Development of a microalgae-based consortium for the bioremediation of sugar mill effluent

Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

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2024

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DECLARATION

Development of a microalgae-based consortium for the bioremediation of sugar mill effluent

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I hereby declare that the thesis represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

	08 April 2025
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2. all references as detailed in the thesis are complete in terms of all personal communications engaged in and published works consulted.

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ABSTRACT

Industrial and agricultural activities have increased exponentially to meet the rising demands for food. The sugar production process is water-intensive and requires high volumes of freshwater that are subsequently discharged as effluent. An average of 1000 L of wastewater is produced per ton of sugarcane processed. The sugarcane industry wastewater is characterized by high chemical oxygen demand (COD: 1752 - 8339 mg L-1), and biochemical oxygen demand (BOD:1052 - 4641 mg L-1) but remains low in nutrients and other minerals. Discharging untreated wastewater into the environment might have negative consequences, thus reusing and treating wastewater is essential. Conventional physiochemical treatment methods including sedimentation, filtration, and coagulation-flocculation have shown limited efficacy. The sugar industry wastewater exhibits high biodegradability, thus biological treatment techniques are preferred due to environmental friendliness and sustainability. In recent years, the co-culturing approach has gained interest as a strategy to improve the biotechnological productivity of microalgae in wastewater treatment. This study aims to develop stable microalgae-based consortia using native, microalgal, bacterial and yeast strains. The steps adopted for achieving the above-stated aim were: (1) isolation, identification, and characterization of indigenous microorganisms from wastewater, and (2) assembling microbial consortia to identify the most effective and compatible strains.

Indigenous microalgal, bacterial, and yeast strains were isolated and screened for growth in synthetic wastewater. The strains exhibiting significant growth were further characterized in real wastewater from the sugar industry to evaluate their performance efficiency based on COD removal efficiencies. To attain higher wastewater treatment efficiencies, different primary and secondary combinations of consortia were constructed from the pool of previously screened and

selected microbial strains guided by the bottom-up approach. The microalgal, bacterial, and yeast microbial strains present in the final consortia were identified using polymerase chain reaction and sequencing. The final microalgal-based consortia were characterised in real effluent to assess wastewater treatment efficiency and elucidate their basic interactions for better application.

Two microalgal, seven bacterial, and four yeast strains were isolated from the sugar industry wastewater. In the primary screening procedure in synthetic wastewater, two microalgal strains (A7 and C12), and four bacterial (B003, B009, B010, and B013) strains were found to grow substantially and thus selected for further studies and subsequent microalgae-consortia development. The two microalgal strains showed high removal efficiency for NO3--N (98–100%), NH4+-N (62-65%), and PO43--P (75-80%), however, the removal rate for COD was mainly observed in bacterial strains ranging between 1–73%. Also, the yeast strain (Y2) reduced COD from 22660 to 11690 mg L-1 (48% removal rate) within 168 h of cultivation. The co-culturing of microalgae with bacteria and yeast could improve the treatment of high-strength wastewater. In this regard, four primary, and three secondary consortia were considered. In all the primary consortia (B009A7, B010A7, B013A7, and Y2A7), improvement in removal efficiency for Total Nitrogen (TN) and Total phosphorus (TP) was recorded in ranges between 75-80% and 84-94%, respectively. However, a significantly lower removal rate (4-7%) was observed for COD. Furthermore, three secondary (B009B010A7, B009B013A7, and B010B013A7) and one tertiary consortia (B009B010B013A7) were considered. All secondary consortia exhibited a prolonged lag phase with reduced COD removal efficiency. In contrast, the tertiary consortia showed improved COD, TN, and TP removal efficiency corresponding to 26%, 85% & 73% (respectively) in synthetic wastewater. The microalgal, bacterial, and yeast strains in the final consortia were

identified as Chlorella sorokiniana A7, Rhodococcus sp B009, Bacillus sp B010, Bacillus sp B013, and Saccharomyces cerevisiae Y2. The wastewater treatment efficiency of the consortia (MBC and MYC) was further evaluated in real sugar industry wastewater. The COD removal efficiency was found to be 86% and 71% after 4 days of cultivation for MBC and MYC, respectively. In addition, the co-culture with S. cerevisiae Y2 markedly increased chlorophyll-a content, photosynthesis, and respiration in microalgae.

The microalgal-based consortia exhibited physical and biochemical interactions, with improved yield parameters and metabolite production between microalgae, bacteria, and yeast. The co-cultivation of Chlorella sorokiniana A7 and Saccharomyces cerevisiae Y2 was observed to have the highest COD removal efficiency from wastewater (100% within 4 d of cultivation). Microalgae and yeast mutually benefited from each other in the MYC system with synergistic cross-feeding between specific parameters such as CO2/O2, and organic acids. In addition, indole-3-acetic acid (IAA) was selected as a marker for evaluating the plant growth-promoting effects of co-cultured partners and determining the communication intensity. All co-cultured strains were found to produce and secrete indole acetic acid (IAA), suggesting plant growth-promoting effects. All the co-culture partners produced different concentrations of IAA under tryptophan ranging between 2 to 129 mg L-1. Meanwhile, IAA production was highest within 24 hr of cultivation in the MBC system, while the MYC exhibited a steady increase in IAA production, with the highest production observed after 72 h. The IAA signals are suggested to facilitate the establishment of mutualistic associations between microalgae and yeast/bacteria under varying environmental conditions. This indicates that yeast/bacteria may promote the growth of the co-existing microalgae through

secretion of IAA, and microalgae would selectively enhance IAA secretion, thus, shaping the physiology and ecology of the partners in the microbial consortia.

The study demonstrated the efficacy of microalgae-based consortia that have potential use in treating high-strength COD wastewater. The results could help improve the performance of the current treatment methods by introducing low-cost and sustainable biological technologies. The study demonstrated that microalgal-based co-cltivation is a promising bioremediation tool for high-strength biodegradable wastewater and presents environmental value in the design of low-energy, small-scale biological treatment systems. An insight into mechanisms of interactions between microalgae and co-cultured microbes still requires further study through integrated omics, studying the ecology and diversity of microbial communities could improve their application in environmental monitoring and bioremediation.

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This work is dedicated to my dear family and the most precious things in my life:

My late mother who never saw this adventure, Thobile Elsie Sibisi-Kubheka

Your compassion, love, and life lessons have been my pillar of support and solace during this journey. This is still in your honour since it was originally intended for you.

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PREFACE

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CHAPTER 1: INTRODUCTION

1.1 Background

Sugarcane is the most cultivated crop in various parts of the world and plays an important role in the socioeconomic development of the local economy. South African Sugar Industry (SASI) comprises 14 sugar factories (mills) located in KwaZulu-Natal and Mpumalanga. The sugar manufacturing process is a water-intensive process, large amounts of freshwater are used in different units of the plant and subsequently discharged as wastewater (Fito *et al.,* 2018). Significant volumes of highly degradable wastewater are generated annually and pose a threat of environmental pollution. For every ton of sugarcane processed, an average of 1 m^3 of wastewater is generated (Sarangi *et al.*, 2008; Hampannavar and Shivayogimath, 2010; Prakash and Capoor, 2018). This amounts to billions of litres per year that require appropriate treatment and disposal. Currently, the sugar industry effluent is either treated and released directly into waterbodies or irrigated on agricultural land, and both practices are subject to strict environmental legislation. In South Africa, these practices are governed by restrictions detailed in Section 39 of the South African National Water Act (Act No. 36 of 1998).

The characteristics of the sugar industry wastewater vary in quality and quantity depending on the feedstock or sugarcane variant, chemicals used in the production, and the quality of the final product (Jadhav *et al.*, 2013; Kushwaha, 2015). Due to the complex nature of this effluent, the development of low-cost technologies for the removal of pollutants remains a challenge. There has been growing interest in developing biological wastewater treatment systems that will enable the reuse of agricultural-industrial wastewater streams (Abegunrin *et al.*, 2015;

Gatta *et al.*, 2015; Libutti *et al.*, 2018; Shannag *et al.*, 2021). Conventional biological wastewater treatment methods rely on the activity of microorganisms to utilize pollutants for their growth and convert the organic substances into CO_2 and water (Kharayat, 2012). Hence, microorganisms such as microalgae, yeast, and bacteria have been used for the biological removal of pollutants from various wastewater streams including municipal, industrial, and agricultural (Oswald *et al.*, 1957; Malandra *et al.*, 2003; Ghosh *et al.*, 2004; Jarboui *et al.*, 2012; Abou-Shanab *et al.*, 2013; Girard *et al.*, 2014). This makes the microorganisms ideal bioremediation tools for the sugar industry wastewater. Their ease of handling and controlling growth conditions is an inexpensive and environmentally friendlier alternative. These microorganisms also provide a sustainable feedstock for diverse bio-products with different biotechnological uses.

There is growing interest in co-cultivation systems involving microalgae, yeast, and bacteria to improve biotechnological productivities in certain parameters such as biomass production and enhanced lipids (de-Bashan *et al.*, 2010; Lananan *et al.*, 2014; Maza-Márquez *et al.*, 2014; Simpson, 2018; Makut *et al.*, 2019; Karim *et al.*, 2021). Microalgal-based co-cultivation strategy offers low-cost and energy-input processes and environmentally friendly biological wastewater treatment technology. Most studies on microalgal-based wastewater treatment relied on monocultures, which are susceptible to contamination and changes in environmental conditions, thus generating low biomass (Suvarna *et al.*, 2011; Rakesh and Karthikeyan, 2019). Therefore, the co-cultivation of multiple species (two or more) provides advantages of improved community functionality and resilience to environmental changes, however, multiple species systems offer low controllability which has limited benefits in wastewater bioremediation (Herrero and Stuckey, 2015). To mitigate this issue of complexity and instability, various studies have constructed or developed microbial consortia with a limited

number of culturable strains for improved biotechnological processes (bottom-up approach) (Kazamia *et al.*, 2014; Padmaperuma *et al.*, 2018). This results in engineered multiple species with minimal but effective community. The co-cultivation systems are designed to improve the natural interaction of partners (growth and survival) and enhance functional/metabolic capabilities leading to the accomplishment of difficult tasks (Brenner *et al.*, 2008; de-Bashan *et al.*, 2016; Dolinšek *et al.*, 2016). The established synthetic interactions between populations result in a cooperative and steady-state microbial community that can perform many biotechnological functions (de-Bashan *et al.*, 2016). The development of microbial consortia via synthetic ecology approaches employing microbial interactions enables the efficient performance of complex tasks. Furthermore, incorporating metagenomics and transcriptomics to explore modes and mechanisms of interactions under controlled environments can improve the rational design of bottom-up synthetic microbial consortia, instead of random combinations.

Several studies have used microalgae-microalgae, microalgae-bacteria, microalgae-yeast, and yeast-bacteria co-cultivation strategies for bioremediation of different domestic, agricultural, and industrial effluents (de-Bashan *et al.*, 2010; Maza-Márquez *et al.*, 2014; Simpson, 2018; Makut *et al.*, 2019; Han *et al.*, 2021; Senith *et al.*, 2021). At present, there are limited reports on the treatment of high-strength COD wastewater from sugarcane processing factories using microalgal co-cultivation strategies. A study by Memon *et al.* (2014) demonstrated that the co-culture of immobilized *Chlorella vulgaris* and *Pseudomonas putida* resulted in 89% removal of COD within 96 h in synthetic sugar factory wastewater (SFW). The authors reported enhanced biomass yields with the addition of 80 ppm of copolymer Polyacrylate polyalcohol (Memon *et al*., 2014). In another study, a mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* showed enhanced lipid yields using sugar molasses wastewater

(Cheirsilp *et al*., 2011). The authors suggested this could be due to various interactions within the consortia, with microalgae providing oxygen to yeast and the yeast releasing CO_2 to microalgae for lipid formation (Cheirsilp *et al*., 2011). Yang *et al.* (2019) used microalgae-fungal co-cultivation between *Chlorella* sp. growing with *Aspergillus* sp. In this system, an exchange of nutrients was observed between the members of the consortium in pre-treated wastewater from the sugar industry. The *Aspergillus* sp hydrolytic enzymes facilitated the conversion of solid organics to soluble low molecular weight nutrients for digestion by *Chlorella* sp. There was a decrease in the colour intensity of the molasses wastewater by 69.98% after 5 days of co-cultivation (Yang *et al.*, 2019). The study has shown that co-cultivation of microalgae and fungi is beneficial in the treatment of sugar industry wastewater.

In addition to the complex nature of the sugar industry wastewater, the microalgal-bacteria systems reported in the literature are based on the co-cultivation of microalgae with activated sludge microorganisms (Su *et al.*, 2012; Mujtaba and Lee, 2017; Zhu *et al.*, 2019; Sepehri *et al.*, 2020). The activated sludges contain very dynamic and diverse microbial populations that offer limited control in treatment processes. The competitive relationship between microalgae and activated sludge microorganisms typically results in low performance. However, wastewater-borne microbes are a promising tool for the design and development of an effective biological wastewater treatment with environmental benefits. Thus, screening for microorganisms that can form a symbiotic relationship with microalgae is a key step in achieving the co-culture for long-term wastewater treatment. This study aimed to develop and assess the potential of a stable microalgal-based consortium using bacterial and yeast strains that are native to the sugar industry wastewater. This could result in an inexpensive low-tech innovation for enhancing the treatment of high-strength COD wastewater.

1.2 **Rationale**

The universal features of sugar-making processes necessitate using huge amounts of freshwater from various sources. During the sugarcane processing, freshwater is utilized for washing in different units of the factory, thus producing significant volumes of wastewater. The wastewater is polluted with high organic loads, suspended solids, and other parameters like oil and grease. The industry is challenged by the appropriate treatment and disposal of a large volume of wastewater, which poses serious threats of environmental pollution. For this reason, the treatment of sugar industry wastewater is essential to reduce the pollution burden on the environment. Several physicochemical methods have shown limited effectiveness in removing all pollutants concurrently. In addition, these methods are costly (require high energy inputs and expensive chemicals) and also generate toxic by-products. Removing pollutants through biological treatment alternatives remains important for environmental and aesthetic values. Particularly, microalgae cultivation in wastewater is characterized by reduced COD and nutrients. Thus, microalgae-based co-cultivation is a promising strategy with significant research interest and has proven to be economical and sustainable through previous studies.

1.3 **Aim and objectives**

1.3.1 **Aim**

To develop a consortium of microalgae, bacteria and yeast for bioremediation of sugar-mill wastewater and evaluate its potential for use as a method of treatment.

1.3.2 **Objectives**

- To isolate native microalgae, bacteria, and yeast from sugar-mill effluent and screen for their abilities to remove organic matter and nutrients from sugar-mill effluent,
- To develop a microalgal-based consortium using growth kinetics and nutrient removal efficiencies of the selected microorganisms,
- To optimize the nutrient and organic matter removal efficiencies of the selected consortium in laboratory trials using sugar-mill effluent,
- To investigate the interactions of microalgae, bacteria, and yeast in the consortium during the remediation of sugar mill wastewater using various chemical and biochemical tests

1.4 **Thesis Outline**

This thesis focuses on the design of microalgal-based co-cultivation strategies for applications in wastewater treatment and evaluating their beneficial interactions for process optimization.

An outline of the thesis structure is given below:

Chapter 1 provides background information and context of the research and motivation for the study carried out in this thesis. A general introduction to sugar industry wastewater, the challenges and limitations of current treatment methods and the superiority of biological wastewater treatment technologies. It also describes the aim and objectives established for the study. Finally, a summary of each chapter of this thesis is also represented.

Chapter 2 presents the background information to microalgal mediated wastewater treatment, it also focuses on the challenges and limitations associated with the use of monocultures for industrial processes over co-cultures. The background information of synthetic microbial

communities for diverse biotechnological applications in wastewater bioremediation. The application of co-cultures and the gaps in knowledge are reviewed.

Chapter 3 represents and highlights the importance of indigenous microbial communities for the design and development of greener technologies for wastewater treatment. This Chapter entails the isolation, screening and characterization of microbial isolates (microalgal, bacterial, and yeast) native to sugar industry wastewater for COD removal. This chapter compares performance and selects the best-performing strains for further studies related to the design and development of microalgal-based co-cultures.

Chapter 4 addresses the second objective of this thesis focusing on the design and development of microalgal co-cultures for the treatment of sugar industry wastewater, which proposed two microalgal-based consortia as well as their COD removal potential in wastewater.

Chapter 5 focuses on deciphering basic interactions influencing the removal of COD in wastewater. Common beneficial interaction mechanisms were investigated including cell-cell contact, substrate exchange, accumulation of biomass and related metabolites, and signal transduction mediated by allelopathic metabolites.

Chapter 6 summarizes the key findings and conclusions of this thesis, it also highlights the novelties of the study as well as offers recommendations for future research

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

All living systems known to biologists exist through symbiosis, this means they interact and depend on each other. Symbiosis entails a relationship between two or more species living directly together or in contact with another (Reece *et al.*, 2017). The mutualistic relationship of lichens is a widely used model to interpret symbiotic associations as they encompass all shades of physiological and behavioural traits (symbiotic systems that are life-supporting systems)(Frank, 1876). The symbiotic relationship includes obligate interactions, whereby both species depend entirely on each other for survival and reproduction, or facultative interactions, whereby symbionts do not require one another to survive and reproduce. These

associations include interactions that are harmful, helpful, or neutral (Figure 2.1). Several factors govern the formation of symbiotic associations in the natural environment.

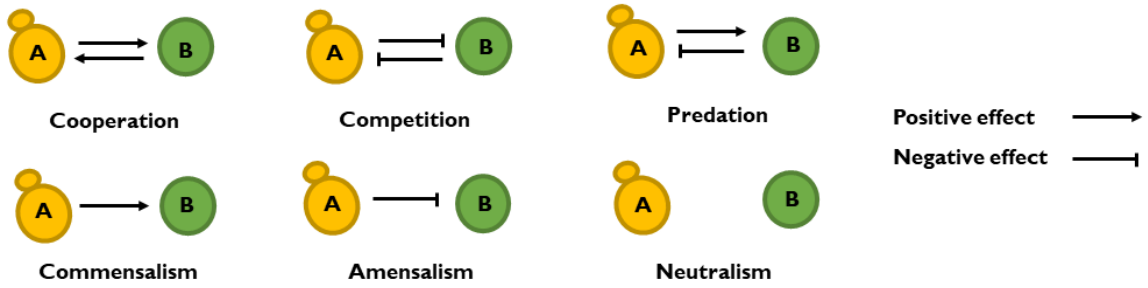

Figure 2.1. Symbiotic relationship types and interactions that are found in nature (Adapted from Wang et al., 2023).

This includes physical and biochemical factors, although nutrients or substrate exchange is mostly documented as the driving force behind most symbiotic relationships (Oksanen, 2006). Mutualistic relationships or mutualisms can be defined as an inter-species interaction that benefits both symbionts or partners (Reece *et al.*, 2017). Mutualistic relationships have received little attention compared to parasitic and predatory associations. This is because mutualistic associations undergo co-evolution of related adaptations in both species, complex ecosystems are challenging and impact the interaction studies between the symbionts and their natural environment (Medina *et al.*, 2022).

The lichens are regarded as highly successful symbiotic associations comprising various kingdoms of life with expandable biotechnological applications (widely studied mutualistic model) (Hawksworth and Grube, 2020; Sanders and Masumoto, 2021). Lichen comprises fungus closely associated with a photosynthetic microorganism (microalgae, filamentous algae, cyanobacteria, or both). In this mutualistic association, the mycobiont (fungus) provides shelter

and protection while providing essential minerals and water. In return, the photobiont's activity leads to the fixation of CO_2 by photosynthesis which supplies the fungus with nutrients (Peters *et al.*, 1986; Oksanen, 2006).

The presence of specific bacterial communities associated with lichens has been linked to various physiological roles such as (1) supply of nutrients (N, P, and S), (2) resistance towards abiotic factors, (3) resistance against biotic stress factors such as pathogens, (4) supply of vitamins and hormones, (5) detoxification of metabolites and (6) the degradation of old lichen thalli (Pankratov *et al.*, 2017). This mutualistic association is essential for stable lichens under changing ecological and environmental conditions. The mutualistic appendages in lichens allow them to strive in extreme environments, where individual partners would not survive (Casano *et al.*, 2011).

The existence of mutualistic associations in nature allows for the production of vast metabolites that can be extracted and used in biotechnological products. Previous studies have mostly focused on the mycobiont as the source of biotechnologically relevant metabolites (Calcott *et al.*, 2018; Pichler *et al.*, 2021). Recently, the algae in lichens has received huge attention for producing unique molecules that differ from that of free-living microalgae or cyanobacteria. The metabolites that originate from lichens include Mycosporine-like amino acids (MAAs), which have diverse applications in personal care products like cosmetics and sunscreens (De la Coba *et al.*, 2009). Lichens have been used as the ideal model for studying mutualistic interactions and demonstrated production of biotechnological metabolites and their persistence in ecological niches that would be unfavourable to individual species (Oksanen, 2006; Hawksworth and Grube, 2020). However, studying the interactions between microalgae/cyanobacteria and yeast/bacteria in their ecosystems or communities is limited,

thus their mutualistic relationship still requires further investigation. Microalgae has high commercial value but is limited by certain bottlenecks for commercial applications (Acién *et al.*, 2016). Moreover, axenic microalgal cultures are more susceptible to contamination, predation, and collapse. Therefore, an improved understanding of mutualistic associations and utilization of co-cultures compromising multiple species that can adapt to specific conditions and cooperative metabolic abilities would improve the costs of microalgae production for many biotechnological processes and applications.

2.2 **Microalgae**

Microalgae are microscopic eukaryotic organisms with cell walls, plasma membranes, cytoplasm, nuclei, and organelles (Harwood, 1998). Microalgae are loaded with chlorophyll consisting of plastids and are capable of photosynthesis like higher plants. Microalgae are found everywhere and in great diversity as free-living, and symbiotic associations forming biofilm mats in different aquatic environments (Spilling, 2017). Microalgae mainly differ in size ranging from a few to hundreds of micrometres (Suganya *et al.*, 2016). Unlike higher plants, every cell in microalgae is capable of photoautotrophic growth with the ability to utilize nutrients. Microalgae also play an essential role in providing atmospheric oxygen, through CO_2 sequestration for photoautotrophic growth (Bhola *et al.*, 2014), making them ideal candidates in the fight against global warming and climate change.

Several value-added products and novel compounds have been derived from different species of microalgal biomass including enzymes, fatty acids, peptides, polymers, etc. (Wu *et al.*, 2001; Vu *et al.*, 2018; Nur and Buma, 2019). Microalgae are primary producers in the aquatic environment, which makes them an ideal source of fatty acids (Perdana *et al.*, 2021). In

addition, algal biomass can be used in energy conversion systems including anaerobic digestion and microbial or membrane fuel cells.

Microalgae have basic growth requirements such as water, sunlight, and nutrients in the form of nitrogen (N) and phosphorus (P) in addition to trace elements like iron to convert CO_2 to organic carbon during photosynthesis. Their physiology allows the heterotrophic utilization of organic compounds as a source of carbon and energy such as glycerol, and acetic acid (Perez-Garcia *et al.*, 2011). Heterotrophic systems are used to overcome some of the challenges associated with phototrophic systems such as less optimal light due to environmental conditions and the requirement of large landmass (Nagarajan *et al.*, 2020). There has been a strong interest in microalgae cultivation (lipids, proteins, and carbohydrates) (Figure 2.2), however this review focuses on microalgal wastewater remediation rather than commercial products.

Their ability to adapt and tolerate environmental conditions and production of value-added biomass makes microalgae superior feedstock for diverse biotechnological applications. However, the production of bio-products from microalgae suffers from certain bottlenecks including huge land requirements and high costs of water and nutrients (Calijuri *et al.*, 2022). Microalgae have been cultivated in various wastewater to provide a low cost of water and nutrients, translating to sustainable algal-biomass generation. Although major interests have previously been on biofuels using axenic cultures, recently the focus has been expanded to designing and developing co-culture systems for numerous biotechnological uses (Goers *et al.*, 2014). This process can be used to mitigate the costs associated with microalgal cultivation for bio-products production.

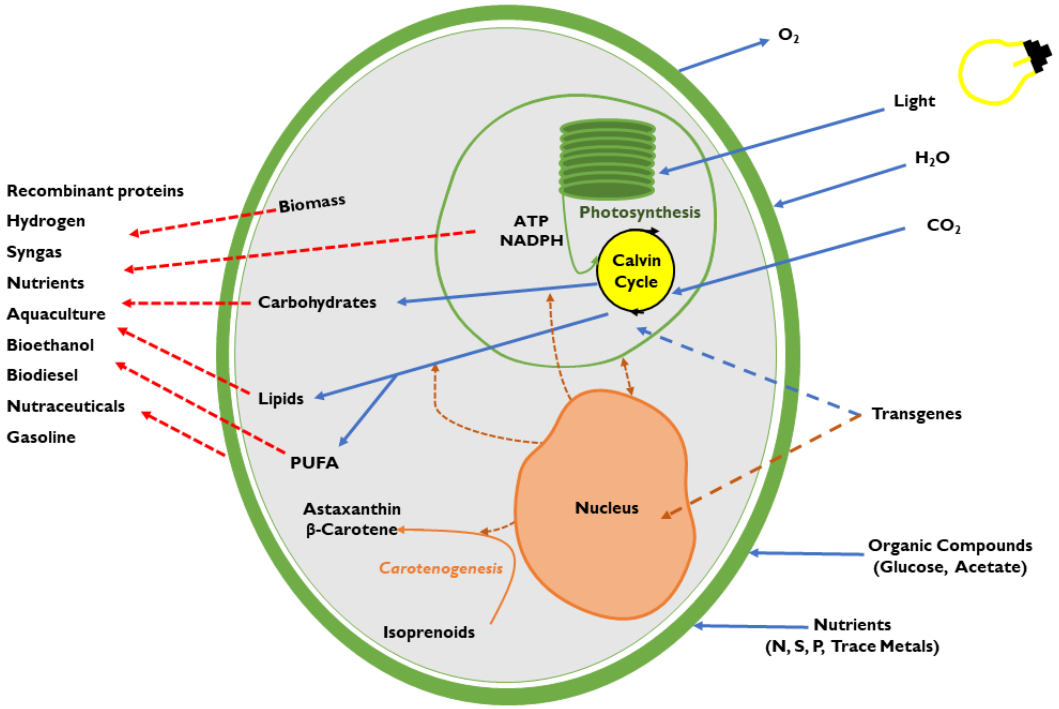

Figure 2.2. Key metabolic processes in microalgae for commercial purposes (Adapted from Rosenberg et al., 2008).

Contamination and vulnerability to environmental changes are challenges associated with the use of monocultures in industrial processes, hence the use of multiple species consortia could provide an advantage of structure and stability in changing environmental conditions and also minimize the risk of invasive species (Subashchandrabose *et al.*, 2011; Natrah *et al.*, 2014). Therefore, the development of multi-species systems including bioprospecting of good candidates for microalgal-based co-cultivation forms part of the integrated strategies to improve the overall biotechnological application of microalgae. Several studies have shown the co-culture of different species of microalgae, however, there is still more to be demonstrated.

