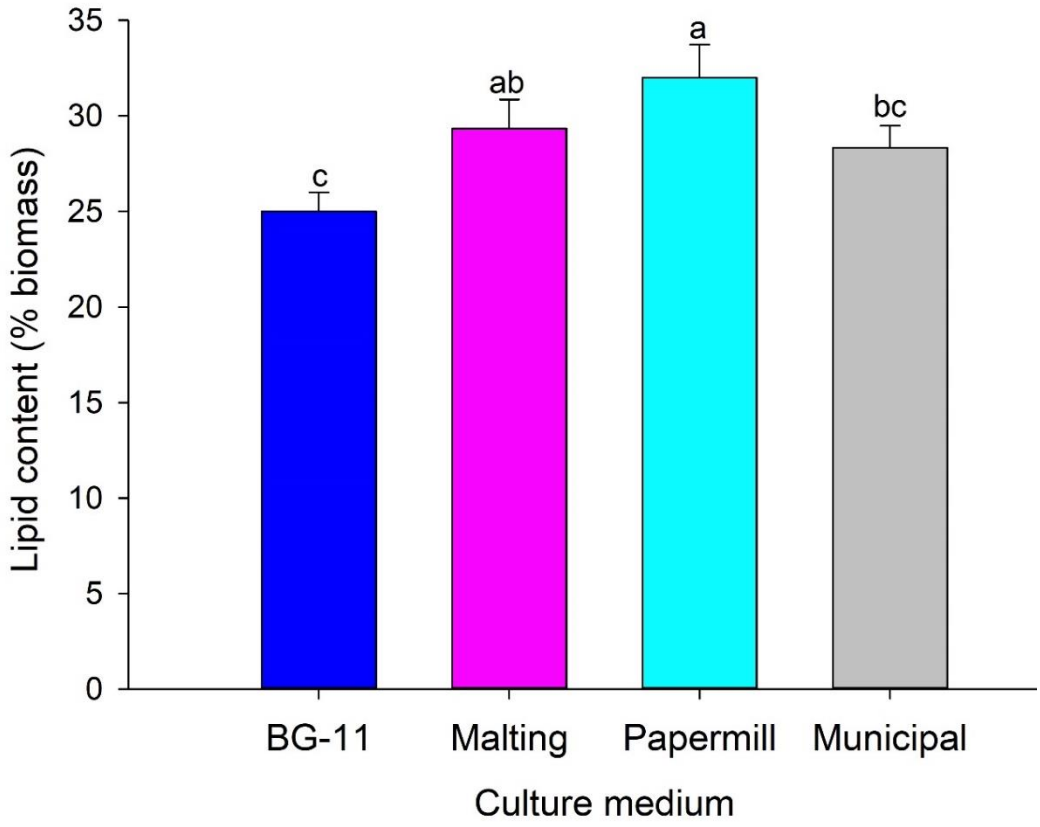

Figure 3.4 Lipid content of *C. vulgaris* cultivated in different growth media. The bar represents the mean lipid content and error bar refers to SD. The statistics derived from one way ANOVA with post-hoc Tukey test. The different letters above the bar denote the significant differences at $P < 0.05$.

Conclusion

Microalgal growth in wastewater contributes to wastewater treatment by removing nutrients and pollutants. This approach not only aids in wastewater treatment but also contributes to biomass production, which can be utilized for various biotechnological applications, including biofuel production. This study optimized the concentration of three different wastewaters: malting effluent, paper mill, and municipal wastewater for microalgal biomass production. Among different dilutions in each wastewater, ME-50 (50 times diluted malting effluent), MC-undiluted (undiluted municipal wastewater), and PM-undiluted (undiluted paper mill wastewater) had the highest cell density, therefore, *C. vulgaris* was cultivated on those conditions for higher biomass and lipid production. Nitrate and phosphate concentrations in wastewaters significantly declined during the cultivation period. Furthermore, our results showed that *C. vulgaris* had the maximum biomass growth and lipid accumulation in ME-50. Therefore, ME-50 was chosen as the optimal culture medium for further study. However, further research should focus on optimizing the conditions for maximum nutrient removal and exploring the economic feasibility of large-scale cultivation.

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Chapter 4

This chapter has been published as:

Khatiwada, J. R., C. Madsen, C, Warwick, S. Shrestha, C. Chio, and W. Qin. 2023. Interaction between polyethylene terephthalate (PET) microplastic and microalgae (*Scenedesmus* spp.): Effect on the growth, chlorophyll content, and hetero-aggregation. Environmental Advances 13, 100399.

4.1 Interaction Between Polyethylene Terephthalate (PET) Microplastic and Microalgae (*Scenedesmus* spp.): Effect on the Growth, Chlorophyll Content, and Hetero-aggregation

Abstract

Microplastics have become a global environmental concern due to their ubiquitous presence and persistence in the environment and have been identified as a major pollutant in aquatic environments. This study aimed to evaluate the effect of various concentrations of polyethylene terephthalate (PET) microplastic (25–200 mg/L) on the growth, chlorophyll content, and toxicity of *Scenedesmus* species from aquatic and terrestrial habitats over a period of 24 days. Our results showed that microplastics at higher concentrations (e.g., 200 mg/L) had a significantly greater inhibitory effect on cell density and chlorophyll content. Also, higher concentrations of extracellular hydrogen peroxide (H_2O_2) extracellular polymeric substance (EPS) were found on microalgae exposed to the microplastic compared to control treatment. Further, Scanning Electron Microscope (SEM) images revealed that microalgae attached to microplastic surfaces and formed hetero-aggregations. Overall, our study provided valuable information for understanding the complex effects of microplastics on microalgae, particularly in comparing the differential impacts on aquatic and terrestrial *Scenedesmus* species.

Keywords: Microplastics, *Scenedesmus* spp., Interaction, Inhibition, Hetero-aggregation

4.1.1 Introduction

Plastics are polymeric materials made from a wide range of synthetic, semi-synthetic, or biobased materials (Ganewatta et al. 2021). Due to their flexibility and lightweight, they have become widely used in the production of various solid objects in different shapes and sizes (Shah et al. 2008). Additionally, plastics are much cheaper than other metals, making them a popular choice for global usage (Bauer et al. 2022). It was estimated that a total of 4,392.5 million metric tonnes of plastic were produced between 1950 and 2020 (Fig. 4.1). If current consumption trends persist, it was projected that by 2050, annual global plastic production will exceed 507.2 million metric tonnes. The increasing production and long-term persistence of plastics in the environment (Gu 2003, 2017), combined with their slow rate of decomposition, are contributing to their accumulation and resulting in adverse impacts on the ecosystem (Gu 2021, Zhou et al. 2022).

Microplastics (plastic particle diameter of 1–5000 μm) are produced from the breakdown of macroplastics into smaller-sized polymer particles by UV radiation from sunlight or mechanical forces (Wang et al. 2018, Croxatto Vega et al. 2021). They are transported to the aquatic system by various means, such as wind, rain, animals, and agricultural runoff (Fig. 4.2).

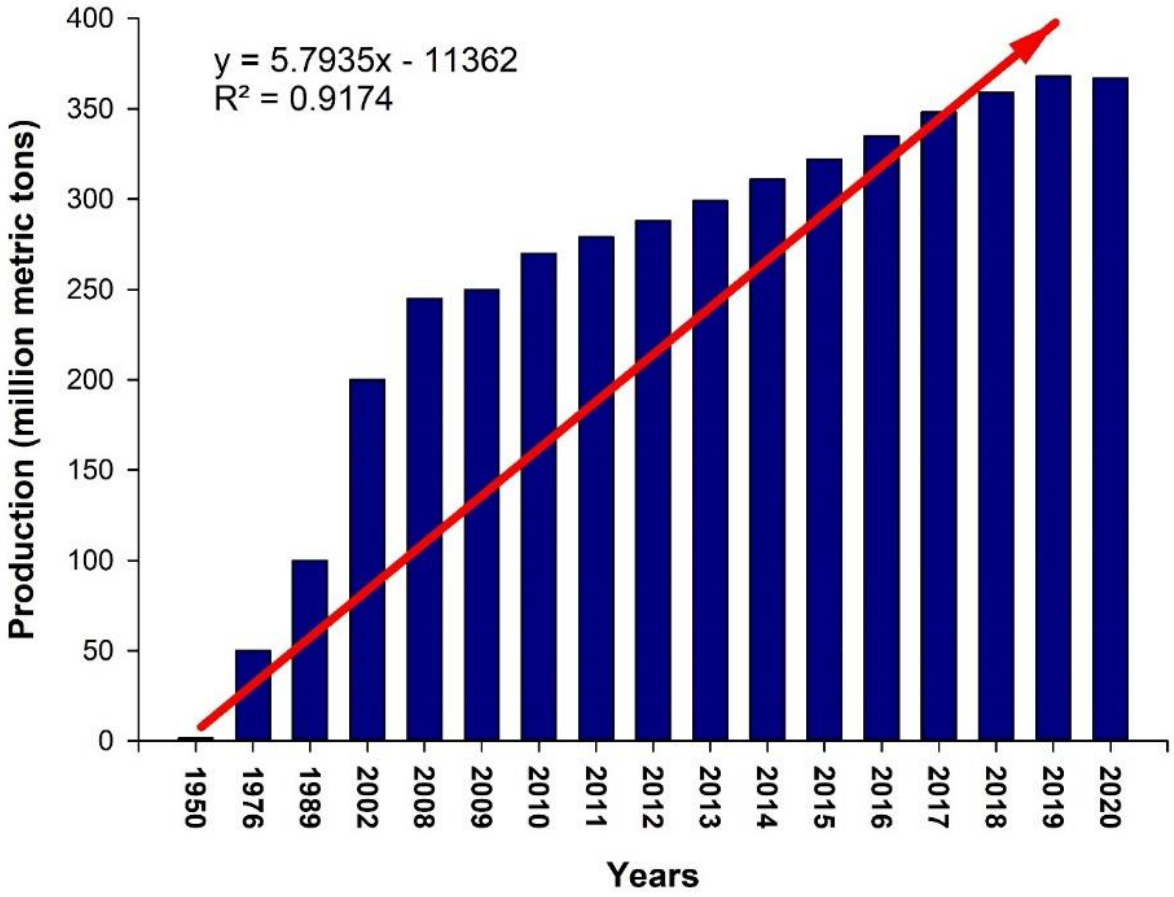

Figure 4.1 Trend of global plastic production. The data derived from Statista (2022). Red line dontes the trend of plastic production.

According to estimates, between 4.8 and 12.7 million tons of plastic waste make their way into the oceans annually (Jambeck et al. 2015). Microplastic pollution has become the biggest challenge for humankind in this decade and is reported from the soil, lakes/reservoirs, rivers, and the Arctic and Antarctic water (Davison et al. 2021). These microplastic particles are present at every level of the food chain and are transferred from primary producers to higher trophic levels (Jaiswal et al. 2022). Microplastics cause several health hazards to humans, such as skin rashes, vision problems, headaches and dizziness, diarrhea and vomiting, respiratory

diseases, cancer, and infertility (Fig. 4.3) (Vázquez and Rahman 2021). However, studies on the effects of microplastics on aquatic ecosystems are still insufficient. Microalgae have a cosmopolitan distribution and have been widely used as an ideal model organism to test the potential effects of microplastics in aquatic ecosystems. Microalgae are primary producers that convert inorganic carbon into organic carbon in the presence of light and produce biomass through photosynthesis.

Microalgal biomass is a crucial source of food for several aquatic animals, such as fish and crustaceans (Pacheco Vega et al. 2015, Seong et al. 2021). Studies showed that microplastics adversely affect microalgae through physical damage to the cell wall resulting in cell death, increasing the shading effect, reducing the photosynthesis rate, and increasing oxidative stress (Zhang et al. 2017, Wang et al. 2021, Yan et al. 2021, He et al. 2022, Wang et al. 2022) (Fig. 3.3). Xiao et al. (2020) carried out the transcriptomic analysis and revealed down-regulated gene expression in microalgae groups treated with polystyrene microplastics.

As a response, microalgae produce an extracellular polymeric substance (EPS) to protect the cell from microplastic toxicity (Li et al. 2020a, Ye et al. 2022).

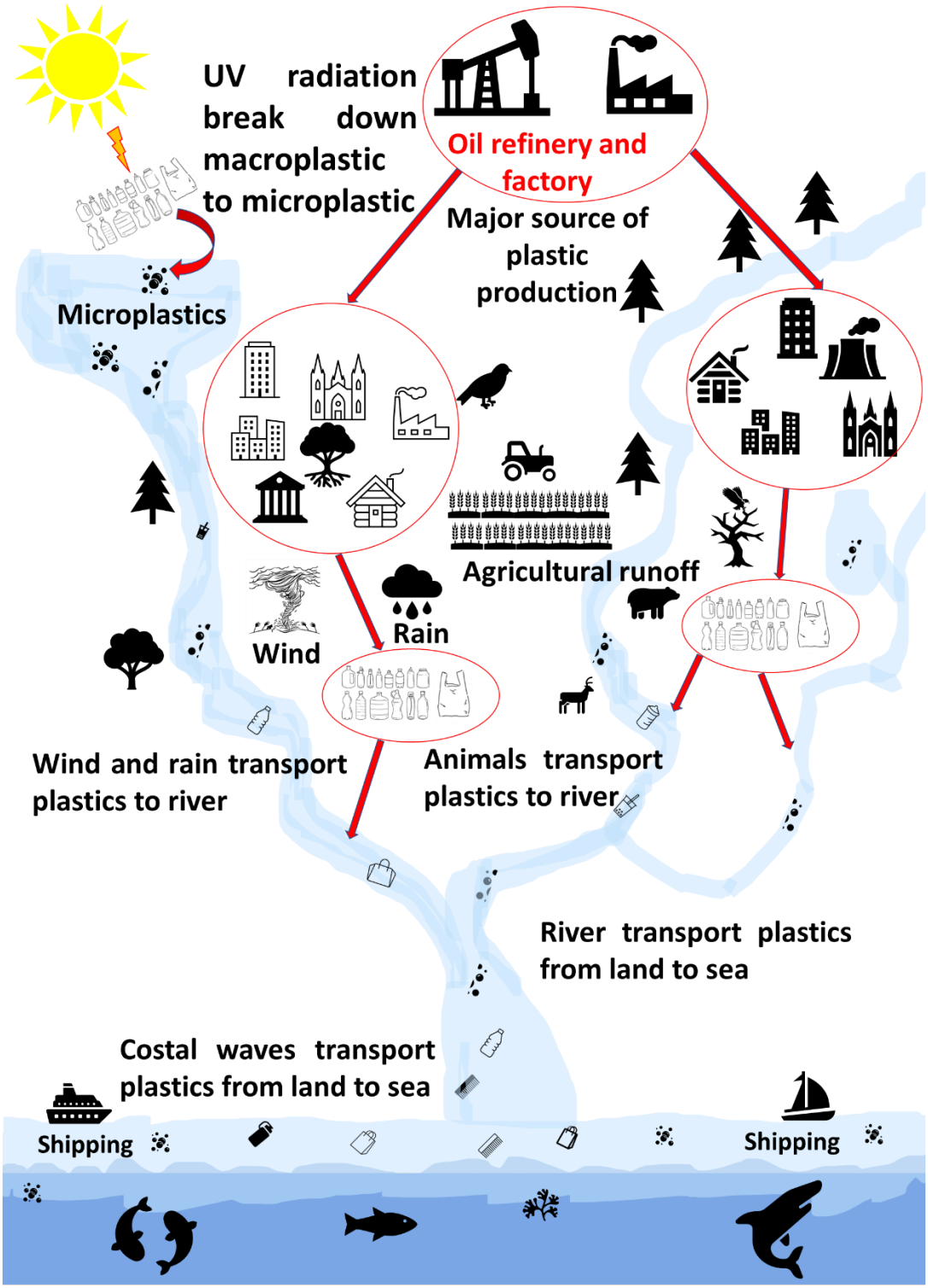

Figure 4.2 Illustration depicting the production and transportation pathways of microplastics.

Furthermore, they form hetero aggregates around microplastic particles (Lagarde et al. 2016) and stimulate the production of reactive oxygen species (hydrogen peroxide) in the cells, resulting in cell death (Lang et al. 2022).

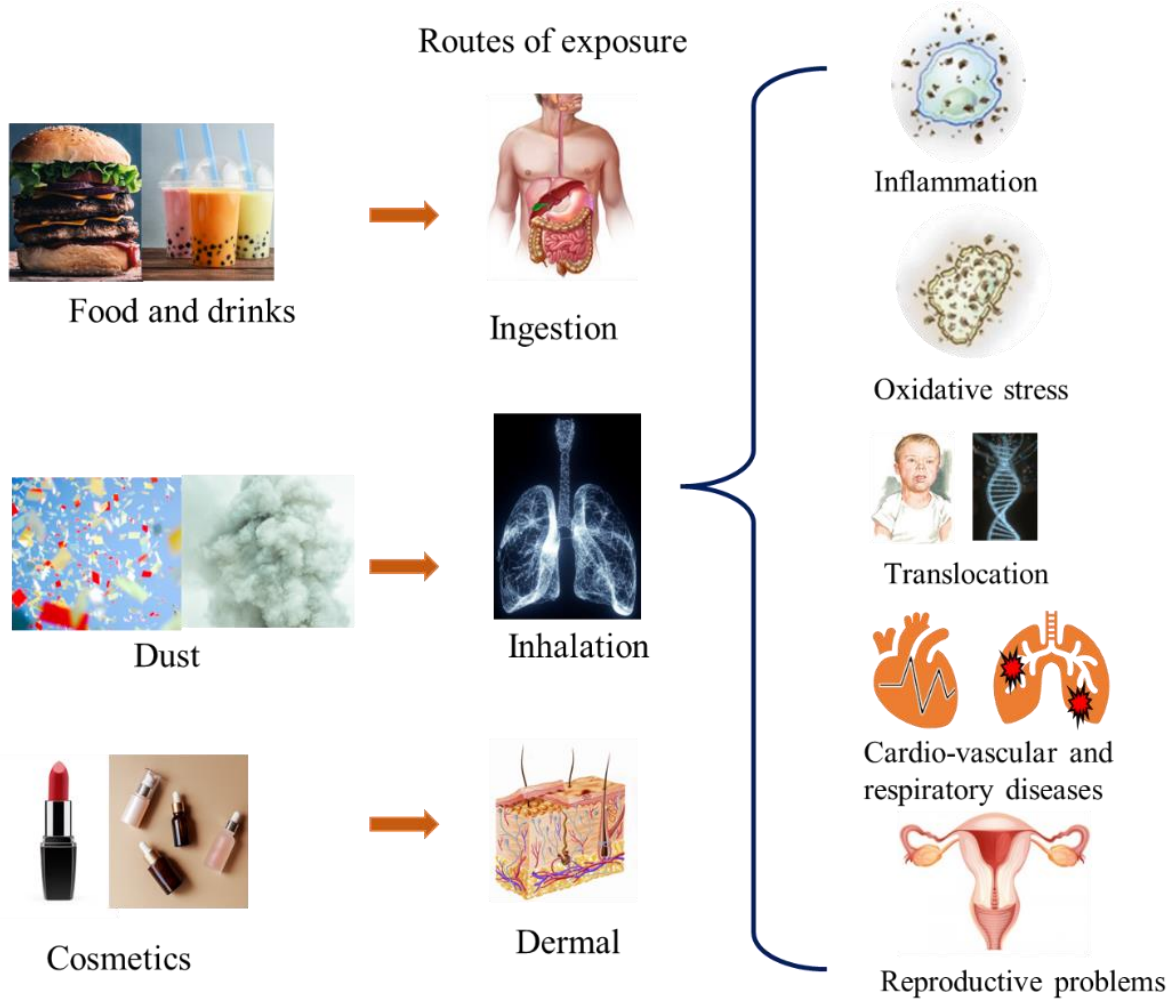

Figure 4.3 Graphical representation of the effects of microplastics on human health.

The interaction between microplastic and microalgae greatly varies within microalgae species and types of microplastic. Literature indicates that PET has growth-promotion effects on most microalgae species studied (Table 4.1). Whether aquatic microalgae are more resistant to

microplastics than terrestrial microalgae remains to be determined. For the first time, this study compared the effects of microplastics on aquatic and terrestrial microalgae. The objectives of this study were: i) to investigate the effects of different PET concentrations on growth and total chlorophyll content, ii) to analyze PET toxicity by measuring the EPS and hydrogen peroxide concentration in algal cells, and iii) to observe the interaction between PET and microalgae.

4.1.2 Materials and methods

4.1.2.1 Microalgae strain

Two microalgae strains (aquatic (JRK08) and terrestrial (JRK09) strains of *Scenedesmus* spp.) were obtained from Lake Superior and Red Maple tree bark (*Acer rubrum*), respectively, in Thunder Bay, Canada. Samples were spread on BG-11 agar plates and cultured at room temperature with continuous illumination for two to three weeks. After the growth of algal colonies, they were further sub cultured on BG-11 agar plates by streak plate technique until a pure isolated colony was obtained. The microalgae strains were identified as *Scenedesmus* sp. using the guidebook (Bellinger and Sigee 2015). A detailed process of the isolation of the culture of microalgae is given in Fig. 2.2. A speck of purely isolated microalgae from a solid culture medium was inoculated into 10 mL of BG-11 medium for 3 to 4 days. In 3 to 4 days, the optical density (OD600) of the cultured medium was observed. Then, 1% v/v of the seed

culture was inoculated into a 2.5 litre glass flask with one litre working volume containing BG11 medium (Stanier et al., 1971).

4.1.2.2 Microplastic

The PET microplastic (300 μm) was purchased from Goodfellow Cambridge Limited, England.

4.1.2.3 Cultivation medium and conditions

To demonstrate the effects of PET on microalgae growth, various concentrations (25 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L) of PET were added to the BG-11 medium. Two-and-a-half-litre glass bottles with a working volume of one litre were used to cultivate microalgae. The bottles were placed next to white, fluorescent lights and subjected to a 16:8-hour day-night cycle with continuous aeration. The experiment was conducted at room temperature of 22 ± 2 ^{o}C.

4.1.2.4 Parameter analysis

Microalgal growth was determined by total algal cell counts using 0.1 mm Tiefe deep Neubauer Improved Haemocytometer (Hamburg, Germany), with the help of a compound microscope (Olympus, Japan). The growth inhibition ratio (IR) was calculated as followed (Khatiwada et al. 2022, Siddique et al. 2022):

$$IR(\%) = \frac{CD_0 - CD_1}{CD_0}$$

Where, CD_0 (cell/ml) and CD (cell/ml) are the cell density values of the control and experimental groups, respectively.

The chlorophyll content was measured following the method of Khatiwada et al. (2022). Two mL of algal solution was centrifuged at 13,000 rpm for 10 minutes to obtain the algal cells. The supernatant was then discarded. The algal cell wall was broken down by brief sonication and the pellet was resuspended in 2 mL of 90% methanol. The solution was incubated at 60−70° C for 10 minutes, kept it in dark for 15 minutes, and then centrifuged again for 10 minutes. The absorbance of the supernatant was recorded at 652 nm and 665 nm via the visible spectrophotometer (BioTek Epoch Microplate Spectrophotometer, Vermont, USA). The total chlorophyll content was calculated using the following equations as described by Siddique et al. (2022):

$$Chlorophyll\ \alpha\ \left(\frac{mg}{L}\right) = 16.82A_{665} - 9.28A_{652}$$

$$Chlorophyll\ \beta\ \left(\frac{mg}{L}\right) = 36.92A_{652} - 16.54A_{665}$$

$$Total\ Chlorophyll\ Content\ \left(\frac{mg}{L}\right) = Chlorophyll\ \alpha + Chlorophyll\ \beta$$

To measure the extracellular polymeric substance (EPS) content, a 2 ml sample of algal culture was centrifuged at 13 000 rpm for 10 minutes, and the supernatant was discarded. The algal pellet was then resuspended in 2 ml of 0.9% Sodium chloride (NaCl) solution and heated at

Table 4.1 Available studies on the interaction between microalgae species and microplastic (PET).

Microalgae species	Particle Size	Media used	Parameter of measurement	Concentration (mg/L)	Major findings	References
Spirulina sp.	0.25–1 mm^2	Nutrition media	Growth rate and morphology	500	− Growth inhibition − Salinity enhances the biodegradation of PET	Hadiyanto et al. (2022)
Scenedesmus vacuolatus	-	GB medium	Growth and morphology	-	− Polyethylene: growth inhibition − PET, polypropylene, and polystyrene: no adverse effects on microalgae	Rummel et al. (2022)
Desmodesmus sp.	0.0065 and 0.013 mm	Modified BG11 medium	Biomass was growth	10, 40, 70, and 100	− PP (polypropylene) and PET do not effect growth	Lin et al. (2022)
Dunaliella salina	250 µm	Sea water	Pyrolysis	10	− Accelerate in the co-pyrolysis process	Chen et al. (2021)
Anabaena sp.	4 mm	Allen & Arnon medium	Growth and chlorophyll a content analysis	3 beads in 20 mL	− No effect on growth and chlorophyll content	Verdú et al. (2021)
Chlorella sp. and *Phaeodactylum tricornutum*	74 µm	BG11 and modified f/2	Measurement of growth, stress enzymes and morphology	200	− Growth promotion	Song et al. (2020)
Chlamydomonas reinhardtii	–	Liquid LB broth	PETase Gene expression and morphology	PET powder	− PETase enzyme catalyzed PET	Kim et al. (2020)
Spirulina sp.	1–2 µm	Nutrition media	Growth and plastic degradations	300, 500 and 550	− Growth inhibition − Tensile strength of PET decreased and microalgae	Khoironi et al. (2019)

60 °C in a water bath for 2 hours, shaking the solution every 15 minutes. After cooling the samples at room temperature for 30 minutes, they were centrifuged at 8 000 rpm for 10 min, and the supernatant was collected for polysaccharide content measurement.

Total polysaccharide content was measured using the Sulphuric acid - Phenol method described by Masuko et al. (2005). Specifically, 50 µl of the supernatant was added to a 96-well plate, followed by the addition of 150 µl of concentrated sulfuric acid and 30 µl of 5% phenol. The microplate was placed in a water bath at 90 °C for 5 minutes and then cooled down at room temperature for 5 minutes. The plate was then reheated at 90 °C for another 5 minutes and cooled again to room temperature. The plate was wiped and dried, and the absorbance of the solution was measured at 490 nm using a spectrophotometer. Control solutions were prepared using distilled water instead of the 50 µl supernatant, and a calibration curve was constructed using different concentrations of glucose (mg/L) as a standard. The EPS content was expressed as mg/L.

Microplastic toxicity was measured by hydrogen peroxide (H_2O_2) content analysis (Danouche et al. 2020). Two ml of sample was collected in Eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded, and 2 ml of 0.1% w/v Trichloroacetic Acid (TCA) solution was added and mixed well. The mixture was then centrifuged at 10,000 rpm for 10 minutes. Next, 0.5 ml of the supernatant was taken and mixed with 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide (KI). The absorbance was

measured at 470 nm, and a calibration curve was constructed using H_2O_2 as a standard with the equation:

Concentration of H_2O_2 = 0.128 + (3.880 * Absorbance), R^2 = 0.972, P<0.001

Settlement rate (ST) was measured after the completion of all toxicological experiments (day 24 and 25). Microalgae cultures were kept without shaking and aeration. On day 24 and 25, a supernatant of a depth of 2 cm was collected, and measured the optical density (OD) at 680 nm (Li et al. 2020b).

$ST\ (\%) = (1- OD_{680}\ (t_2)) / OD_{680}\ (t_1)) \times 100$

Where, OD_{680} (t_2 = day 25) is the final and OD_{680} (t_1 = day 24) is the initial OD values.

3.1.2.5 Sample preparation for SEM

The interaction between microplastic and microalgae cells was portrayed by a SEM. The samples were prepared for SEM as described by Siddique et al. (2022) and (Zhao et al. 2019). Two drops of each treatment solution were placed on carbon adhesive tape attached to aluminum mountain pins and samples were placed in a freezer at -80° C for 6 hours. Then, samples were placed in a freeze drier for 24 hours to ensure the samples were completely dry. Detailed information on SEM can be found elsewhere (Zhao et al. 2019, Song et al. 2020).

3.1.2.6 Statistical analysis

For the parametric test, normality was checked for all numerical variables. Linear regression analysis was conducted to investigate the trend of algal cell density and total chlorophyll versus the cultivation days. Mean difference among the variables were calculated using one-way analysis of variance (ANOVA) with Tukey's HSD post hoc test. All statistical analyses were carried out in R v. 4.2.2 software (R Development Core Team 2022).

4.1.3. Results

4.1.3.1 Effects of PET on microalgae growth

The cell density of both aquatic and terrestrial *Scenedesmus* sp. exhibited significant linear growth with increasing cultivation days under different concentrations of PET (Fig. 4.4). A one-way ANOVA indicated no significant difference in mean cell density among the treatment groups in aquatic *Scenedesmus* sp. In contrast, a significant difference was noticed in terrestrial *Scenedesmus* sp. ($F = 6.79$, $P<0.001$). The highest average cell density was noted in the control treatment (54.20 cells/mL), followed by the 50 mg/L treatment (41.58 cells/mL), the 25 mg/L treatment (35.01 cells/mL), the 100 mg/L treatment (30.12 cells/mL), and the 200 mg/L treatment (23.13 cells/mL) in terrestrial *Scenedesmus* sp.

All four concentrations of PET (25, 50, 100, and 200 mg/L) exhibited inhibitory effects in terms of cell density on both aquatic and terrestrial *Scenedesmus* species (Table 4.2). After 24 days

of exposure, PET showed the highest inhibitory effect at 200 mg/L in both species (Table 4.2). The average order of growth inhibition of PET was 200 mg/L ≥ 25 mg/L ≥ 100 mg/L ≥ 50 mg/L and 200 mg/L > 25 mg/L ≥ 100 mg/L > 50 mg/L on aquatic and terrestrial *Scenedesmus* sp. respectively.

3.1.3.2. Effects of PET on the chlorophyll content of microalgae

The total chlorophyll content showed a significant linear increase from day 1 to day 24 in all the treatment groups. Compared to the control group, the algal cells exposed to microplastics showed lower chlorophyll content (Fig. 4.5). In aquatic species of *Scenedesmus*, there was an inhibitory effect of PET on chlorophyll content until day 14. From day 16, species exposed to a higher concentration of PET showed a growth promotion effect. In contrast, overall inhibition was noticed in terrestrial *Scenedesmus* sp. along the cultivation days. There were marked variations in the inhibition rate of chlorophyll content in the different treatment groups in aquatic species ($F = 4.18$, $P = 0.01$) and terrestrial species ($F = 3.88$, $P = 0.01$). The IR of chlorophyll content order in aquatic *Scenedesmus* sp. was as follows: 25 mg/L (43.6 %) ≥ 100 mg/L (28.39 %) ≥ 200 mg/L (23.74 %) ≥ 50 mg/L treatment (22.57 %). In terrestrial *Scenedesmus* sp., the inhibition rate was as follows: 50 mg/L (51.53 %) ≥ 200 mg/L (41.17 %) ≥ 100 mg/L (36.38 %) ≥ 25 mg/L (33.53 %) respectively (Table 4.2).

