INTEGRATED WASTE MANAGEMENT FOR SUSTAINABLE LIVESTOCK AND AQUACULTURING SYSTEMS USING MICROALGAE

by

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ABSTRACT

INTEGRATED WASTE MANAGEMENT FOR SUSTAINABLE LIVESTOCK AND AQUACULTURING SYSTEMS USING MICROALGAE

As the world population continues to grow, the demand for high quality food will be on the rise. To meet the demands for safe and sustainable food, urgent agricultural practices are needed utilizing water, energy, land, and nutrient resources more efficiently. In this study, 10 different strains of microalgae from *Chlorella* and *Scenedesmus* genera were screened to test their capabilities to remove the nutrients of agricultural wastewater collected from a dairy farm. The most successful strain was cultivated in a custom designed reusable plastic photobioreactors operated in batch mode and fed with coagulated, filtered and 1/4 diluted farm wastewater. Physical and chemical parameters including total nitrogen, total phosphorus, chemical oxygen demand and total solids of photobioreactor influent and effluent were measured. Removal efficiencies of 59.4%, 64.2% and 95.7% were obtained for total phosphorus, total nitrogen, and chemical oxygen demand parameters, respectively. Biomass concentration of 1.8 g/L was achieved at reactor closure. To demonstrate a circular bioeconomy case by reusing agricultural wastewater nutrients, generated microalgal biomass was further explored as potential feed formulations for livestock and/or aqua-culturing operations by analyzing protein, carbohydrate, lipid, vitamins, amino-acids, and fatty acid profiles using visible spectrophotometry, LC-MS/MS and GC-FID. Overall, the study aimed to optimize general and specific parameters of a low-cost microalgae-based agricultural wastewater treatment application and generate value added bio-feed products for sustainable livestock production and aquaculturing operations.

ÖZET

SÜRDÜRÜLEBİLİR HAYVANCILIK VE SU ÜRÜNLERİ SİSTEMLERİ İÇİN MİKROALGLER İLE ENTEGRE ATIK YÖNETİMİ

Dünya nüfusu artmaya devam ettikçe, yüksek kaliteli gıda talebi giderek artmaktadır. Güvenli ve sürdürülebilir gıda taleplerini karşılamak için su, enerji, toprak ve besin kaynaklarını daha verimli kullanan tarımsal uygulamalarına acil olarak ihtiyaç duyulmaktadır. Bu çalışmada, *Chlorella* ve *Scenedesmus* cinslerinden 10 farklı mikroalg türü, bir süt çiftliğinden toplanan tarımsal atık suda bulunan besinleri (nutrient) giderme potansiyelleri test edilmek üzere tarandı. En başarılı tür, koagüle edilmiş, filtrelenmiş ve 1/4 oranında seyreltilmiş çiftlik atık suyu ile beslenen özel tasarlanmış, yeniden kullanılabilir plastik fotobiyoreaktörlerde büyütüldü. Toplam azot, toplam fosfor, kimyasal oksijen ihtiyacı ve toplam katı olmak üzere fiziksel ve kimyasal parametreler ölçüldü. Toplam fosfor, toplam azot ve kimyasal oksijen ihtiyacı parametreleri için sırasıyla %59,4, %64,2 ve %95,7 oranında giderim elde edildi. Tarımsal atıksu besinlerini yeniden kullanarak döngüsel bir biyoekonomi örneği göstermek üzere, hayvancılık ve su ürünleri-balıkçılık operasyonları için potansiyel yem formülasyonları yaratmak amacıyla elde edilen mikroalgal biyokütlesi içeriğindeki protein, karbonhidrat, yağ analizleri ölçüldü; vitamin, amino asit ve yağ asidi profilleri spektrofotometrik ve kromatografik yöntemler kullanarak belirlendi. Bu çalışma, düşük maliyetli mikroalg tabanlı tarımsal atıksu arıtma uygulamalarının genel ve spesifik parametrelerini optimize etmeyi ve sürdürülebilir hayvancılık üretimi ve su kültürü tesisleri için katma değerli biyo-yem ürünleri üretmeyi amaçlamıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
%	Percent	
λ	Lambda	nm
µ	Micro	
µL	Microliter	
µm	Micrometer	
µg	Microgram	
°C	Degree Celsius	
~	Approximately Equal	
α	Alpha	
β	Beta	
ω	Omega	
C:N	Carbon to Nitrogen Ratio	
C:P	Carbon to Phosphorus Ratio	
N:P	Nitrogen to Phosphorus Ratio	

Abbreviation	Explanation	Unit
BBM	Bold Basal Medium	
BOD	Biological Oxygen Demand	
BSA	Bovine Serum Albumin	
CCAP	Culture Collection of Algae and Protozoa	
COD	Chemical Oxygen Demand	
cPBR	Closed Photobioreactors	
CVS	Calibration Verification Standard	
DIW	Deionized Water	
DTT	Dithiothreitol	
EBR	Erlenmeyer Flask Photobioreactors	
FA	Formic Acid	
FAME	Fatty Acid Methyl Ester	

FAO	Food and Agriculture Organization of UN
FWW	Farm Wastewater
GC	Gas Chromatography
GHG	Greenhouse Gas
LC-MS/MS	Liquid Chromatography-Mass spectrometry
M	Molarity
MB3N	Modified Bold 3N Medium
MBTH	3-methyl-2-benzothiazolinone hydrazine
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MWW	Manure Wastewater
N	Normality
Na_2EDTA	Ethylenediaminetetraacetic Acid
NED	N-(1-naphtyl)-ethylenediamine dihydrochloride
nm	Nanometre
ORP	Open Raceway Pond
PBR	Photobioreactor
rpm	Revolutions Per Minute
SDG	Sustainable Development Goals
SIM	Stable Isotope Mix
sMRM	Scheduled Multiple Reaction Monitoring
TN	Total Nitrogen
TP	Total Phosphorus
TS	Total Solids
UN	United Nations
UPH_2O	Ultrapure Water
US	United States
UTEX	Culture Collection of Algae at the University of Texas at Austin
UV	Ultraviolet
WSP	Waste Stabilization Pond

1. INTRODUCTION

With 7.7 billion estimated people living on Earth, according to the recent "World Population Prospects 2019" report of United Nations (UN), the World population could grow to near 9.7 billion levels by 2050 (UN, 2019). Furthermore, Sustainable Development Goals (SDGs) are challenged by the rapid population growth becoming a burden on finite resources (UN, 2019). Even if the world population stabilizes at some point, the pressure on food supply system will increase due to developing wealth in developing countries (Godfray et al., 2018; Godfray et al., 2010; Sans and Combris, 2015). As a result, the demand for meat, dairy and fish products will increase accordingly (Bajželj et al., 2014). In accordance with these increases in food demand, higher livestock production will lead to competition and environmental risks for natural resources (Alexandratos and Bruinsma, 2012).

Though livestock production and aquaculturing activities support the food demands of billions of people worldwide, they also have significant environmental impacts that cannot be ignored (Capper et al., 2013). Besides competing for valuable resources such as water (both freshwater and marine), and land (both on-shore and off-shore), livestock production and aquaculturing also have adverse effects on air, soil, and water quality due to several pollutants during operational practices (de Vries and de Boer, 2010). According to Food and Agriculture Organization (FAO) of the UN, 14.5 % of the anthropogenic greenhouse gas (GHG) emissions are produced by livestock operations (equaling 9% of anthropogenic carbon dioxide 37% of anthropogenic methane, 65% of anthropogenic nitrous oxide, and 64% of anthropogenic ammonia) (Steinfeld et al., 2006). In addition, 35% of all the global arable land is used for livestock production and agriculture indicating the immensity of ecological footprint (Bajželj et al., 2014). Within the context of livestock operations, carbon dioxide, methane and nitrous oxide emissions could be reduced by integrated waste management approaches, defined as sustainable intensification (Godfray et al., 2010). Similarly, depletion of resources could also be controlled and minimized by sustainable management of land, water resources, and energy (Steinfeld et al., 2006).

Manure waste generated during livestock production is a rich source of nutrients and organic carbon utilized as fertilizer and biogas generation by farmers since the beginning of the 20th century (Burton and Turner, 2003). In recent years, more systematic approaches not only enabled better manure management,

but also more sustainable usage of resources such as water, energy and land (Herrero et al., 2010; Peoples, et al., 1995; Tilman et al., 2002).

Apart from livestock production, aquaculturing activities also compete with feed, water, energy, land, and marine resources as well (Dosdat, 2009). With increasing demand for fish and other aquatic animals in food market, aquaculturing practices get intensified worldwide (Cao et al., 2007). Compared to captured fishery production, aquaculture supplied 7% fish for human food consumption in 1974, 26% in 1994 and 39% in 2004. In 2015, aquaculture contributed to 53% by outrunning capture production (Subasinghe, 2017). Freshwater aquaculturing in artificial or natural ponds generate nutrient dense waste which results in eutrophication in natural water bodies or wastewater in artificial aquaculture ponds. Both cases cause water pollution which leads to negative environmental impacts of aquaculturing in addition to its competition with scarce resources (Cao et al., 2007). Freshwater aquaculturing practices accounted for over 60% of global aquaculture production in 2014, implying that its water consumption competes with agriculture, livestock production, urbanization, and overall natural protection (Subasinghe, 2017). Although some promising technologies for aquaculturing are present such as closed water systems and recirculated water systems, their applicability is limited to industrial facilities (Subasinghe, 2017). As for the feed competition, external aquaculture feed production grows along with increased aquaculturing practices which reflects that sustainable feed production should be of immediate concern (Subasinghe, 2017).

The ultimate goal of this study was to assist sustainable farms by offering low-cost biological management options for the management of livestock production waste by recycling valuable resources such as water and manure nutrients into value-added products: bioenergy and animal feed. For the preliminary work, manure collected from a veterinary school cattle lot was homogenized with distilled water and pretreated by separating its solid components to form a cultivation medium for microalgae species to be tested. Six out of 10 microalgae species were selected for further trials based on their growth performances when cultivated in manure wastewater. These pre-selected microalgae species were then cultivated in coagulated, filtered, and diluted farm wastewater, which was collected from a dairy farm located in Silivri, Turkey, with a capacity of nearly 200 dairy cows including their calves. Following a second elimination of species, one of the best performing species with respect to growth and nutrient removal was cultivated in reusable plastic photobioreactors (PBRs) that were custom designed to mimic floating over a pond near livestock production farms (Figure 1.1). Harvested microalgal biomass was

analyzed for its nutritional profile to be utilized as feed formulations for livestock production and/or aquaculturing practices.

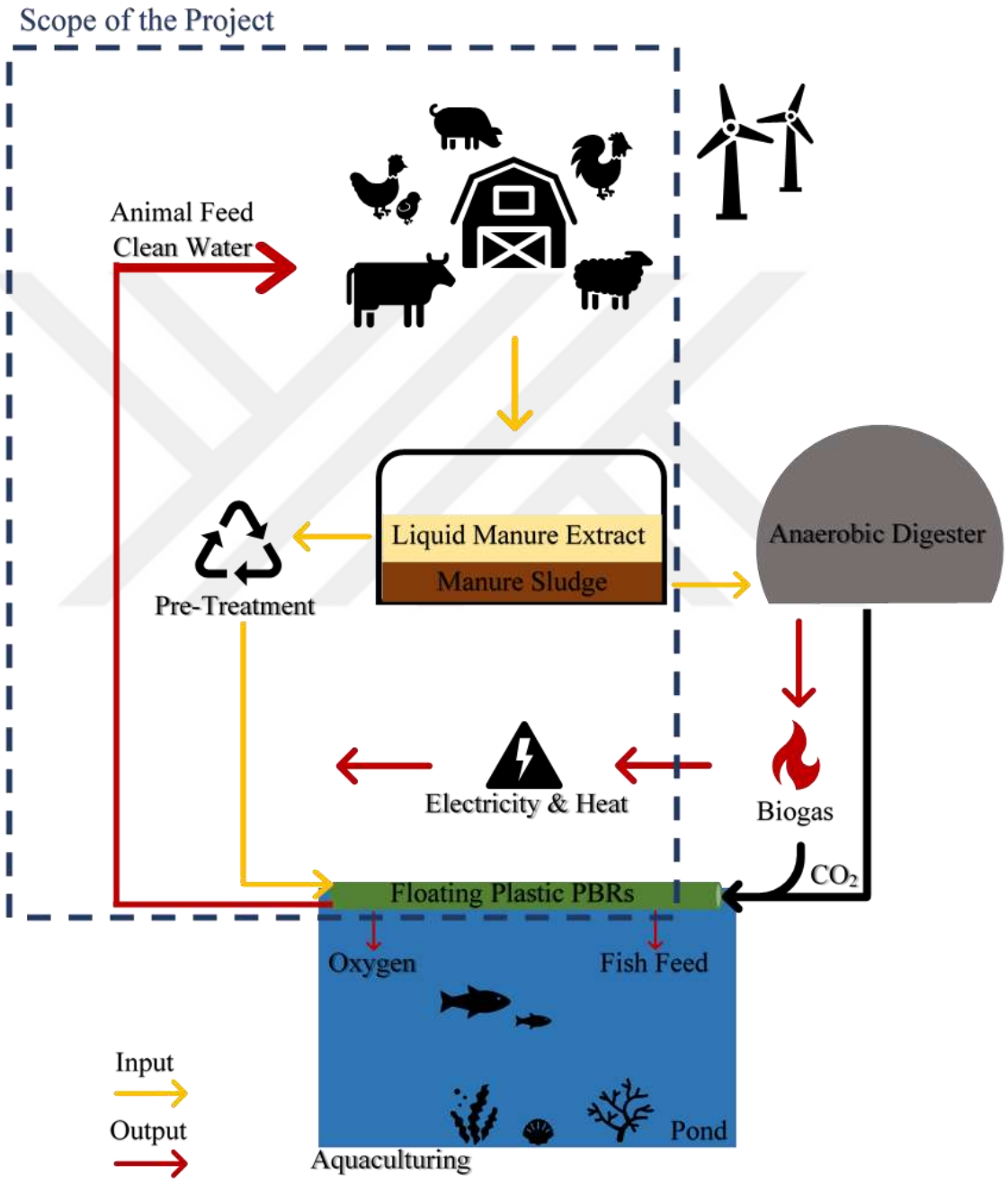


Figure 1.1. Scheme of integrated waste management project for livestock production facilities.

In this integrated waste management scheme, at pilot scale (Figure 1.1), solid component of the farm wastewater is digested by anaerobic microorganisms in an anaerobic digester and generated methane is used as biogas, as an energy source to produce electricity and heat, or to pasteurize possible pathogens

present in manure. Carbon dioxide collected from the anaerobic digester is diverted into the plastic PBRs to enhance the growth of the microalgae. Treated wastewater is redirected into the farm where it could be repurposed as irrigation water. Wastewater treatment with microalgae and generated biomass valorization for feed applications is the scope of this thesis and covers a significant portion of the integrated waste management scheme for livestock production mentioned above (Figure 1.1).

In this grand waste management scheme, suggested treatment system not only creates sustainable farming practices by recycling resources but also decreases the GHGs generated during the livestock production. Methane present in biogas which has approximately 23 times more global warming potential than carbon dioxide, can be utilized in the form of electricity for the farm and/or heat pretreatment for manure as well as pasteurization of digestate sludge. In addition, methane converted into carbon dioxide can be fed into floating reactors to be sequestered by the algae growing with manure nutrients. At the end of each treatment cycle, generated algae biomass can be converted into value-added bioproducts such as livestock and/or aquaculturing feed. By creating sustainable animal and aquaculture farming systems, it is possible to improve the overall efficiency which will eventually lead to better economics for the farmers as well as lower environmental impacts. As a result, a sustainable farming approach for livestock and aquaculture to cope with both future demand for food and lessen the environmental impact of these two sectors will be achieved.

2. LITERATURE REVIEW

2.1. Impact of Livestock Production and Aquaculturing on Environment

Human diet increasingly includes animal-based food, while livestock production has more significant footprints on environment, such as land degradation, water pollution and biodiversity loss, as well as instabilities in the climate due to carbon emissions (Frenette et al., 2017; Sans and Combris, 2015). With tremendously increasing human population, growth in income and increased urbanization, the demand for livestock production is on the rise (Sans and Combris, 2015). Although high meat consumption rates per capita in developed countries-initiated pressures on environmental systems, the increasing trend of shifting from traditional diet to more pre-processed and meat-based eating diet in developing countries may add extra pressure due to higher demand on intensive livestock production. (Vranken et al., 2014). Livestock production contributes to 17% of calorie consumption and 33% of protein consumption (Thornton, 2010). Although the technological advances in breeding, nutrition and health, the potential of livestock production are enhanced, their negative impacts on the environment are not zeroed (Herrero et al., 2010).

The ever-increasing growth of the livestock production causes extensive forest clearings in the Amazon Basin, South Asia and West and Central Africa alongside with biodiversity loss, soil erosion, and increased carbon emissions (Ilea, 2009). Moreover, the increased livestock production can also lead to interference and competition with crops, relation to land sharing and to agro-ecological impacts. It was estimated that 14.5% of all anthropogenic carbon dioxide equivalent emissions is caused by livestock supply-chain sector (Gerber et al., 2013). This indicates that it is the second largest contributor of GHG emissions following the energy sector (Gerber et al., 2013; Steinfeld et al., 2006). Beside natural carbon dioxide released from the animal metabolism, the livestock production is also responsible for 68% of nitrous oxide which is a GHG having a 296-fold higher impact on ozone layer than carbon dioxide (Dopeltet al., 2019). Being the main source of the 64% of total ammonia emissions, it is also the leading cause of acid rains and the acidification of the aquatic ecosystems (Gerber et al., 2013; Steinfeld et al., 2006). As being a more potent gas than carbon dioxide on the climate change, 40% of the methane is released via the livestock production (Gerber et al., 2013; Steinfeld et al., 2006). Livestock production does not imply only meat production, but also dairy products and eggs, comprising of cattle, small

ruminants, pig, and poultry breeding (Pelletier and Tyedmers, 2010; Thornton, 2010). Arable land is extensively used for this indirect agricultural production; one-third is exploited for feed production and a third of overall cereal production goes directly as animal feed, competing with straightforward human consumption and reducing energy conservation (Godfray et al., 2018). In terms of the cultivated products, the exploitation is so high that when grass is included, 60 % of world's total food crop production in dry matter tons is used only for animal feed (Bijl et al., 2017). Thus, it is followed by consequences; in the US, animal agriculture is accountable for 55 % of soil erosion, 37 % of pesticide utilization, half of total antibiotic use, and one third of nitrogen and phosphorus run-off into freshwater systems (Steinfeld et al., 2006). Water scarcity can be another problem caused by the livestock production as irrigation of feed crops comprise 7% of the global water usage (Gerber et al., 2013; Steinfeld et al., 2006).

On the other hand, even though 40% of the world-wide agricultural output comes from the livestock production which supports the security of food and livelihoods of nearly one billion of the human population, though that the child labor in this sector should not be ignored (Hardaker, 1997). The aggressiveness of the animals, the likelihood to come across with diseases due to poultry-pathogens, the exposure to dusts, fumes, and chemicals such as disinfectants which are used in livestock production and the leakage of wastes into water resources are the factors that need to be considered.

2.2. Phycoremediation of Wastewater

Wastewater and waste gas such as flue gas, carbon dioxide and methane have a potential to pollute freshwater resources if released without proper treatment. Furthermore, those waste streams have a high potential to feed microalgae with their abundant nitrogen, phosphorus, organic carbon, and carbon dioxide content (Javed et al., 2019). Microalgae not only use nutrients such as phosphorus, nitrogen, and carbon but they also use macro- and micro-nutrients in the wastewaters such as sulphur, magnesium, calcium, potassium, iron, manganese, zinc, and molybdenum (Javed et al., 2019; Mohan, et al., 2015).

Microalgae have gained popularity in recent years due to their dual potential of providing phycoremediation solutions by capturing nutrients such as nitrogen and phosphorus, and producing valuable biomass which can be used as a feedstock for animal feed, biofertilizers, biofuels, food supplements, and pharmaceuticals (Chandra et al., 2019). Besides their phycoremediation and value-added bioproduct generation potential, they provide GHG mitigation by sequestering carbon dioxide (Chandra et al., 2019; Kumar et al., 2010). Cultivation of microalgae is also possible in non-arable lands

so that agricultural crop production is not disrupted. These beneficial features of microalgae make them advantageous among low-cost wastewater treatment technologies (Sheehan et al., 1998; Woertz et al., 2009).

As studied extensively for decades, wastewater contains significant amounts of recoverable nutrients (Abdel-Raouf et al., 2012). It is crucial to recover these nutrients from the wastewater for several reasons. Firstly, it requires substantial amounts of energy to generate nitrogen-based fertilizers by costly Haber-Bosch process which is used for the production of more than 90% of the World's nitrogen based fertilizers (Gálvez et al., 2007). Similarly, it requires significant amount of time and energy to mine phosphate rocks which are non-renewable resources and expected to be depleted in roughly 100 years (Cordell et al., 2009). As they are usually the limiting nutrients of terrestrial ecosystems, agricultural activities heavily depend on phosphorus and nitrogen as fertilizers for plants to grow (Ågren et al. 2012). Therefore, it is utmost important to recover these nutrients from potential sources such as wastewater and reuse them in agricultural practices to resolve the anticipated food demand in the future. The other reason for nutrient recovery is when nutrient rich wastewater is discharged into receiving water bodies, it decreases the quality of aquatic life and creates eutrophication problems. Similar to land plants and agricultural crops, cultivation of algae also requires nutrients which supports the idea of their integration into wastewater treatment systems (Grobbelaar, 2004).

The idea of using microalgae in wastewater treatment was proposed in 1950s in US as their potential of nutrient removal has been discovered (Oswald and Gotaas, 1957). Significant benefits of using microalgae in wastewater treatment include providing oxygen to the aerobic heterotrophic microorganisms, while sequestering carbon dioxide, and uptake nutrients (Oswald and Gotaas, 1957). Generated biomass can also be used in anaerobic digesters to produce biogas (Abdulsada and Kadhim, 2014). Nitrogen removal is carried out by the assimilation of ammonium or nitrate uptake into the algal biomass (Arita et al., 2015). Phosphorus is removed from wastewater by assimilation to polyphosphate by microalgae (Wang et al., 2010). The nutrient removal provided by algae is useful especially when it is used as an advanced nutrient removal process in wastewater treatment facilities (Wang et al., 2010). In addition to nutrient removal, algae can provide oxygen to the aerobic microorganisms to facilitate the degradation of organic matter present in wastewater (Lembi and Waaland, 1988). Thus, using microalgae for wastewater treatment not only enhances the nutrient removal but also aids the Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) removal from the wastewater if it is used as an advanced treatment technology (Lembi and Waaland, 1988). Removal of coliform bacteria and heavy

metals are other advantages of using microalgae in wastewater treatment (Abdel-Raouf et al., 2012). By using wastewater as a growth media for microalgae, it is also possible to reduce the energy and cost of growing microalgae for a large-scale production of algae-based bio-products (Lizzul et al., 2014).

Several examples of utilizing algal biomass for wastewater treatment are present in literature. Notably, in a study conducted by Palmer to see which genera of microalgae is abundant in waste stabilization ponds (WSPs), it was observed that *Chlorella, Ankistrodesmus, Scenedesmus, Euglena, Chlamydomonas, Oscillatoria, Micractinium,* and *Golekinia* genera were frequently seen (Palmer, 1974). Palmer also showed that the most organic pollution tolerant eight genera of microalgae were *Chlorella, Scenedesmus, Euglena, Chlamydomonas, Oscillatoria, Nitzschia, Novicula,* and *Stigeoclonium* (Palmer, 1969)

In another study by Wang et al., a wild strain of *Chlorella sp.* was cultivated in four different wastewater media and the nitrogen, phosphorus and COD removal efficiencies were analysed. The centrate from sludge centrifuge which had the highest levels of total nitrogen (TN), total phosphorus (TP) and COD among other wastewater types collected from a local municipal wastewater treatment plant resulted with more algal growth (Table 2.1). In other words, increased amount of nitrogen, phosphorus and COD aided the algal growth in the wastewater (Wang et al., 2010).

Table 2.1. Nutrient concentrations in different types of wastewaters.

Parameters (mg/L)	**Wastewater before primary settling**	**Primary settled wastewater**	**Effluent from aeration tank**	**Centrate from sludge centrifugation**
TN	40.65±0.07	38.95±0.1	19.1±0.1	131.5±2.1
TP	5.66±0.08	6.86±0.05	0.32±0.04	201.5±10.6
COD	231.0±4.2	224.0±4.2	42.2±1.9	2250.0±99.0

In a study conducted by Sawayama et al., *Botryococcus braunii* was cultivated in the secondary treatment effluent from domestic wastewater treatment plants at laboratory scale. Microalgae in this study grew well and it was concluded that they could be used for nutrient removal purposes (Sawayama et al., 1992). In another study, urban wastewater after secondary treatment was used as a cultivation medium for *B. braunii* and it was discussed that secondary treated wastewater effluent could be used as a growth medium for this species (Órpez et al., 2009).

Chlorella sorokiniana was cultivated in two wastewater system (secondarily treated wastewater and centrate produced from the separation of solids from an anaerobic digester) in a research study conducted in United Kingdom (Lizzul et al., 2014). As a result, microalgae showed comparable growth yield with commercial medium when it was cultivated in centrate and secondarily treated wastewaters (Lizzul et al., 2014). Removal of carbon monoxide, carbon dioxide, and nitrogen oxides were 20-30%, 30-45%, 95-100% levels, respectively. It was also noted that supplying 12% of carbon dioxide showed significant increase in terms of biomass yield and lipid production of microalgae (Lizzul et al., 2014). Addition of carbon dioxide also improved the removal rate of nutrients of the two wastewater types (Lizzul et al., 2014). In another research study, *C. sorokiniana* was able to grow in suboptimal temperatures in bio-industrial wastewater medium (Podevin et al., 2017).Maximum specific growth rate was reported as 0.48/day (Podevin et al., 2017). It was also observed that algal growth in suboptimal temperatures were consistent and comparable with literature (Podevin et al., 2017).

2.2.1. Biotic Factors Affecting Phycoremediation of Wastewater

In aquatic environments, microorganisms compete with each other for limited nutrients. When microalgae are cultivated in open ponds, they are exposed to other microorganisms such as bacteria, fungi, and protozoa. Wastewater treatment in open ponds are prone to contamination of these organisms as well as predators which can be lethal for certain microalgae cultures and cause culture crashes (Larsdotter, 2006).

2.2.2. Abiotic Factors Affecting Phycoremediation of Wastewater

2.2.2.1. Nitrogen. Nitrogen is one of the growth limiting factors for plants and microalgae and it is abundant in the atmosphere in gaseous form. nitrogen-fixing microorganisms (some cyanobacteria and certain bacteria and archaea), lightning, industrial processes such as Haber-Bosch process and ambient nitrogen reduction are ways to convert nitrogen in the atmosphere into a bioavailable form (Peoples et al., 1995).

Nitrogen is utilized in the microalgal cells in nitrite, nitrate, ammonia and gaseous nitrogen forms and their assimilation in the cell are dependent on the N:P ratio in the culture medium (Arita et al., 2015;

Xin et al., 2010). It was reported that for a maximum nitrogen removal efficiency, 5:1 to 12:1 N:P ratio for *Scenedesmus sp.* and 7:1 N:P for *Chlorella vulgaris* (Xin et al., 2010). With 11:1 to 15:1 N:P ratio, *C. vulgaris* (FC-16) showed maximum TN removal. 99% removal of TN by *N. oleoabundans* was reported in an artificial piggery wastewater with 3:1 N:P ratio (Wang and Lan, 2011). *S. obliquus* showed best TN removal performance with a N:P ratio range of 9:1 to 13:1 (Arbib et al., 2013).

2.2.2.2. Phosphorus. Phosphorus is another important growth limiting factor for plants and microalgae as it is the major constituent of several organic molecules in plant and microalgal cells such as adenosine triphosphate, nucleic acids, and phospholipids (Cordell et al., 2009). Phosphorus is a finite source in the environment, and it is mostly scarce in aquatic systems. Moreover, it is found in the earth's crust and mined for commercial uses such as fertilizer and detergent production (Cordell et al., 2009).

Microalgae assimilate phosphorus in the form of orthophosphate (Larsdotter, 2006). When orthophosphate is short in the growth medium, organic phosphates are converted into orthophosphate species with phosphatase enzyme (Larsdotter, 2006; Larsdotter, 2006). Microalgae produce volutin that enables them to store polyphosphates to use in the case of phosphate-deficient growth medium (Cai et al., 2013). Phosphorus shortage in the growth media prevents the proliferation of microalgae (Choi and Lee, 2015). In a study, deformation of mitochondria and chloroplast was reported for *C. reinhardtii* cultivated in a phosphorus-deficient medium (Nagarajan et al., 2019).

2.2.2.3. Carbon. Microalgae are autotrophic/heterotrophic/mixotrophic microorganisms, and both inorganic and organic forms can be a carbon source for microalgae. Utilized carbon source is dependent on the metabolism of which microalgae uses; heterotrophic and mixotrophic microalgae can utilize glucose, organic acids, glycerol, and acetate in wastewater (Gera et al., 2015; Suganya et al., 2016; Woertz et al., 2009). Green microalgae such as *Scenedesmus* and *Chlorella*, carbon utilization mode may differ depending on the carbon source in the growth medium (Larsdotter, 2006).

C:N and C:P ratios of the wastewater are related to biomass productivity. Carbon deficiency in the wastewater leads to inefficient removal of nitrogen, phosphorus, and other nutrients (Woertz et al., 2009). Most wastewater types lack the sufficient carbon sources for microalgae to grow resourcefully (Gera et al., 2015). Flue gas of industrial plants are diverted into the biological reactors as an extra carbon source that enables microalgae to proliferate effectively (Suganya et al., 2016).

2.2.2.4. Light. Microalgae react distinctly to varied light intensity. If the light intensity reaches above the optimum levels, it can cause photoinhibition by disrupting the light-dependent reactions of oxygenic photosynthesis (Gonzalez-Camejo et al., 2018).

Light intensity and light cycles have a direct effect on the removal of nutrients from wastewater. *Chlorella kessleri* (strain UTEX 398) was cultivated in continuous light and light/dark cycles and removed 18.8% and 8.3% of nitrate respectively in an artificial wastewater (Lee et al. 2001)

Chlorella sorokiniana (strain Shih. Et Krauss) was reported to be the most resistant to excessive heat and light intensities amongst all *Chlorella* species (Morita et al., 2000). An environmental *Chlorella sorokiniana* (strain UTEX 2805) isolate was showed to reduce ammonium levels in wastewater from 10 mg/L NH_4^+ to untraceable levels in four days of cultivation (de-Bashan et al., 2008).

When cultivated in wastewater with increased intensity of light, four strains of microalgae showed higher biomass yields in a recent study (Nzayisenga et al., 2020). Also, fatty acid content of two strains were increased with reduction of proteins (Nzayisenga et al., 2020). Nutrient removal efficiency of more than 75% (TN and TP) were observed in the same study when cultivated in wastewater (Nzayisenga et al., 2020).

Bohutskyi and colleagues reported that with increased light intensity from 30 to 140 µmol photon/m^2/s, growth rate and biomass concentrations of *Chlorella sorokiniana* (CCTCC M209220), *Chlorella vulgaris* (UTEX 2714), *Chlorella sorokiniana* (UTEX B 3010), *Chlorella sorokiniana* (UTEX 1230), *Scenedesmus acutus f. alternans* (UTEX B 72) and *Scenedesmus dimorphus* (UTEX 1237) were increased (Bohutskyi et al., 2016).

2.2.3. Phycoremediation of Manure Wastewater

Manure is a good fertilizer that contains nutrients such as nitrogen, potassium and phosphorus (Pettygrove et al., 2010). It also contains micronutrients such as copper, zinc, manganese and many others. Thus, it is possible to cultivate algae in manure wastewater by providing the nutrient removal treatment stage at the same time (Pettygrove et al., 2010).

Various manure wastewater from livestock production are tested for microalgae cultivation (González et al., 1997). A study was conducted in order to test the nutrient removal efficiencies of two microalgae in agro-industrial wastewater of a dairy industry and pig farming: *Chlorella vulgaris* and *Scenedesmus dimorphus. S. dimorphus* was more efficient in terms of ammonia removal yet total ammonia removal efficiency of two microalgae at the end of each cultivation was similar (González et al., 1997). Phosphorus removal of two species were lower than *Scenedesmus obliquus* which has a P removal efficiency of 75% (Lavoie and De la Noüe, 1985).

In a similar study, *Chlorella vulgaris* was cultivated in both undigested and digested dairy manures (Wang, et al., 2010). It was noted that undigested dairy manure which had higher organic carbon concentration compared to digested dairy manure had significantly increased the algal productivity (Wang et al., 2010). It was suggested that undigested dairy manure is a preferable feedstock for wastewater treatment in a dairy farm (Wang et al., 2010).