2.3 **Microalgal-based co-cultures**

Microalgae-based co-cultivation is a promising strategy to enhance microalgal biotechnological application. Co-culture refers to the cell cultivation setup involving multiple species within the same system, two or more different microorganisms are cultured with some degree of contact between them (Pacheco and Segrè, 2019). This setup seeks to mimic or simulate natural ecosystems and interactions not observed in single-culture systems. Exploiting interspecies interactions results in improved productivity and stability. In nature, diverse microbial cells or appendages grow and survive in close associations either through mutualistic or antagonistic interactions (Wang *et al.*, 2014; D'Souza *et al.*, 2018; Islam *et al.*, 2018). Mutualism entails a prolonged and stable long-term association between two or more different organisms that benefits all individuals in the association (Reece *et al.*, 2017). These interactions shape the physiology and ecology of the community (Zhou *et al.*, 2016a).

Several recent studies have demonstrated the beneficial impacts of mutualistic associations in the ecosystems, thus conferring certain advantages to the members involved. Mutualistic co-culture offers the advantages of resilience in environmental changes, antagonistic effects on invasive species, and longevity in nutrient-limited environments (Brenner *et al.*, 2008; Hays *et al.*, 2015; Dolinšek *et al.*, 2016; Gong *et al.*, 2017). This is all possible due to various interactions between microorganisms leading to diffusion of molecular precursors such as quorum sensing molecules, cross-fed metabolites, and antimicrobial agents thus leading to numerous behaviours including divisions of labour (Figure 2.3)(Kouzuma *et al.*, 2015; Chen *et al.*, 2017; You *et al.*, 2021). This highlights the potential for more sustainable and efficient biotechnological application of microbial consortia over monoculture systems.

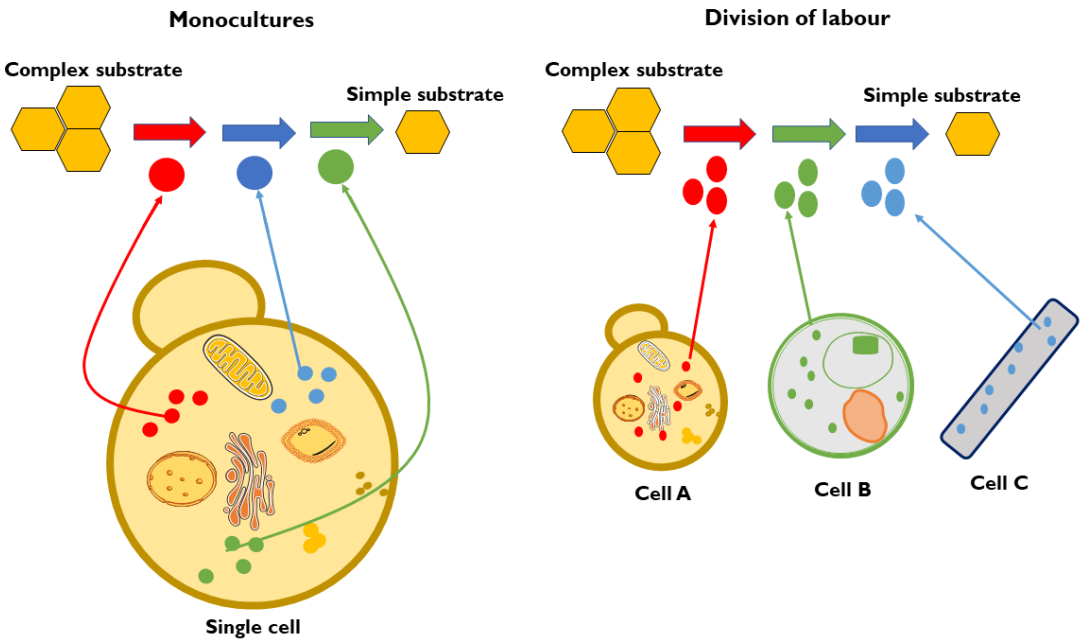

Figure 2.3. Division of labour for complex substrate utilization and degradation (Adapted from Atkinson et al., 2022).

Despite the positive advantages of mutualistic interactions, previous studies have also shown the evolution of mutualistic dependency in one partner due to the loss of adaptive genes, while the other species remains minimal dependent on the other partner (Morris *et al.*, 2012). Changes in abiotic and biotic factors influence partners' mutualistic dependency levels. In some mutualistic interactions, certain biological processes are omitted by certain partners if they can be provided by other members of the community (Morris *et al.*, 2012; Kazamia *et al.*, 2016). Therefore, co-evolution sometimes leads to mutualistic dependency and loss of fitness (one species member loses its ability to perform without the other partner). Mutualistic associations can break down when the costs of the interaction outweigh its benefits for one or both partners enough to drive evolutionary changes (Sachs and Simms, 2006). Mutualistic associations are also subjected to disease spreading amongst one or both members of the community. The disease of one partner is most likely to affect other community members.

The death of one partner by disease or killing via predation will harm the remaining members of the association.

Elucidating fitness in beneficial microbial systems is challenging and complex due to everchanging abiotic and biotic factors, and also since beneficial fitness can be provided by multiple species within the natural communities or ecosystems (Reece *et al.*, 2017). One problem is that the closeness in mutualistic associations is poorly defined, encompassing terms such as obligate and facultative mutualism often used to confer mutual dependency. Obligate mutualistic interaction entails two organisms that are completely or entirely dependent on each other and the removal of one partner leads to loss of growth and reproduction (survival). Facultative mutualistic interactions are widely encountered in nature and require prolonged association for co-evolution as both partners can survive and reproduce independently (not completely dependent on each other)(Reece *et al.*, 2017). Beneficial interactions between microalgae/cyanobacteria and yeast/bacteria will be discussed in the following sections owing to the growing body of knowledge with the potential to improve microalgal biotechnological processes and productivity.

2.4 **Microalgae-Bacteria co-cultures**

Microalgae/cyanobacteria are ubiquitous and commonly form mutualistic consortia or relationships with bacteria. This microalgae-bacterial system becomes beneficial because the microalgae stimulate bacterial growth through the production of organic compounds via photosynthesis, conversely, the bacterial cells also stimulate algal growth in a mutualistic fashion (Figure 2.4)(Huo *et al.*, 2020; González-González and de-Bashan, 2021; Scognamiglio *et al.*, 2021). Many previous studies have demonstrated specific mutualistic interactions with different microalgae-bacteria systems. However, studying such interaction in natural consortia

is often challenging because complex microbial appendages and laboratory studies do not represent definite interactions as the co-partners are often disregarded or discarded. Many studies have shown beneficial interaction between microalgae and bacteria (González-Fernández *et al.*, 2011; Hernández *et al.*, 2013; Udaiyappan *et al.*, 2020; Wang *et al.*, 2020; Biswas *et al.*, 2021; dos Santos Neto *et al.*, 2021; You *et al.*, 2021).

The increasing costs and energy consumption in conventional wastewater treatment require the development of low technology that is both sustainable and environmentally friendlier. In the past, there has been great interest in using pure microalgal strains for wastewater treatment, however, the use of symbiosis of microalgae and bacteria is gaining momentum, especially in wastewater treatment (Dolinšek *et al.*, 2016). Microalgae-bacterial systems offer the advantages of cost-effective aeration, stable removal of pollutants, and removal of pathogens like viruses (Oswald *et al.*, 1957).

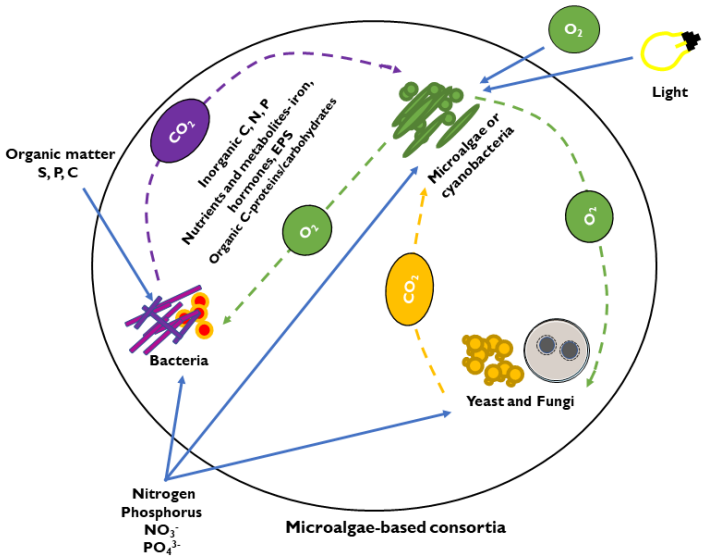

Figure 2.4. Diagram showing the symbiotic interactions in microalgal-based consortia. Microalgae/cyanobacteria can interact with yeast, fungi, bacteria, and other microorganisms (Adapted from Zhu et al., 2023).

Previous reports focus on substrate/nutrients and gaseous exchange as the most documented form of interactions in microalgal-bacterial mutualistic systems. In the following section, studies that explored interactions between microalga and bacteria in wastewater treatment are discussed and important beneficial impacts of the symbiotic systems are highlighted.

2.5 **Microalgal-Bacteria interactions in wastewater treatment**

Various wastewater streams have been used to cultivate microalgal-bacterial consortia including municipal, agricultural, and industrial effluents (e.g., textile, paper, and rubber industries) (Hernández *et al.*, 2013; Arcila and Buitrón, 2016; Makut *et al.*, 2019; Ji *et al.*, 2020; Kohlheb *et al.*, 2020; Udaiyappan *et al.*, 2020; dos Santos Neto *et al.*, 2021; Qu *et al.*, 2021). Wastewater treatment aims to reduce COD, BOD, total suspended solids, nutrients, and other wastewater parameters to levels deemed safe for disposal or recycling. These effluents typically contain significant amounts of organic materials and inorganic elements which makes them suitable for the cultivation of microalgae and their consortia for bioremediation processes (Shahid *et al.*, 2020). The intrinsic nature of the wastewater stream influences the design and operations of the wastewater treatment method. For example, typical domestic wastewater contains an average of 500-800 mg L^{-1} COD, 10-50 mg.L^{-1} TN, 4-10 mg L^{-1} TP could have energy consumption between 0.3–0.8 kWh·m^{-3} (with an associated cost of 0.14 USD/kW-h)(Tchobanoglus *et al.*, 2003; Shizas *et al.*, 2004). These effluents have been successfully used raw or as a digestate (anaerobically digested), however in some cases may be inhibitory due to the intrinsic nature of the wastewater (composition) like a high concentration of ammonia (Gutierrez *et al.*, 2016). Thus, most of the studies have adopted pre-treatment processes, which involve anaerobic digestion, dilution, filtration, centrifugation, and autoclaving of food and agro-industrial wastewater (Gupta *et al.*, 2019).

Microalgae-bacterial systems have shown huge environmental and commercial potential due to their diverse metabolites and high biodegradation potential in wastewater treatment. Oswald *et al.* (1957), first proposed and demonstrated the sustainable cultivation of microalgae in wastewater using high-rate ponds (HRP). Thereafter, many studies have elucidated the microalgae-bacterial bioremediation of various agro-industrial wastewaters. Microalgae and heterotrophic bacteria are well-documented models of interactions. It has been demonstrated that mixotrophic-grown microalgae use oxygen generated during high lights to remove COD (Rezvani *et al.*, 2018). In microalgae-bacterial systems, the microalgae produce organic materials via photosynthesis that provide dissolved carbon for bacterial species in low-carbon loading wastewater (Ahmad *et al.*, 2017). The heterotrophic bacteria stimulate microalgal growth through substrate exchange and signal transduction mechanisms (Figure 2.4). Higgins *et al.* (2018) demonstrated that bacteria utilize organic photosynthase produced by microalgal activities during winery wastewater treatment. This study showed that an increase in COD was observed in microalgal monoculture systems, suggesting that heterotrophic activity was responsible for COD removal in the periods of increased DO and may result from denitrification in low-oxygen environments, especially at night (Higgins *et al.*, 2018).

The metabolic interactions of microalgal-bacterial systems mitigated the aeration (oxygen) requirements in the oxidation ponds (Oswald *et al.*, 1957). The microalgal photosynthetic activities supply oxygen-dependent processes such as nitrification. Nitrifying bacteria have high DO requirements, microalgae-bacterial systems could supply over 74% of DO requirements leading to rapid nitrification and removal of ammonia (76%)(Wang *et al.*, 2015). These are typical examples of symbiotic relations between microalgae and bacteria whereby both

organisms benefit from each other's CO_2 and O_2 exchange (Kouzuma *et al.*, 2015; Praveen and Loh, 2015).

Microalgae requires nitrogen in high quantities for growth and pigment production. Nitrogen fixed by bacterial communities in the psychosphere provides a rich source of nitrogen in the form of ammonia for microalgal cells (Llamas *et al.*, 2023). Co-cultivation of *Chlorella sorokiniana* and activated sludge in poultry litter digestate improved nutrient removal (Bankston *et al.*, 2020). In another study, the maximum ammonium removal rate (100% within 8 days) was achieved with the inoculum ratio of 10:90 *C. vulgaris* to nitrifier-enriched activated sludge (NAS) (Sepehri *et al.*, 2020). *Chlorella vulgaris* and nitrifying bacterial community exhibited the highest ammonium removal rate of 133 mg NH_4^+-N. L^{-1}. Nitrifying bacteria greatly removed ammonia, whilst the microalgae provided support (Sepehri *et al.*, 2020). The bacterial communities utilize organic nitrogen, thereby producing ammonium and preventing nitrogen starvation. Also, the presence of microalgae could enhance bacterial activities through the exchange of certain metabolites or substrates required for their nutrient utilization. Microalgae can provide the inorganic substance for chemoautotrophic denitrifying bacteria required to reduce nitrate, such as hydrogen gas (Rezvani *et al.*, 2019). This further demonstrates the vast number of synergistic interactions in microalgae-bacteria systems, which makes them superior to monoculture systems. This is all due to various physical and metabolic interactions that lead to improved performance in wastewater treatment and bio-products production.

Phosphorus is an essential nutrient for the growth of photosynthetic microorganisms (Borchardt and Azad, 1968). Phosphorus is provided by bacterial cells via the decomposition of phosphorus into organic products that can be assimilated by the microalgae. Assimilation of phosphorus is largely done by microalgae and several mechanisms are proposed for bacterial-induced growth (Larsdotter *et al.*, 2010). The cultivation of microalgae-bacteria

consortia is an additional sustainable method to improve the removal efficiency of phosphorus. Rezvani & Sarrafzadeh (2020) demonstrated that in comparison to monocultures of microalgae (4.5 mg $L^{-1}d^{-1}$) and bacteria (2.6 mg $L^{-1}d^{-1}$), the microalgae-bacteria consortium resulted in a greater phosphate removal rate of 6.34 mg. L^{-1}. d^{-1}. Similar observations were drawn from the microalgae-activated sludge consortium (Bankston *et al.*, 2020), these findings suggest that bacteria and microalgae may work together to remove phosphorus. Bacteria can promote microalgal growth through the production of essential vitamins, co-factors, and phytohormones (Croft *et al.*, 2005; De-Bashan *et al.*, 2008; Kazamia *et al.*, 2012). Vitamin cofactor provision by bacterial cells has been shown to improve growth and nutrient uptake (Higgins *et al.*, 2018). This suggests bacterial processes or activity are also important for microalgal growth and physiology. The close association is also important in pathogen resistance and competition exclusion (Santos and Reis, 2014). This is particularly important in open microalgal cultivation systems, which are more susceptible to contamination by bacteria, viruses, and parasites.

Besides high performance in biological wastewater treatment, microalgae-bacteria systems have demonstrated potential in producing value-added biomass from which bio-products such as carbohydrates, lipids, proteins, and pigments can be sourced (Rosero-Chasoy *et al.*, 2021). Co-culture of *Chlorella sorokiniana* and aerobic bacteria exhibited maximum biomass productivity of 26.3 mg and 18.3 mg when cultivated in potato processing industry wastewater and pre-treated pig manure, respectively (Hernández *et al.*, 2013). In addition, numerous abiotic and biotic factors affect the performance of microalgal-bacterial consortia in wastewater treatment including light intensity, C/N, and algal-bacterial inoculum ratios, this information has been extensively reviewed by other authors (Swati Rani *et al.*, 2020; Fallahi *et al.*, 2021; Amaro *et al.*, 2023). Ongoing research focuses on optimizing these factors and

parameters to maximize nutrient removal and biomass production for diverse bio-products. Table 2.1 summarizes the studies done on microalgal-bacterial systems for the biological treatment of various wastewater streams and highlights the modes of interactions.

Table 2.1. Interaction between microalgae and bacteria exploited for research and diverse biotechnological applications.

Microalgae	Bacteria	Wastewater /Substrate	Symbiotic exchange	Applications/remarks	References
Chlorella minutissima	*Escherichia coli*	N8-NH_4 media enriched with glucose, glycerol, or sodium acetate	Carbon and dissolved organic carbon (DOC)	Production of Biofuel Precursors Improved total lipid and starch productivity	Higgins and VanderGheynst (2014)
Botryococcus braunii	*Rhizobium* sp.	Freshwater supplemented (JM: F/2 medium)		Hydrocarbons for biofuel	Rivas *et al.* (2010)
Tetradesmus ob liquus AARL G022	*Piscicocus intestinalis*	Biogas digestate effluent	Oxygen and carbon dioxide	Production of microalgal biomass and lipid for biodiesel	Kumsiri *et al.* (2021)
Dunaliella salina	*Alteromonas* sp., *Muricauda* sp	Artificial seawater (ASW)	Carbon and Nitrogen and Phosphorus	Enhanced assimilation of ammonium under nitrogen-limited conditions	Chevanton *et al.* (2013)
Chlorella vulgaris or *C. sorokiniana*	*Azospirillum brasilense* strain Cd	Municipal wastewater		Remove nutrients (P & N)	de-Bashan *et al.* (2002) de-Bashan *et al.* (2016)
Amphidinium operculatum	*Halomonas* sp.	Synthetic growth medium	Vitamin B12 and photosynthate	Model algae for interaction assays	Croft *et al.* (2005)
Lobomonas rostrata	*Mesorhizobium*	Synthetic growth medium			Kazamia *et al.* (2012)

Dunaliella salina	*Marinobacter* sp., *Halomonas* sp., *Pelagibaca* sp	Synthetic growth medium	Iron/ siderophore and DOC	Promote the growth of microalgae under iron-limited conditions.	Baggesen *et al.* (2014)
Scippsiella trochoidea	*Marinobacter* sp.	Synthetic seawater medium,		Sustain microalgae-bacteria equilibrium in ocean communities	Amin *et al.* (2015)

2.6 **Microalgae-Yeast co-cultures**

Microalgae and yeast are major cell factories for a wide range of biotechnological and commercially relevant products. Yeasts are the subject of extensive research and have been predominately used for alcoholic products, baking, biofuels, probiotics, and food supplements (Rani and Soni, 2007; Branduardi and Porro, 2012; Hittinger *et al.*, 2018; Jach and Serefko, 2018; Ahuja *et al.*, 2023). Yeasts have attracted significant interest as wastewater bioremediatory agents. For instance, *Saccharomyces cerevisiae* displays high biosorption activities with strong COD removal abilities. *Saccharomyces cerevisiae* reduced 69% of COD over 48 h of cultivation in winery wastewater under aerated conditions (Malandra *et al.*, 2003). Various microalgae-yeast consortia have been exploited to improve biotechnological productivity processes and several studies describing interactions are on the rise. These studies emphasize the importance of the symbiotic relationship of the partners in enhancing the production of targeted outputs (Table 2.2). In the following section, previous studies investigating microalgae and yeast interactions with different industrial applications are discussed and the important benefits of these co-culture systems are highlighted.

2.6.1 **Microalgal-Yeast interactions in wastewater treatment**

Microalgae and yeast co-cultivation can be harnessed for diverse industrial and environmental applications. When microalgae and yeast are cultured together, the microalgae will absorb CO_2 from the yeast`s activities and the yeast utilizes the O_2 released by the microalgae (Figure 2.5). In addition, the microalgae absorb organic acids produced by the yeast activity and alleviate their detrimental effect on the yeast cells (Yen *et al.*, 2015). In yeast monoculture, the production of organic acids inhibits yeast in the later stages of growth. The growth of yeast favours the production of an acidic environment that ends up being detrimental to its growth. According to Xue *et al.* (2010), the photosynthetic activity of microalgae converts CO_2 into bicarbonate that is utilized as substrate for growth resulting in the production of OH-ions in the medium thus establishing an alkaline environment. The yeast has the metabolic capabilities of breaking down complex carbohydrates into simple sugar molecules that could be utilized by microalgae for its growth. The important interactions between microalgae and yeast involve the interplay of essential metabolites resulting in the steady-state of factors such as CO_2/O_2, pH, and DO in the medium (Figure 2.5)(Arora *et al.*, 2019). These stable and steady conditions result in improved growth for both partners. Several research on the co-culture of various microalgae and yeasts using various feedstocks are represented in Table 2.2.

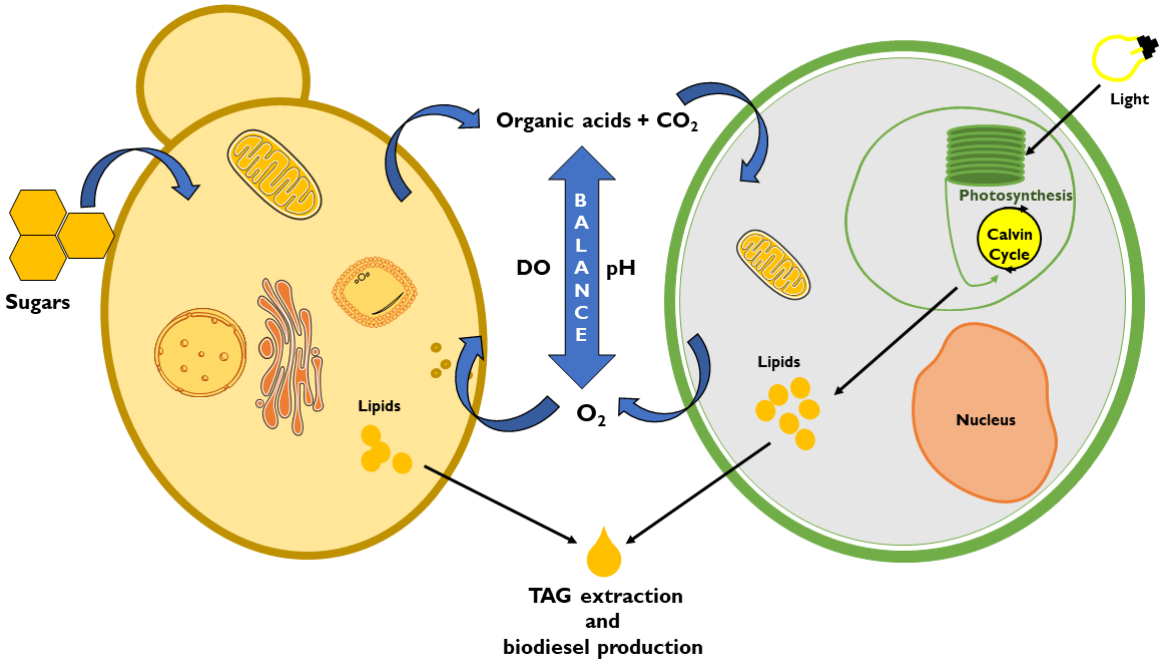

Figure 2.5. Diagram showing interactions in the co-culture of yeast and microalgae (Adapted from Arora et al., 2019).

The co-culture of microalga *Chlorella* sp. KKUS2 and the oleaginous yeast *Torulaspora maleeae* or *Torulaspora globose* showed high lipid yield (22.90%), when compared to monoculture systems (Papone *et al.*, 2012). Here, the microalgae supplied oxygen to the yeast and the yeast subsequently generated CO_2 for microalgal growth. The maximum lipid production of 920 $mg.L^{-1}/d^{-1}$ was observed in the co-culture of *C. vulgaris* and *R. glutinis* cultivated in diluted seafood processing effluent (Cheirsilp *et al.*, 2011). The synergistic utilization of CO_2/O_2 by co-cultured species mitigated the accumulation of toxic by-products such as organic acids (Cheirsilp *et al.*, 2011). Similarly, Liu *et al.* (2018) reported that co-cultured *Chlorella pyrenoidosa* and *Rhodotorula glutinis* in cassava hydrolysate attained lipid productivity and content of 1.54 $g.L^{-1}/d^{-1}$ and 42.27%, respectively. These studies have demonstrated the beneficial interactions of co-cultures as compared to axenic systems. These interactions can be explained by the metabolic complementarity of co-partners that enables the use of diverse substrates and by-

products that could otherwise be detrimental or growth-limiting, thus creating a stable environment (Xue *et al.*, 2010; Kitcha and Cheirsilp, 2014; Ling *et al.*, 2014). Recent studies on microalgae and yeast co-cultivations have focused mainly on biofuel production, feed, and the generation of value-added products like astaxanthin (Table 2.2).

Yeasts have diverse metabolic capabilities that enable them to utilize various sugar substrates as nutrient sources such as lignocellulosic and agricultural wastes. Secondary and tertiary wastewater treatment contains significant amounts of nutrients that can be utilized by the microalgae and yeast for their growth, thus offering a platform for biological wastewater treatment (Xie *et al.*, 2013). Axenic microalgal strains can utilize nitrogen, phosphorus, and CO_2 from wastewater, however high COD loading rates lead to retarded growth (Ling *et al.*, 2014). On the contrary, the yeasts can effectively remove COD ranging from 150 to 500 mg L^{-1} but show limited removal of nutrients (N and P). Thus, the co-culture of microalgae and yeast is a promising bioremediation strategy for reducing COD/TOC and nutrients in wastewater (Ling *et al.*, 2014). Reciprocation of carbon and nitrogen interaction has been documented in the co-culture of *Chlamydomonas reinhardtii* and yeast *Saccharomyces cerevisiae* (Hom and Murray, 2014). In addition, physical contact and interactions were observed between the microalgae *Chlamydomonas* sp. and filamentous fungi (Hom and Murray, 2014). Table 2.2 represents recent studies utilizing microalgal and yeast consortia for diverse applications. Major studies in microalgal-yeast systems focused on improving lipid and pigment productivity, and more research should be conducted on the interactions between microalgae and yeast for specific applications such as wastewater bioremediation processes.