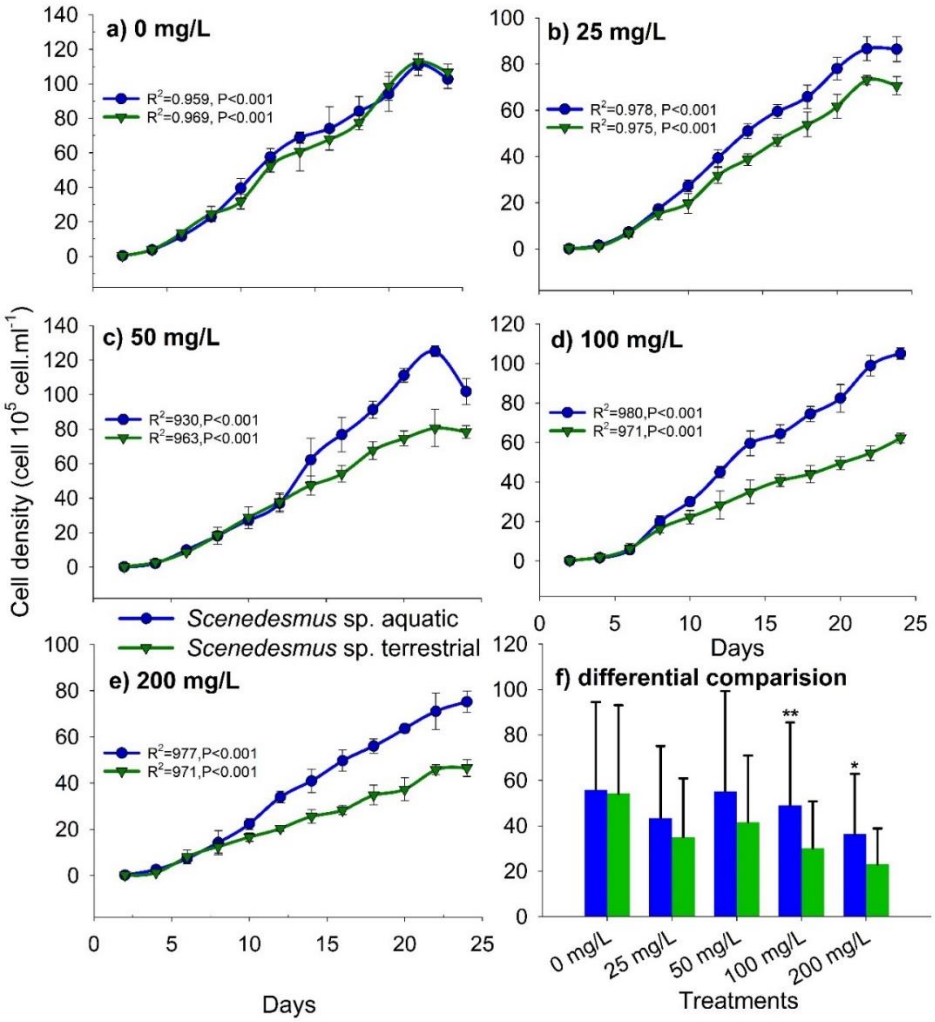

Figure 4.4 Variation of cell density under different concentrations of PET microplastic versus cultivation days (a -e) and differential comparison of cell density between the treatments (f). Blue line refers to aquatic *Scenedesmus* sp. and the green line refers to terrestrial *Scenedesmus* sp. The dots, inverted triangles and bars correspond to the mean cell density and the error bars represent standard deviations. R^2 and P values were derived from linear regression analysis whereas differential comparison was carried out by two-sample t-test (*P<0.05, **P<0.01).

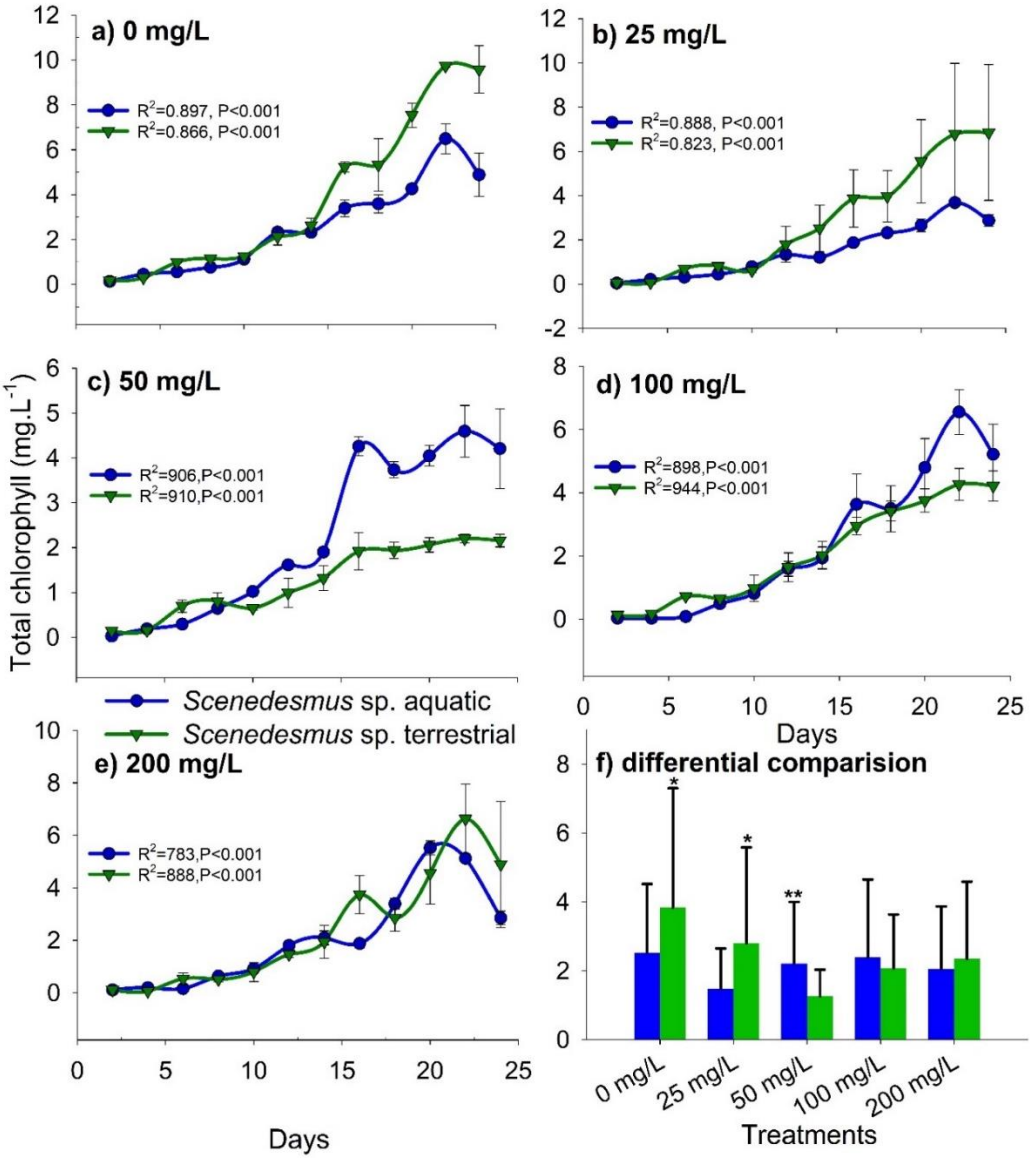

Figure 4.5 Variation of total chlorophyll content under different concentrations of PET microplastic versus cultivation days. Blue line refers to aquatic *Scenedesmus* sp. and green line refers to terrestrial *Scenedesmus* sp. The dots, inverted triangles and bars correspond to the mean cell density and the error bars represent standard deviations. R^2 and P values were derived from linear regression analysis whereas differential comparison was carried out by two-sample t-test (*P<0.05, **P<0.01).

3.1.3.3. Production of hydrogen peroxide

The concentration of extracellular hydrogen peroxide production gradually increased during the early stages of the experiment. For aquatic *Scenedesmus* sp., there were similar levels of hydrogen peroxide content in all treatments on day 0 and day 2. One way-ANOVA test revealed that other experimental groups have elevated levels of H_2O_2 compared to the control group from day 4 onwards (Fig. 4.6). When pooling data from day 0 to 24, there was a significant difference in H_2O_2 content between the treatments ($F = 9.43$, $P = 0.001$) (Fig. 4.8a). Further analysis using multiple comparison tests revealed that the mean order of H_2O_2 concentration was 25 mg/L ≥ 50 mg/L ≥ 200 mg/L ≥ 100 mg/L ≥ 0 mg/L (Table 4.2). A similar trend was observed in terrestrial *Scenedesmus* sp. (Fig. 4.8b). In the early stages (day 0 and day 2), all treatments showed similar levels of H_2O_2 content. However, a one-way ANOVA test revealed that, from day 4 onwards, the H_2O_2 content in the four experimental groups (25 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L) was significantly higher than that of the control group (0 mg/L) (Fig. 4.8b). The overall data showed a significant difference in the H_2O_2 concentration among the treatment groups ($F = 12.15$, $P = 0.001$).

Further analysis using multiple comparison tests revealed that the mean H_2O_2 concentration was in the following order: 100 mg/L ≥ 50 mg/L ≥ 25 mg/L ≥ 200 mg/L > 0 mg/L (Table 4.2).

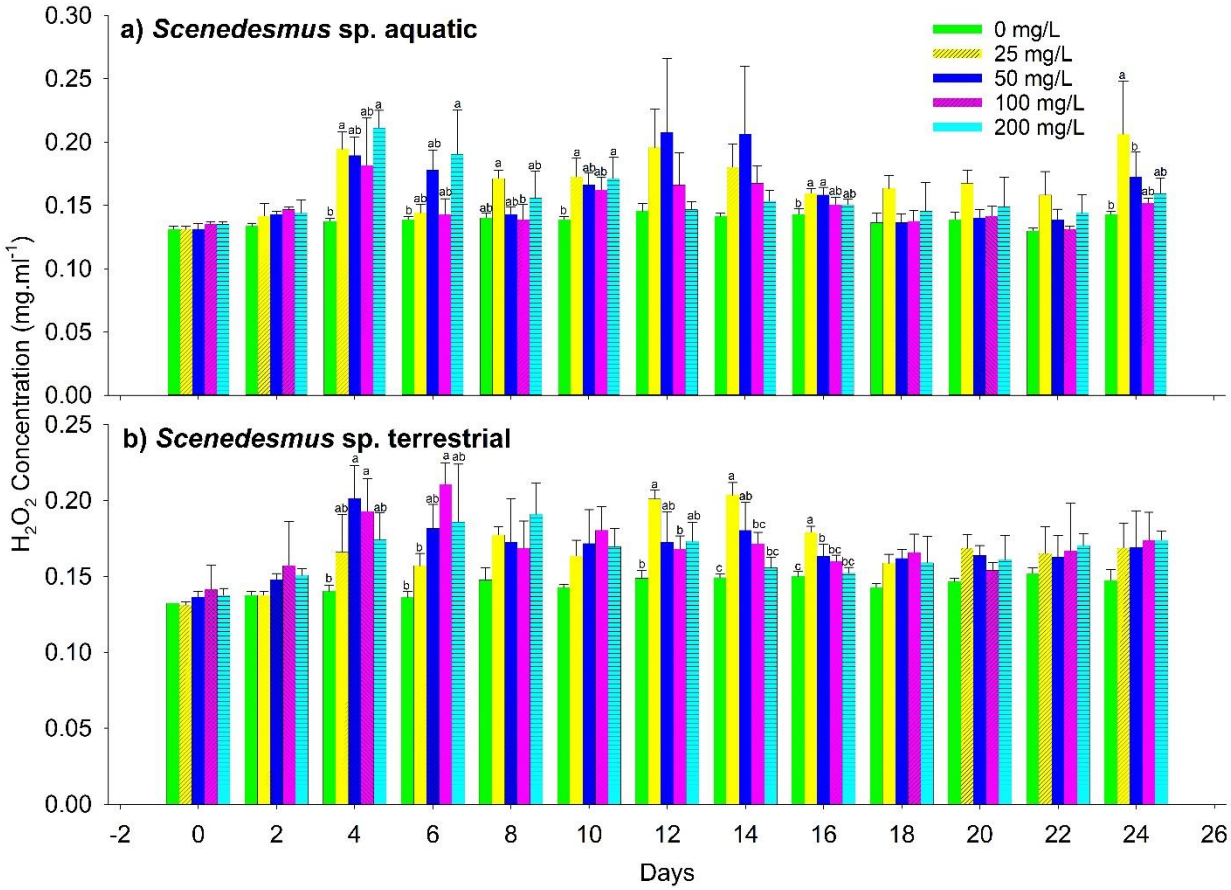

Figure 4.6 Variation of H_2O_2 concentration under different concentrations of PET versus cultivation days. a) *Scenedesmus sp*. aquatic sp. b) *Scenedesmus sp.* terrestrial sp. The bars show the mean H_2O_2 content and errors refer to standard deviation. The statistic derived from a one-way ANOVA with post-hoc Tukey's test. The different letters above the bars signified the differences between treatments at $P < 0.05$.

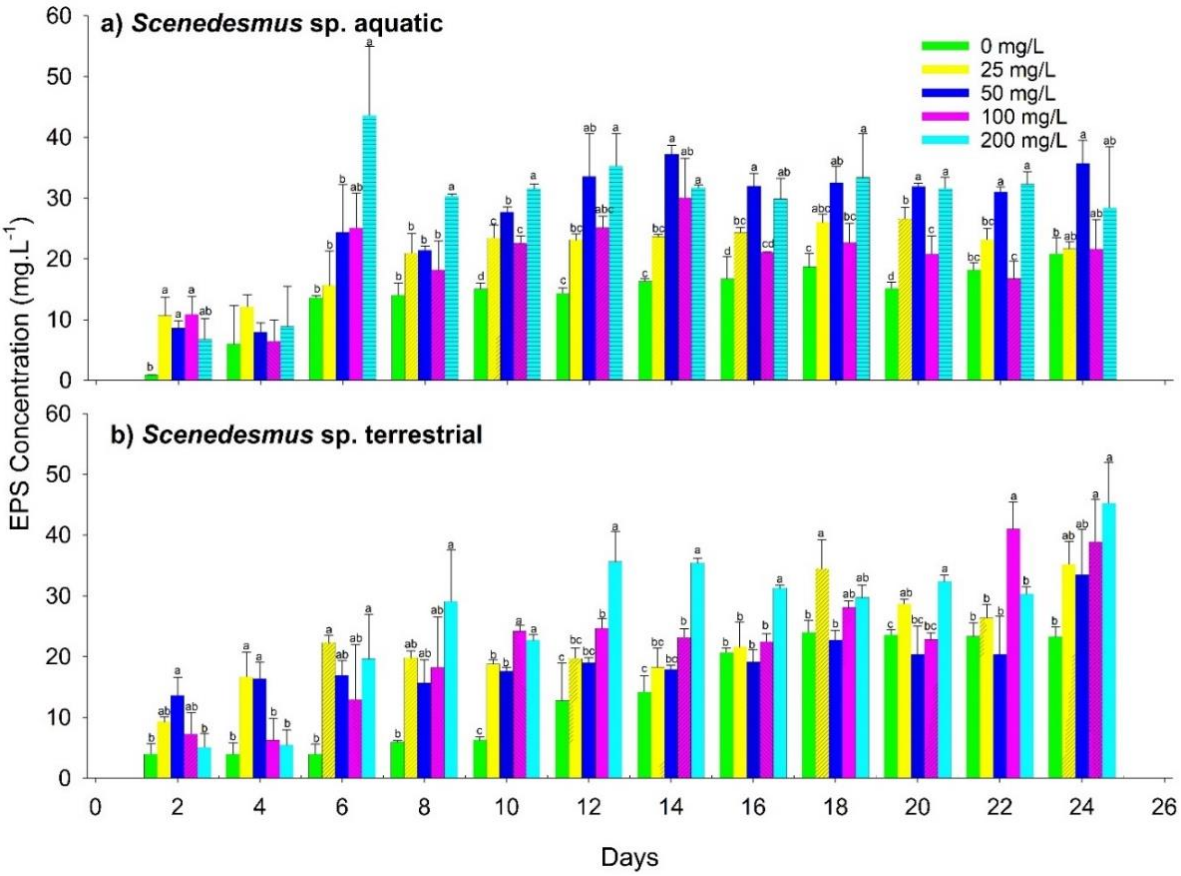

Figure 4.7 Variation of EPS under different concentrations of PET versus cultivation days. a) *Scenedesmus* sp. aquatic sp. b) *Scenedesmus* sp. terrestrial sp. The bars show the mean EPS and errors refer to standard deviation. The statistic derived from a one-way ANOVA with post-hoc Tukey's test. The different letters above the bars signified the differences between treatments at $P < 0.05$.

3.1.3.4. Variation of EPS content

During the experiment, EPS production was assessed for control and microplastic-exposed microalgae. Results presented in Fig. 4.7a and 4.7b indicate a significantly higher extracellular

carbohydrate production in experimental groups on most of the cultivation days when compared to the control for both aquatic and terrestrial *Scenedesmus* sp.

Table 4.2 Effect of PET concentrations on the growth inhibition (%) on cell density and chlorophyll content and variation on extracellular hydrogen peroxide and EPS of *Scenedesmus* sp. The data presented as mean ± SD. The statistic derived from a one-way ANOVA with post-hoc Tukey's test. The different letters in the superscript signified the differences between treatments at $P < 0.05$.

	Aquatic *Scenedesmus* sp.			
PET concentration	Cell density	Chlorophyll	H_2O_2	EPS
0 mg/L	-	-	0.14 ± 0.00^{c}	14.14 ± 5.76^{c}
25 mg/L	29.63 ± 17.96^{ab}	43.6 ± 11.64^{a}	0.17 ± 0.02^{a}	20.95 ± 5.49^{b}
50 mg/L	13.16 ± 27.53^{c}	22.57 ± 28.32^{b}	0.16 ± 0.03^{ab}	27.00 ± 9.96^{a}
100 mg/L	22.12 ± 20.32^{bc}	28.39 ± 39.05^{ab}	0.15 ± 0.01^{bc}	20.09 ± 70^{b}
200 mg/L	35.53 ± 12.4^{a}	23.74 ± 27.76^{b}	0.16 ± 0.02^{ab}	28.64 ± 11.20^{a}
	Terrestrial *Scenedesmus* sp.			
0 mg/L	-	-	0.14 ± 0.01^{b}	13.82 ± 8.70^{c}
25 mg/L	38.81 ± 13.7^{b}	51.53 ± 22.31^{a}	0.17 ± 0.02^{a}	22.6 ± 7.60^{ab}
50 mg/L	23.94 ± 14.18^{c}	36.38 ± 21.81^{b}	0.17 ± 0.02^{a}	19.43 ± 5.75^{bc}
100 mg/L	41.68 ± 16.38^{b}	41.17 ± 21.28^{ab}	0.17 ± 0.02^{a}	22.50 ± 1.05^{ab}
200 mg/L	52.32 ± 15.22^{a}	33.53 ± 28.83^{b}	0.17 ± 0.02^{a}	26.82 ± 2.10^{a}

When pooling data from day 0 to 24, there was a significant difference in the EPS content between the treatments ($F = 18.11$, $P = 0.001$, $df = 3$) (Fig. 4.7). Further analysis using multiple comparison tests revealed that the mean order of EPS concentration was 200 mg/L ≥ 25 mg/L

≥ 100 mg/L ≥ 50 mg/L > 0 mg/L (Table 4.2). Similarly, a significant difference was noticed in EPS content on terrestrial *Scenedesmus* sp. ($F = 9.58$, $P<0.001$). Post-hoc comparison revealed that the mean order of EPS concentration was 200 mg/L ≥ 100 mg/L ≥ 25 mg/L ≥ 50 mg/L ≥ 0 mg/L (Table 4.2).

3.1.3.5. Structural characterization and settlement rate

To further investigate the interactions of microalgae with PET at different concentrations, the morphological properties were studied by SEM imaging (Fig. 4.8). As shown in 4.8 a), a single cell of Scenedesmus sp. has a regular bean shape with a smooth surface in the control treatment. On day 24, microplastic aggregations were seen on microalgae surfaces (Fig. 4.8b-d). On the PET-exposed cell, the cell wall became rough and irregular (Fig. 4.8d).

The results of a One-way ANOVA indicated a significant difference in settlement rate among different treatments of aquatic *Scenedesmus* sp. ($F = 23.46$, $P < 0.000$). Post-hoc multiple comparisons revealed that the mean settlement rate was highest in the control group (94%), followed by the groups treated with 25 mg/L and 50 mg/L PET. The lowest settlement rate (50%) was observed in the 100 mg/L and 200 mg/L treatment groups (Fig. 4.9a). The settlement ratio for terrestrial *Scenedesmus* sp. varied significantly among the treatments ($F = 10.82$, $P = 0.001$). Further comparisons of the means showed that the settlement order was as follows: 0 mg/L ≥ 25 mg/L ≥ 50 mg/L ≥ 100 mg/L ≥ 200 mg/L (Fig. 4.9b).

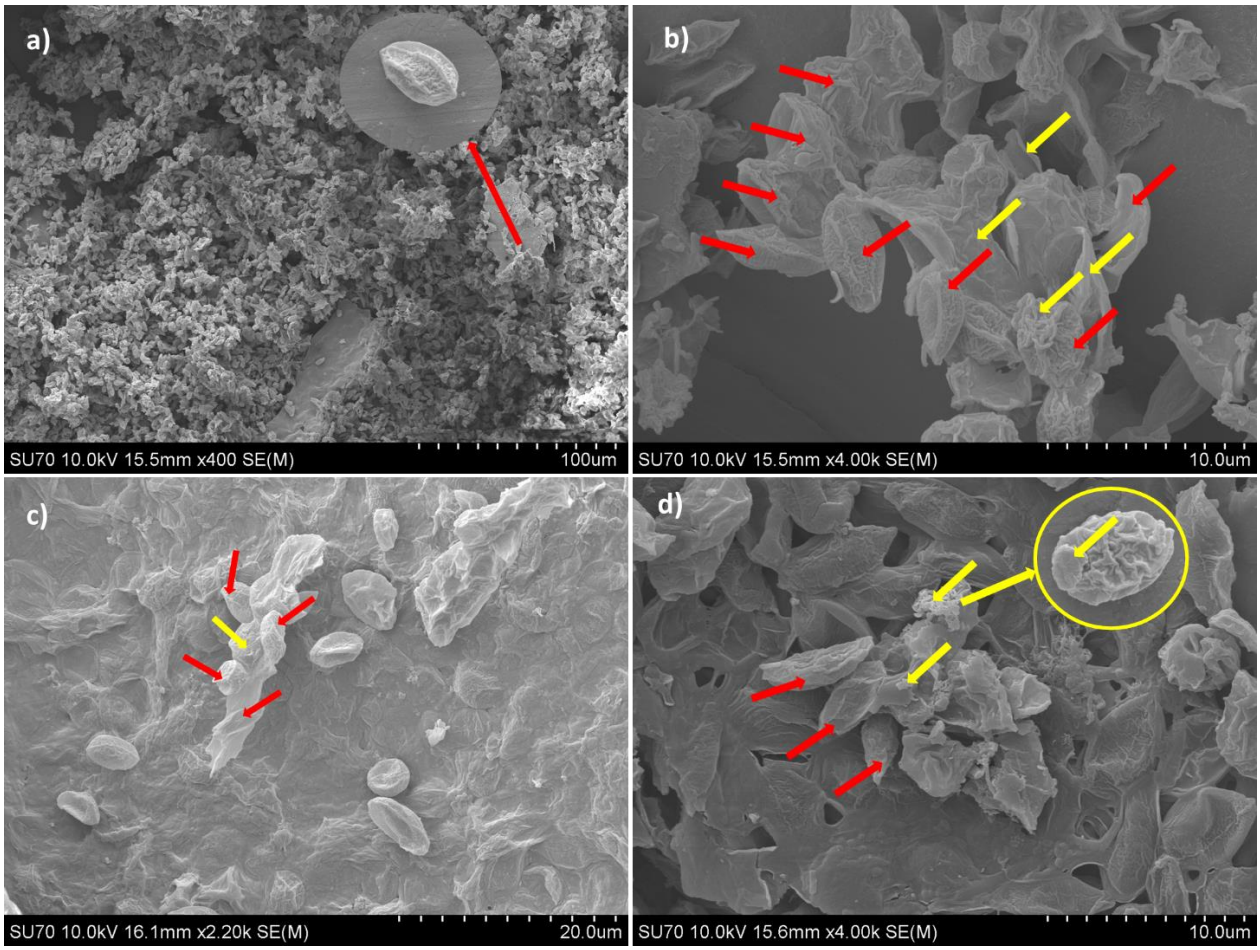

Figure 4.8 SEM images of *Scenedesmus* sp. cultivated in different concentrations of PET (a) microplastics colonized by *Scenedesmus* sp. cells (X400), (b) PET trapped in between *Scenedesmus* sp. cells, (X4000), (c) Aquatic *Scenedesmus* sp. forming homo-aggregates (X2200), and (d) Terrestrial *Scenedesmus* sp. forming homo-aggregates (X4000). Red arrows refer to *Scenedesmus* sp. and yellow arrow refer to PET microplastic.

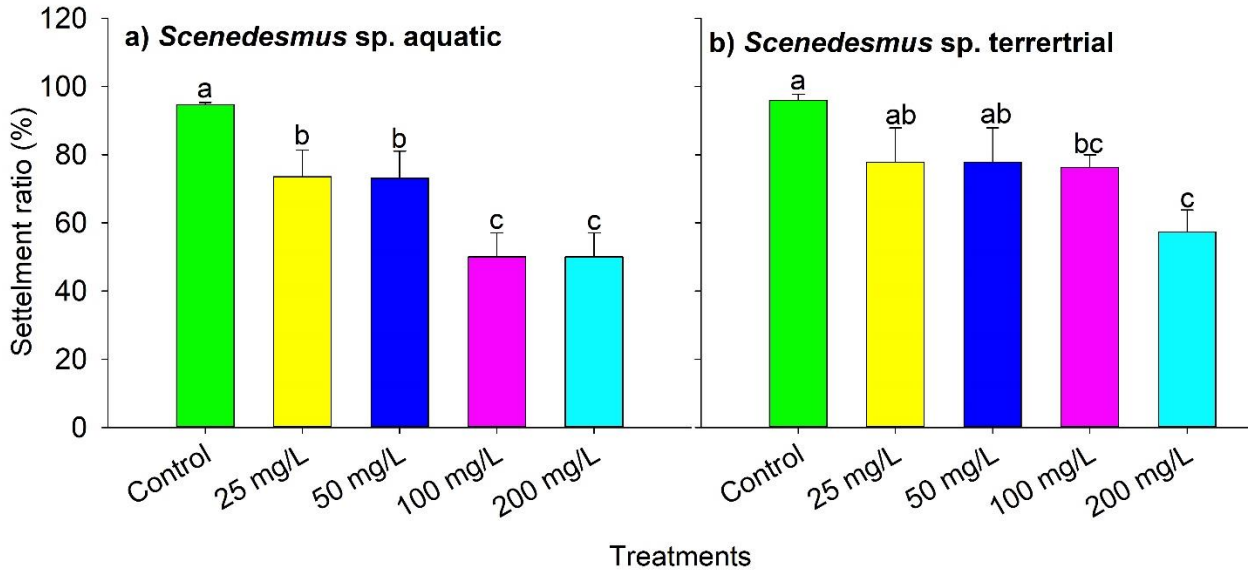

Figure 4.9 The settlement rate (ST) of different treatment groups in day 24 and 25 (24 hours). The statistic derived from a one-way ANOVA with post-hoc Tukey's test. The different letters above the bar signified the differences between treatments at $P < 0.05$.

4.1.4 Discussion

4.1.4.1 PET-induced growth inhibition

The effects of microplastics on microalgae growth have been investigated in several studies and have shown that microplastics can reduce the growth rate, biomass, and chlorophyll content (Song et al. 2020, Issac and Kandasubramanian 2021, Lang et al. 2022). The effects of microplastics on microalgae growth can have significant consequences for the aquatic food chain, as microalgae are an important food source for many aquatic organisms (Pacheco Vega et al. 2015, Seong et al. 2021). Furthermore, the accumulation of microplastics in aquatic systems can also lead to the transfer of these particles through the food chain, potentially

causing further ecological damage (Huang et al. 2021, Jaiswal et al. 2022). The result suggests that the growth of *Scenedesmus* sp., both aquatic and terrestrial, was lowered compared to the control group. However, the effect was more significant in terrestrial *Scenedesmus* sp. than in aquatic *Scenedesmus* sp.. The terrestrial microalgae used in this study were isolated from tree bark, therefore, it has less exposure to different pollutants than aquatic species. For instance, Lagarde et al. (2016) reported an increasing trend of cell number of *Chlamydomonas reinhardtii* in polypropylene (PP) and high-density polyethylene (HDPE) microplastic along the cultivation days. Similarly, Cunha et al. (2019) observed the increasing trend of cell density for *Microcystis panniformis* and *Scenedesmus* sp. but the cell density was significantly lower in microplastic-treated groups compared to the control group. In terms of cell density, our study revealed that aquatic microalgae are more tolerant to microplastic pollution than terrestrial ones. During the early cultivation period (1 − 6 days), *Scenedesmus* sp. exhibited an increased level of inhibition due to the unfavorable environment created by the PET on the microalgal cells. Our study is in line with the findings of other studies. Ye et al. (2023) studied the interaction of microplastic and 12 species of microalgae and revealed growth inhibition by microplastics. He et al. (2022) reported the increasing trend of growth inhibition (7−17%) in *Chlorella pyrenoidosa* during the cultivation days. However, PET microplastic showed mixed patterns of effect on microalgae (Table 1). Studies by Hadiyanto et al. (2022) and Khoironi et al. (2019) found that PET particles can inhibit the growth of *Spirulina* sp. In another study, Song et al. (2020) demonstrated that PET can provide a suitable substrate for the growth of *Chlorella* sp.

and *Phaeodactylum tricornutum*. However, high concentrations of PET particles had a negative effect on the growth of *Scenedesmus vacuolatus* (Rummel et al. 2022).

4.1.4.2 Inhibition in chlorophyll content

Chlorophyll is a major photosynthetic pigment in microalgae. The study findings showed that the total chlorophyll content of all treatment groups linearly increased from day 1 to day 24, indicating the growth of microalgae. However, compared to the control group, microalgae exposed to microplastics showed lower chlorophyll content. Lower chlorophyll content in microplastic exposed groups further suggested that photosynthesis was hindered, possibly due to microplastic blocking the pores for cellular respiration (Bhattacharya et al. 2010) and the buildup of reactive oxygen species within the cell (Song et al. 2020). This could lead to cellular damage and impede the synthesis of chlorophyll. These findings are consistent with previous studies that reported the inhibitory effects of microplastics on the growth and photosynthetic activities of microalgae (Yang et al. 2020b, Xu et al. 2023). Xu et al. (2023) found that the chlorophyll content of *Skeletonema costatum* decreased by 20% under 50 mg/L PVC microplastics. But, the study by Song et al. (2020) demonstrated that 200 mg/L concentrations of PP, PE, PET, and PVC microplastic did not affect the chlorophyll content of *Chlorella* sp. and *Phaeodactylum tricornutum* within 96 hours. The microplastic toxicity greatly varies between the species, microplastic type, and exposure time (Lagarde et al. 2016, Yang et al. 2020b, Xu et al. 2023). The findings suggest that microplastic pollution can negatively impact

the photosynthetic activities of microalgae, which can have implications for the functioning of aquatic and terrestrial ecosystems.