In a study by An et al., secondarily treated piggery wastewater obtained from a wastewater treatment plant was used to cultivate *Botryococcus braunii* (UTEX 572). Wastewater was pre-treated by a membrane bioreactor via acidogenic fermentation and it contained considerable amounts of nitrate (An, Sim, Lee, & Kim, 2003). Total nitrogen removal efficiency of the microalgae was 43%, ammonia removal was more than 98% and 80% removal efficiency was obtained for nitrate (An et al., 2003).

In another study, *Chlorella sorokiniana* strains CS-01, UTEX 1230 and UTEX 2714 were tested in anaerobically digested cattle manure effluent. Biomass productivity of the strains were compared with Bold's Basal Medium (BBM) (Kobayashi et al., 2013). CS-01 and UTEX 1230 showed similar or higher biomass productivity in anaerobically digested cattle manure effluent compared to BBM (Kobayashi et al., 2013). It was suggested that high yields of protein and starch (34%, 23% respectively) in UTEX 1230 strain would be suitable for animal feed production (Kobayashi et al., 2013).

Recent studies in the field concentrates on how to increase sustainability of the system by using microalgal strains that efficiently removes nutrients in the wastewater with an increased biomass and lipid production. Biological reactor design and downstream processing challenges are in the focus, as well.

2.3. Reactor Types Used in Phycoremediation of Wastewater

Two main types of reactors are used for the wastewater treatment with microalgae: open ponds and closed photobioreactors (cPBR). Both reactor types are used in wastewater treatment with microalgae research and their nutrient removal efficiencies are compared.

Open systems such as open raceway ponds (ORP) and WSP are widely used for biological wastewater treatment. ORPs are considered as effective in nutrient removal from wastewater and relatively economic biological reactors yet they are prone to microbial contamination, lack of resilience to weather and environmental conditions, low light penetration, relatively lower productivity and high water footprint are some of the operational problems (Mohan et al., 2015; Rawat, 2013; Suganya et al., 2016).

Removing up to 80% of nitrogen and phosphorus from wastewater is possible with a well-designed ORP, moreover, it is possible to remove over 90% of the BOD (Oswald and Gotaas, 1957). Increased pH values in the culture cause indirect nutrient removal by phosphate precipitation and ammonia stripping where ammonia is removed by the provided aeration through continuous mixing (Larsdotter, 2006).

Photobioreactors are classified as closed microalgal growth systems that are more controlled and refined relatively to open systems. Biomass productivity, light penetration and less retention time for higher biomass yield are some of the beneficial aspects of closed cultivation systems despite their higher operating costs and energy consumption (Larsdotter, 2006).

2.4. Bioproducts of Biomass from Phycoremediation

Considering microalgae as a feedstock for bio-products has multiple advantages; cultivating algae does not require very much freshwater input like agricultural crops does, non-productive lands could be used for microalgae cultivation so that it does not compete with agricultural crop lands. Furthermore, the bio-products produced using microalgae could be used in agricultural activities as supplemental food for livestock and aquaculture. Thus, using microalgae for production of bio-products is a promising sustainable alternative than agricultural crops in terms of water and land usage (Pittman etal., 2011).

To ensure animal and aquaculture feed security for the years to come, it is inevitable to search for sustainable production practices while ensuring minimum harm to the environment. Microalgae has been an adequate candidate of food supply for human and animal consumption for at least 60 years because of their nutritional potential (Grau and Klein, 1957; Milner, 1953). However, high costs of production of microalgae has limited the applicability of it as a feed source (Soeder, 2017). Yet, the need for sustainable and efficient algal biomass production led to intensive research in this field. It is widely recognized that by using wastewater as a cultivation media, it is possible to eliminate the costly chemicals and water consumption for producing algal biomass (Hu et al., 2012).

Algae is a fine candidate of feed for human and animal consumption in terms of its nutritional composition. It mostly consists of proteins, carbohydrates, lipids, vitamins, antioxidants and trace elements (Yaakob et al., 2014). Although, percentage composition of macro and micro-nutrients varies between algae species, their carbohydrate, lipid and protein contents could be defined as outstanding compared to conventional animal feed formulations (Table 2.2) (Lum et al., 2013).

Table 2.2. Nutrient composition of conventional animal feed and several microalgae species (% dry weight) adopted from (Becker, 2007; Lum et al., 2013; Shields and Lupatsch, 2012).

Source	Crude Protein	Carbohydrates	Lipids
Soybean	37	30	20
Fish Meal	63	-	11
Corn	20	85	4
Wheat	14	84	2
C. vulgaris	51-58	12-17	14-22
A. maxima	60-71	13-16	6-7
S. obliquus	50-56	10-17	12-14

Microalgae not only provides protein, amino acids, fatty acids, vitamins, minerals and other bio-active compounds (Pulz and Gross, 2004) but also contributes to improved immune system (Turner et al., 2002), lipid metabolism (Güroy et al., 2011; Nakagawa, 1997), antiviral & antibacterial action, gut function (Michiels et al., 2012) and stress resistance (Nath et al., 2012; Sheikhzadeh et al., 2012) even when supplied in low concentrations as livestock and aquaculture feed (Shields and Lupatsch, 2012).

Lysine is the first limiting amino acid among dietary amino-acids which can be synthesized in relatively large amounts by various microalgae species (Lum et al., 2013). However, other limiting amino acids such as methionine and cysteine are found to be deficient in most of the algae species (Becker, 2007). Thus, mixing microalgae with conventional animal and aquaculture feed would be an option to increase the utilization of various amino acids in the feed (Lum et al., 2013).

It remains unanswered, even if recent studies indicate that 30% of the global algal production is used by the animal feed industry, whether microalgae will be a bulk aquaculture and animal feed that supplies energy and bioactive compounds or will remain only as a supplement because of the lacking efficient large-scale microalgae production and costs (Becker, 2007; Shields & Lupatsch, 2012). However, it is possible to use microalgae even as a supplement to improve the diet of livestock and aquaculture animals for human consumption (Lum et al., 2013).

As a conclusive remark for this section, studies in recent years showed that a microalgae production is a burden to the environment in terms of water footprint, energy and fertilizer usage for microalgae growth and harvesting (Benemann and Oswald, 1996; Woertz et al., 2009). It is also economically not

feasible because of the same reasons. Integrating microalgal biomass production for a variety of products with wastewater treatment and carbon dioxide mitigation is reported to be feasible when coupled with value-added bio-product generation (Sheehan et al., 1998).

3. MATERIALS AND METHODS

In this chapter, experimental methods to prepare manure wastewater for cultivation of microalgae, details on culture conditions and microalgal biomass analyses are described in detail. The chapter expands upon four main sections on cultivation specifications, pretreatment of wastewater, nutrient removal efficiencies of microalgae and characterization analyses of the harvested biomass.

3.1. Microalgae Species Selection and Cultivation

Species selected from the literature were cultivated in Erlenmeyer flask photobioreactors (EBRs) at specific conditions for the determination of their growth curves.

3.1.1. Species Selection

Ten different green microalgae species were selected for preliminary screening tests. Species previously obtained from Culture Collection of Algae at the University of Texas at Austin (UTEX) and maintained at Boğaziçi University – İstanbul Microalgae Biotechnologies and Research Unit (İMBİYOTAB) are *Chlorella sorokiniana* (strain UTEX1230), *Chlorella vulgaris* (strain UTEX 395), *Chlorella zofingiensis* (strain UTEX 32), *Scenedesmus* sp. (strain UTEX 1589), *Scenedesmus dimorphus* (strain UTEX 1237), and *Scenedesmus obliquus* (strain UTEX 393). *Chlorella vulgaris* (strain CCAP 211/11B) was obtained from the Culture Collection of Algae and Protozoa (CCAP) located within the Scottish Association for Marine Science.

Main rationale for selecting these species was based on the established nutrient removal capabilities of both Chlorella and Scenedesmus genera from different wastewater systems as reported in literature (Asadi, Rad, Qaderi, & Research, 2019; González et al., 1997; Gupta et al., 2016). In addition, three environmental isolates were included in the study two of which were previously isolated from Ergene Watershed (EWS) heavily impacted by industrial and agricultural pollution and one from the wastewater lagoons of Kayseri Şeker (KS) Factory Plant located in Kayseri, Central Anatolia, Turkey. These environmental isolates were designated as *Chlorella* sp. (strain EWS-N66), *Scenedesmus* sp. (strain EWS-N50) and *Chlorella* sp. (strain KS-1) respectively.

All microalgae species were maintained at 25°C under 1200 lux light intensity provided by cool white fluorescent bulbs, under 12:12 hour light:dark cycle in a plant growth chamber (Model GC401, Nüve, Turkey). Two aliquots of each microalgae species were maintained in Modified Bold's 3N (MB3N) agar culture medium and two aliquots were cultivated in liquid MB3N culture medium (Table 3.1). All aliquots were sub-cultured every 15-20 days for maintaining healthy cultures. Soil water GR+ medium was prepared by mixing one tablespoon of greenhouse soil with 200 mL of water and pasteurizing for two consecutive days for 3 hours on each day at 98°C. P-IV Metal solution recipe is given in Table 3.2.

Table 3.1. MB3N culture medium ingredients adopted from (UTEX, 2020c).

#	Component	Amount (mL/L)	Stock Solution Concentration (g/mL dH_2O)	Final Concentration (mM)
1	$NaNO_3$	30	10/400	8.8
2	$CaCl_2.2H_2O$	10	1/400	0.17
3	$MgSO_4.7H_2O$	10	3/400	0.3
4	K_2HPO_4	10	3/400	0.43
5	KH_2PO_4	10	7/400	1.29
6	NaCl	10	1/400	0.43
7	P-IV Metal Solution	6	-	
8	Soil water: GR+ Medium	40	-	
9	Vitamin B12	1	-	
10	Biotin vitamin solution	1	-	
11	Thiamine vitamin solution	1	-	

Table 3.2. P-IV metal solution ingredients adopted from (UTEX, 2020d)

#	Component	Amount (g/L)	Final Concentration (mM)
1	$Na_2EDTA.2H_2O$	0.75 g/L	2 mM
2	$FeCl_3.6H_2O$	0.097 g/L	0.36 mM
3	$MnCl_2 \cdot 4H_2O$	0.041 g/L	0.21 mM
4	$ZnCl_2$	0.005 g/L	0.037 mM
5	$CoCl_2 \cdot 6H_2O$	0.002 g/L	0.0084 mM
6	$Na_2MoO_4 \cdot 2H_2O$	0.004 g/L	0.017 mM

3.1.2. Growth Curve Assays

Microalgae show similar growth profiles with other microorganisms in four main phases: lag, exponential, stationary and death phases. In order to achieve actively growing synchronous cultures for each experimental condition, growth profiles of each species were individually determined, and their mid-exponential phases were identified. Mid-exponential phase ensured maximum cell viability, and also remarked the harvesting time of the microalgal culture for inoculating reactors.

For the determination of growth profiles, selected species were cultivated in 1L EBRs operated as batch reactors at room temperature (~22 °C) (Figure 3.1.). Working volume of each reactor was 700 mL, and MB3N was used as cultivation medium for all of the species. Reactors were kept under 8000 lux of light intensity with LED daylight source, supplied with 0.5 L/min of dry air flow passed through a 0.45 µm filter and dispersed with diffuser stones. Constant air pressure was ensured with air flowmeters. Two exhaust outlets were secured with 0.45 µm air filters to sustain atmospheric pressure and each EBR were continuously stirred with a magnetic stirrer at 150 rpm. Glassware used for EBRs were cleaned thoroughly with hydrogen peroxide and autoclaved for 15 min at 121°C for sterilization prior to experiments. All growth curve assays were conducted with biological triplicates inoculated with a cell concentration of 10^6 cells/mL.

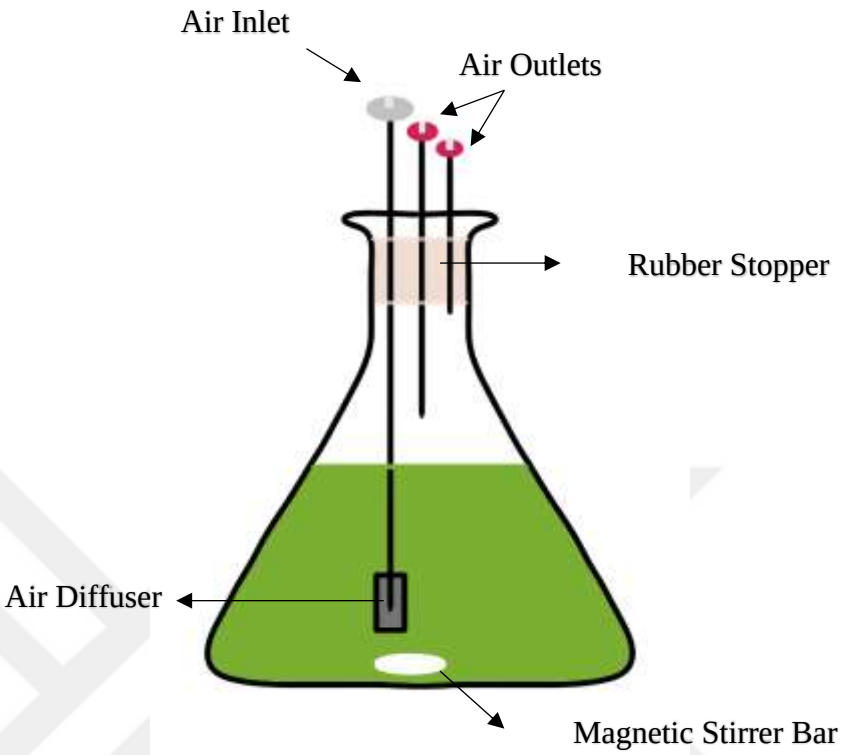


Figure 3.1. Erlenmeyer flask photobioreactors used in the study.

Growth profiles of each culture were determined by measuring optical density (OD) of the EBRs daily at 680 nm with a UV-Visible spectrophotometer (DR3900, Hach, Colorado, USA). Cell counts were also determined every other day using a Neubauer improved hemocytometer and binocular light microscope (Boeco, Germany). pH values of EBRs were collected everyday by a multimeter (Handylab, SI Analytics, UK). For these measurements, three mL of samples from each EBRs were taken and replaced with fresh MB3N media to sustain constant working volumes.

3.2. Preliminary Screening of Microalgae Species

3.2.1. Preparation of Manure Wastewater

Manure wastewater (MWW) to be utilized for the preliminary screening of microalgae species was collected from a male cattle, herded by İstanbul University Veterinary Medicine Faculty facilities. Manure samples were collected with a clean autoclave bag and stored at 4°C till processing. All glassware used in the MWW preparation experiments were soaked in acid bath overnight (6N hydrochloric acid),

rinsed with ultrapure water (UPH_2O) and dried in the oven (Daihan Scientific, South Korea) at 105°C for at least half an hour. 100 g of wet manure was mixed with 400 mL of ultrapure water and homogenized for six rounds with 10 second intervals in a household blender (B1680 Premium Inox, Kenwood, Tokyo, Japan) (Figure 3.2). Homogenized MWW was poured into Imhoff cones for equilibration for 16 ±1 hours (Figure 3.2). At the end of the equilibration, liquid and coarse solid phases in the wastewater was separated by prefiltering through a cheese cloth, washed with UPH_2O and dried till all of the water content was percolated from the filter into the beaker (Figure 3.2). Prefiltered MWW was autoclaved for 15 minutes at 121°C.



Figure 3.2. Homogenization (left), equilibration (center) and filtering (right) of MWW.

3.2.2. Pretreatment of Manure Wastewater

3.2.2.1. Coagulation and flocculation. In order to decrease colloidal solid particles present in prefiltered MWW and achieve lower turbidity values, coagulation/flocculation and sand filtering was applied. As the first step, colloidal particles were converted into flocs via chemical coagulation using $FeCl_3$ after determining the best $FeCl_3$ concentration for MWW with a jar test.

The jar test was conducted with $FeCl_3$ concentrations at 1100, 1200, 1300, 1400 and 1500 mg/L. After the addition of the coagulant, MWW was initially stirred for 5 minutes at 200 rpm, and 15 to 20 minutes at 80 rpm. Coagulation and flocculation were observed at concentrations starting from 1400 mg/L, which was selected for pretreatment procedure (Figure 3.3).

3.2.2.2. Sand filtration. Following coagulation and flocculation steps, MWW supernatant was subjected to sand filtering. Three different sizes of quartz sand were used: 0.5-1.2 mm, 1-3 mm, and 3-5 mm. Sand were washed with tap water to get rid of the bulk dirt and rinsed with deionized water for three times. Following, sand was acid-treated in 1M hydrochloric acid for 24 hours in a borosilicate, screw capped glass bottle. Hydrochloric acid was discarded and sand was rinsed with 1M hydrochloric acid to clean the residual impurities such as cations. At the final step, acid-washed sand was re-equilibrated by rinsing with UPH_2O and dried at 105°C for 18 to 24 hours.

For the filtration procedure, a custom made Büchner Funnel with porous plate of pore size one that had one L capacity (Borucam, Turkey) was used as filtering unit headpiece. Büchner flask was used for the filtering basin and two pieces were connected with a rubber bung. For rapid filtration, vacuum was applied to the filtering unit with a diaphragm vacuum pump (Model 622.12.001, Isolab, Germany). A Grade 4 Whatman filter was placed on the porous plate of Büchner Funnel in order to protect the pores from clogging with fine sand. Later, 627.7 grams of fine sand, 505.7 grams of medium and 210 grams of coarse sand were loaded into the Büchner Funnel. Sands were layered from fine to coarse bottom to top and used for filtering MWW. Following sand filtration, MWW was filtered through Grade 4 Whatman filter once more.

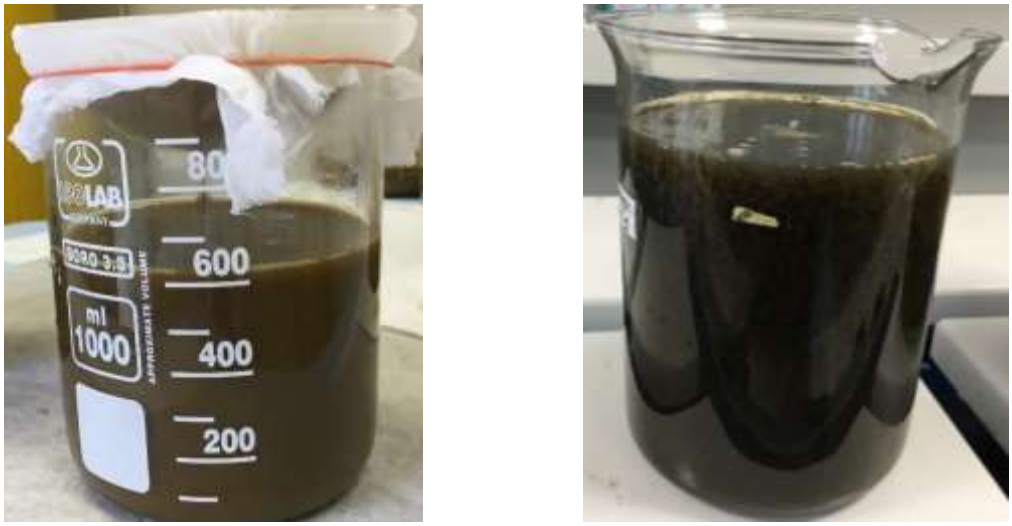


Figure 3.3. MWW before (left) and after (right) coagulation.

3.2.3. Characterization of Manure Wastewater

3.2.3.1. Total nitrogen determination. For the determination of influent and effluent total nitrogen (TN) present in MWW, a TN kit (LCK 338, Hach, Colorado, USA) was used with a UV-Visible spectrophotometer (DR3900, Hach, Colorado, USA) following manufacturer's recommendations. As a standard solution, NH_4Cl was used (0.1486 g/L = 38.8925 mg/L TN).

3.2.3.2. Total phosphorus determination. For the determination of influent and effluent total phosphorus (TP) as total orthophosphate present in MWW, a TN kit (LCK 350, Hach, Colorado, USA) was used with a UV-Visible spectrophotometer (DR3900, Hach, Colorado, USA) following manufacturer's recommendations. Standard solution was prepared by dissolving 15.9 mg/L KH_2PO_4 in 1L of UPH_2O (15.9 mg/L KH_2PO_4 = 3.6189 mg/L PO_4-P). All glassware was soaked with acid overnight (6N hydrochloric acid) and dried at 105°C before TP measurements.

3.2.3.3. Chemical oxygen demand determination. For the determination of influent and effluent chemical oxygen demand (COD) of the MWW, a COD kit (LCK 014, Hach, Colorado, USA) was used with a UV-Visible spectrophotometer (DR3900, Hach, Colorado, USA) following manufacturer's recommendations. Standard solution was prepared by drying 1 g of potassium hydrogen phthalate KHP solid at 110°C, then, 1,708 mg of KHP was dissolved in 1 L of UPH_2O (1,708 mg/L KHP = 2,009.4118 mg/L COD). Three different standard samples were tested with this kit. Procedure that was provided by the kit was followed directly for all samples.

3.2.3.4. Total solids determination. For the determination of total solids (TS) content of the MWW, Standard Methods TS procedure was followed (Eaton et al., 2005). Briefly, evaporating dishes (Eds) were prepared by igniting them at 550°C for one hour in muffle furnace (Daihan Scientific, South Korea). Following, dishes were cooled down to room temperature and stored in the desiccator. Before the sample processing, tare masses of dishes were determined. MWW sample was continuously stirred at 120 rpm with a magnetic stirrer to ensure homogeneous sampling. 10 mL of MWW was pipetted and poured into the evaporating dishes. Samples were evaporated to dryness in oven at 105°C. After evaporation is done, samples were dried in the oven (Daihan Scientific, South Korea) for at least 1 hour. Then, the sample was cooled, kept in the desiccator for half an hour, and weighed. Cycle of drying, cooling, desiccating, and weighing was repeated until a constant weight was obtained. Samples were processed as duplicates. Results were calculated according to the Equation 3.1 given below.

$$\text{mg Total Solids/L} = \frac{(A-B)\times 1000}{\text{sample volume, mL}} \qquad (3.1.)$$

where:

A = weight of dried residue + dish, mg

B = weight of dish, mg.

3.2.4. Challenge Tests with Manure Wastewater

Challenge tests were conducted with ten selected species mentioned in Section 3.1.1 with pretreated raw MWW and 1/2 dilution with UPH_2O. For each species, control, raw MWW and MWW 1/2 dilution was tested (Figure 3.4).

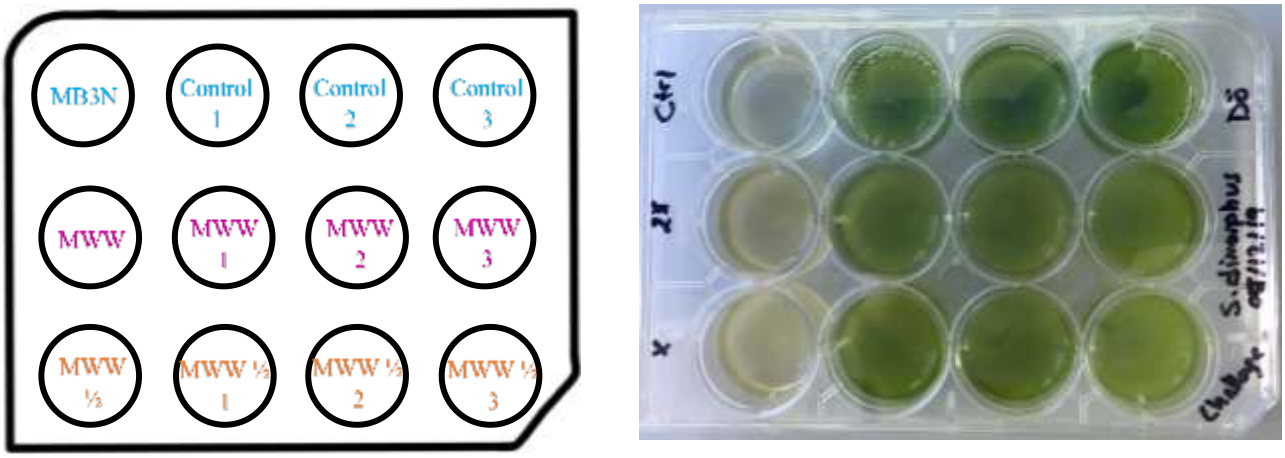


Figure 3.4. Multi-well plate design of the challenge tests of MWW.

Inoculum for the challenge tests were prepared by cultivating the tested species in EBRs till their mid-exponential phase given in Chapter 4 (Table 4.1).

After harvesting the cultivated microalgae by centrifuging the medium for five minutes at 4500 rpm in 50 mL conical bottom centrifuge tubes, biomass pellet was resuspended in 20 mL BBM (Table 3.3) by vortexing at least for 30 seconds, then re-centrifuged for two minutes at 3200 rpm to wash out impurities without biomass loss. Washing procedure was repeated twice. BBM medium, similar to MB3N, was preferred for seed preparation due to its low nitrogen and phosphorus content, in order to prevent high amounts of nitrogen and phosphorus load for the challenge tests. After washing the biomass, microalgal pellet was resuspended in 20 mL BBM and used as an inoculum for challenge tests. 10^7 cells/mL were inoculated into microwell plates with a working volume of 3 mL containing MB3N, raw MWW and 1/2 MWW. “Control” row of multi-well plates consisted of MB3N and 10^7 cells/mL in 3 mL, “MWW” row contained MWW and 10^7 cells/mL in 3 mL and “MWW 1/2” row contained 1/2 diluted MWW and 10^7 cells/mL in 3 mL. Bold trace stock recipe in BBM is given in Table 3.4.

Table 3.3. BBM growth medium ingredients adopted from (UTEX, 2020b).

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	$NaNO_3$	10 mL/L	10 g/400mL dH2O	2.94 mM
2	$CaCl_2•2H_2O$	10 mL/L	1 g/400mL dH2O	0.17 mM
3	MgSO4•7H2O	10 mL/L	3 g/400mL dH2O	0.3 mM
4	K_2HPO_4	10 mL/L	3 g/400mL dH2O	0.43 mM
5	KH_2PO_4	10 mL/L	7 g/400mL dH2O	1.29 mM
6	NaCl	10 mL/L	1 g/400mL dH2O	0.43 mM
7	EDTA Stock	1 drop (0.05 mL)	8.5 µM	
8	Iron Stock	1 drop (0.05 mL)	0.9 µM	
9	Boron Stock	1 drop (0.05 mL)	9 µM	
10	Bold Trace Stock	1 drop (0.05 mL)		

Table 3.4. Bold trace stock ingredients adopted from (UTEX, 2020a).

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	H_2SO_4 conc.	10-mL		
2	$ZnSO_4•7H_2O$	8.82 g	0.44 mg/L	1.50 µM
3	$MnCl•4H_2O$	1.44 g	0.072 mg/L	0.36 µM
4	MoO_3	0.71 g	0.38 mg/L	0.26 µM
5	$CuSO_4•5H_2O$	1.57 g	0.78 mg/L	0.31 µM
6	$Co(N0_3)_2•6H_2O$	0.49 g	0.025 mg/L	0.084 µM

For the challenge test setup, day-light fluorescent projector lamps providing 8000 lux were used. Daily OD data at 680 nm were taken with a plate reader (SpectraMax i3, Molecular Devices, San Jose, CA, USA). OD vs. time graphs were plotted to be able to compare the growth performances in MWW.

3.3. Farm Wastewater Study

For the preliminary study, manure generated from one cattle was mixed with water to create a representative wastewater for the screening tests. However, in order to obtain more results from the experiments, it was necessary to use real farm wastewater that included not only manure waste but also the flushing water coming from the barn. Using a real farm wastewater enabled observing the growth results of microalgae in a real setup.

3.3.1. Collection of Farm Wastewater

Farm wastewater (FWW) was collected from a dairy farm (Gündönümü Çiftliği, Silivri, Turkey). Manure, urine, and flushing water coming from the barn hosting nearly 200 dairy cows, some of which are calves (Figure 3.5), are directed into a concrete pit stirred with propellers (Figure 3.5). Wastewater is sucked into a solid-liquid separator where solid manure is collected and used as fertilizer (Figure 3.5). Liquid part of the wastewater is re-directed into the pit.

FWW sample was collected from the effluent of the separator after waiting for a while for the wastewater to be separated (separator was started before the wastewater was collected). For FWW sample collection 5 L polyethylene terephthalate and 19 L polycarbonate containers were used (Figure 3.5). Collected wastewater was stored in +4°C cold room until processing.

Prior to experiments, all glassware was soaked in 6N hydrochloric acid overnight, rinsed with UPH_2O and dried in drying oven (Daihan Scientific, South Korea) at 105°C for at least half an hour. FWW was decanted into 5 L glass bottles and autoclaved at 121°C for 30 minutes (Figure 3.7). After the sterilization, FWW was filtered through 20 and 60 mesh sieves (Figure 3.7).



Figure 3.5. Concrete waste pit (top left), solid liquid separator (top right), barn (bottom left) and sample collection (bottom right).

3.3.2. Pretreatment of Farm Wastewater

For the pretreatment of FWW, chitosan was selected as a coagulant as $FeCl_3$ was not an efficient chemical coagulant for larger volumes of wastewater. Chitosan is a natural polysaccharide that coagulates the solid particles in water or wastewater with pH higher than 8. Chitosan (low molecular weight, 448869, Sigma Aldrich) was dissolved in 2% acetic acid to achieve 0.45% final concentration (w/v). After a jar test, for 50 mL of wastewater, 12 mL of chitosan solution was used. Following the addition of chitosan solution, FWW was initially stirred for 5 minutes at 200 rpm, and 15 to 20 minutes at 80 rpm (Figure 3.7). After coagulation, FWW was sieved through 20, 60 and 200 mesh sieves and filtered through Whatman Grade 4 Filter to eliminate remaining solid particles (Figure 3.6). Filtered FWW was diluted 1/4 for plastic PBR experiments (Figure 3.7). pH of the FWW was 4.91 at 23.4°C

after the pretreatment steps, 18.970 mL of 1 M sodium hydroxide was added into the wastewater in order to adjust the pH value of it to 8.00.

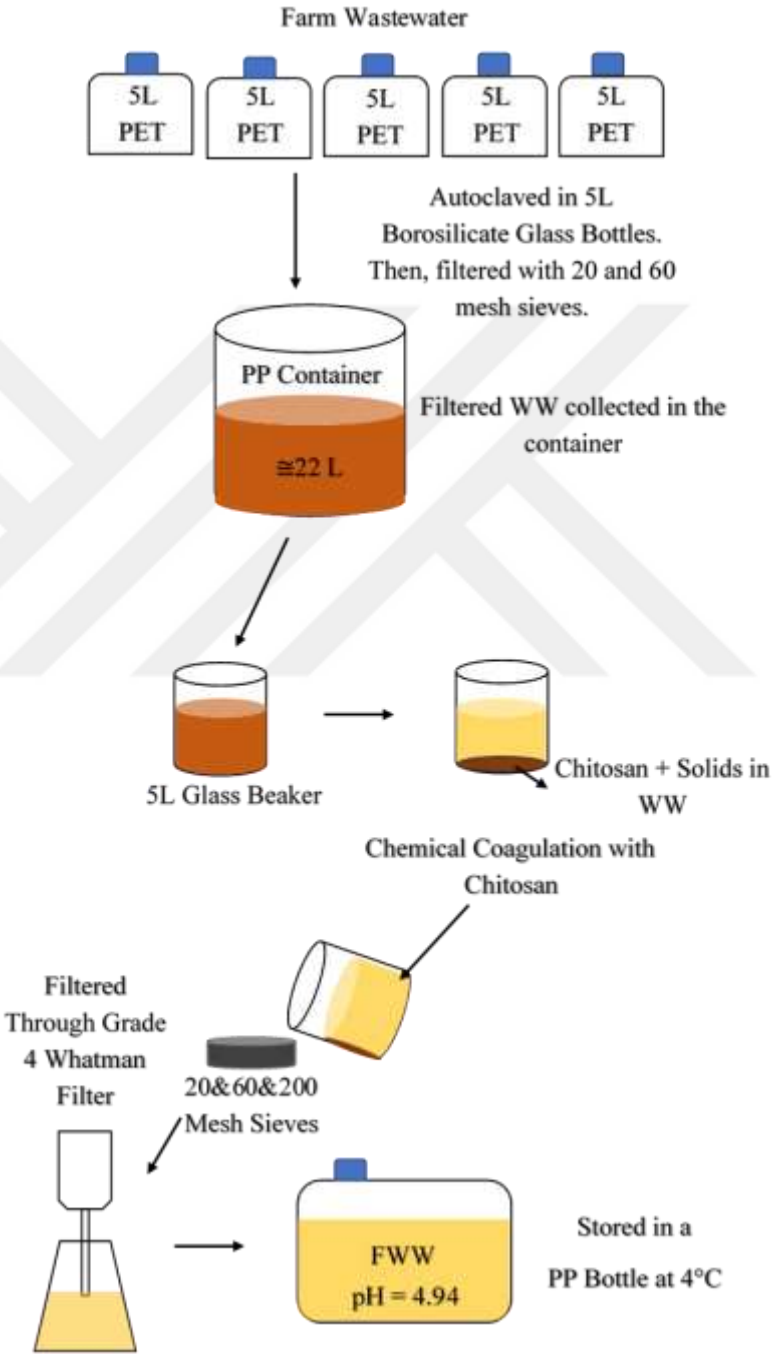


Figure 3.6. Pretreatment steps of FWW.