Table 2.2. Interaction between microalgae and yeast exploited for research and diverse biotechnological applications

Microalgae	Yeast	Wastewater/ Substrate	Symbiotic exchange	Applications/remarks	References
***Chlorella* sp. KKUS2**	*Torulaspora maleeae* or *Torulaspora globose*	Sugarcane juice	Oxygen and carbon dioxide	Lipids for biodiesel	Papone *et al.* (2012)
Chlorella vulgaris	*Rhodotorula glutinis*	Industrial wastes			Cheirsilp *et al.* (2011)
Spirulina platensis	*Rhodotorula glutinis*	Glutamate wastewater			Xue *et al.* (2010)
Chlorella pyrenoidosa	*Rhodotorula glutinis*	Cassava bagasse hydrolysate		Wastewater treatment	Liu *et al.* (2018)
Haematococcus pluvialis	*Phaffia rodozyma*	BBM medium		Astaxanthin production	Dong and Zhao (2004)
Chlamydomonas reinhardtii	*Saccharomyces cerevisiae*	Synthetic growth medium	Carbon and Nitrogen	Model of micro-ecosystem	Hom and Murray (2014)
Chlorella sorokiniana	*Saccharomyces cerevisiae*	Synthetic growth medium		Synthetic ecology study	Naidoo *et al.* (2019)

2.7 **Synthetic Microbial Ecology**

Synthetic ecology is a developing "synthetic biology" subfield that designs and creates artificial biological systems using engineering concepts (Dunham, 2007; Brenner *et al.*, 2008). Specific molecules, cell components, entire cells, tissues, and organisms can all be treated using these engineering principles (Drubin *et al.*, 2007; Serrano, 2007). The field of synthetic microbial ecology has sparked intense research interest in mutually beneficial symbioses in ecosystems and engineered habitats. To facilitate, regulate, or optimize a particular biotransformation, artificial microbial systems are designed rationally and manipulated theoretically. This is known

as synthetic ecology, a subfield of synthetic biology. Synthetic ecology focuses on designing, building, measuring, and forecasting the dynamic behaviours of ecological circuits (De Roy *et al.*, 2014; Stenuit and Agathos, 2015; Dolinšek *et al.*, 2016). Synthetic biology designs, builds, analyzes, and predicts the dynamic behaviours of metabolic and regulatory circuits to comprehend how molecular interactions lead to the development of cellular-level traits. On the contrary, the goal of synthetic microbial ecology is to comprehend how microbial interactions give rise to certain traits at the community level. Building a simplified environment with essential functions and characteristics of a more complex microbial ecosystem is the goal of many synthetic microbial ecology studies (Dolinšek *et al.*, 2016). Synthetic ecology aims to apply engineering principles to the behaviour and design of artificial communities that could be used to enhance biotechnological productivity (Dolinšek *et al.*, 2016). This method could be utilized to create systems that provide valuable ecosystem services while also acting as self-regulating climax communities that are resistant to biological invasion (de-Bashan *et al.*, 2016).

The complex systems and dynamic environmental elements in the natural ecosystems present difficulties in understanding and characterization of microbial interactions. To overcome this, synthetic microbial ecology relies on the selection of compatible strains, engineering of environments and/or beneficial/mutual symbiosis, and tailored characteristics (physical properties, nutrient compositions, etc.) (Kazamia *et al.*, 2014). According to Bujara & Panke (2010), the artificial microbial engineering concept or design series is utilized in processes that require control, performance predictability, and efficiency. It involves the following steps: (1) measuring, (2) modelling, (3) manipulating, and (4) manufacturing. Artificial microbial communities can be designed based on informed decisions where conditions are controlled, monitored, and manipulated. It is important to understand the procedures and expectations (results) of synthetic microbial systems before devoting time and resources to application on

an industrial level (Simpson, 2018). Gaining in-depth knowledge of synthetic microbial systems could help us understand microbial interactions, and address some of the major issues in single-culture systems and genetically modified strains in biotechnological processes and industrial applications (Pandhal and Noirel, 2014).

Numerous studies have designed artificial microbial communities including microalgae-bacteria, microalgae-yeast, microalgae-microalgae, and yeast-bacteria for the bioremediation of various industrial, agricultural, and domestic effluents (de-Bashan *et al.*, 2010; Lananan *et al.*, 2014; Maza-Márquez *et al.*, 2014; Simpson, 2018; Makut *et al.*, 2019; Karim *et al.*, 2021). Their beneficial interactions produce high-value bio-products with diverse biotechnological uses in addition to wastewater treatment (Rosero-Chasoy *et al.*, 2021). Enhanced or improved cell size, biomass, growth rate, and productivity are advantages of microalgal-based co-cultivation strategies used in wastewater bioremediation (de-Bashan *et al.*, 2002; Choix *et al.*, 2012). Synthetic mutualistic models have fewer variables, and controlled laboratory conditions enable an easier understanding of beneficial interactions (main trade-offs). There is still much to elucidate about the nature of mutualistic interactions in synthetic microalgal-based consortia. Some of the early studies on synthetic mutualism focused on *Chlorella* sp. co-cultivated with *A. brasilense*. The bacteria cells promoted the growth and metabolism of the microalgae, resulting in improved pigment production and lipid content (de-Bashan *et al.*, 2002). In another study, methanotrophs were grown in monoculture and co-cultivated with green microalgae to utilize and convert residual nutrients to single-cell protein (Rasouli *et al.*, 2018). Other research focuses on isolating specific yeast/bacteria strains as candidates for symbiotic co-culture from wastewater containing target microalgae species. For instance, growth-promoting bacteria were found in a variety of wastewater effluents and following exposure to these bacterial isolates, the growth rates of three distinct microalgal strains (*C. vulgaris*, *C. reinhardtii*, and

Euglena gracilis) increased by 2.8 times (Toyama *et al.*, 2018). These studies have demonstrated that enhanced biotechnological productivity and our understanding of complex ecosystems could be expanded by combining synthetic ecological principles, such as artificial symbiosis with reciprocal metabolic capacities (de-Bashan *et al.*, 2016; Dolinšek *et al.*, 2016).

Recent studies have focused on the engineering of microbial symbiotic co-cultures, identifying potential strains of microalgae, bacteria, or yeast. However, the underlying interactions such as causal mechanisms, dynamics and stability, spatial organization, and the role of other microorganisms remain unexplored. Further investigation is needed to understand the causal connections between microbial interactions and emergent behaviours. For instance, metagenomics and meta-transcriptomics offer insights into consortia composition and functions, but understanding causal relationships between microbial interactions and specific functions requires further studies. Also, long-term studies with high resolution are needed to better understand the temporal dynamics, stability, and factors governing natural consortia's assembly and succession. The distribution, and arrangement of microbes within consortia is crucial for their function but often overlooked. Advanced imaging techniques and spatial omics approaches are needed to map and understand these micro-environments. The complexity of natural consortia hinders the development of accurate predictive models, necessitating the integration of multi-omics data with advanced computational approaches. The impact of pollutants and stress-inducing factors on the structure and function of natural microbial consortia is not fully understood and necessitates further investigation.

2.8 **Sugar-industry wastewater**

SASI generates significant volumes of wastewater, on average 2184 KL of wastewater are discharged for every ton of sugarcane processed (Ndobeni, 2017). This corresponds to one billion litres of sugar industry effluent that needs to be disposed of annually. Washing sugarcane, including water from tanks containing processing residues, accounts for over 75% of the total volumes of wastewater generated by the sugarcane industry. This effluent is characterized by high chemical oxygen demand (COD: 1752 - 8339 mg L^{-1}), and biochemical oxygen demand (BOD: 1052 – 4641 mg L^{-1}) with low nutrients and other minerals (Fito *et al.*, 2018; Nájera-Aguilar *et al.*, 2021). Wastewater from the sugar industry fluctuates greatly in quantity and quality, making it challenging to design and develop sustainable cost-effective treatment solutions due to challenges associated with conventional methods of treatment. The cane variety (feedstock), the season (soil-specific qualities), and process-specific treatments (chemicals utilized) influence the wastewater composition. Sucrose, bagacillo, oil, and grease from the mill's bearing house contribute to the organic content of the effluent (Kaur *et al.*, 2010; Kumar and Srikantaswamy, 2015). The organic content of the wastewater originates from the cane processing (cutting and pressing), evaporation, crystallization, and refining processes.

The COD of sugar industry effluent ranges between 1752 and 8339 mg L^{-1}. The South Africa National Water Act (Act No. 36 of 1998) states that the pH of sugar industry wastewater should be between 5 and 9.5, and COD (<75 mg L^{-1}) upon discharge (Ndobeni, 2017). Most sugar industry wastewater exceeds the regulatory limits, pH range of 5.5 – 8.5 and COD values as low as 35 mg $L^{-1}$and as high as 7432 mg L^{-1} have been reported in other countries (Parande *et al.*, 2009; Siddiqui and Waseem, 2012; Shivayogimath and Jahagirdar, 2013; Saranraj

and Stella, 2014; Qureshi and Mastoi, 2015). Wastewater discharge standards in South Africa are determined by COD limits (as well as other quality parameters) applied through irrigation per day volume. The law of South Africa stipulates that per daily volume, the COD limits should range from 5000 mg L^{-1} for volumes up to 50 kL/day or < 400 mg L^{-1} for volumes up to 500 kL/day (Government Gazette number 19182 of 2013). The majority of wastewater from sugar factories has quality criteria that are higher than permitted, which puts the environment's water ecosystems, soil characteristics, and soil microflora at risk of toxicity (Kumar and Chopra, 2010). To prevent negative environmental impact, sugar industry wastewater should be treated before being released into waterbodies and the environment via fertigation (Fuess and Garcia, 2014).

The formation of organic compounds as liquid effluents (sugar juice, syrup, and molasses) from the process units is one of the variables influencing COD in wastewater (Solomon, 2005). Fresh wastewater samples for experiments are unavailable in certain places due to the lack of sugar refineries. For reliable comparative testing, most researchers create synthetic wastewater recipes from sugar mills. A synthetic sugar industry wastewater recipe was created by Ndobeni (2017) using molasses, it is an appropriate substrate due to its carbohydrate and cysteine content. Singh *et al.* (2019), have extensively reviewed the treatment methods for sugar industry effluents ranging from traditional to more advanced technologies. Different techniques for the treatment of sugar industry wastewater are grouped into 5 categories, namely physicochemical, biological, membrane filtration and separation, advanced oxidation processes, and combined biological and advanced oxidation processes (Figure 2.6).

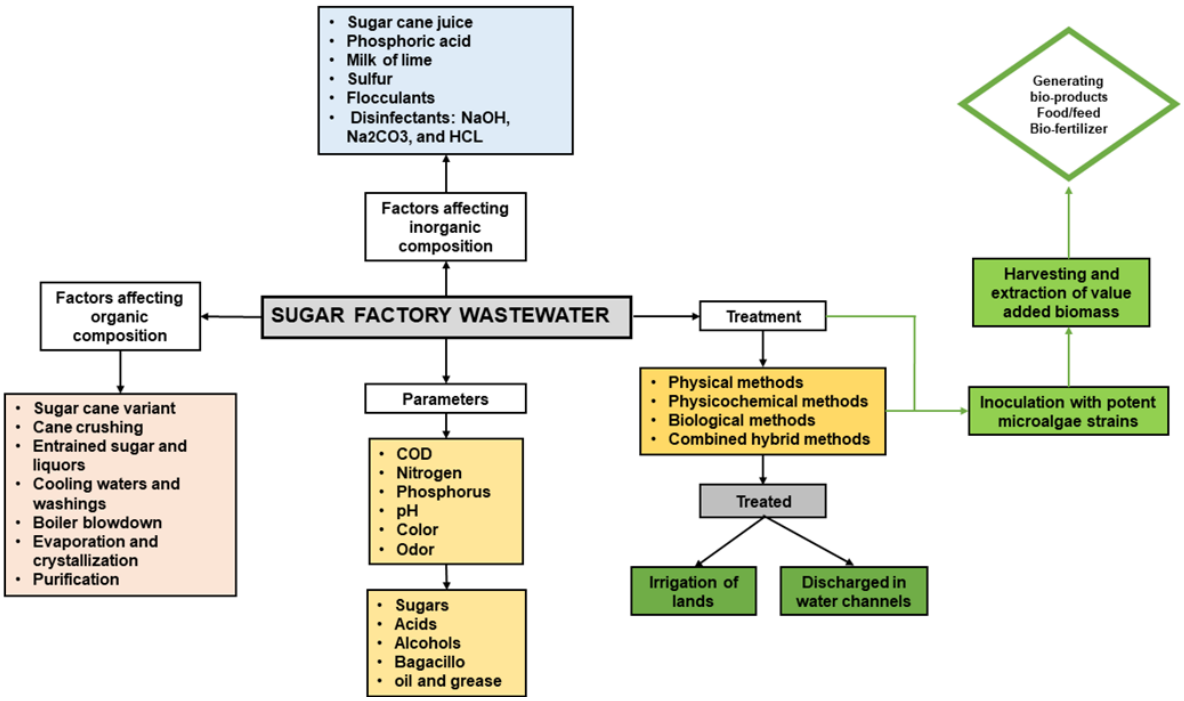

Figure 2.6. Major components of sugar industry wastewater.

Singh *et al.* (2019), reviewed and discussed the benefits and drawbacks of each treatment method. Table 2.3 provides a summary of the characteristics of sugar industry wastewater. Despite stringent laws governing wastewater disposal, globally many sugar factories discharge their effluent without adequate treatment due to a lack of the necessary funds, expertise, or sometimes even the lack of desire to spend money on wastewater treatment.

Table 2.3. The average physicochemical properties of the cane industrial effluents (Adapted from Fito et al., 2018).

Parameter	Unit	Min	Max	Average
Temperature	°C	29.3	44.3	36.8
Ph		6.7	8.4	7.55
Electric conductivity	µS/cm	540.3	925.9	732.9
BOD 5	mg L^{-1}	654.6	1968.5	1311.5
COD	mg L^{-1}	1100.3	2148.9	1624.6
Chloride	mg L^{-1}	30.5	866.6	463.8
Total hardness	mg L^{-1}	356.2	2493.1	1424.6

Calcium	mg L^{-1}	365.4	468.0	416.7
Magnesium	mg L^{-1}	214.8	341.0	277.9
Total solid	mg L^{-1}	2452.3	3050.6	2751.4
Total dissolved solids	mg L^{-1}	1480.2	1915.1	1697.6
Total suspended solids	mg L^{-1}	220.3	790.7	505.5
Nitrates	mg L^{-1}	0.4	0.9	0.85
Organic –N	mg L^{-1}	24.3	36.4	42.5
Ammonia –N	mg L^{-1}	0.0	4.2	2.1
Total nitrogen	mg L^{-1}	11.1	40.6	25.85
Phosphate	mg L^{-1}	1.2	9.6	6.00
Sulfate	mg L^{-1}	21.5	51.7	47.35
Oil and greases	mg L^{-1}	88.7	134.4	155.9

Waste stabilization ponds (WSPs) are often used to treat sugar industry wastewater in South Africa (Ndobeni, 2017). These are typically designed for low COD and BOD loading rates and a retention time of 5 – 50 days (Nähle, 1990). In these systems, the treatment processes are carried out by various types of bacterial species, algae, and zooplankton (Mara, 2009). A consortium of microorganisms is usually associated with removing broad ranges of different pollutant types to acceptable levels before discharge into surrounding water environments. However, in most cases, a single process is not enough, WSPs have been plagued with many problems from design and operational-related ones. WSPs cannot sustain shock loads without significantly losing their ability to remove pollutants (Pearson *et al.*, 1996). Due to the lengthy biological treatment process and the size of the required land area, this takes an exorbitant amount of resources.

To date, there is limited studies focusing on the synthetic ecology approach for the treatment of high-strength COD wastewater from the sugar industry. Recent studies have shown the feasibility of using microalgae and yeast/bacteria (monoculture and consortia) to treat various wastewater streams. Combined with their biotechnological potential, these systems are ideal candidates for the bioremediation of sugar industry wastewater and the development of

valuable bioproducts (Cheirsilp *et al.*, 2011; Memon *et al.*, 2014; Yang *et al.*, 2019). Developing an integrated treatment system that could reduce nutrient load while boosting production and developing a self-sufficient biological process that uses wastewater as a source of nutrition. Better design and development of environmentally friendly wastewater treatment systems are made possible by understanding the interactions in artificial microbial communities. The interaction mechanisms between cells within co-culture systems could provide insight into structure and function for further development and implementation in wastewater treatment processes.

CHAPTER 3: ISOLATION AND CHARACTERIZATION OF INDIGENOUS MICROALGAL, BACTERIAL, AND YEAST STRAINS FROM SUGAR INDUSTRY WASTEWATER

3.1 Introduction

Microorganisms like bacteria, fungi, and microalgae have demonstrated potential in the development of low-tech and sustainable methods for the treatment of various wastewater streams (Parmar *et al.*, 2022). Naturally occurring bacteria can easily use the pollutants in wastewater for their proliferation and growth (Xie *et al.*, 2012; Nájera-Aguilar *et*

al., 2019; Nájera-Aguilar *et al.*, 2021). Their high surface area-to-volume ratio, small size, and extensive environmental interaction allow them to rapidly adapt to adverse environmental conditions (Chandran *et al.*, 2020). They also possess a wide array of intracellular and extracellular enzymes capable of breaking down a variety of substances, due to their specific and non-specific substrate affinity (Gallert and Winter, 2005; Cammarota and Freire, 2006). In addition, they undergo fast genetic adaptation, giving them access to novel metabolic pathways that could degrade even the pollutants of emerging concern (Gallert and Winter, 2005). Previous reports suggest wastewater-borne microorganisms are superior bioremediation tools due to their adaptability and advantages of overcoming ecological barriers (Baduru Lakshman Kumar and Gopal, 2015; Zahedi, 2018). Environmental adaptation is generally higher in microorganisms isolated from the same environment. Native microorganisms play an essential role in biogeochemical processes, their presence considerably facilitates the degradation of organic loads in wastewater. Characterization of specific strains may assist in selecting the best bioremediation agents for reducing the pollution burden on the ecosystem.

This chapter focuses on the isolation, screening, and evaluation of indigenous microalgal, bacterial, and yeast strains for the treatment of high-strength COD wastewater from the sugar industry under nutrition support. This study seeks to reduce the number of native microbial isolates by selecting the best-performing strains and assessing their ability to co-exist for inclusion in the final consortia.

3.2 Materials and Methods

3.2.1 Wastewater collection

Wastewater for isolating indigenous microorganisms was collected by random sampling of various oxidation ponds in the sugarcane processing mill located in KwaZulu-Natal, South Africa. The grab sampling technique was used, whereby the sampling bottle was dipped directly into the wastewater. Approximately 1000 mL of wastewater was collected and transferred into sterile labelled 2000 mL plastic sampling bottles. Sampling details such as locality details, date, time, and sample type were also recorded during the time of sampling. The samples were transported to the laboratory in a dark thermo box for enrichment within 24 h of collection.

3.2.2 Isolation and purification of microalgal strains

Isolation and purification of the microalgal isolates were carried out by enrichment of wastewater samples with TAP-medium (Tris-Acetate-Phosphate) (Gorman and Levine, 1965). The pH of the medium was adjusted to 7.5 with 1 M NaOH before autoclaving at 121°C for 15 min. A volume (10 mL) of the wastewater samples (10% v/v) was transferred to a 250 ml conical Erlenmeyer flask containing 90 mL of the sterile enrichment medium. The flasks were illuminated by Sylvania® Gro-Lux® lightbulbs (μmol/m^{-2}/s^{-1}),16 h light: 8 h dark cycles at 29 ± 2°C for 28 days. The flasks were hand-shaken 2-3 times daily to resuspend the culture. The cultures showing growth were purified by conventional serial dilution and spread plating on TAP agar medium, pH 7.0. Wet mounts were prepared and examined using light microscopy (Zeiss Axio microscope) to ensure the purity of the culture. Single colonies were picked from agar plates and inoculated into a liquid 10 mL TAP medium. Cultures were maintained in 2 L Erlenmeyer flasks containing 1 L of TAP media under continuous agitation at 100 rpm.

3.2.3 **Isolation of bacterial and yeast isolates**

Bacterial and yeast strains were isolated by inoculating wastewater samples (10% v/v) in sterile nutrient broth (NB) and yeast extract peptone dextrose (YPD) broth. The flasks were incubated in an orbital shaker (OrbiShake Shaker, Labotec, South Africa) at 130 rpm, 32 ± 2 °C (Bacteria), and 25 ± 2 °C (Yeast) for 48 h. The cultures showing growth were purified by conventional streaking technique followed by serial dilution and spread plating on solid-agar plates. The pure bacterial, and yeast strains were maintained on solid agar plates for species identification and bioremediation studies.

3.2.4 **Colonial morphology and grams-staining**

Morphological properties such as shape, colour, surface, margin, elevation, opacity, and consistency were determined to ensure the selection of morphological distinct colonies. The bacterial isolates were subjected to Gram-staining (Coico, 2006). Once the cells were appropriately stained, slides were viewed using light microscopy (Zeiss Axio microscope) at 400x and 1000x magnification to substantiate the cultures as either gram-positive or gram-negative.

3.2.5 **Light microscopy for microalgal strains**

To examine the morphological characteristics, wet mounts were prepared and observed using a Zeiss Axio microscope equipped with transmitted light and phase contrast illumination (Axio

Imager A1; Carl Zeiss, Germany). Micrographs were taken with a digital camera (Axiolab) using the Zen software. The major characteristics examined were cell shape, cell dimensions (diameter, length), the type of cell division, colour of cells, and motility.

3.2.6 **Screening and selection of microalgal and bacterial strains in synthetic wastewater**

3.2.6.1 Inoculum preparation

Microalgal starter cultures (inoculum) were prepared in 50 mL TAP medium containing NH_4Cl and incubated in an orbital shaker (OrbiShake Shaker, Labotec, South Africa) as described in Section 3.2.2 (till mid-log) with agitation at 110 rpm.

Pure bacterial colonies obtained in Section 3.2.3 were used to prepare inoculum for growth and nutrient removal studies. The inoculum was prepared by aseptically taking a loopful of colonies from agar plates of each isolate and inoculating each isolate separately into 100 mL conical Erlenmeyer flasks containing 30 mL of the sterile NB (Merck, Darmstadt, Germany). The flasks were then incubated at 32°C in an orbital shaker (OrbiShake Shaker, Labotec, South Africa) at 180 rpm for 24 h until an exponential growth phase was obtained. The growth of the flasks was monitored by turbidity at an OD of 600 nm. When the mid-exponential growth phase was reached, the concentration of cells was standardized to a final concentration of 1×10^8 cells per mL^{-1}.

3.2.6.2 Cultivation of microalgal and bacterial strains in synthetic wastewater

The ability of the selected microbial isolates to grow in wastewater, remove COD and nutrients, and produce hydrolytic enzymes (bacterial) was assessed by growth studies using synthetic sugar industry wastewater. The synthetic wastewater served to operate by creating controlled environments with suitable substrates and growth nutrients for microorganisms to proliferate. The synthetic wastewater was prepared by mixing all components in Table 3.1 in 2000 mL flasks containing 1000 ml of medium. The prepared medium was sterilized by autoclaving at 121°C for 15 min. The initial concentrations of the parameters were; 7790 mg L^{-1} COD, 0.294 mg L^{-1}, NO_3^- -N, 99.24 mg L^{-1}, NH_4^+-N, and 28.126 mg L^{-1} PO_4^{3-} -P.

Table 3.1. Synthetic SIWW composition used for the growth of bacterial and microalgal strains

Compound	Amount	Unit
Sucrose	5.0	g L^{-1}
$(NH_4)_2$ SO_4	0.1	g L^{-1}
K_2HPO4	0.052	g L^{-1}
KH2PO4	0.04	g L^{-1}
(NH_4) HCO_3	0.4	g L^{-1}
$NaHCO_3$	3.2	g L^{-1}
$KHCO_3$	3.2	g L^{-1}
Yeast extract	0.02	g L^{-1}
Macronutrients solution I	20	mL
Micronutrients solution II	5	mL

Macronutrients and micronutrients: NH_4Cl, 10.0 g L^{-1}; K_2HPO_4, 2.0 g L^{-1}; H_3BO_3, 0.05 g L^{-1}; $FeCl_2$. $2H_2O$, 2.00 g L^{-1}; $ZnCl_2$, 0.05 g L^{-1}; $MnSO_4$, 0.5 g L^{-1}; $CuCl_2$. $2H_2O$, 0.03 g L^{-1}; $(NH_4)_6$ Mo_7 O_{24}. $4H_2O$, 0.05 g L^{-1}; $AlCl_3$. $6H_2O$, 0.05 g L^{-1}; $CoCl_2$. 6H2O, 2.00 g L^{-1}; $MnCl_2$, 0.25 g L^{-1}; $MgCl_2$, 1.00 g L^{-1}; EDTA, 0.05 g L^{-1}; $NiCl_2$. $6H_2O$, 0.25 g L^{-1}; HCl, 1.00 mL.

Minerals and trace metals were adapted from methods outlined by Gonzalez *et al.* (1998) and were prepared once in a stock solution which was used for the duration of the study.

The microalgal and bacterial cultures obtained in Section 3.2.6.1 were used to inoculate 250 mL Erlenmeyer flasks containing 50 mL synthetic wastewater medium with a starting optical density (OD 600 nm) of 0.05 for bacterial, and OD 680 nm of 0.2 for microalgal isolates. The inoculated bacterial flasks were then incubated at 32°C for 12 h on an orbital shaker (OrbiShake Shaker, Labotec, South Africa) at a speed of 180 rpm. Microalgal cultures were incubated as described in Section 3.2.2 with agitation at 110 rpm. Thereafter, a volume of 10 mL samples was harvested at 24 h intervals from each flask for analysis described in Section 3.2.7 below. A negative control was used and contained no culture addition.

3.2.6.3 Analysis of growth and bioremediation potential of isolates

The samples obtained in Section 3.2.6.2 were analyzed for turbidity, the reduction of waste ions, and COD, along with the ability to synthesize enzymes such as amylase, cellulase, protease, and lipase. Turbidity was assessed by dispensing 2 mL of sample into a cuvette and measuring optical density (OD 600 nm for bacterial and OD 680 nm for microalgal strains) using a Jenway 7205 UV/Visible Spectrophotometer (Lasec, South Africa) to determine the growth of each isolate in synthetic wastewater. The growth profile was calculated using the following equation (Levasseur *et al.*, 1993).

$$\mu = d \ln OD (600/750 \text{ nm}) / dt \qquad \text{(Equation 3.1)}$$

The ability of the isolates to break down waste ions and COD was assessed by dispensing 5 mL of sample from 3.2.6.2 into Falcon conical centrifuge tubes and centrifuged at 10 000 x *g* for 5 min. The resultant supernatant was used to assess the reduction of COD, ammonium, nitrate, and phosphates. The wastewater analysis was conducted according to the methods

outlined respectively in Section 3.2.7.1 and Section 3.2.7.1. Enzyme assays were conducted according to methods outlined in Section 3.2.7.3 to Section 3.2.7.6. The percentage removal of nutrients was calculated using the following equation

$$\text{Removal Efficiency (\%)} = C_i - C_n/C_i \times 100 \qquad \text{(Equation 3.2)}$$

Where C_i is the initial nutrient concentration and C_n is the nutrient concentration on day n.

3.2.6.4 COD analysis

The HACH COD test (HACH method, High Range Plus) is based on the amount of oxygen originating from potassium dichromate that reacts with the oxidizable substances contained in a sample. The supernatant (200 µL) from each filtered sample from section 3.2.4.2 was slowly added to sample vials found in the kit. Each vial contained a sulphuric solution of potassium dichromate, with silver sulphate as the catalyst. The vials were homogenized using a vortex, after which they were placed in a thermoreactor for 120 min at 148°C. After the incubation period, the vials were removed from the thermoreactor and allowed to cool to room temperature, thereafter COD was measured by spectrophotometry (DR6000, HACH, America). The resultant measurement appearing on the DR6000 screen was recorded and multiplied by the dilution factor for a final concentration.

3.2.6.5 Nutrient analysis

Nutrients (NO_3-N; NO_2-N; NH_3-N and PO_4) were analyzed by the Gallery™ Discrete Analyzer (Thermo Scientific, Germany). Tests were conducted as single measurements and were validated against a known standard. Analysis within 5% error was accepted.