4.1.4.3 Toxicity of PET on microalgae

Hydrogen peroxide concentration

Hydrogen peroxide (H_2O_2) is one of the ROS that is produced during oxidative stress and plays a key role in signaling pathways and defense mechanisms in plants and algae (Mullineaux et al. 2018). The production of extracellular H_2O_2 can be induced by various environmental stressors, including exposure to environmental stress such as salinity, pollutants, and microplastics (Xu et al. 2019, Thiagarajan et al. 2022, Zhang et al. 2022, Kholssi et al. 2023). The increment of hydrogen peroxide in the algal cells can have a severe effect on cellular metabolism which ultimately leads to cellular damage and death (Xu et al. 2019, Song et al. 2020). In our study, the increased level of H_2O_2 in the algal cells that where exposed to microplastic reflected the oxidative stress caused by the microplastic. The gradual increase in H_2O_2 levels in the early stages of the experiment may be due to the initial stress response of the algal cell, followed by a sustained production of H_2O_2 as the stress persisted. The findings of this study are consistent with previous studies that have reported an increase in H_2O_2 levels in response to environmental stressors in algae (Zhang et al. 2017, Song et al. 2020, Xiao et al. 2020) and other plants (Khan et al. 2023). For example, exposure to heavy metals has been

shown to induce H_2O_2 production in various algae species, including *Scenedesmus* sp. (Danouche et al. 2020). Similarly, exposure to pollutants such as polycyclic aromatic hydrocarbons (PAHs) has been shown to induce oxidative stress and H_2O_2 production in plants (Molina and Segura 2021).

Variation of EPS content

Extracellular polymeric substances (EPS) are a complex mixture of biopolymers secreted by microorganisms that play an essential role in their attachment, aggregation, and biofilm formation (Mahto et al. 2022). EPS production has been observed in various microorganisms, including microalgae, in response to environmental stressors such as pollution, heavy metals, and microplastics (Song et al. 2020). In our study, EPS production markedly increased in microalgae exposed to microplastic. Similar results were observed in *Microcystis aeruginosa* when exposed to polystyrene microplastics (0.1 μm and 5 μm) at a concentration of 100 mg/L (Xiao et al. 2020). Notably, EPS production appears to increase markedly upon exposure to stress factors such as microplastics (Senousy et al. 2023). Zheng et al. (2022) revealed that when *Microcystis aeruginosa* was exposed to nylon microplastics at a concentration of 100 mg/L, it significantly affected the TCA cycle which is associated with polysaccharides, lipid, and protein decomposition. Once the TCA cycle gets disturbed, the decomposition of organic matter gets delayed and organic matter starts to deposit on the algal cell wall (Yang et al. 2020a).

This is a possible self-defence mechanism of microalgae to protect themselves from the toxicity of microplastics (Senousy et al. 2023).

4.1.4.4 Interaction between microplastic and microalgae

This study examined the morphological changes in *Scenedesmus* sp. cells using Scanning Electron Microscope (SEM) imaging. We found that the control cells had a regular bean shape with a smooth surface. However, when exposed to PET microplastics, the cell wall became rough and irregular. This might be due to the production of EPS by algal cell and the attachment of PET to the microalgae cell wall (Lagarde et al. 2016). Studies showed microalgal cells formed hetero-aggregates with microplastics which cause physical impairment, exerting a negative influence on the growth of microalgae and eventually reducing photosynthesis (Zheng et al. 2022). A higher concentration of microplastic can block microalgae cells to absorb nutrients and light (Zhao et al. 2019, Xiao et al. 2020). Wang et al. (2020) and Zheng et al. (2022) also reported microplastic aggregation on microalgae surfaces and subsequently declined growth of microalgae as the concentration of microplastic increased.

Once microalgal growth reaches the stationary phase, they begin to settle due to an increase in biomass, lipids, and EPS (Zhu et al. 2018, Li et al. 2020a). Microalgae form hetero-aggregates with microplastics, which makes them more buoyant and delays settlement in PET-exposed microalgae. In natural aquatic ecosystems, these hetero-aggregates of microplastic and

microalgae may float for longer periods of time and could be transferred to other primary consumers which further threatens the health of aquatic organisms.

Conclusion

This study investigated the effects of PET microplastic on native microalgae isolated from northern Ontario, Canada, and explored the potential as a biological agent for treating microplastic pollution. The results showed that PET microplastic inhibited the growth and chlorophyll content and induced the production of extracellular hydrogen peroxide in microalgae. However, the production of EPS in microalgae cells assisted in the adsorption of microplastic particles and formation of hetero-aggregation and facilitated the sedimentation. These findings shed light on the ecological risks of microplastics on aquatic organisms and highlight the need for effective management strategies to reduce their input into the environment. The limitation of this study is that we were unable to compare the effects of different sizes of PET microplastics on various algal species. Further research is necessary to understand the impact of microplastics on microbial communities and ecosystem health.

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Chapter 5

5.1 Optimizing Pretreatment Parameters for Enhanced Bioethanol Production from Native Freshwater Microalgae Using Response Surface Methodology

Abstract

The pretreatment of microalgal biomass is a major hurdle for biofuel production from microalgae. Various pretreatment methods have been used to convert microalgal polysaccharides into simple sugar, which is later converted to bioethanol using fermentation. This study aimed to optimize the reducing sugar production by using different pretreatment parameters such as: algal biomass, concentration of hydrolytic agent, and autoclave time, which have a significant impact on the reducing sugar yield. Response surface methodology (RSM) was used to optimize the reducing sugar extraction from three independent factors: algal biomass, sulfuric acid concentration, and autoclave time using a central composite design (CCD). Our results showed that microalgal biomass hydrolyzed with H_2SO_4, followed by the autoclave, yielded a higher reducing sugar than NaOH and H_2O hydrolysis. The optimal conditions for biomass hydrolysis for the maximum reducing sugar production (247.55 mg/g) were biomass weight of 10.2% biomass (v/w), H_2SO_4 concentration of 6.6%, and autoclave time of 35 min. The highest concentration of bioethanol was obtained at 0.134 g ethanol/biomass at 72 hours of fermentation. This study highlights the potential for producing

bioethanol from native freshwater microalgae cultivated on malting effluent followed by acid hydrolysis and autoclave pretreatment.

Keywords: Microalgae, Pretreatment, Reducing sugar, Response surface methodology, Bioethanol

5.1.1 Introduction

The ongoing depletion of fossil fuel, surging prices, and its potential effect on global warming by releasing greenhouse gases pose significant challenges in the 21st century (Abbasi et al. 2022, Rehman et al. 2023). It brings attention to finding an alternative, green, sustainable, and eco-friendly energy source. Most biofuels, enzymes, and biomolecules have been produced from the first (cereals, corn, and canola) and second (lignocellulosic biomass) generation of biofuels (Mat Aron et al. 2020). In the scenario of limited availability of arable land and a global food resource crisis, numerous challenges arise in ensuring the long-term sustainability of biofuel production. This applies to both first (crops) and second generation (lignocellulosic biomass) biofuels (Singh et al. 2020). Moreover, there is an increasing interest in the production of third-generation biofuels using microalgae (Debnath et al. 2021). Algal biomass is recognized as a vital source for biofuel production because of its accelerated cellular growth rate and higher biomass yield in contrast to terrestrial plants. Furthermore, its capacity to thrive in various wastewater environments and its non-competitive relationship with food resources

make it well suited for biofuel production. Microalgae offers a potential substitute for biofuels, value-added components, and bioactive compounds. However, algal biofuel production is still in the rudimentary stages and has faced several hurdles to producing cost-competitive biofuel compared to petroleum fuels (Debnath et al. 2021). Proper lighting conditions, nutrient supplements, harvesting algal biomass, and dewatering are critical factors affecting the commercialization of generating biofuel and bioproducts from algal biomass (Ananthi et al. 2021).

Microalgal biomass contains various biomolecules, including lipids, proteins, carbohydrates, and value-added products (Khoo et al. 2023). As seen in *Chlorella* sp., *Scenedesmus* sp., *Chlamydomonas* sp., *Chlorococcum* sp., and *Desmodesmus* sp., which contain higher polysaccharide content in their biomass, and signifies the optimal conditions for the production of biofuels (Goswami et al. 2022). Among the varieties of biofuels, bioethanol draws substantial interest as a renewable energy source because of its capacity to replace fossil fuels and aid in mitigating greenhouse gas emissions. Therefore, algal biomass is considered a promising source to produce bioethanol. Several microalgae species have been identified for commercial-scale cultivation and production of bioethanol (Bibi et al. 2022, Yirgu et al. 2023). Compared to other lignocellulosic biomasses, algal cells are rich in cellulosic content and devoid of hemicellulose and lignin, simplifying the pretreatment process during biofuel production. Pretreatment of microalgal biomass facilitates in the conversion of intracellular

polysaccharides into simple sugars, and different microorganism converts sugar into bioethanol (Sanchez Rizza et al. 2017, Yirgu et al. 2023). Yeast (*Saccharomyces cerevisiae*) fermentation have been used in many studies and for commercial scale bioethanol (Bader et al. 2020). This fungus can ferment various sugars into ethanol and exhibits higher tolerance to ethanol concentrations, as well as higher ethanol productivity (Kong et al. 2021). Pretreatment process significantly affect the ethanol production. The *Chlorella* sp. pretreated with diluted sulfuric acid (5%) produce 0.28 g of ethanol/biomass (Phwan et al. 2019). El-Mekkawi et al. (2019) found that *Microcystis* sp. can produce 0.18 g of ethanol/microalgae biomass treated with 0.5 N sulfuric acid for 4 hours at 120 °C. Similarly, *Desmodesmus* sp. grown in BG-11 medium with various nitrogen concentrations can produce 0.24 g of ethanol/biomass (Sanchez Rizza et al. 2017).

This study aimed to optimize the reducing sugar production by using three key parameters: algal biomass, acid/base/water bath hydrolysis, and autoclave time, which have a significant impact on the fermentation yield (Kaur et al. 2022, Yirgu et al. 2023). Previous studies often investigated these parameters individually, keeping one constant while varying the others, limiting the understanding of potential interactions among the factors. To overcome this limitation, we utilized response surface methodology (RSM) to investigate the simultaneous impact of multiple parameters on reducing sugar production. RSM enables the identification

of nonlinear relationships between the independent variables. Additionally, a predictive model for bioethanol production was developed to further enhance the understanding of the process.

5.1.2 Materials and Methods

5.1.2.1 Microalgae cultivation and biomass production

Chlorella sorokiniana was cultivated in malting effluent (hereafter ME). The effluent was kept at 4 °C until the experiment. The particulate matter was removed by sedimentation followed by filtration. Based on our previous study Khatiwada et al. (2022), diluted malting effluent (ME-50) (one volume of ME mixed with one volume of distilled water) yielded higher biomass production, therefore, this study used malting effluent diluted for biomass production and nutrient removal experiment. A four-liter glass bottle with three liters of working volume was used for microalgae cultivation. The bottles were illuminated with white LED lights with 1850 lumens and with a 16-hours light and 8-hours dark light cycle with continuous air supply (CO_2 aeration of 2%). The experiment was carried out at a uniform room temperature of 22 °C (±2 °C).

5.1.2.2 Microalgal biomass pretreatment for reducing sugar production

In this study, microalgal biomass was pretreated with sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), and a water bath followed by an autoclave. Previous studies have widely used acids and bases for releasing reducing sugar from microalgal biomass. Five different parameters:

algal biomass (5 to 30% w/v), H_2SO_4 and NaOH concentration (2 to 10%), water bath (90 °C for 20 min), and autoclave time (5 minutes to 55 minutes) were manipulated to produce optimal reducing sugar.

After pretreatment, samples were placed into an ice bath, pH neutralized using HCl and NaOH, and then centrifuged to separate the algal pellet and the supernatant. The concentration of reducing sugars in the supernatant was determined using the 3,5-dinitrosalicylic acid (DNS) method. Specifically, 10 µL of the supernatant sample was combined with 20 µL of 0.05 M phosphate buffer (pH 7.0) and transferred to a 96-well plate. The plate was then incubated in a water bath at 50 °C for 10 minutes. Following this incubation, 60 µL of DNS solution was added to each well. The plate was subsequently placed in a 90 °C water bath for 5 minutes to terminate the reaction. After this, the reaction mixture was cooled in ice water, and 200 µL of distilled water was added. Absorbance was measured at 540 nm using a UV-spectrophotometer. The amount of reducing sugars released was quantified using a glucose calibration curve as a standard.

5.1.2.3 Optimization of pretreatment methods from RSM

Based on the single factor analysis 6% H_2SO_4 concentration, 10% biomass (w/v), and autoclave time (35 minutes) yielded the maximum reducing sugar (see details in the 5.1.3.3). Therefore, the RSM methodology was used to find the optimal reducing sugar extraction process. A central

composite design (CCD) within the RSM framework was utilized to explore the optimal production levels, focusing on three independent variables: H_2SO_4 concentration, biomass weight, and autoclave time using Design-Expert 13. Table 5.1 provides a detailed list of these independent variables.

Table 5.1 Independent variables with actual and coded values.

Variable	**Coded value**		
	Low (-1)	**Center (0)**	**High (+1)**
Acid concentration (%)	2	6	10
Biomass (% of acid volume)	5	10	15
Autoclave time (minutes)	5	30	55

5.1.2.4 Parameter estimates

On the final day of the experiment (day 22), the dry weight of the microalgal samples was measured. The biomass was collected by centrifuging 50 mL of the microalgal culture at 4000 rpm for 10 minutes. To ensure the removal of salts and other contaminants, the resulting microalgal pellet was washed twice, then dried in an oven at 80 °C for 48 hours. The total chlorophyll content was assessed following the method described by Khatiwada et al. (2023). Specifically, 2 mL of the culture was centrifuged at 13 000 rpm for 10 minutes to obtain the algal pellet. Two millilitres of methanol were added to the pellet, followed by brief ultrasonication to disrupt the algal cell walls. The mixture was then incubated at 65 °C for 10 minutes and subsequently kept in the dark at room temperature for 15 minutes followed by 10-

minute centrifugation. The absorbance of the supernatant was measured at 652 nm and 665 nm using a UV-spectrophotometer.:

Chlorophyll α (mg/L) = $16.82A_{665}$-$9.28A_{652}$

Chlorophyll β = $36.92A_{652}$-$16.54A_{665}$

Total Chlorophyll Content (mg/L) = Chlorophyll α + Chlorophyll β

The total carbohydrate concentration was measured following the method outlined by Masuko et al. (2005), with few modifications. A sample of 0.01 grams of dry biomass was combined with 150 µl of concentrated sulfuric acid, and then 30 µl of 5% phenol solution was added. This mixture was heated in a water bath at 90 °C for 10 minutes, cooled in an ice bath for another 10 minutes, reheated at 90 °C for 10 minutes, and cooled again in an ice bath. The absorbance of 100 µl of the reaction mixture was then measured at 490 nm using a UV-spectrophotometer. Glucose served as the standard for estimating the total carbohydrate content.

For protein content measurement, the Bradford Assay was employed. A 0.01 gram sample of algae was mixed with 200 µl of distilled water and subjected to ultrasonication for ten minutes. The samples were centrifuged for 5 minutes. Then, 150 µl of the supernatant was mixed with 150 µl of Bradford reagent. This mixture was incubated at room temperature for 10 minutes, and absorbance was measured at 595 nm using a spectrophotometer, with bovine serum albumin as the standard.

Total lipids from the algal biomass were extracted according to the Bligh and Dyer (1959) method. In brief, 0.1 g of dry algal biomass was mixed with a mixture of chloroform, methanol, and distilled water in a 1:2:0.8 (v/v/v) ratio. The algal cells were disrupted via ultrasonication for 10 minutes, followed by centrifugation at 13,000 rpm for 10 minutes. The supernatant was transferred to a pre-weighed Eppendorf tube (W1 g), and the cell pellet was re-extracted with a chloroform and methanol mixture (1:2, v/v). After another round of centrifugation, the supernatant was added to the same pre-weighed Eppendorf tube. Chloroform and water were then added to achieve a final ratio of 1:1:0.9 (chloroform:methanol:water, v/v/v). The mixture was homogenized by vortexing and centrifuged again at 13 000 rpm for 5 minutes. The top layer was discarded, and the bottom layer was evaporated and dried at 80 °C until a constant weight (W2 g) was achieved. The total lipid content was determined by subtracting W1 from W2 and expressed as a percentage of the dry weight.

5.1.2.5 Fourier transform infrared spectroscopy (FTIR)

The effectiveness of the acid pretreatment on algal cell wall degradation was determined by FTIR analysis. Using distilled water, the acid-pretreated biomass was washed twice, followed by the drying of the biomass at 80 ºC for 24 hours. Once dried, the biomass was ground with mortar to obtain a fine particle. The FTIR spectra were captured using a Perkin Elmer FTIR system spectrum. The absorbance was measured between 3,500 cm^{-1} and 500 cm^{-1} at a spectral resolution of 4 cm^{-1}.

5.1.2.6 Preparation of yeast inoculum for fermentation and bioethanol analysis

Saccharomyces cerevisiae was cultivated in a Yeast Peptone Dextrose (YPD) medium to prepare the yeast inoculum, which comprised 1% yeast extract, 2% peptone, and 2% glucose. This culture was grown in a liquid medium and incubated at 35°C for 24 hours. The fermentation of microalgal hydrolysate was performed in triplicate using 125-ml conical flasks, each containing 50 ml of working volume. Each flask was supplemented with the following components: 6 g/L Na_2HPO_4, 3 g/L KH2PO_4, 5 g/L $NaNO_3$, 1 g/L NaCl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.5 g/L yeast extract. Aseptically, 0.5 ml of pre-cultured yeast was added to each flask. The flasks were sealed with rubber stoppers and purged with nitrogen gas for 5 minutes to create anaerobic conditions. The flasks were then incubated at 30°C with shaking at 200 rpm for 96 hours. Samples were collected every 24 hours for ethanol measurement.

5.1.2.7 Bioethanol quantification

Bioethanol production at every 24 hours were determined using acidic potassium dichromate ($K_2Cr_2O_7$) reagent (Miah et al. 2017, Sriariyanun et al. 2019, El-Sheekh et al. 2023). The dichromate reagent was prepared by dissolving 15 g of $K_2Cr_2O_7$ in 100 ml of 5 M H_2SO_4. Ethanol was extracted using tri-n-butyl phosphate (TBP). In brief, 2 ml of the sample was centrifuged at 13 000 rpm for 10 minutes. One millilitre of the supernatant was mixed with an equal volume of TBP and vortexed vigorously for 1 minute. The mixture was then centrifuged

at 13 000 rpm for 5 minutes. The upper layer (500 µl) was combined with 500 µl of the dichromate reagent and vortexed until the phases separated. The lower phase, which appeared blue-green, was diluted ten folds, and its absorbance was measured at 595 nm using a spectrophotometer. Bioethanol concentration was estimated by constructing a standard curve using known volumes of absolute ethanol.

5.1.2.7 Statistical analysis

The differences in biomass, chlorophyll, carbohydrate, and lipid content variation along the cultivation day and the reducing sugar content from different pretreatment methods were tested using one-way ANOVA with post hoc Tukey test. The correlation between the observed and expected reducing sugar content was analyzed by linear regression. All the experiments were performed in triplicates, and statistical analysis was conducted in the R-language (R Development Core Team 2022).

5.1.3 Results and Discussion

5.1.3.1 Biomass production and nutrient removal

This study used malting steep wastewater for the nutrient removal experiment. The nitrate and phosphate removal rates for *Chlorella sorokiniana* were 94.11% and 72.78%, respectively (Fig. 5.1). These findings showed that microalgae can efficiently eliminate nutrients from malting

steep wastewater and simultaneously producing biomass. There was a linear increment of algal biomass with increasing cultivation days (Fig. 5.1).

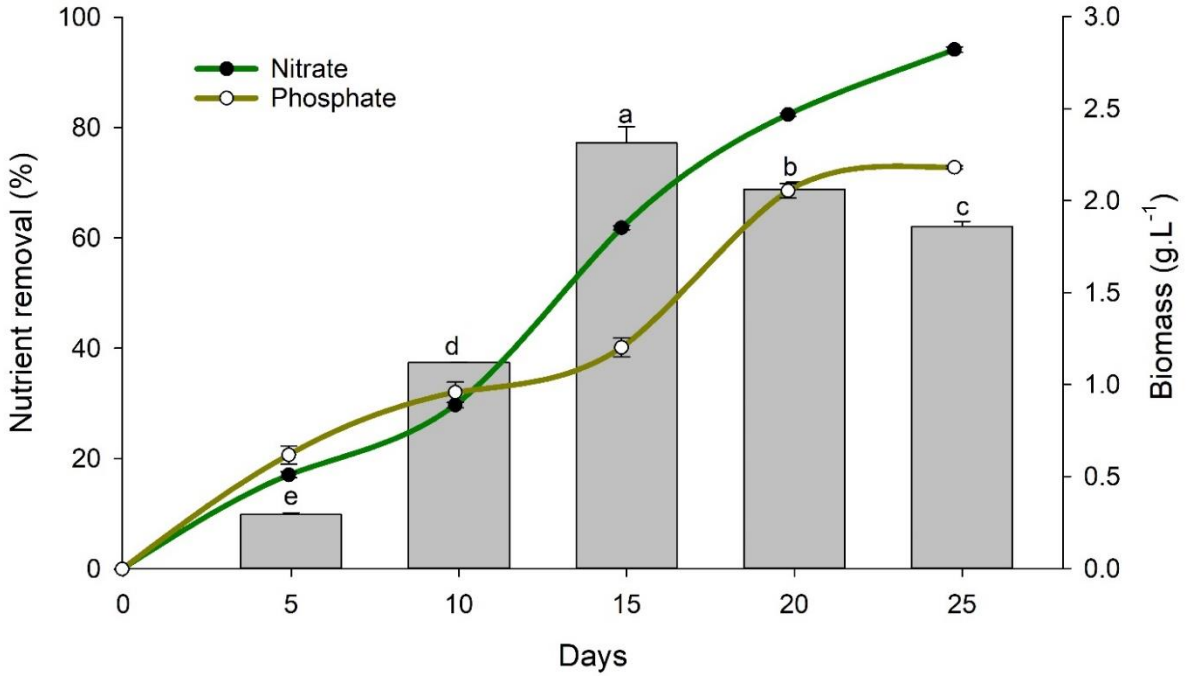

Figure 5.1 Variation of biomass production and nutrient removal by *C. sorokiniana* sp. along the cultivation days. The bar represents the mean cell density and error bar represents the SD. The letters above the bars are derived from on way ANOVA with posthoc Tukey test. The different letter above the bars signifies the significant different at $P < 0.05$. The lines represents the trend of nutrient removal (%).

The maximum dry weight (2.31 ±0.08 $g.L^{-1}$) produced on days 15 and growth of *C. sorokiniana* declined afterwards. Yirgu et al. (2023) (Yirgu et al. 2023) reported that nitrate and phosphate removal efficiencies of *Scenedesmus* sp. were 94.52% and 69.42% with the utilization of

brewery effluent. Another study by Ferreira et al. (2017) (Ferreira et al. 2017) demonstrated 88.5% and 40.8% of total nitrogen and phosphate removal after cultivation. Our study aligned with previous studies and reported the higher nutrient removal by microalgae from brewery wastewater (Darpito et al. 2015, Ferreira et al. 2017, Yirgu et al. 2023). Nitrate and phosphate are major nutrients for microalgae for growth and development. The malting steep water contains higher nutrient availability (Khatiwada et al. 2022) compared to other wastewater sources, for example, municipal, pulp and paper industries and industrial wastewater.

5.1.3.2 Biomolecule composition

The biochemical composition of *C. sorokiniana* grown in malting effluent was carried out on day 25 and found to be 18.38% proteins, 22.70% lipids, 33.87% carbohydrates, and 2.94 mg/L of chlorophyll content (Table 5.2). Microalgae stimulate their metabolic processes by storing carbohydrates and lipids as energy reserves to endure nitrogen stress (Ran et al. 2019). Peptides and proteins undergo conversion into energy stores for this purpose, while protein synthesis is inhibited due to nitrogen being a key element required for this process (Su 2021).

Chlorophyll is a significant pigment of microalgae and valuable understanding regarding the physiological reactions to the environment (Khatiwada et al. 2023). *C. sorokiniana* showed a consistent linear rise in total chlorophyll content with increasing days with the highest concentration on day 15. The chlorophyll content in microalgae can vary significantly among

different species and under different environmental conditions. This variation is influenced by factors such as nutrient availability, light intensity, temperature, and the growth phase of the microalgae (Pérez-Morales et al. 2023, Singh Chauhan et al. 2023).

Table 5.2 Biomolecule composition of *C. sorokiniana* grown in malting effluent.

Biomolecule	**Concentration**
Carbohydrate (% of biomass)	31.67 ± 0.33
Protein (% of biomass)	18.54 ± 0.74
Lipid (% of biomass)	23.04 ± 1.60
Chlorophyll ($mg.L^{-1}$)	2.75 ± 0.14

It is revealed that lipid was the dominant biomolecule component of *C. sorokiniana*, followed by carbohydrate content. Yirgu et al. (2023) outlined a higher carbohydrate concentration in *C. sorokiniana* cultivated using brewery effluent compared to domestic and municipal wastewater. In response to stress conditions, algal cells accumulate carbohydrates and lipids. This study revealed elevated lipid and carbohydrate levels after 15 days of the experiment, confirming nutrient depletion in the growth medium. Kaur et al. (2022) reported increased carbohydrate content during nitrogen deficiency conditions. They further revealed that limiting the nitrogen sources markedly increased the carbohydrate content by 50% in *Chlorella sorokiniana*. Liu et al. (2022) disclosed a higher lipid content of *Chlorella vulgaris* by 31.33% during nutrient starvation. They also reported increased cell size, numbers, and starch granules. Lipids are essential for biofuel production and contain fatty acids for diverse industrial processes. Higher

lipid content might make *C. sorokiniana* a promising candidate for biofuel production, contributing to sustainable energy solutions.

5.1.3.3 Effect of pretreatment on reducing sugar production

In this study we used H_2SO_4, NaOH, and H_2O for the pretreatment of dry microalgal biomass. Among them H_2SO_4 was the most effective hydrolytic agent for releasing reducing sugar in different autoclave times. H_2SO_4 pretreated algal biomass released higher amounts of reducing sugar (282.32 ± 0.42 mg/g) compared to NaOH (60.26 ± 0.93 mg/g) and H_2O (9.72 ± 0.62 mg/g) (Fig. 5.2 a–c). Therefore, H_2SO_4 was selected for the hydrolysis of microalgal biomass. Furthermore, autoclave timing also significantly affects reducing sugar production. The highest quantity of reducing sugar was acquired from 10% biomass with 6% H_2SO_4 at 30 minutes autoclave compared to 10 and 55 minutes, respectively (Fig. 5.3). Our study aligned with other studies that reported that sulfuric acid is the best hydrolytic agent for reducing sugar extraction from microalgae (Yu et al. 2020, Kaur et al. 2022). Studies have shown that H_2SO_4 was more efficient than hydrochloric acid and NaOH at releasing sugar from different microalgae species (Miranda et al. 2012). Moreover, Yirgu et al. (2023) (Yirgu et al. 2023) performed acid, base, and H_2O_2 hydrolysis on microalgal biomass, and findings indicated that NaOH and H_2O_2-assisted hydrolysis yielded lower sugar compared to acid-assisted hydrolysis.

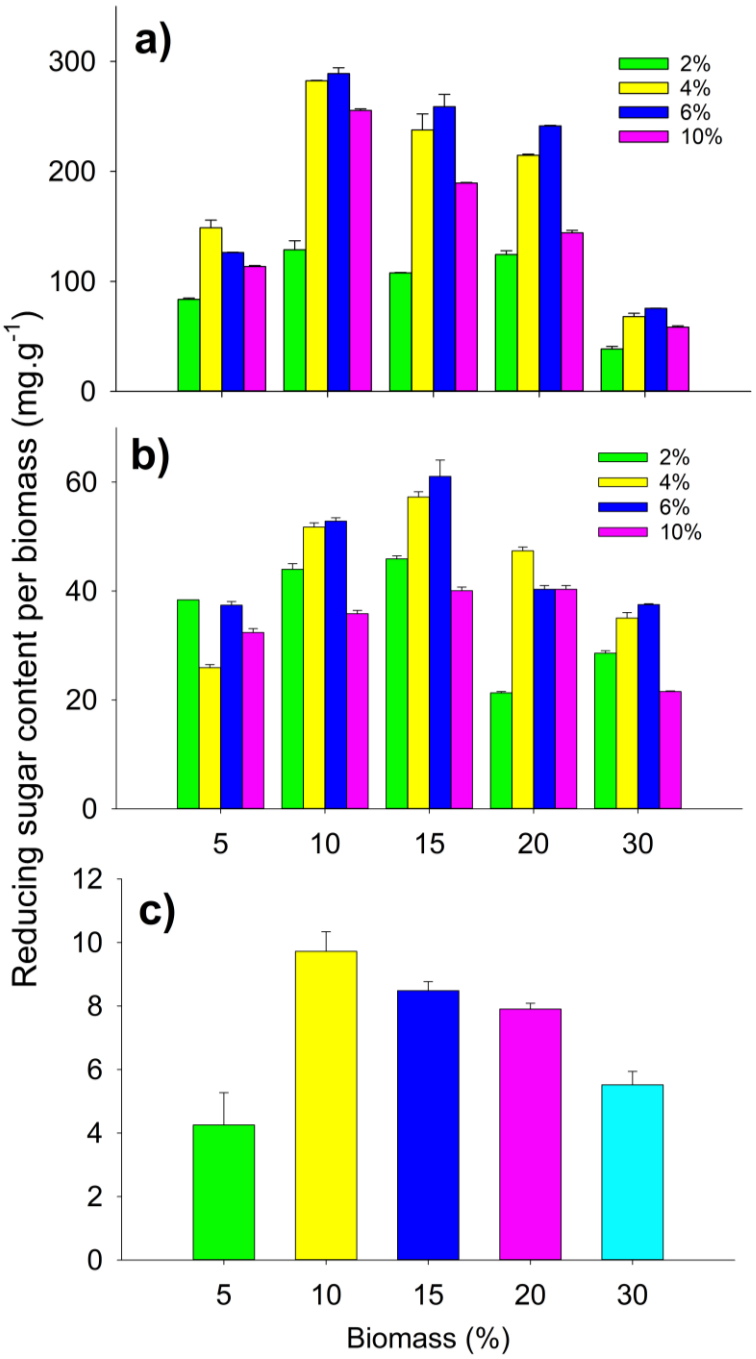

Figure 5.2 Amount of reducing sugar production using (a) sulphuric acid, (b) sodium hydroxide, and (c) water as hydrolytic agent.