Figure 3.7. FWW before autoclaving (left), after the addition of chitosan (center) and after filtration with sieves (right).

3.3.3. Characterization of Farm Wastewater

Total nitrogen, TP, COD and TS measurements of the FWW were conducted similar to characterization of MWW as described in Section 3.2.3.

3.3.4. Challenge Tests with Farm Wastewater

Challenge Tests with MWW were done with 10 species of microalgae (Section 3.2.4). Four out of these species were eliminated due to lack of resistance to the wastewater. Microalgae species subjected to MWW and passed the first challenge test were subjected to a second stage of elimination with the FWW in order to ensure resilience against different manure wastewater.

Inoculum for the challenge tests were prepared by cultivating the tested species in EBRs till mid-exponential phase at 8000 lux light source, 0.5 L/min dry air supply in 700 mL of MB3N culture media with a starting cell concentration of 10^6 cells/mL at room temperature (~22 °C). Biomass was resuspended with BBM after the washing procedure. Cell concentration was determined as described in Section 3.2.4 and 10^7 cells/mL of biomass were inoculated into six different culturing media which are MB3N, raw FWW, FWW 1/2, FWW 1/4, FWW 1/8, FWW 1/10 (Figure 3.8). Due to unknown concentrations of ammonia in the FWW, more dilutions were tested to be sure of viability in the tests.

For the challenge test setup, day-light fluorescent projector lamps providing 8000 lux were used. Daily OD data at 680 nm were taken with a plate reader (SpectraMax i3, Molecular Devices, San Jose, CA, USA). OD vs. time graphs were plotted to be able to compare the growth performances in FWW.

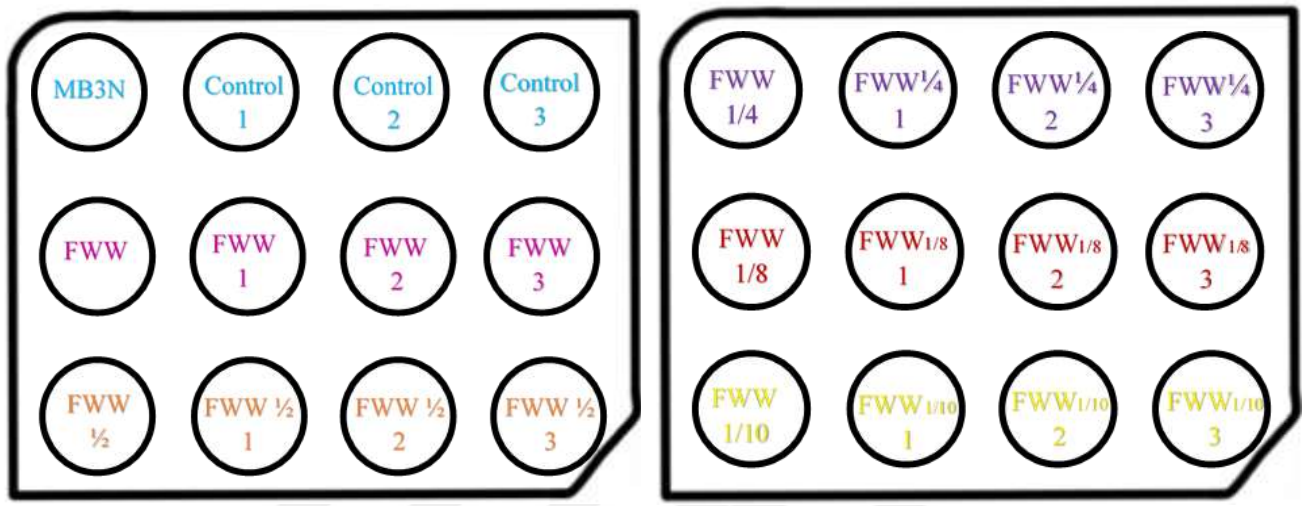


Figure 3.8. Multi-well Plate Design of Challenge Tests of FWW.

3.4. Plastic Photobioreactor Study

As a result of two comprehensive challenge tests, best performing microalgal strain *Chlorella sorokiniana* (strain UTEX 1230) was selected for demonstration of FWW treatment in plastic PBRs. Microalgae were cultivated in the plastic PBRs until the culture reached the stationary phase. Daily TN, TP, OD, and pH data were taken. Cell density and dry weight data were collected every other day. For value-added animal/aquaculturing feed applications, nutrient profile of the biomass was analysed after harvesting the PBRs.

3.4.1. Design of Plastic Photobioreactors

Plastic PBR setup was designed with a low-density polyethylene transparent agricultural irrigation hose (Figure 3.9). Reactors with 1.3 m height and 20 cm wide dimensions were washed with hydrogen peroxide, then, rinsed with distilled water and hanged for drying. Before the inoculation, reactors were sealed with an impulse sealer (PCS-400, Brother, Texas, USA), sterilized with UVC for one hour, then, septum, exhaust and diffuser hoses, which were autoclaved before, were added on with a hot silicone gun. Autoclaved media was loaded with a funnel from the hole that was pierced for the diffuser hose.

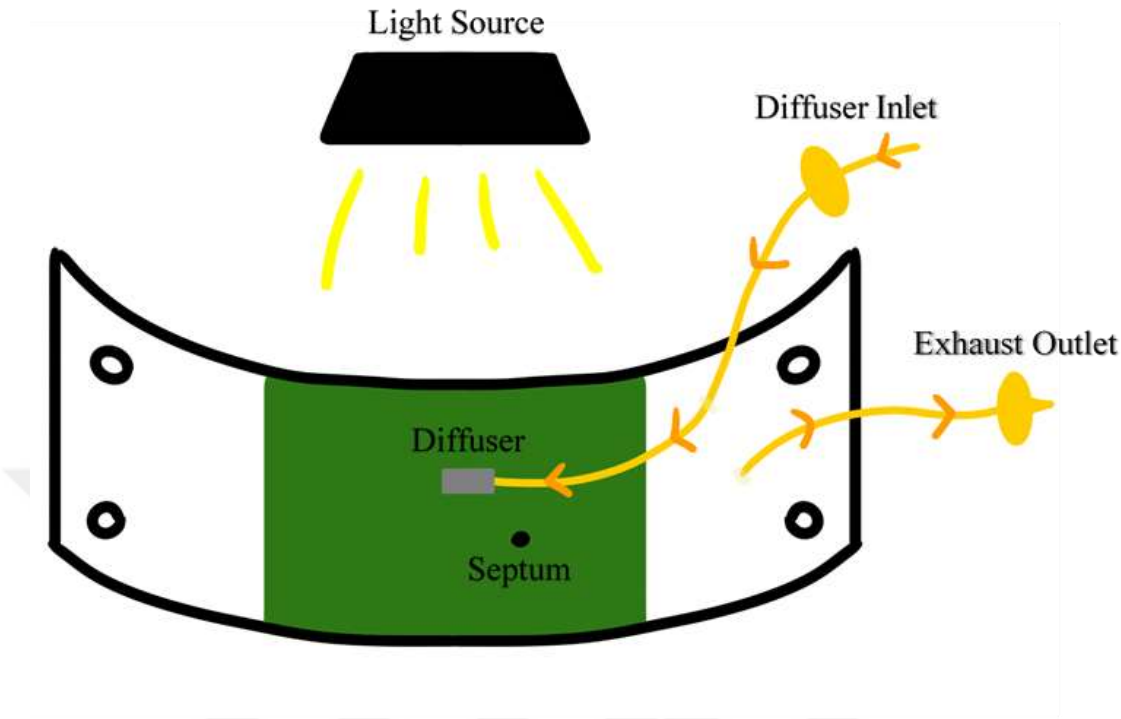


Figure 3.9. Design of plastic PBRs.

After loading, sealing, and hanging the reactor, the biomass seed was inoculated into the reactor through the septum. Plastic PBRs were hanged on a custom setup that enabled them to get direct light from above (Figure 3.10).





Figure 3.10. Plastic PBRs with FWW (top row), plastic PBRs with control medium (bottom row).

3.4.2. Cultivation of Microalgae in Plastic Photobioreactors

The PBRs were started at a cell concentration of 10^7 cells/mL, inoculated with cultures at mid-exponential phase (Figure 3.10). Air flow to the reactors was 0.5 L/min, supplied from a dry air compressor (DMD75C, Sönmez, Turkey) and passed through 0.45 µm air filter. Incoming air was dispersed into the liquid growth medium with an aquarium air diffuser stone and constant air pressure was ensured with air flowmeters. An exhaust outlet was also secured with 0.45 µm air filter to ensure axenic growth conditions. Reactors were placed onto an orbital shaker (3017, Lauda Gesellschaft für Labortechnik mbH, Germany) operating at 70 rpm and illuminated atop with a LED daylight source providing 8000 lux.

In total, there were six PBRs operated in batch mode with a working volume of 2 L, three of them were allocated for FWW treatment, and the rest were control (Figure 3.10). Treatment reactors were fed with FWW diluted with distilled water at 1/4 ratio and control reactors were fed with MB3N culture medium. Daily samples were taken through the septa located on top of the PBRs. Reactors were spiked with 100 µL of polyethylene glycol (202339, Sigma, Germany) to eliminate the foams that were generated due to the bubbling of the organic content in FWW.

3.4.3. Nutrient Removal in Plastic Photobioreactors

Total phosphorus and TN parameters were measured daily to determine the nutrient removal efficiency of microalgae from FWW. For TP determination, peroxodisulfate digestion method was followed in couple with ascorbic acid and ammonium molybdate spectrophotometry (Staff, 2004). For TN determination, peroxodisulfate digestion method coupled with vanadium trichloride spectrophotometry procedure was followed (Koistinen et al., 2019).

3.4.3.1. TP determination in plastic PBRs. International standard of the determination of phosphorus with ammonium molybdate spectrophotometric method was used for the daily nutrient removal profiling (Staff, 2004). In principal, orthophosphate ions react with antimony and molybdate ions and form antimony phosphomolybdate complex in acidic medium (Staff, 2004). Reducing this complex with ascorbic acid results in molybdenum blue complex. Absorbance of the solution gets measured to determine the concentration of orthophosphate in wastewater. Most of the organophosphorus compounds are digested with peroxodisulfate and converted into orthophosphate (Staff, 2004).

All glassware used in the experiments were soaked in acid bath for at least 2 days, rinsed with UPH_2O and dried at 105°C for at least an hour. Samples were stored at 4°C with 1 mL 4.5 mol/L sulfuric acid (pH≅1) per 100 mL of test sample until the day of analyses. 5 mL of samples were pipetted into a 100 mL Erlenmeyer flask and diluted to approximately 40 mL. 4 mL of 50 g/L potassium peroxodisulfate solution was added into the samples and boiled gently for 30 mins. Samples were cooled down to room temperature and pH was adjusted to approximately 3.4 with 2 M sodium hydroxide solution and 2 M sulfuric acid. Samples, then, diluted to 40 mL and transferred into 50 mL volumetric flasks. Standards and blanks were treated just as samples. 1 mL of ascorbic acid solution was added, and flasks were swirled for 30 seconds. Two mL of acidic molybdate solution was added, and samples were diluted to the mark of 50 mL volumetric flasks. The colorization process of the samples was completed in 10-15 minutes. Absorbance values of the samples were taken at 880 nm in quartz cuvettes with a UV-Visible spectrophotometer (DR3900, Hach, Colorado, USA).

Orthophosphate standard stock solution was prepared by dissolving 0.2197 g of pre-dried (at 105°C to constant weight) potassium dihydrogen phosphate (KH_2PO_4) in 800 mL UPH_2O and diluted to 1 L in a volumetric flask. 20 mL of orthophosphate stock solution was diluted with 480 mL of UPH_2O and used as a working standard solution with a concentration of 2 mg/L PO_4-P. Calibration curve standards that are prepared with the working standard solution are given in Table 3.5. Orthophosphate concentrations

of unknown samples were calculated by using the equation of the calibration curve (Figure 3.11, Equation 3.2)

Table 3.5. Concentrations of calibration curve standards of KH_2PO_4 standard.

#	Working Standard Solution Volume (mL)	Final Concentration (mg/L PO_4 - P)	Absorbance at 880 nm
1	0	0.00	0.002
2	1	0.04	0.030
3	2	0.08	0.060
4	3	0.12	0.082
5	4	0.16	0.116
6	5	0.20	0.150
7	6	0.24	0.177
8	7	0.28	0.206
9	8	0.32	0.237
10	9	0.36	0.257
11	10	0.40	0.292

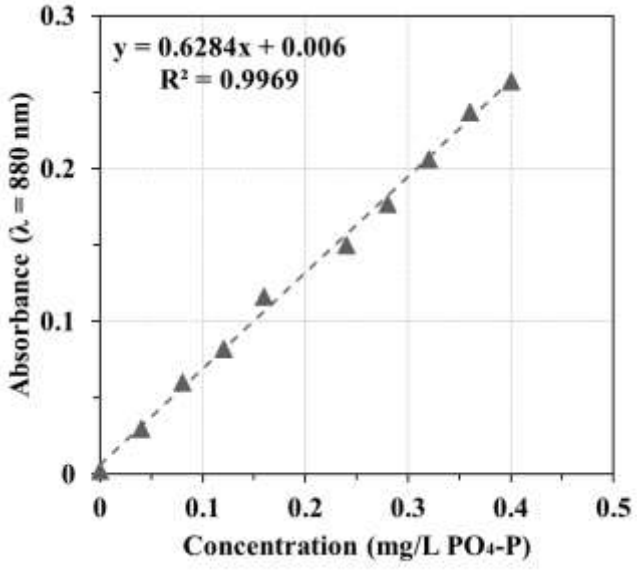


Figure 3.11. Calibration curve of KH_2PO_4 standard.

$$y = 0.6284x + 0.006 \quad (3.2.)$$

where,

y = Absorbance of sample at 880 nm

x = Orthophosphate concentration in the sample

3.4.3.2. TN determination in plastic PBRs. Total nitrogen determination by a spectrophotometric method developed by (Koistinen et al., 2019) was used for the determination of nitrogenous species in the wastewater effluent. Total nitrogen consists of nitrates, nitrites, ammonia, dissolved and particulate organic nitrogen. In principle, the method enables nitrogenous compounds to be oxidized into nitrate in an alkaline medium. Then, nitrate is reduced to nitrite with acidic vanadium chloride reagent. Amines in the acidic vanadium chloride reagent form an azo dye that can be measured with the help of spectrophotometry (Koistinen et al., 2019).

All glassware used in the experiments were soaked in acid bath for at least two days, rinsed with UPH_2O and dried at 105°C for at least an hour. Oxidizing reagent solution was prepared by dissolving 10 grams of potassium peroxodisulfate and 6 grams of boric acid in 1 L of 0.075 M sodium hydroxide solution. TN mix was prepared by mixing solutions of sulfanilamide, N-(1-naphtyl)-ethylenediamine dihydrochloride (NED) and vanadium trichloride together with a ratio of 1:1:5 respectively. Sulfanilamide solution was prepared by dissolving 1 gram of sulfanilamide in 85 mL ofUPH_2O mixed with 14.5 mL concentrated hydrochloric acid. NED solution was prepared by dissolving 0.07 grams of NED in 100 mL UPH_2O. Vanadium trichloride solution was prepared by dissolving 1.6 grams of vanadium trichloride in 170 mL of UPH_2O and 16.8 mL conc. hydrochloric acid mixture, then UPH_2O was added to a total final volume of 200 mL. Calibration curve standards and blanks were treated as samples. Five mL of sample and five mL of oxidizing reagent solution were pipetted into 20 mm screw capped reaction tubes and autoclaved for 30 minutes at 121°C. After the digestate cooled down to room temperature, 0.5 mL of digested samples and three mL of TN mix were pipetted into new reaction tubes, mixed, and incubated for one hour at 45°C. Absorbance values of the samples were taken at 545 nm with a UV-Visible spectrophotometer (DR3900, Hach, Colorado, USA) in disposable polystyrene cuvettes with a light path of 10 mm.

Total nitrogen standard that contains 140 µg/mL N was prepared by dissolving 0.1862 grams of disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA) in 100 mL. Working solutions of 50, 70, 100, 140, 200, 280, 400 and 560 µg/L were prepared from TN standard on the day of analyses for the

calibration curve (Figure 3.12). Equation 3.3 was used for the calculations of total nitrogen concentrations of samples.

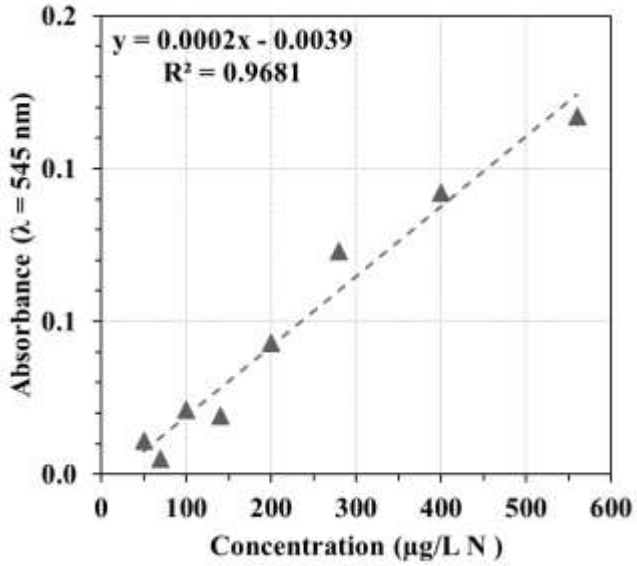


Figure 3.12. Calibration curve of Na_2EDTA.

$$y = 0.0002x - 0.0039 \quad (3.3.)$$

where,

y = Absorbance of sample at 545 nm

x = Total nitrogen concentration of sample

3.4.4. Biochemical Characterization of Harvested Biomass

Harvested biomass was characterized for its nutritional profiling, in order to determine if selected microalgae species is suitable for animal and aquaculture feed production after going through wastewater treatment processes or not. In this section of the study, methods of total proteins, carbohydrates and lipids are elaborated. In addition, fatty acid methyl ester (FAME), vitamin, and amino acid profiling of the microalgal biomass are expressed in detail.

3.4.4.1. Total protein analysis. For the total protein analysis of the biomass, alkaline extraction developed by Safi et al. and quantification with Lowry assay was coupled (Lowry, et al., 1951; Safi et al., 2013). Lowry reagent A was prepared by dissolving 2 g of anhydrous sodium carbonate in 100 mL of 0.1 N sodium hydroxide prepared with UPH_2O. Lowry reagent B was prepared by dissolving 1 g of Na K tartrate tetrahydrate in 100 mL UPH_2O. Lowry reagent C was prepared by dissolving 0.5 g of $CuSO_4.5H_2O$ in 100 mL in UPH_2O. In the day of the analysis, Lowry reagents A, B and C were mixed with a 48:1:1 ratio respectively to prepare Lowry reagent D.

The hydro-soluble protein content of microalgae was extracted from freeze-dried biomass. For 30 mg biomass, 1.5 mL of UPH_2O of pH 12 (adjusted with 2N sodium hydroxide) was added in 2 mL polypropylene microtubes (Sarstedt). Vials were incubated in a digital precise shaking water bath (MaxTurdy-30, Daihan Scientific, South Korea) at 40 °C for 1 hour and vortexed every 10 minutes. Vials were centrifuged at 5000X g for 10 minutes and 200 µL of supernatants were transferred into clean new polypropylene microtubes (Sarstedt). 1 mL of Lowry reagent D was added into each vial, vortexed and incubated for 10 minutes. After the incubation was complete, 100 µL od 1 N Folin-Ciocalteu's phenol reagent was added into the vials, vortexed and then, incubated for 30 minutes. Absorbance values of samples were measured at 750 nm and inserted into the calibration curve equation produced from standard solution of bovine serum albumin (BSA) (Sigma P536) with a concentration range of 0 to 3 mg/mL. Calibration curve standards' concentrations prepared with 5 mg/mL stock are given in Table 3.6 and calibration curve produced from BSA standard is shown in Figure 3.13. Hydro-soluble protein contents of the microalgal biomass were determined by using Equation 3.4.

Table 3.6. Concentrations of calibration curve standards of BSA standard.

#	UPH_2O Volume (µL)	Stock BSA Volume (µL)	Final Concentration (mg/mL)
1	200	0	0.0
2	180	20	0.5
3	160	40	1.0
4	140	60	1.5
5	120	80	2.0
6	100	100	2.5
7	80	120	3.0

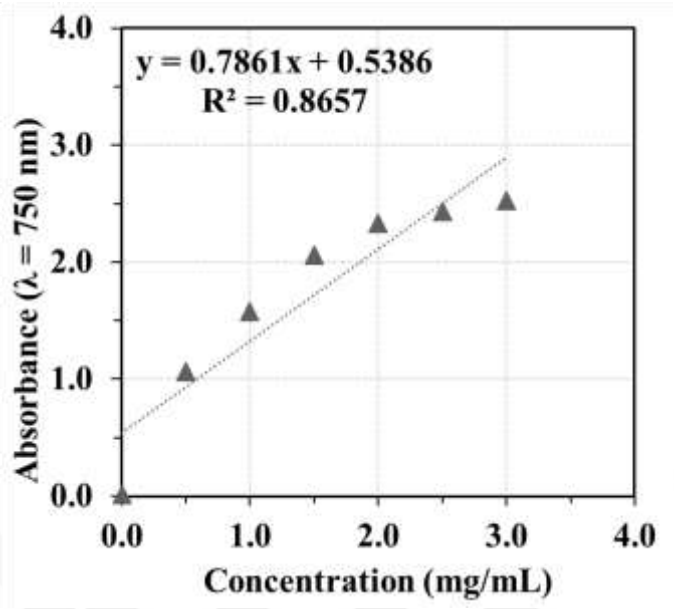


Figure 3.13. Calibration curve of BSA.

$$y = 0.7861x + 0.5386 \quad (3.4.)$$

Where,

y = Absorbance of sample at 750 nm

x = Concentration of hydro-soluble protein in the solution.

3.4.4.2. Total carbohydrate analysis. Total carbohydrate content of biomass was determined by using the two-step sulfuric acid hydrolysis and soluble carbohydrate determination by spectrophotometry method developed by (Van Wychen and Laurens, 2020). This method was chosen instead of commonly used phenol sulfuric acid method because of its consistency under visible spectroscopy when 3-methyl-2-benzothiazolinone hydrazine (MBTH) dye is used as colouring agent, which gives close response for different aldehyde sugars in the microalgal carbohydrate extract (Van Wychen and Laurens, 2020).

0.5 M 200 mL sodium hydroxide solution, 3 mg/mL MBTH solution, 1 mg/mL dithiothreitol (DTT) solution, 0.25 M 200 mL hydrochloric acid solution and ferric solution that consists of 200 mg 0.5% ferric ammonium sulfate dodecahydrate, 200 mg 0.5% sulfamic acid and 0.25 M 40 mL hydrochloric acid reagents were prepared before experiments as instructed by Van Wychen and Laurens, 2020.

Prior to sample preparation, tare weights of 10 mL screw capped glass tubes were recorded. Biomasses of each plastic PBR were homogenized with porcelain mortar and pestle. 25 ± 2.5mg lyophilized microalgal biomass were weighed in pre-weighed 10 mL screw capped glass tubes. Biomass were dried in oven at 40°C overnight and dehumidified in glass vacuum desiccator (Isolab, Germany) for 24 hours. Following dehumidification, moisture contents of oven-dried microalgal biomass were measured. Ten percent of moisture content for microalgal biomass was ensured.

For the hydrolysis part, 250 µL of 72% (w/w) sulfuric acid solution was added into each glass tube and incubated in water bath for 1 hour at 30 ± 3°C and vortexed every 10 minutes. Following incubation, 7 mL UPH_2O was added into glass tubes to obtain a final concentration of 4% sulfuric acid in the solution. Samples were sealed and autoclaved at 121°C for 1 hour in liquid mode. Samples were allowed to cool down to room temperature and filtered through 0.2 µm nylon syringe filters with disposable and sterile syringes.

Calibration curve standards were prepared with a glucose standard stock solution that was prepared by dissolving 25 mg GC grade glucose with ≥ 99.5% purity (Sigma G8270, Germany) in 100 mL UPH_2O. Glucose standard solution with 0.25 mg/mL concentration was diluted to the concentrations as shown in Table 3.7.

Table 3.7. Concentrations of calibration curve standards of glucose standard.

#	**UPH_2O Volume (µL)**	**Stock Glucose Volume (µL)**	**Final Concentration (mg/mL)**
1	500	0	0.0000
2	480	20	0.0100
3	470	30	0.0150
4	450	50	0.0250
5	425	75	0.0375
6	400	100	0.0500

Hydrolysates from samples were diluted to 1:10 and 1:20 with 50 µL and 25 µL of filtered hydrolysates respectively to a total volume of 500 µL. For quality control test, calibration verification standard (CVS) was prepared by dissolving 75 µL in 425 µL of UPH_2O. Preparation of MBTH working solution was done, just before adding it to the samples, by mixing 3 mg/mL MBTH and 1 mg/mL DTT in a 1:1 (v/v) ratio. First, 500 µL of 0.5 M NaOH, then, 500 µL of MBTH working solution was added

into each glass tube. Glass tubes were vortexed and placed into a dry thermostat (LT-200, Hach, Colorado, USA) pre-heated to 80°C for 15 ± 1 minutes, immediately after the reagents were added. Following the incubation, dry thermostat was turned off, 1 mL of ferric solution was added immediately while the glass tubes were still in the thermostat. When the reaction with ferric solution was completed, 2.5 mL UPH_2O was added into each tube and vortexed. Absorbance values of samples were measured with a UV-Visible spectrophotometer (DR3900, Hach, Colorado, USA) at 620 nm in disposable polystyrene cuvettes with a light path of 10 mm. Concentration of the monomeric sugars in the biomass were calculated according to the Equation 3.5 that was derived from the calibration curve (Figure 3.14).

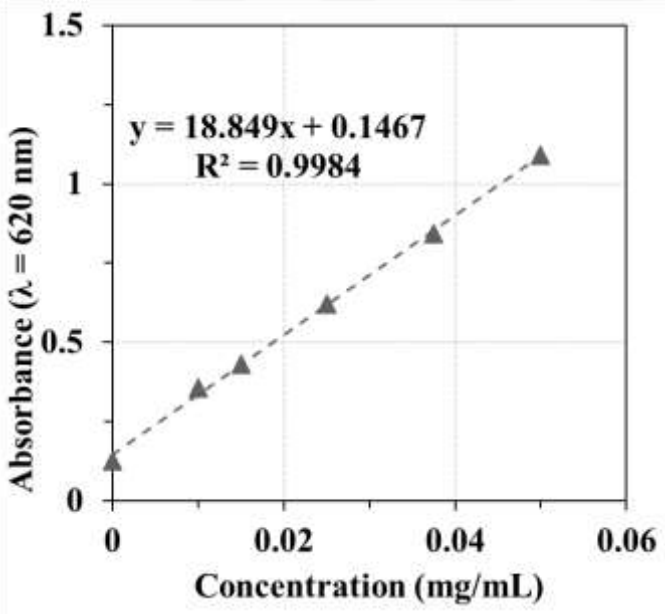


Figure 3.14. Calibration curve of glucose standard.

$$y = 18.849x + 0.1467 \quad (3.5.)$$

where,

y = Absorbance of sample at 620 nm

x = Concentration of monomeric sugars in the solution.

3.4.4.3. Total lipid analysis. Lipid extraction and quantification of microalgal biomass was done by a cell disruption method followed by a gravimetric analysis developed by Breuer et al. (Breuer et al., 2013). 300 mg of 0.1 mm and 100 mg of 0.5 mm diameter glass beads were weighed in polypropylene microtubes (Sarstedt) and 50±1 mg of lyophilized microalgal biomass from each plastic PBR were weighed separately in the microtubes. 50 mg/L of nonadecanoic acid (C19:0) (Sigma N5252) was dissolved in chloroform:methanol 4:5 (v/v) as an internal standard stock solution. 1 mL of internal standard solution was added into each microtube. Prior to bead beating, glass beads were kept in 1 M HNO_3 bath overnight in order to remove impurities. Glass beads were rinsed with deionized water (DIW) five times, dried in an oven (Daihan Scientific, South Korea) at 105°C overnight and stored in a desiccator. Bead beating was done at 4500 rpm at 10°C with six cycles of 60 second intervals with a pause of 15 seconds in a sample homogenizer (Precellys Evolution with Cryolys Unit, Bertin Technologies, France). Following homogenization, extracts were poured into 50 mL glass centrifuge tubes with Teflon inset screw caps. Microtubes that were used in bead beating were washed three times with 1 mL chloroform:methanol 4:5 (v/v) and poured into glass tubes. Extracts were vortexed and kept in sonication bath (Sonorex Super RK 102 H, Bandelin, Germany) for 10 minutes. A solution of 50 mM Tris and 1 M NaCl was prepared with DIW, pH of the solution was set to 7 by using 1 N hydrochloric acid and 2.5 mL of that solution was added into glass centrifuge tubes to ensure the transfer of lipids into chloroform layer. Glass tubes were vortexed and sonicated for 10 minutes and centrifuged for 5 minutes at 1200X g. Clean glass vials were weighed for the gravimetric analysis. Chloroform layer (bottom layer) was transferred into pre-weighed clean glass vials with a plastic bulb and a glass Pasteur pipette. For full recovery of lipids, solvent extraction part was repeated with chloroform for several times. Following, extraction, chloroform was evaporated under N_2 gas flow. When the evaporation was complete, glass vials were weighed for the determination of total lipid content of microalgal biomass gravimetrically. Mass of extracted lipid was calculated by subtraction of tare mass of glass vials from dried lipid extract in glass vials and percent lipid content was calculated as shown in Equation 3.6. Extracts were stored at -20°C until FAME analysis.

$$\% = \frac{L\ x\ 100}{M} \tag{3.6.}$$

where,

% = Percent lipid content in biomass

L = Mass of lipid extract (mg)

M = Mass of biomass weighed (mg)

3.4.4.4. Fatty acid methyl ester analysis. In order to determine the fatty acid profile of microalgal biomass, extracted lipids were subjected to transesterification step. 50 mL methanol containing 5% (v/v) sulfuric acid was prepared, 3 mL of this solution was added into each glass vial after the drying step, and vials were kept in water bath (MaxTurdy-30, Daihan Scientific, Wonju, South Korea) at 70°C for 3 hours and vortexed every 30 minutes. Following incubation, samples were cooled down to room temperature, 3 mL of DIW and 3 mL of hexane was added to each tube. Test tubes were vortexed and kept in test tube rotator (Wisemix RT-10, Witeg, Wertheim, Germany) for at 15 rpm 15 minutes. Samples were centrifuged at 1200X g for five minutes and 2 mL of hexane layer (top layer) were collected from each sample and transferred into clean glass vials. 2 mL of DIW was added to each collected part of samples as a washing step. Tubes were vortexed, centrifuged at 1200X g for 5 minutes and hexane layers were transferred into GC vials for Gas Chromatography Analysis.

Qualification and quantification of FAMEs were done on a gas chromatography system (GC-2014, Shimadzu, Japan) with a flame ionization detector (FID). FAME samples were injected into the instrument via autosampler (AOC-20i, Shimadzu, Japan). SBL-IL11 polar ionic liquid column (Sigma) with 100 m length x 0.25 mm diameter x 0.20 µm film thickness was used for the analyses. Oven temperature was initially 140°C for 5 minutes, then, gradually increased with 8°C/min rate to 180°C, and gradually increased with 5°C/min rate to 260°C. Detector (FID) temperature was at 260°C, carrier gas (H_2) flow was set to 40 cm/sec, and 1 µL of injection was done with 100:1 split ratio. A certified reference material (CRM47885, Supelco, Inc., Bellefonte, Pennsylvania, United States) was used as a calibration curve standard and concentrations of the standards are given in Table 3.8. Certified reference material included 37 components, however, only six components were included in this section as they were qualified in the microalgal biomass. Calibration curves of six components are given in figures 3.15 to 3.20. Representative chromatogram for FAME analysis is given in Figure 3.21.

Table 3.8. Concentrations of certified reference materials used in calibration curves.