3.2.6.6 Amylase activity

This method was adapted from methods outlined by Alariya *et al.* (2013). Amylase activity was detected using a starch agar basic medium which was prepared by adding 1 g soluble starch and 28 g nutrient agar into 1000 mL of distilled water and sterilized for 15 min at 121°C. After cooling to approximately 60°C, the agar was aseptically poured into sterile plastic Petri dishes and left to solidify. After solidifying a well was made in the agar using the back end of a sterile pipette tip after which 100 µL of culture of each test isolate, was added to the well in triplicate plates. The plates were then incubated at 32°C for 24 h. After the incubation period, the plates were flooded with Grams of iodine solution for 3 to 5 minutes (Grams of iodine was prepared by adding 2 g potassium iodide and 1 g iodine in 300 mL distilled water). Zones of clearing were measured and an average of the three plates was recorded.

3.2.6.7 Cellulase activity

This method was adapted from methods outlined by Kasana *et al.* (2008). Screening for cellulase-producing isolates was done using nutrient agar plates containing 1 g carboxymethyl cellulose (CMC) and 28 g nutrient agar in 1000 mL of distilled water. The agar was then sterilized, inoculated, and incubated as described above (Section 3.2.7.3). After the incubation period, zones of clearing around each well were measured and an average of the three plates was recorded.

3.2.6.8 3.2.7.5 Lipase activity

Lipase activity was measured using a modified version of the method published by Kouker & Jaeger (1987). The growth medium containing nutrient agar was supplemented with gum Arabic (10 g L^{-1}), Rhodamine B solution (10 g L^{-1}), and olive oil (10 mL L^{-1}). The media was

sterilized in an autoclave at 121°C. for 15 min and cooled to approximately 60°C. Thereafter, calcium chloride ($CaCl_2$) solution was added into the medium, mixed gently then poured into plastic petri dishes and left to solidify. Single wells were made on triplicate agar plates and 100 µL of culture was added into each well for each of the test isolates. The plates were incubated at 32°C for 48 h. The presence of the lipase enzymes was observed by zones of clearing when viewing under UV light. Zones of clearing were an indicator that the lipase enzyme had degraded the olive oil in the media (Haba *et al.*, 2000). Due to the sensitivity of the assay when viewing under UV light, zones are seen within seconds and vanish just as easily therefore results are noted as positive or negative.

3.2.6.9 Protease activity

This method was adapted from methods outlined by Vijayaraghavan & Vincent (2013). To assess the production of proteases 28 g nutrient agar supplemented with casein (10 g) and milk powder (10 g) were mixed in 1000 mL of distilled water. The agar was sterilized, inoculated, and incubated as described above (Section 3.2.7.3). After incubation, the plates were flooded with a 25% trichloroacetic acid (TCA) solution and incubated for 15 min at 45°C. In the presence of the protease enzyme, zones of clearing were observed around the wells, these zones were measured and an average of the three plates was recorded.

3.2.7 **Evaluation of bioremediation potential of selected isolates in real wastewater**

3.2.7.1 Sample collection and pre-treatment

Wastewater samples were collected by random sampling from the same sugarcane processing factory located in KwaZulu-Natal, South Africa as described in Section 3.2.1. Wastewater physical parameters such as pH, electrical conductivity (EC), temperature, and dissolved

oxygen (DO) were measured using a YSI MPS 556 multiparameter system (Yellow Spring Instrument Co.; USA). The suspended solids, alkalinity, and chemical oxygen demand (COD), following the American Public Health Association (Apha, 2007). Large solid particles were removed by sedimentation followed by filtration using Whatman No.1 filter paper. The samples were then autoclaved for 15 min at 121°C for controlled conditions and stored at 4°C until required for analysis. Before cultivation, wastewater samples were supplemented with macronutrient solution I (20 mL) and micronutrient solution II (5 mL) per liter as recommended by Gonzalez et al. (1998).

3.2.7.2 Sample collection and pre-treatment

Wastewater samples were collected by random sampling from the same sugarcane processing factory located in KwaZulu-Natal, South Africa as described in Section 3.2.1. Wastewater physical parameters such as pH, electrical conductivity (EC), temperature, and dissolved oxygen (DO) were measured using a YSI MPS 556 multiparameter system (Yellow Spring Instrument Co.; USA). The suspended solids, alkalinity, and chemical oxygen demand (COD), following the American Public Health Association (Apha, 2007). Large solid particles were removed by sedimentation followed by filtration using Whatman No.1 filter paper. The samples were then autoclaved for 15 min at 121°C for controlled conditions and stored at 4°C until required for analysis. Before cultivation, wastewater samples were supplemented with macronutrient solution I (20 mL) and micronutrient solution II (5 mL) per liter as recommended by Gonzalez *et al.* (1998).

Table 3.2. Characteristics of the sugar industry wastewater used in this study.

Parameter	Oxidation Pond	Inlet (High strength COD WW)
Color	Grey-black	Brown-reddish
Odor	Unpleasant	Worty or stale
pH	6.92 ± 0,28	11.85 ± 0,58
Temp (°C)	29.09 ± 0,66	28.69± 0,86
Conductivity (µS/cm)	3947 ± 861,2	7688 ± 61,2
TDS (g/L)	2.56 ± 0,55	4.99 ± 0,75
Salinity (%)	2.09 ± 0,46	4.27 ± 1,56
COD (mg L^{-1})	7025 ± 45,47	22660 ± 4180
N-NH_3 (mg L^{-1})	0.00 ± 0.00	0.00 ± 0.00
N-NO_2^- (mg L^{-1})	0.00 ± 0.00	0.00 ± 0.00
N-NO_3^- (mg L^{-1})	0.00 ± 0.00	0.00 ± 0.00
P-PO_4^{3-} (mg L^{-1})	2.56 ± 0.50	0.604 ±0.10

Data represents mean ± standard deviation of three independent determinations.

3.2.7.3 Inoculum preparation

Agar plates containing purified selected isolates (B003, B009, B010, and B013) were used to inoculate and prepare an inoculum as described in Section 3.2.6.1. Following incubation and growth at mid to end of the exponential growth phase based on tracking OD 660nm measurements, cell concentration was determined at the endpoint, and standardization of the inoculum to a cell concentration of 1×10^8 cells per mL^{-1} before use.

3.2.7.4 Preparation of bacterial consortium

To enhance bioremediation potential, the bacterial consortium was constructed using the selected performing strains (B003, B009, B010, and B013). The inoculum was prepared by growing each strain on NB (Merck, Darmstadt, Germany), and then after cells were collected by centrifugation (4000 *x g*, 10 min at 4°C). The pellets were resuspended and diluted with 0.85% (w/v) NaCl solution to a cell concentration of 1×10^8 cells per mL^{-1}. The inoculums of each selected strain were mixed in equal proportion to construct the bacterial consortium.

3.2.8 Cultivation of microalgal and bacterial isolates in real wastewater

The experimental setup, analytical data, and measurements were performed as described in Sections 3.2.7.1 and 3.2.7.1. Chlorophyll-*a* content, biomass yields, and productivity were conducted according to methods outlined in Sections 3.2.9.1 and 3.2.9.2 below.

3.2.8.1 Quantification of chlorophyll-a content

For chlorophyll determination, 5 mL of the culture was taken in 24 h intervals in each flask. The chlorophyll-*a* was extracted using 90 % methanol at 50°C in a water bath overnight and centrifuged again for 5 min at 12 000 *x g*. The absorbance of the solution was measured at 665 and 652 nm using a Jenway 7205 UV/Visible Spectrophotometer (Lasec, South Africa), and chlorophyll-*a* concentration was calculated using the following equation (Porra *et al.*, 1989).

$$\text{Chlorophyll-}a\ (\mu g\ mL^{-1}) = 16.29 \times A_{665} - 8.45 \times A_{652} \qquad \text{(Equation 3.3)}$$

3.2.8.2 Biomass yields and biomass productivity

Biomass yield was determined by taking 10 mL of the well-mixed culture and centrifuging at 2000 x *g* at 4°C for 10 min. The supernatant was discarded, and the pellet was washed with

distilled water. The culture was further centrifuged to remove the water, and the pellet was transferred to dried pre-weighed watch glasses and kept in the oven at 70°C overnight. The dry cell weight was determined gravimetrically using an analytical balance. The biomass productivity was determined according to the following equation.

Biomass productivity mg d = (Biomass yield (mg L)/(Number of days) (Equation 3.4)

3.2.9 **Cultivation of yeast isolates in high-strength COD wastewater**

Four yeast strains (Y1, Y2, Y4, and Y5) previously isolated from wastewater samples were evaluated for their growth potential in high-strength COD wastewater (Table 3.2). The yeast strains starter cultures were prepared as described in Section 3.2.6.1 using YPD broth. Each strain was inoculated to an OD 600 nm of 0.05 in a 250 mL Erlenmeyer flask containing 50 mL of wastewater medium and incubated in an orbital shaker (OrbiShake Shaker, Labotec, South Africa) at 25 ± 2°C for 168 h with agitation at 120 rpm. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error. Growth rates and COD removal were determined as per Section 3.2.7 above. Total carbohydrates, total reducing sugars, and specific gravity (SG) were conducted according to methods outlined in Section 3.2.10.1.

3.2.9.1 Total carbohydrates, reducing sugars, and specific gravity

Total carbohydrates were quantified using the phenol-sulfuric acid method. In brief, 0.1 mL of supernatant was diluted to 1 mL and then mixed with 1 mL of phenol (5% w/v) and 5 ml of 96 % H_2SO_4. After cooling to 25 - 30°C, the absorbance of this solution was measured at 490 nm using a spectrophotometer (DR6000, HACH, America). Total carbohydrates were quantified by referring to a calibration curve prepared using glucose as a standard. The residual sugar concentration was analyzed by the DNS (dinitrosalicylic acid) method with glucose as the

standard for the calibration curves. After mixing 0.75 mL of medium with 0.5 mL of DNS reagent, the samples were heated at 100°C for 5 min. The samples were cooled to room temperature, and then 3 mL of water was added. The absorbance of this solution was measured at 540 nm using a spectrophotometer (DR6000, HACH, America). The specific gravity expressed in °P was monitored using a Handheld Brix Gravity Refractometer with automatic temperature compensation (ATC).

3.2.10 **Evaluation of molasses decolourization potential by bacterial and yeast strains**

Crude molasses was diluted to 40 g L^{-1} in distilled water and supplemented with 4.0 g L^{-1} peptone and 4.0 g L^{-1} yeast extract (Seyis and Subasioglu, 2009). The pH was adjusted to 7.0 before sterilization. Freshly prepared inoculum (10%) of selected bacterial and yeast strains was inoculated into 250 mL Erlenmeyer flasks containing 50 mL of sterile molasses medium. The flasks were incubated at 25 °C (Yeast) and 32 °C (Bacteria) for submerged cultivation for 7 d. Thereafter, a volume of 10 mL samples was centrifuged at 6000x *g* for 10 min, filtered through a 0.2 µm syringe filter and the colour intensity was measured at 475 nm using Jenway 7205 UV/Visible Spectrophotometer against the original stock medium. The decolourization yield was calculated according to the following equation:

$$\text{Decolorization (\%)} = (\text{Initial absorbance} - \text{Final absorbance}) / (\text{Final absorbance}) \times 100 \qquad \text{(Equation 3.5)}$$

3.2.11 **Data analysis**

The data were analyzed by one-way ANOVA at a 95% confidence limit ($\alpha = 0.05$). All statistical tests were performed using GraphPad Prism Version 9 for Windows (GraphPad Software, San

Diego, California, USA). $p < 0.05$ denotes a statistically significant difference. The values were expressed as the means ± standard deviation.

3.3 **RESULTS AND DISCUSSION**

3.3.1 **Sampling and isolation of native microalgal, bacterial, and yeast isolates**

Overall, 3 liquid samples (triplicates) were collected from each pond of the treatment points in the sugarcane processing mill located in KwaZulu-Natal as shown in Table 3.3.

Table 3.3. Sample type, source, and location of samples collected.

Sample #	Type of sample	Source
1	Liquid	Oxidation Pond 1
2	Liquid	Oxidation Pond 2
3	Liquid	Oxidation Pond 3

Seven bacterial, 4 yeast, and 2 microalgal strains were isolated from the samples presented in Table 3.3. Presumptively identified and exhibited characteristics listed in Table 3.4. All the microbial isolates were assessed for cell and colony morphology. The gram reaction was assessed for only bacterial strains, in which all bacterial isolates gave a Gram-positive reaction, spore-forming rods as depicted in Table 3.4. Most bacterial isolates exhibited the basic characteristics of *Bacillu*s sp such as being gram-positive, having rods, and the ability to form spores.

Table 3.4. List of pure bacterial, yeast, and microalgal isolates used in the selection study

Isolate	Microscopic Morphology	Colony Morphology	Gram reaction
B001	Short rods appearing in pairs	Circular, small, smooth, cream-white, translucent Flat, and even	+
B003	Medium-sized rods in chains	Circular, medium, smooth, cream-white, opaque, flat, and even	+
B007	Long thick rods in pairs	Punctiform, small, smooth, white, opaque, flat, and even	+
B008	Short rods appear in pairs	Circular, large, smooth, yellow, translucent, umbonate, and even	+
B009	Short rods, forming long chains	Circular, medium, shiny, pink, opaque, raised, and even	+
B010	Thick rods, forming long chains	Irregular, small, rough, pink, opaque, raised, and wavy	+
B013	Thick and short rods	Circular, large, shiny or glistening, cream-white,	+

		translucent, flat, and even	
Y1	Oval cells	Filamentous, large, wrinkled, cream-white or dusty white, opaque, umbonate, and even	ND
Y2	Oval or cylindrical cells	Circular, large, smooth, cream-white, opaque, umbonate, and even	ND
Y4	Round or oval-shaped cells	Circular, large, smooth, cream-white, opaque, flat, and even	ND
Y5	Round or oval budding cells	Circular, medium, shiny or glistening, pink-red, opaque, flat, and even	ND

Note: ND - not determined

After purification and incubation for two weeks visible colonies appeared, and the colonies' purity was examined under the microscope. Purified isolates appeared unicellular, green, and oval with a cylindrical shape, depending on the stage of the cell division cycle. Cells appeared to have smooth topology.

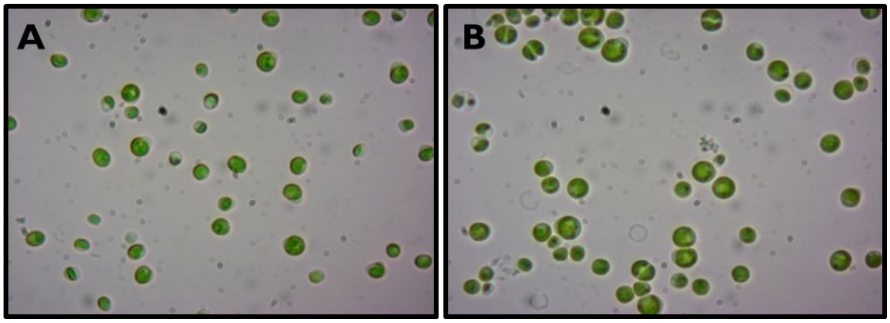

Figure 3.1. Light micrograph showing spherical or round single cells undergoing binary fission (1000x), A) microalgal strain A7, and B) microalgal strain C12.

3.3.2 Screening and selection to determine the bioremediation potential of isolates

3.3.2.1 Growth rates, COD, and nutrient removal by microbial isolates

In the present study, microalgal strains (A7 and C12), and seven bacterial strains (B001, B003, B007, B008, B009, B010, and B013) were isolated from sugar industry wastewater. The growth, and nutrient removal efficiencies of microalgal, and bacterial isolates were evaluated using synthetic wastewater during 96 h (bacterial) and 168 h (microalgal) cultivation period. The specific growth rates, COD and nutrient removal, as well as the production of hydrolytic enzymes by the bacterial strains, were compared between microbial isolates (Table 3.5). The growth curves of microalgal and bacterial isolates grown in synthetic wastewater are represented as optical density over time (Figure A1 and Figure A2, Appendix 2). Rapid growth and utilization of organic nutrients are critical parameters for selecting microbial strains for the wastewater treatment processes. Microalgal and bacterial strains that could grow on TAP medium and nutrient broth (respectively) were considered. However, bacterial isolates that grew on TAP agar plates were considered advantageous for co-cultivation studies with microalgal strains. The selection of ideal microalgal and bacterial strains is essential for the establishment of microbial consortia for the treatment of diverse wastewater streams. Subsequently, all isolated strains were characterized for their potential to treat wastewater in terms of removal efficiency for COD, NH_4^+-N, NO_3^-N, and PO_4^{3-}-P as represented in Table 3.5. The microalgal strains exhibited a high growth rate ranging between 0.33 to 0.37 d^{-1}, which suggests their potential to survive and proliferate in the medium. In addition, the microalgal

strains showed high removal efficiency for NO_3^--N (98–100%), NH_4^+-N (62-65%), and PO_4^{3-}-P (75-80%), however, the removal rate of COD was somehow low ranging between 32–37%. The pollutant removal rates in microalgae-based phytoremediation processes usually vary depending on the type of wastewater, initial concentration of parameters, type of microalgae strain, and retention time. For instance, de Mattos & Bastos (2016) in a Stirred tank batch reactor observed that the *Desmodesmus* sp. strain reduced COD in high-strength sugar vinasse wastewater (27 100 mg L^{-1} COD) by about 36.2% after 30 h of cultivation period in the dark. According to Scherer *et al.* (2017), a consortia of two *Scenedesmus* species reduced 1165.7 mg L^{-1} of COD by about 54% after 10 days of cultivation in cattle manure effluents. Also, approx. a 92% reduction in COD was observed in 96 h using *Coelastrella* sp. strain in piggery effluent (Lee *et al.*, 2021). The initial COD concentrations in previous reports are significantly lower (< 8000 mg L^{-1} COD) than those used in the current study. The reported studies are analogues due to a lack of available literature on the topic of bioremediation of sugar milling wastewater using native microorganisms. Other food processing or agricultural wastewater containing comparatively high COD was used in some instances. Nonetheless, microalgal strains meet requirements in utilizing nitrogen and phosphorus for growth, thus being incorporated into numerous biological processes such as peptides, proteins, enzymes, chlorophylls, energy transfer molecules, and genetic materials (Luo et al., 2016).

All bacterial strains exhibited exceptional removal of 99.24 mg L^{-1} NH_4^+-N with efficiency ranging between 99 to 100%, indicating a strong nitrification capacity as represented in Table 3.5. The ability of bacterial strains to produce a wide range of enzymes like lipase, amylase, cellulase, and protease is an advantageous characteristic and is important for treating wastewater. Small molecules produced by these hydrolytic enzymes are absorbed by other organisms as a source of energy.

Table 3.5. Specific growth rate (μ) and doubling time (k), nutrient removal efficiencies, and enzyme production by microalgal and bacterial strains isolated from the sugarcane processing plant wastewater

Microalgal/bacterial strains	Specific growth rates		COD removal efficiency (%)	NH_4^+-N removal efficiency (%)	NO_3^- -N removal efficiency (%)	PO_4^{3-} -P removal efficiency (%)	Enzyme Intensity (EI)	
	μ, hr/d^{-1}	k, hr/d^{-1}					Proteolytic	Cellulolytic
A7	0.37±0.01	0.53±0.02	32	65.05±1.11	100± 0.13	75.09±0.13	ND	ND
C12	0.33±0.01	0.48±0.02	37	62.55±0.70	98.33±0.22	80.14±1.94	ND	ND
B001	0.15 ±0.01	0.22±0.02	46	99.97±0.118	92.45± 0.89	92.07±0.36	2.22±0.01	2.17±0.07
B003	0.09±0.01	0.13±0.01	65	100 ±0.03	89.54±4.42	61.10±2.400	2.63±0.52	00±0,00
B007	0.11±0,00	0.15±0.00	1	100±0.001	90.70±1.14	19.00±0.07	2.90±0.14	2.17±0.08
B008	0.00±0.01	0.00±0.00	48	99.92±0.05	27.45±6.11	3.68±0.69	00±0.00	2.34±0.09
B009	0.02±0.01	0.03±0.01	51	99.25±0.02	57.75±2.81	75.46±3.85	3.47±0.04	2.23±0.09
B010	0.18±0.02	0.26±0.03	73	100±0.26	9.65±1.89	49.31±1.80	2.21±0.10	2.16±0.00
B013	0.12±0.01	0.17±0.01	45	100±0.15	77.92±5.84	82.13±0.40	2.95±0.70	2.23±0.02

Most bacterial strains showed both proteolytic and cellulolytic activities, except for B003 and B008, which produced only cellulase and protease enzymes, respectively. However, the enzymatic levels did not vary significantly ($p < 0.05$) among tested bacterial strains. Isolate B010 exhibited the highest growth rate of 0.18 ± 0.02 h-1, which was also capable of removing COD (73%) and PO_4^{3-}-P (49.31%) at sufficient amounts. Isolate B001 exhibited the highest NO_3^--N (92.45%) and PO_4^{3-}-P (92.07%) removal efficiencies compared to other isolates. Moreover, the removal efficiencies of COD (45%), $NO3^-$-N (77.92%), and PO_4^{3-}-P (82.13%) by isolate B013 were notable. Despite the low growth rates by isolates B003 and B009, both strains showed significant removal efficiencies for COD (51-65%), NH_4^+-N (57-89%), and NO_3^--N (61-75%). Ghosh *et al.* (2004) isolated and identified bacteria that were able to effectively remove COD 44% (axenic) and 55% (consortia) from molasses spent wash. These microbes play a crucial role in the recycling of nutrients in the soil, thus the biodegradation of organic loads in the wastewater would be greatly aided by indigenous microorganisms. In contrast, isolate B008 showed poor growth rates with an extended lag phase. In the present study, two microalgal and four bacterial strains were selected based on their growth profile with improved pollutant removal efficiency in synthetic wastewater, which often underrepresents the complexity of natural wastewater treatment processes.

Also, most bacterial isolates displayed both proteolytic and cellulolytic activities, except for B003 and B008, which produced either cellulase or protease enzymes. The enzymes have wide degradation potential due to their specific substrate affinity, however, the enzymatic levels did not vary significantly (ANOVA, $p < 0.05$) among tested isolates. Carbohydrates need to be broken down into simpler compounds that can be easily assimilated by the microorganisms (Burgess and

Pletschke, 2008). The production of intracellular and extracellular enzymes is primarily linked to the assimilation of organic matter. Antioxidant enzymes are released to lessen cellular damage due to the oxidative stress induced by high nutrient loads in microbial strains. In addition to breaking down the organic matter in wastewater, bacterial and fungal cells also release enzymes that break down big molecules like proteins, polysaccharides, and other macromolecules into smaller ones for better absorption and use (Alam *et al.*, 2022; Chan *et al.*, 2022). Phosphorus is also removed from wastewater by the enzymes released by the fungal strains, which also break down phosphate precipitates and enhance phosphorus absorption (Zhang *et al.*, 2020).

The observations from the current study complied with previous studies that have demonstrated that *Bacillus* spp. are known to be good enzyme producers and have been widely used to produce enzymes for different applications (Schallmey *et al*., 2004; Fábio *et al*., 2013; Pant *et al*., 2015; Singh and Kumar, 2017). For instance, *Bacillus subtilis* was utilized by Barros *et al*. (2013) to produce lipases, proteases, and amylases, among other enzymes. Their observation also showed cassava wastewater produced higher enzymatic activity in contrast to the synthetic medium (Barros *et al.*, 2013). Pant *et al.* (2015) employed skimmed milk, casein, and gelatine media, respectively, to isolate extracellular protease from *Bacillus subtilis*, yielding activity/clear zones of 15, 17, and 22 mm. Other previous research has demonstrated further uses of *Bacillus* for the synthesis of enzymes of industrial importance (Singh and Kumar, 2017; Cai *et al*., 2019; Caulier *et al*., 2019; Horak *et al*., 2019). These studies have also shown that hydrolytic enzymes may benefit the overall bioremediation process by enhancing the transformation of complex organic substances into simpler, more easily absorbed forms.

3.3.3 **Cultivation of selected microalgal, bacterial, and yeast isolates in real wastewater**

Two microalgal (A7 and C12) and four bacterial strains (B009, B003, B010, B013, and their consortia) were selected from the preliminary screening studies and were then characterized for their growth and COD removal efficiency in real wastewater environment. The growth curves of microalgal strains in high-strength wastewater after 168 hours cultivation period is represented by Figure A3 (Appendix 2). Both A7 and C12 contributed towards the removal of COD (24–30% efficiency) in high-strength wastewater (Figure 3.2). Microalgal strain A7 showed 30.79% COD removal efficiency with chlorophyll-*a* content (26.29 ± 0.55 μg mL^{-1}) and biomass (1.56 ± 0.02 g L^{-1}) as represented in Table 3.6. In the present study, bacterial strains and their consortia contributed predominantly towards removing COD with an 83–88% removal rate after 96 h of cultivation (Figure 3.2).

The growth profiles of axenic bacterial strains and their consortia showed a shorter lag phase suggesting optimal adaptation/acclimatization to the environmental conditions (Figure A4, Appendix 2). Their consortia exhibited a high growth rate of 0.1 ± 0.00 h^{-1} and was also capable of removing COD (87%) under optimal conditions when compared to 0.01 ± 0.01 h^{-1} and COD (19%) in the control experiments using wastewater without nutritional supplementation.

Table 3.6. Specific growth rate, chlorophyll-a concentration, and biomass yield of microalgal strains cultivated in wastewater

Microalgae Strain	Growth rate (d^{-1})	Chlorophyll-a (μg mL^{-1})	Biomass (g L^{-1})	Biomass productivity (mg L^{-1} day^{-1})

A7	0.28 ± 0.00	26.29 ± 0.55	1.56 ± 0.02	22.4 ± 0.29
C12	0.12 ± 0.02	24.41 ± 1.55	0.913 ± 0.00	13.04 ± 0.04

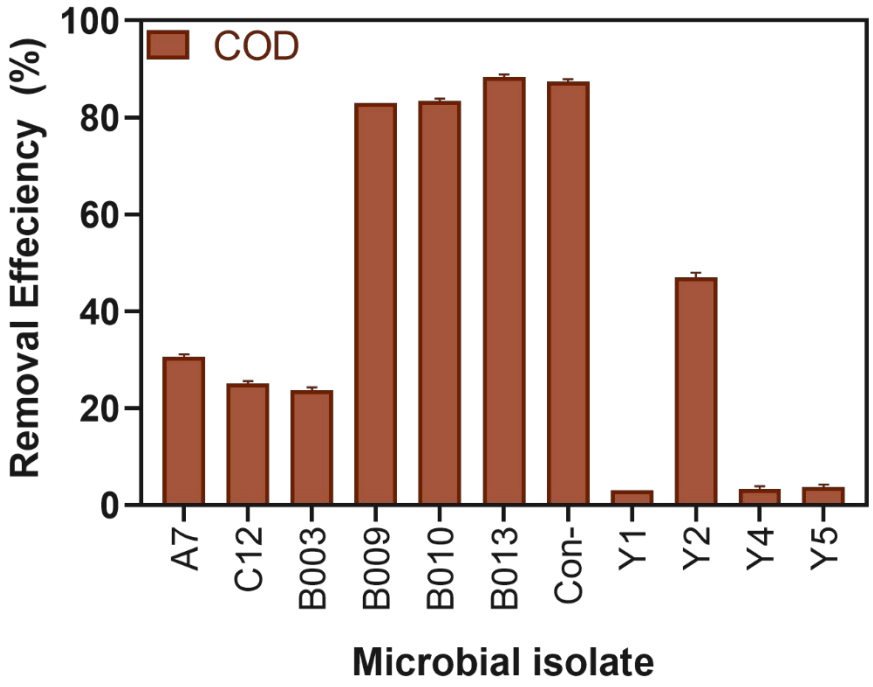

Figure 3.2. COD removal efficiency in high-strength wastewater by indigenous microbial strains.