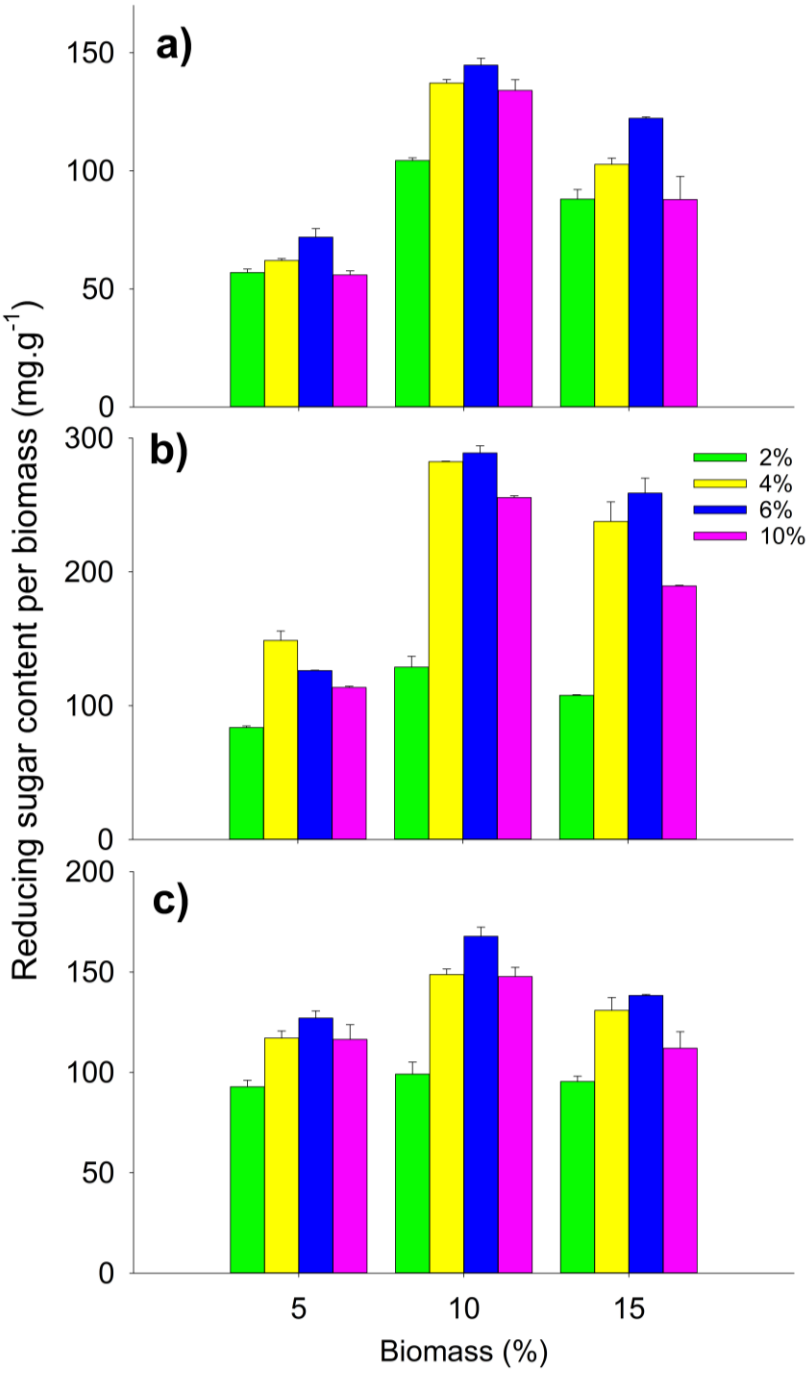

Figure 5.3 The amount of reducing sugar released from microalgal using different concentrations of sulfuric acid in different autoclave time (a) 5 minutes, (b) 30 minutes and (c) 55 minutes.

However, they further revealed that HCl hydrolysis was more efficient than H_2SO_4 hydrolysis for *Scenedesmus* sp. Thus, hydrolytic agent for reducing sugar extraction might vary between the microalgal species.

5.1.3.4 RSM for the optimization of H_2SO_4 concentration, microalgal biomass, and autoclave time

The synergistic effect of algal biomass, H_2SO_4 concentration, and autoclave time were optimized for the optimal reducing sugar extraction using a face-centered CCD through RSM. The model generated from RSM was validated using regression model (R^2), ANOVA statistics (not significant lack of fit), and response surface plots. The relations between reducing sugar content and experimental variables (autoclave time (A), algal biomass (B), and H_2SO_4 concentration (C)) were investigated through multiple regression analysis. Table 5.3 showed the 15 sets of responses for reducing sugar content production were employed. The results depicted the quadratic relationship between response and experimental variables and were estimated using the following equation.

Reducing sugar content (mg/g) = − 481.11 + 44.9A + 3008.57B+ 14.84C + 21.52AB + 0.059AC − 5.21BC -3.97A^2 − 7220.09B^2 − 0.19C^2

A, B, and C refer to the linear terms for acid concentration, autoclave time, and biomass weight, respectively. AB, AC, and BC are interaction terms, whereas A^2, B^2, and C^2 are the quadratic terms of experimental variables.

Table 5.3 Face-centered central composite design matrix with actual and predicted values of reducing sugar production.

Trail	**Acid Concentration**	**Biomass**	**Time**	**Reducing sugar content Per biomass (mg/g)**	**Predicted Reducing sugar (mg/g)**
1	2	10	55	98.98 ± 5.95	104.96
2	2	10	15	104.17 ± 1.13	80.32
3	2	15	35	109.05 ± 2.42	94.56
4	2	5	35	105.03 ± 4.01	98.32
5	6	15	55	138.07 ± 0.56	111.82
6	6	5	55	126.78 ± 3.58	119.2
7	6	10	35	259.01 ± 29.36	246.67
8	6	10	35	266.01 ± 27.05	246.67
9	6	10	35	250.01 ± 53.91	246.67
10	6	15	15	122.01 ± 0.55	98.58
11	6	5	15	71.73 ± 3.2	64.28
12	10	10	55	147.47 ± 4.58	143.43
13	10	10	15	133.78 ± 4.54	99.91
14	10	10	35	165.4 ± 8.13	140.81
15	10	5	35	126.96 ± 3.44	110.13

Thus, the maximum reducing sugar (mg/ml) can be predicted by modifying the autoclave time, concentrations of the algal biomass, and H_2SO_4 concentration in the above equation. A positive

sign before the term suggests a synergistic effect, whereas a negative sign implies an antagonistic effect.

Table 5.4 Statistics of analysis of variance for the polynomial quadratic response surface model with respect to reducing sugar production from microalgal biomass hydrolysis with different concentration of sulfuric acid in different autoclave time.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	1.740E+05	9	19328.73	67.63	< 0.0001
A	9170.13	1	9170.13	32.09	< 0.0001
B	4986.25	1	4986.25	17.45	0.0002
C	3429.96	1	3429.96	12.00	0.0013
AB	889.31	1	889.31	3.11	0.0852
AC	267.19	1	267.19	0.9349	0.3393
BC	1827.71	1	1827.71	6.40	0.0154
A^2	45373.17	1	45373.17	158.76	< 0.0001
B^2	59013.60	1	59013.60	206.48	< 0.0001
C^2	68594.64	1	68594.64	240.01	< 0.0001
Residual	11717.85	41	285.80		
Lack of Fit	1835.55	3	611.85	2.35	0.0874
Pure Error	9882.30	38	260.06		
Cor Total	1.857E+05	50			

In a synergistic effect, there will be a positive correlation between the terms and reducing sugar production; a negative correlation is expected between the terms and reducing sugar production

in the antagonistic effect. All quadratic terms (A^2, B^2, and C^2) showed a negative coefficient, indicating an antagonistic effect on reducing sugar content. There was a positive interaction between the terms AC and BC, indicating a synergistic effect, while AB showed a negative interaction, reflecting an antagonistic effect on reducing sugar content. ANOVA result indicated the regression model was significant ($F = 79.38$, $R^2 = 95.53\%$, $P < 0.0001$) and accounted for 95.53% of the variability in reducing sugar production can be predicted by the model (Table 5.3). Furthermore, the lack of fit was not significant, further validating the model ($F = 2.44$, $P < 0.0826$) (Kaushal et al. 2022).

The interaction between three parameters on the reducing sugars extraction was obtained by generating the three-dimensional response surface plots (Fig. 5.4). RSM offers a significant advantage in the experimental design by including the center points, which visualize the shape of the response curvature (Latha et al. 2017). In this study, increasing the acid concentration from 2% to 6.6% significantly increases the reducing sugars (245.65 mg/g) (Fig. 5.4). However, when further increasing the acid concentration from 6.6%, it decreases the reducing sugar content. According to the graphical analysis in Fig. 5.4, optimum reducing content was obtained when the autoclave time was 35 minutes with 10% of the biomass.

Furthermore, there was a significant correlation between the predicted and experimental reducing sugar content values (R^2=0.989, P<0.001, Fig. 5.5). The straight regression line

indicated that the RSM model predicted the response (sugar content) adequately (Yirgu et al. 2023).

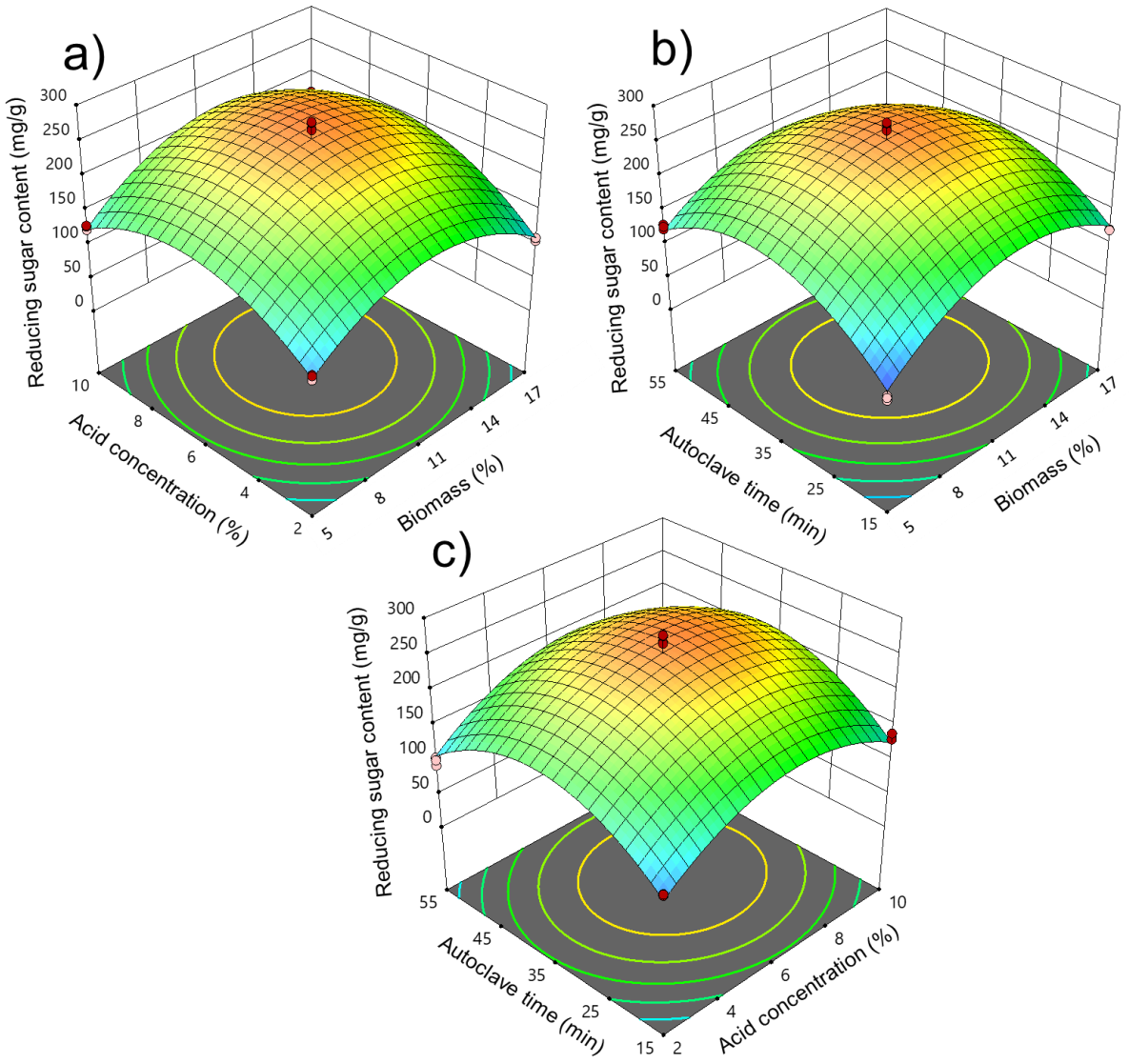

Figure 5.4 CCD matrix from RSM plots (a) effect of acid concentration and algal biomass on the reducing sugar content, (b) effect of the autoclave time and algal biomass on the reducing sugar content, and (C) effect of the autoclave time and acid concentration on the reducing sugar content.

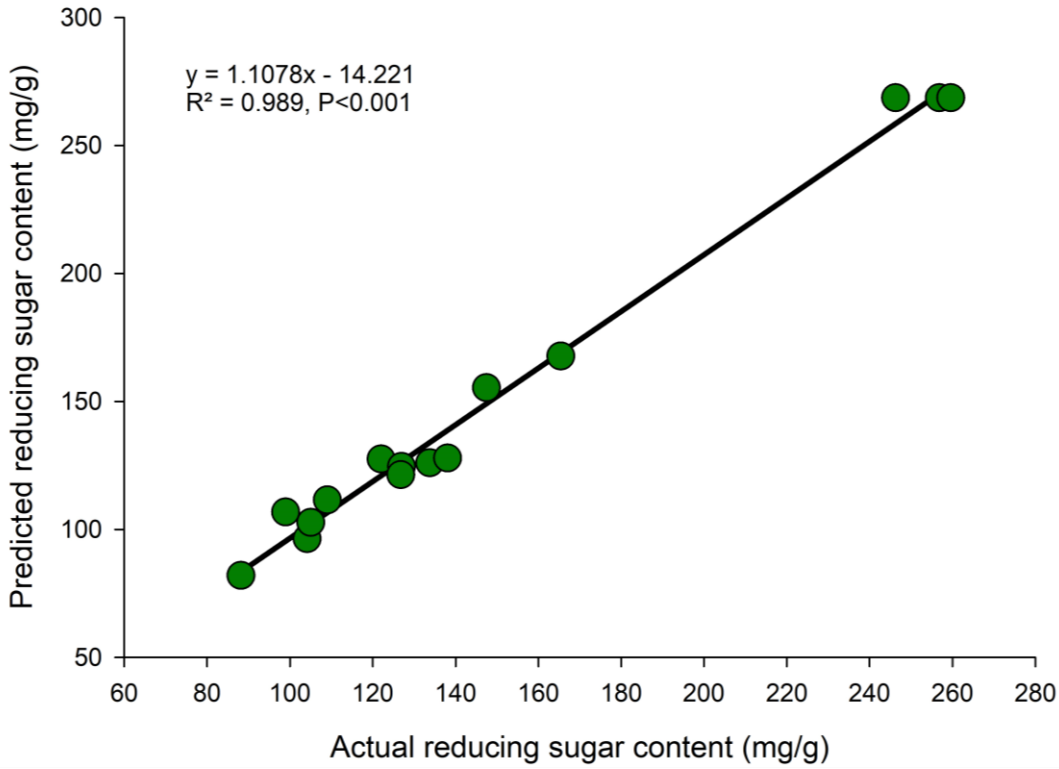

Figure 5.5 Relationship between experimental and predicted reducing sugar from acid hydrolysis.

5.1.3.5 Optimization result

The amount of reducing sugar content was optimized from RSM using three variables. This study identified the optimal condition for reducing sugar production from 10.2% algal biomass (v/w), 6.6% H_2SO_4, and 35 minutes of autoclave time. Our optimization efforts aimed to identify the ideal treatment conditions that yield the highest reducing sugar extraction and the outcomes obtained by utilizing a Design Expert to define all the criteria that led to the optimal solution using RSM methodology. This study used a locally adapted *Chlorella* sp. and found a

higher concentration of reducing sugar than other studies (Giang et al. 2019, Abou El-Souod et al. 2021). Giang et al. (2019) used 5% H_2SO_4 as a hydrolytic agent for the extraction of reducing sugar from *Chlorella* sp. and obtained a yield of 151.8 mg/g. Similarly, Abou El-Souod et al. (2021) extracted 231.0 mg/g of reducing sugar from *Chlorella* sp. biomass hydrolyzed with 5.56% H_2SO_4.

5.1.3.6 FTIR analysis

Functional group analysis was performed on acid-pretreated and untreated biomass using FTIR analysis. The FTIR spectroscopy of pretreated and untreated algal samples are demonstrated in Fig. 5.6. The band near 900 − 1300 cm^{-1}, 1570 − 1800 cm^{-1}, 2250 - 2400 cm^{-1}, and 3100 - 3500 cm^{-1}. The band near 900 − 1300 cm^{-1} indicates the presence of polysaccharides and stretching vibrations of C-H bonds (Marcilla et al. 2009). The spectra between 1570 and 1800 cm^{-1} represent absorption bands related to functional groups and chemical bonds like ketones, aldehydes, conjugated alkenes, amines, alkynes, and some aromatic compounds (Giordano et al. 2001, Kumar et al. 2018). The region between 2200 and 2400 cm^{-1} typically corresponds to the stretching vibrations of carbon triple bonds (C≡C) and carbon-nitrogen triple bonds (C≡N). This region is often referred to as the nitrile region due to the presence of nitrile functional groups (C≡N) in many organic compounds, such as nitriles, isocyanates, and some alkynes (Nandiyanto et al. 2019). The spectra peaks near 3000 − 3800 cm^{-1} may be indication of a functional group known as a C-H stretch. This is often seen in compounds containing alkanes

(saturated hydrocarbons) or other organic molecules with CH bonds. This spectral region corresponds to the stretching vibrations of the carbon-hydrogen bonds (Kumar et al. 2023).

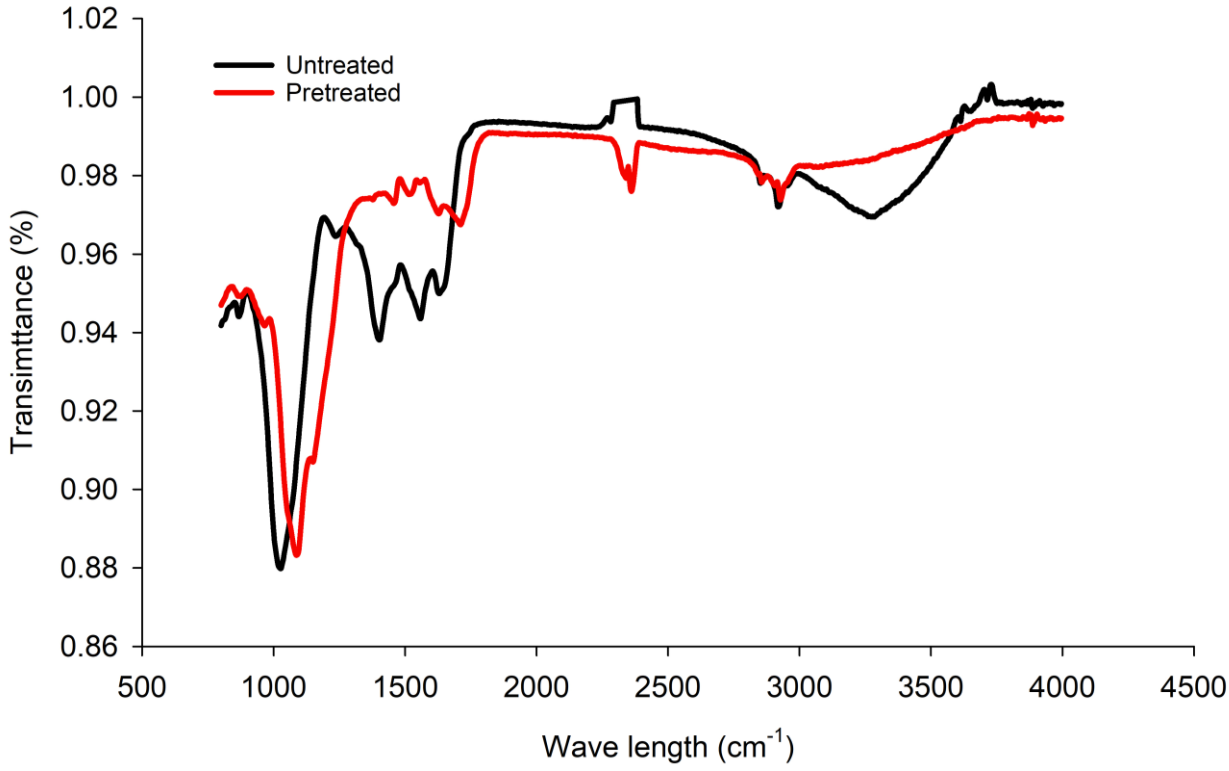

Figure 5.6 The FTIR spectra of pretreated and untreated algal biomass.

5.1.3.7 Bioethanol production

The liquid broth of reducing sugar was obtained from the optimized condition of microalgal biomass hydrolysis using H_2SO_4. Then, *Saccharomyces cerevisiae* was used to ferment the liquid broth into bioethanol. There was a gradual decrease in sugar with increased bioethanol production. Higher bioethanol yield (0.13 ± 0.01 g/g) was obtained after 72 hours of fermentation, then sharply decreased the bioethanol concentration (Fig. 5.7). The maximum

theoretical ethanol yield was 72.82% from the reducing sugar obtained from acid hydrolysis of microalgal biomass (the theoretical ethanol yield is calculated by assuming that 1 g of glucose can produce 0.51 g of ethanol).

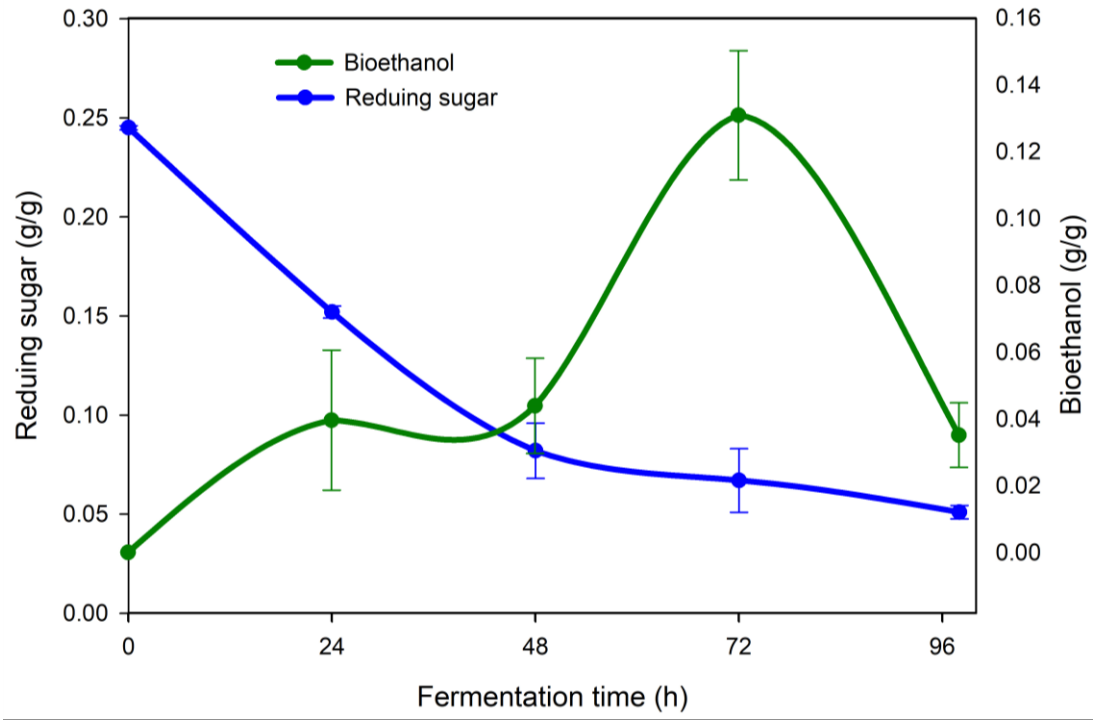

Figure 5.7 Variation of reducing sugar content and bioethanol concentration with increasing fermentation time.

Overall, this study resulted in a ratio of 0.134 grams of ethanol per gram of biomass (78% of theoretical ethanol yield), corroborating the findings of the other studies (Acebu et al. 2022, Yirgu et al. 2023). Our study reported higher ethanol yields after 72 hours of fermentation. Acebu et al. (2022) reported a maximum ethanol production (84.2%) after eight hours of fermentation, whereas (Abou El-Souod et al. 2021) the highest ethanol yield was achieved on

the sixth day of fermentation. The growth condition of *Saccharomyces cerevisiae* is a major factor responsible for the conversion of glucose/mono-sugar to ethanol. Several factors such as temperature, nutrient availability, and pH are major stressors that affect the growth of yeast cells [41]. This ultimately affects the bioethanol production process (Tse et al. 2021). Further study on the optimization of bioethanol production is essential.

Conclusions

This study employed acid pretreatment for reducing sugar extraction from the carbohydrate-rich microalga *C. sorokiniana* and subsequent bioethanol production. Among the three hydrolytic agents (H_2SO_4, NaOH, H_2O), H_2SO_4 was the most effective pretreatment method for extracting reducing sugars from *C. sorokiniana*. This study further optimized the concentration of H_2SO_4, biomass (w/v), and autoclave time using RSM-CCD method. The optimal concentration for maximum sugar extraction was 6.6% H_2SO_4, 10.2% (w/v) for biomass and 35 minutes of autoclave time respectively. By employing a 6.6% H_2SO_4 for biomass hydrolysis, efficiently breaks down complex polysaccharides into simple sugar, enabling a successful bioethanol fermentation process. This study obtained maximum ethanol concentrations of 0.13 ± 0.01 g/g biomass. This study highlights the importance of the selection of native microalgae species for nutrient removal from wastewater and subsequent biomass and bioethanol production.

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5.2 Ultrasonic Pretreatment of Wet Microalgal Biomass for Biomolecules and Bioethanol Production

Abstract

This study investigated the effect of ultrasonic pretreatment of wet biomass of five different microalgae species cultivated in diluted malting effluent for biomolecule extraction. The results indicated that *Chlorella vulgaris* exhibited the significantly highest polysaccharide (55.63 ± 0.32 %) and lipid (22.33 ± 0.49 %) and phenolic (9.01 ± 0.39 mg GAE g−1) and ash (19.13 ± 1.91 %) content and therefore suitable candidate for biofuel production. Whereas *Scenedesmus dimorphus* had the highest protein (35.43 ± 3.60 %) and chlorophyll content (10.42 ± 0.49 mg/L), making it a potential protein source for various applications. Furthermore, the conditions for higher reducing sugar production from wet biomass of *C. vulgaris* were optimized using response surface methodology (RSM). The maximum ethanol yield was achieved at 30°C (0.14 g ethanol/biomass) within 72 hours of fermentation of reducing sugar obtained from sonication. Ultrasonication effectively breaks down the rigid cell walls of microalgae, making inner cellar compounds more accessible and enhancing the extraction of valuable biomolecules.

Keywords: Microalgae, Ultrasonic pretreatment, Biomolecule composition, reducing sugar optimization, Ethanol production

5.2.1 Introduction

There is a growing need for alternative fuels because of the volatility in prices of fossil fuels and the strict regulation of greenhouse gas emissions (Islam Rony et al. 2023). Biofuel could be a potential replacement for fossil fuels because it produces less greenhouse gases and pollutants compared to fossil fuels and therefore more environmentally friendly, sustainable, and renewable. Chemically, biofuels contain shorter carbon chains and less sulfur content, leading to cleaner burning and reduced sulfur oxide emissions (Maleki and Ashraf Talesh 2022). Fossil fuels, with longer hydrocarbon chains, provide higher energy density but contribute more significantly to environmental pollution and global warming (Styring et al. 2021). Among the different renewable energy options, microalgal biofuel could be one of the promising alternatives. Biomolecules derived from microalgae have applications in pharmaceuticals, nutraceuticals, and cosmetics (Deng et al. 2022), while lipids and carbohydrates are potential source of biodiesel and bioethanol and serves as a renewable energy source that can reduce reliance on fossil fuels (Ramachandra and Hebbale 2020). Efficient harvesting of microalgal biomass is one of the major hurdles in microalgal biorefineries. Microalgal cultures are typically constituted of low biomass concentrations (Kumar et al. 2023). Harvesting such dilute cultures requires processes that are both energy-efficient and capable of concentrating the biomass without significant losses. Common harvesting techniques include centrifugation,

filtration, flocculation, and flotation (Kumar et al. 2023). Each method has its trade-offs in terms of cost, energy consumption, and scalability (Soudagar et al. 2024).

Another challenge is efficient pretreatment of microalgal biomass. Microalgal cell walls are composed of complex compounds for example, cellulose, hemicellulose, pectin, agar, phospholipids, and alginate which poses a challenge in extracting lipids and carbohydrates (Rana and Prajapati 2021). Conventional methods such as mechanical disruption and chemical treatments often require significant energy inputs or chemical usage, which can limit their practicality and sustainability (Hernández et al. 2015, Loureiro et al. 2023). Ultrasonic pretreatment has emerged as a promising technique for the effective disruption of microalgal cell walls and enhance sugar extraction efficiency (Rokicka et al. 2021). This method utilizes high-frequency sound waves to disrupt cell structures, leading to increased permeability and improved accessibility of intracellular compounds (Liu et al. 2022). Studies have demonstrated the efficacy of ultrasonication in weakening the cell walls of microalgae, thereby facilitating the release of sugars. Sivaramakrishnan and Incharoensakdi (2019) reported the significant increase in lipid production from 0.77 to 1.31 $g.L^{-1}$. They also revealed the increment of different biomolecules such as chlorophyll content and carotenoid. Another study by Eldalatony et al. (2016) highlighted the importance of sonication followed enzymatic hydrolysis of microalgae facilitated the release of reducing sugar by fourfold (from 74 to 280 $mg.g^{-1}$). Ultrasonic pretreatment offers several advantages over traditional methods. It is a

relatively low-energy process that can be easily scaled up for industrial applications (Liu et al. 2022). Additionally, it does not require the use of harsh chemicals and also easy to adjust parameters such as ultrasonic frequency, power density, and treatment time to optimize the release of target biomolecules (Gerde et al. 2012).

Efficient pretreatment requires optimization of ultrasonication parameters such as frequency, intensity, and duration (Chia et al. 2019). The application of ultrasonic pretreatment facilitating to release fermentable sugars (Chen et al. 2020) and, consequently, increased bioethanol production. The objectives of this study were: i) to investigate the effectiveness of ultrasonic pretreatment for the extraction of different biomolecules, ii) to find the optimal ultrasonic conditions from wet microalgal biomass (the biomass volume, amplitude, and time) for higher reducing sugar production using response surface methodology, and iii) to optimize the fermentation condition for bioethanol production.