Fatty Acid Name	Certified Value (µg/mL)	Analytical Value (µg/mL)	CAS Number
Methyl palmitate (C16:0)	601 ± 15	606	112-39-0
Methyl stearate (C18:0)	401 ± 9	402	112-61-8
Cis-9-oleic acid methyl ester (C18:1n9c)	401 ± 14	404	112-62-9
Linolelaidic acid methyl ester (C18:2n6t)	201 ± 9	202	2566-97-4
Methyl linoleate (C18:2n6c)	201 ± 10	205	112-63-0
Methyl linolenate (C18:3n3)	201 ± 10	208	301-00-8

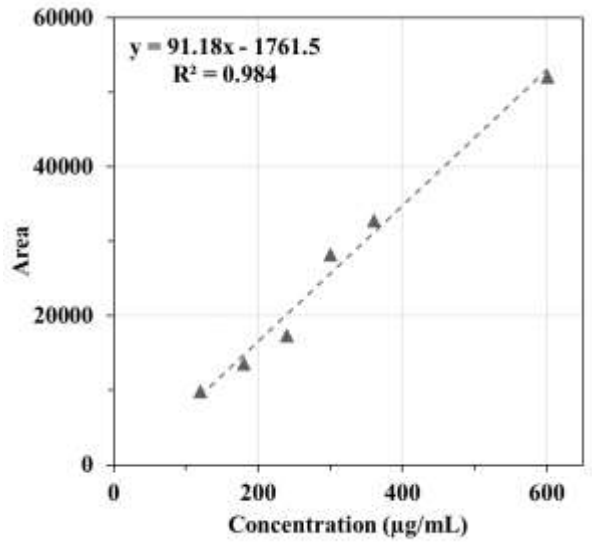


Figure 3.15. Calibration curve of C16:0

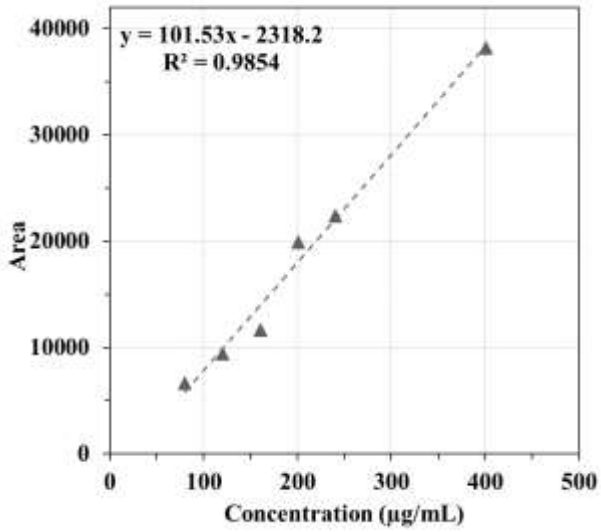


Figure 3.16. Calibration curve of C18:0.

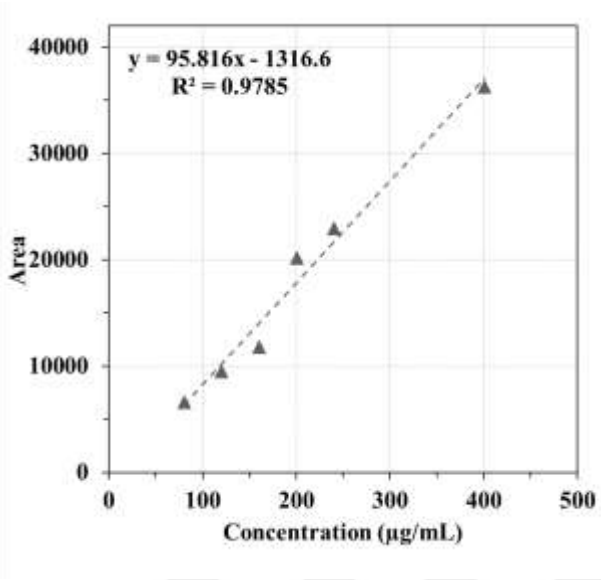


Figure 3.17. Calibration curve of C18:1n9c.

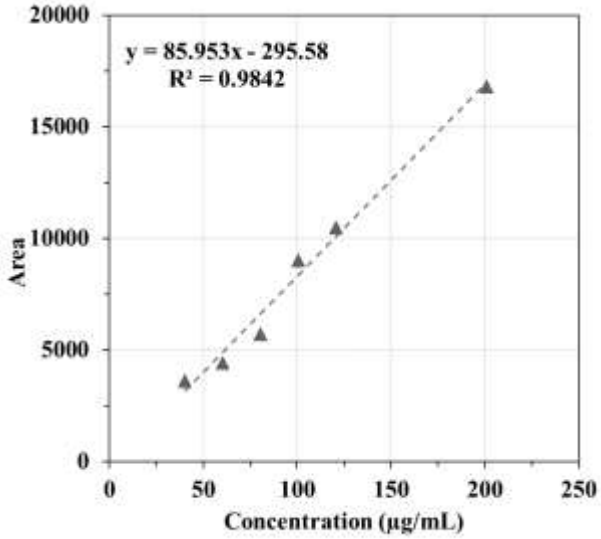


Figure 3.18. Calibration curve of C18:2n6t.

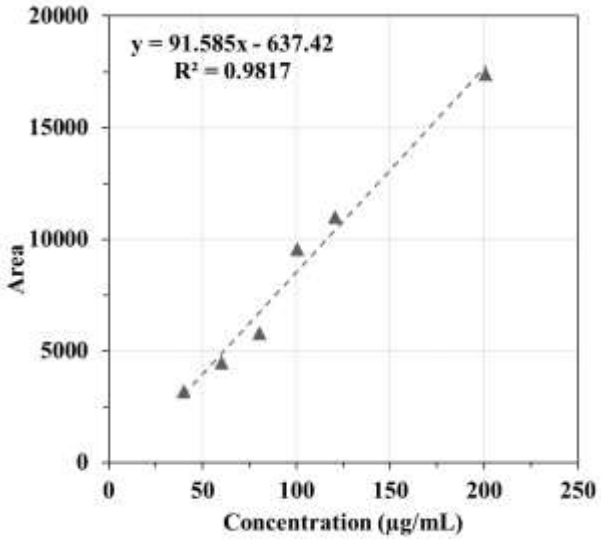


Figure 3.19. Calibration curve of C 18:2n6c.

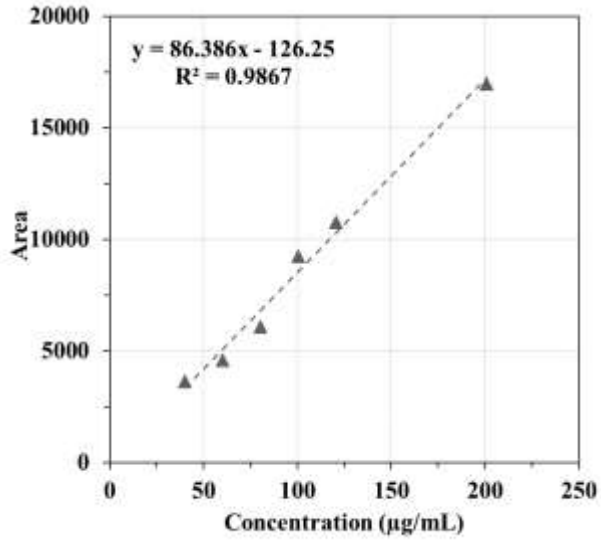


Figure 3.20. Calibration curve of C18:3n3.

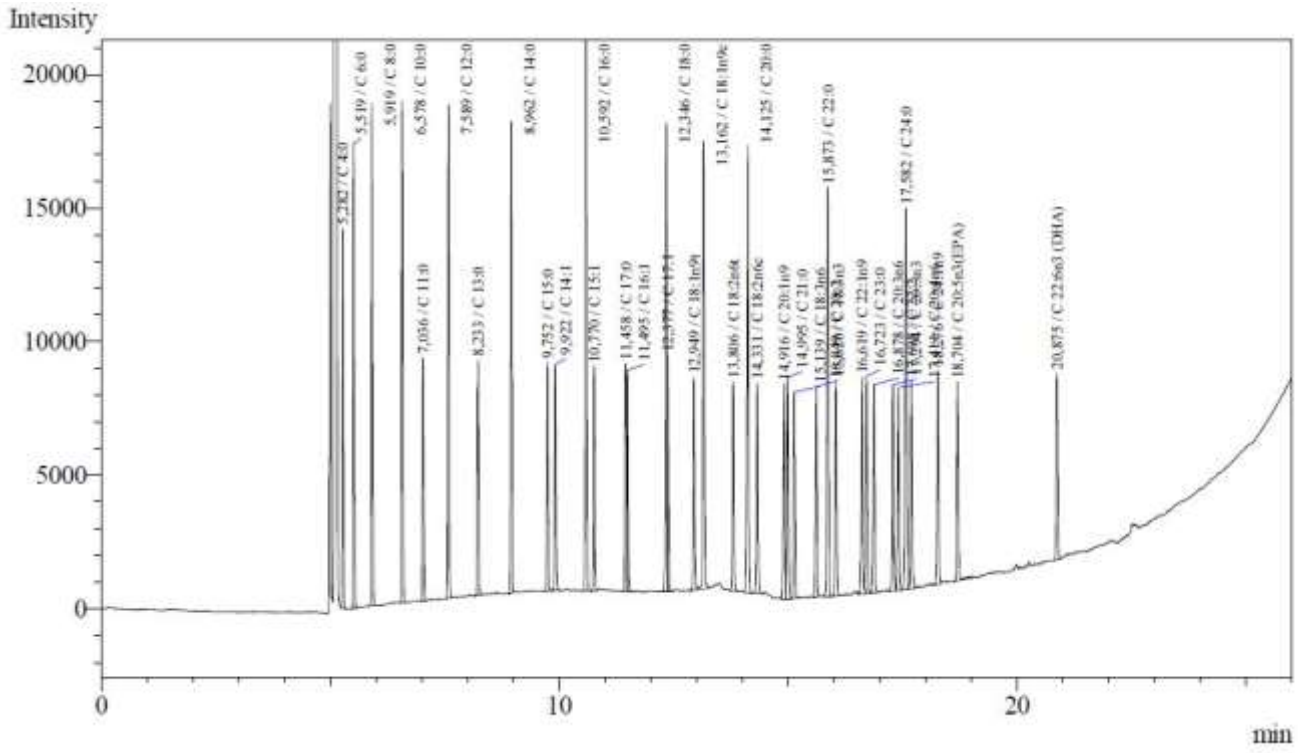


Figure 3.21. Representative chromatogram of FAME compounds.

3.4.4.5. Vitamins analysis. A modified version of the vitamin extraction method of Lock et al. was performed for fat- and water-soluble vitamins in microalgal biomass (Lock & Noestheden, 2013). In order to extract the vitamins, physical cell disruption and chemical extraction methods were used.

For the water-soluble vitamin extraction, 150 ± 1 mg of 0.1 mm and 50 ± 1 mg of 0.5 mm glass beads and 125 ± 1 mg microalgal biomass were weighed into polypropylene microtubes (Sarstedt). One mL of UPH_2O were added into each microtube and bead beating was done for six rounds at 4500 rpm with a pause of 15 seconds at 10 °C in a sample homogenizer (Bertin Technologies). Extracts were transferred with the glass beads into 15 mL plastic conical centrifuge tubes (Isolab) and wrapped with aluminum foil to prevent light driven oxidation. Microtubes were washed with one mL of UPH_2O and 2 mL of acetonitrile (ACN). 80 µL of formic acid were added into each conical centrifuge tube and tubes were vortexed briefly. Conical centrifuge tubes were kept in test tube rotator (Wisemix RT-10, Witeg, Wertheim, Germany) at 45 rpm for 10 minutes. Following rotation, tubes were centrifuged at 4500 rpm for 15 minutes at 18°C. Supernatants were filtered through polypropylene 25 mm 0.45 µm pore sized syringe filters (729004, Macherey-Nagel, Düren, Germany). 950 µL of extracts and 50 µL of 10 mg/L cyanocobalamin as an internal standard were added into chromatography vials with screw capped septa

(Isolab). Samples were put into the auto-sampling unit of triple-quadrupole liquid chromatography-mass spectrometer (LC-MS/MS) system (AB SciEX 4500 equipped with Eksigent Ultra LC 110, Toronto, Canada) for comprehensive qualitative and quantitative vitamins analysis.

For fat soluble vitamin extraction, 150 ± 1 mg of 0.1 mm and 50 ± 1 mg of 0.5 mm glass beads and 125 ± 1 mg microalgal biomass were weighed into polypropylene microtubes (Sarstedt). One mL of methanol (MeOH) were added into each microtube and bead beating was done for six rounds at 4500 rpm with a pause of 15 seconds at 10°C in a sample homogenizer (Bertin Technologies). Extracts were transferred with the glass beads into 15 mL plastic conical centrifuge tubes (Isolab) and wrapped with aluminum foil to prevent light driven oxidation. Microtubes were washed with 0.5 mL MeOH and 1 mL hexane for two times. Five mL of additional hexane was added into centrifuge tubes and vortexed for 30 seconds. Tubes were centrifugated at 3000 rpm for 5 minutes. Five mL of the hexane (top) layers were pipetted and placed in new amber glass vials. Hexane in the amber vials was evaporated under N_2 atmosphere. To reconstitute the fat-soluble vitamins, 2 mL of MeOH was added into vials and vortexed for 10 seconds, filtered through polypropylene 25 mm 0.45 µm pore sized syringe filters (729004, Macherey-Nagel, Düren, Germany) and diluted into 1/50 with MeOH as diluent. 950 µL of fat-soluble vitamin extracts and 50 µL of 10 mg/L cyanocobalamin as an internal standard were added into chromatography vials with screw capped septa (N9, ISOLAB, Germany). Samples were injected into a Linear Ion Trap mass spectrometer equipped with a TurboV IonSpray probe connected to ultrahigh performance liquid chromatography system (LC-MS/MS) (AB SCIEX QTRAP 4500 equipped with Eksigent Ekspert UltraLC 110, Framingham, MA, USA) for comprehensive qualitative and quantitative vitamins analysis.

LC-MS/MS analyses were done principally by introducing vitamin standards to the equipment via manual injection with compound optimization feature. For qualitative and quantitative analyses, multiple reaction monitoring (MRM) of vitamin standards and retention times of the analytes were identified (Appendix A.1). Following the introduction of vitamin standards, prepared vitamin mixture of various concentrations, shown in Table 3.9, was injected into the LC-MS/MS to be able to draw calibration curves of the analytes. Calibration curves (Figures 3.22 to 3.32) of vitamins were plotted by using area ratio calculated by peak area of analyte over peak area of internal standard (cyanocobalamin) versus actual concentrations of the analytes. For the LC/MS-MS method, sample injection volume was 10 µL, oven temperature was set to 40°C, aqueous mobile phase was MS grade water (Pump B) with 0.1% FA and organic mobile phase was MS grade MeOH (Pump A) with 0.1% FA. Separation of the analytes

were done using a polar C18 column with dimensions of 50 mm length x 2.1 mm internal diameter and 2.6 µm of particle size (Kinetex C-18, Phenomenex, Torrance, California, USA) The gradient elution with constant 0.6 mL/min flow rate started at pump A fraction of 12% for 1.5 minutes, followed by 75% of pump A for 2 minutes and maintained at 100% of pump A for the rest of the 9.5 minutes. Analytes were detected based on the scheduled MRM was done in positive and negative ionization modes. Instrument control and data acquisition was done using Analyst 1.6.2 (AB Sciex) and peak integration and data analysis were done using MultiQuant 3.0.1 software. Representative chromatogram for vitamins is given in Figures 3.33 and 3.34.

Table 3.9. Calibration curve standard concentrations of vitamins.

Standard	**Concentration of standard (µg/mL)**						
Tag	**Level 1**	**Level 2**	**Level 3**	**Level 4**	**Level 5**	**Level 6**	**Level 7**
Thiamine (B1)	5.0	10.0	25.0	50.0	100.0	250.0	-
Riboflavin (B2)	5.0	10.0	25.0	50.0	100.0	250.0	500.0
Nicotinic Acid (B3)	5.0	10.0	25.0	50.0	100.0	250.0	500.0
Pantothenic Acid (B5)	5.0	10.0	25.0	50.0	100.0	250.0	500.0
Pyridoxine (B6)	5.0	10.0	25.0	50.0	100.0	250.0	500.0
Biotin (B7)	5.0	10.0	25.0	50.0	100.0	250.0	500.0
Folic Acid (B9)	10.0	25.0	50.0	100.0	250.0	500.0	1000.0
Methylcobalamin (B12)	25.0	50.0	100.0	250.0	500.0	1000.0	-
Ascorbic Acid (C)	50.0	100.0	250.0	500.0	1000.0	2000.0	5000.0
β-carotene	50.0	100.0	250.0	500.0	1000.0	2000.0	5000.0
α-tocopherol (E)	1.0	2.5	5.0	10.0	25.0	50.0	100.0

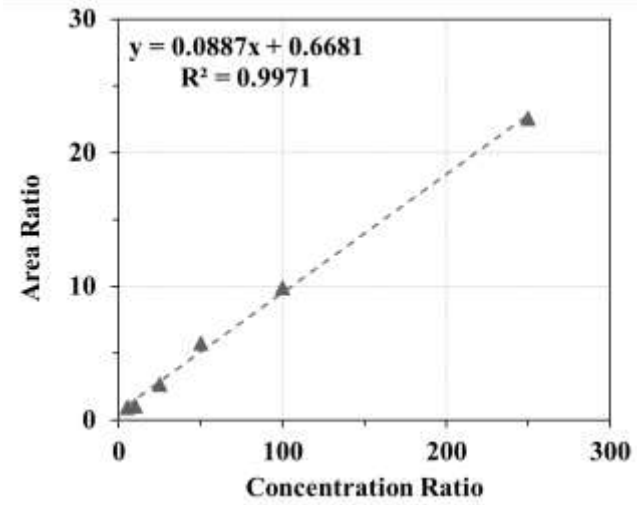


Figure 3.22. Calibration curve of vitamin B1.

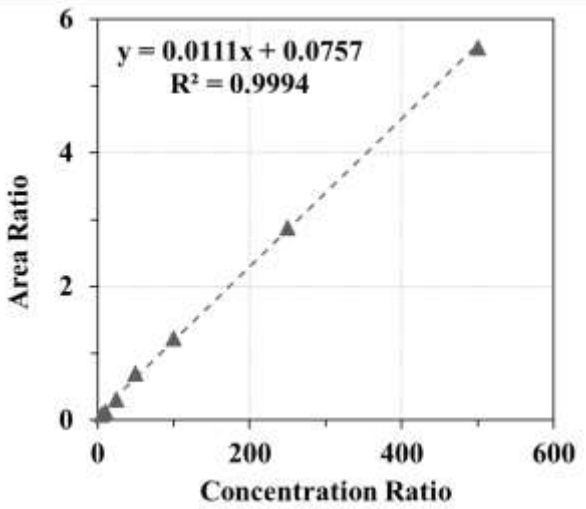


Figure 3.23. Calibration curve of vitamin B2.

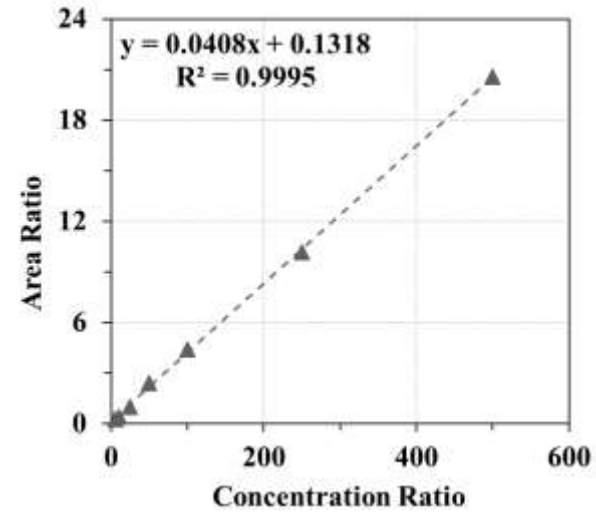


Figure 3.24. Calibration curve of vitamin B3.

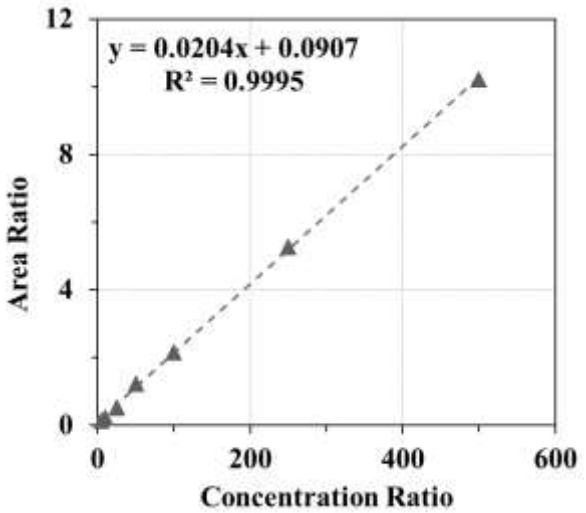
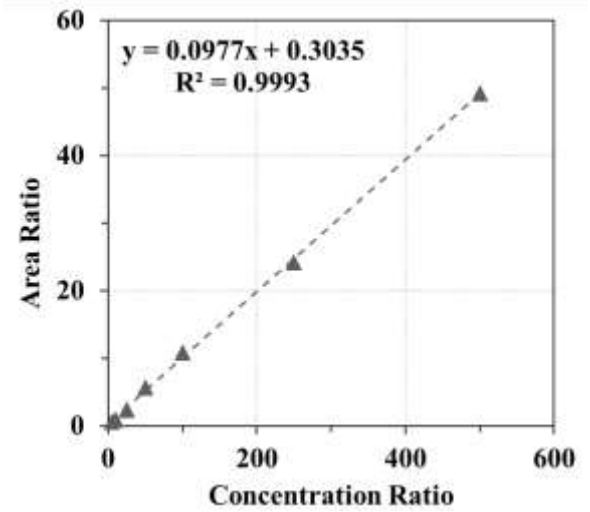


Figure 3.25. Calibration curve of vitamin B5



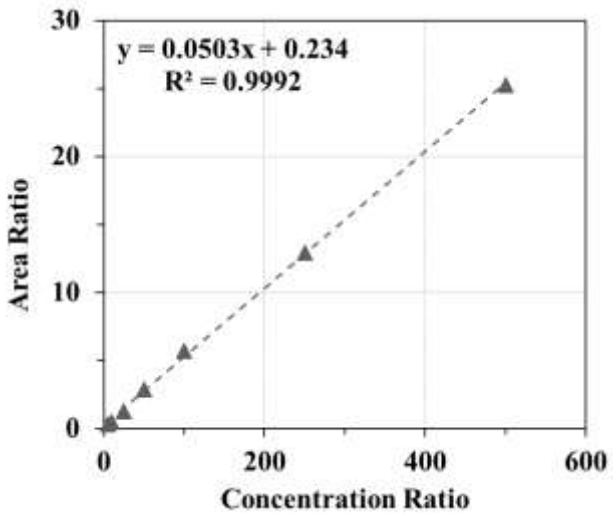


Figure 3.26. Calibration curve of vitamin B6.

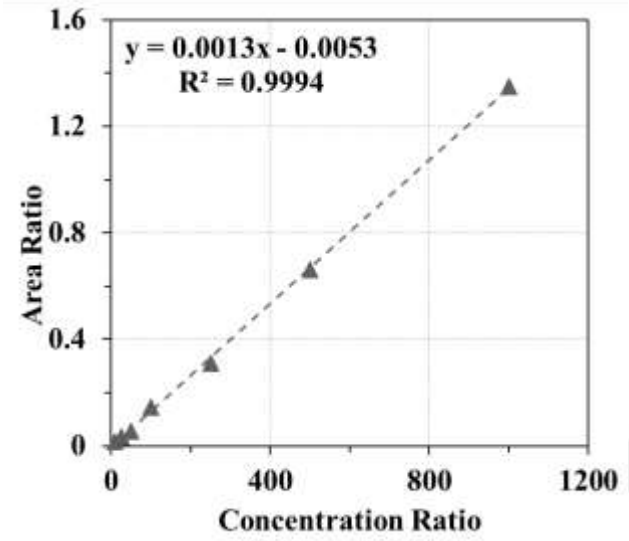


Figure 3.27. Calibration curve of vitamin B7.

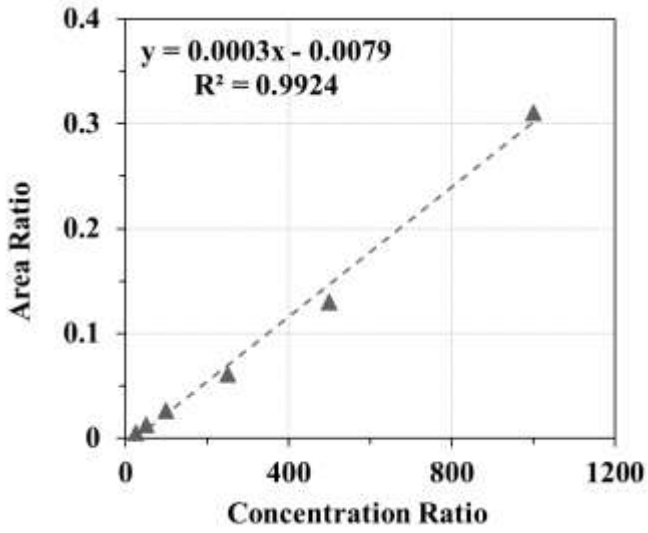


Figure 3.28. Calibration curve of vitamin B9.

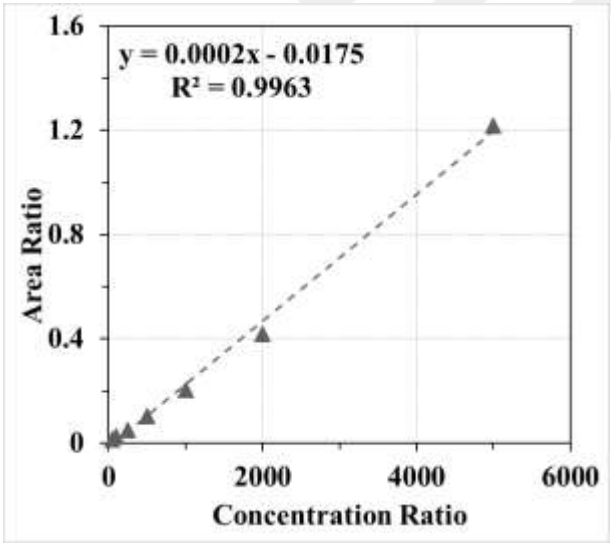


Figure 3.29. Calibration curve of vitamin B12.

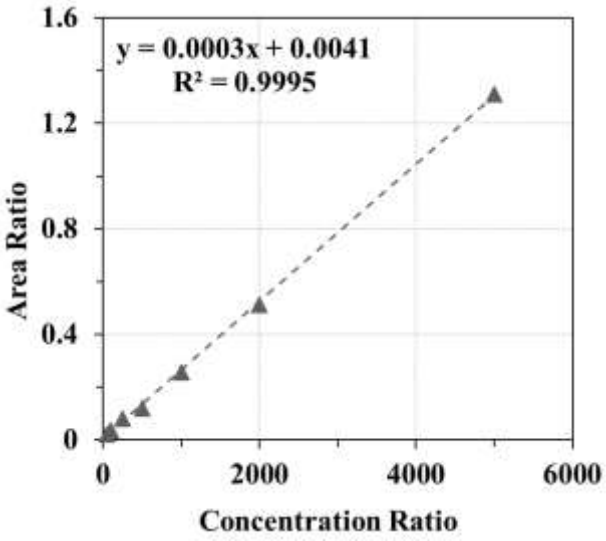


Figure 3.30. Calibration curve of vitamin C.

Figure 3.31. Calibration curve of β-carotene.

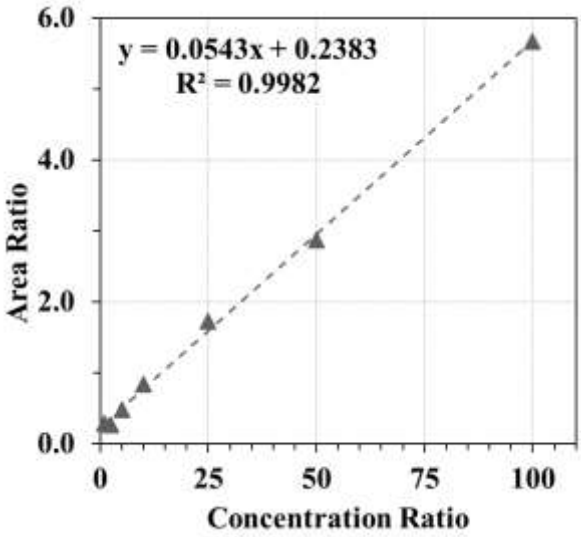


Figure 3.32. Calibration curve of vitamin E.

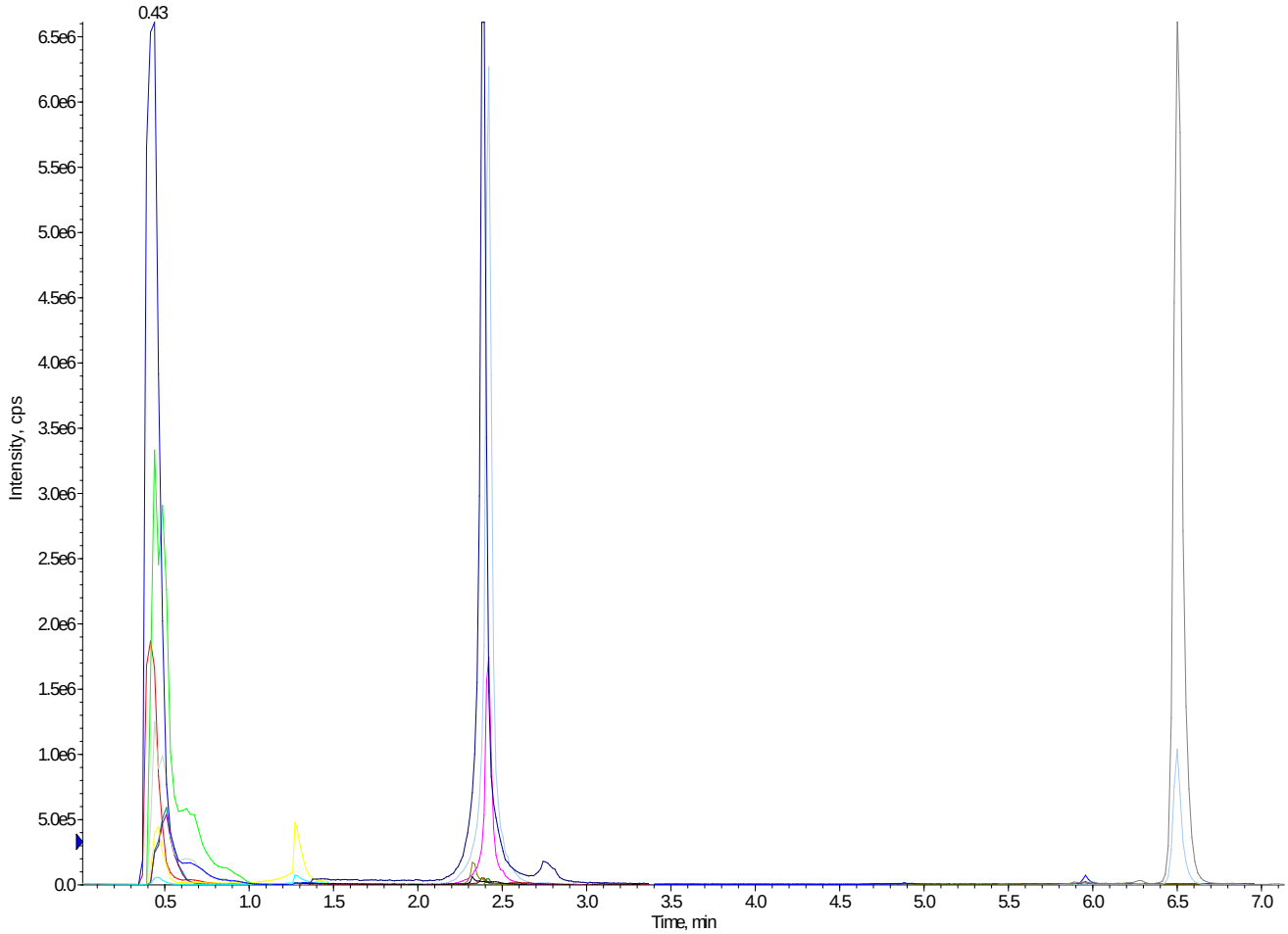


Figure 3.33. Representative positive ionization chromatogram for vitamins.