Isolate B009 had the highest specific growth rate of 0.21 ± 0.01 h^{-1}, followed by B003 with 0.11 ± 0.01 h^{-1}. A similar trend was observed in COD removal efficiencies. Three bacterial strains (B009, B010, and B013) attained COD removal efficiencies between 83 and 88% after 96 h of growth in SIWW. Amongst the selected bacterial strains, B009 exhibited the highest COD removal of 88%, while B003 had the lowest COD removal of 24% after 96 h of cultivation (Figure 3.2). This could be attributed to its growth rate, as B003 had the slowest growth rate when compared to the other three isolates. In addition, isolate B003 did not obtain the highest waste

ion removal rate, and COD removal rate, thus for these reasons, B003 was omitted from the consortia leaving B009, B010, and B013. The result demonstrated the beneficial impact of utilizing bacterial consortia for the biodegradation of organic materials in wastewater. These findings were consistent with previous reports, Saranraj & Stella (2012) showed reductions in BOD, COD, and sulphates in sugar mill effluent using immobilized bacterial consortia. In another study, Mohana *et al.* (2007) showed that bacterial consortium could remove 67% and 51% of colour and COD, respectively, in distillery spent wash. The bacterial consortia exhibited high removal efficiencies of 89.97% and 84.71% for colour and COD respectively in textile wastewater (Lamia *et al.*, 2012). In addition, Lina Liu *et al.* (2021) reported that the bacterial consortia showed high removal efficiency for NH_4, TN, and COD corresponding to 70.06%, 58.12%, and 81.48% in livestock wastewater. This outcome may be a result of the interaction between a wide range of metabolic activities and physiological variables that led to the removal of various pollutants in wastewater, which are not present in monocultures (Mthethwa, 2019).

The previously isolated yeast strains were able to proliferate in pH 11.85 ± 0.58 of high-strength COD effluent when inoculated at an OD600 nm of 0.05. The growth curve of the tested yeast isolates is represented as OD values over time (Figure 3.3). The best-performing yeast strain Y2 was able to reduce COD from 22660 to 11690 mg L^{-1} corresponding to a 48% removal rate after 168 h of cultivation (Figure 3.2). Other yeast strains showed significantly lower ($p < 0.05$) COD removal rates between 3-4% after 168 h of cultivation in sugar industry wastewater.

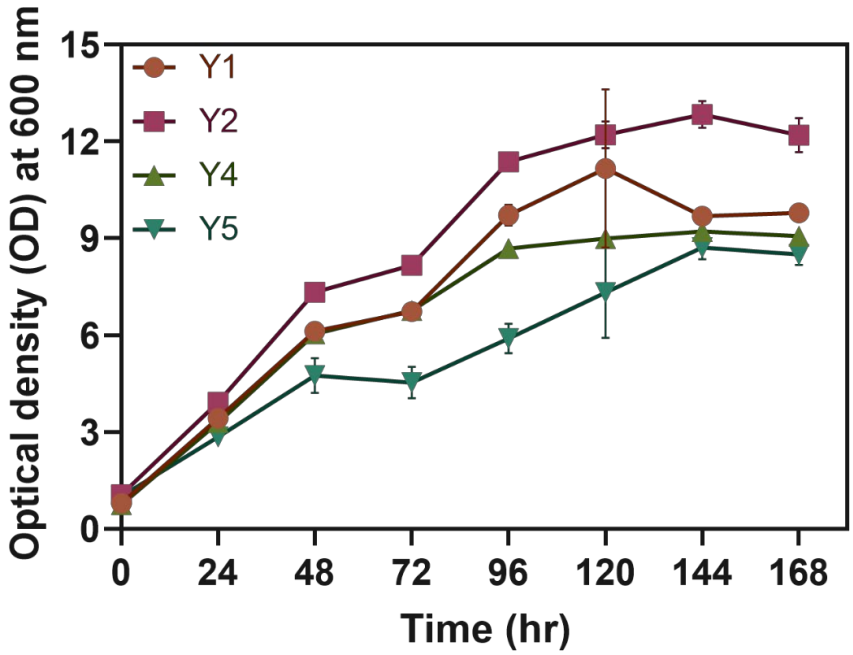

Figure 3.3. Growth of yeast isolates in high strength COD SIWW (pH 11.8) at 25°C. Data represent the mean ± standard error (n=3).

The yeast Y2 could reduce COD more efficiently in high-strength COD wastewater, this was validated by the reduction in total carbohydrates content, reducing sugars, and specific gravity in the wastewater corresponding to 3023.33 ± 428.18 µg mL^{-1}, 1418.28 ± 422,16 µg mL^{-1}, and 1.025 ± 0.00, respectively (Table 3.7).

Table 3.7. Biodegradation potential of native yeast strains in high-strength COD wastewater

Isolate	Total carbohydrates (µg mL^{-1})	Total reducing sugars (µg mL^{-1})	Specific gravity (SG)
Control WW	8172.22 ± 518.30	8063.10 ± 1367.74	1.045 ± 0.00

Y1	8176.67 ± 404.15	2147.01 ± 187.05	1.033 ± 0.00
Y2	3023.33 ± 428.18	1418.28 ± 422.16	1.022 ± 0.00
Y4	8083.33 ± 657.30	3247.01 ± 497.63	1.035 ± 0.00
Y5	7883.33 ± 808.29	8033.22 ± 409.64	1.037 ± 0.00

The biotechnological application of yeast strains in wastewater treatment has been previously reported (Jarboui *et al.*, 2012; Weng *et al.*, 2018), however, little is known about their adaptation in these environments. Both standard and non-traditional yeasts can be utilized to remediate liquid wastes, some non-conventional genera like *Pichia* and *Kluyveromyces*, as well as conventional *Saccharomyces* strains, can remove organic compounds, nutrients, and heavy metals present in the wastewater, are known to be effective in the treatment of wastewater. The current study demonstrated that yeast strains could present a benefit in removing COD and potentially other nutrients in wastewater. Thus, the induction of yeast could enhance COD removal efficiency in wastewater treatment. Zhang *et al.* (2021), reported that the Candida tropicalis strain exerted the best removal capability of COD (90%), nitrogen (89%), and phosphate (82%) for brewery wastewater. Lim *et al.* (2003), showed that yeast strain could reduce the COD of industrial wastewater by 70% in 6.3 h. In another study, Malandra *et al.* (2003) demonstrated that after 120 h of aerated growth, *S. cerevisiae* MEA9 reduced COD by 88% in synthetic wine wastewater (SWW). This is attributed to their extensive carbon metabolic capacities with an aptitude to tolerate environmental changes. This study demonstrated the biodegradation potential of native yeast strains of high-strength COD wastewater. There are limited reports on the bioremediation of sugar industry wastewater using yeast strains. According to our findings, the sugar industry wastewater environment was favorable for yeast strain Y2 explaining the increased growth and

improved COD removal rates suggesting that our strain can use sugar and other organic acids as carbon sources.

3.3.4 **Decolorization potential of selected bacterial and yeast strains**

Individual bacterial strains, their consortia, and yeast isolates were assessed for their ability to degrade natural melanoidins present in molasses medium with an initial COD (mg L^{-1}) of 28 590. In this study, B010 had the highest removal efficiency for colour (25.02 ± 2.66) and COD (24%), which was accompanied by a drop in pH 5.71 (Table 3.8). The bacterial consortia attained a decolorization efficiency of 11.41 ± 2.84%, and the COD removal of 23% was also notable. The decolorization potential and COD removal varied significantly (ANOVA, $p < 0.05$) among the bacterial isolates. None of the bacterial treatments could achieve a final pH above 8 units. The COD removal efficiencies in molasses medium further support that selected bacterial isolates are robust and have a high potential to degrade organic matter present in the wastewater (Table 3.8).

Similarly, Ruhi *et al.* (2017), isolated four bacterial strains from sugar-mill effluents with the potential to degrade synthetic melanoidins. Their consortia yielded maximum COD reduction and decolorization which were around 75% and 60%, respectively. Moreover, bacterial isolate *Bacillus cereus* strain H3 previously isolated from the sugar-mill effluent showed the highest reduction of COD (93%) and removal of colour (60%) (Saha *et al.*, 2018). On another hand, yeast strains showed significantly higher decolorization potential and COD removal when compared to bacterial strains (ANOVA, $p < 0.05$). High decolorization potential and COD removal were observed in yeast strain Y4 with 36.58 ± 2.98% and 64%, respectively.

Table 3.8. Decolourization potential and COD removal efficiency by bacterial and yeast strains cultivated in molasses medium.

Isolate number	Changes in OD 475 nm (%)	Reduction of COD (%)	Final pH
Bacterial cultures:			
B009	6.14 ± 3.04	21	7.22 ± 0.06
B010	25.02 ± 2.66	24	5.71 ± 0.03
B013	11.88 ± 2.47	24	7.49 ± 0.04
Consortium	11.41 ± 2.84	23	7.31 ± 0.07
Yeast cultures			
Y1	29.13 ±5.53	54	7.07 ± 0.01
Y2	31.64 ± 2.83	64	6.32 ± 0.01
Y4	36.58 ± 2.98	61	5.57 ± 0.05
Y5	16.43 ±1.34	65	6.59 ± 0.07

Data represents the mean ± standard deviation of three independent determinations. COD was conducted as single measurements and validated against a known standard.

This was also associated with considerable drops in the final pH to 5.5 (Table 3.8). This was followed by the yeast Y2 with colour removal of 31.64 ± 2.83%. A COD removal efficiency of 64% was noticed for Y2. The yeast strains used in this study were notable for high COD removal (>50%), suggesting that these strains are ideal for the biodegradation of pollutants in wastewater. Due to their rapid growth, yeasts have been explored for the treatment of sugarcane distillery wastewater (Akaki *et al.*, 1981).

Notably, the bacterial isolate B010 and yeast strain Y4 exhibited high decolorization potential complemented by a downshift in the final pH reaching 5.71 and 5.57 units (respectively). Some reports have discovered that melanoidins-degradation tends to increase with alkaline pH, indicating that melanoidins are more soluble in alkaline pH than in acidic pH (Hayase *et al.*, 1984; Mohana *et al.*, 2007; Agarwal *et al.*, 2010). Contrary to these studies, we found more decolourization of molasses medium occurred in acidic pH (Table 3.8). This acidification of the medium could be attributed to ethanol production from the aerobic fermentation of sugars in the wastewater. We have also observed there is a link between decolourization and pH in the case of these two microbial isolates. However, acidification of wastewater is not recommended due to cost implications. The cost of wastewater treatment might increase to adjust the pH to optimal before discharge. In addition, the acidic environment might impede microbial growth, thus affecting the overall performance of the treatment processes.

3.4 **CONCLUSIONS**

In the present study, the sugar industry wastewater rich in COD was treated using indigenous microalgal, bacterial, and/or yeast strains. The isolation of indigenous microorganisms was the first step in the design and development of biological treatment for sugar industry wastewater. During the bioremediation process, nutrition support was used to improve growth and pollutant removal efficiency. Under optimal conditions, axenic bacterial strains and their consortia achieved an exponential phase within 8 h and showed good pollutant removals (COD >85%) after 96 h of cultivation. Enhanced growth, COD removal efficiency (44%), and reduction of total carbohydrates, reducing sugars, and SG were recorded in a yeast-based treatment system. The findings could help improve the performance of the current wastewater treatment plants by introducing low-cost and sustainable biological technologies. Therefore, the present study

demonstrated that wastewater-borne microorganisms are promising bioremediation tools for sugar industry wastewater and showed environmental value in the development of low-energy, small-scale biological treatment systems. Further studies are required to gain more insight into mechanisms of interactions between *Chlorella* sp., and co-cultured microbial strains grown in sugar industry wastewater.

CHAPTER 4: DEVELOPMENT AND EVALUATION OF MICROALGAL-BASED CONSORTIA FOR WASTEWATER TREATMENT

4.1 INTRODUCTION

Microalgal-based wastewater remediation and biorefinery are usually carried out using single-cell cultures (monocultures), which are prone to contamination and are susceptible to environmental changes (Suvarna *et al.*, 2011; Rakesh and Karthikeyan, 2019). Researchers have noted that microalgae exist side-by-side with different microorganisms in the natural environment. The focus has now shifted to co-culture approaches for biofuel production, wastewater remediation, and the generation of high-value-added products (Das *et al.*, 2021). In this system, two or more different microorganisms are cultivated with some contact between them (Pacheco and Segrè, 2019). Co-cultivation systems are designed to improve the natural interaction of partners (growth and survival) and enhance functional/metabolic capabilities leading to the accomplishment of difficult tasks (Brenner *et al.*, 2008; Dolinšek *et al.*, 2016). Generally, the interplay in microalgal-microbe consortia mostly involves substrate exchange, signal transduction, physical contact, and horizontal gene transfer (Kouzuma *et al.*, 2015; You *et al.*, 2021).

This chapter focuses on developing different types of microalgal-based consortia from the pool of selected microalgal, bacterial, and yeast isolates. The designed microbial consortia were evaluated for their bioremediation potential in synthetic wastewater and the best-performing consortia were selected based on growth, COD, and nutrient removal efficiencies.

4.2 MATERIALS AND METHODS

4.2.1.1 Development and evaluation of microalgal-based consortia in synthetic wastewater

The ability of the selected microalgal, bacterial, and yeast strains to co-exist in wastewater, and remove COD and nutrients was assessed using synthetic wastewater. The synthetic wastewater was prepared as per Section 3.2.6.2 of Chapter 3. The initial concentrations of the parameters were; 9630 mg L^{-1} COD, 193 mg L^{-1} Total Nitrogen (TN), and 224 mg L^{-1} Total Phosphorus (TP). The performance of the microalgal-based consortia was evaluated in terms of growth and wastewater treatment efficiency. Four primary, three secondary, and one tertiary combination were considered by pairing one microalgal with one, two, and three bacterial and/or yeast strains (respectively) from the pool of selected isolates from Chapter 3 (as shown in Table 4.1). The selection of suitable combinations was guided by the bottom-up approach which involves consecutive stages of defining the preferred objective function or output i.e. nutrient removal and subsequently the selection of the suitable pairs or matrix (Padmaperuma *et al.*, 2018; Makut *et al.*, 2019).

Table 4.1. Different criteria of primary and secondary combinations for consortia development from the selected microbial strains

Primary combinations	Algal-bacteria/yeast ratio	Microalgae isolate ($\times 10^5$ cell/mL)	Bacterial or yeast isolates ($\times 10^5$ CFU/mL)
MBC-1	1:1	A7	B09
MBC-2	1:1	A7	B010
MBC-3	1:1	A7	B013
MYC	1:1	A7	Y2
Secondary combinations			
MBC-1	1:1	A7	BC-1 (B09 + B010)

MBC-2	1:1	A7	BC-2 B09 + B013)
MBC-3	1:1	A7	BC-3 (B010 + B013)
MBC-4	1:1	A7	BC-4 (B09 + B010 + B013)

BC, Bacterial consortium; MBC, microalgae-bacterial consortium; MYC, microalgae-yeast consortium.

The inoculum was prepared as per section 3.2.6.1 of the previous chapter. The inoculum of each selected strain was mixed in equal proportion to construct different types of consortia. Each consortium was inoculated in a 250 mL Erlenmeyer flask containing 50 mL of wastewater medium and incubated in an orbital shaker (OrbiShake Shaker, Labotec, South Africa) at 25 ± 2°C and light intensity of 120 μmol/m^{-2}/s^{-1} for 7 d with agitation speed of 110 rpm. All the experiments were conducted in triplicate and the data were expressed as mean ± standard deviation. Growth rates and COD removal were determined as per Section 3.2.7 of Chapter 3. Total nitrogen HACH method #10071 (persulfate digestion method) and Total phosphorus HACH method #8190 (acid persulfate digestion) were conducted according to the methods outlined on HACH test kits following the manufacturer's instructions.

4.2.1.2 Cultivation of the selected microalgal-based consortia in real wastewater

The wastewater collection, pre-treatment, analytical data, and measurements were performed as per Section 3.2.8.1 of Chapter 3. The samples were then autoclaved for 15 min at 121°C and supplemented with fertilizer solution comprising (g L^{-1}) 1.5 NH_4NO_3; 0,5 KH_2PO_4; 0,5 K_2HPO_4. The inoculum and consortia preparations were performed as described in Section 4.2.1 above. The experiment was carried out in a 500 mL Erlenmeyer flask with a working volume of 200 mL. The flasks were incubated as described in Section 4.2.1 above. Growth and chlorophyll-*a* content, and COD removal were determined as per Sections 3.2.7, and 3.2.9.1 of Chapter 3.

4.2.1.3 Identification of isolates in the final consortia by 16S rDNA and ITS sequencing

Single colonies of each of the isolates from mono-septic and purified plate cultures were re-streaked on fresh nutrient agar plates and incubated for 24 h at 32 ± 2°C (Bacteria), and 25 ± 2°C (Yeast). The isolates that were part of the final consortia were visually verified as mono-septic cultures and then sent to Inqaba Biotechnical Industries (Pty) LTD, Pretoria, South Africa for sequencing and analysis. At Inqaba, the DNA was extracted using the ZR Fungal/Bacterial DNA kit™ (Zymo Research). The 16S target region was amplified using the primers shown in Table 4.2. PCR products were gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit), and sequenced in the forward and reverse directions on an ABI PRISM™ 3500 xl Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™) were analysed using CLC Main Workbench 7 followed by a BLAST search (NCBI).

Table 4.2. 16S rDNA and ITS Primers sequence primer sequences (Inqaba Biotechnical Industries, Pretoria, South Africa).

Name of primer	Target	Sequence (5' to 3')
27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT
ITS1	Internal Transcribed Spacer Region	TCCGTAGGTGAACCTGCGG
ITS4	Internal Transcribed Spacer Region	TCCTCCGCTTATTGATATGC

4.2.2 **Data analysis**

Data and statistical analysis were performed as per Sections 3.2.11 of Chapter 3.

4.3 RESULTS AND DISCUSSION

4.3.1 Design and development of microalgal-based consortia in synthetic wastewater

Microalgae have the proficiency to establish mutualistic symbiotic relationships in nature with either bacteria or fungi (yeast), with interactions that improve the performance capabilities of microalgae. This approach has been widely used for the biotreatment of different wastewater streams ranging from municipal, industrial, and agro-industries. In the present study, four different primary combinations (MBC-1, MBC-2, MBC-3, and MYC), and four secondary combinations (MBC-1, MBC-2, MBC-3, and MBC-4) were developed from the pool of selected microalgal and bacteria/yeast strains, thus evaluated for their growth and nutrient removal in synthetic wastewater (Table 4.1). For the development of an effective microalgal-based consortium for wastewater treatment, the growth and COD removal of all primary consortia is represented in Figure 2(a) and Figure 2(b). In all primary consortia, the initial optical density was 0.2 at 750 nm, which decreased to 0.1 or less after 24 h of cultivation in synthetic wastewater [Figure 2(a)].

The monocultures (controls) exhibited a similar trend, particularly the microalgal strain showed a prolonged lag phase, thus exhibiting uniform growth over 168 days of cultivation. In contrast, an increase in OD 600 from 0.05 to 1.142 was observed with B009. The growth profile data indicates that there was no significant increase in growth in primary consortia and controls after a cultivation period of 168 h in synthetic wastewater. This might be due to primary consortia cultures being unable to acclimatize quickly to the wastewater environment, thus requiring longer incubation times to overcome the extended lag growth profile or increase in growth for appropriate consortia selection. Lack of adaptability of all primary consortia, the removal

efficiencies for COD, TN, and TP ranged between 4.8-7%, 78-80%, and 84-94%, respectively [Figure 2(b)]. Similar removal efficiencies were also observed in the axenic microalgal strains, and the inclusion of bacteria should have resulted in enhanced COD and nutrient removal. Most axenic microalgal strains do not affect COD removal but exhibit excellent N, and P removal in wastewater (Su *et al.*, 2012).

To improve growth and COD removal, four secondary combinations were developed and characterized in synthetic wastewater as depicted in Figure 3(a) and Figure 3(b). Similarly, a decrease in optical density was observed for all secondary consortia after 48 h as shown in Figure 3(a). However, an increase in OD was observed for axenic microalgae A7 after 24 h of the cultivation period, which thereafter maintained a steady state over the cultivation period. An improved but insignificant increase in COD was observed in all secondary consortia ranging between 19% and 22%, compared to controls and primary consortia Figure 3(b). All secondary pairs showed exceptional removal for N and P, a similar trend was observed in all primary consortia.

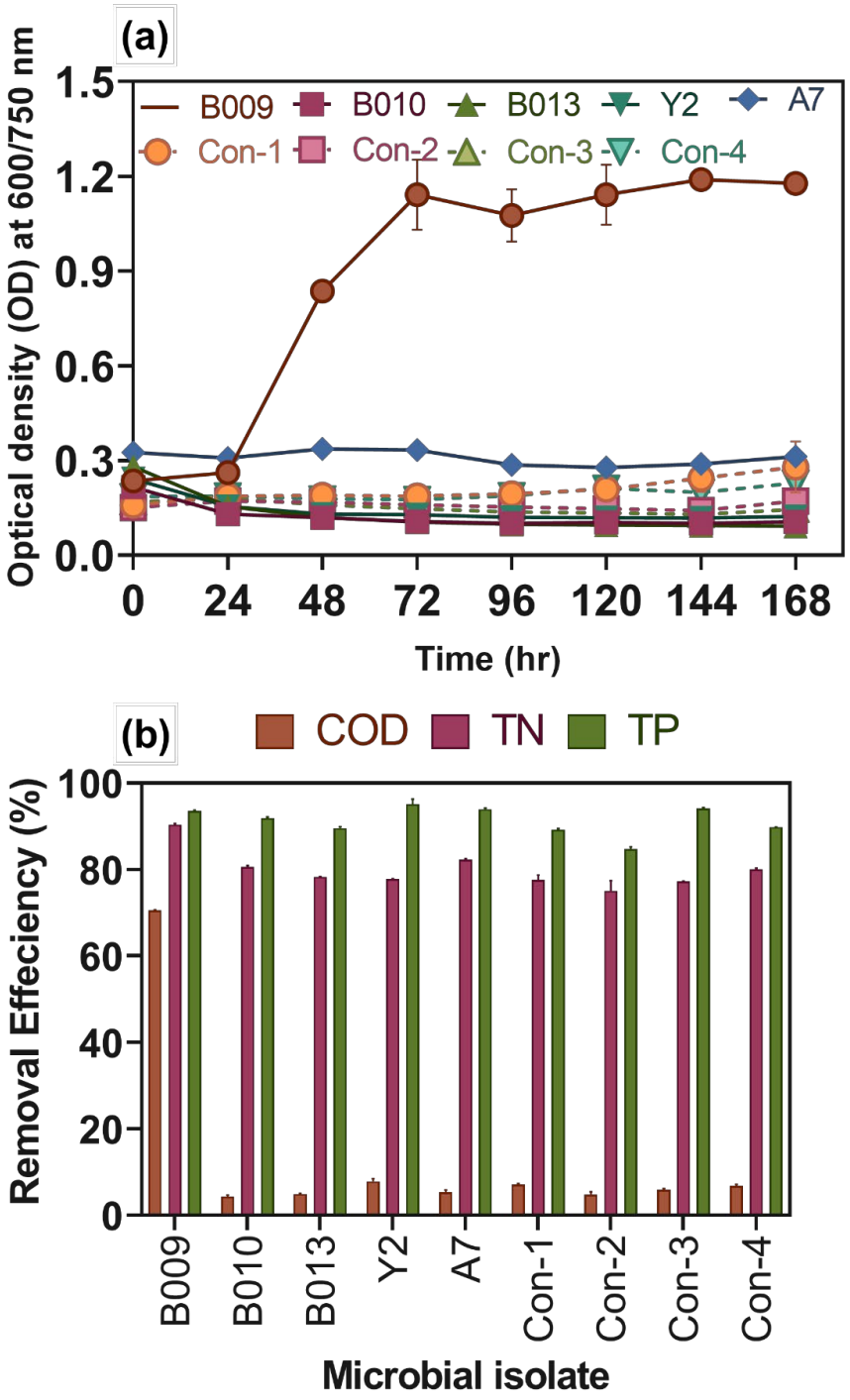

Figure 4.1. Comparative growth (OD 600 nm and 750 nm), (b) COD removal efficiency (%), TN removal efficiency (%), and TP removal efficiency (%) of four primary consortia of microalgae and bacteria and/or yeast cultivated on synthetic wastewater. Data represent the mean ± standard error (n=3).

The slow adaptation of microbial isolates in synthetic wastewater yielded differences in growth and nutrient removal efficiency. The findings of this study suggest that microbial respiration and photosynthesis were inhibited, which in turn affected the growth rates of both bacteria and microalgae (Gupta *et al.*, 2019). Also, the co-cultivation of microorganisms may lead to the excretion of diverse metabolites, or allelochemicals, which have a detrimental effect on the co-cultivated partners (Fergola *et al.*, 2007; Bacellar Mendes and Vermelho, 2013). Abiotic factors such as nutritional deprivation, low temperatures, light intensities, and high pH either promote or inhibit allelochemical production. Several studies have reported improved growth and nutrient removal efficiency in the co-cultivation of microalgae and bacteria or yeast in various synthetic wastewater (Memon *et al.*, 2014; Ji *et al.*, 2018; Russel *et al.*, 2020). Contrary to previous studies, slow development and COD removal rates were observed in this study, the intrinsic characteristics of the effluent could influence the overall performance of intended biological treatment systems.

Therefore, the final MBC was formed with one microalgae strain and all three bacterial strains and was designated as MBC-4 [Figure 3(a)] and [Figure 3(b)]. The MBC-4 showed increased growth with improved COD, TN, and TP removal efficiency observed to increase to 26%, 85% & 73% (respectively) compared to primary and secondary consortia pairs as shown in Figure 3(b). Based on microalgal-consortia development and screening experiments in synthetic wastewater, the MBC (microalgae A7 with three bacterial isolates B009, B010, and B013) and MYC (*microalgae A7* with yeast Y2) systems were considered for further characterization and process development.