5.2.2 Material and methods

5.2.2.1 Microalgae culture and preparation of microalgae wet biomass

Chlorella vulgaris was cultivated in malting effluent (ME50: 50 times diluted malting effluent) based on the previous experiment (Chapter 3). Microalgae culture with three different concentrations (100 ml, 200 ml, and 300 ml) were prepared by centrifuging at 4000 rpm for 5 minutes. Parts of samples were oven dried at 80º C for dry weight estimation.

5.2.2.2 Pretreatment of microalgal biomass using ultrasonication

The cell wall of microalgae was breakdown using ultrasonication using the Fisher Scientific Model 50 Sonic Dismembrator, operating at a frequency of 20 kHz for durations of 2, 10, and 18 minutes, each with varying amplitudes of 20, 60, and 100. This procedure was conducted in 50 mL tubes submerged in an ice bath to prevent sample overheating. Ultrasonication involved alternating cycles of 30 seconds of activity and 10 seconds of rest. The samples were centrifuged at 4000 rmp for 10 minutes, and supernatant (reducing sugar) was collected for the bioethanol production experiment.

5.2.2.3 Effect of sonication on biomolecule composition of microalgae

Total polysaccharide content

Total polysaccharide content was measured using sulphuric acid – phenol method. In brief, 0.01 grams of dry biomass were subjected to ultrasonication for 10 minutes and centrifuged at 13000 rpm for 10 minutes, and 50 µL of supernatant was added to a 96-well plate. Then, 150 µL of concentrated sulfuric acid and 30 µL of 5% phenol was added to the reaction mixture. The plate was then subjected to a water bath at 90 °C for 5 minutes followed by cooled down in an ice-water bath for 5 minutes. Then, the plate was placed back to 90 °C water bath for 5 minutes, followed by an ice-water bath. The absorbance of reaction mixture (100 µL) was

measured at 490 nm using a spectrophotometer. Reference solutions were prepared similarly using different concentrations of glucose, and carbohydrate content was expressed in mg/L.

Protein content

The Bradford reagent method was used for the total soluble protein measurement (Bradford 1976). Initially, 0.01 grams of dry biomass were subjected to ultrasonication for 10 minutes and centrifuged at 13 000 rpm for 10 minutes, and 20 µL of supernatant was added to a 96-well plate followed by 200 µL of Bradford reagent. The reaction mixture was left to stand for 10 minutes at room temperature. The absorbance of the reaction mixture was measured at 595 nm, and the protein concentration was determined using Bovin serum as standard.

Lipid content

The lipid content of was measured using the Sulfo-Phospho-Vanillin (Mishra et al. 2014). Fresh Phospho-Vanillin reagent was prepared prior to the test being conducted. In glass tubes, 0.01 g of samples were added, followed by 2 mL of sulfuric acid. The glass tubes were then placed in a 100°C water bath for 10 minutes and immediately placed in an ice bath for 5 minutes afterwards. 5 mL of the Phospho-Vanillin reagent was added, and the samples were left to stand for an additional 15 minutes. The 200 µl of each sample were then transferred to a 96-well plate and the absorbance was measured at 540 nm using a spectrophotometer. The standard curve for this reaction was also prepared alongside the test. The standards were prepared using

various concentrations of canola oil. The canola oil was initially dissolved in chloroform, and heated until the chloroform evaporated (approximately 80 °C for 10 min), and then the same steps as mentioned above were followed.

Total phenolic content

The Folin-Ciocalteau reagent was employed to determine the total phenolic content. The Folin-Ciocalteau reagent was diluted into a 1:10 ratio with distilled water. The dried 0.01 g of microalgal biomass was mixed with 1 ml of distilled water and sonicated for 10 minutes. Then, 200 µL of algal suspension was mixed with 1 ml of diluted reagent and added 800 µL of sodium carbonate solution. The reaction mixture was placed in 50 °C water bath for 30 minutes and absorbance was measured at 750 nm using spectrophotometer. The different concentrations of gallic acid were used as the standard to construct the calibration curve.

Ash content

One gram of oven dried microalgae biomass was placed in a muffle furnace and gradually increased the temperature to 500 – 600 °C. The ash content was calculated as a percentage of the original dry weight of the microalgae sample using the formula:

Ash Content (%) = (Initial Dry Weight of Sample – Weight of Ash) / Initial Dry Weight of Sample ×100%

5.2.2.4 Optimization of ultrasonic pretreatment of wet biomass for reducing sugar production using RSM

The DNS (3,5-dinitrosalicylic acid) assay was employed to quantify reducing sugars in the supernatant after sonication. Initially, 10 µL of the supernatant and 20 µL of 0.05 M phosphate buffer (pH 7.0) were transferred into a 96-well plate. This mixture was then subjected to a 10-minute incubation at 50°C in a water bath. Subsequently, 60 µL of DNS solution was added to the reaction mixture, followed by incubation in a water bath at 90°C for 5 minutes. The 96-well plate was transferred to an ice bath to cool the reaction and 200 µL of distilled water was added into the reaction mixture. Finally, the absorbance of the reaction mixture (100 µL) was measured at 540 nm using a spectrophotometer. The quantification of reducing sugars was determined based on a calibration curve constructed using glucose as the standard.

The effect of amplitudes (20, 60, and 100), time (2, 10, and 18 min) and biomass volume (100, 200 and 300 ml) were evaluated for the reducing sugar production. Further, Central Composite Design (CCD) in RSM was employed to explore the optimal conditions for maximizing reducing sugar production. The design matrix consisted of three factors, each at three levels: low, medium, and high (Table 5.5).

A total of 15 experiments were conducted using the three factors based on the experimental design obtained from DesignExpert version 13. Quadratic regression was used to evaluate the optimal reducing sugar production.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_{i2} + \Sigma\beta_{ij} X_i X_j$$

Where, Y is response, β_0 refers to regression coefficient, β_i, $\beta_{ii,}$ and β_{ij} are linear, quadratic and interaction term, and X = independent variables.

Table 5.5 Code and levels of independent variables used in CCD.

Independent variables	**Level**		
	-1	**0**	**1**
Amplitude	20	60	100
Biomass (ml)	100	200	300
Time (min)	2	10	18

5.2.2.5 Yeast inoculum preparation for the process of biomass fermentation

Pretreated biomass was autoclaved and supplemented with following nutrients: Na_2HPO_4 – 6 g/L, KH_2PO_4 – 3/L g, $NaNO_3$ – 5/L g, NaCl – 1/L g, $MgSO_4 \cdot 7H_2O$ – 0.5/L g and yeast extract – 0.5 g/L. The fermentation was carried out in 125-ml conical flasks with 50 ml of working volume. Precultured yeast (*Saccharomyces cerevisiae*) was inoculated aseptically, the flasks were sealed by the rubber stopper and flushed with nitrogen gas to ensure anerobic conditions.

Incubation was carried out at 35°C with shaking at 200 rpm for 96 hours. Samples were collected at 24-hour intervals for ethanol measurement.

5.2.2.6 Bioethanol determination

The acidic potassium dichromate ($K_2Cr_2O_7$) reagent was used to determine the bioethanol concentration (Bibi et al. 2021). The distillation was carried out to extract the ethanol from the fermented samples. The extracted 1 mL of ethanol was mixed with equal volume of tri-n-butyl phosphate (TBP) and vortexed. The sample mixture was centrifuged at 13,000 rpm for 5 minutes. Two distinct layers were developed and 500 µl of the upper layer was collected and mixed with an equal volume of dichromate reagent and mixed by vortexing. Again, two distinct layers were developed. The lower layer (blue-green) was collected and diluted to 10 times and the absorbance was measured at 595 nm using a spectrophotometer. The bioethanol content was determined by constructing a standard curve using known volumes of absolute ethanol.

5.2.2.7 Statistical analysis

The differences in biomass, carbohydrate, protein, lipid, phenolic and ash variation between pretreated and untreated samples were tested using student t-test. The correlation between the observed and expected reducing sugar content was analyzed by linear regression. All the experiments were performed in triplicates, and statistical analysis were carried out in the R-language (R Development Core Team 2024).

5.2.3 Results and Discussions

5.2.3.1 Effect of ultrasonic pretreatment on biomolecule composition of microalgae

The results of sonication treatment on biomolecule composition of the five different microalgae are summarized in Table 5.6. The biomolecule composition of microalgae is greatly affected by the culture medium and condition. In this study microalgae were grown in 50 times diluted malting effluent. The wet algal biomass was pretreated with ultrasonication (optimized condition for sonication is given in the next section). The result revealed that ultrasonic pretreatment significantly affected the polysaccharides, proteins, lipids, chlorophyll, phenolic compounds, and ash in these microalgae.

Polysaccharide

The total polysaccharide content significantly varied between the microalgae species ($F = 202.84$, $P < 0.001$, $df = 4$ and Table 5.6.). *Chlorella vulgaris* exhibited the highest polysaccharide content (55.63 ± 0.32 %). The average order of polysaccharide content was *C. vulgaris* > *Chlamydomonas* sp. > *C. sorokiniana* ⩾ *S. dimorphus* > *S. acutus* (Table 5.6). The cultivation medium and conditions are critical factors affecting the polysaccharide content in microalgae species (Markou et al. 2012). The enhanced polysaccharide content in microalgae due to ultrasonic pretreatment holds significant implications for their use in biofuel production. High polysaccharide levels can improve the fermentation process, leading to higher bioethanol

yields (Sudhakar et al. 2021). Additionally, polysaccharides have applications in the food and pharmaceutical industries as thickeners, stabilizers, and bioactive compounds (Caetano et al. 2022).

Protein

Microalgae proteins offer a sustainable, nutritious, and versatile alternative to traditional protein sources. Their high protein content, balanced amino acid profile, and additional health benefits make them suitable for human consumption, animal feed, and biotechnological applications (Ahmad and Ashraf 2023). Our study revealed a high protein content in *Scenedesmus dimorphus* (35.43 ± 3.60 %) compared to other microalgae species ($F = 73.72$, $P < 0.001$, $df = 4$ and Table 5.6). The average order of protein content was *S. dimorphus* > *S. acutus*. > *Chlamydomonas* sp. ⩾ *C. sorokiniana* ⩾ *C. vulgaris*. Wang et al. (2013) reported the versatility of *Scenedesmus dimorphus* for protein and other biomolecule production.

Lipid

The lipid content of microalgae is particularly important for biofuel production, as higher lipid content translates to better oil yields (Sajjadi et al. 2018). The result indicated that ultrasonic pretreatment significantly increased the lipid content across all microalgae species examined. *Chlorella vulgaris* (22.33 ± 0.49 %) and *Chlorella sorokiniana* (22.9 ± 1.37) both exhibited higher lipid contents compared to other species ($F = 114.69$, $P < 0.001$, $df = 4$ and Table 5.6),

making them promising candidates for biodiesel production. The enhancement of lipid content through ultrasonic pretreatment suggests that this method can effectively optimize microalgal biomass for biofuel applications (Rokicka et al. 2021).

Chlorophyll content

Chlorophyll is the pigment responsible for photosynthesis in plants and algae, playing a crucial role in converting light energy into chemical energy (Simkin et al. 2022). This study compared total chlorophyll content before and after ultrasonic and found a significant increase after pretreatment in all microalgae species. *Scenedesmus dimorphus* had the highest chlorophyll content (10.42 ± 0.49 mg.L^{-1}), followed by *Scenedesmus acutus* (8.71 ± 0.1 mg.L^{-1}) ($F = 38.46$, $P < 0.001$, $df = 4$ and Table 5.6). This makes ultrasonic pretreatment a promising method to boost microalgal productivity for bioenergy and bioproducts (Milledge 2013).

Phenolic content

Phenolic compounds are secondary metabolites widely found in plants and known for their significant biological activities, particularly their protective effects against oxidative damage (Mutha et al. 2021). These compounds are crucial in neutralizing free radicals and preventing oxidative stress, which can cause cellular damage (Hassan et al. 2021). This study investigated the phenolic contents of various microalgae strains and found a significant variation in phenolic content across different strains ($F = 109.40$, $P < 0.001$, $df = 4$, and Table 5.6).

Table 5.6 Effect of ultrasonic pretreatment on composition of different biomolecules

Microalgae	Polysaccharide (%)	Protein (%)	Lipid (%)	Chlorophyll ($mg.L^{-1}$)	Phenolic content	Ash (%)
Before pretreatment						
Chlorella vulgaris			11.4 ± 0.17a	1.34 ± 0.06c	4.3 ± 0.38a	
Chlorella sorokiniana			7.93 ± 0.38b	1.61 ± 0.2c	3.88 ± 0.63ab	
Chlamydomonas sp.			7.63 ± 1.17b	3.45 ± 0.18c	2.79 ± 0.32c	
Scenedesmus dimorphus			8.8 ± 0.62b	5.85 ± 0.52a	3.15 ± 0.36ab	
Scenedesmus acutus			3.53 ± 1.5c	5.18 ± 0.11b	2.91 ± 0.59ab	
After pretreatment						
Chlorella vulgaris	55.63 ± 0.32a	12.55 ± 0.16d	22.33 ± 0.49a	5.85 ± 0.52c	9.01 ± 0.39a	19.13 ± 1.91a
Chlorella sorokiniana	36.85 ± 2.21c	15.04 ± 1.46cd	22.9 ± 1.37a	6.49 ± 0.68c	7.49 ± 0.12b	16.24 ± 1.25abc
Chlamydomonas sp.	50.26 ± 0.41b	19.32 ± 0.96c	22.7 ± 2.52a	6.49 ± 0.67c	5.78 ± 0.47c	12.04 ± 1.19c
Scenedesmus dimorphus	40.46 ± 0.97c	35.43 ± 3.60a	14.7 ± 0.35b	10.42 ± 0.49a	5.44 ± 0.2cd	17.7 ± 1.36ab
Scenedesmus acutus	31.01 ± 1.14d	26.58 ± 1.25b	6.93 ± 0.76c	8.71 ± 0.1b	4.66 ± 0.28d	13.88 ± 2.01bc

Among them, *Chlorella vulgaris* exhibited the significantly highest phenolic content (9.01 ± 0.39 mg GAE g^{-1}). This high phenolic content indicates a robust antioxidant potential, making *C. vulgaris* a promising candidate for applications requiring high antioxidant activity followed by *C. sorokiniana* (7.49 ± 0.12 mg GAE g^{-1}). Martínez-Sanz et al. (2020) reported that the sonication greatly increased the polyphenol content in microalgal species. Therefore, microalgae can be a valuable source of natural antioxidants.

Ash

Ash content in biomass represents the total mineral content remaining after complete combustion and indicates the inorganic matter present (de Souza et al. 2020). This study revealed a significant difference in ash content among the microalgae species ($F = 9.78$, $P < 0.01$, $df = 4$, Table 5.6). The average order of ash content was *C. vulgaris* ⩾ *S. dimorphus* ⩾ *C. sorokiniana* ⩾ *S. acutus*. ⩾ *Chlamydomonas* sp. High ash content in *C. vulgaris* (19.13 ± 1.91 %) indicates their potential for use as mineral-rich supplements in agriculture and aquaculture. The minerals in ash can provide essential nutrients for plant growth and animal health, making microalgae a sustainable source of natural fertilizers and feed additives (Liu et al. 2020).

5.2.3.2 Effect of ultrasonication on reducing sugar extraction

Based on the above experiment, *C. vulgaris* possessed the higher total polysaccharides. Therefore, this study investigated the effect of sonication on reducing sugar extraction from *C. vulgaris* biomass at different volumes (100 ml, 200 ml, and 300 ml), amplitudes (20, 60, and 100), and durations (2, 10, and 18 minutes). Results indicated that sonication significantly enhances sugar extraction, with the highest yields (197.25 mg/g) observed at 10 minutes, 60 amplitude, and 200 ml biomass (Table 5.7).

Table 5.7 The effects of sonication pretreatment on reducing sugar production from *C. vulgaris* biomass at different volumes (100 ml, 200 ml and 300 ml), amplitudes (20, 60, and 100) and time intervals (2, 10, and 18 minutes). The data presented as mean ± SD. The statistics derived from one way ANOVA with posthoc multiple comparison test. The different letter in the superscript signified the significant different at $P < 0.05$.

Biomass	Amplitude	Time (min)		
		2	10	18
100 ml	20	76.28 ± 3.06^{a}	84.7 ± 3.92^{a}	61.51 ± 2.26^{b}
	60	83.52 ± 1.73^{a}	87.89 ± 2.23^{a}	63.95 ± 2.45^{b}
	100	79.31 ± 2.16^{a}	74.21 ± 2.06^{a}	56.86 ± 1.34^{b}
200 ml	20	117.74 ± 4.45^{a}	126.35 ± 6.30^{a}	103.38 ± 2.15^{b}
	60	121 ± 1.68^{b}	197.25 ± 4.92^{a}	129.97 ± 3.73^{c}
	100	104.87 ± 2.73^{b}	114.94 ± 2.68^{a}	58.43 ± 3.17^{c}
300 ml	20	92.6 ± 3.59^{a}	111.38 ± 4.06^{a}	98.54 ± 3.76^{b}
	60	81.98 ± 3.37^{a}	128.19 ± 2.87^{a}	86.66 ± 4.47^{b}
	100	85.68 ± 2.96^{a}	105.56 ± 3.85^{a}	83.38 ± 1.74^{b}

Ultrasonication can disrupt cell walls and enhance intracellular content, including reducing sugars (Martínez-Sanz et al. 2020). However, prolonged sonication can lead to the formation of free radicals, causing sugar degradation (Zinoviadou et al. 2015). Based on the above results, this study further optimized the sonication parameters to maximize sugar extraction using RSM.

RSM optimization of reducing sugar production from sonication

Among the physical pretreatment of microalgae, sonication is considered as a promising method for the extraction of reducing sugar from microalgae. It has been shown that amplitude, biomass volume, and sonication time can affect reducing sugar production (de Farias Silva et al. 2020, Uzuner et al. 2024). Hence, these parameters were optimized using the CCD method for the maximum reducing sugar production. Table 5.8 shows the experimental and predicted reducing sugar production from the optimization process.

Following second order polynomial regression equation was obtained from the model:

Reducing sugar production = – 212.89 + 3.33 Amplitude + 2.46 Biomass + 14.46 Time + 0.0003 Amplitude x Biomass + 0.005 Amplitude x Time − 0.008 Biomass x Time − 0.031 $Amplitude^2$ -0.005 $Biomass^2$

The ANOVA result revealed the quadratic regression equation generated from the CCD was highly significant ($F = 44.12$, $P < 0.001$) and validated the model. Furthermore, the lack of fit

in ANOVA statistics was not significant, indicating that the model predictions were accurate (Table 5.9).

Table 5.8 CCD of three factors (amplitude, biomass, and time) for maximum reducing sugar content with experimental and predicted values.

Run	Amplitude	Biomass (ml)	Time (min)	Reducing sugar (mg/ml)	
				Experimental	Predicted
1	20	100	10	104	98.51
2	60	100	18	87	92.83
3	60	100	2	89	90.27
4	100	100	10	70	73.71
5	20	200	18	103	112.23
6	20	200	2	119	125.67
7	60	200	10	192	201.51
8	60	200	10	208	201.51
9	60	200	10	196	201.51
10	100	200	18	93	93.03
11	100	200	2	102	100.07
12	20	300	10	108	111.71
13	60	300	18	88	95.63
14	60	300	2	118	118.67
15	100	300	10	79	91.71

Table 5.9 Result of ANOVA statistics. A refers to amplitude, B refers to Biomass and C refers to Time.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	27234	9	3026	44.12	< 0.001
A-A	1012.5	1	1012.5	14.76	< 0.01
B-B	231.13	1	231.13	3.37	0.125
C-C	406.13	1	406.13	5.92	< 0.05
AB	6.25	1	6.25	0.0911	0.774
AC	12.25	1	12.25	0.1786	0.690
BC	196	1	196	2.86	0.151
A^2	9169.33	1	9169.33	133.7	< 0.001
B^2	12672	1	12672	184.77	< 0.001
C^2	7339.1	1	7339.1	107.01	< 0.001
Residual	342.92	5	68.58		
Lack of Fit	204.25	3	68.08	0.982	0.5403
Pure Error	138.67	2	69.33		
Cor Total	27576.9	14			

The model explained 98.76% of the variance in reducing sugar production ($R^2 = 0.98$) and the low coefficient of variation (7.07) indicated the reliability of the statistical modeling. The coefficients of the linear terms for amplitude ($P < 0.01$) and times ($P < 0.05$) appear to be very significant whereas the interaction between these terms were not significant. The quadratic terms were of amplitude, biomass and time were highly significant ($P < 0.001$). RSM plots visualized the 3D model for the maximum production of reducing sugar with interaction between the independent variables: amplitude and biomass (Fig. 5.8). There was a higher production of reducing sugar when biomass volume increased from 100 - 200 ml with sonication amplitude at 60 (Fig. 5.8a). At 10 min of time and 60 sonication amplitude and 200

ml biomass volume obtained higher reducing sugar content (Fig. 5.8b – 1c). For the model validation, additional experiments were conducted in triplicate as predicted by the RSM (amplitude = 53, biomass volume = 184 and time = 11 min). The predicted value (198 mg/ml) and the experimental value (184.6 ± 1.63 mg/ml) were very close to each other indicated the fitness of the model.

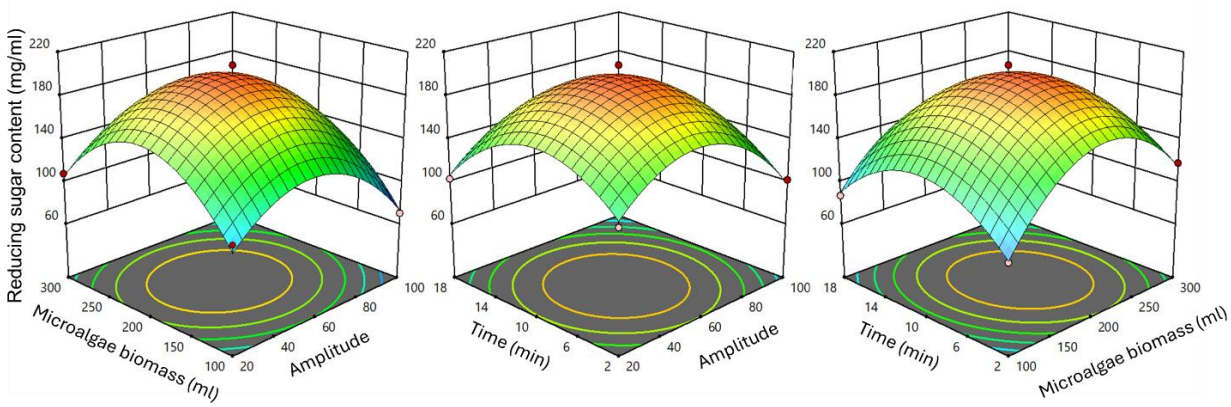

Figure 5.8 RSM plots (a) effect of algal biomass and amplitude on the reducing sugar content, (b) effect of the time and amplitude on the reducing sugar content, and (C) effect of the time and algal biomass on the reducing sugar content.

5.2.3.3 Bioethanol production

The *C. vulgaris* biomass pretreated with sonication was utilized to produce bioethanol. The reducing sugar was obtained from the optical condition identified by RSM (amplitude = 53, biomass volume = 184 ml and time = 11 min). *Saccharomyces cerevisiae* was used for the fermentation of reducing sugar obtained after sonication pretreatment of biomass. After the

yeast fermentation, maximum ethanol concentration was obtained in 72 hours for all treatments ($F = 3.47$, $P < 0.01$, $df = 3$, Fig. 5.9).

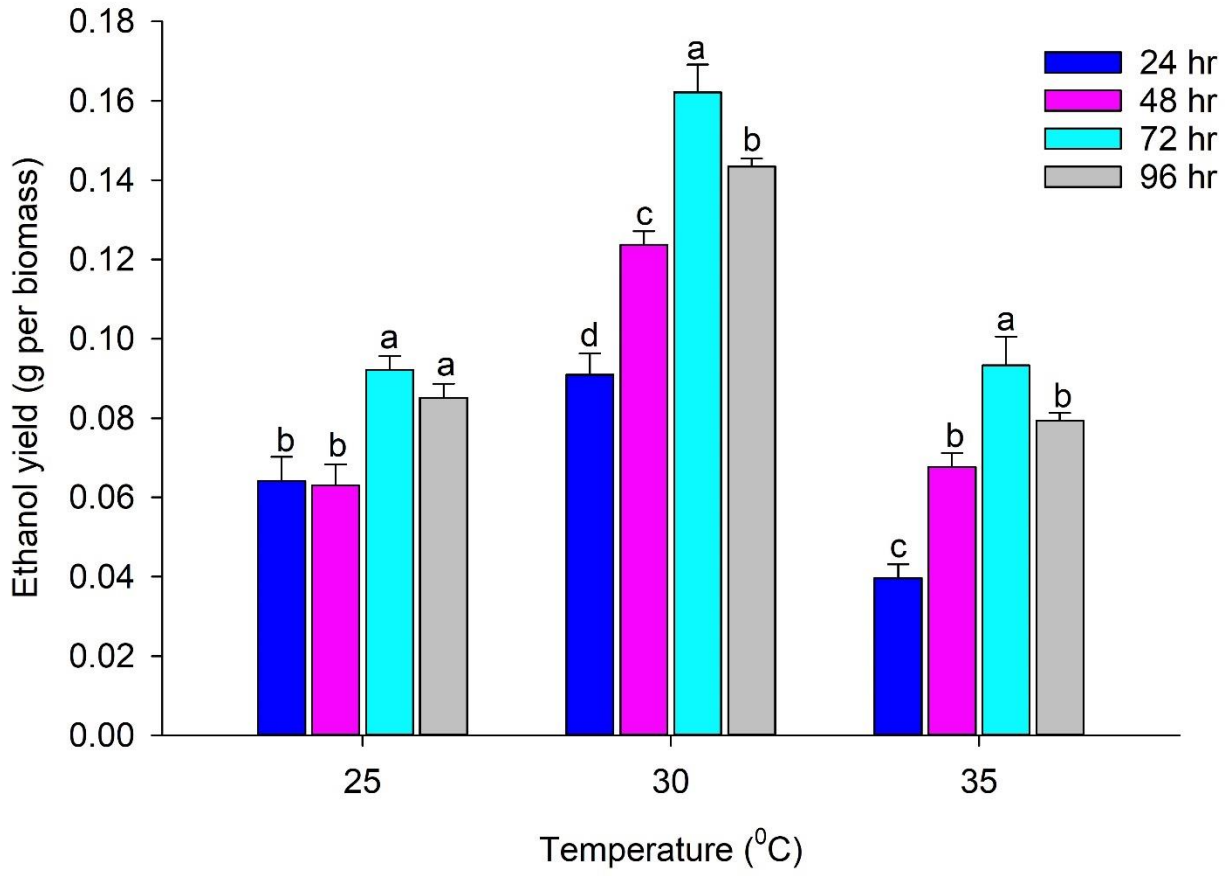

Figure 5.9 Effect of fermentation temperature and time on bioethanol yield. The bar represents the ethanol yield and error bar represents the SD. The letters above the bars are derived from on way ANOVA with posthoc Tukey test. The different letter above the bars signifies the significant different at $P < 0.05$.

The significantly higher ethanol (0.14 g ethanol/biomass) yield was obtained in 72 hours at 30 ºC of fermentation temperature compared to 25 and 35 ºC treatments, respectively (Fig. 2). Our result aligned with the results from Hashem et al. (2021), who demonstrated that 30 °C is often

the optimal temperature for the fermentation of *P. barkeri* biomass, leading to maximum ethanol production. The consistently high yield at this temperature highlights its effectiveness for ethanol fermentation. Shrestha et al. (2023) reported the higher ethanol production from agro-waste at 30 °C.

Conclusion

The study demonstrated that ultrasonic pretreatment significantly enhances the biomolecule composition of microalgae, making it a promising method for optimizing biofuel production. *Chlorella vulga*ris exhibited the highest polysaccharide content, crucial for improving fermentation and maximizing bioethanol yields. *Scenedesmus dimorphus* showed the highest protein content, underscoring its potential as a sustainable protein source for various applications. The lipid content was also significantly increased across all microalgae species, with *Chlorella vulgaris* and *Chlorella sorokiniana* showing the highest levels, making them ideal candidates for biodiesel production. Additionally, *Chlorella vulgaris* had the highest phenolic content, highlighting its antioxidant potential. For bioethanol production, the study confirmed that 30°C is the optimal fermentation temperature, achieving the highest ethanol yield of 0.14 g per biomass within 72 hours. Future research should focus on scaling up the ultrasonic pretreatment process to industrial levels to validate its feasibility and cost-effectiveness. Investigating the long-term effects of ultrasonic pretreatment on different microalgae species and optimizing fermentation conditions for each species could further

enhance biofuel yields. Additionally, exploring the integration of ultrasonic pretreatment with other renewable energy technologies may provide a comprehensive solution for sustainable energy production.

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5.3 Enhancing Lipid Extraction from Microalgal Biomass using Bacterial Cocktail Enzyme Pretreatment

Abstract

Microalgae have gained significant attention as a promising feedstock for biofuel production due to their high growth rates and lipid content. However, efficiently extracting valuable components such as lipids and sugars from microalgal biomass is a critical challenge. Enzymatic pretreatment emerges as a promising strategy to enhance the accessibility of microalgal biomass, facilitating the release of sugars and lipids. In this study, we generated a cocktail enzyme from lignocellulosic biomass and employed it to degrade microalgal cell walls. Among the five lignocellulosic biomasses tested, the bacterial strain cultivated in wheat straw biomass exhibited the highest enzyme activities and was selected for wet microalgal biomass degradation. The cocktail enzyme significantly improved the lipid extraction efficiency across all five microalgal biomass samples by breaking down the cell wall, leading to a higher lipid yield in the pretreated algal biomass. These results were further validated from the Fourier Transform Infrared (FT-IR) spectroscopy which revealed marked differences in peaks within the region 830–1475 cm^{-1}, 1745 cm^{-1}, and 3050 to 2800 cm^{-1} indicating changes in the chemical structure of the pretreated algal biomass. It was revealed that the cocktail enzymes weakened the cell wall and facilitated the extraction of lipids and other intracellular molecules.

Keywords: Soil Bacteria, Lignocellulosic Biomass, Cocktail Enzyme, Microalgal Biomass, Lipid Extraction

5.3.1 Introduction

Microalgae have emerged as a promising alternative for biofuel production, offering numerous advantages over lignocellulosic biomass (Narayanan 2024). These microscopic photosynthetic organisms have higher growth rates and lower harvesting time. They can be cultivated in various environments, including wastewater and non-arable land, making them a versatile biofuel feedstock (Shuba and Kifle 2018). The utilization of biomass as a renewable resource for bioenergy production has gained significant attention in recent years due to environmental concerns and the need to reduce dependency on fossil fuels. However, the efficient conversion of biomass into biofuels faces challenges because of its complex and rigid cell wall structure (Singhvi and Gokhale 2019). The pretreatment process facilitates the accessibility of cellulose and hemicellulose and intracellular polysaccharides (Agarwalla et al. 2023). Among the different pretreatment methods such as mechanical (bead milling, high-pressure homogenization), ultrasonication, chemical disruption (surfactants or detergents, organic solvents, acids, and bases), thermal shock (freeze-thaw cycling), electroporation (electric pulses), and microwave treatments have been widely used for the breakdown of microalgal cell walls (Sirohi et al. 2021, Krishnamoorthy et al. 2022, Agarwalla et al. 2023). Many physical methods, such as ultrasonication, microwave irradiation, and mechanical disruption, require

significant energy (Li et al. 2021). This can lead to high operational costs and may reduce the overall energy efficiency of the biofuel production process (Krishnamoorthy et al. 2022). The chemical methods use acids or bases for biomass hydrolysis, resulting in the formation of residues in the final biofuel product. They also have negative environmental impacts and may require additional steps for removal or neutralization (Zhou et al. 2021). Some chemical pretreatment methods may result in losing valuable nutrients from microalgae biomass, affecting its potential use as a byproduct in animal feed or fertilizer (Demuez et al. 2015).