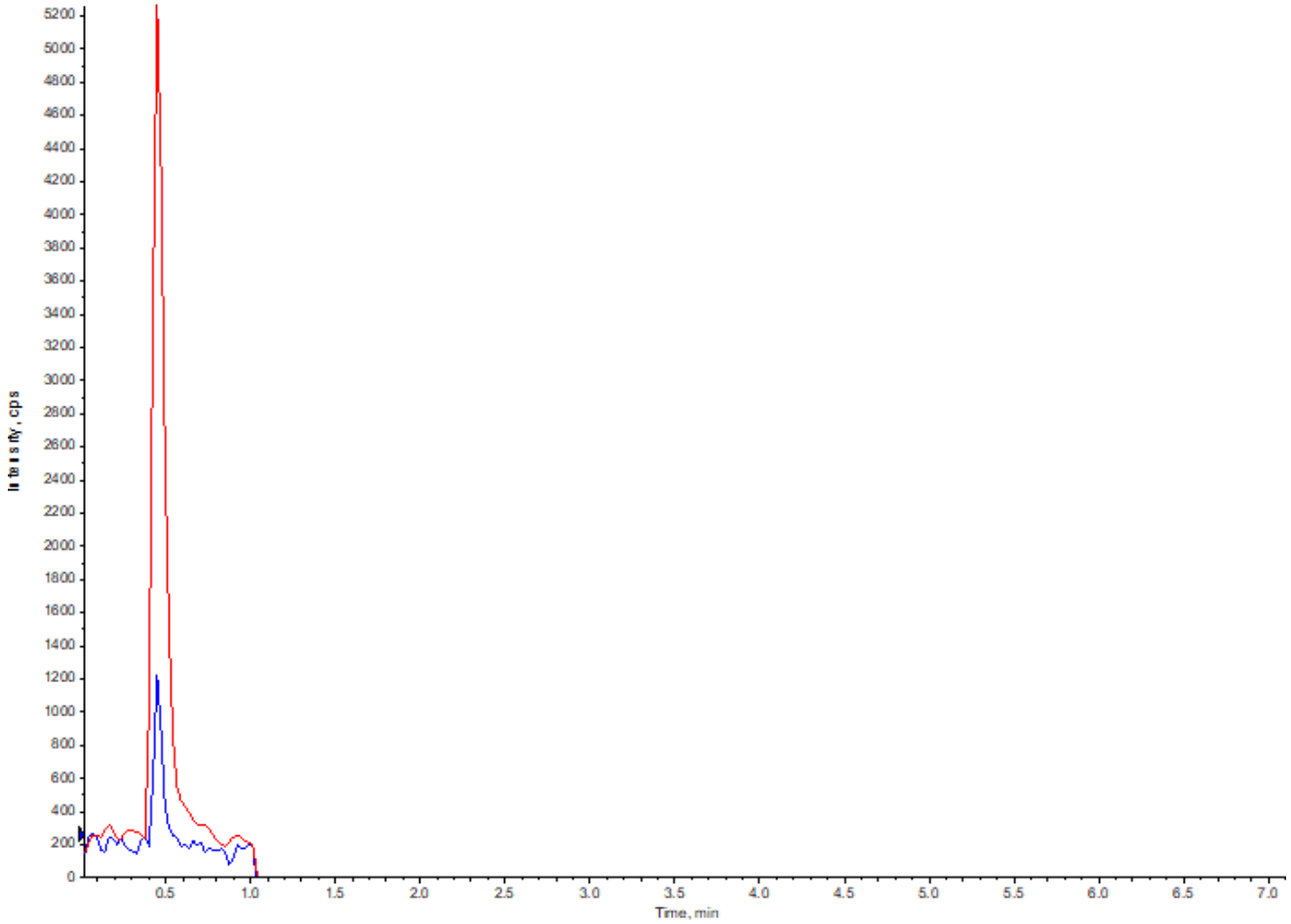


Figure 3.34. Representative negative ionization chromatogram for vitamin C.

3.4.4.6. Amino-acids analysis. Similar extraction techniques in vitamin analyses were used for the amino acids in the microalgal biomass. First, mechanical cell disruption was done and then, chemical extraction was performed.

120 ± 1 mg of 0.1 mm and 40 ± 1 mg of 0.5 mm glass beads and 50 ± 1 mg microalgal biomass were weighed into polypropylene microtubes (Sarstedt). One mL of UPH_2O were added into each microtube and bead beating was done for six rounds at 4500 rpm with a pause of 15 seconds at 10 °C in a sample homogenizer (Bertin Technologies). Extracts were transferred with the glass beads into 15 mL plastic conical centrifuge tubes (Isolab) and wrapped with aluminum foil to prevent light driven oxidation. Microtubes were washed with three mL's of UPH_2O and 4 mL's of ACN 80 µL of FA were added into each conical centrifuge tube and tubes were vortexed briefly. Conical centrifuge tubes were kept in test tube rotator (Witeg) at 45 rpm for 10 minutes. Following rotation, tubes were centrifuged at 4500 rpm for 15 minutes at 18°C. Supernatants were filtered through polypropylene 25 mm 0.45 µm

pore sized syringe filters (Düren). 10 µL amino acid extract, 10 µL 1/20 Stable Isotope Mix (SIM) (NSK-A, Cambridge Isotope Laboratories, MA, United States) as internal standard and 980 µL of 10% MeOH were added into chromatography vials with screw capped septa (Isolab). Amino acids concentrations in the SIM were given in Table 3.10. Samples were injected into a Linear Ion Trap mass spectrometer equipped with a TurboV IonSpray probe connected to ultrahigh performance liquid chromatography system (LC-MS/MS) (AB Sciex) for comprehensive qualitative and quantitative amino acids analysis.

Table 3.10. Concentrations of amino acid standards in SIM.

Reference Standard	Concentration (nmol/mL)
^{15}N; 2-^{13}C-Glycine	2500
$^{2}H_4$-Alanine	500
$^{2}H_8$-Valine	500
$^{2}H_3$-Leucine	500
$^{2}H_3$-Methionine	500
$^{13}C_6$-Phenylalanine	500
$^{13}C_6$-Tyrosine	500
$^{2}H_3$-Aspartate	500
$^{2}H_3$-Glutamate	500
$^{2}H_2$-Ornithine	500
$^{2}H_2$-Citrulline	500
$^{2}H_4$; 5-^{13}C-Arginine	500

LC-MS/MS analyses were done principally by introducing amino acid standards (apart from the SIM) to the equipment via manual injection with compound optimization feature. For qualitative and quantitative analyses, multiple reaction monitoring (MRM) of amino acid standards and retention times of the analytes were identified (Appendix A.2). Following the introduction of the standards, prepared amino acids mixture of various concentrations, shown in Table 3.11, was injected into the LC-MS/MS to be able to draw calibration curves of the analytes. Calibration curves (Figures 3.35 to 3.51) of amino acids were plotted by using area ratio calculated by peak area of analyte over peak area of internal standard (SIM) versus actual concentrations of the analytes. For the LC/MS-MS method, sample injection volume was 10 µL, oven temperature was set to 40°C, aqueous mobile phase was MS grade water (Pump B) with 0.1% FA and organic mobile phase was MS grade MeOH (Pump A) with 0.1% FA. Separation of the analytes were done using a polar C8 column with dimensions of 150 mm length x 3.9

mm internal diameter and 4 µm of particle size (Nova-Pak C8, Waters, Milford, Massachusetts, USA) The gradient elution with constant 0.6 mL/min flow rate started at pump A fraction of 12% for 1.5 minutes, followed by 75% of pump A for 2 minutes and maintained at 100% of pump A for the rest of the 9.5 minutes. Analytes were detected based on the scheduled MRM (sMRM) was done in positive ionization mode. Instrument control and data acquisition was done using Analyst 1.6.2 (AB Sciex) and peak integration and data analysis were done using MultiQuant 3.0.1 software. Representative chromatogram for amino acid analysis is given in Figure 3.52.

Table 3.11. Concentrations of calibration curve standards of amino acids.

Standard	**Concentration of standard (µg/mL)**						
Tag	**Level 1**	**Level 2**	**Level 3**	**Level 4**	**Level 5**	**Level 6**	**Level 7**
Alanine (Ala)	50	100	250	500	1000	-	-
L-Arginine (Arg)	5	10	25	50	100	250	500
Aspartic acid (Asp)	2	5	10	25	50	100	-
Glutamic acid (Glu)	10	25	50	100	250	500	1000
Histidine (His)	2	5	10	25	50	100	-
Isoleucine (Iso)	2	5	10	25	50	100	-
Leucine (Leu)	2	5	10	25	50	100	250
Lysine acetate (Lys)	2	5	10	25	50	100	250
Methionine (Met)	2	5	10	25	50	100	-
L-Ornithine (Orn)	2	5	10	25	50	100	250
Phenylalanine (Phe)	2	5	10	25	50	100	250
Proline (Pro)	5	10	25	50	100	250	-
L-Serine (Ser)	5	10	25	50	100	250	500
Threonine (Thr)	2	5	10	25	50	100	250
Tryptophan (Trp)	2	5	10	25	50	100	250
Tyrosine (Tyr)	2	5	10	25	50	100	250
Valine (Val)	2	5	10	25	50	100	250

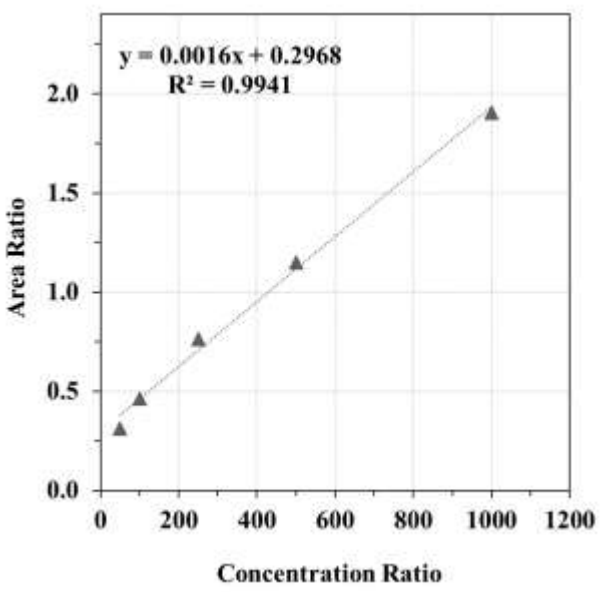


Figure 3.35. Calibration curve of Ala.

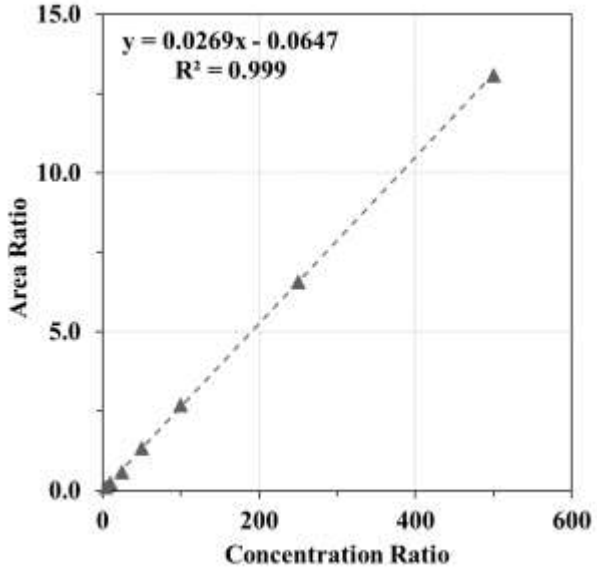


Figure 3.36. Calibration curve of Arg.

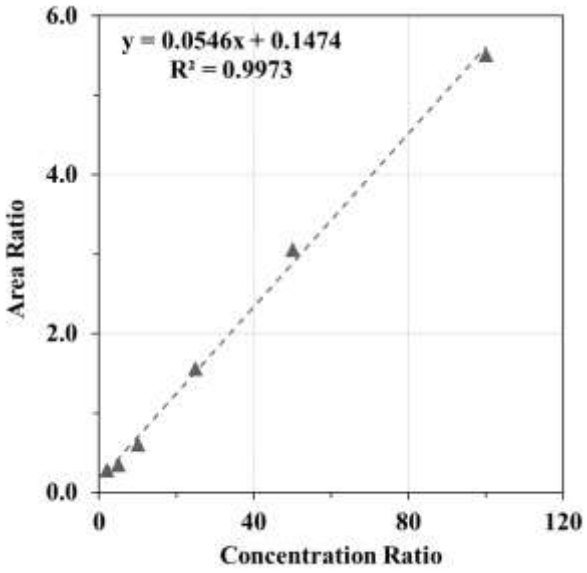


Figure 3.37. Calibration curve of Asp.

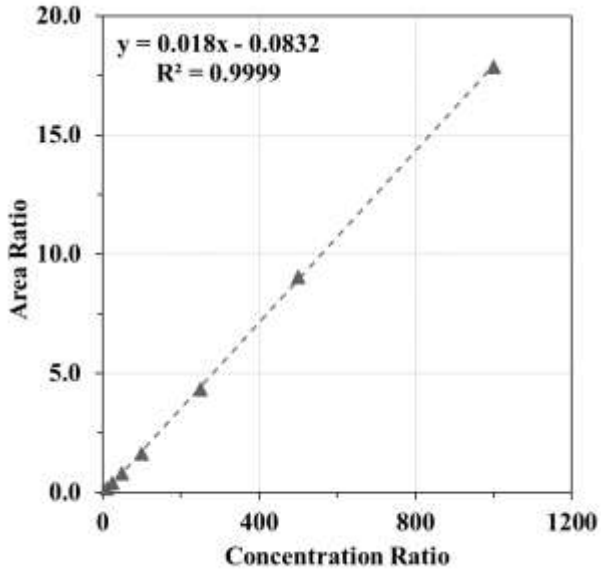


Figure 3.38. Calibration curve of Glu.

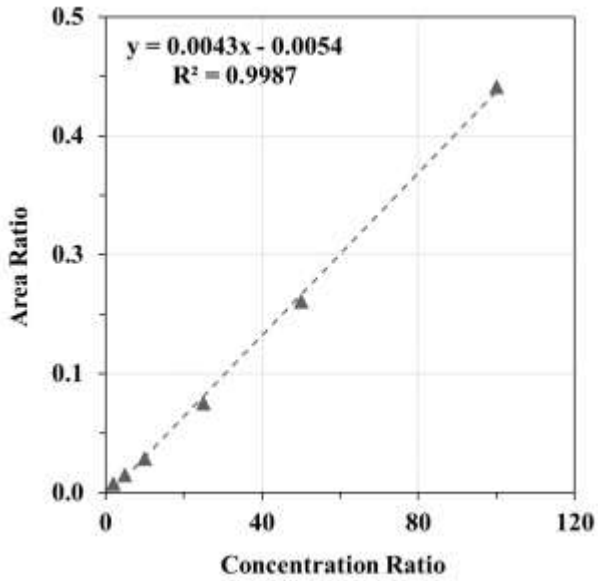


Figure 3.39. Calibration curve of His.

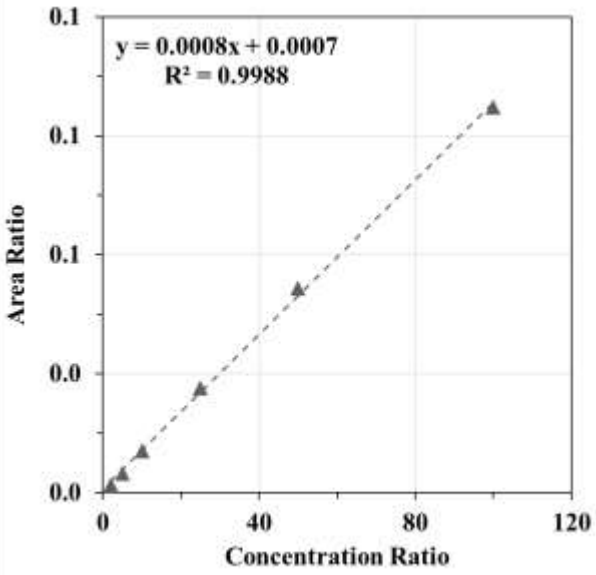


Figure 3.40. Calibration curve of Iso.

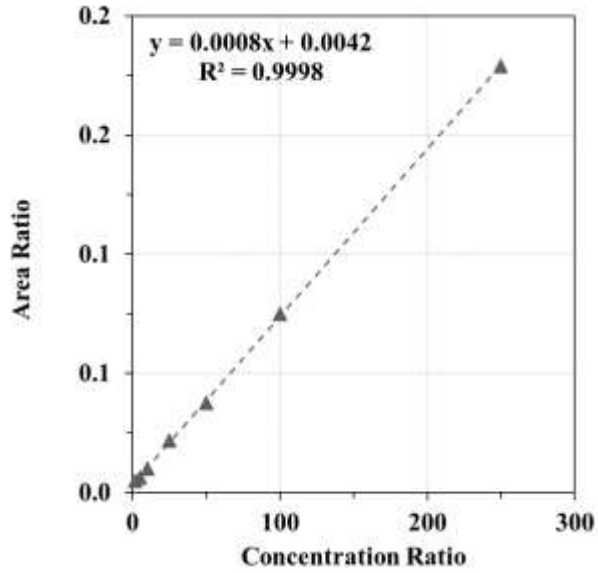


Figure 3.41. Calibration curve of Leu.

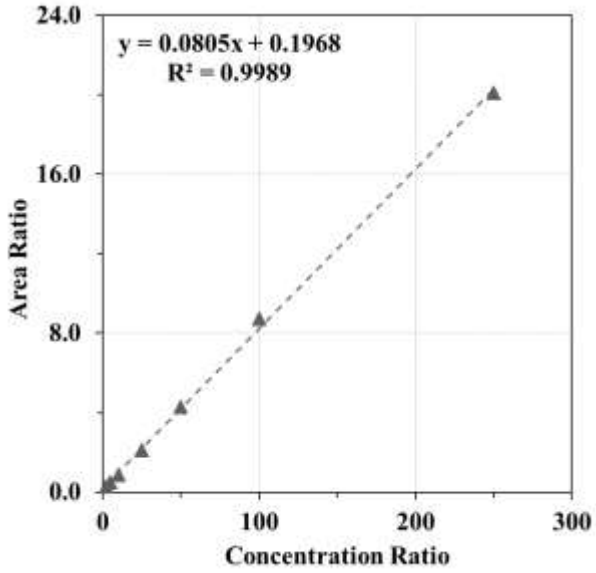


Figure 3.42. Calibration curve of Lys.

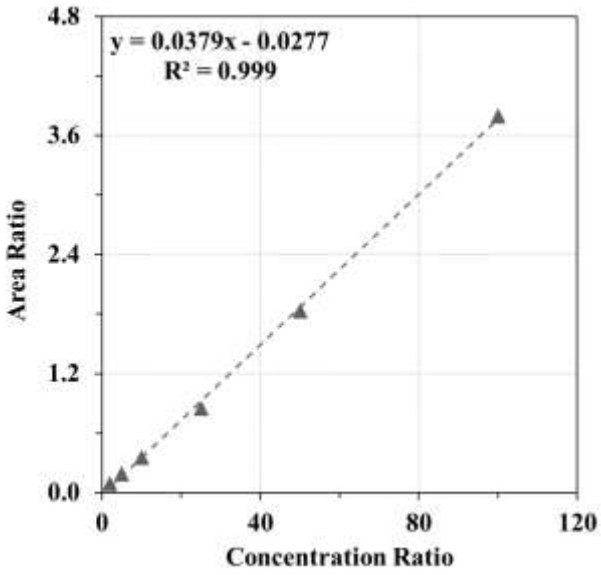


Figure 3.43. Calibration curve of Met.

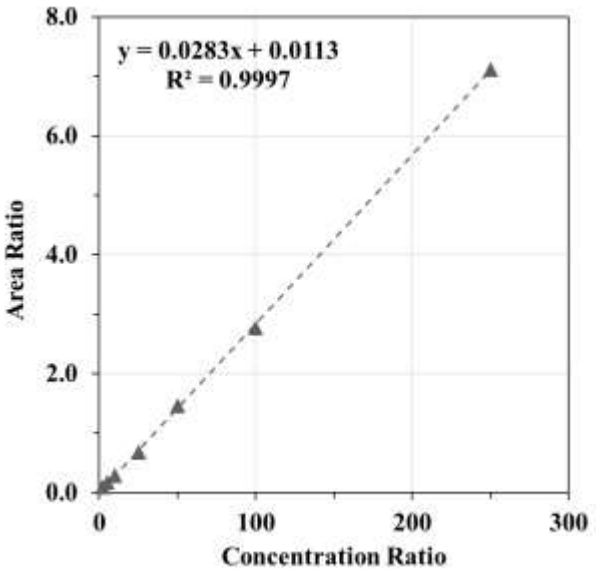


Figure 3.44. Calibration curve of Orn.

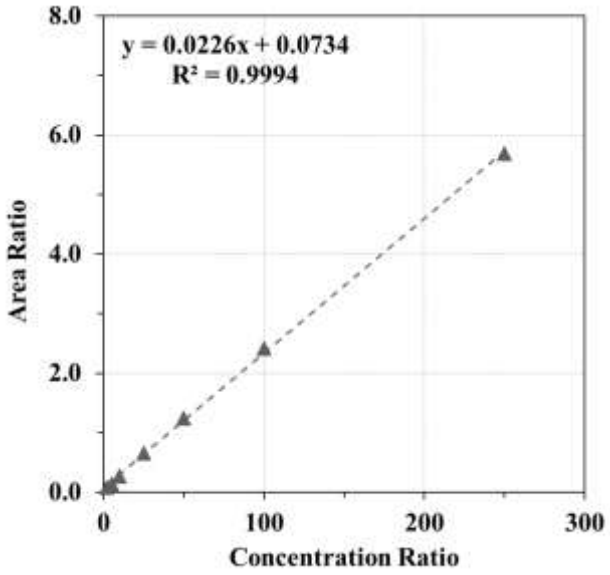


Figure 3.45. Calibration curve of Phe.

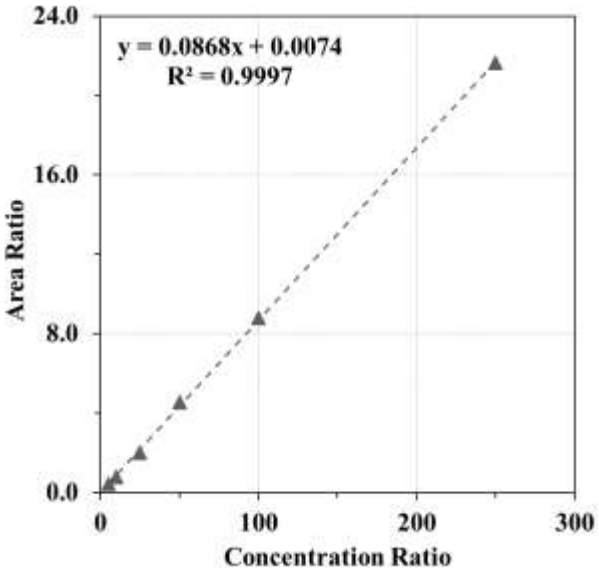


Figure 3.46. Calibration curve of Pro.

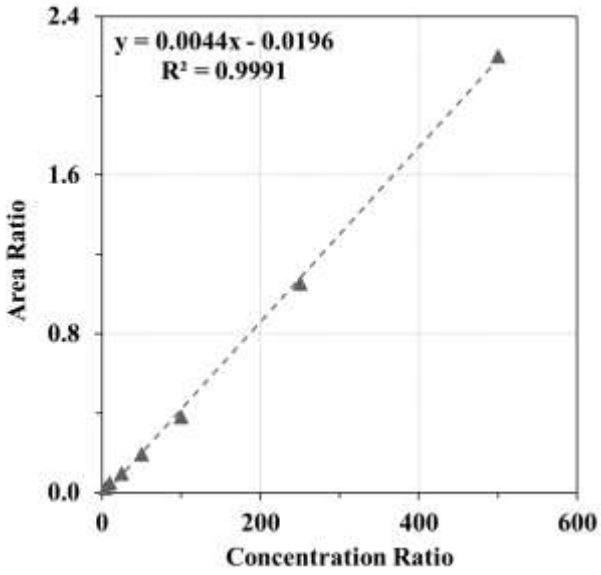


Figure 3.47. Calibration curve of Ser.

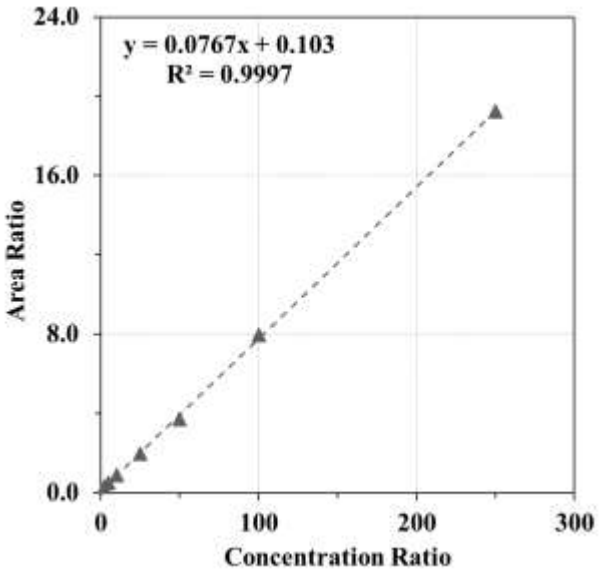


Figure 3.48. Calibration curve of Thr.

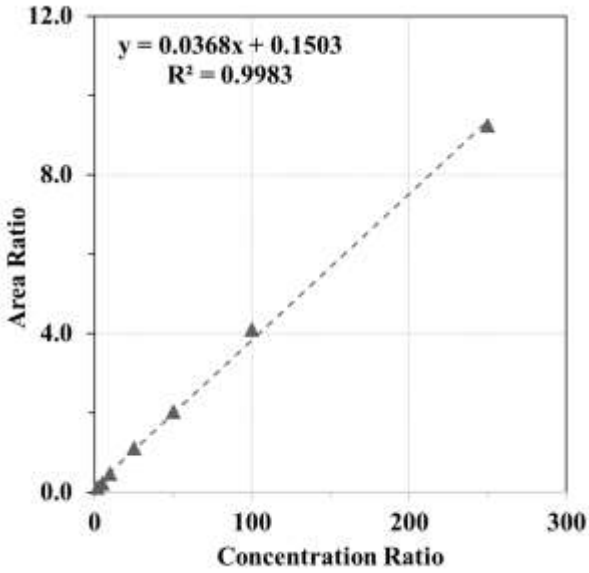


Figure 3.49. Calibration curve of Trp.

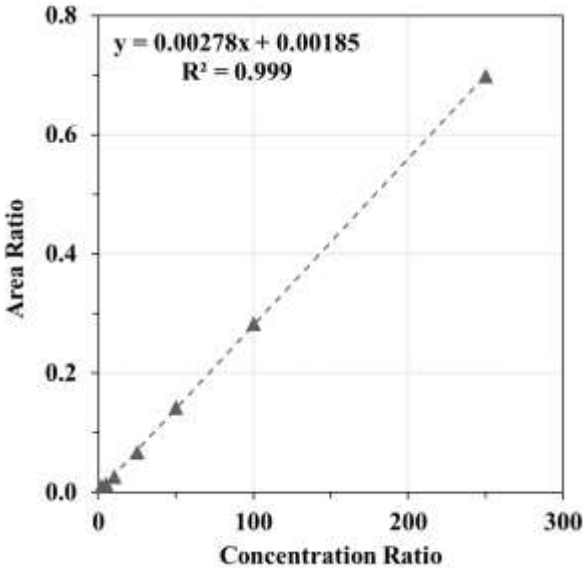


Figure 3.50. Calibration curve of Tyr.

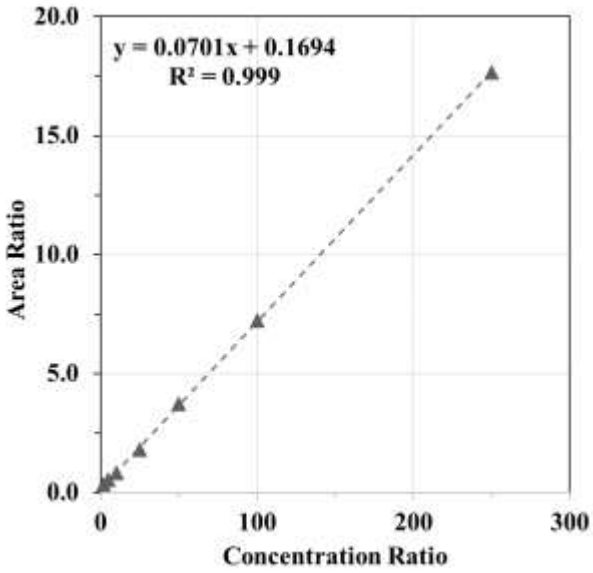


Figure 3.51. Calibration curve of Val.

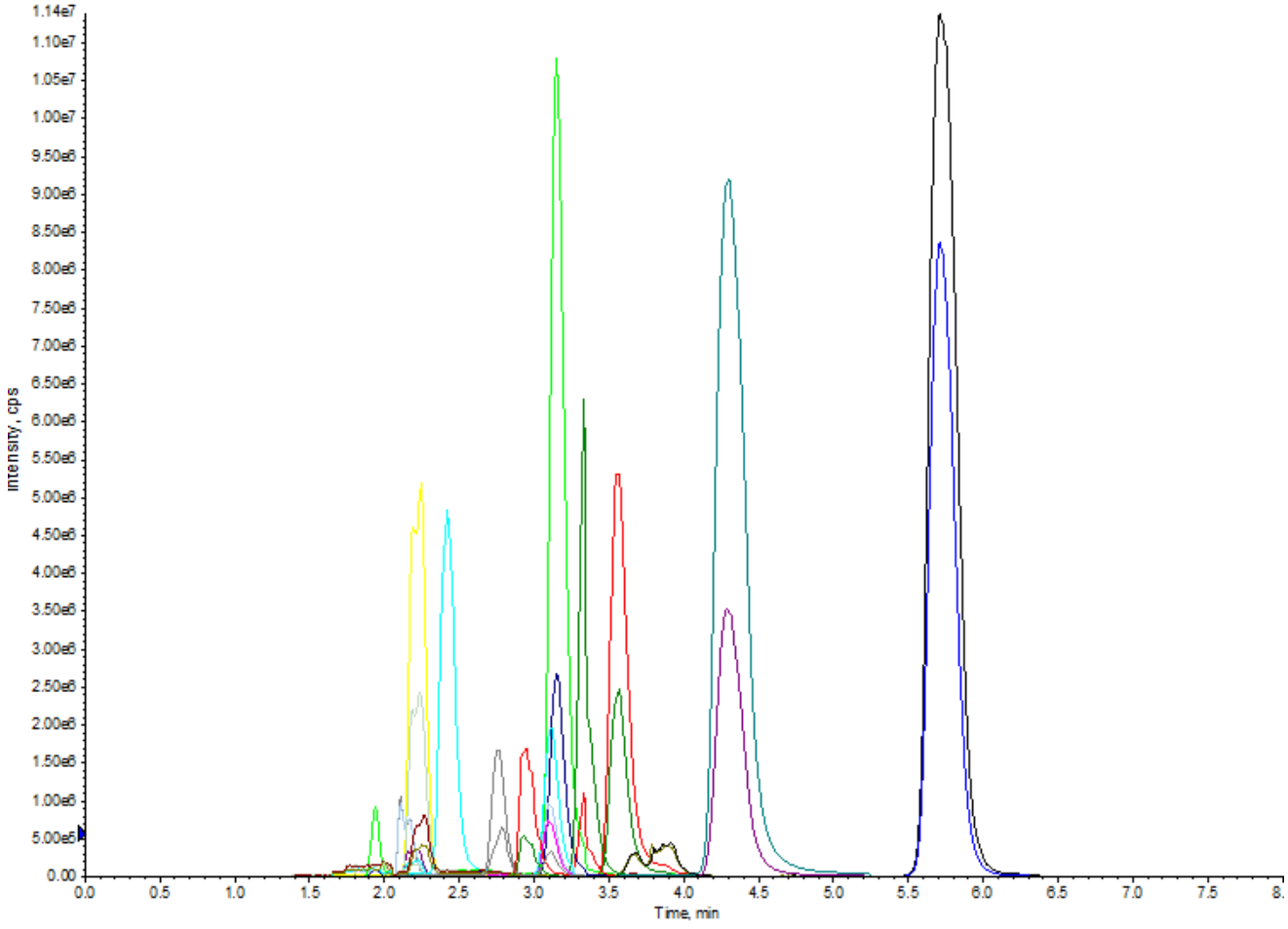


Figure 3.52. Representative chromatogram for amino acids.

4. RESULTS AND DISCUSSION

This chapter contains the results of growth profiles of microalgae species, MWW characteristics, MWW challenge tests, FWW characteristics, FWW challenge tests, plastic PBR growth profiles, biomass nutrition values and nutrient removal profiles.

4.1. Growth Profiles

Ten different microalgal strains were used for the growth profiling and all of them were cultivated in EBRs described in Chapter 2. There were minor differences in microalgal growth due to ambient conditions. Some of the strains have more differences between their triplicates because of their tendencies to aggregate in the liquid medium. OD and cell density data were used for plotting growth curves for selected species. OD versus time and cell density versus time graphics were shown below at Figures 4.1 to 4.20.

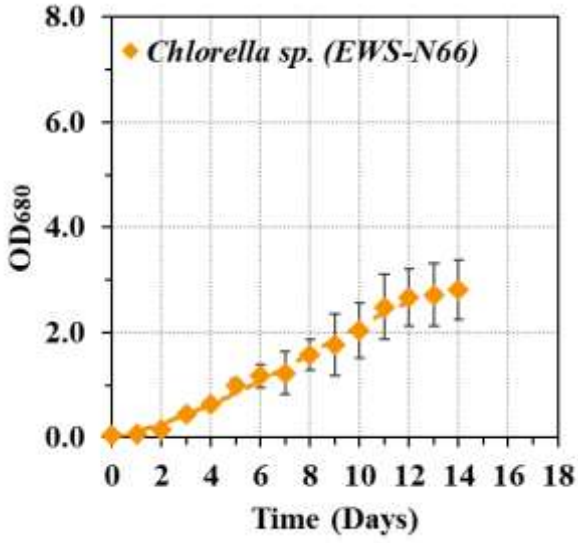


Figure 4.1. Growth profile of EWS-66.