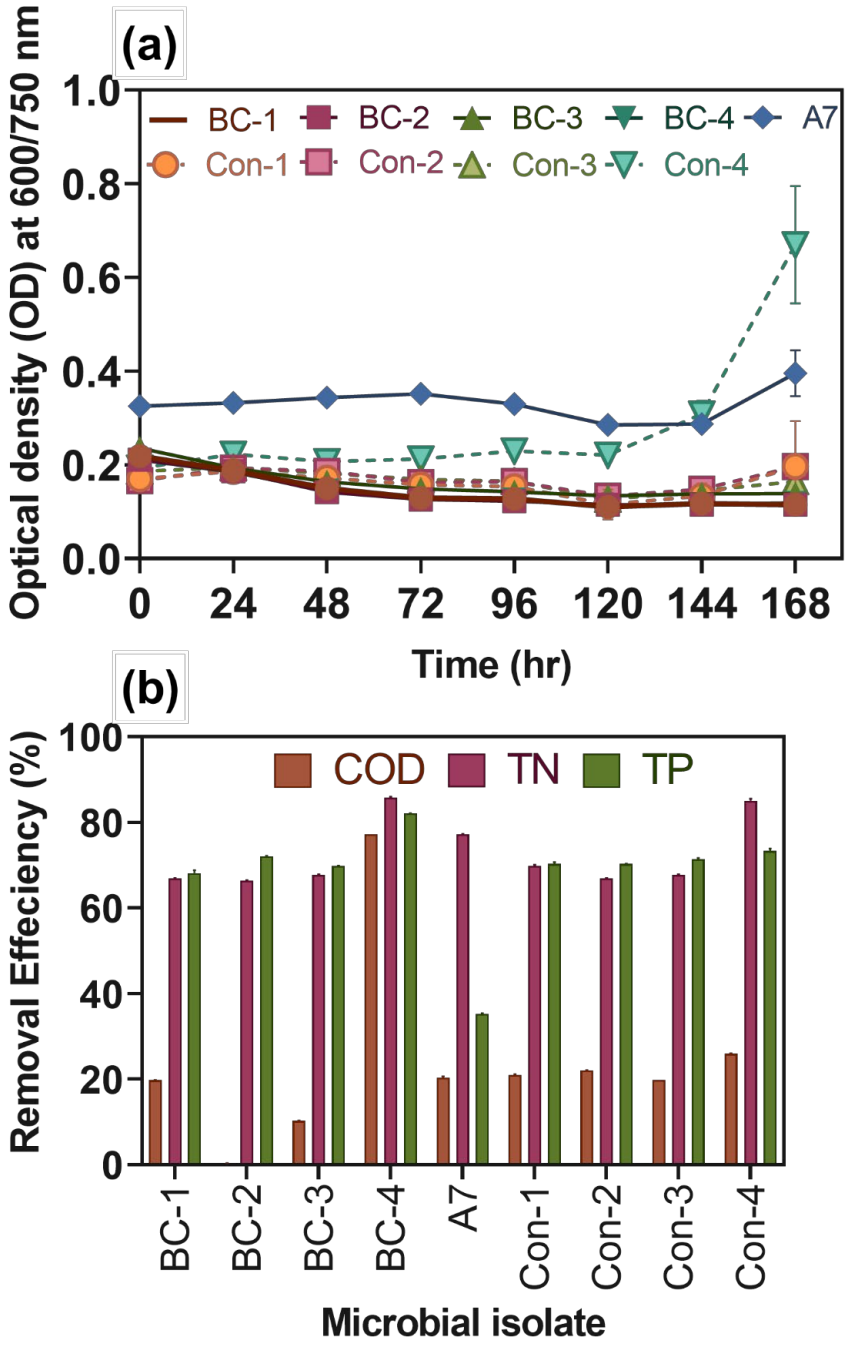

Figure 4.2. (a) Comparative growth (OD 600 nm and 750 nm), (b) COD removal efficiency (%), TN removal efficiency (%), and TP removal efficiency (%) of four secondary consortia of microalgae and bacteria cultivated on synthetic wastewater. Data represent the mean ± standard error (n=3).

4.3.2 **Identification of microalgal, bacterial, and yeast strains by 16S rDNA and ITS sequencing**

Based on the growth and bioremediation potential, the identity of the selected microbial isolates was confirmed using sequencing and sequence analysis. The partial 18S rDNA gene (745 bp in length for A7, 679 bp in length for C12, and 832 bp n length for Y2) sequences of the microalgal and yeast strains were submitted to GenBank (Accession Number: MN788610.1 for A7, ON668126.1 for C12, and MZ373068.1 for Y2). BLAST analysis in the nucleotide database revealed that microalgal strain A7 and C12 share the closest relative to *C. sorokiniana* with a maximum similarity of 97.46% and 95.58%, respectively, whereas Y2 shared the closest relative to *Saccharomyces cerevisiae* with a maximum similarity of 99.40% (Table 4.3).

The biology and industrial applications of *Chlorella* sp. are well documented, particularly in the wastewater treatment process (Liu and Chen, 2016). *Chlorella* genus is a well-known robust, high-biomass-producing green algae taxon and their species can produce phytohormones making *Chlorella* strains popular in various biotechnological industries, including wastewater treatment due to their versatile nature (Liu and Chen, 2016). Among other microorganisms, yeast is one of the most promising in biotechnological applications. Yeasts belonging to the genera *Saccharomyces*, *Candida, Cryptococcus,* and *Rhodotorula* are frequently found in wastewater treatment systems (Yang *et al.*, 2013). High organic load wastewater and lignocellulosic biomass are important substrates for the growth of yeast for the production of products like lipids (Arora *et al.*, 2019; Das *et al.*, 2021; Osman *et al.*, 2022). Yeasts are efficient in treating high-strength organic wastewater from pharmaceutical industries (Gulmez *et al.*, 1998).

The partial 16S rDNA gene sequences (1452 bp in length for B003, 847 bp in length B009, 1445 bp in length B010, and 889 bp in length B013) of the bacterial strains were deposited in the GenBank, and the accession numbers MT393628.1, CP050952.1, MT393628.1, and MT393628.1. BLAST analysis showed that B003, B010, and B013 shared the closest relative to *Bacillus* sp. with a maximum similarity ranging between 99-100%, whereas B009 appeared to be the closest relative to *Rhodococcus* sp with a maximum similarity of 99.76% (Table 4.3).

Table 4.3. Molecular identification of selected microbial strains based on the partial amplification and analysis of the18S and 16S rRNA gene sequences.

Strain code	Accession number	Sequence length (nt)	Microalgal identity	Similarity (%)
B003	MT393628.1	1452	*Bacillus sp.*	100
B009	CP050952.1	847	*Rhodococcus sp*	99.76
B010	MT393628.1	1445	*Bacillus sp.*	100
B013	MT393628.1	889	*Bacillus sp.*	99.68
A7	MN788610.1	745	*Chlorella sorokiniana*	97.46
C12	ON668126.1	679	*Chlorella sorokiniana*	95.58
Y2	MZ373068.1	832	*Saccharomyces cerevisiae*	99.40

Although many microbial strains have been identified for bioremediation of wastewater, *Bacillus* and *Rhodococcus* spp specialize in the breakdown of different nutrients (COD, NH_4^+-N, NO_3^-N, and PO_4^{3-}P) for their growth in the wastewater treatment system. This is due to their fast growth rate, and tolerance to a wide range of physiological conditions that enable them to carry out their roles as water quality modulators. The aliphatic and aromatic hydrocarbons, oxygenated and halogenated chemicals, nitroaromatics, heterocyclic compounds, nitriles, as well as numerous

types of emerging pollutants have all been targets for bioremediation using *Bacillus* sp and *Rhodococcus* sp (Horak *et al.*, 2019; Nazari *et al.*, 2022).

4.3.3 Evaluation of growth and COD removal from real wastewater by MBC and MYC

Several microalgal symbiotic systems with activated sludge have been investigated for their ability to remove pollutants in wastewater. Also, there has been an increasing number of studies conducted on defined microbial consortia, which easily allow control under laboratory conditions. However, there is limited research on microalgal-bacteria/yeast consortia for certain types of effluents, particularly high-strength COD wastewater streams. In the present study, two *Chlorella sorokiniana-based* symbiotic systems were evaluated for their growth, Chl-*a,* and COD removal in sugar industry wastewater after 168 h cultivation period. The growth curves and chlorophyll-*a* content of the two symbiotic systems, along with the control are represented as optical density values over time as shown in Figure 4(a) and Figure 4(b). Both symbiotic systems achieved the highest specific growth and COD removal rates compared to axenic. For instance, the two co-cultures of *C. sorokiniana* A7 with the bacterial consortia and the yeast *S. cerevisiae* Y2 attained the highest specific growth rates of 0.23 d^{-1} & 0.18 d^{-1} respectively, as compared to 0.096 d^{-1} for single microalgae culture. In contrast, a high concentration of chlorophyll-*a* was obtained in MYC (24.31 μg mL^{-1}), followed by the single microalgae culture (21.71 μg mL^{-1}), however, MBC showed lower Chl-a content of 9.49 μg mL^{-1}.

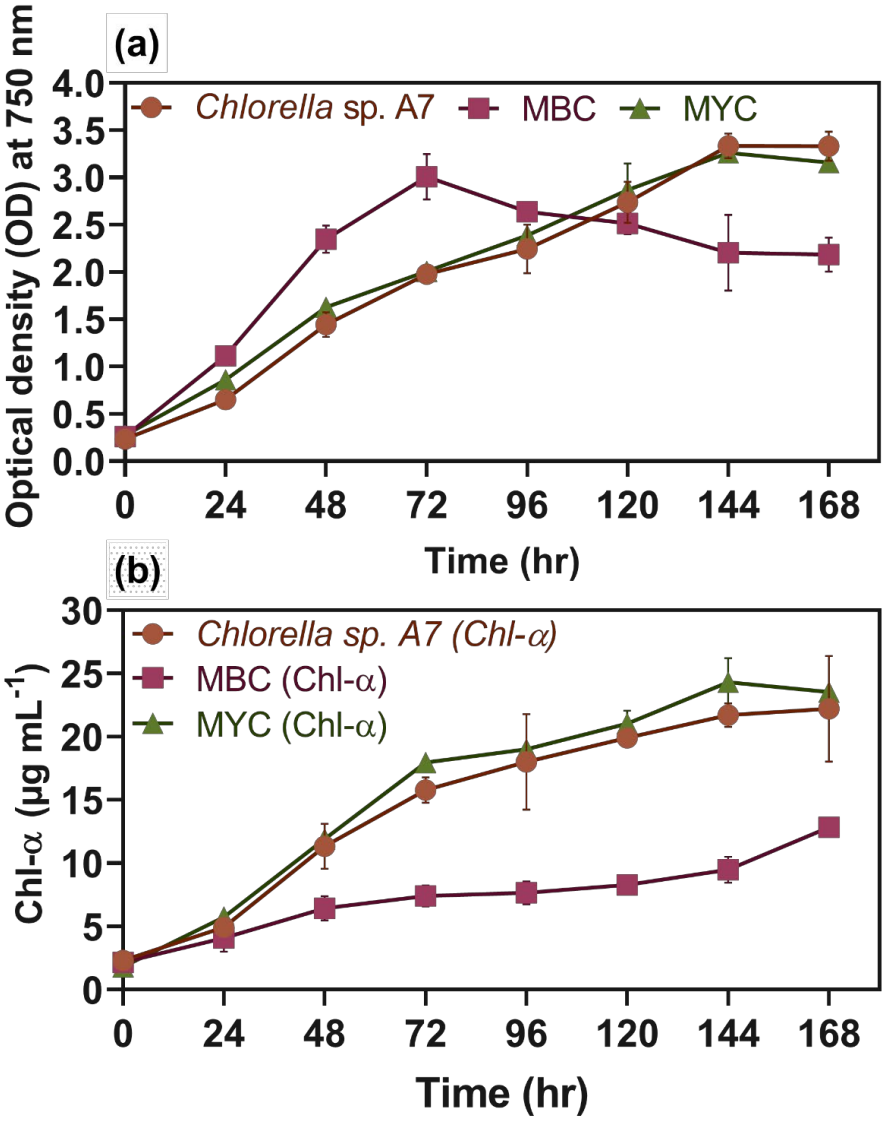

Figure 4.3. (a) The growth profile, (b) chlorophyll-a of Chlorella sp., symbiotic systems (microalgae-bacteria consortia and microalgae-yeast consortia), and axenic Chlorella sp., cultivated in sugar industry wastewater for 168 h. Data represent the mean ± standard error (n=3).

Due to the faster growth rate of the MBC system, there was an improved and rapid removal of COD from the sugar industry wastewater. In the MBC system, the concentration of COD decreased from 9400 mg L^{-1} to 1225 mg L^{-1} (86.97% removal rate) after 96 h of cultivation

(Figure 4.4). COD is removed through the oxidation of organic matter by bacteria, utilizing the oxygen supply provided by microalgae through photosynthesis (Ferreira *et al.*, 2018; Ji *et al.*, 2020). This finding revealed the positive impact of using microalgae-bacterial consortia for the degradation of organic matter in wastewater. The same phenomenon was observed in the MYC system with a 71.12% COD removal rate. After 7 days of the cultivation period, the COD removal rate of both *Chlorella* sp., symbiotic systems had COD removal efficiency ≥ 90% compared to 45% observed in a single microalgae culture.

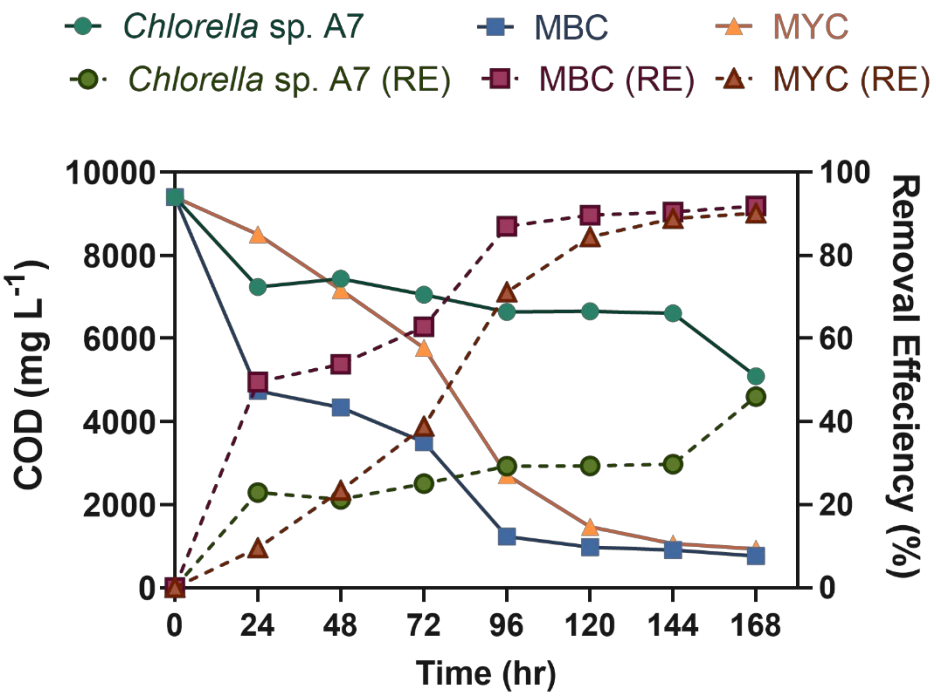

Figure 4.4. COD removal by Chlorella sp., symbiotic systems (microalgae-bacteria consortia and microalgae-yeast consortia), and axenic Chlorella sp., cultivated in sugar industry wastewater for 168 h. The COD of the treated effluent was measured at every 24 h interval. Data represent the mean ± standard error (n=3).

Other researchers have reported comparable COD removal (> 80%) in different wastewaters using microalgae and bacteria consortia (Bankston *et al.*, 2020; Udaiyappan *et al.*, 2020; Ashadullah

et al., 2021; Nagabalaji *et al.*, 2023). For instance, Udaiyappan *et al.*, 2020 reported highest COD removal (> 60%) by *Scenedesmus* sp. UKM9 inoculated at different concentrations in unsterilized palm oil mill effluent (POME). High COD removal in unsterilized POME was attributed to the presence and interactions between *Scenedesmus* sp. UKM9 and indigenous bacteria supported the reductions of COD and nutrients compared to sterilized POME. In another study, Bankston *et al.*, (2020) reported co-culturing of bacteria and microalgae was superior to bacterial monoculture in removing COD in the treatment of poultry litter anaerobic digestate. The results suggest that interactions between *C. sorokiniana* and bacterial populations (heterotrophs, nitrifiers, and phosphate-accumulating bacteria) improved treatment of poultry litter anaerobic digestate. In addition, COD removal effectiveness has been reported to be greater than 80% in microalgae and bacterial consortia in the treatment of synthetic substrate (Ashadullah *et al.*, 2021). The innate ability of microalgae-bacteria consortia to use organic compounds as an energy source and carbon dioxide may be the cause of the rapid decrease in COD content (Abdelfattah *et al.*, 2023). The current study has shown that using microalgae-bacteria consortia rather than pure microalgal cultures for wastewater treatment is undoubtedly more favourable from an application standpoint. The synergistic interaction between microalgae and bacteria offers a variety of metabolic routes and physiological variables for the removal of COD in wastewater, which are not present in monocultures. Also, the microalgal-bacterial consortia system shortened the period of degradation by maintaining process stability and sustaining appropriate performance despite changes in environmental conditions, this is particularly important for outdoor applications.

4.4 **CONCLUSIONS**

In this study, the removal rates of COD were significantly improved by the MBC and MYC systems. The two proposed *Chlorella* sp. symbiotic systems may provide a potentially feasible and efficient biological approach for sugar industry wastewater treatment. Our study further enhances the widely accepted premise of co-culture being more beneficial than monoculture in algal biotechnology. Co-cultivation of microalgae with bacterial and/or yeast strains using a synthetic ecology approach may further enhance the benefits of co-culture and improve efficacy in the treatment of wastewater containing a high organic loading. This could serve as the basis for the development of low-energy, small-scale biological treatment systems with significant environmental benefits. The main trade-offs in microalgae-consortia are clear to interpret because of controlled laboratory conditions. However, the nature of mutualistic interactions in an algal-based consortium remains underexplored. Understanding the interactions in microalgae-based co-cultivation models will give insight into structure and function for further development, scaling up, and implementation in wastewater treatment.

CHAPTER 5: THE INTERACTIONS OF MICROALGAE-BASED CONSORTIA AND THEIR EFFECTS ON COD REMOVAL FROM HIGH-STRENGTH WASTEWATER

5.1 INTRODUCTION

Microalgal consortia including microalgal–bacterial consortia, and microalgal–fungi or yeast can either occur via natural associations or be engineered artificially for a specific use. Microorganisms are grown near one another and rely on each other for a variety of functions, such as nutrients, protection, division of labour, etc. (Boucher, 1985). For instance, heterotrophic bacteria stimulate algal growth by releasing phytohormones, fixed nitrogen, and exogenous sources of thiamine (B1), cobalamin (B12), and biotin (B7) (Croft *et al.*, 2005). Microalgae can also release organic sources, like carbohydrates, which bacteria can use as an energy source. In addition to the direct exchange of nutrients, AHLs (N-acyl-homoserine lactones) and indole-3-acetic acid (IAA), are two distinct chemical signals produced by bacteria (Amin *et al.*, 2015). These chemical signals aid in the formation of biofilms, mediate group behaviours and shape ecological functions between partners. Interactions between microorganisms in consortia are not well understood, and co-cultivation strategies are believed to induce both cooperative and competitive interactions. Autotrophic-heterotrophic interactions in natural microbial communities can be better understood by considering the interactions in microalgae-based consortia. This can also help improve microalgae large-scale cultivation strategies.

Therefore, this chapter focuses on evaluating the interactions in the microalgae-bacteria consortia (MBC) and microalgae-yeast consortia (MYC) systems and their effects on COD removal from the sugar industry wastewater.

5.2 **MATERIALS AND METHODS**

5.2.1 Sample collection, analysis and pre-treatment

Analytical data and measurements were performed as per sections 3.2.8.1 of Chapter 3. The characteristics of wastewater employed in this study are provided in Table 5.1.

Table 5.1. Characteristics of the sugar industry wastewater used in this study

Parameter	Wastewater quality
Colour	Grey-black
Odour	Unpleasant
pH	5.66 ± 0,072
Temp (°C)	25.14 ± 0,04
Conductivity (µS/cm)	5310 ± 117,6
TDS (g/L)	3.45 ± 0,07
Salinity (%)	2.86 ± 0,06
COD (mg L^{-1})	9816 ± 473,84
N-NH_3 (mg L^{-1})	0.00 ± 0.00
N-NO_2^- (mg L^{-1})	0.00 ± 0.00
N-NO_3^- (mg L^{-1})	0.00 ± 0.00
P-PO_4^{3-} (mg L^{-1})	1.50 ± 0.50

5.2.2 **Experimental design and analysis**

Microalgal and yeast/bacterial i***noculum*** was prepared and standardized as described in Section 3.2.4.1. The experiment was carried out in a 500 mL Erlenmeyer flask with a working volume of 200 mL. The flasks were incubated at room temperature (22 ± 2 °C), with an agitator speed of 130 rpm, and light intensity of 120 μmol/m^{-2}/s^{-1} for 7 d. Analytical data and measurements such as growth and chlorophyll-*a* content, biomass production, and COD removal were performed as per sections 3.2.7 and 3.2.9.1 of Chapter 3.

5.2.2.1 Scanning electron microscopy (SEM)

Scanning electron microscopy was conducted to examine the morphological characteristics of microalgal-consortia (cell-to-cell contact, and the presence of a sheath, polymeric substances) and distribution of cells at high magnification. Pre-treatment of the microalgal consortia was completed as previously described (Wu *et al.*, 2010). For SEM analysis microalgal-consortia were harvested on the 7th day and samples were washed 3 times using distilled water to remove impurities. Samples were immersed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4°C for 12 h, thereafter, rinsed twice with sodium phosphate buffer (pH 7.2). This was followed by a series of dehydration steps using 30, 50, 75, 85, 95, and 100% ethanol for 5 min and drying at a critical point for 3 h under a CO_2 environment. The samples were subsequently mounted on stubs (Ø12.7mm) and sputter-coated with gold, using Quorum (Q 150R ES). The coated samples were then examined with a ZEISS LEO 1450 scanning electron microscope operated at 10 or 20 kV and with working distances of 7–9 mm.

5.2.2.2 Extraction of extracellular polymeric substances (EPS)

The extracellular polymeric substances (EPS) were extracted from the biomass after the cultivation period according to the method of Strieth et al. (2020). Briefly, 0.5 g wet biomass was re-suspended in 5 mL of pre-heated (60°C) 0.05% NaCl solution and incubated for 30 minutes at 60°C in an orbital shaker (100 rpm). The suspension was centrifuged at 10000 x g for 15 minutes, and the supernatants were transferred to a new 50 mL tube. The analysis of chemical composition was determined using Bradford assay (PN- proteins), phenol sulfuric acid method (PS- polysaccharides), and Fourier Transform Infrared (FTIR).

5.2.2.3 Carbohydrates analysis in biomass

Total carbohydrates in algae biomass were determined by the sulphuric acid method (DuBois *et al.*, 1956). An aliquot (100 mg) of dry cell biomass was mixed with diluted sulphuric acid (2% v/v) and hydrolysis was carried out by autoclaving for 30 min at 121°C. Afterward, the mixture was neutralized by adding 1M H_2SO_4 until the effervescence ceased. To obtain supernatant, the mixture was subsequently centrifuged at 1509 x g for 10 min. 0.1 ml of supernatant was transferred into the test tube and diluted to 1 ml. Consequently, 1 ml of phenol solution (5% w/v) and 5 ml of 96% H_2SO_4 were added. The mixture was kept at 30°C in a water bath for 30 min, and the absorbance of the solution was measured at 490 nm using a spectrophotometer (Spectroquant Pharo300, Merck). Glucose was used as a standard for the analysis (Kassim *et al.*, 2014). The % carbohydrate yield was calculated according to the following equation.

$$Carbohydrate\ content\ (\%) = \frac{C}{V} \times M \qquad \text{(Equation 5.1)}$$

Where C is the carbohydrate content (mg/mL) obtained from the calibration curve, V is the volume (mL) of the supernatant used for the analysis and M is the total volume (mL) of the microalgal sample solution.

5.2.2.4 Proteins analysis in biomass

Protein estimation from algal biomass was carried out as per (Lowry *et al.*, 1951). Reagents were prepared as per (López *et al.*, 2010). A 25 ml lysis buffer solution per 100 mg of dried biomass was added and ground with mortar and pestle for five mins, and then vortexed for two mins. The supernatant was collected after centrifugation at 3000 x *g* for 10 min. The pellet was resuspended in the same amount of lysis buffer solution, then ground and vortexed. Finally, the supernatant was collected by centrifugation and pooled. Approximately 0.5 ml of SDS solution was mixed with 0.5 ml of pooled supernatant and vortexed. This mixture was then added to 5 ml of reagent-C and vortexed. After 10 min, 0.5 ml of Folin reagent was added and left to rest for 30 min. A spectrophotometer (Spectroquant Pharo300, Merck) was used to measure the absorbance of this mixture at 750 nm. Standards for calibration were prepared by dissolving bovine serum albumin in lysis buffer, and the calibration curve was used for protein quantification and yield was quantified according to Equation (López *et al.*, 2010).

$$\text{Protein yield } (\%) = CVDm \times 100 \qquad \text{(Equation 5.2)}$$

Where C is the protein concentration (mg L^{-1}) obtained from the calibration curve, V is the volume (L) of the lysis buffer used to resuspend the biomass, D is the dilution factor and m is the amount of biomass (mg).

5.2.2.5 Lipids determination in biomass

Total lipids were extracted using a 2:1 (v/v) mixture of chloroform and methanol as per the method of (Folch *et al.*, 1957) with microwave-assisted cell disruption. A total of 20 ml of solvent was added to 1 g of dried biomass and digested in a microwave digester (Milestone S.R.L., Italy; 1200 W of output power) at 1000 W and 100°C for 10 min. The solvent containing extracted lipids was centrifuged and vacuum-filtered. The solvent was evaporated to dryness in an oven at 60°C. The crude microalgal lipid was measured gravimetrically and the lipid yield was calculated according to Equation 6.3 (Talukder *et al.*, 2012).

$$Lipid\ yield\ (\%) = \frac{Extracted\ lipid}{Total\ lipid\ in\ biomass} \times 100 \qquad \text{(Equation 5.3)}$$

5.2.3 **Indole acetic acid (IAA) production assay**

Screening of IAA production by axenic microbial strains was performed using tryptophan (0.15% w/v) added to the nutrient broth (NB) and yeast-extract peptone dextrose (YPD) broth. A colorimetric test was performed with the Salkowaski reagent (Glickmann and Dessaux, 1995). The supernatant (1 ml) was mixed with 2 ml of Salkowaski reagent. IAA production in *Chlorella sorokiniana* A7 co-cultures grown in wastewater, and monocultures were monitored at different times using a spectrophotometer (DR6000, Hach) at 535 nm. The concentration of IAA produced in each supernatant was estimated using the standard curve and expressed in μg mL^{-1}.

5.2.4 **Data analysis**

Data and statistical analysis were performed as per Sections 3.2.11 of Chapter 3.

5.3 **RESULTS AND DISCUSSIONS**

To examine the beneficial interactions of the co-cultured bacterial and yeast strains on microalgal growth, biomass yields, pollutant removal (COD), biomass metabolite formation, and secretion of biomolecules like IAA and EPS, two *Chlorella sorokiniana* A7 co-cultures were proposed and cultivated in sugarcane processing wastewater.

5.3.1 **Structure and morphology of algal-based co-cultures**

Scanning Electron Microscopy (SEM) was performed to visualize and evaluate the interactions (structural or morphological variations, and formation of biofilms) in microalgal co-cultures during the biotreatment of sugar industry wastewater on different days (0, 4, and 7) as shown in Figure 5.1. Visualization of the microalgal-bacterial consortia (MBC) by SEM revealed suspended microalgal cells that could be in contact with rod-shaped bacterial cells. At day 0, most microalgal cells appeared with smooth and intact cell surface morphology, while some cells showed rough/wrinkled cell surfaces.