Among the different pretreatment methods, enzymatic pretreatment has emerged as a promising approach to address the challenges associated with traditional chemical and physical pretreatment methods. Enzymatic pretreatment involves using enzymes to break down complex polysaccharides in biomass, primarily cellulose, hemicellulose, and lignin into fermentable sugars (Demuez et al. 2015). Cellulases, hemicellulases, and ligninolytic enzymes play pivotal roles in this process (Masran et al. 2016). Cellulases, including endoglucanases, exoglucanases, and β-glucosidases, target cellulose chains, cleaving them into soluble sugars (Sharma et al. 2016). Hemicellulases, such as xylanases and mannanases, act on hemicellulose, depolymerizing it into monomeric sugars (Houfani et al. 2020). Ligninolytic enzymes, like laccases and peroxidases, aid in lignin degradation, improving the accessibility of cellulose and hemicellulose to subsequent enzymatic and microbial actions (Bhatt et al. 2022, Zhang et al.

2022). Moreover, the modification of lignin enhances the accessibility of enzymes to cellulose, leading to increased hydrolysis rates.

The available studies on enzymatic pretreatments of microalgal biomass have employed commercial enzymes for biomass hydrolysis. From the economic perspective, commercial enzymes may not be a sustainable approach for biomass pretreatment. As an alternative, microorganisms like bacteria and fungi are commonly employed for enzyme production. Guo et al. (2017) used *Cellumonas xylanilytica* and *Escherichia coli* JM109 for crude enzyme production for the microalgal biomass hydrolysis and reported the higher production of reducing sugar and facilitated in lipid productivity. Prajapati et al. (2015) reported the efficient pretreatment of microalgae species by using the crude enzyme (20%: v/v) produced by *Aspergillus lentulus*. Enzymatic pretreatment induces significant changes in the structure and composition of biomass (Prajapati et al. 2015), affecting its susceptibility to subsequent conversion processes. Optimization of culture conditions, substrate composition, and fermentation parameters are crucial for enhancing enzyme yields.

Lipid extraction from wet algal biomass has been suggested as a cost-effective method since it reduces the additional cost required for concentration and drying processes (Das et al. 2022). There are limited studies on the pretreatment of microalgal biomass by the crude enzyme produced by the selected bacterial strain. The objectives of this study were: i) to isolate, identify and characterized the bacteria strain with multienzyme activities from forest soil, ii) to find the

suitable lignocellulosic biomass and method (LSF and SSF) for the crude enzyme production, and iii) to compare the lipid content before and after the pretreatment of microalgal biomass by crude enzyme.

5.3.2 Materials and methods

5.3.2.1 Microalgae biomass production

The microalga was cultivated in BG-11 medium within a 16-hour dark and 8-hour light cycle provided by LED lighting. The cultivation was conducted in four-litre glass flasks with a working volume of three liters. Continuous aeration was maintained throughout the 21-day cultivation period. The detailed method of microalgae isolation and culture is given in Khatiwada et al. (2022). Microalgae biomass was collected by centrifuging the biomass in 13 000 rmp for 5 minutes and discarded the supernatant. The biomass was washed twice with distilled water and stored in 4 ºC. Some biomasses were dried in 80 ºC for the dry biomass production experiment.

5.3.2.2 Bacterial isolation and characterization

Soil samples were collected from various locations within the forest of Lakehead University, Thunder Bay, Canada. Serial dilutions of soil samples were performed, and 100 µL of appropriate dilutions were spread onto nutrient agar plates (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl and 15 g/L agar) for bacterial isolation. Plates were incubated at 30°C for 24-48

hours to allow bacterial growth. Individual bacterial colonies were selected based on morphological differences (e.g., size, shape, color) and streaked onto fresh agar plates to obtain pure cultures. All the bacterial strains were cultured overnight in LB medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl) and stored in -82 ºC. These strains were further tested for their degradation ability of microalgal biomass. Microalgal agar plates were made in mineral salt medium containing 5 g/L NaNO3, 3 g/L KH2PO4, 6 g/L Na2HPO4, 1 g/L NaCl, 0.5 g/L MgSO4, 0.5 g/L yeast extract, 5 g/L microalgal biomass and 15 g/L agar. All the strains had the ability to degrade algal biomass with clear halo region around the bacterial colony (Fig. 5.10). Among them, bacterial strain JRK4 (*Bacillus* sp. – See Chapter 6) was selected for lignocellulosic biomass degradation and crude enzyme production.

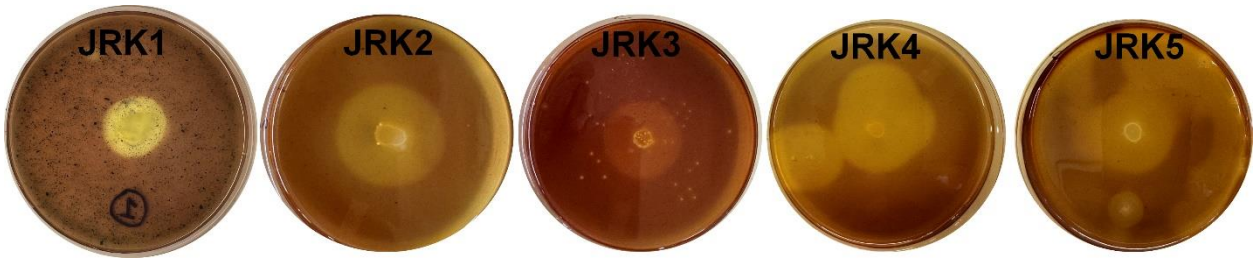

Figure 5.10 Hydrolysis ability of microalgal biomass by the bacterial isolates.

5.3.2.3 Production of cocktail enzyme

Five different biomasses (algal biomass, rice husk, barley spent grain, wheat straw and golden rod stem) were tested for the cocktail enzyme production. The fermentation was carried out by two different methods: liquid state fermentation (LSF) and solid-state fermentation (SSF). In

LSF, biomass was autoclaved and mixed with mineral salt medium containing 5 g/L $NaNO_3$, 3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 1 g/L NaCl, 0.5 g/L $MgSO_4$, 0.5 g/L yeast extract and 5 g/L biomasses and 100 µl of bacterial inoculum was inoculated in the culture medium (100 ml) and incubated at 35 ºC at 200 rmp for 120 hours. In SSF, 5% of autoclaved biomass (w/v) was mixed with culture medium with 100 µl of bacterial inoculum and incubated at 35 ºC for 120 hours. Among the five different biomasses, spent grain and wheat straw yielded higher cellulase, xylanase, amylase, and pectinase enzyme activities at 72 hours of incubation time. Then, spent grain was selected for the crude enzyme production for microalgal degradation.

5.3.2.4 Inoculum preparation for microalgal pretreatment

Pretreatment assays were conducted in 50 mL glass flask containing 20 ml of crude enzyme and the wet algal biomass obtained from centrifuging 200 ml of algal culture (about 0.38 ± 0.05 g of dry algal biomass from 200 ml of algal culture). Control samples were prepared by substituting the enzyme with 0.5 M sodium phosphate buffer.

5.3.2.5 Analytical methods

Measurement of enzyme activity

Quantitative assays were performed by measuring the release of reducing sugars from cellulase, xylanase, amylase, and pectinase enzymes using the dinitrosalicylic acid (DNS) method (Bailey et al. 1992). Enzyme activity corresponded to the release of 1 µM of reducing sugar

equivalent per minute from the substrate (carboxymethyl cellulase, xylose, starch and polygalacturonic acid).

Algal cell disruption quantification via hemocytometry microscopy

Microalgal cell density was examined by counting the cell number. The cell density was measured by 0.1-mm Tiefe deep Neubauer Improved haemocytometer under a compound microscope. Lieca microscope was used to capture the microalgal images at magnification of 100 × to verify the effect of crude enzyme pretreatment on the structure of algal cell wall.

Lipid qualifications

Total lipids were extracted from the algal biomass using a modified Bligh and Dyer (1959) method. In brief, 0.1 g of dried algal biomass was mixed with chloroform, methanol, and distilled water in a 1:2:0.8 (v/v/v) ratio. The culture was thoroughly homogenized by vortexing and centrifuged at 13 000 rpm for 10 minutes. The supernatant was collected in a pre-weighed Eppendorf tube (W1 g), and the cell pellet was re-extracted with a chloroform (1:2, v/v) mixture. After another round of centrifugation, the supernatant was combined with the first extract in the same pre-weighed tube. Chloroform and distilled water were added to achieve a final ratio of 1:1:0.9 (chloroform:methanol:water, v/v/v). This mixture was thoroughly homogenized by vortexing and centrifuged at 13 000 rpm for 5 minutes. The upper phase was discarded, and the lower phase was evaporated and dried at 80°C until a constant weight (W2 g) was obtained.

The total lipid content was determined by subtracting W1 from W2 and expressed as a percentage of the dry weight.

5.3.3 Results and Discussion

5.3.3.1 Hydrolyzing capability of microalgal cell wall by biomass-decomposing bacteria

The capability of hydrolysis of microalgal cell wall by five different bacterial isolates was screened using microalgal biomass as a sole carbon source. The algae-agar plate was stained with potassium iodide solution. The microalgal polysaccharides react with iodine, resulting in the formation of a blue-black complex (Kasana et al. 2008). The clear halo zone around the bacterial colony signified the hydrolyzing ability of bacterial strain microalgal cell wall by the bacterial strain used. The bacterial strains JRK1, JRK2, JRK3, JRK4, and JRK5 had the ability to degrade the microalgal cell wall (Fig. 5.10). The hydrolysis ability of different bacterial strains ranged from 12.2 to 21.4 (Table 1). Among them, JRK4 (*Bacillus* sp.) isolates had a significantly higher hydrolysis ability ($F = 34.5$, $df = 4$, $P < 0.001$) of microalgal biomass compared to other species isolated from soil and was chosen for the further lignocellulosic biomass degradation and production of multi-cocktail enzymes. The selected strains had shown the ability to produce four different enzymes (xylanase, pectinase, amylase, and cellulase). Guo et al. (2017) reported the lignocellulosic biomass degradation abilities and multienzyme production capabilities of *Bacillus* sp.

Table 5.10 Bacterial strains with microalgal biomass hydrolysis capacity. The statistics derived from One way ANOVA with post-hoc multiple comparisons. The similar letters on the superscript signified the significance difference at $P < 0.05$.

Bacterial strains	**Halo diameter (cm) n=3**	**Colony diameter (cm) n=3**	**Hydrolysis ability n=3**
JRK1	2.6 ± 0.1^{c}	0.8 ± 0.1^{ab}	12.0 ± 2.6^{b}
JRK2	2.7 ± 0.1^{c}	0.7 ± 0.1^{b}	16.2 ± 2.3^{ab}
JRK3	2.4 ± 0.2^{c}	0.6 ± 0.1^{b}	15.9 ± 2.7^{ab}
JRK4	4.0 ± 0.2^{a}	0.9 ± 0.1^{a}	21.4 ± 1.0^{a}
JRK5	3.2 ± 0.2^{b}	0.9 ± 0.1^{a}	14.0 ± 1.2^{b}

5.3.3.2 Characterization of crude cocktail enzyme produced from LSF and SSF

Five different lignocellulosic biomasses (microalgae, rice husk, spent grain, wheat straw, and goldenrod stem) were used to produce cocktail enzymes for microalgal biomass degradation from LSF and SSF. In LSF, the enzyme activities were higher in 72 hours of incubation. Among the different biomass used spent grain (pectinase: 1.97 ± 0.006 u/ml, amylase: 1.41 ± 0.006 u/ml, xylanase: 3.84 ± 0.27 u/ml and cellulase: 0.22 ± 0.01 u/ml) and wheat straw (pectinase: 1.97 ± 0.006 u/ml, amylase: 1.41 ± 0.006 u/ml, xylanase: 3.84 ± 0.27 u/ml and cellulase: 0.22 ± 0.01 u/ml) (Fig. 5.11).

Similar trend was observed in SSF with higher enzyme activities after 72 hours of incubation. The spent grain (pectinase: 1.20 ± 0.02 u/ml, amylase: 1.23 ± 0.03 u/ml, xylanase: 2.80 ± 0.03

u/ml and cellulase: 0.22 ± 0.01 u/ml) and wheat straw (pectinase: 1.85 ± 0.01 u/ml, amylase: 1.11 ± 0.02 u/ml, xylanase: 2.85 ± 0.26 u/ml and cellulase: 0.25 ± 0.035 u/ml) produced higher enzymes SSF (Fig. 5.12).

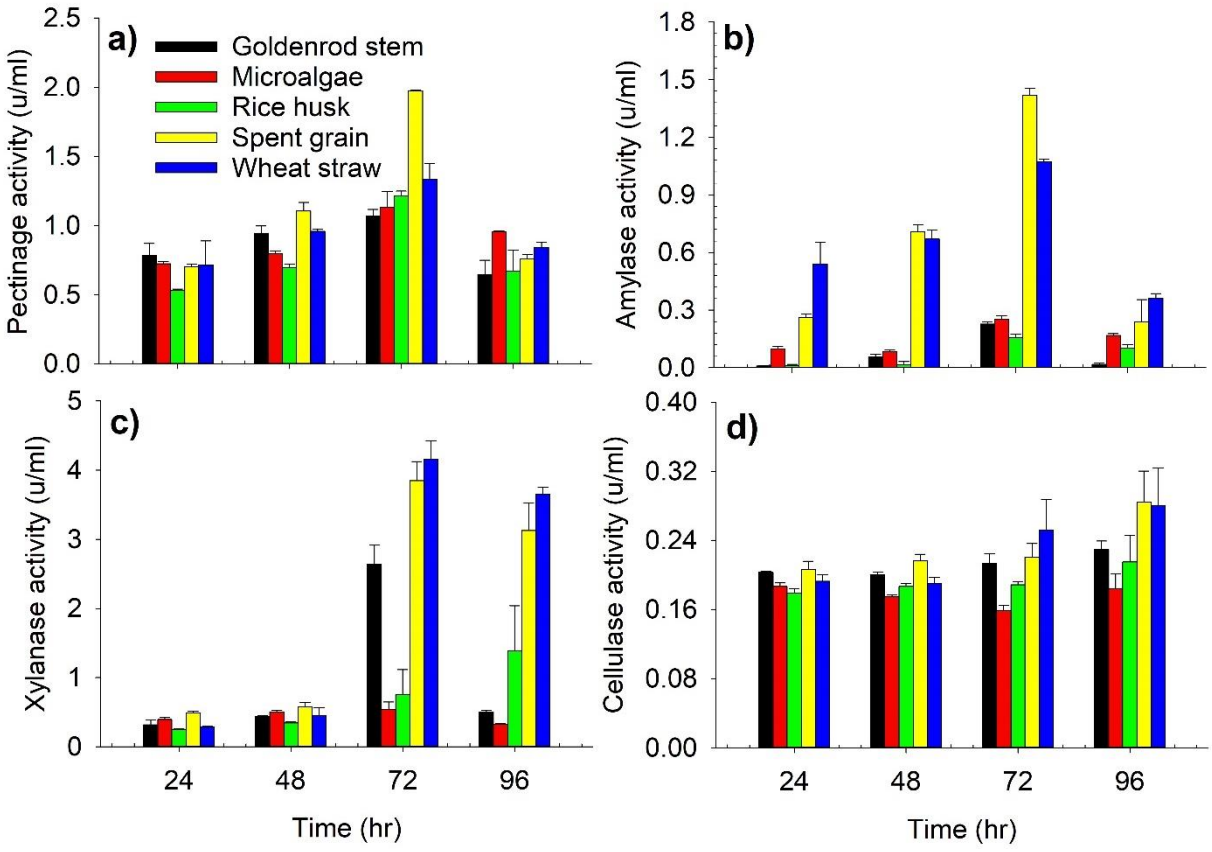

Figure 5.11 Multienzyme produced from LSF a) pectinase b) amylase c) xylanases and d) cellulase.

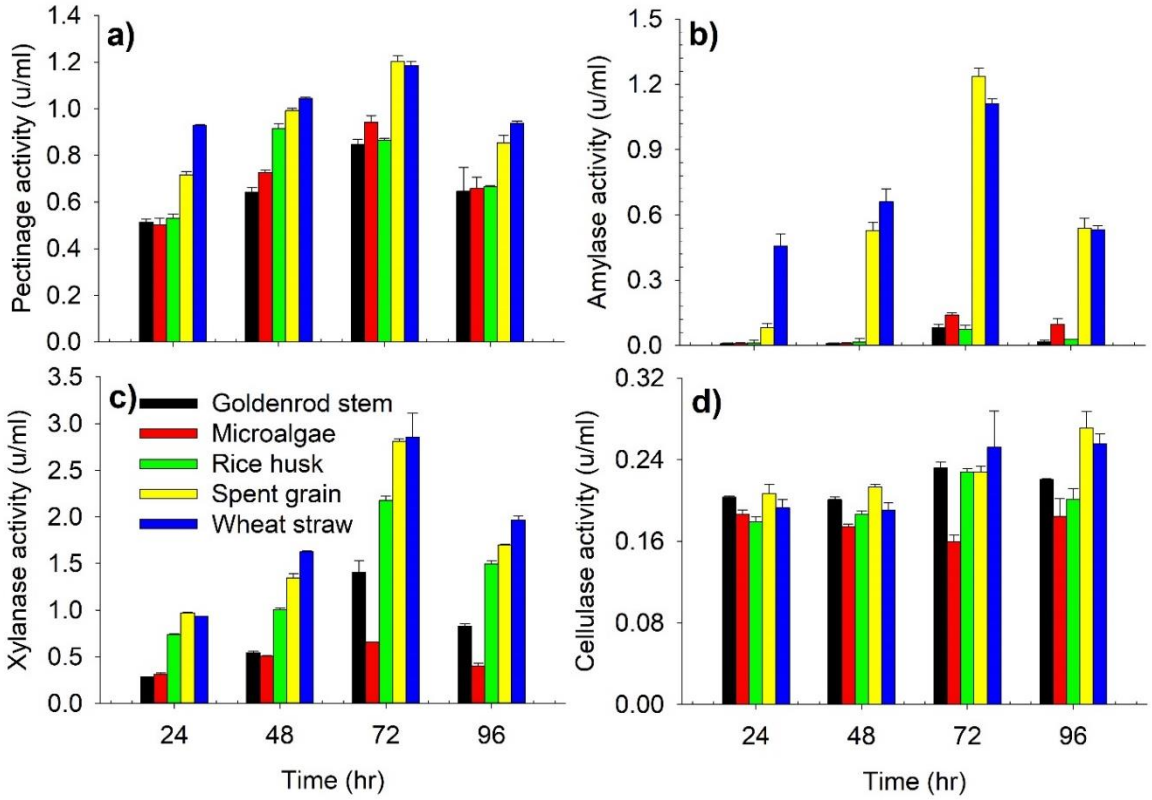

Figure 5.12 Multienzyme produced from SSF a) pectinase b) amylase c) xylanases and d) cellulase.

5.3.3.3 Effect of cocktail crude enzyme on algal cells

In our study, the cell number was significantly reduced after the enzymatic pretreatments of microalgae with cocktail enzyme (Fig. 5.13). The cell death and cell wall degradation were further visualized with microscopic examinations of pretreated microalgae with cocktail enzymes. The microscopic images revealed that cell structure was changed and disruption of cell wall after the pretreatment. Some algal cells were completely broken and formed the cell debris. In comparison with the pretreated algal cells, untreated algal cells remained intact and

smoother in shape. Our results coincided with the previous finding, which reported the breakdown of algal cell walls treated with crude enzymes produced by bacteria and fungi (Prajapati et al. 2015, Guo et al. 2017). Guo et al. (2017) pretreated the wet algal biomass with crude enzyme produced from *Bacillus* sp. and reported the maximum 88.6% of algal cell death. Olena et al. (2022) used *Pseudoalteromonas* sp. bacterial strain to proteases enzyme to degrade the *Chlorella protothecoides*. They reported the crude enzyme with higher antioxidant activity and facilitated the lipid extraction. Microalgae cell walls vary in composition and structure among different species. Generally, microalgae cell walls consist of complex polysaccharides, proteins, and glycoproteins (Spain and Funk 2022).

Enzymes play a crucial role in breaking down cellular structures, initiating the process of cell death in microalgae. Xylanase enzymes, for instance, cleaving the β-1,4-glycosidic bonds between xylose units (Šuchová et al. 2022) and allowing access to intracellular contents for extraction and utilization. Similarly, cellulase enzymes are capable of breaking down a β-1,4-linked glucose polymer into smaller oligosaccharides and glucose monomers (Thapa et al. 2020). Pectinases is another important enzyme produced by the *Bacillus* sp. and facilitates in cleaving the glycosidic bonds within pectin polysaccharides, leading to the depolymerization of the pectin network and subsequent weakening of the cell wall structure. Likewise, amylase enzyme degrades α-1,4 glycosidic bonds between glucose monomers and helps to degrade cellulases and hemicelluloses.

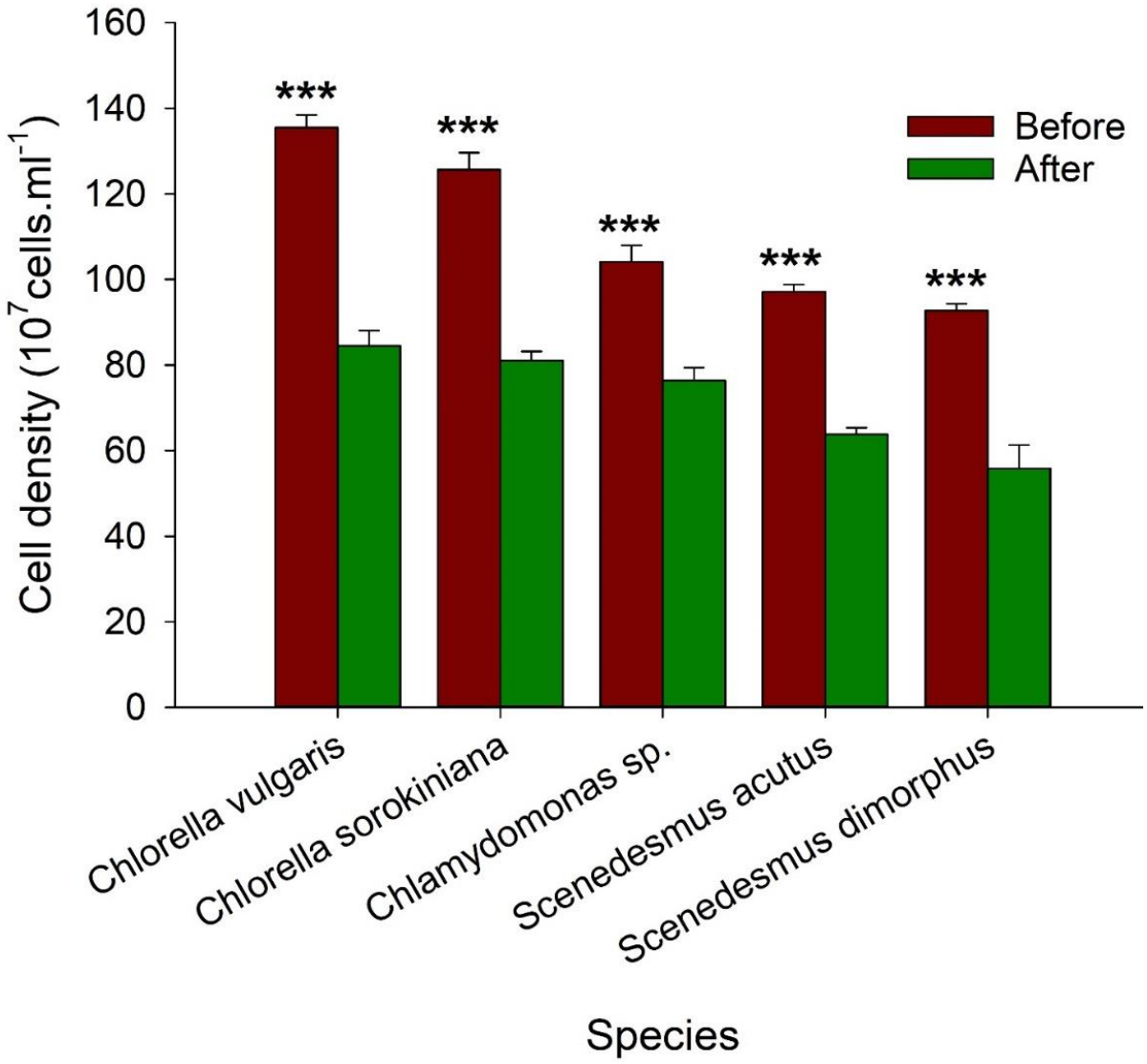

Figure 5. 13 Effect of crude enzyme on cell density. The statistics derived from paired t-test (***P < 0.001).

5.3.3.4 FTIR

The Fourier Transform Infrared (FT-IR) spectroscopy was used to analyze the functional groups and chemical composition of a microalgae biomass before and after ultrasound

treatment by measuring the absorption of infrared radiation (Fig. 5.14). There were major differences in peaks appearing within the region 830–1475 cm^{-1}. This region is associated with common functional groups such as carbohydrates (e.g., cellulose, starch) and peaks around 1000-1200 cm^{-1} (C-O stretching) (Martínez-Sanz et al. 2020). Moreover, there were differences between the pretreated and untreated biomass around the spectrum between 3050 to 2800 cm^{-1} and 1745 cm^{-1}. The 3050 to 2800 cm^{-1} region corresponds to the lipid bands associated with hydrocarbons and 1745 cm^{-1} regions associated with esters of triacylglycerols (TAGs) (Grace et al. 2020, Zhao et al. 2020). It reveals that the cocktail enzymes weakened the cell wall and facilitated in lipid extraction process.

5.3.3.5 Lipid production

Cocktail crude enzyme is comprised with a mixture of different enzymes and used for microalgae cell wall degradation which facilitates the lipids extraction process. In this study, the lipid contents were significantly higher in microalgal biomass treated with cocktail enzymes (Fig. 5.15). One-way ANOVA result revealed the mean difference in the lipid extraction efficiency among the microalgal strains ($F = 11.88$, $df = 4$, $P = 0.001$). Among them, *Chlorella vulgaris* (33.32 %), *Chlorella sorokiniana* (31.60 %), *Chlamydomonas* sp. (30.77%) had the highest lipid extraction efficiency followed by *Scenedesmus dimorphus* (25.18 %) and *Scenedesmus acutus* (16.43%) respectively. Cell wall degradation by bacterial enzymes enhances lipid extraction efficiency primarily by facilitating the release of lipids trapped within

the cell wall matrix. Bacterial enzymes, such as cellulases, xylanase, amylase, and pectinases, break down structural components of the cell wall, including cellulose, hemicellulose, and pectin, respectively. This enzymatic degradation weakens the integrity of the cell wall and allows lipid molecules to be more easily accessed and extracted from the cell. Several studies have demonstrated the effectiveness of enzymatic cell wall degradation in improving lipid extraction efficiency. For example, a study by Guo et al. (2017) investigated the use of bacterial crude enzymes to enhance lipid extraction from microalgae.

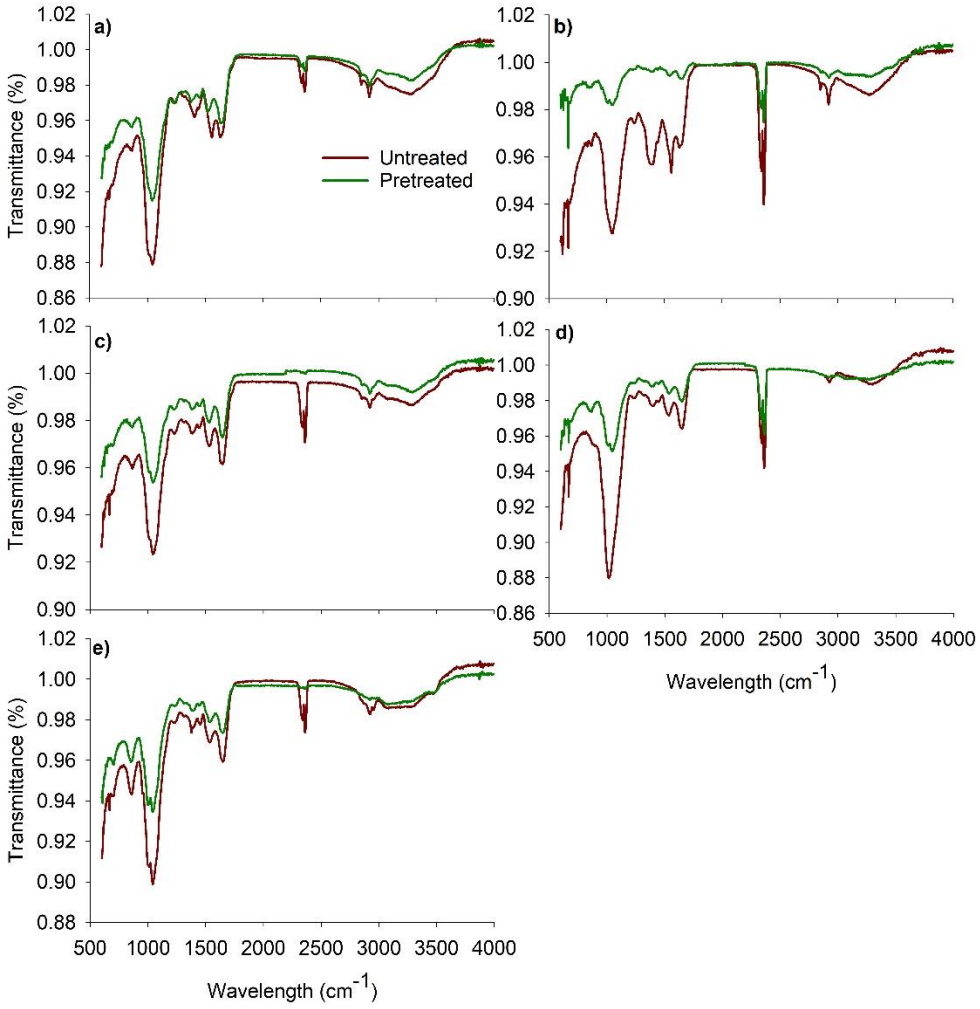

Figure 5. 14 FTIR spectroscopy analysis shows the effect of enzymatic pretreatment on microalgal biomass for lipid production a) *Chlorella vulgaris* b) *Chlorella sorokiniana* c) *Chlamydomonas* sp. d) *Scenedesmus acutus* and, e) *Scenedesmus dimorphus*.