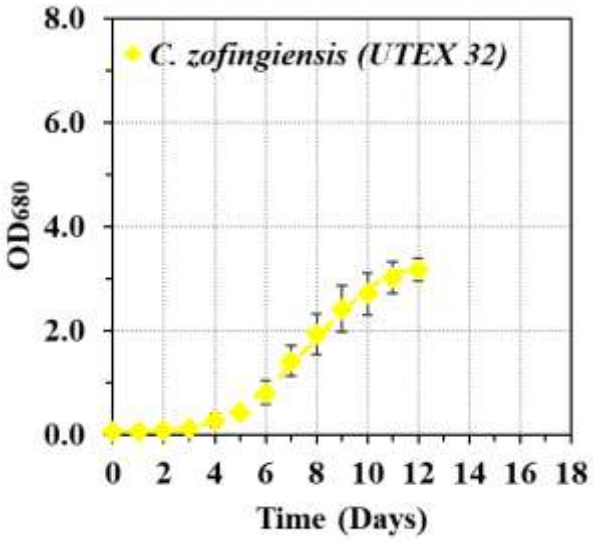


Figure 4.2. Growth profile of UTEX 32.

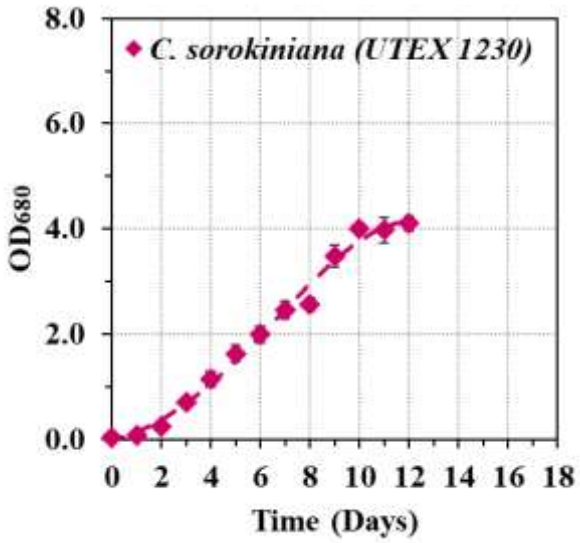


Figure 4.3. Growth profile of UTEX 1230.

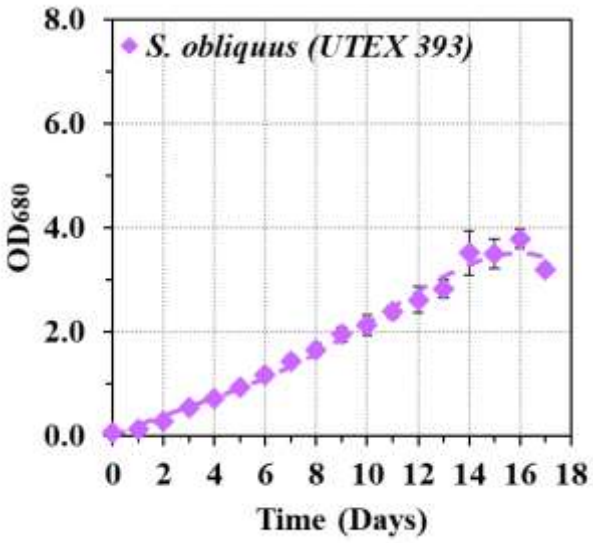


Figure 4.4. Growth profile of UTEX 393.

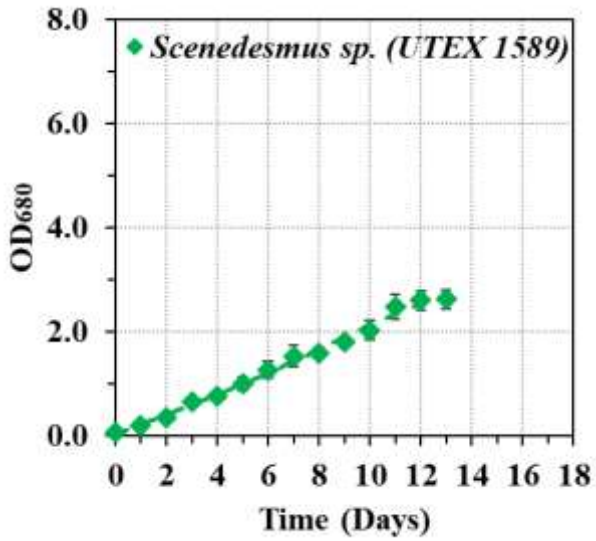


Figure 4.5. Growth profile of UTEX 1589.

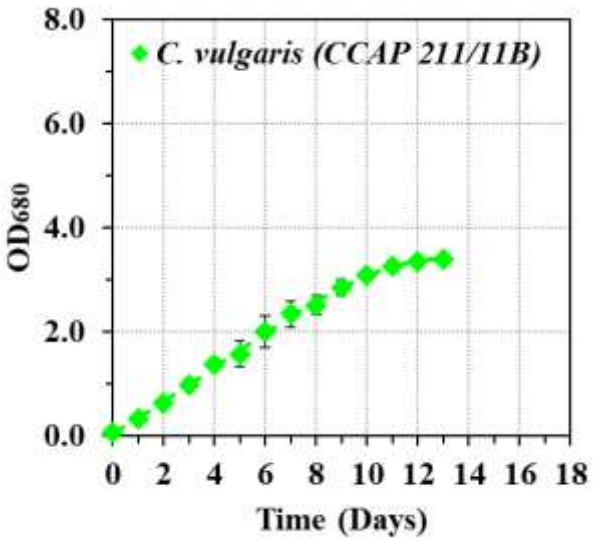


Figure 4.6. Growth profile of CCAP 211/11B.

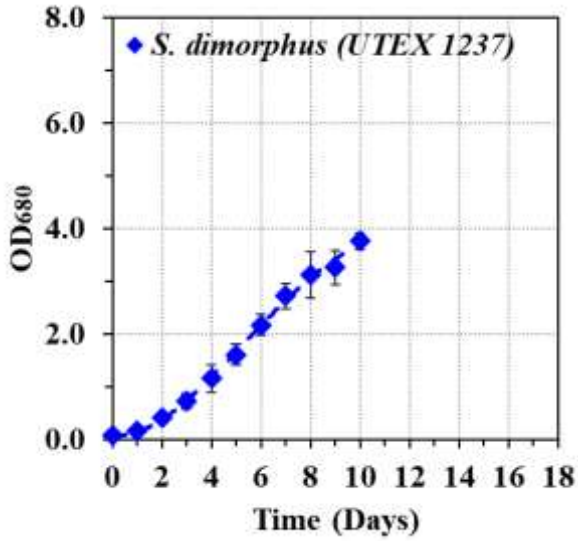


Figure 4.7. Growth profile of UTEX 1237.

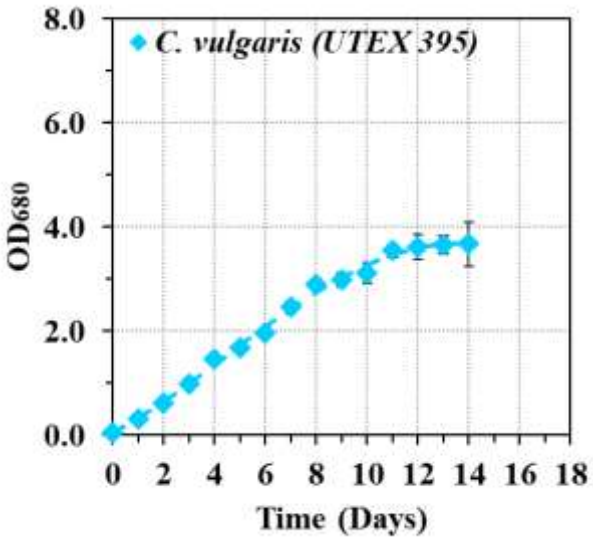


Figure 4.8. Growth profile of UTEX395.

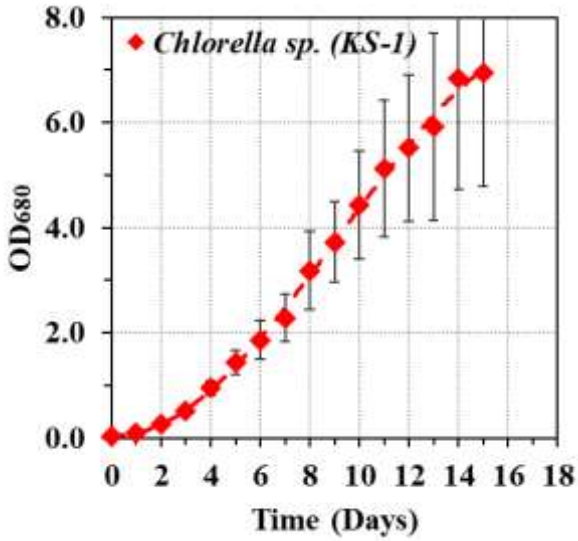


Figure 4.9. Growth profile of KS-1.

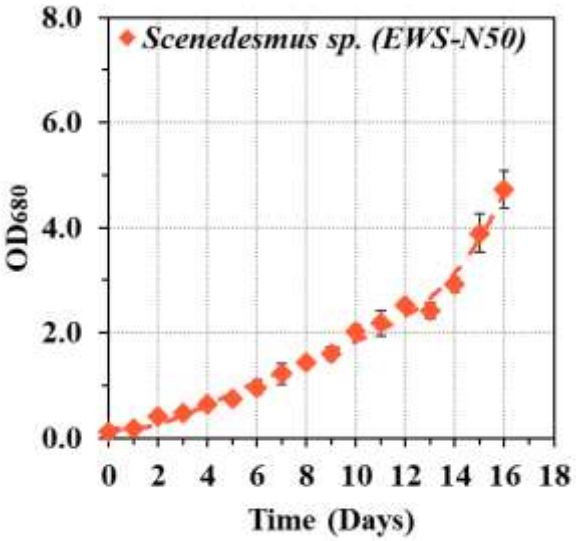


Figure 4.10. Growth profile of EWS-N50.

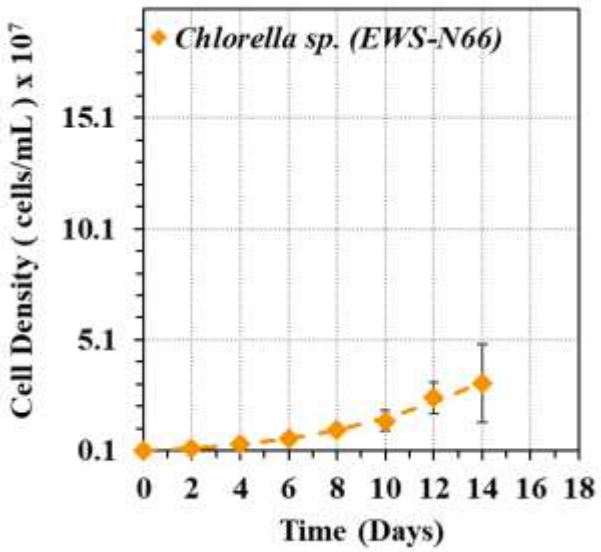


Figure 4.11. Cell density vs time of EWS-N66.

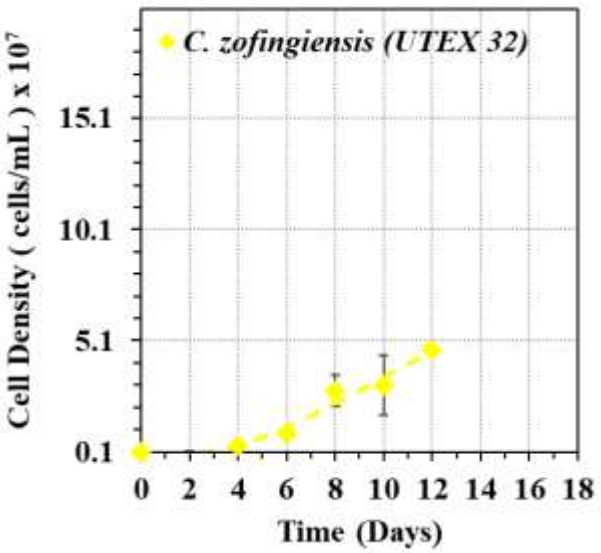


Figure 4.12. Cell density vs time of UTEX 32.

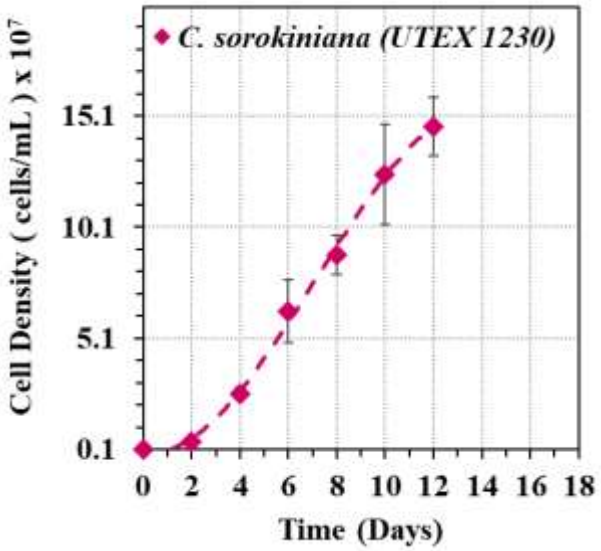


Figure 4.13. Cell density vs time of UTEX1230.

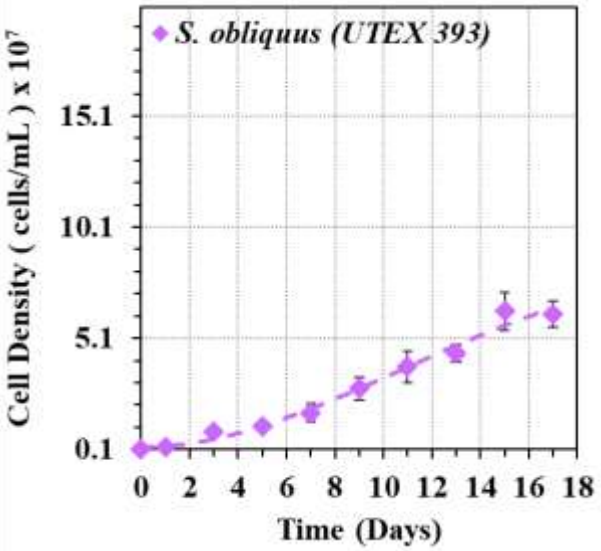


Figure 4.14. Cell density vs time of UTEX 393.

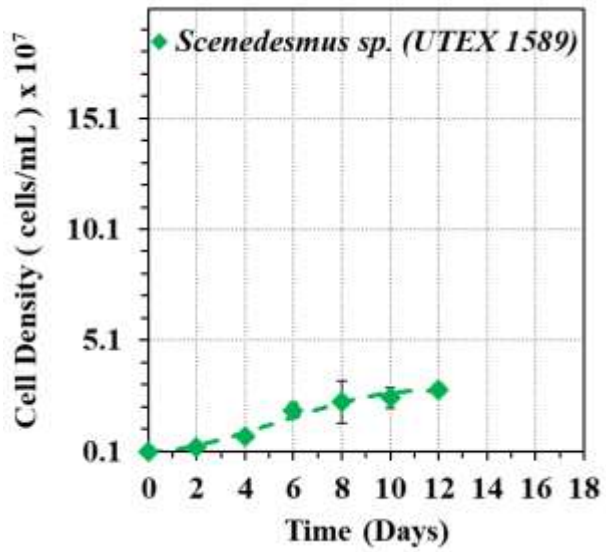


Figure 4.15. Cell density vs time of UTEX 1589.

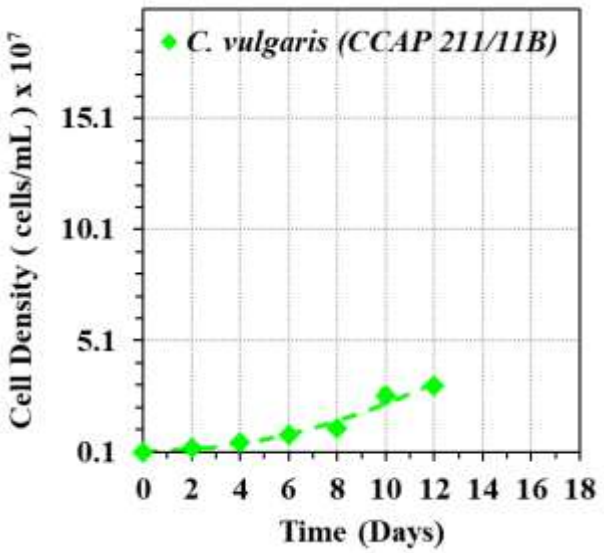


Figure 4.16. Cell density vs time of CCAP 211/11B.

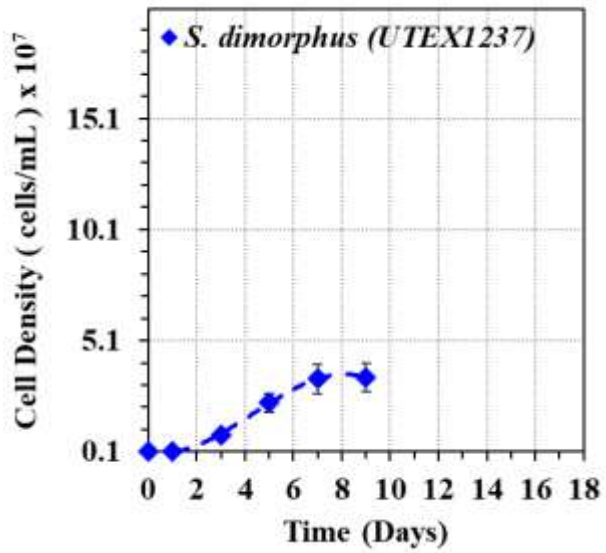


Figure 4.17. Cell density vs time of UTEX 1237.

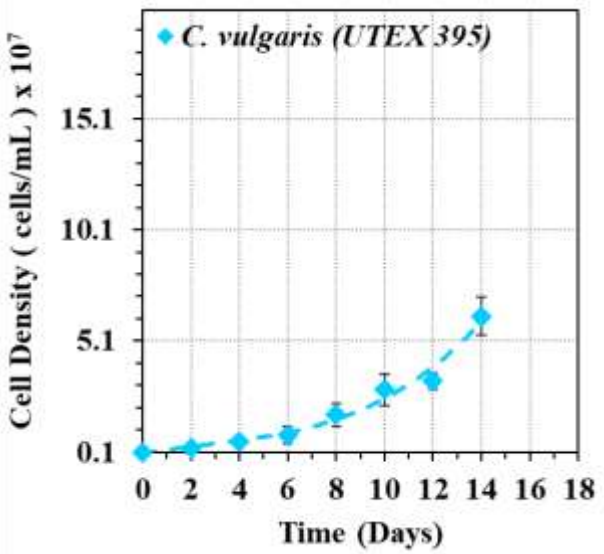


Figure 4.18. Cell density vs time of UTEX 395.

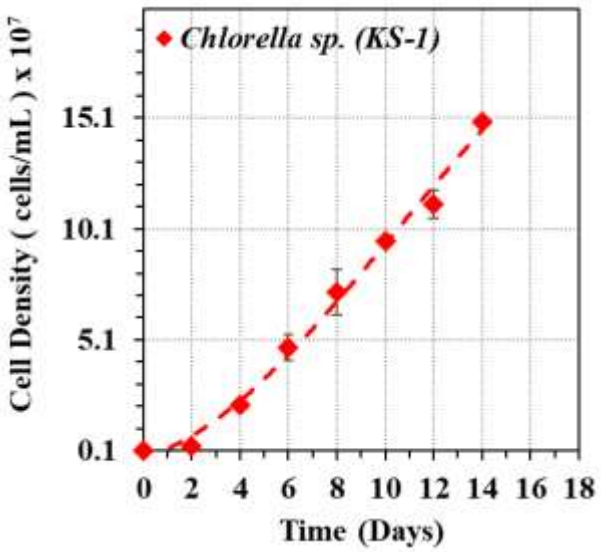


Figure 4.19. Cell density vs time of KS-1.

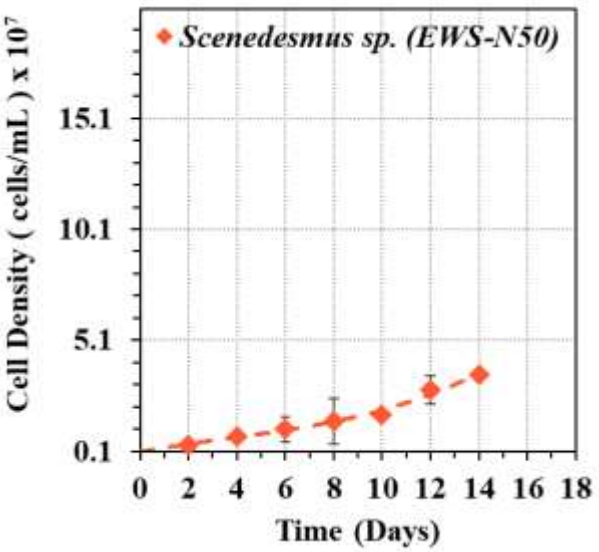


Figure 4.20. Cell Density vs Time of EWS-N50.

After the determination of growth profiles of microalgae, mid-exponential days of growth in EBRs were identified by determining the days of exponential growth phase (explained in Chapter 2) and average day value of the exponential growth phase was taken (Table 4.1).

Table 4.1. Mid-exponential days of microalgal strains.

Species Name	Strain ID	Mid-Exponential Day
Chlorella vulgaris	CCAP 211/11B	7
Chlorella vulgaris	UTEX 395	7
Chlorella zofingiensis	UTEX 32	8
Chlorella sorokiniana	UTEX 1230	5
Scenedesmus obliquus	UTEX 393	8
Scenedesmus dimorphus	UTEX 1237	5
***Scenedesmus* sp.**	UTEX 1589	5
***Chlorella* sp.**	KS-1	7
***Chlorella* sp.**	EWS-N66	7
***Scenedesmus* sp.**	EWS-N50	10

4.2. Manure Wastewater Study

Preliminary screening of microalgal strains were done in prepared MWW. Following the characterization of prepared wastewater, microalgal strains were cultivated in multi-well plates with control medium (MB3N), MWW and 1/2 MWW with triplicates for each. This screening showed which microalgal strains were more resilient to MWW. Characteristics of MWW and growth profiles of microalgae in challenge tests were given in sections 4.2.1 and 4.2.2.

4.2.1. Characteristics of Manure Wastewater

Total nitrogen result of 63.5 mg/L N, TP result of 4.5 mg/L PO_4-P and COD result of 1.140 mg/L COD was obtained after the pretreatment of MWW (Table 4.2, 4.3 and 4.4). Also, total solid content of the MWW was measured to be 0.97% (Table 4.5). The nutrient results of the wastewater are slightly higher than municipal wastewater before primary settling (an example is shown in Table 2.1) that indicates it could be a suitable medium for microalgae to grow.

Table 4.2. Total nitrogen results of manure wastewater.

Sample	TN Before Pretreatment (mg/L TN)	TN After Pretreatment (mg/L TN)	% Removal of Pretreatment
Reagent Blank	3.20*	-	-
Standard Solution (38.8925 mg/L TN)	42.3*	-	-
Manure Wastewater	392.4	63.5	83.8

Table 4.3. Total phosphorus results of manure wastewater.

Sample	TP Before Pretreatment (mg/L PO_4-P)	TP After Pretreatment (mg/L PO_4-P)	% Removal of Pretreatment
Reagent Blank	0.058*	-	-
Reagent Blank 2	0.057*	-	-

Standard Solution (3.6189 mg/L PO_4-P)	3.81*	-	-
Manure Wastewater	73.2	4.5	93.9

Table 4.4. COD results of manure wastewater.

Sample	COD Before Pretreatment (mg/L COD)	COD After Pretreatment (mg/L COD)	% Removal
Reagent Blank	-31.1*	-	-
Standard Solution 1 (425.4 mg/L KHP = 500.4706 mg/L COD)	490	-	-
Standard Solution 2 (850.4 mg/L KHP = 1,000.4704 mg/L COD)	957	-	-
Standard Solution 3 (1,708 mg/L KHP =2,009.4118 mg/L COD)	1,992	-	-
Manure Wastewater	11,486	1.140	90.1

**If the result of the sample specific blank is below the measuring range, it can be ignored (Hach, 2020).*

Table 4.5. Total solids results of manure wastewater.

Sample	ED Tare Mass (g)	ED + Sample 1st weighing(g)	ED + Sample 2nd weighing(g)	Average of 1st and 2nd weighing (g)	Total Solids (mg/L)	Total Solids %
1st Duplicate	110.4636	110.5613	110.5616	110.5615	9,790	0.97
2nd Duplicate	102.7645	102.8625	102.8629	102.8627	9,800	0.97

4.2.2. Results of Challenge Tests with Manure Wastewater

Challenge test growth profile results were used for eliminating weak microalgal strains. In the same growth conditions, microalgae showed different growth profiles. Some of the strains were deteriorated and some of them were bleached and it was not possible to identify the reason for it because of the limited working volume of 3 mL's. Thus, experiments were kept up by eliminating those bleached microalgal strains which were *C. vulgaris* (strain UTEX 395), *C. vulgaris* (strain CCAP 211/11B), *C. zofingiensis* (strain UTEX 32), and *Scenedesmus sp.* (strain UTEX 1589). Growth profiles of ten microalgal strains were given in Figures 4.21 to 4.30.

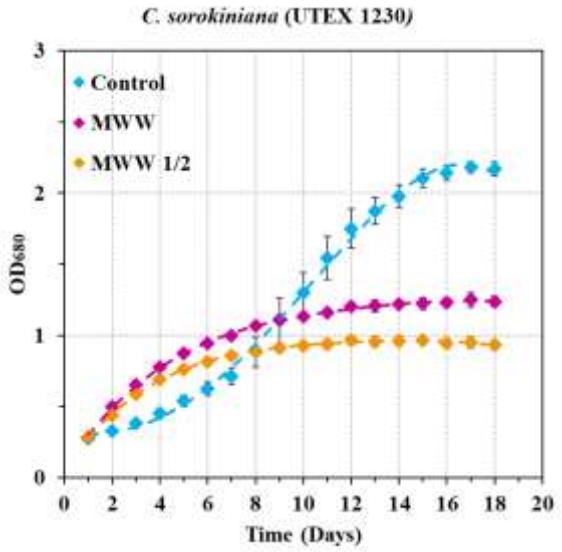


Figure 4.21. Growth profile of UTEX 1230 in MWW and control medium.

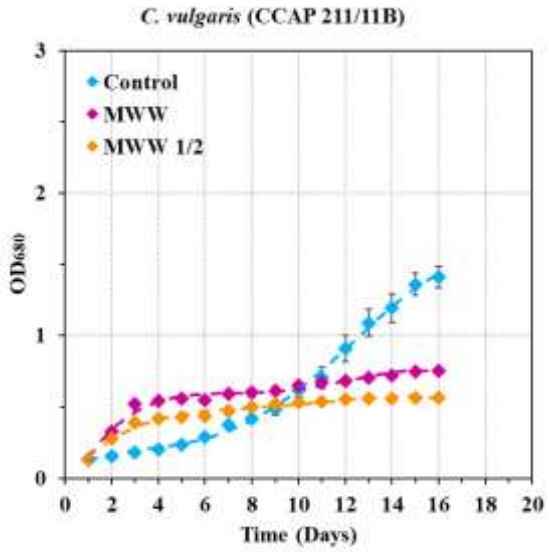


Figure 4.22. Growth profile of CCAP 211/11B in MWW and control medium.

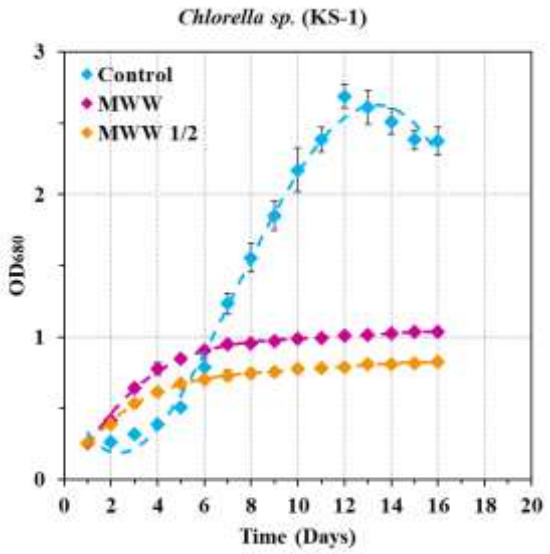


Figure 4.23. Growth profile of KS-1 in MWW and control medium.

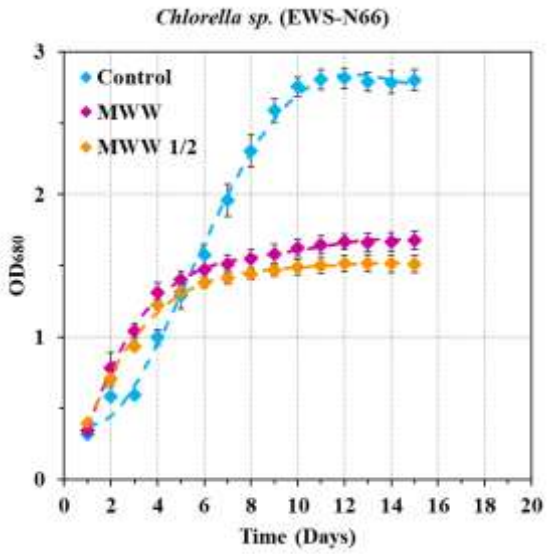


Figure 4.24. Growth profile of EWS-N66 in MWW and control medium.

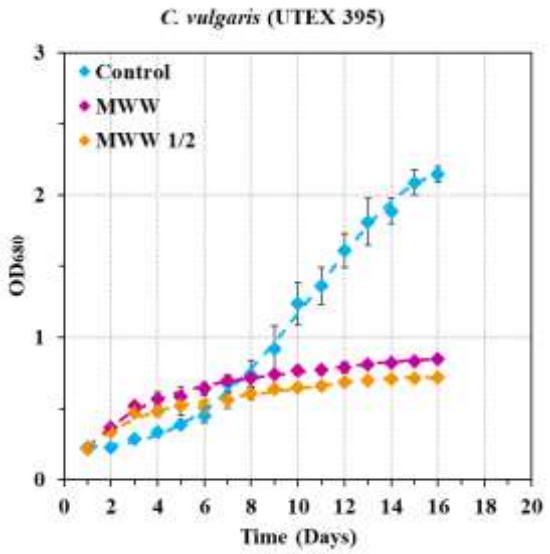


Figure 4.25. Growth profile of UTEX 395 in MWW and control medium.

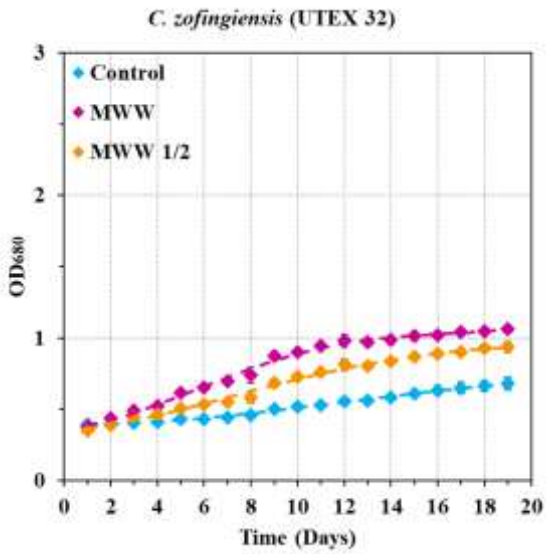


Figure 4.26. Growth profile of UTEX 32 in MWW and control medium.

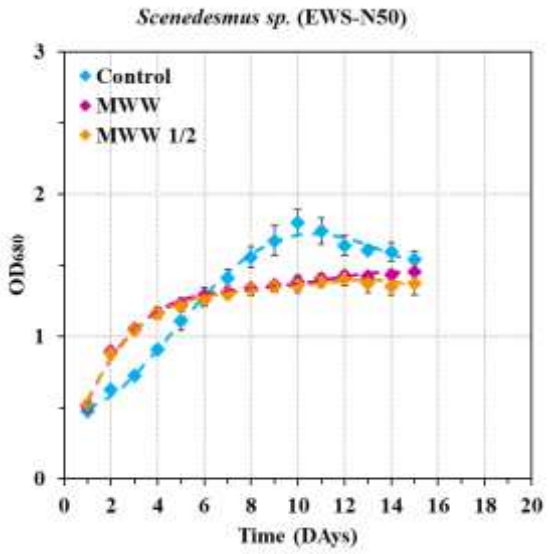


Figure 4.27. Growth profile of EWS-N50 in MWW and control medium.