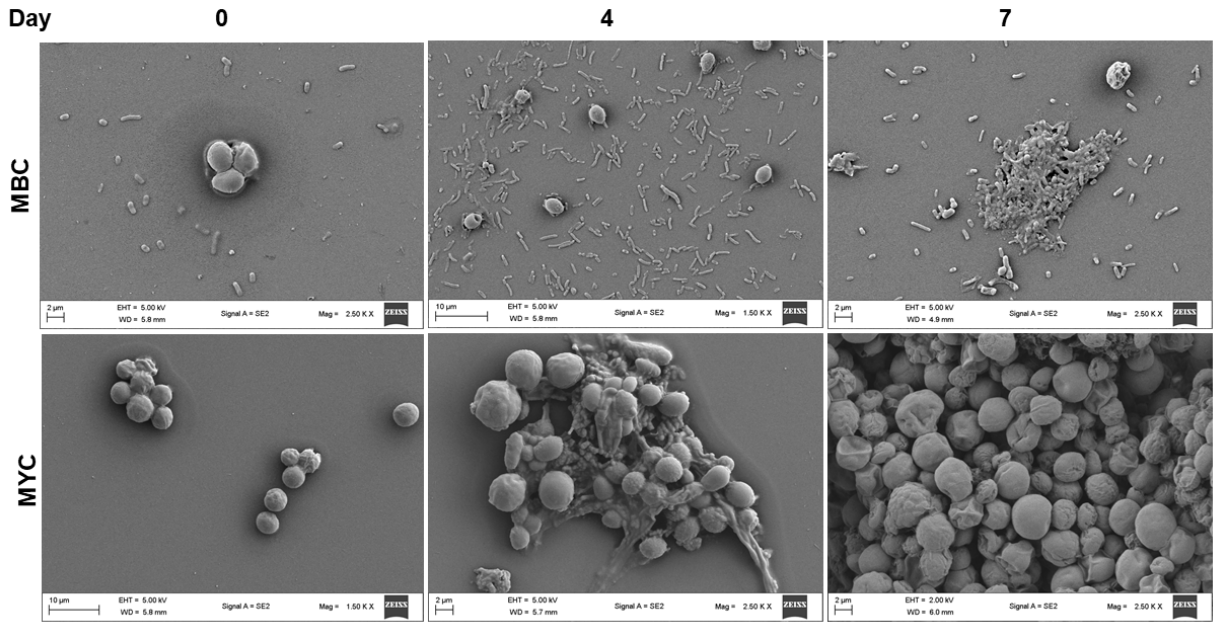

Figure 5.1. SEM analysis of Chlorella sorokiniana A7-based co-cultures. Microalgae-Bacterial Consortium (MBC), and Microalgae-Yeast consortium (MYC) were the applied symbiotic systems. Images were made using FESEM (Zeiss Ultra Plus 5S) on days (0, 4, and 7) of cultivation in sugar industry wastewater.

At day 4, the dominance of bacterial cells was observed with relatively loose adhered to the surface of *Chlorella* sp., cells, resulting in more scattered rod-shaped structures Figure 5.1. The surface topography of algal cells was not affected by the presence of bacterial cells or their contact and adherence. Microalgal aggregates and bacterial biofilm were observed on day 7 as clusters of cells held together by an extracellular polymeric substance that is produced by both members of the consortia. However, the SEM analysis did not show the phenomena of bacterial coating of algal cells (flocs), this might be attributed to interrupted or distorted microbial interactions during the sample preparation stages and time of sampling. The evaluation and visualization of the microalgae-yeast consortia (MYC) showed the dominance of oval-shaped cell structures with wrinkled cell surface morphology, and distinguishing microalgae cells in co-culture with yeast was

difficult as shown in Figure 5.1. At day 4, some yeast cells were distinguished by the presence of bud scars on the surface, which are not observed in the smooth *Chlorella* sp., cells. The EPS in the biofilm interacts with various substances and pollutant particles present in the wastewater environment leading to adsorption or bio-transformation, which could be explained by the presence of rough or wrinkled cell surface topography in some cells within the biofilms at day 7 (Bano *et al.*, 2021). These findings suggested that *Chlorella sorokiniana* A7 cells could co-exist with bacterial and yeast cells under conditions provided by this experiment. The presence of either bacterial or yeast cells did not affect the algal cell surface. Similar cell morphology observations and interactions were reported in the *Chlorella vulgaris-Bacillus licheniformis* symbiotic system, and *Chlorella sorokiniana* UUIND6-*Saccharomyces cerevisiae* UUIND1 consortium (Ji *et al.*, 2018; Bisht *et al.*, 2023). Moreover, *Chlorella sorokiniana A7* did not release extracellular products (antibacterial substances) to regulate attachment or interaction explaining the high density of bacterial and yeast cells at the end of the cultivation period (Zhang *et al.*, 2014; Borowitzka, 2016).

5.3.2 **Analysis of Extracellular Polymer Substances (EPS)**

EPS are cellular substances made up of proteins and polysaccharides; they are produced and discharged in response to physiological phenomena and/or protection in suboptimal conditions (Moreno *et al.*, 1998; Rossi and De Philippis, 2015; Han *et al.*, 2017; Shujuan Meng *et al.*, 2020b). The chemical and functional group composition (structure-function relationships) of the microbial EPS produced by *Chlorella sorokiniana* A7 co-cultures were analyzed using the quantitative calorimetric procedures (with specific standards) and Fourier Transform Infrared Spectroscopy (FTIR) after day 7 of the cultivation as depicted by Figure 5.2(a) – Figure 5.2(b). In this study, the average protein content (PN) produced by the co-cultures, MBC, MYC, and *Chlorella* sp. A7 were 15.61 ± 0.83, 33.37 ± 1.27, and 25.12 ± 2.91 mg/g DCW respectively, suggesting that the proteins

were a major component in the soluble EPS extracts accounting for the majority of the organic matter (COD) [Figure 5.2(a)]. High protein content supports the formation of matured biofilm or granular structure formation with high stabilizing capacity. Several studies have reported elevated protein content in *Chlorella* sp., under antibiotic, pesticide, and surfactant exposure (Chen *et al.*, 2020; Cheng *et al.*, 2020). High protein content might be attributed to increased response mechanisms (protein synthesis and regulatory elements) to overcome specific stress factors (Tang *et al.*, 2021). The polysaccharide contents (PS) were significantly lower ($P < 0.05$) than protein content (PN) in MBC, MYC, and *Chlorella* sp. A7 with an average of 125.5 3.31 ± 0.43, 9.59 ± 0.47, and 6.76 ± 1.07 mg/g DCW respectively [Figure 5.2(a)]. Reports suggest that the polysaccharide content decreases as a result of being used up as a carbon and energy source by co-cultured bacteria or yeast strains (Zhao *et al.*, 2020).

Stress factors influence the amount, composition, and structure of EPS produced by algal-co-culture systems. A higher protein (PN) content ratio to polysaccharide (PS) has been reported by some researchers in microalgal-bacterial systems upon exposure to pollutants such as nanoparticles and antibiotics (Hou *et al.*, 2019; Oberoi *et al.*, 2019). The protein content is crucial for binding pollutants and organic compounds via carboxyl and amino groups.

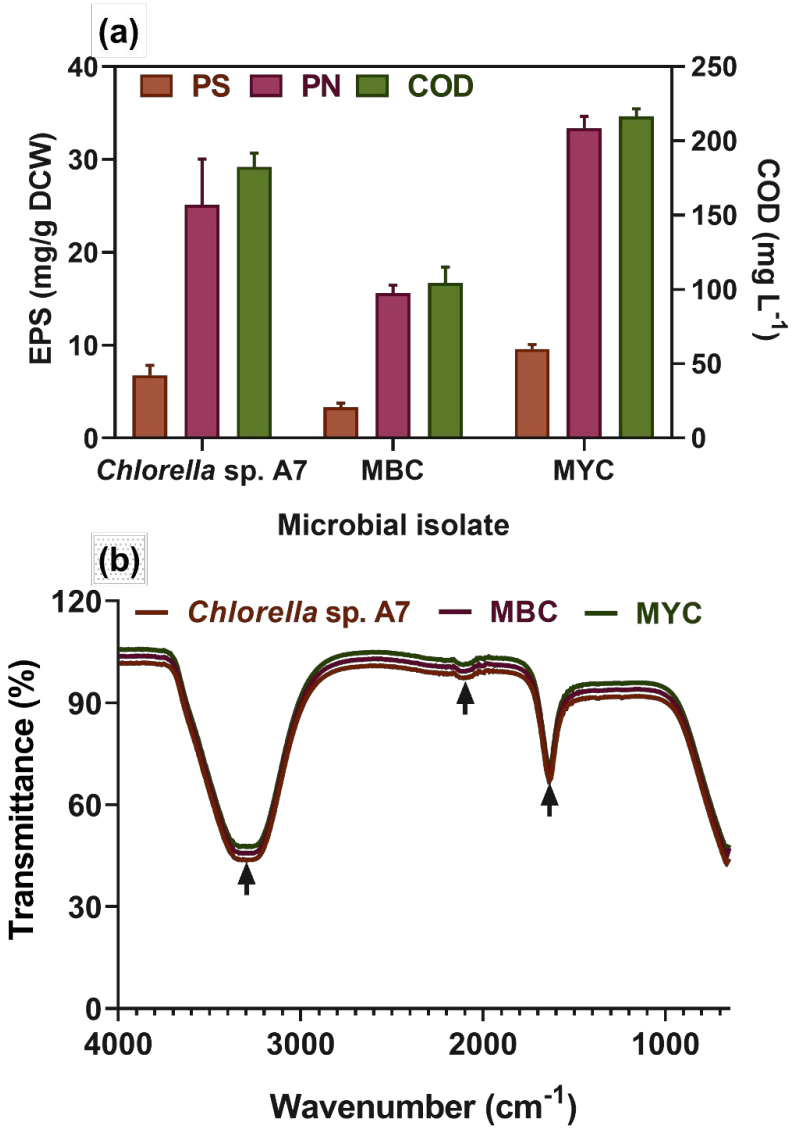

Figure 5.2. Analysis for extracellular protein (PN) and polysaccharides (PS) content (a), and FTIR spectrum (b) of EPS extracts of axenic Chlorella sp. A7 and co-cultures (MBC and MYC) grown in sugar industry wastewater for a cultivation period of 7 days. Data represent the mean ± standard error (n=3).

EPS consists of a plethora of functional groups including carboxyl, amino, hydroxyl, and carbonyl in polysaccharides, proteins, lipids, and nucleic acids (Xiao and Zheng, 2016). In this study, the spectra of the derived EPS extracts from the *Chlorella sorokiniana* A7 co-cultures are depicted in

Figure 5.2(b). A broad-spectrum band between 3332.24 and 2320.41 cm^{-1} indicates the presence of specific alcohols or phenols (inter-molecular O–H stretch) in algal co-cultures and axenic microalgae. Also, specific small stretching peaks were detected at 1938.21 cm^{-1}, 2023.94 cm^{-1}, and 2113.40 cm^{-1}, indicating the presence of carbohydrate and lipid functional groups (=C–H and C–H). In addition, the presence of medium bands ranging from 1636.30 cm^{-1} and 1625 cm^{-1} was identified in all treatments, implying –NH bending vibrations of amide I and the amide II groups from the cell wall structures. The findings suggest the *Chlorella sorokiniana* A7 co-cultures and the axenic monoculture exhibited similar functional groups in the extracted EPS. However, compared to axenic *Chlorella* sp., no changes or differences were observed in the chemical composition of the generated EPS extracts in response to bacterial and yeast co-cultivations. The findings suggest the EPS produced by the axenic *Chlorella* sp., contained similar constituents (functional groups) in the extracted EPS. However, the report suggests that microbial interaction between co-cultured partners improves the variability and heterogeneity of the EPS compared to single monocultures. This provides more binding sites for pollutants to adsorb, thus limiting their bioavailability and toxicity (Tang *et al.*, 2018).

5.3.3 **Growth and COD removal**

5.3.3.1 Growth, chlorophyll-a, and biomass yields

The growth performance of *Chlorella sorokiniana* A7 co-cultures (MBC and MYC), and axenic control(s) in sugar industry wastewater was monitored by optical density (OD) at 750 nm, chlorophyll-*a* content, and biomass production during 7 days cultivation period. The specific growth rates, chlorophyll-*a*, and biomass production were compared between treatment systems. The growth curves are represented as optical density values and chlorophyll-a

concentrations versus time for MBC, MYC, and axenic *Chlorella sorokiniana* A7 as shown in supplementary materials (Figure A6, Appendix 4). Also, the biomass production (yield) by *Chlorella sorokiniana* A7 co-cultures and axenic control was determined and compared after 7 days of cultivation (Table 5.2).

Table 5.2. Specific growth rate, chlorophyll-a concentration, and biomass yield of axenic Chlorella sp. A7 and co-cultures (MBC and MYC) after 7 days of cultivation in the sugar industry wastewater

Treatment	Growth rate (d^{-1})	Chlorophyll-a ($\mu g\ mL^{-1}$)	Biomass ($g\ L^{-1}$)	Biomass productivity ($mg\ L^{-1}\ day^{-1}$)
A7	0.097 ± 0.08	15.08 ± 0.51	0.91 ± 0.03	26.17 ± 0.95
MBC	0.14 ± 0.07	24.74 ± 0.64	1.82 ± 0.07	52.21 ± 2.22
MYC	0.33 ± 0.01	22.81 ± 0.54	2.32 ± 0.13	66.30 ± 3.75

In this study, the MYC system was able to utilize sugar industry wastewater achieving the highest specific growth rates of 0.33 d^{-1} higher, followed by the MBC exhibiting 0.14 d^{-1} in a real sugar wastewater environment (Table 5.2). A significantly lower specific growth rate of 0.08 d^{-1} ($p < 0.05$) was observed in axenic *Chlorella sorokiniana* A7 culture, suggesting that symbiotic co-cultures provide several advantages including high specific growth rates, enhanced biomass productivity, resistance to growth stresses; and better operational stability. The reduced growth in monocultures was characterized by changes in the lag or log phases, thus requiring longer incubation times for optimal performance.

A similar trend was observed in the quantification of chlorophyll-*a* content, high chlorophyll-*a* concentration was obtained in the MYC system reaching 24.74 μg mL^{-1}, and axenic *Chlorella sorokiniana* A7 achieved 22.81 μg mL^{-1} after 7 days (Table 5.2). Significantly lower chlorophyll-*a* concentration of 15.08 ± 0.5 μg mL^{-1} ($p < 0.05$) was obtained in the MBC system compared to axenic *Chlorella* sp. The MYC system also accumulated high biomass of 2.3 ± 0.13 g L^{-1} followed by the MBC system with 1.8 ± 0.07 g L^{-1} at the end of the 7-day cultivation period in sugar industry wastewater. Significantly lower biomass yields of 0.91 ± 0,03 g L^{-1} ($p < 0.05$) were obtained in the axenic *Chlorella sorokiniana* A7 compared to the co-cultures (Table 5.2). Similarly, high biomass productivity of 66.3 ± 3.75 mg L^{-1} day^{-1} was recorded in the MYC, followed by MBC (52.2 ± 2.22 mg L^{-1} day^{-1}) with the lowest value (26.1 ± 0.95 mg L^{-1} day^{-1}) ($p < 0.05$) obtained in the control system (Table 5.2). After 7 days of cultivation period, higher biomass yields and productivity were observed in co-cultures compared to monocultures.

The findings are coherent with several previous studies for both symbiotic systems; the presence of *Pseudomonas aeruginosa* in an algal-bacterial symbiotic system enhanced the weight of the dry cells (Talapatra *et al.*, 2023). Synergistic co-cultivation of microalga *Chlorella vulgaris* and *Rhodotorula glutinis* showed enhanced biomass and lipid concentrations compared to pure cultures (Zhang *et al.*, 2014; Zhang *et al.*, 2017). Several studies have shown that microalgal-yeast symbiotic systems exhibit heterotrophic-autotrophic growth complementarity and are better suited for sugar-containing industrial waste effluents despite limited knowledge of their true potential (Cheirsilp *et al.*, 2011; Papone *et al.*, 2012; Zuccaro *et al.*, 2019). In the current study, *Chlorella sorokiniana* A7 synergistically co-existed with *Saccharomyces cerevisiae* Y2 and both species contributed to the increase in biomass, which would benefit the long-term stable operation of

biological wastewater treatment. The yeast has the metabolic capabilities of breaking down complex carbohydrates into simple sugar molecules that could be utilized by microalgae for its growth. The microalgae absorb organic acids produced by the yeast`s activities and alleviate their detrimental effect on the yeast cells (Yen *et al.*, 2015). According to Xue *et al.* (2010), the photosynthetic activity of microalgae converts CO_2 into bicarbonate that is utilized as substrate for growth resulting in the production of OH-ions in the medium thus establishing an alkaline environment. The important interactions between microalgae and yeast involve the interplay of essential metabolites resulting in the steady state of factors such as CO_2/O_2, pH, and DO in the medium (Arora *et al.*, 2019). The co-culture of microalga *Chlorella* sp. KKUS2 and the oleaginous yeast *Torulaspora maleeae* or *Torulaspora globose* showed high lipid content (96%), when compared to monoculture systems (Papone *et al.*, 2012). Here, the microalgae supplied oxygen to the yeast and the yeast subsequently generated CO_2 for microalgal growth. The maximum lipid production of 920 mg $L^{-1}d^{-1}$ was observed in the co-culture of *C. vulgaris* and *R. glutinis* cultivated in diluted seafood processing effluent (Cheirsilp *et al.*, 2011). The synergistic utilization of CO_2/O_2 by co-cultured species mitigated the accumulation of toxic by-products such as organic acids (Cheirsilp *et al.*, 2011). These interactions can be explained by the metabolic complementarity of ideal co-partners that enables the use of diverse substrates and by-products that would be detrimental to each other, thus creating a stable environment (Xue *et al.*, 2010; Kitcha and Cheirsilp, 2014; Ling *et al.*, 2014).

5.3.3.2 Biomass metabolites

The cultivation of microalgae in wastewater is characterized by the assimilation of nutrients, which are converted into value-added biomass that has diverse biotechnological applications. The

biomass metabolite compositions of *Chlorella sorokiniana* A7 co-cultures and control showed varied content after extractions (Figure 5.3). The lipids, proteins, and carbohydrates yield obtained by *Chlorella sorokiniana* A7 co-cultures after primary extraction of biomass ranged between 6.2-6.8%, 24.9-40.5%, and 10-34% per g DCW respectively (Figure 5.3).

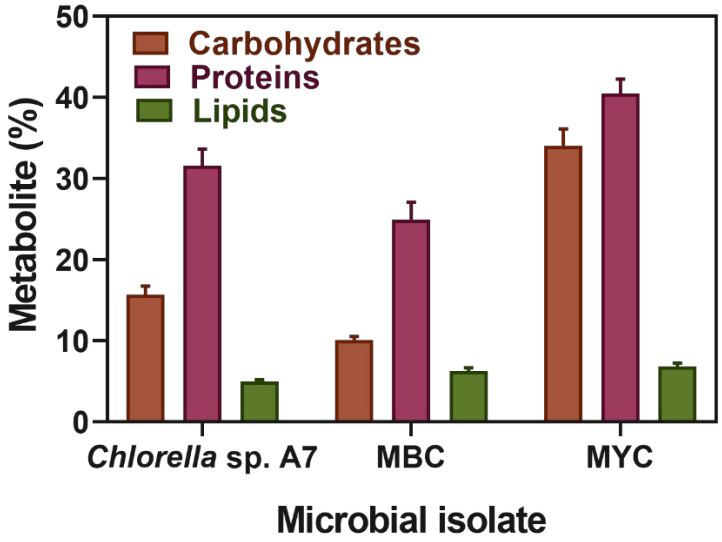

Table 5.3. Biomass metabolites of axenic Chlorella sp. A7 and co-cultures (MBC and MYC) after 7 days of cultivation in the sugar industry wastewater. Data represent the mean ± standard error (n=3).

The axenic *Chlorella* sp., A7 exhibited high yields for proteins (31.6%), and carbohydrates (15.6%) compared to the MBC system. The biomass composition in this study was dominated by proteins and carbohydrates rather than lipids. The lipid percentage in all treatments was lower in comparison to previous reports utilizing microalgae-bacterial consortia and microalgae-yeast consortia in different wastewater streams (de-Bashan *et al.*, 2002; Choix *et al.*, 2012). The varied biomass composition could be attributed to the conversion of nutrient availability from abundance to limitation due to biomass growth modifying the microalgae cell composition (i.e.,

proteins, lipids, and carbohydrates). In this study, the *Chlorella sorokiniana* A7 co-cultures obtained a higher percentage of proteins and carbohydrates than the axenic *Chlorella* sp., A7 when grown in sugar industry wastewater. Several microalgal strains have been documented to store carbohydrates (carbon source) in suboptimal conditions, particularly in nutritional stress, and are subsequently broken down and transformed into lipids (Ran *et al.* 2019). A similar strain-specific phenomenon has been reported for proteins, which may be transformed into lipids in N-limited cultures (Griffiths *et al.*, 2014; Morales-Sánchez *et al.*, 2016). The findings suggest that biomass accumulation and related metabolites vary depending on the type of wastewater (initial concentration of parameters), microalgal strain, and the interaction of several intrinsic and extrinsic factors.

5.3.3.3 COD removal from wastewater

The dynamic COD removal profiles by *Chlorella sorokiniana* A7 co-cultures and axenic controls were evaluated at 24 h intervals over the cultivation period of 7 days in sugar industry wastewater (Figure 5.4). In this study, the MYC system reduced COD concentration from 11120 mg L^{-1} to 975 mg L^{-1} (91% removal rate) after 4 days of cultivation. The COD was removed through the carbon metabolism of the yeast *S. cerevisiae* Y2; thus, the microalgae utilized the carbon dioxide provided by yeast through fermentation.

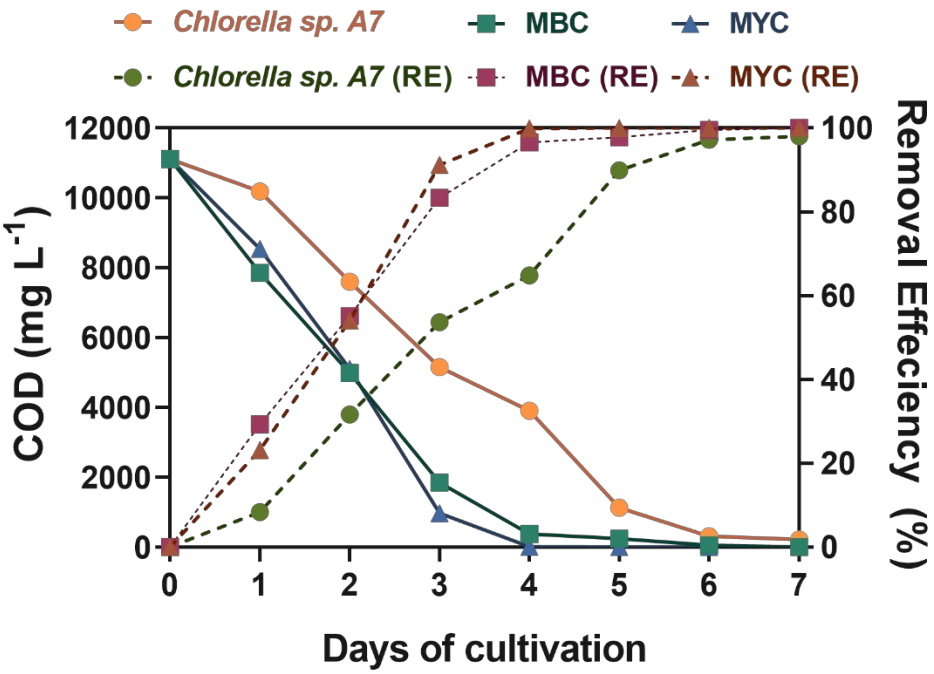

Figure 5.4. COD removal by axenic Chlorella sp., A7, and co-cultures (MBC and MBC) cultivated in sugar industry wastewater for 7 days. The COD of the treated effluent was measured at every 24 h interval. Data represent the mean ± standard error (n=3).

The same phenomenon was observed in the MBC system with an 83.4% COD removal rate. A complete COD removal rate of 100% was observed in the MYC after 4 days of cultivation. The results have demonstrated that co-cultures enhanced COD removal by maintaining process stability and sustaining appropriate performance despite changes in environmental conditions. After 7 days of cultivation period, the COD removal rate of axenic *C. sorokiniana* A7 was close to complete removal. Despite microalgal-yeast co-cultures being tailored for biomass and lipid enhancement, researchers have reported comparable COD removal between 35-95% amongst other nutrients in different wastewater streams (Xue *et al.*, 2010; Cheirsilp *et al.*, 2011; Huang *et al.*, 2011; Cheirsilp *et al.*, 2012; Zhou *et al.*, 2013; Ren *et al.*, 2014; Simpson, 2018). In a study by Cea Barcia *et al.* (2020) attained a COD removal efficiency of 84.7 ± 0.5% using microalgae-yeast

flocs cultivated in tequila vinasse diluted with tequila process water. In another study, *C. sorokiniana* UUIND6 and *Saccharomyces cerevisiae* UUIND1 consortia biofilm exhibited removal of COD (58.88 ± 0.01%), and TP (66.37 ± 0.34%) (Bisht *et al.*, 2023). The co-culture of *C. vulgaris* and *R. sphaerocarpum* enhanced the removal of over 90% of NO_2^-, PO_4^{3-}, and COD (Luo *et al.*, 2021). Also, a mixed culture of *Rhodosporidium toruloides* and *Chlorella pyrenoidosa* showed nutrient and SCOD removal efficiencies of 95.34 ± 0.07% from distillery and domestic mixed wastewater (Ling *et al.*, 2014). High COD causes retarded growth in pure microalgal strains, while yeasts can effectively remove COD ranging from 15 to 50 g L^{-1} (Xue *et al.*, 2010; Peng *et al.*, 2013; Zhou *et al.*, 2013). Thus, the co-culture of microalgae and yeast is a promising bioremediation strategy for reducing high-strength COD and other nutrients in wastewater (Ling *et al.*, 2014). The findings of this study have demonstrated that *Chlorella sorokiniana* A7 co-cultures are beneficial for the biodegradation of high-strength COD wastewater. The findings suggest that the sugar industry wastewater environment was more favorable towards the MYC corresponding to increased growth, and excellent COD removal rates, signifying the metabolic exchange of sugars and other organic acids between *Chlorella sorokiniana* A7 and *Saccharomyces cerevisiae* Y2. In addition, the reciprocation of carbon and nitrogen interaction between *Chlamydomonas reinhardtii* and yeast *Saccharomyces cerevisiae* has been previously reported (Hom and Murray, 2014).