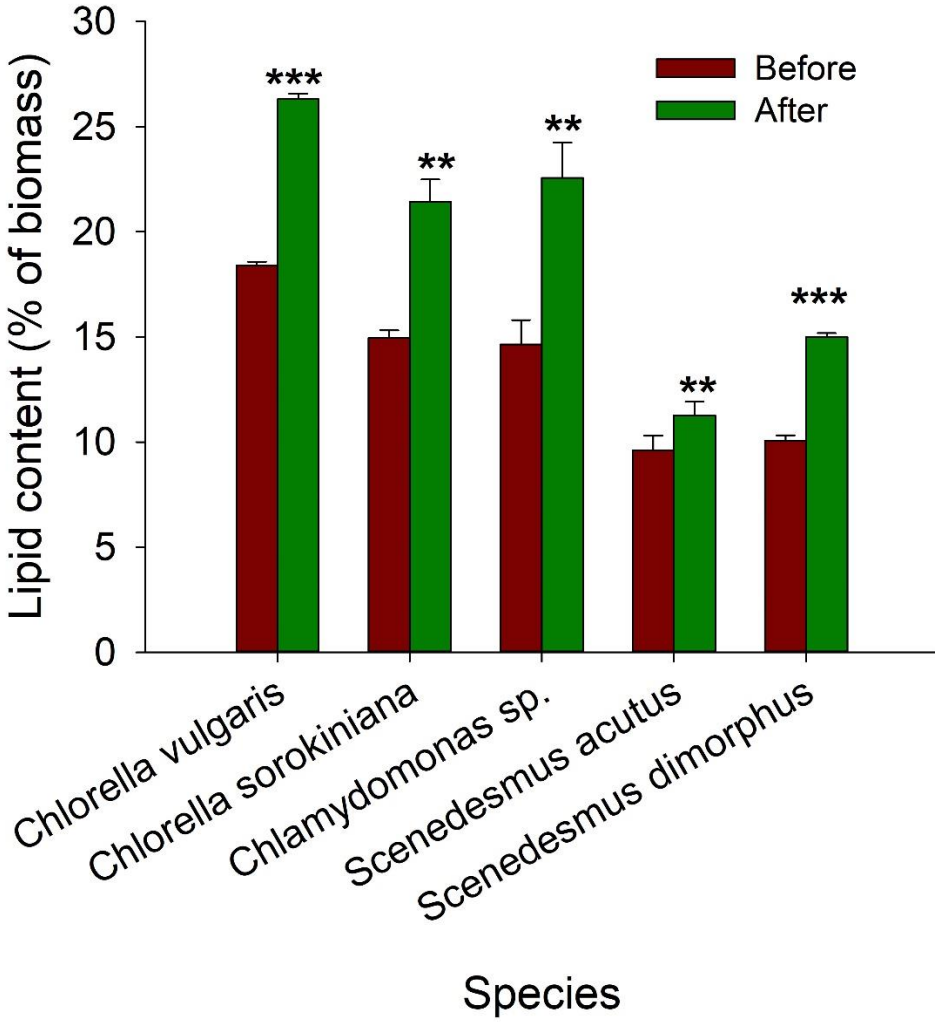

Figure 5. 15 Effect of enzymatic pretreatment on lipid production. The statistics derived from paired t-test (***P < 0.001).

reported the maximum of 43.1% of increment of lipid production in microalgae after the bacterial crude enzyme pretreatment. Zhang et al. (2018) examined the impact of enzymatic treatment (cellulase, xylanase, and pectinase enzymes) on lipid extraction from microalgae biomass. They found that enzymatic hydrolysis of the cell wall prior to lipid extraction resulted in higher lipid yields compared to untreated samples, indicating that enzymatic degradation of the cell wall enhances lipid accessibility and extraction efficiency.

Conclusion

This study demonstrated that cocktail enzymes produced lignocellulosic degrading bacterial isolate can efficiently degrade the microalgal cell wall and enhanced biomolecule extraction. *Bacillus* sp. produced higher xylanase, pectinase, amylase, and cellulase activities from spent grain and wheat straw biomass using LSF. The crude enzyme produced from the *Bacillus* sp. facilitated lipid extraction and increased the maximum yield by 33%. Our study revealed that enzymatic hydrolysis of the cell wall prior to lipid extraction results in higher lipid yields, indicating that enzymatic degradation of the cell wall enhances lipid accessibility and extraction efficiency.

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Chapter 6

6.1 Bioelectricity and Xylanase Enzyme Production from Wet Microalgal Biomass Employing Microalgae Microbial Fuel Cell (MMFC)

Abstract

The pretreatment and bioconversion processes in microalgae-based biofuel and biomolecule production pose major challenges to biomass production, bioproduct recovery, and economic viability. This study aimed to explore integrated approach of bioconversion and bioelectricity production, coupled with bioproducts recovery from multifunctional microalgae microbial fuel cell (MMFCs). Microalgal biomass was cultivated in a MMFC and pretreated with ultrasonication followed by xylanase-producing bacterial hydrolysis to maximize xylanase production. Dual-chambered and single-chambered MMFCs were used for bioelectric generation experiments. The maximum current generation was 875 mA and highest chemical oxygen demand (COD) removal efficiency was 79.82% for Dual-Chambered Fuel Cell (DCFC) and 515 mA with 81.53% COD removal efficiency for Single-chambered fuel cell (SCFC). The anodic chamber with 50% dilution treatment (50ME) yielded higher biomass (0.68 ± 0.016 g/l) in DCFC and (0.61 ± 0.009 g/l) in SCFC. Maximum xylanase activity of 4.537 u/ml was achieved using response surface methodology. MMFC represents an exciting frontier in the realm of renewable energy and offers a unique and sustainable approach to electricity

generation. This study offers the potential to diminish the carbon footprint of industrial waste, conserve resources, aid in climate change mitigation, and protect the environment while facilitating the production of biomaterials and renewable green energy for more sustainable future.

Keywords: Microalgae Microbial Fuel Cell, Bioelectricity, Biomass, Response surface methodology, Xylanase production, Wastewater treatment

6.1.1 Introduction

The surge in demand driven by rapid population growth and industrialization has led to the overexploitation of natural resources, causing pollution and increased environmental concerns (Wang and Azam 2024). Global wastewater production is one of the major environmental challenges as urbanization and industrial activities continue to increase. While efforts have been made to minimize environmental pollution, the conventional methods of wastewater treatment often come with drawbacks such as high energy consumption and hazards of chemical usage (Al-Hazmi et al. 2023). As the world struggles with climate change and depleting fossil fuel reserves, sustainable energy production has become a critical area of research and development (Qureshi et al. 2022). Sustainable energy solutions have led researchers to explore innovative approaches that can reduce pollution and generate clean energy by natural processes. Microalgae Microbial Fuel Cells (MMFC) have emerged as a

promising solution with multifaceted applications, encompassing wastewater treatment, microalgae cultivation for biofuel production, and the generation of sustainable bioelectricity (Nayak and Ghosh 2019, Hadiyanto et al. 2022, Ribeiro et al. 2022).

In MMFC, microalgae and bacteria are pivotal in converting complex organic compounds into chemical energy to generate electrical power. Wastewater with higher organic matter contains greater microbial activities. When these microbes consume organic compounds through metabolic processes, they release electrons as byproducts. In microbial fuel cells, these electrons can be harnessed to generate electricity through electrochemical reactions at the anode (Butti et al. 2016). Therefore, the higher the organic content in wastewater, the higher the microbial activities, resulting in greater electron flow and enhanced bioelectricity production. Various types of wastewater, including municipal solid waste landfill leach, (Khatiwada et al. 2022) have been utilized in MMFCs to produce electricity. We selected malting wastewater as it is enriched with organic materials (Khatiwada et al. 2022) and thus could be used as one of the best substrates for bioelectricity production. While both dual-chamber and single-chambered MMFCs have been widely used for bioelectricity generation (Kusmayadi et al. 2020), the fundamental components of MMFCs include an anode, a cathode, and a membrane/bridge separating the two chambers. The anode serves as the site for the oxidation reaction, where electrons are released during the metabolic processes. These electrons travel through an external circuit to the cathode, combining with protons and oxygen

to produce water. The flow of electrons through the external circuit generates electrical power (Butti et al. 2016).

The microalgae biomass produced through cultivation in MMFCs is enriched with carbohydrates, proteins, and lipids, which are highly sought after for their potential in biofuels and various commercial enzyme production (Siddiki et al. 2022). However, the microalgal cell wall composed of complex compounds for example, cellulose, hemicellulose, pectin, phospholipids, etc. (Jothibasu et al. 2022) which poses a challenge in extracting carbohydrates. Therefore, out of different known biomass pretreatment methods we selected ultrasonication as a promising pretreatment method to enhance sugar extraction efficiency (Sharma et al. 2021). Studies have demonstrated the efficacy of ultrasonication in weakening the cell walls of microalgae, thereby facilitating the release of sugars (de Farias Silva et al. 2020, Krishnamoorthy et al. 2023). The mechanical forces involves in the application of high-frequency sound waves disrupt the cell wall structures, leading to increased permeability and improved accessibility of intracellular compounds such as extraction of sugars from microalgae biomass. (Liu et al. 2022).

The sugars acquired through ultrasonication can serve as a carbon source for bacterial growth and the synthesis of enzymes, like xylanase, which facilitate the further degradation of recalcitrant lignocellulosic components within microalgal biomass. While the xylanase enzymes are synthesized by various microorganisms, including bacteria, fungi, and yeast,

selecting the right microorganism and optimizing culture conditions are essential to maximize enzyme production. The bacterial xylanase plays a pivotal role in the degradation of hemicellulose, a major component of lignocellulosic biomass (Wang et al. 2022). While the agricultural residues, forestry by-products, and industrial waste are commonly utilized as a substrate for xylanase production (Wang et al. 2022), the studies utilizing microalgal biomass for the xylanase production is lacking. Further optimization of fermentation condition plays a pivotal role in maximizing xylanase production. Moreover, we intended to investigate an integrated approach to maximize resource utilization for producing bioproducts, enhancing sustainability and economizing the bioprocess. The objectives of this study were: i) to determine the optimal dilution conditions for malting effluent to enhance biomass and bioelectricity production ii) to compare bioelectricity production between DCFC and SCFC systems, and iii) to optimize the fermentation condition for higher xylanase production from wet microalgal biomass.

6.1.2 Material and methods

6.1.2.1 MMFC setup

Dual-chamber MMFCs consist of two compartments volume of 3.5 L connected by a salt bridge. The salt bridge was made according to Hadiyanto et al. (2022) with few modifications. In brief, 2M potassium chloride and 2% agar was dissolved in 200 ml distilled water and autoclaved for

35 minutes. The mixture was transferred to an 8 cm long PVC pipe with an internal diameter of 5 cm. Graphite rods were used as electrodes with a total area of 0.000464 m^2 (Fig. 6.1a). The configuration of single chamber MMFC is given in Fig 6.1b.

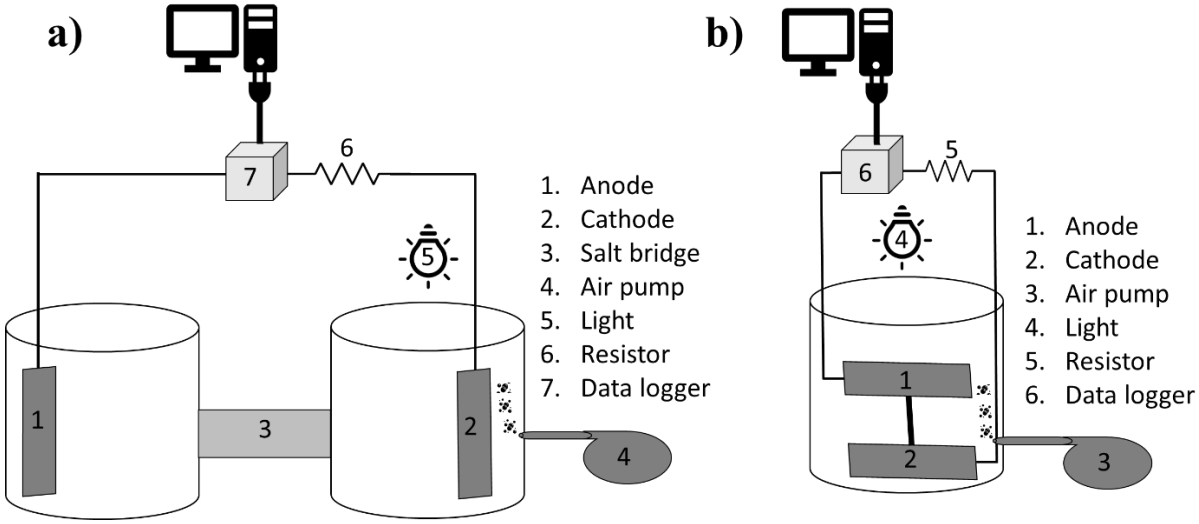

Figure 6.1 Setup of microalgae microbial fuel cell a) dual chamber fuel cell and b) single chamber fuel cell.

The anode and cathode were connected by a copper wire with a 1000 ohms resistor. The voltage generated by the MMFC was recorded by the data logger. The current, current density, and power density were calculated using the following formulae (Raja et al. 2022):

Current (I) = V / R

Current density (CD) = V/(R*A)

Power density (P) = V*I/A

Where I is the current, V is the voltage, R is resistance (1000 Ω), and A is the area of the electrode.

6.1.2.2 Microalgae culture in cathodic chamber

The microalga (*Scenedesmus dimorphus*) was cultivated in a 3.5 L cathodic compartment with 2.5 L working volume using a malting effluent as growth medium. Malting effluent was obtained from Canada Malting, Thunder Bay, Canada. The malting effluent contains different microorganisms such as fungi and bacteria. These microorganisms could influence the growth of microalgae, so, the effluent was sterilized in an autoclave at 121 °C for 35 minutes. Subsequently, the autoclaved effluent was transferred into the cathode chamber and inoculated with *S. dimorphus*. The growth chamber was illuminated with LED lights with 16:8 hours light/dark cycle and provided continuous aeration. The growth of *S. dimorphus* was continuously monitored using a microscopic cell count method. At the end of the experiment, microalgal culture was centrifuged for 5 min at 4,000 rpm and supernatant was discarded.

6.1.2.3 Malting effluent in anodic chamber

The anodic chamber was loaded with autoclaved malting effluent. Microorganisms present in malting effluent were identified by spread plate technique. A total of five bacterial (JRK-M1, JRK-M2, JRK-M3, JRK-M4 and JRK-M5) strains with different morphology were isolated from malting effluent. All the isolated bacterial strains were cultured using LB media and

preserved in 40 % glycerol at -80 ºC. inoculated (0.1% v/v) into the anodic chamber for the bacterial fermentation of organic matters. The morphology and biochemical character are given in Table 6.1.

Table 6.1 Biochemical characteristics of bacterial isolates

Bacterial strains	Gram staining	Shape
JRK-M1	+	Spherical
JRK-M2	+	Rod
JRK-M3	-	Rod
JRK-M4	+	Rod
JRK-M5	-	Spherical

6.1.2.3 Bacterial isolation and screening for xylanase activity

The bacterial strains having optimal xylanase activity were isolated from soil samples collected from Lakehead University, Thunder Bay, Canada. Samples were spread on an LB agar plate, and individual colonies were isolated from the streaking method. The isolates bacterial strains were grown overnight into an agar plate containing 1% of xylene and subjected to a xylanase enzyme screening test by flooding the plate with Congo red (Meddeb-Mouelhi et al. 2014). Following bacterial isolation, these bacterial strains with higher xylanase activity were further screened for microalgal biomass degrading ability. For this purpose, bacterial strains were grown overnight into plates containing 2% microalgal biomass as a sole carbon source, and the plate was flooded with iodine solution. The halo zones around the bacterial colony indicated

that two bacterial strains (JRK10) had xylanase activities and efficiently hydrolyzed the microalgal biomass (Fig. 6.2) and were identified based on 16S rRNA sequences.

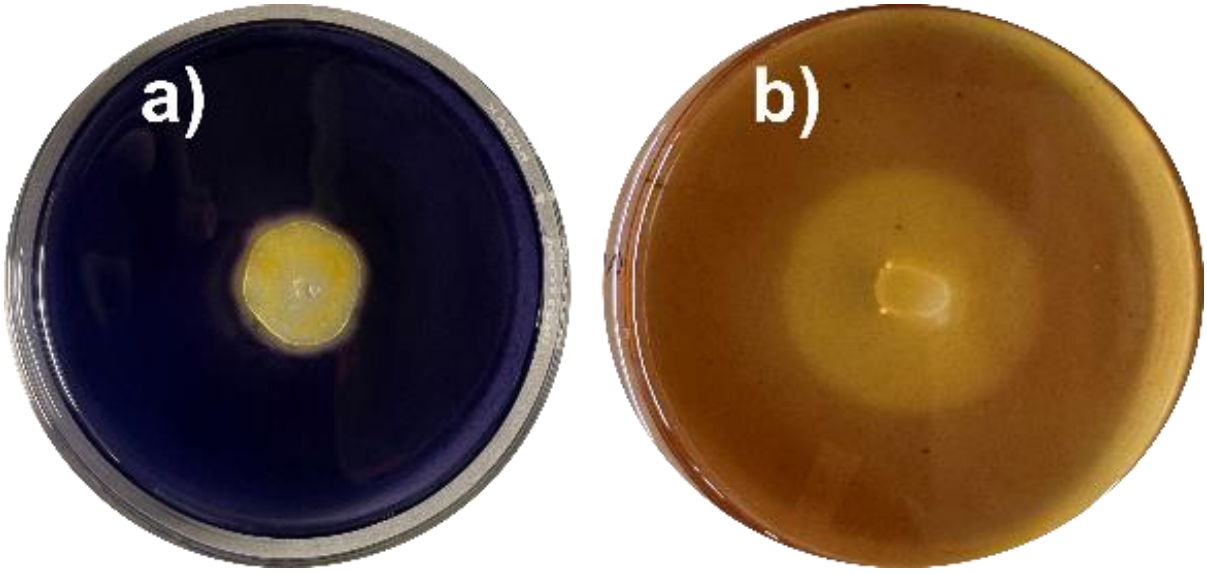

Figure 6.2 Bacterial strain (JRK10) with a) xylanase activity and b) hydrolysis capabilities of microalgal biomass

6.1.2.4 Optimization of xylanase enzyme production from wet algal biomass

The microalgae culture obtained from the cathodic chamber was centrifuged for 10 min at 4000 rpm, and the supernatant was discarded. Microalgae pellets were washed twice with distilled water. Some biomasses were dried at 80 ºC for 24 hours and used for dry biomass and other biomolecule measurements. Wet biomass (200 ml) was subjected to ultrasonication (Fisher Scientific Model 50 Sonic Dismembrator) at 20 kHz frequency for 10 min with 65 amplitudes (see details in Chapter 5.2). This process was carried out in 50 mL tubes immersed in an ice bath to avoid overheating the sample. Samples were autoclaved for 35 minutes for sterilization.

Then, 20 ml of growth medium containing the following constituents: Na_2HPO_4 – 6 g/L, KH_2PO_4 – 3 g/L, $NaNO_3$ – 5 g/L, NaCl – 1 g/L, $MgSO_4 \cdot 7H_2O$ – 0.5 g/L, yeast extract – 0.5 g/L, and wet algal biomass (100 ml, 200 ml and 300 ml) as sole carbon source was added into 50 ml flask. The bacterial inoculum (20 µl) prepared by overnight culturing in the LB medium at 35 °C, 150 rpm for 12 h, was inoculated into the growth medium. Then culture samples were placed into the rotating shaker at 150 rpm with different temperatures (30, 35, and 40 °C) and pH (4, 5, 6, 7, 8, 9, and 10) for 96 hr.

At every 24 hours, 200 µl of sample was collected and centrifuged for 10 min at 13 000 rpm. The supernatant (crude enzyme) was subjected for a xylanase activity test. In brief, 10 µl of crude enzyme was transferred into 96-well plate and added 20 µl of substrate (1% beechwood Xylan dissolved in 0.05 M phosphate buffer at 7.0 pH) and plate was placed in hot water bath at 55 °C for 10 min. The DNS (3,5-Dinitrosalicylic acid) solution (60 µl) was added into the mixture and placed in boiling water bath for 5 min to stop the reaction. Then, the 96-well plate was removed from boiling water and kept in an ice-water bath (5 min) and 200 µl of distilled water was added in it. The absorbance of the reaction mixture (100 µl) was measured at 540 nm in UV-spectrophotometer. The xylanase activity was quantified using standard curve of xylose after subtracting the absorbance of enzyme blank from substrate blank. The linear relationship between xylose released and enzyme dilution factors was considered to estimate

the enzyme activity. Where, one unit of xylanase enzyme corresponds to the release of 1 µM of reducing sugar equivalent per minute from substrate (xylose).

Xylanase activity (IU/ml) was calculated using following formula (Guo et al. 2017):

$$\text{Xylanase activity (IU/ml)} = \frac{\textbf{Dilution} \times \textbf{xylose(mg/ml)} \times \textbf{Total reaction volume (ml)}}{\textbf{0.15} \times \textbf{Reaction time}}$$

The effect of incubation temperature (30, 35, and 40 °C), time (48, 72, and 96) and pH (5, 7, and 9) were evaluated for the xylanase production. Further, Central Composite Design (CCD) in response surface methodology (RSM) was employed to explore the optimal conditions for maximizing xylanase enzyme production. The design matrix consisted of three factors, each at three levels: low, medium, and high (Table 6.2).

Table 6.2 Code and levels of independent variables used in CCD

Independent variables	Level		
	-1	0	1
Temperature (ºC)	30	35	40
Time (min)	48	72	96
PH	5	7	9

A total of 15 experiments were conducted using the three factors based on the experimental design obtained from DesignExpert version 16 (Table 6.3). The quadratic polynomial regression was used to evaluate the optimal Xylanase activity.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_{i2} + \Sigma\beta_{ij} X_i X_j$$

Where Y is a response, β0 refers to the regression coefficient, βi, βii, and βij are linear, quadratic and interaction terms, and X = independent variables.

Table 6. 3 CCD of three factors (temperature, time and pH) for maximum xylanase activity with experimental and predicted values.

Run	Temperature (ºC)	Time (min)	pH	Xylanase activity (u/ml)	
				Experimental	Predicted
1	30	72	9	1.04	1.217048
2	30	72	5	1.14	1.094536
3	30	96	7	1.33	1.320704
4	30	48	7	1.95	1.820528
5	35	96	9	1.55	1.374473
6	35	48	9	2.47	2.419337
7	35	72	7	4.66	4.534989
8	35	72	7	3.96	4.534989
9	35	72	7	4.99	4.534989
10	35	96	5	1.87	1.916961
11	35	48	5	1.23	1.401825
12	40	72	9	1.14	1.182128
13	40	72	5	1.01	0.829616
14	40	96	7	1.28	1.405744
15	40	48	7	1.43	1.435648

6.1.2.5 Statistical analysis

All experiments were performed in triplicates and data were presented as mean ± SD. One way analysis of variance with post-hoc Tukey test was carried out to evaluate the mean difference among the treatments. The statistical analysis was performed in R language (R Development Core Team 2024).

6.1.3 Results and discussions

6.1.3.1 Growth characteristics of *S. dimorphus*

The cell density of the *S. dimorphus* in MMFCs was measured by the cell count method. The highest cell density of microalgae sp. cultured in 30 ME (68.83 ± 3.51 x 10^5 cells/ml), 50 ME (98.57 ± 2.33 x 10^5 cells/ml), and undiluted (62.33 ± 3.51 x 10^5 cells/ml) malting effluent was recorded on day 17 and declined subsequently in DCFC (Fig. 6.3a). In SCFC, there was a linear increasing trend of cell density till day 17 and declined thereafter (Fig. 6.3b). The highest cell density was in 30 ME (68.83 ± 4.60 x 10^5 cells/ml), 50 ME (88.41 ± 4.11 x 10^5 cells/ml), and undiluted (60.41 ± 4.60 x 10^5 cells/ml) malting effluent in SCFC respectively.

The biomass production was highest in microalgae species grown in 50 ME malting effluent (0.66 ± 4.032 g/L), followed by 30 ME (0.58 ± 0.02 g/L) and undiluted (0.40 ± 0.01 g/L) malting effluent treatment in DCFC whereas 0.61 ± 0.00 g/L in 50 ME, followed by 0.52 ±

0.01 g/L in 30 ME and 0.38 ± 0.01 g/L in undiluted ME respectively. This study aligned with our previous findings with higher cell density and biomass production in 50 ME. Malting wastewater is rich in organic compounds and nutrients. However, to facilitate optimal nutrient absorption by microalgae, it is necessary to dilute the malting wastewater (Khatiwada et al. 2022).

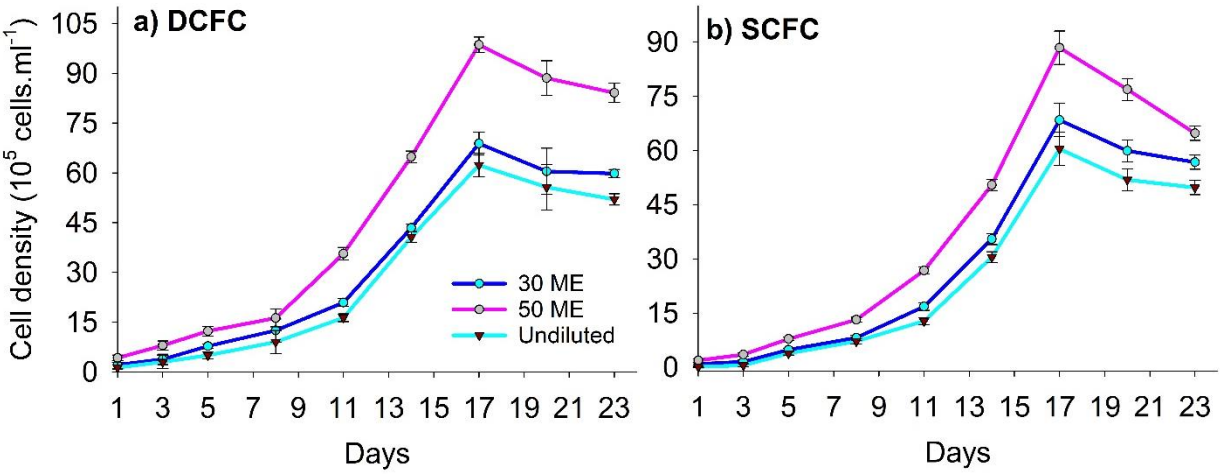

Figure 6.3 Variation of cell density along cultivation days a) dual-chambered fuel cell and b) single-chambered fuel cell.

This dilution process likely facilitates light penetration inside the growth chamber, enhancing the microalgal photosynthesis process and potentially improving the absorption and utilization of nutrients from wastewater. Consequently, this can lead to increased growth and productivity of microalgae, followed by the bioremediation of wastewater (Khatiwada et al. 2022).

6.1.3.2 The comparison of bioelectricity generation between DCFC and SCFC

We found that the MMFC showed a high variation in voltage production between DCFC and SCFC. The voltage was generated under the closed-circuit of 1000 ohms. In DCFC, 50 ME produced maximum voltage (885.33 ± 2.054 mV), followed by 30 ME (781.33 ± 1.2 mV), 100 ME (685.66 ± 2.01 mV) and control (162.66 ± 0.47 mV). The voltage of all treatments showed a quadratic relationship ($R^2 = 0.946$, $P < 0.001$ in 30 ME; $R^2 = 0.947$, $P < 0.001$ in 50 ME; $R^2 = 0.935$, $P < 0.001$in 100 ME; and $R^2 = 0.979$, $P < 0.001$ in Control) with cultivation days (Fig. 6.4a).

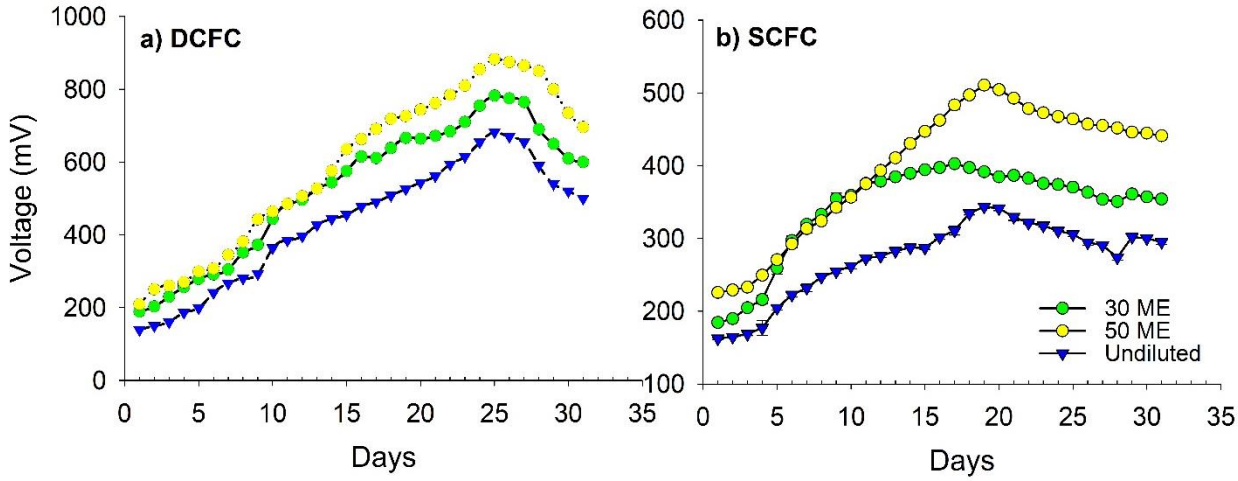

Figure 6.4 Variation of cell voltage along cultivation days a) dual chamber fuel cell and b) single chamber fuel cell.

In SCFC, 50 ME produced maximum voltage (510.66 ± 2.08 mV), followed by 30 ME (402.66 ± 2.08 mV 781.33 ± 1.2 mV), 100 ME (334.66 ± 2.30 mV) and control (144.34 ± 2.51 mV). The voltage of all treatments in SCFC exhibited a quadratic relationship ($R^2 = 0.959$, $P < 0.001$

in 50 ME; $R^2 = 0.932$, $P < 0.001$in 30 ME; $R^2 = 0.942$, $P < 0.001$ in 100 ME; and $R^2 = 0.569$, $P < 0.001$ in Control) with the number of cultivation days (Fig. 6.4b).