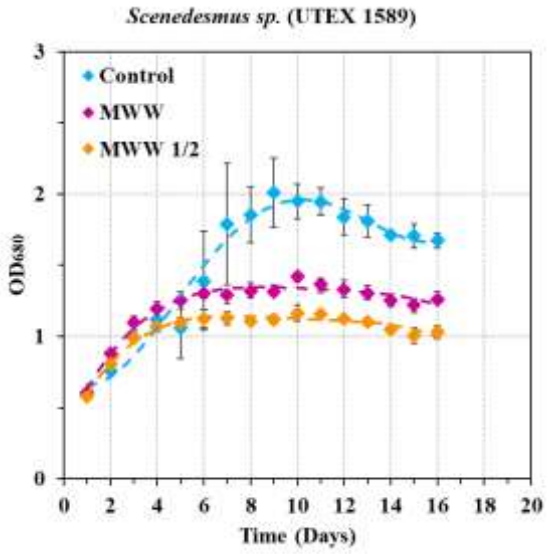


Figure 4.28. Growth profile of UTEX 1589 in MWW and control medium.

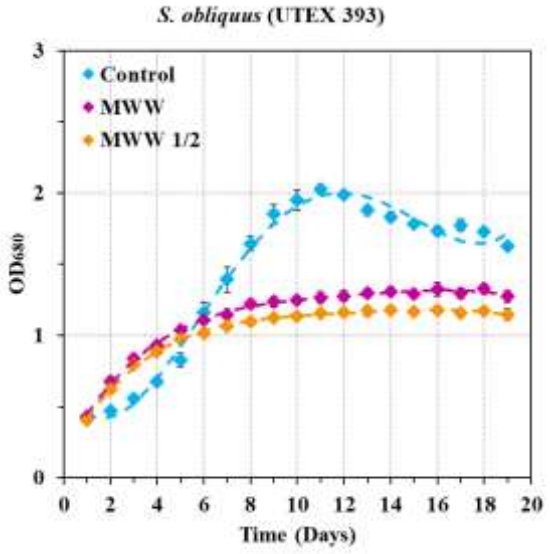


Figure 4.29. Growth profile of UTEX 393 in MWW and control medium.

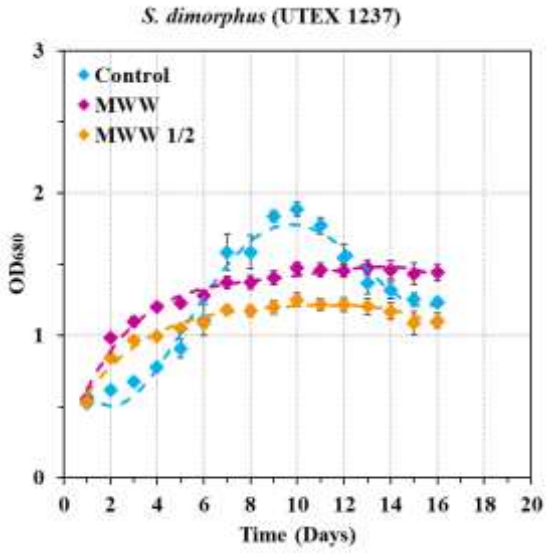


Figure 4.30. Growth profile of UTEX 1237 in MWW and control medium.

4.3. Farm Wastewater Study

For authenticity, a secondary screening was done with a real farm wastewater which was denoted as FWW. It was collected from a dairy farm, autoclaved, and pre-treated with a natural coagulant. Following pretreatment, microalgal strains were cultivated in multi-well plates with control medium (MB3N), FWW, 1/2 FWW, 1/4 FWW, 1/8 FWW and 1/10 FWW with triplicates for each. More dilutions

of wastewater were included in the trials because there are approximately ten times more nutrients in FWW (even after the pretreatment step) than MWW. Characterization results of nutrients in the FWW and challenge test growth profiles are given in sections 4.3.1 and 4.3.2.

4.3.1. Characteristics of Farm Wastewater

Total nitrogen result of 532.5 mg/L N, TP result of 69.6 mg/L PO_4^-P and COD result of 10,626 mg/L COD was obtained after the pretreatment of FWW (Table 4.6). Nutrient content of FWW shows similarities between reported dairy manure anaerobic digestion effluents in literature and some of the studies with chlorophyte, cyanobacteria, and diatom groups of microalgae showed promising nutrient removal efficiencies in these type of wastewaters (Cai et al., 2013).Thus, in the light of literature, FWW was used in the challenge tests as a further elimination step for the plastic PBRs.

Table 4.6. TN, TP, and COD results of FWW before and after pretreatment.

	Before Coagulation	**After Coagulation**	**% Removal**
Total Nitrogen (mg/L TN)	1,202	532.5	55.7
Total Phosphorus (mg/L PO_4^{3-}P)	156	69.6	55.4
COD (mg/L COD)	20,784	10,626	48.9

4.3.2. Results of Challenge Tests with Farm Wastewater

Despite starting from nearly the same OD values, microalgae showed different growth profiles when cultivated in FWW. Most efficient growth was seen *C. sorokiniana* (strain UTEX 1230) as it showed similar growth with its control when cultivated in 1/2 FWW. *Chlorella sp.* (strain KS-1) showed the second-best performance in 1/2 FWW, however the growth profile of it was not as good as its control. Thirdly, *S. dimorphus* (strain UTEX 1237) and *S. obliquus* (UTEX 393) showed moderately higher growth profiles compared to EWS-N66, EWS-N50 strains, when cultivated in 1/4 FWW and 1/2 FWW, respectively. *S. obliquus* (UTEX 393) showed higher growth with respect to its control, however it was not as efficient as *C. sorokiniana* (strain UTEX 1230) when compared to their OD results. Growth profiles of six microalgal strains were given in Figures 4.31 to 4.36.

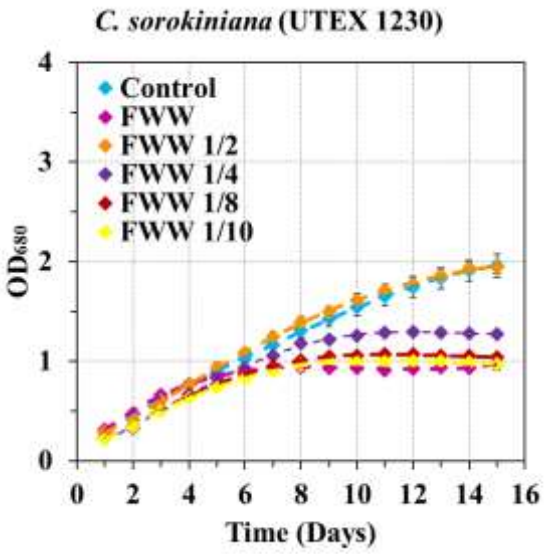


Figure 4.31. Growth profile of UTEX 1230 in FWW and control medium.

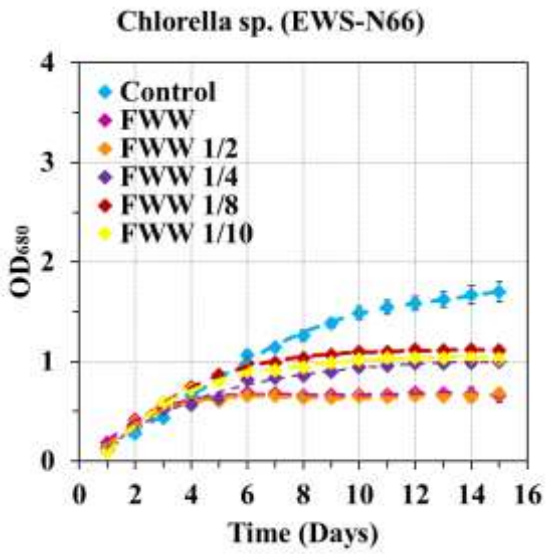


Figure 4.32. Growth profile of EWS-N66 in FWW and control medium.

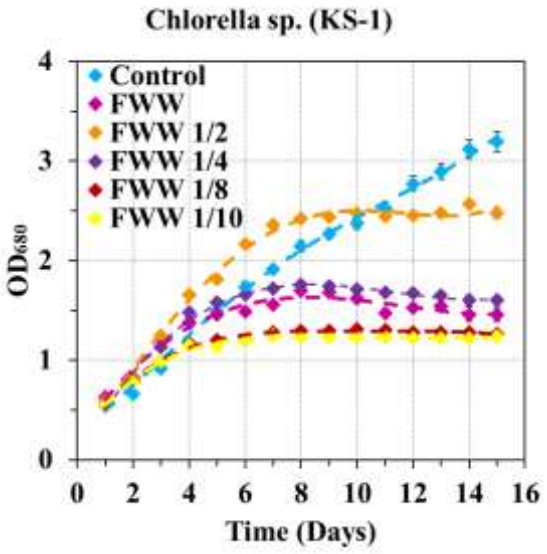


Figure 4.33. Growth profile of KS-1 in FWW and control medium.

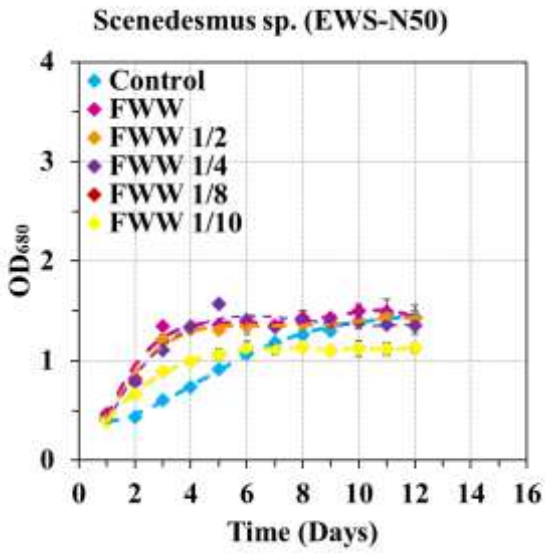


Figure 4.34. Growth profile of EWS-N50 in FWW and control medium.

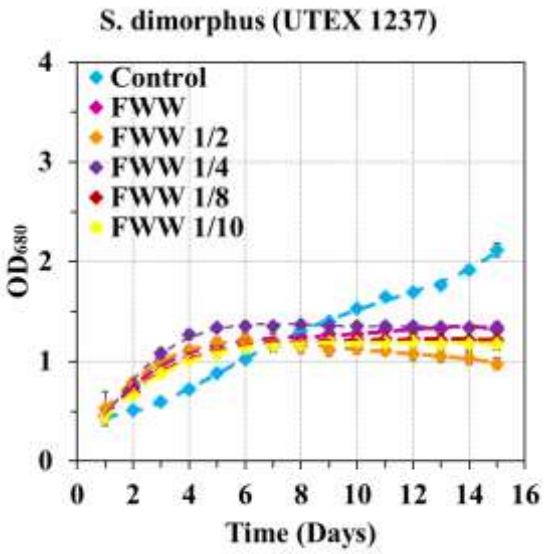


Figure 4.35. Growth profile of UTEX 1237 in FWW and control medium.

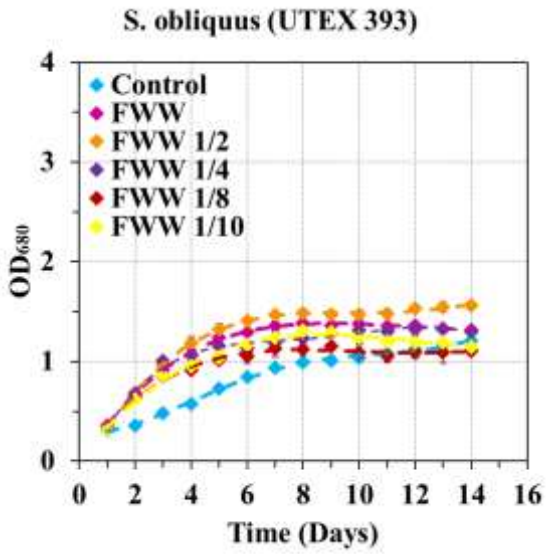


Figure 4.36. Growth profile of UTEX 393 in FWW and control medium.

4.4. Plastic Photobioreactor Study

As a result of two comprehensive challenge tests, best performing microalgal strain *Chlorella sorokiniana* (strain UTEX 1230) was selected for demonstration of FWW treatment in plastic PBRs. Microalgae were cultivated in the plastic PBRs until the culture reached the stationary phase. Daily TN, TP, OD, and pH data were taken. Cell density and dry weight data were collected every other day. For value-added animal/aquaculturing feed applications, nutrient profile of the biomass was analysed after harvesting the PBRs.

Plastic PBRs were operated with triplicates that were denoted as A, B, and C. Control PBRs were also operated in triplicates and denoted as D, E, and F. Growth and nutrient removal profiles of the reactors were drawn with the mean values of triplicates. Both individual PBR and mean values of triplicates are presented for nutritional profile of harvested biomass from plastic PBRs.

4.4.1. Plastic Photobioreactor Growth Profiles

Although *C. sorokiniana* (strain UTEX 1230) showed better growth performance in 1/2 FWW than its control reactor in challenge tests (Section 4.3.2), it showed apparent worse growth than its control reactor in plastic PBRs (Figure 4.37, 4.38 and 4.39). The most obvious reason for this growth gap

between two cultures could be the air supply. Challenge tests were conducted in multi-well plates with no air supply and limited working volume of 3 mL, whereas, plastic PBRs had air supply, 2L working volume and a wide surface area for light penetration. Nevertheless, final biomass concentration of 1.8 g/L shows promising results compared to reported literature results (Table 4.7) (Abinandan et al., 2015). Different biomass concentration data reported with different type of wastewaters and biological reactors for *Chlorella* genera are given in Table 4.8.

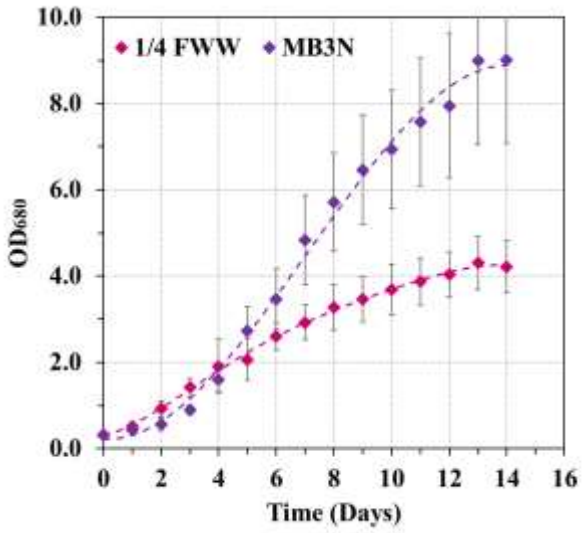


Figure 4.37. Growth profile of UTEX 1230 in plastic PBRs.

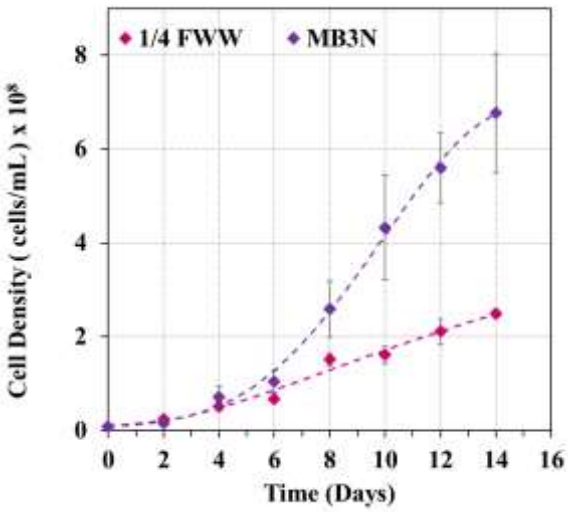


Figure 4.38. Cell density over time of UTEX 1230 in plastic PBRs.

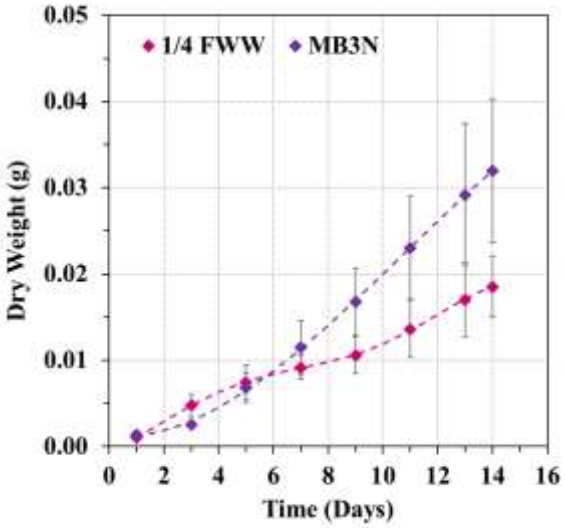


Figure 4.39. Dry weight over time of UTEX 1230 in plastic PBRs.

Table 4.7. Biomass concentration results of individual plastic PBRs.

PBR	Harvest Dry Weight (g/10 mL)	Concentration (g/L)	Mean	Std Dev
A	0.0167	1.7		
B	0.0163	1.6	1.8	0.3
C	0.0225	2.3		
D	0.0377	3.8		
E	0.0224	2.2	3.2	0.8
F	0.0358	3.6		

Table 4.8. Biomass concentrations of Chlorella genera in different type of wastewater.

Species Name	Wastewater Type	Biomass Concentration	Cultivation Mode	References
Chlorella pyrenoidosa	Dairy WW	6.8 g/L	Batch	(Abinandan et al., 2015)
Chlorella vulgaris	Piggery WW	0.2-1.2 g/L	Batch	(Abinandan et al., 2015)
Chlorella vulgaris	Undigested dairy manure	3.8 g/L	Batch	(Abinandan et al., 2015)
Chlorella vulgaris	Digested dairy manure	1.4 g/L	Batch	(Abinandan et al., 2015)
Chlorella sorokiniana	Swine WW	5.5 g/L	Cell Immobilization	(Chen et al., 2020)
Chlorella sorokiniana	Shrimp Culture WW	4.4 g/L	Semi-continuous	(Chen et al., 2019)

4.4.2. Nutrient Removal Profiles

Daily TN and TP data were taken from the plastic PBRs and daily nutrient removal profiles were drawn (Figure 4.40 and 4.41). For COD parameter, only influent and effluent analyses for FWW were done. An average removal of 64.2%, 59.4%, and 95.7% were seen in plastic PBRs for TN, TP, and COD, respectively (Tables 4.9, 4.10, and 4.11). Microalgae can remove TN and TP up to 99% when optimal conditions are reached. It is reported that with initial concentrations of 100 to 400 mg/L TN, *Chlorella* genera showed up to 89% removal in different types of wastewater (Cai et al., 2013). Similarly, with initial concentrations of 5 to 112 mg/L TP, *Chlorella* genera removed up to 100% of phosphorus from different kinds of wastewater (Cai et al., 2013).

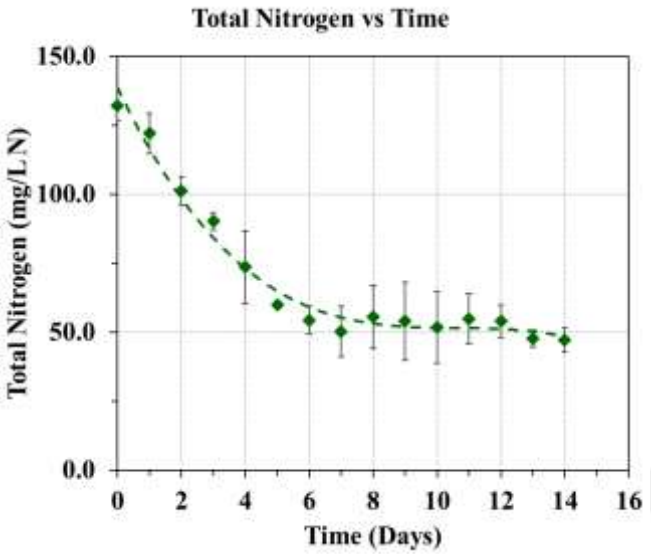


Figure 4.40. Total nitrogen over time profile of plastic PBRs Operated with FWW.

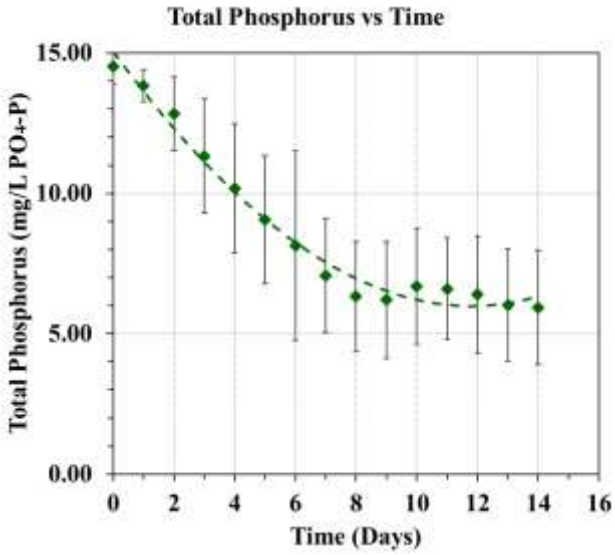


Figure 4.41. Total Phosphorus over time profile of plastic PBRs operated with FWW.

Table 4.9. Total nitrogen removal of plastic PBRs (only FWW reactors).

PBR	TN of Raw FWW (mg/L N)	TN of FWW After Pretreatment (mg/L N)	TN of FWW After Phycoremediation (mg/L N)	% Removal with Phycoremediation	Mean % Removal with Phycoremediation	Total % Removal
A	300.5	130.4	51.1	60.8		83.0
B	300.5	127.8	47.8	62.6	64.2	84.1
C	300.5	138.2	42.5	69.2		85.9

Table 4.10. Total phosphorus removal of plastic PBRs (only FWW reactors).

PBR	TP of Raw FWW (mg/L PO4-P)	TP of FWW After Pretreatment (mg/L PO4-P)	TP of FWW After Phycoremediation (mg/L PO4-P)	% Removal with Phycoremediation	Mean % Removal with Phycoremediation	Total % Removal
A	39.0	14.64	4.9	66.5		87.4
B	39.0	13.84	4.65	66.4	59.4	88.1
C	39.0	15.12	8.27	45.3		78.8

Table 4.11. Chemical oxygen demand removal of plastic PBRs (only FWW reactors).

PBR	COD of Raw FWW (mg/L COD)	COD of FWW After Pretreatment (mg/L COD)	COD of FWW After Phycoremediation (mg/L COD)	% Removal with Phycoremediation	Mean % Removal with Phycoremediation	Total % Removal
A	5196	2744	134.0	95.1		97.4
B	5196	2704	88.6	96.7	95.7	98.3
C	5196	2713	129.0	95.2		97.5

4.4.3. Nutritional Profile of Biomass

In this section, hydro-soluble protein, monomeric sugar, total lipid, FAME, amino acid, and vitamin contents of harvested microalgal biomass from FWW compared with control reactors are given in Tables 4.12 to 4.20. Mean values of triplicates are compared with recent studies in Tables 4.21. to 4.22. FAME profiling reports are given in Appendix B.

Microalgae cultured in FWW shows slightly higher total protein content, slightly lower carbohydrate content and lower lipid content compared to its control. When compared to *Chlorella* species (mostly *C. vulgaris*), *C. sorokiniana* (strain UTEX 1230) shows much higher carbohydrate content, much lower protein content and slightly higher lipid content. *Chlorella* genus is promising in terms of animal feed production when used in small amounts in feed. It is reported to enhance the growth of poultry when used in feed formulations.

Table 4.12. Hydro-soluble protein content of harvested biomass from FWW (A, B and C) and control (D, E and F) PBRs.

Plastic PBR	Biomass Weight (mg)	Absorbance at λ=750nm	Calculated Concentration (mg/mL)	Hydro-soluble protein (mg)	Hydro-soluble Protein %	Mean %	Std Dev
A	29.0	1.395	1.09	10.3	35.4		
B	30.8	1.644	1.41	14.1	45.7	28.7	21.2
C	30.3	0.660	0.15	1.5	5.0		
D	29.0	1.009	0.60	5.6	19.4		
E	30.2	1.145	0.77	7.6	25.1	25.2	5.7
F	30.0	1.287	0.95	9.3	30.9		

Table 4.13. Monomeric sugar content of harvested biomass from FWW (A, B and C) and control (D, E and F) PBRs.

Plastic PBR	Biomass Weight (mg)	Absorbance at λ=750nm	Calculated Concentration (mg/mL)	Monomeric Sugars (mg)	Total Carbohydrate %	Mean %	Std Dev
A	26.1	0.598	0.96	11.3	43.1		
B	26.0	0.567	0.89	10.5	40.3	47.3	9.8
C	24.9	0.731	1.24	14.6	58.5		
D	25.4	0.644	1.06	12.4	48.8		
E	24.4	0.583	0.93	10.9	44.6	50.8	7.4
F	24.2	0.719	1.21	14.3	59.0		

Dilution Factor: 40

Table 4.14. Total lipid content of harvested biomass from FWW (A, B and C) and control (D, E and F) PBRs.

Plastic PBR	Biomass Weight (mg)	Tare Mass of Vials (g)	Vials + Lipid Extract	Total Lipids (mg)	Lipids in Biomass %	Mean %	Std Dev
A	99.3	32.4360	32.4555	19.5	19.6		
B	100.7	32.3734	32.3921	18.7	18.6	22.6	6.0
C	99.4	32.4878	32.5171	29.3	29.5		
D	100.8	32.3307	32.3594	28.7	28.5		
E	99.6	32.3857	32.4081	22.4	22.5	32.9	13.1
F	100.8	32.7050	32.7530	48	47.6		

Table 4. 15. Concentration of FAMEs in samples.

Plastic PBR	Concentration (µg/mL)					
	C16:0	C18:0	C18:1n9c	C18:2n6t	C18:2n6c	C18:3n3
A	278.9	39.8	93.4	45.7	282.4	71.6
B	347.3	39.6	154.8	71.6	460.0	131.0
C	874.3	154.3	261.1	119.9	904.4	229.6
D	476.7	50.4	206.6	144.8	531.5	220.1
E	349.6	39.4	148.0	160.3	405.2	246.2
F	852.9	93.9	257.1	227.0	1086.3	386.1

Table 4.16. FAME content in individual plastic PBRs.

FAME Tag	Plastic PBR	Concentration (µg/mL)	Weight of FAME in sample (mg)	Total lipid in biomass (mg)	FAME content (% total lipid)	Mean %
C16:0	A	278.9	0.84	19.5	4.3	6.3
	B	347.3	1.04	18.7	5.6	
	C	874.3	2.62	29.3	9.0	
	D	476.7	1.43	28.7	5.0	5.0
	E	349.6	1.05	22.4	4.7	
	F	852.9	2.56	48	5.3	
C18:0	A	39.8	0.12	19.5	0.6	0.9
	B	39.6	0.12	18.7	0.6	
	C	154.3	0.46	29.3	1.6	
	D	50.4	0.15	28.7	0.5	0.5
	E	39.4	0.12	22.4	0.5	
	F	93.9	0.28	48	0.6	
C18:1n9c	A	93.4	0.28	19.5	1.4	2.2
	B	154.8	0.46	18.7	2.5	
	C	261.1	0.78	29.3	2.7	
	D	206.6	0.62	28.7	2.2	1.9
	E	148.0	0.44	22.4	2.0	
	F	257.1	0.77	48	1.6	
C18:2n6t	A	45.7	0.14	19.5	0.7	1.0
	B	71.6	0.21	18.7	1.1	
	C	119.9	0.36	29.3	1.2	
	D	144.8	0.43	28.7	1.5	1.7
	E	160.3	0.48	22.4	2.1	
	F	227.0	0.68	48	1.4	
C18:2n6c	A	282.4	0.85	19.5	4.3	7.0
	B	460.0	1.38	18.7	7.4	
	C	904.4	2.71	29.3	9.3	
	D	531.5	1.59	28.7	5.6	5.9
	E	405.2	1.22	22.4	5.4	
	F	1086.3	3.26	48	6.8	
C18:3n3	A	71.6	0.21	19.5	1.1	1.9
	B	131.0	0.39	18.7	2.1	
	C	229.6	0.69	29.3	2.4	
	D	220.1	0.66	28.7	2.3	2.7
	E	246.2	0.74	22.4	3.3	
	F	386.1	1.16	48	2.4	

Table 4.17. Concentrations of vitamins in samples.

Vitamin Tag	Concentration in sample (µg/L)							
	A	B	C	Mean	D	E	F	Mean
B1	5.9	5.9	< 5.0	5.9	< 5.0	< 5.0	< 5.0	-
B2	33.3	5.6	< 5.0	19.4	< 5.0	< 5.0	< 5.0	-
B3	178.3	75.8	35.4	96.5	114.3	133.2	83.7	110.4
B5	nd.	nd.	nd.	-	nd.	nd.	nd.	-
B6	< 5.0	< 5.0	< 5.0	-	< 5.0	< 5.0	< 5.0	-
B7	< 5.0	< 5.0	< 5.0	-	< 5.0	< 5.0	< 5.0	-
B9	66.2	30.3	11.4	36.0	12.8	15.4	21.3	16.5
β carotene	3081.4	765.1	179.1	1341.8	103.6	228.7	106.8	146.4
Vitamin C	17451.3	4989.5	7127.8	9856.2	7253.5	10927.8	6840.1	8340.5
B12	1048.0	495.3	97.8	547.0	325.1	259.0	157.4	247.2
Vitamin E	27.7	31.2	5.0	21.3	10.0	2.3	< 1.0	6.2

nd.: Not detected

< 5.0: Peak was observed yet under the limit of quantification according to calibration curve.

< 1.0: Peak was observed yet under the limit of quantification according to the calibration curve.

Table 4.18. Vitamin and β carotene content in harvested biomass.

	PBR	C in sample (µg/L)	Weight of vitamin in Sample (µg)	Biomass Weight (mg)	Vitamin content (mg/kg)	Mean (mg/kg)
B1	**A**	5.9	0.02	124.8	0.2	0.2
	B	5.9	0.02	125	0.2	
B2	**A**	33.3	0.14	124.8	1.1	0.6
	B	5.6	0.02	125	0.2	
B3	**A**	178.3	0.73	124.8	5.8	3.2
	B	75.8	0.31	125	2.5	
	C	35.4	0.14	125.2	1.2	
	D	114.3	0.47	125.7	3.7	3.6
	E	133.2	0.54	125	4.3	
	F	83.7	0.34	124.9	2.7	
B9	**A**	66.2	0.27	124.8	2.2	1.2
	B	30.3	0.12	125	1.0	
	C	11.4	0.05	125.2	0.4	
	D	12.8	0.05	125.7	0.4	0.5
	E	15.4	0.06	125	0.5	
	F	21.3	0.09	124.9	0.7	
β carotene	**A**	3081.4	12.57	124.8	100.7	43.8
	B	765.1	3.12	125	25.0	
	C	179.1	0.73	125.2	5.8	
	D	103.6	0.42	125.7	3.4	4.8

	E	228.7	0.93	125	7.5	
	F	106.8	0.44	124.9	3.5	
Vitamin C	**A**	17451.3	71.20	124.8	570.5	321.9
	B	4989.5	20.36	125	162.9	
	C	7127.8	29.08	125.2	232.3	
	D	7253.5	29.59	125.7	235.4	271.9
	E	10927.8	44.59	125	356.7	
	F	6840.1	27.91	124.9	223.4	
B12	**A**	1048.0	4.28	124.8	34.3	17.9
	B	495.3	2.02	125	16.2	
	C	97.8	0.40	125.2	3.2	
	D	325.1	1.33	125.7	10.6	8.0
	E	259.0	1.06	125	8.5	
	F	157.4	0.64	124.9	5.1	
Vitamin E	**A**	27.7	0.11	124.7	0.9	0.7
	B	31.2	0.13	125	1.0	
	C	5.0	0.02	124.1	0.2	
	D	10.0	0.04	124.6	0.3	0.2
	E	2.3	0.01	124.6	0.1	

Table 4.19. Concentrations of amino acids in samples.

Amino acid Tag	**Concentration in sample (µg/L)**							
	A	**B**	**C**	**Mean**	**D**	**E**	**F**	**Mean**
Ala	189.9	132.6	nd.	161.3	< 50.0	< 50.0	< 50.0	-
Arg	80.1	26.0	11.3	39.1	15.4	16.2	5.3	12.3
Asp	34.0	3.9	14.9	17.6	4.9	< 2.0	8.9	6.9
Tyr	40.5	9.8	9.3	19.9	5.3	5.0	2.2	4.2
Glu	102.5	43.7	64.6	70.3	20.4	41.9	8.6	23.6
His	17.7	5.2	4.7	9.2	2.8	3.4	< 2.0	3.1
Iso	29.2	10.0	3.5	14.3	6.9	13.7	3.9	8.2
Leu	66.0	5.6	12.6	28.1	10.1	18.5	3.4	10.7
Lys	95.0	56.4	9.0	53.4	16.5	43.9	2.1	20.9
Met	22.9	6.7	5.0	11.5	6.1	7.4	2.5	5.3
Orn	37.3	< 2.0	2.7	20.0	6.3	< 2.0	< 2.0	6.3
Phe	50.8	7.2	< 2.0	29.0	< 2.0	2.8	< 2.0	2.8
Pro	89.5	71.1	32.0	64.2	46.4	67.6	17.3	43.8
Ser	161.3	46.5	144.1	117.3	117.8	104.0	55.7	92.5
Thr	134.0	89.2	112.1	111.8	180.3	59.9	45.3	95.1
Trp	17.9	< 2.0	< 2.0	17.9	< 2.0	< 2.0	< 2.0	-

Val	56.9	17.8	18.6	31.1	15.4	19.8	4.9	13.4

nd.: Not detected

< 50.0: Peak was observed yet under the limit of quantification according to calibration curve.