5.3.4 **Mechanisms of interactions - IAA production and communication patterns**

Some plant growth-promoting microorganisms may release phytohormones to establish cross-kingdom signalling mechanisms between algae and bacteria or yeast (Zhou *et al.*, 2016b). Among the most prevalent phytohormones is IAA, and previous studies have demonstrated that IAA could promote the growth and lipid accumulation of microalgae strains (Amin *et al.*, 2015; Palacios *et al.*, 2016a; Palacios *et al.*, 2016b; Chen *et al.*, 2019; Meng *et al.*, 2020). In this study, the ability

of co-cultured strains to produce IAA that may directly affect the growth of microalgae was studied under tryptophan and controls (no addition) [Figure 5.4(a)]. All the tested strains could produce IAA in varying concentrations, three isolates with the highest IAA concentrations 127.4 ± 0.39 µg mL^{-1} (*Bacillus* sp. B013), 48.46 ± 0.22 µg mL^{-1} (*S. cerevisiae* Y2), 23.68 ± 0.32 µg mL-1 (*Bacillus* sp. B010), and 3.97 ± 0.024 µg mL^{-1} (*Rhodococcus* sp. B009) under tryptophan induction, suggesting that these strains may influence auxin levels in the wastewater environments. Without tryptophan, *Bacillus* sp. B013 achieved a significantly high IAA concentration of 29.10 ± 0.86 µg mL^{-1} compared to other tested strains ($p < 0.05$).

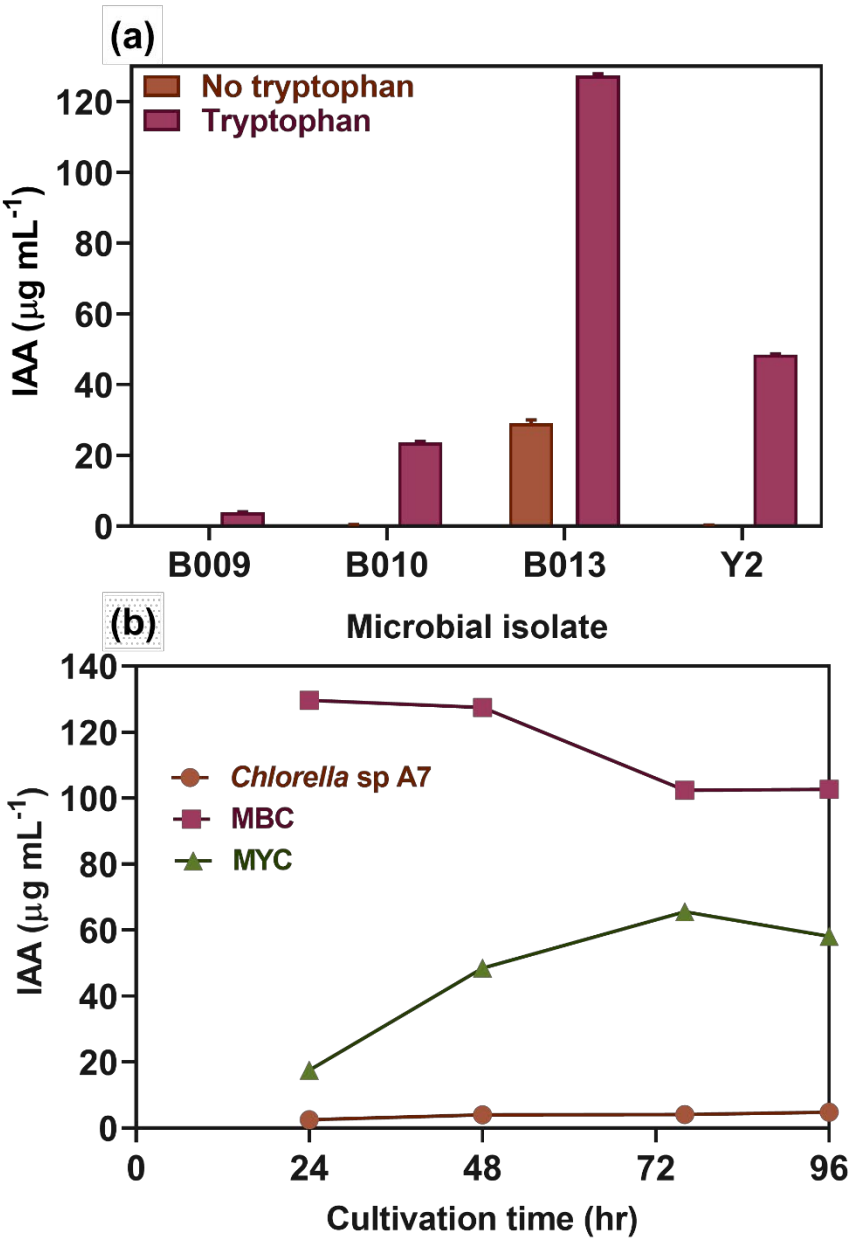

Figure 5.5. (a) IAA production by co-cultured bacterial and yeast strain(s) after 48 h of cultivation in medium supplemented with (0.15% w/v) tryptophan, (b) IAA production by Chlorella sorokiniana A7 co-cultures and axenic control from 24-96 h of cultivation in sugar industry wastewater. Error bars represent the standard deviation

IAA production in *Chlorella sorokiniana* A7 co-cultures and control was quantified after 24 to 96 h of cultivation time [Figure 5.4(b)]. The highest production of IAA was detected in the MBC with 129.5 µg mL^{-1} produced after 24 h of growth, then decreased to 102.62 µg mL^{-1}at 96 h of cultivation in wastewater. In MYC, a steady increase in production from 17.4 µg mL^{-1} to the highest of 65.54 µg mL^{-1}at 96 h was observed. The findings imply that the growth and metabolism of microalgae may be influenced by IAA released by co-cultured strains. Zhang *et al.* (2021) demonstrated that the major bacterial species in the *Ochromonas* and *Chlorella* systems could produce IAA, with *Agrobacterium* and *Rhizobium* with the highest levels of IAA production. Growth promotion in terms of chlorophyll-*a* content and enhanced biomass accumulation was observed in the co-culture system of microalgae and the supernatant of IAA high-yielding bacteria. In another study, 26 microalgae growth-promoting bacteria were identified from *Scenedesmus* sp. LX1 was cultured in domestic wastewater, in which ten strains secreted indole acetic acid (IAA). IAA produced had a stimulatory effect on the growth of microalgae and also reports suggest that microalgae release signals that enhance IAA secretion by the bacterial strains due to alleviated levels of tryptophan in the environment (Dao *et al.*, 2018). *Sulfitobacter* sp. promoted the growth of diatom cells by stimulating the diatoms to synthesize endogenous tryptophan. The tryptophan and IAA biosynthesis-regulating genes of bacteria were shown to be upregulated, thus increasing the quantity of IAA that algae could utilize (Amin *et al.*, 2015).

In addition to the direct transfer of nutrients, signal transduction is another mechanism in which bacteria and microalgae cooperate in co-cultures (Zhang *et al.*, 2020). Reports suggest that bacteria and microalgae can interchange small molecules in the culture medium without contact. Numerous microalgae require specific vitamins as auxotrophs (Croft *et al.*, 2005). For instance,

nitrogen-fixing bacteria could supply microalgae with vitamin B12, which aided the functioning of the methionine synthase in the microalgae (Kazamia *et al.*, 2012). The release of phytohormones by bacteria or algae influences substrate and gaseous exchange within symbiotic systems. Signal exchange is an essential element of synergistic mutualism in which chemically based substances are employed in communication to alter the metabolic processes within cells (Tong *et al.*, 2023). The signalling molecule (IAA) secreted by bacteria influences the growth of microalgae, and in return, the microalgae produce signal molecules (tryptophan) that might stimulate bacteria to release more IAA. This leads to the development of a positive feedback mechanism of signal exchange between the bacteria and microalgae, thus intensifying their relationship. More research is required to fully comprehend the underlying mechanisms of the signal molecules, which could be valuable for microalgal large-scale cultivation.

5.4 **CONCLUSIONS**

In this study, two microalgae-based consortia were selected to construct a feasible and efficient biological approach for the treatment of high-strength wastewater. This study demonstrated that the MYC system achieved better removal efficiencies of COD than the single algae or microalgae-bacteria system did. In the MYC coculture system, the growth of *Chlorella* sp. A7 and *S. cerevisiae* Y2 were both enhanced, indicating the synergistic relationship between the two species, which results in enhanced COD removal. The increased abundance of co-cultured strains in both consortia was the key to improving COD removal. The production of bound EPS and its soluble extracts plays a role in stress mitigation in wastewater treatment. In addition, the production of IAA by co-cultured strains could be proposed as a signalling mechanism, thus the formation of a stable microalgal consortia. These findings will contribute to a better understanding of the interactions in microalgal-consortia for wastewater treatment studies. The results from this study

could be used as a basis for future research focusing on the characterization of microbial interactions based on genetic elements, which was not yet covered by this study and is essential for large-scale microalgal cultivation. The interactions described here illustrate how co-cultured strains influence microalgae physiology and may be linked to natural processes such as algal bloom formation.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The application of microalgal-based consortia has been demonstrated and shown to be an ideal approach for accelerated high-strength COD wastewater treatment. This has great potential to address the cost and energy factors associated with conventional wastewater treatment methods. Therefore, it is desirable to select co-culture partners from the existing natural associations and monitor their performance towards pollutant degradation and their overall influence on the ecosystem. Microorganisms isolated from their natural wastewater stream tend to strive in their natural environment, thus consortia design should be tailored for each target pollutant or wastewater stream. This study focused on developing a microalgal-based co-culture system as a bioremediation tool to reduce COD in high-strength effluents.

The following conclusions can be drawn from this study:

The screening of efficient microalgal, bacterial, and yeast strains is critical factor for maximum nutrient removal from wastewater. Identifying microbial strains that can tolerate extreme biotic and biotic factors is still necessary for process optimisation. Most biological wastewater treatment processes involve several phases, each potentially using different species responsible for wastewater bioremediation. However, the selection of optimum isolates to accomplish high bioremediation performance and produce beneficial enzymes, whilst being environmental safe, still requires further investigations.

The sugarcane industry wastewater is characterized by high chemical oxygen demand and biochemical oxygen demand with low nutrients and other minerals. The treatment of raw sugar

industry wastewater by microalgae presents a critical challenge. The sugar industry wastewater is dark in colour with high turbidity that can hinder light penetration in the wastewater for microalgae growth. Moreover, a high COD ratio to nutrients (nitrogen and phosphates) may pose risks of inhibition and toxicity without pre-treatments. There are various pre-treatment methods like filtration, autoclaving, centrifugation, use of chemical additives to improve microalgae performance in wastewater treatment. The sugar industry wastewater required nitrogen and phosphate supplementation to be a viable medium for microbial cultivation. Supplemented wastewater achieved similar results to conventional medium with increased growth and biomass production which is beneficial due to lower cost.

Most available literature focuses on microalgal-activated sludge co-culture for nutrient removal and biomass production. Symbiosis is a prerequisite for long-term wastewater treatment by microalgal-based co-cultivation strategies. A competitive relationship between microalgae and microorganisms usually leads to low performance in wastewater treatment, thus screening for microorganisms that can form symbiotic relationships with microalgae is a key step in achieving the co-culture. A large percentage of the bacterial population detected in the ponds were predominantly beneficial families involved in mutualistic relationships such as the supply of vitamins and siderophores or were involved in the degradation of organic matter and mineralization of compounds making them more available to algae. It is necessary to investigate the interaction mechanism between microalgae and co-cultured microorganisms for improved application of co-cultures.

Microalgal-based co-cultures were developed and compared to monoculture concerning COD removal potential, growth, biomass, and biomolecule productivity. Developing suitable microalgae-bacterial and microalgae-yeast consortia could show a synergistic effect that enhances

nutrient removal potential, growth, and survivability in varying environmental conditions would be achieved. Nutrient removal from wastewater has been widely studied at laboratory scale. Scale-up of the process still requires further investigation for commercial and economic viability. There are limited studies that have been reported on a sub-pilot scale.

Shifts in the microbial populations in co-cultures, although expected can be controlled to some degree in favour of cultures producing the desired product. The propagation of a specific microalgal co-cultures towards wastewater treatment and biomass productivity is influenced by several factors that are cannot be feasibly controlled. Changes in environmental factors and microalgal population can affect the performance of wastewater treatment. Multi-stage optimization is required for optimization as laboratory and pilot-scale results do not translate well to large scale. Therefore, the stability of microbial communities should be monitored and regulated to improve stable wastewater treatment performance for extended periods.

To promote the use of microalgal-based co-cultivation strategies in wastewater treatment on a commercial scale, optimization of the equipment configuration for consortia proliferations is critical. Factors including the reactor configuration, light source, nutrient supply and harvesting system, are necessary considerations for sustainable and efficient large-scale wastewater treatment. Furthermore, a comprehensive quality monitoring system for microalgae growth status, nutrient content, and interactions to ensure optimal performance in wastewater treatment should be established.

6.2 **RECOMMENDATIONS**

For process development, the designed technologies could be used in addressing the challenges of a wider range of different effluents such as agricultural and industrial wastewater.

The study was operated under controlled environmental conditions, however slight changes in the conditions could alter the behaviour of the consortia thus destabilizing the synergistic interactions and leading to the loss of desired response. Therefore, potential reactor design could be a better tool for monitoring, prediction, and optimization of microalgal-based co-culture systems.

This technology could be upscaled to assess the bioremediation potential of the designed co-culture systems at the sub-pilot level using larger volumes of effluents.

The interactions in the consortia could be further studied using different tools including the multi-omics approach. The generated information (database) could provide a framework in which co-cultures could be further utilized. Metagenomics and meta-transcriptomics provide insights into the composition and potential functions of consortia. Further studies are needed to elucidate the underlying mechanisms driving these interactions at the molecular level.

The wastewater treatment could be coupled with biomass production and valorisation for diverse applications in bioenergy/biofuels, food and feed, and biofertilizer amongst other uses.

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APPENDICES

Appendix 1: Statistical optimization of COD removal by selected microalgal-consortium systems for effective wastewater treatment

Table. A1. Analysis of variance (ANOVA) for the quadratic model of COD removal rate

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value
Model	9238,41	9	1026,49	1,63	0,2285
A-Ammonium nitrate	1583,15	1	1583,15	2,51	0,1439
B-Dipotassium phosphate	1090,5	1	1090,5	1,73	0,2175
C-Bacterial inoculum	1027,77	1	1027,77	1,63	0,2302
AB	119,47	1	119,47	0,1898	0,6724
AC	65,43	1	65,43	0,1039	0,7538
BC	170,59	1	170,59	0,271	0,614
A^2	694,55	1	694,55	1,1	0,3183
B^2	2739,87	1	2739,87	4,35	0,0635
C^2	2632,82	1	2632,82	4,18	0,0681
Residual	6295,74	10	629,57		
Lack of Fit	3674,57	5	734,91	1,4	0,36
Pure Error	2621,17	5	524,23		
Cor Total	15534,15	19			
R^2	0,5947	Adjusted R^2	0,2299	Predicted R^2	-1,0531

Appendix 2: Growth curves of microalgal, bacterial, and yeast strains cultivated in synthetic and real sugar industry wastewater

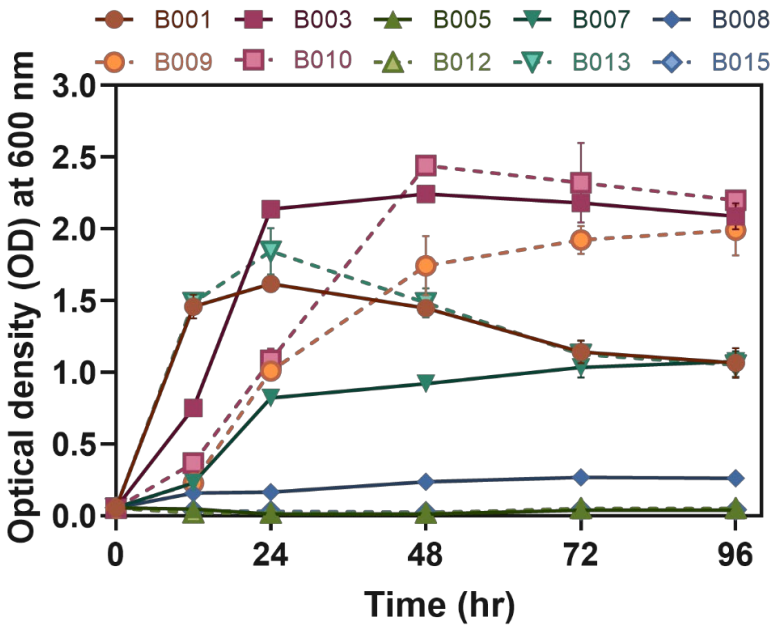

Figure A1. Growth profile of indigenous bacterial isolates expressed as optical density (OD) versus time in synthetic wastewater after 96 h of cultivation period. Data represent the mean ± standard error (n=3).

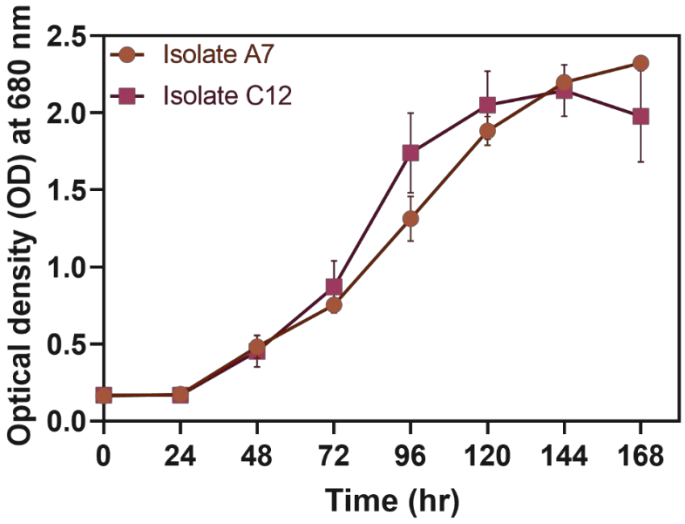

Figure A2. Growth profile of indigenous microalgal isolates expressed as optical density (OD) versus time in synthetic wastewater after 168 h of cultivation period. Data represent the mean ± standard error (n=3).

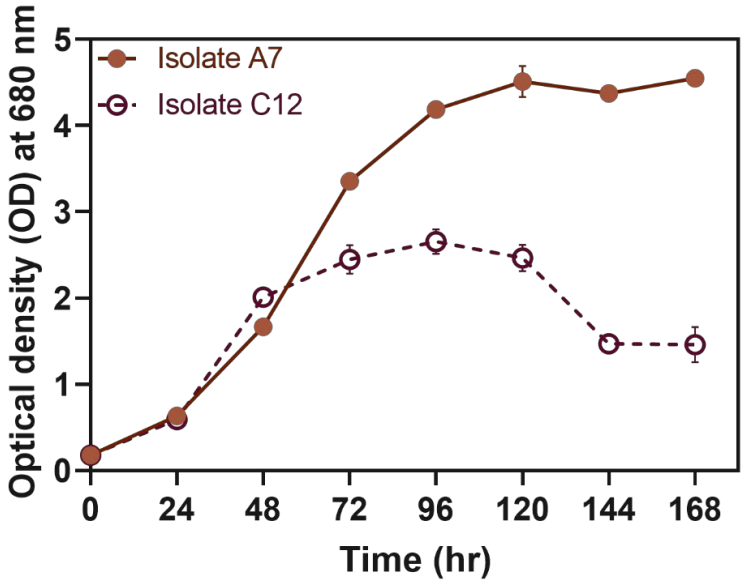

Figure A3. Growth profile of indigenous microalgal isolates expressed as optical density (OD) versus time in real wastewater (supplemented with nutrients) after 168 h of cultivation period. Data represent the mean ± standard error (n=3).

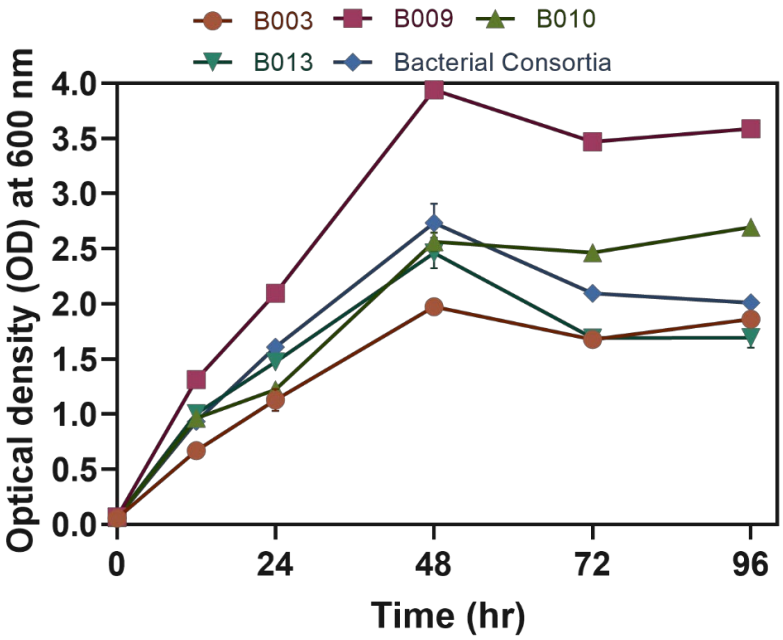

Figure A4. Growth profile of indigenous bacterial isolates expressed as optical density (OD) versus time in real wastewater (supplemented with nutrients) after 96 h of cultivation period. Data represent the mean ± standard error (n=3).

Appendix 3: Morphologies and microscopic analysis of the selected microbial strains

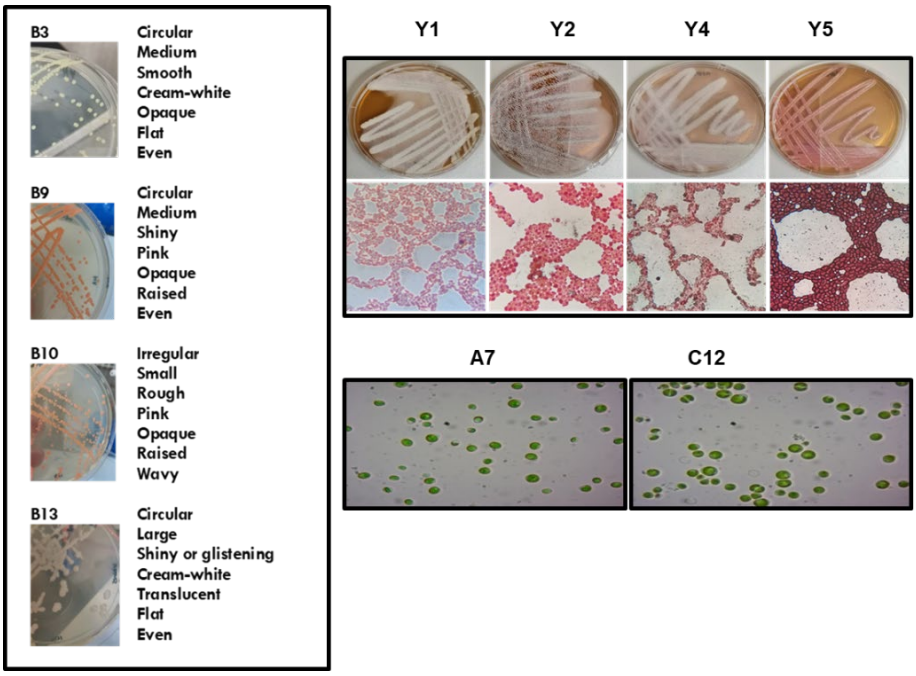

Figure A5. Indigenous microbial isolates from the sugar industry wastewater were used in this study.

Appendix 4: Growth curve of microalgal-based consortia cultivated in sugar industry wastewater

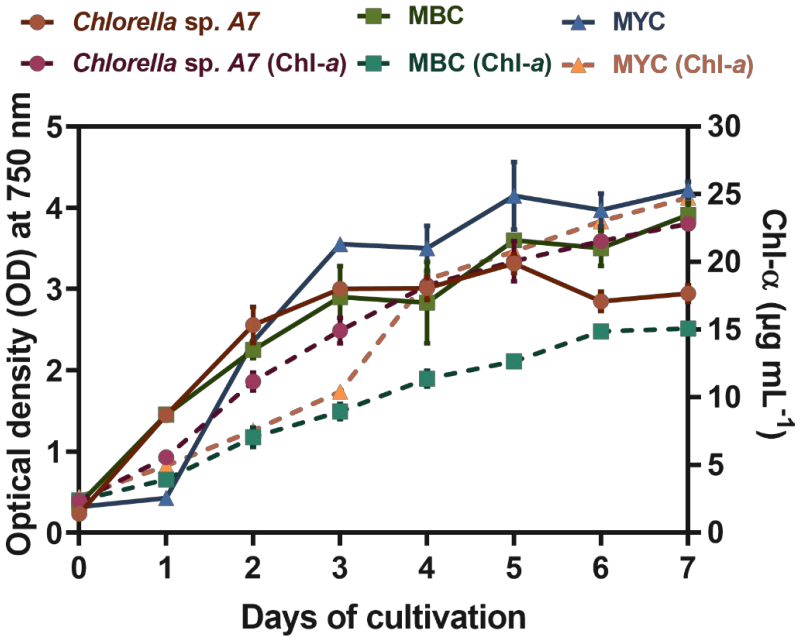

Figure A6. Comparative growth (OD750 nm and Chlorophyll-a) of axenic Chlorella sp., A7 and co-cultures (MBC and MYC) grown in the sugar industry wastewater for 7 days. Data represent the mean ± standard error (n=3).

Appendix 5: Article 1, Published in Algal Research (2024)

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Development and performance of microalgae-based symbiotic systems for high-strength chemical oxygen demand wastewater treatment from the sugar mills

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ABSTRACT

Agricultural and agro-industrial activities have risen exponentially to meet the ever-growing demand for food, energy, and other important resources. High freshwater consumption occurs in these sectors and is discharged as effluent containing excessive organic loads that require treatment. In this study, microalgal, bacterial, and fungal (yeast) isolates native to the sugar industry effluent were screened for effective chemical oxygen demand (COD) removal from wastewater when co-cultured. The microalgae-bacteria consortium (MBC) comprised *Chlorella sorokiniana* A7 and three bacterial strains including *Rhodococcus* sp. B009, *Bacillus* sp. B010, and B013; whilst the microalgae-yeast consortium (MYC) consisted of *Chlorella sorokiniana* A7 and *Saccharomyces cerevisiae* Y2. When the *Chlorella sorokiniana*-based symbiotic systems were characterized in sugar industry wastewater, excellent COD removal efficiencies were achieved compared to the axenic *Chlorella sorokiniana* A7. The COD removal efficiencies were 86 %, and 71 % after 96 h of cultivation for MBC, and MYC, respectively. After 168 h of cultivation in wastewater, ≥90 % of COD removal efficiency was observed in both MBC and MYC systems. The MYC also showed improved chlorophyll-*a* content, photosynthesis, and respiration in *Chlorella sorokiniana* A7. This study has demonstrated the efficiency of *Chlorella sorokiniana*-based consortium systems that could be used as eco-friendly and sustainable bioremediation tools for high-strength COD wastewater streams. An insight into mechanisms of interactions between *Chlorella* sp., and co-cultured microbial strains grown in sugar industry wastewater still needs further studies.

Appendix 6: Article 2, Submitted in Chemosphere (2024)

Simple mechanisms of interactions influencing COD removal by *Chlorella sorokiniana* symbiotic systems from the sugar industry wastewater

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