Fig. 6.5 shows the variation of power density for all three concentrations of malting effluent treatments. There was a steady increase in power density and attained a maximum value of 1.69 ± 0.009 W/m^2 on day 25 DCFCs and 0.562 ± 0.004 W/m^2 for SCFCs on day 19, respectively for 50 ME treatment. Our result showed that the 50% dilution of malting effluent (50 ME) generated the highest power density compared to 30% dilution and undiluted malting effluent. This could be related to adequate nutrient content in the 50 ME that facilitated the higher algal growth followed by improvement in the oxygen reduction reaction, where oxygen is reduced to water, releasing protons and electrons in the cathodic chamber (Estrada-Arriaga et al. 2021). However, the lower power output in 30 ME is due to its low nutrient content, while undiluted malting effluent, which contains higher nutrient levels, hindered algal growth, and did not result in higher current or power density. Microbial fuel cells rely on the activity of microorganisms to generate electricity through the microbial oxidation of organic compounds (Prathiba et al. 2022). The voltage generation in DCFC in our study was similar to other previous works. Nayak and Ghosh (2019) demonstrated the maximum voltage of 745.96 mV through DCFC utilizing the pharmaceutical wastewater. Lakshmidevi et al. (2020) used 50% leachate and produced maximum of 1.02 V from duel chambered fuel cell. This study is in line

with the study by Akinwumi et al. (2022) and produced 820 mV of maximum open circuit voltage from brewery wastewater.

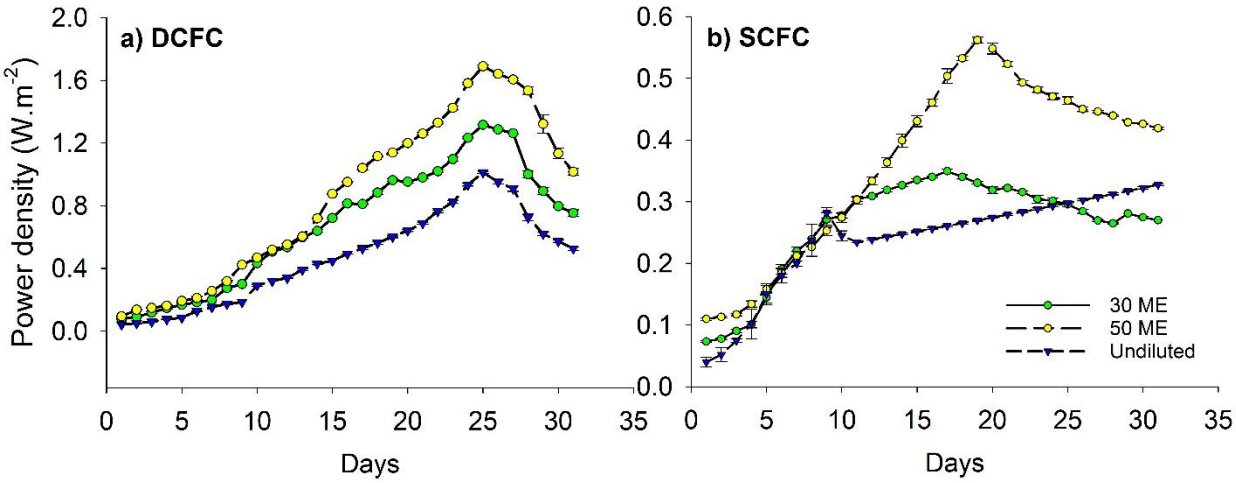

Figure 6.5 Variation of power density along cultivation days a) dual-chamber fuel cell and b) single chamber fuel cell.

6.1.3.3 COD removal

It is evident that COD was significantly reduced in both DCFCs and SCFCs in all treatments (Fig. 6.6). The COD removal efficiency increased gradually, and maximal removal efficiency (%) was observed in 30 ME (78.79 ± 0.99 mg/L), followed by 50 ME (72.56 ± 0.47 mg/L), and undiluted malting effluent (34.28 ± 0.34 mg/L) for DCFC. A similar trend was observed in SCFC, with maximal removal efficiency (%) in 30 ME (80.77 ± 0.71 mg/L), followed by 50 ME (76.36 ± 0.71 mg/L), and undiluted malting effluent (40.01 ± 0.61 mg/L) respectively.

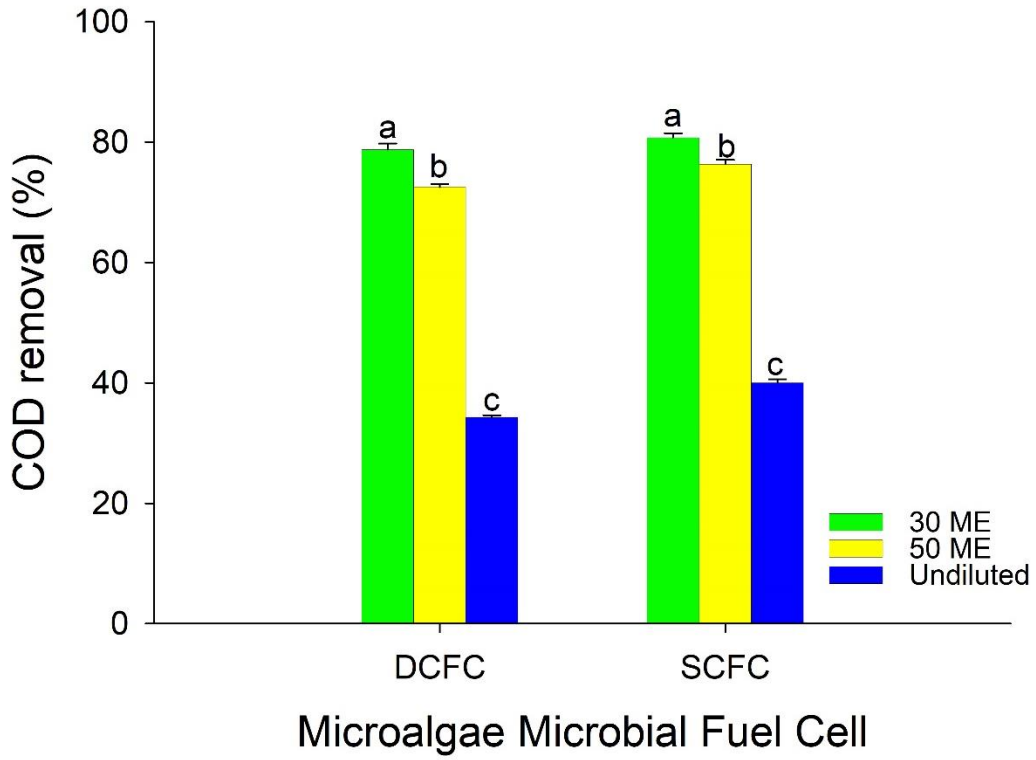

Figure 6.6 COD removal by different MMFCs. The bar represents mean COD removal amd error bar refers to SD. The letters above the bars are derived from on way ANOVA with posthoc Tukey test. The different letter above the bars signifies the significant different at $P < 0.05$.

The relationship between COD removal and power generation in MMFCs is complex and interconnected. As organic matter is degraded by microbial communities, electrons are released during microbial metabolism and transferred to the anode electrode, thereby generating electricity (Ishaq et al. 2024). The result of this study is consistent with other previous studies. Nayak and Ghosh (2019) reported 97.24% of COD removal efficiency by photosynthetic microbial fuel cells. Jiang (2017) demonstrated the 67% COD removal from domestic wastewater treatment by the SCFC. The efficiency of COD removal in MMFCs is influenced by various factors, including substrate composition, microbial diversity, reactor design, and

operating conditions (Prathiba et al. 2022). Organic substrates with high biodegradability and readily available electron donors tend to enhance COD removal rates in MMFCs (Pan et al. 2021). Higher concentrations of organic pollutants typically result in an increased electron flux to the anode, leading to greater power generation (Lin et al. 2022). However, excessive organic loading (undiluted malting effluent) may exceed the capacity of microbial communities to metabolize substrates efficiently, potentially inhibiting COD removal and compromising MMFC performance.

6.1.3.4 Effect of temperature, pH and fermentation time on xylanase production

This study explored the effect of temperature, pH, and fermentation time to produce xylanase enzyme utilizing the microalgal biomass as sole carbon source. The bacterial strain used for the fermentation of microalgal biomass was identified as a *Bacillus* sp. (Fig. 6.7). One-way ANOVA revealed that significantly higher xylanase activity (4.53 ± 0.52) at 35 ºC at 72 hours compared to 30 ºC and 40 ºC (Fig. 6.8).

6.1.3.4 RSM optimization of xylanase production

The optimal conditions for the higher xylanase production from *Bacillus* sp. were further optimized using RSM. The results of the model response, including the actual experimental and predicted value of xylanase activity are shown in Table 6.3. Xylanase activity was the highest during run no. 5, reaching 4.53 IU/mL, under conditions of a 35 °C incubation

temperature at 72 minutes of incubation time and pH 7. The lack of fit in ANOVA was not significant (p = 0.889), signifying that the RSM model was validated.

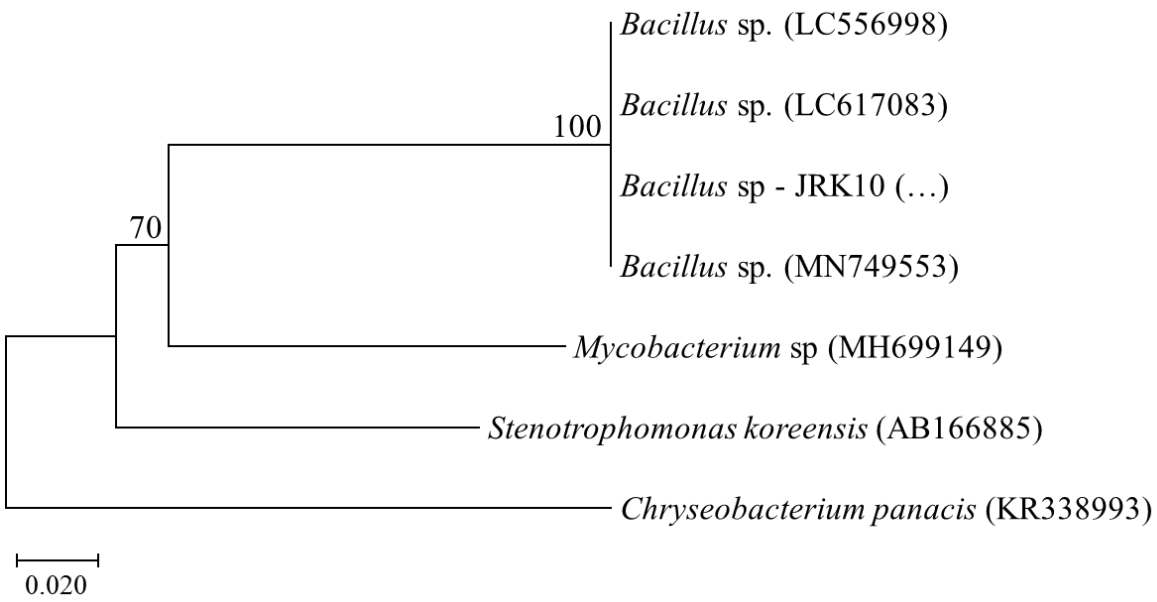

Figure 6.7 Phylogenetic analysis of 16S RNA sequences using neighbor-joining (NJ) method. Numbers at nodes represent bootstrap percentages (1000 replicates). Letters in parentheses represent the accession number.

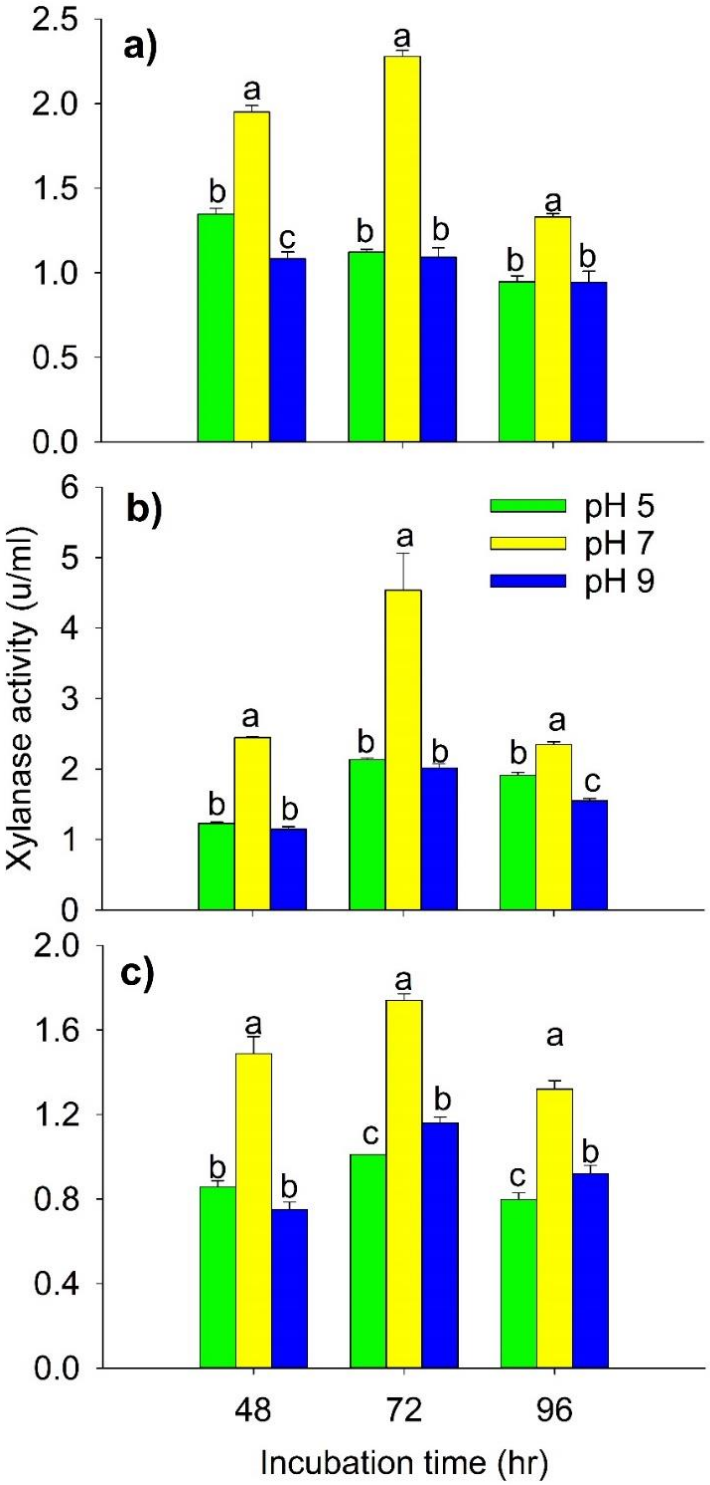

Figure 6.8 Xylanase enzyme production from different incubation temperature a) 30 ºC b) 35 ºC and c) 40 ºC.

The RSM model explained the 97.18% (R^2 = 0.971) of variance in xylanase production. The model with an adjusted R^2 value of 0.921 predicted an R^2 value of 0.847, and the adjusted precision of 11.96, further validating the effectiveness of the model.

The results illustrated a quadratic relationship between the response and experimental variables, with xylanase activity estimated using the following equation:

Xylanase activity = -116.69 + 5.10*A + 0.30*B +5.99*C + 0.0009*A*B + 0.005*A*C - 0.008*B*C - 0.074*A^2 - 0.002*B^2 - 0.396*C^2

The terms A, B, and C refer to incubation temperature, incubation time, and pH respectively. AB, AC, and BC are interaction terms, whereas A^2, B^2, and C^2 are the quadratic terms of independent variables. The ANOVA result revealed an overall model significant (F = 19.15, P = 0.00205). The variables were insignificant in linear and interaction effects, suggesting no influence in xylanase production. In quadratic terms, all of variables showed significance ($P < 0.001$) indicating a substantial interaction among incubation temperature, incubation time and pH, impacting xylanase production. The interaction between three independent variables on the xylanase production was further illustrated by plotting the three-dimensional response surface plots (Fig. 6.9). RSM experiments offers a significant advantage by aiding in visualizing the shape of the response curvature, detecting nonlinearity, estimating pure error,

improving model fitting, and enhancing the robustness and reliability of the experimental results (Latha et al. 2017).

Table 6.4 ANOVA table showing the summary statistics.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	24.77	9	2.75	19.15	0.0023	significant
A-A	0.045	1	0.045	0.313	0.6	
B-B	0.1378	1	0.1378	0.9587	0.3725	
C-C	0.1128	1	0.1128	0.7848	0.4162	
AB	0.0552	1	0.0552	0.3842	0.5625	
AC	0.0132	1	0.0132	0.092	0.7739	
BC	0.6084	1	0.6084	4.23	0.0948	
A^2	12.89	1	12.89	89.66	0.0002	
B^2	5.06	1	5.06	35.21	0.0019	
C^2	9.29	1	9.29	64.6	0.0005	
Residual	0.7187	5	0.1437			
Lack of Fit	0.1655	3	0.0552	0.1994	0.8895	insignificant
Pure Error	0.5533	2	0.2766			
Cor Total	25.49	14				

Figure 6.9 illustrates the interaction between incubation temperature and time for the xylanase activity, showing a maximum activity at 35 °C temperature and 72 h incubation time. Temperature played a crucial and significant effect on xylanase production. The decline in xylanase activity at higher temperatures can be attributed to a ctors including enzyme denaturation, suboptimal thermal stability, substrate instability, and altered reaction kinetics (Motta et al. 2013, Dalagnol et al. 2017). Figure 7c depicted higher xylanase activity at pH 7.

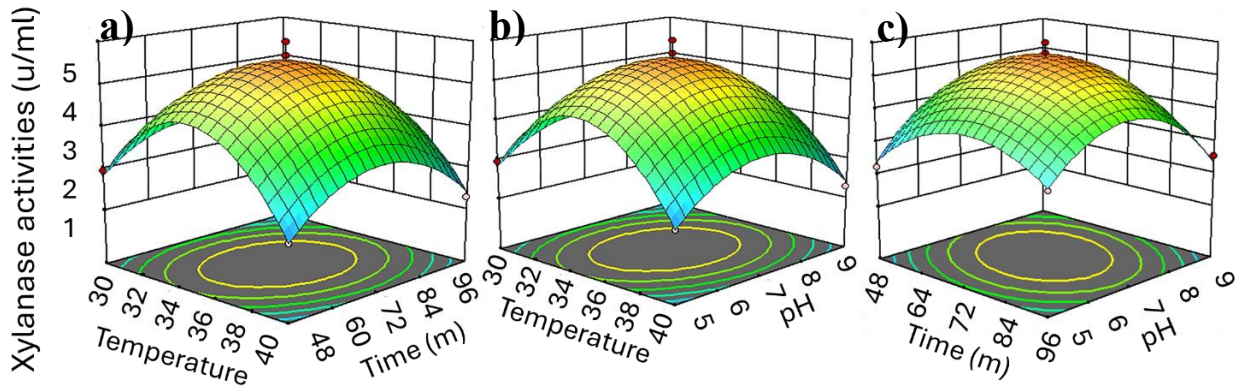

Figure 6.9 RSM plots (a) effect of temperature and incubation time on the xylanase activities, (b) effect of the temperature and pH on the xylanase activities, and (C) effect of the time and pH on the xylanase activities.

A multivariable optimal conditions analysis was performed with the RSM data to predict the bacterial response for higher xylanase production. This study identified the optimal parameters for maximizing xylanase production were 35 ºC of incubation temperature, 72 hours of incubation time and pH 7 with expected xylanase production of 4.53 (u/ml). Validation experiments conducted under these conditions demonstrated a high level of agreement between the predicted and actual bacterial xylanase production. The result for experimental validation achieved the xylanase production of 4.37 ± 0.10 u/ml as it was predicted by the RSM data. The neutral pH promotes bacterial growth and enhance xylanase activity by creating an optimal environment for enzyme production, stability, substrate availability, and overall metabolic activity (Bajaj and Singh 2010). The significant importance of efficient xylanase enzyme production spans across multiple industries such as biofuel, paper and pulp, and food processing (Chaudhary et al. 2021), with bacterial strains such as *Bacillus subtilis* and

Streptomyces spp. also making substantial contributions to the array of xylanase-producing microorganisms (Chandra et al. 2023). Furthermore, incubation time is another important industrial parameter regarding cost efficacy. The bacterium utilized in this study achieved optimal growth during the stationary phase at 72 hours, correlating with higher xylanase activity at the same time point. While prolonged incubation times beyond the peak enzyme production can escalate operational costs, including labor, energy, and raw materials (Ravindran and Jaiswal 2016), achieving optimal enzyme production within 72 hours, incubation time could be a cost-effective approach in industrial settings.

Conclusion

This study demonstrated the multifunctionality of MMFCs as an eco-friendly and economically viable option for decentralized wastewater treatment, bioelectricity generation, microalgal biomass, and commercial enzyme production. The growth characteristics of *S. dimorphus* in malting effluent was investigated, with the highest biomass production observed in cultures grown in 50% diluted malted effluent, highlighting the suitability of malting wastewater as a growth medium due to its rich organic compounds and nutrients. The MMFCs demonstrated varied voltage production between DCFC and SCFC. The maximum voltage generation was 875 mA and highest COD removal efficiency was 79.82% for DCFC and 515 mA of volage with 81.53% COD removal efficiency for SCFC, with 50% diluted malted effluent showing the highest voltage power. The study highlighted a significant reduction in COD in both DCFCs

and SCFCs across all treatments, with higher organic loading leading to greater power generation. Similarly, the microalgal biomass production was also highest in 50 % diluted malting effluent in DCFC (0.66 ± 4.032 g/L) and SCFC (0.61 ± 0.00 g/L). The RSM optimization identified 35°C incubation temperature, 72 hours incubation time, and pH 7 as the optimal conditions (35°C temperature, 72 hours incubation, and pH 7) for achieving the highest xylanase production. It revealed a quadratic relationship between these factors, emphasizing the importance of neutral pH for optimal enzyme activity. Such integrated approach of bioconversion and bioelectricity generation, alongside the recovery of bioproducts from microalgal and bacterial cultivation in MMFCs using malting effluent, showcases a promising research endeavor. This approach not only offers the potential to reduce the environmental impact of industrial effluent, conserve resources, contribute to climate change mitigation, and safeguard the environment, but also enables the generation of renewable energy and biomaterials, marking a substantial advancement toward a more sustainable and environmentally responsible society.

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Chapter 7

7.1 Summary

In this study, we explored and isolated microalgae for bioremediation, biomolecule and biofuel production. We also identified the suitable culture condition of microalgae for higher biomass production and optimized pretreatment and fermentation conditions for the lipid and bioethanol production.

This study (Objective 1) isolated and screened microalgae strains with higher lipid and growth from more than 75 strains of native microalgae from the different freshwater system of northern Ontario Canada. Five microalgae species were selected and identified using both molecular and morphological methods and were identified as: *Chlorella vulgaris, Chlorella sorokiniana, Scenedesmus acutus, Scenedesmus dimorphus* and *Chlamydomonas* sp. Among four different photoperiods, 16L:8D light/dark cycle produced significantly higher biomass and lipids. Therefore, 16L:8D photoperiod was selected for further experiments. This research contributes valuable insights into the potential of native freshwater microalgae for sustainable renewable energy production.

Moreover, microalgae utilized nutrients from wastewater and promote microalgal growth and contribute to wastewater treatment by removing nutrients and pollutants. This approach not only aids in wastewater treatment but also contributes to biomass production, which can be

utilized for various biotechnological applications, including biofuel production. This study optimized the concentration of three different wastewaters: malting effluent, papermill, and municipal wastewater for microalgal biomass production. Among the different dilution in each wastewater, ME-50 (50 times diluted malting effluent), MC-undiluted (undiluted municipal wastewater) and PM-undiluted (undiluted papermill wastewater) had the highest cell density, therefore*, C. vulgaris* was cultivated on those condition for biomass and lipid production. Nitrate and phosphate significantly declined during the cultivation days. Further, the results showed that *C. vulgaris* had the maximum biomass growth and lipid accumulation in ME-50. Therefore, ME-50 was chosen as the optimal culture medium for further study. Wastewater provides numerous benefits, including high nutrient availability, cost savings, and environmental advantages. Further research should focus on optimizing the conditions for maximum nutrient removal and exploring the economic feasibility of large-scale cultivation.

We investigated the effects of PET microplastic on native microalgae isolated from northern Ontario, Canada, and explored the potential as a biological agent for treating microplastic pollution (Objective 2). The results showed that PET microplastic inhibited the growth and chlorophyll content and induced the production of extracellular hydrogen peroxide in microalgae. However, the production of EPS in microalgae cells assisted in the adsorption of microplastic particles and formation of hetero-aggregation and facilitated the sedimentation. These findings shed light on the ecological risks of microplastics on aquatic organisms and

highlight the need for effective management strategies to reduce their input into the environment.

Microalgal pretreatment is a major hurdle for biomolecules and value-added product production. We employed chemical, physical and enzymatic pretreatment for microalgal cell wall disruption for extraction of biomolecules and subsequent biofuel production (Objective 3). Among the three hydrolytic agents (H_2SO_4, NaOH, H_2O), H_2SO_4 was the most effective pretreatment method for extracting reducing sugars from *Chlorella* sp. This study further optimized the concentration of H_2SO_4, biomass (w/v), and autoclave time using RSM-CCD method. The optimal concentration for maximum sugar extraction was 6.6% H_2SO_4, 10.2% (w/v) for biomass and 35 minutes autoclave time, respectively. By employing 6.6% H_2SO_4 for biomass hydrolysis, it efficiently breaks down complex polysaccharides into simple sugar, enabling a successful bioethanol fermentation process. This study obtained maximum ethanol concentrations of 0.13 ± 0.01 g/g biomass.

Physical pretreatment (ultrasonic) significantly enhances the biomolecule composition of microalgae, making it a promising method for optimizing biofuel production. *Chlorella vulgar*is exhibited the highest polysaccharide content, crucial for improving the fermentation process and maximizing bioethanol yields (Objective 3). *Scenedesmus dimorphus* showed the highest protein content, underscoring its potential as a sustainable protein source for various applications. The lipid content significantly increased across all microalgae species, with

Chlorella vulgaris and *Chlorella sorokiniana* displaying the highest levels, making them ideal candidates for biodiesel production. Additionally, *Chlorella vulgaris* had the highest phenolic content, highlighting its antioxidant potential. For bioethanol production, the study confirmed that 30 °C is the optimal fermentation temperature, achieving the highest ethanol yield of 0.14 g per biomass within 72 hours. Future research should focus on scaling up the ultrasonic pretreatment process to industrial levels to validate its feasibility and cost-effectiveness. Investigating the long-term effects of ultrasonic pretreatment on different microalgae species and optimizing fermentation conditions for each species could further enhance biofuel yields. Additionally, exploring the integration of ultrasonic pretreatment with other renewable energy technologies may provide a comprehensive solution for sustainable energy production.

The cocktail enzyme produced lignocellulosic degrading bacterial isolate can efficiently degrade the microalgal cell wall and enhanced biomolecule extraction. *Bacillus* sp. produced higher xylanase, pectinase, amylase, and cellulase enzymes from spent grain and wheat biomass using LSF. The crude enzyme produced from the *Bacillus* sp. facilitated lipid extraction and increased the maximum yield by 33%. Our study revealed that enzymatic hydrolysis of the cell wall prior to lipid extraction results in higher lipid yields, indicating that enzymatic degradation of the cell wall enhances lipid accessibility and extraction efficiency.

We designed a multifunctionality of MMFCs for wastewater treatment, bioelectricity generation, microalgal biomass and commercial enzyme production (Objective 4). The

MMFCs demonstrated varied voltage production between DCFC and SCFC. The maximum voltage generation was 875 mA, and the highest COD removal efficiency was 79.82% for DCFC and 515 mA of voltage with 81.53% COD removal efficiency for SCFC, with 50% diluted malted effluent showing the highest voltage power. The study highlighted a significant reduction in COD in both DCFCs and SCFCs across all treatments, with higher organic loading leading to greater power generation. Similarly, the microalgal biomass production was also highest in 50 % diluted malting effluent in DCFC (0.66 ± 4.032 g/L) and SCFC (0.61 ± 0.00 g/L). The RSM optimization identified 35°C incubation temperature, 72 hours incubation time, and pH 7 as the optimal conditions (35°C temperature, 72 hours incubation, and pH 7) for achieving the highest xylanase production. It revealed a quadratic relationship between these factors, emphasizing the importance of neutral pH for optimal enzyme activity. Such integrated approach of bioconversion and bioelectricity generation, alongside the recovery of bioproducts from microalgal and bacterial cultivation in MMFCs using malting effluent, showcases a promising research endeavor. This approach not only offers the potential to reduce the environmental impact of industrial effluent, conserve resources, contribute to climate change mitigation, and safeguard the environment, but also enables the generation of renewable energy and biomaterials, marking a substantial advancement toward a more sustainable and environmentally responsible society.

7.2 Further recommendations

Based on the results obtained from this study, the following recommendations are suggested:

1. Compare the economic feasibility of cultivating microalgae under natural sunlight conditions in northern Ontario

Our analysis indicated that the primary cost associated with microalgae cultivation was electricity. By utilizing natural sunlight for growth, we can significantly reduce electricity expenses. In Thunder Bay, northern Ontario, the region experiences a maximum of 16.25 ± 0.35 hours of sunlight in June and a minimum of 8.25 ± 0.10 hours in December. A recent study by Chen et al. (2024) demonstrated that outdoor light conditions are economically beneficial for microalgae cultivation. Consequently, evaluating the feasibility and cost-effectiveness of using sunlight for microalgal growth and biomolecule production is crucial.

2. Optimization of large-scale microalgal cultivation and biomass production using malting wastewater as a culture medium

The nutrient-enriched malting effluent enriched with nutrients and suitable for microalgae cultivation. Our study revealed that 50 times diluted malting effluent is suitable for microalgae cultivation. Therefore, large-scale cultivation experiments is essential to evaluate the economic feasibility using malting effluent as a cultivation medium of microalgae. Evaluating nutrient removal efficiencies is crucial, focusing on the uptake of nitrogen, phosphorus, and other

pollutants by microalgae. This contributes to biomass yield and improved wastewater quality. Additionally, the environmental impact assessment should highlight the dual benefits of wastewater treatment and resource recycling while addressing potential risks like eutrophication and impacts on local biodiversity.

3. Investigate the ecological impacts of microplastics on microalgae and enhancing bioremediation strategies

Based on our study, microplastic higher concentration of PET microplastic posed cellular damaged, growth and chlorophyll content, further research should focus on understanding the long-term ecological impacts of microplastic pollution on aquatic ecosystems. It is recommended to explore the differential effects of various sizes and types of microplastics on multiple microalgae species to develop a comprehensive risk assessment.

4. Advanced pretreatment methods

We found that chemical, physical, and enzymatic pretreatment of microalgal biomass enhanced the biomolecule extraction and production of biofuels. Studies revealed that physical pretreatment followed by enzymatic hydrolysis enhanced the extraction of biomolecules and bioethanol production from microalgae (Kim et al. 2014, Hernández et al. 2015). The comprehensive study on ultrasonic followed by microbial enzyme pretreatments could provide valuable insights.

5. Optimization and scale-up of multifunctional microalgae microbial fuel cells (MMFCs) for enhanced wastewater treatment, bioelectricity generation, and bioproduct recovery

Comparative analysis of different wastewater types, including municipal, industrial, and kitchen wastes, would determine the most effective substrates for MMFCs. A comprehensive assessment and economic feasibility studies are critical to evaluate the environmental benefits, potential drawbacks, and cost-effectiveness of scaling up MMFC technology.

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