< 2.0: Peak was observed yet under the limit of quantification according to the calibration curve.

Table 4.20. Amino acid content in harvested biomass.

AA Tag	PBR	C in sample (µg/L)	Biomass weight (mg)	Weight of aa in sample (µg)	AA content (µg/mg)	Mean (µg/mg)
Ala	**A**	189.9	50.0	153.47	3.1	
	B	132.6	50.0	107.13	2.1	2.6
Arg	**A**	80.1	50.0	64.72	1.3	
	B	26.0	50.0	21.02	0.4	
	C	11.3	50.0	9.10	0.2	0.6
	D	15.4	50.0	12.45	0.2	
	E	16.2	50.0	13.12	0.3	
	F	5.3	50.0	4.32	0.1	0.2
Asp	**A**	34.0	50.0	27.49	0.5	
	B	3.9	50.0	3.11	0.1	
	C	14.9	50.0	12.05	0.2	0.3
	D	4.9	50.0	3.96	0.1	
	F	8.9	50.0	7.19	0.1	0.1
Glu	**A**	102.5	50.0	82.83	1.7	
	B	43.7	50.0	35.33	0.7	
	C	64.6	50.0	52.22	1.0	1.1
	D	20.4	50.0	16.45	0.3	
	E	41.9	50.0	33.85	0.7	
	F	8.6	50.0	6.95	0.1	0.4
His	**A**	17.7	50.0	14.28	0.3	
	B	5.2	50.0	4.17	0.1	
	C	4.7	50.0	3.81	0.1	0.1
	D	2.8	50.0	2.25	0.0	
	E	3.4	50.0	2.71	0.1	0.0
Iso	**A**	29.2	50.0	23.63	0.5	
	B	10.0	50.0	8.09	0.2	
	C	3.5	50.0	2.85	0.1	0.2
	D	6.9	50.0	5.57	0.1	
	E	13.7	50.0	11.03	0.2	
	F	3.9	50.0	3.15	0.1	0.1
Leu	**A**	66.0	50.0	53.32	1.1	
	B	5.6	50.0	4.52	0.1	
	C	12.6	50.0	10.18	0.2	0.5
	D	10.1	50.0	8.17	0.2	
	E	18.5	50.0	14.95	0.3	
	F	3.4	50.0	2.71	0.1	0.2

Lys	**A**	95.0	50.0	76.73	1.5	
	B	56.4	50.0	45.56	0.9	
	C	9.0	50.0	7.25	0.1	0.9
	D	16.5	50.0	13.36	0.3	
	E	43.9	50.0	35.51	0.7	
	F	2.1	50.0	1.69	0.0	0.3
Met	**A**	22.9	50.0	18.51	0.4	
	B	6.7	50.0	5.40	0.1	
	C	5.0	50.0	4.05	0.1	0.2
	D	6.1	50.0	4.95	0.1	
	E	7.4	50.0	5.95	0.1	
	F	2.5	50.0	2.01	0.0	0.1
Orn	**A**	37.3	50.0	30.13	0.6	
	C	2.7	50.0	2.20	0.0	0.3
	D	6.3	50.0	5.13	0.1	0.1
Phe	**A**	50.8	50.0	41.08	0.8	
	B	7.2	50.0	5.80	0.1	0.5
	E	2.8	50.0	2.30	0.0	0.0
Pro	**A**	89.5	50.0	72.31	1.4	
	B	71.1	50.0	57.44	1.1	
	C	32.0	50.0	25.85	0.5	1.0
	D	46.4	50.0	37.51	0.8	
	E	67.6	50.0	54.64	1.1	
	F	17.3	50.0	13.94	0.3	0.7
Ser	**A**	161.3	50.0	130.35	2.6	
	B	46.5	50.0	37.60	0.8	
	C	144.1	50.0	116.40	2.3	1.9
	D	117.8	50.0	95.22	1.9	
	E	104.0	50.0	84.06	1.7	
	F	55.7	50.0	45.03	0.9	1.5
Thr	**A**	134.0	50.0	108.26	2.2	
	B	89.2	50.0	72.10	1.4	
	C	112.1	50.0	90.61	1.8	1.8
	D	180.3	50.0	145.66	2.9	
	E	59.9	50.0	48.38	1.0	
	F	45.3	50.0	36.59	0.7	1.5
Trp	**A**	17.9	50.0	14.44	0.3	0.3
Tyr	**A**	40.5	50.0	32.76	0.7	
	B	9.8	50.0	7.91	0.2	
	C	9.3	50.0	7.55	0.2	0.3
	D	5.3	50.0	4.30	0.1	
	E	5.0	50.0	4.07	0.1	
	F	2.2	50.0	1.74	0.0	0.1
Val	**A**	56.9	50.0	45.94	0.9	
	B	17.8	50.0	14.40	0.3	0.5

C	18.6	50.0	15.00	0.3	
D	15.4	50.0	12.48	0.2	
E	19.8	50.0	16.01	0.3	
F	4.9	50.0	3.99	0.1	0.2

Table 4.21. Nutritional profile of *Chlorella* and *Scenedesmus* genera used in aqua and animal feed adopted from (Dineshbabu et al., 2019; Madeira et al., 2017).

Parameter	**Plastic PBR results (FWW)**	**Plastic PBR results (Control)**	***Chlorella* sp. (Madeira et al., 2017)**	***Chlorella* (Dineshbabu et al., 2019)**	***Scenedesmus* (Dineshbabu et al., 2019)**
Total proteins (%dw)	28.7	25.2	37.7-47.8	46.5	43.7
Total carbohydrates (%dw)	47.3	50.8	18.1-27.5	15.8	25.4
Total lipids (%dw)	22.6	32.9	13.3-20.9	15.8	16.0
Vitamins (mg/kg)					
B1	0.2	-	15-24	17.5	-
B2	0.6	-	-	-	-
B3	3.2	3.6	-	82.5	-
B5	-	-	-	-	-
B6	-	-	10-17	7.7	-
B7	-	-	-	-	-
B9	1.2	0.5	269	-	-
Vitamin C	321.9	271.9	-	-	-
B12	17.9	8.0	-	0.6	-
Vitamin E	0.7	0.2	200	434.5	-
Carotenoid (mg/kg)					
β carotene	43.8	4.8	8-80 (total carotenoids)	0.014 (%dw)	0.07 (%dw)

Table 4.22. Comparison of fatty acid composition of *C. sorokiniana* with literature adopted from (Yun et al., 2020).

Fatty Acid Composition (%)	**Plastic PBR results (FWW)**	**Plastic PBR results (Control)**	***C. sorokiniana* strain KNUA104 (25°C)**	***C. sorokiniana* strain KNUA114 (25°C)**	***C. sorokiniana* strain KNUA122 (25°C)**
C16:0	6.3	5.0	24.6	26.5	25.5
C16:1	-	-	0.1	2.3	1.5
C16:2	-	-	6.2	10.0	9.6
C16:3	-	-	8.9	13.9	12.6
C18:0	0.9	0.5	1.6	0.6	0.8
C18:1 ω9	2.2	1.9	10.0	2.3	3.2
C18:2 ω6	8.0	7.6	21.5	21.4	21.2
C18:3 ω3	1.9	2.7	27.1	22.2	24.7

5. CONCLUSIONS

Microalgae has a potential to remove nutrients from wastewater, as well as sequestering carbon dioxide from waste gas stream (flue gas or similar). In this project, microalgae were purposed as a feedstock for phycoremediation of small-scale farm wastewater coupled with potential livestock and aquaculturing feed application. In summary, obtained farm wastewater was pretreated, ten different microalgae species were screened for their resilience against wastewater, a floating plastic PBR was designed and used for the phycoremediation of the farm wastewater with the best performing microalgal strain, nutrient removal profile of phycoremediation was measured, and biochemical characterization of the biomass were reported for livestock and aquaculturing feed production.

This study showed that *C. sorokiniana* (UTEX 1230) removed 64.2%, 59.4%, and 95.7% of TN, TP, and COD, respectively from pretreated dairy farm wastewater (1/4 dilution) when cultivated in custom designed plastic PBRs. Although lower when compared to similar studies, final biomass concentration of 1.8 g/L was achieved. However, modes of operation, biological reactor types and cultivation conditions are highly influential for nutrient removal and biomass productivity, as well as N:P ratio of wastewater. Thus, for this particular plastic PBR designed for livestock production facilities, optimization studies could improve both N and P removal efficiencies and biomass productivity.

As a final conclusive remark, a detailed nutritional profiling was done for *C. sorokiniana* (UTEX 1230) to be used in future studies, considering it as an input for livestock and aquaculture feed formulations. Comparing with the control's nutritional profile, it is possible to say that FWW is sufficiently nutritious for *C. sorokiniana* (UTEX 1230) that a slightly more protein, FAME, amino acid and vitamin content was observed. It is also possible to repurpose microalgal biomass for bio-fertilizer or other value-added products with a detailed analysis derived from this thesis study.

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UTEX. 2020c. Modified Bold 3N Medium. Web Page: https://utex.org/products/modified-bold-3n-medium?variant=30991514763354

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APPENDIX A

Table A.1. Scheduled MRM table for vitamin standards.

Vitamin	Q1 (Da)	Q3 (Da)	RT (min)	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
B1 Quantifier	265.1	80.9	0.39	6	10	43	6
B1 Qualifier	265.1	122.0	0.40	6	10	23	6
B2 Quantifier	376.9	172.0	2.39	111	10	51	10
B2 Qualifier	376.9	243.0	2.39	111	10	33	8
B3 Quantifier	123.8	79.9	0.48	61	10	29	8
B3 Qualifier	123.8	77.9	0.48	61	10	31	8
B5 Quantifier	220.1	90.0	0.44	61	10	20	6
B5 Qualifier	220.1	98.0	0.45	61	10	20	8
B6 Quantifier	170.0	134.0	0.44	16	10	19	8
B6 Qualifier	170.0	152.0	0.44	16	10	19	6
B7 Quantifier	245.1	226.9	2.36	51	10	21	10
B7 Qualifier	245.1	98.9	2.36	51	10	35	10
B9 Quantifier	442.2	294.9	2.31	71	10	23	12
B9 Qualifier	442.2	176.0	2.31	71	10	57	12
B12m Quantifier	673.0	971.5	2.40	50	10	38	14
B12m Qualifier	673.0	665.0	2.40	50	10	25	4
B12c Quantifier	678.4	147.0	2.31	91	10	71	12
B12c Qualifier	678.4	358.9	2.31	91	10	33	16
C Quantifier	172.9	142.8	0.51	-65	-10	-14	-7
C Qualifier	172.9	112.5	0.51	-65	-10	-14	-9

E Quantifier	431.4	165.1	6.45	-120	-10	-40	-15
E Qualifier	431.4	137.1	6.46	-120	-10	-68	-19
B carotene Quantifier	536.0	280.9	5.95	71	10	39	12
B carotene Qualifier	536.0	502.9	5.95	71	10	21	20

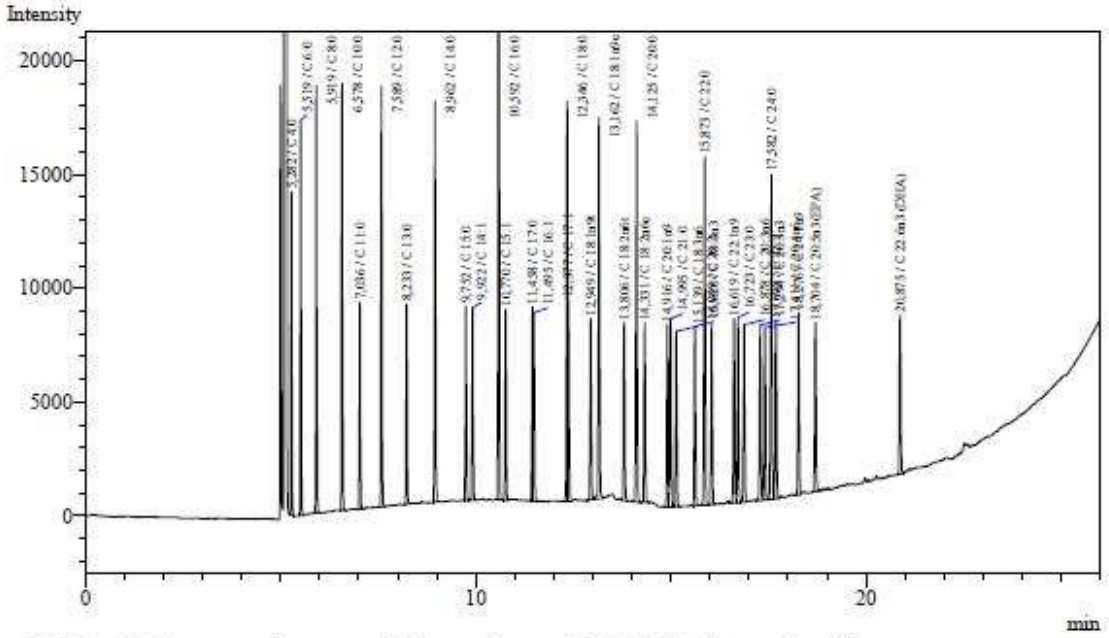
Table A.2. Scheduled MRM table for amino acid standards.

Amino Acid	Q1 (Da)	Q3 (Da)	RT (min)	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
Ala Quantifier	90.1	44.2	2.36	151	10	15	4
Ala Qualifier	90.1	71.9	2.36	151	10	13	6
Arg Quantifier	175.0	115.9	3.54	56	10	19	6
Arg Qualifier	175.0	69.9	3.54	56	10	31	6
Asp Quantifier	134.2	87.8	2.11	26	10	13	6
Asp Qualifier	134.2	73.9	2.11	26	10	19	6
Tyr Quantifier	182.0	76.9	3.01	66	10	49	6
Tyr Qualifier	182.0	90.9	3.01	66	10	37	8
Glu Quantifier	148.0	129.8	2.22	56	10	13	6
Glu Qualifier	148.0	83.8	2.21	56	10	21	6
His Quantifier	156.0	109.8	3.14	16	10	19	8
His Qualifier	156.0	82.8	3.14	16	10	33	8
Iso Quantifier	132.0	68.9	3.77	6	10	23	6
Iso Qualifier	132.0	85.9	3.69	6	10	15	6
Leu Quantifier	132.0	43.1	3.88	41	10	35	4
Leu Qualifier	132.0	85.8	3.88	41	10	15	6

Lys Quantifier	147.1	83.9	3.38	51	10	21	8
Lys Qualifier	147.1	129.9	3.38	51	10	19	8
Met Quantifier	150.1	103.8	2.76	51	10	15	8
Met Qualifier	150.1	60.9	2.76	51	10	31	6
Orn Quantifier	132.8	69.9	3.12	6	10	23	6
Orn Qualifier	132.8	116.0	3.13	6	10	13	6
Phe Quantifier	166.0	120.1	4.30	56	10	17	6
Phe Qualifier	166.0	102.8	4.30	56	10	35	6
Pro Quantifier	115.9	69.9	2.41	6	10	19	6
Pro Qualifier	115.9	67.9	2.41	6	10	39	6
Ser Quantifier	106.0	60.0	2.20	31	10	15	6
Thr Quantifier	120.0	73.9	2.24	21	10	15	6
Thr Qualifier	120.0	56.0	2.25	21	10	21	6
Trp Quantifier	204.9	187.9	5.68	51	10	13.0	8
Trp Qualifier	204.9	146.0	5.67	51	10	23.0	6
Val Quantifier	118.0	54.9	2.84	51	10	45	6
Val Qualifier	118.0	71.9	2.84	51	10	15	6

APPENDIX B

Analysis Date & Time	: 7.09.2020 16:54:34
User Name	: Admin
Vial#	: 6
Sample Name	: FAME_Cal-L6
Sample ID	: NoIS
Sample Type	: Standard
Injection Volume	: 1,00
ISTD Amount	:
Data Name	: C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Data\07092020\DO_FAME_Cal-L6_NoIS_1.g
Method Name	: C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Data\07092020\FAME-SupelcoMethod_Calibr

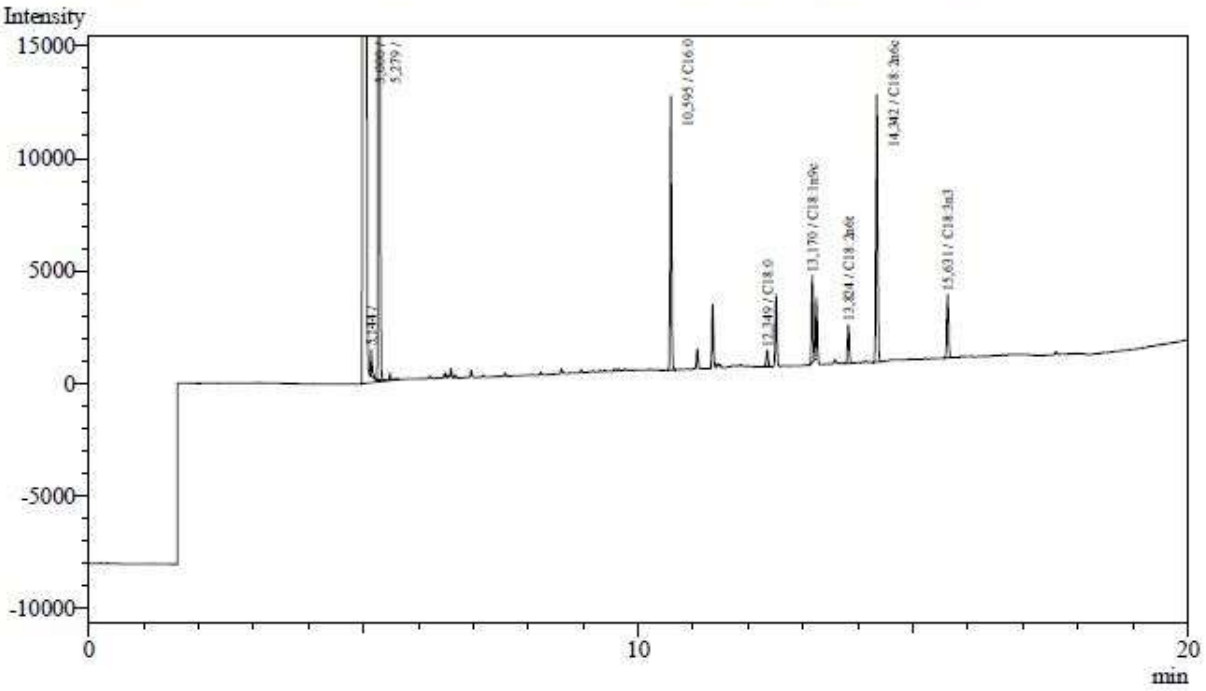


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	5,282	20930	14073	387,095	ppm		1	C 4:0
2	5,519	26028	17210	387,872	ppm		2	C 6:0
3	5,919	29107	18359	389,859	ppm		3	C 8:0
4	6,578	30884	18587	389,267	ppm		4	C 10:0
5	7,036	15614	9032	389,349	ppm		5	C 11:0
6	7,589	32564	18010	389,271	ppm		6	C 12:0
7	8,233	16229	8628	388,587	ppm		7	C 13:0
8	8,962	33174	17413	389,846	ppm		8	C 14:0
9	9,752	16661	8510	390,825	ppm		10	C 15:0
10	9,922	16638	8268	388,582	ppm	V	9	C 14:1
11	10,592	52083	26043	389,932	ppm		12	C 16:0
12	10,770	16613	8353	389,180	ppm		11	C 15:1
13	11,458	16237	8370	388,624	ppm		14	C 17:0
14	11,495	17137	8185	389,237	ppm	V	13	C 16:1
15	12,346	38187	17469	395,058	ppm		16	C 18:0
16	12,377	14037	8838	377,701	ppm	V	15	C 17:1
17	12,949	17128	7937	387,672	ppm	V	18	C 18:1n9t
18	13,162	36278	16618	387,269	ppm	V	17	C 18:1n9c
19	13,806	16801	7770	392,738	ppm		20	C 18:2n6t
20	14,125	36195	16645	391,023	ppm		23	C 20:0
21	14,331	17387	7828	388,147	ppm		19	C 18:2n6c
22	14,916	17426	7892	391,146	ppm	V	24	C 20:1n9
23	14,995	17714	8124	390,124	ppm	V	30	C 21:0
24	15,139	16695	7595	389,607	ppm	V	21	C 18:3n6
25	15,620	16982	7794	391,544	ppm		22	C 18:3n3
26	15,873	36057	15171	390,560	ppm		31	C 22:0

Figure B.1. FAME mix GC report.

Analysis Date & Time : 30.08.2020 18:52:23
User Name : Admin
Vial# : 11
Sample Name : SampleA_FAME
Sample ID : UNK-0001
Sample Type : Unknown
Injection Volume : 1,00
ISTD Amount :

Data Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Data\30082020\Sample_FAME_A.gcd
Method Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Methods\FAME-SupelcoMethod_.gcm

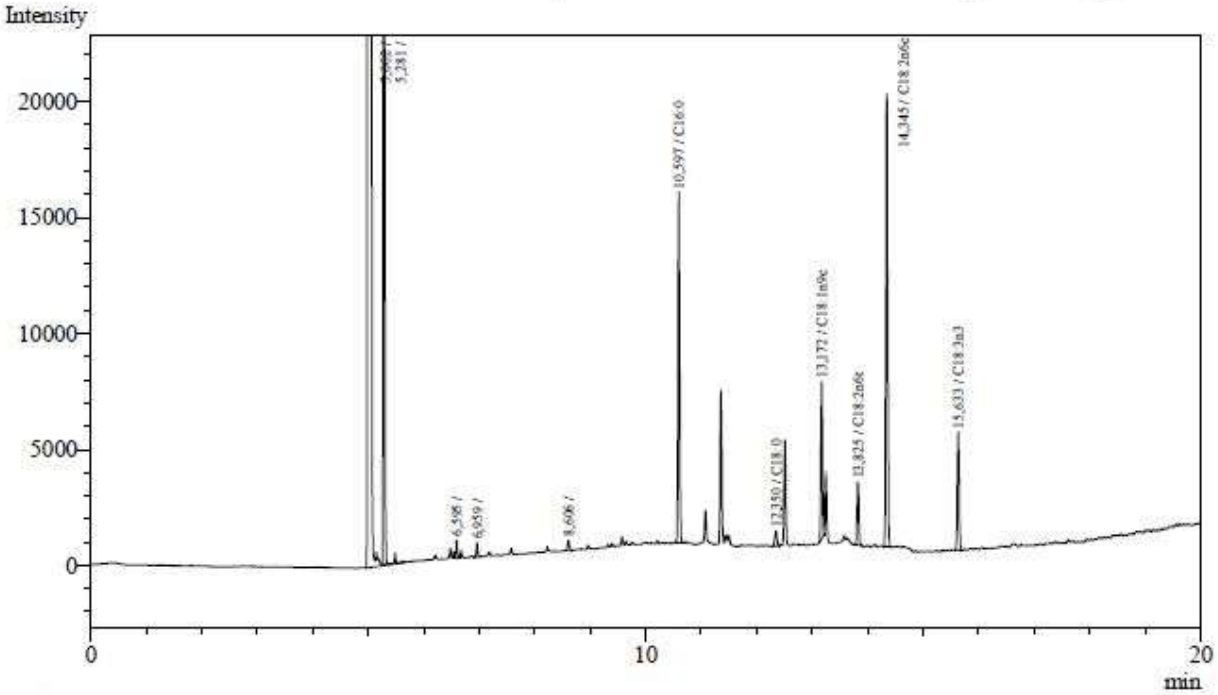


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	5,000	67032598	47835847	0,000		S		
2	5,144	1930	1135	0,000		T		
3	5,279	327875	212916	0,000		T		
4	10,595	23665	11994	0,000	ppm		1	C16:0
5	12,349	1719	706	0,000	ppm		2	C18:0
6	13,170	7628	3796	0,000	ppm		3	C18:1n9c
7	13,824	3630	1686	0,000	ppm		4	C18:2n6t
8	14,342	25225	11725	0,000	ppm		5	C18:2n6c
9	15,631	6056	2784	0,000	ppm		6	C18:3n3
Total		67430326	48082589					

Figure B.2. Gas chromatography report for PBR A.

Analysis Date & Time : 30.08.2020 19:26:01
User Name : Admin
Vial# : 12
Sample Name : SampleB_FAME
Sample ID : UNK-0002
Sample Type : Unknown
Injection Volume : 1,00
ISTD Amount :

Data Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Data\30082020\Sample_FAME_B.gcd
Method Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Methods\FAME-SupelcoMethod_.gcm

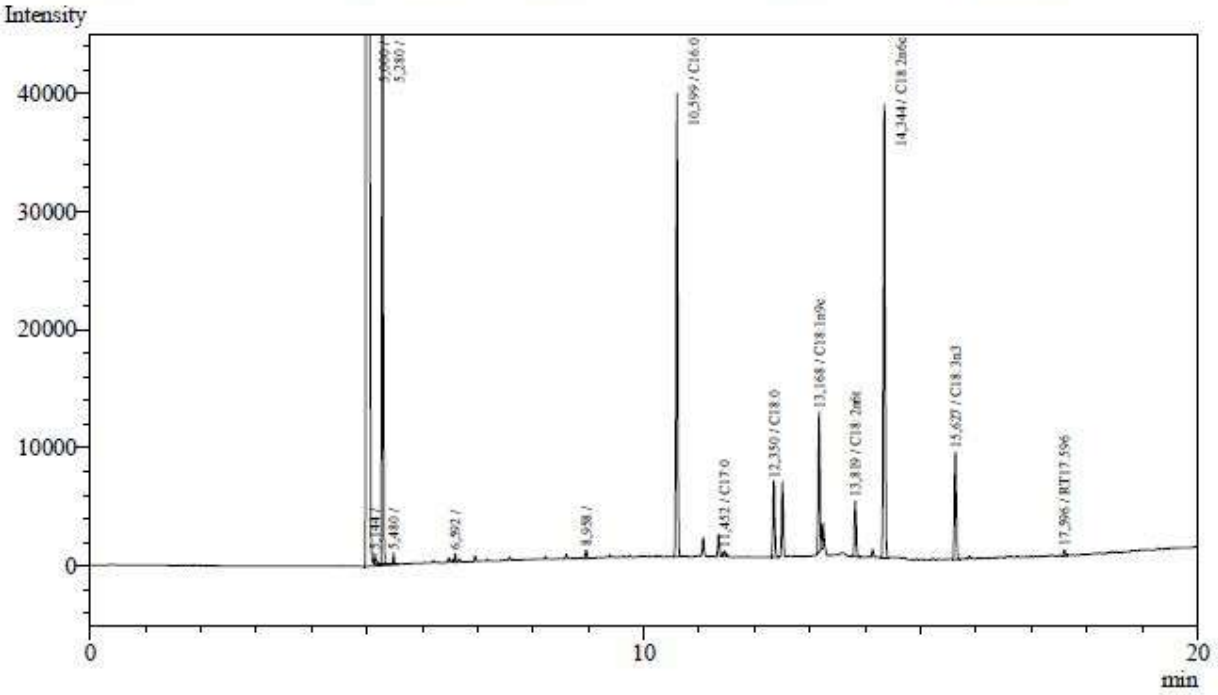


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	5,002	68212049	49467459	0,000		S		
2	5,281	97027	64352	0,000		T		
3	6,595	1223	749	0,000		V		
4	6,959	1103	610	0,000		V		
5	8,606	1069	445	0,000		V		
6	10,597	29904	15068	0,000	ppm	V	1	C16:0
7	12,350	1704	640	0,000	ppm		2	C18:0
8	13,172	13516	6726	0,000	ppm		3	C18:1n9c
9	13,825	5856	2695	0,000	ppm		4	C18:2n6t
10	14,345	41496	19384	0,000	ppm		5	C18:2n6c
11	15,633	11194	5076	0,000	ppm		6	C18:3n3
12	22,098	1184	325	0,000		V		
13	22,621	2044	440	0,000		V		
14	22,671	1067	358	0,000		V		
15	24,519	1140	88	0,000				
Total		68421576	49584415					

Figure B.3. Gas chromatography report for PBR B.

Analysis Date & Time : 30.08.2020 19:59:35
User Name : Admin
Vial# : 13
Sample Name : SampleC_FAME
Sample ID : UNK-0003
Sample Type : Unknown
Injection Volume : 1,00
ISTD Amount :

Data Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Data\30082020\Sample_FAME_C.gcd
Method Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Methods\FAME-SupelcoMethod_.gcm

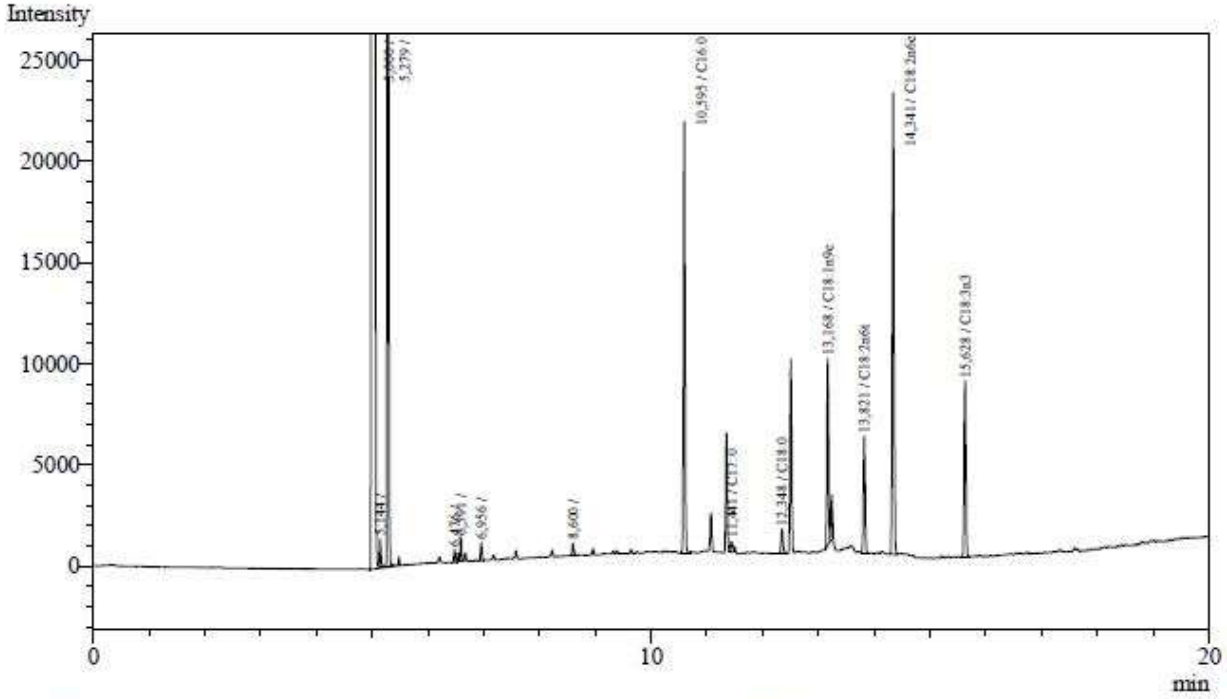


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	5,000	67724505	48879367	0,000		S		
2	5,144	1570	831	0,000		T		
3	5,280	107550	70861	0,000		T		
4	5,480	1370	947	0,000		TV		
5	6,592	1225	717	0,000		V		
6	8,958	1386	705	0,000		V		
7	10,599	77954	38577	0,000	ppm		1	C16:0
8	11,452	1226	455	0,000	ppm		2	C17:0
9	12,350	13347	6411	0,000	ppm	V	3	C18:0
10	13,168	23699	11771	0,000	ppm		4	C18:1n9c
11	13,819	10010	4661	0,000	ppm		5	C18:2n6t
12	14,344	82190	38074	0,000	ppm		6	C18:2n6c
13	15,627	19712	9069	0,000	ppm		7	C18:3n3
14	17,596	1262	513	0,000	ppm		8	RT17.596
15	22,089	1177	322	0,000		V		
16	22,592	1793	425	0,000		V		
17	22,635	1388	465	0,000		V		
18	23,091	1224	103	0,000		V		
Total		68072588	49064274					

Figure B.4. Gas chromatography report for PBR C.

Analysis Date & Time	: 30.08.2020 20:33:10
User Name	: Admin
Vial#	: 14
Sample Name	: SampleD_FAME
Sample ID	: UNK-0004
Sample Type	: Unknown
Injection Volume	: 1,00
ISTD Amount	:

Data Name	: C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Data\30082020\Sample_FAME_D.gcd
Method Name	: C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Methods\FAME-SupelcoMethod_.gcm

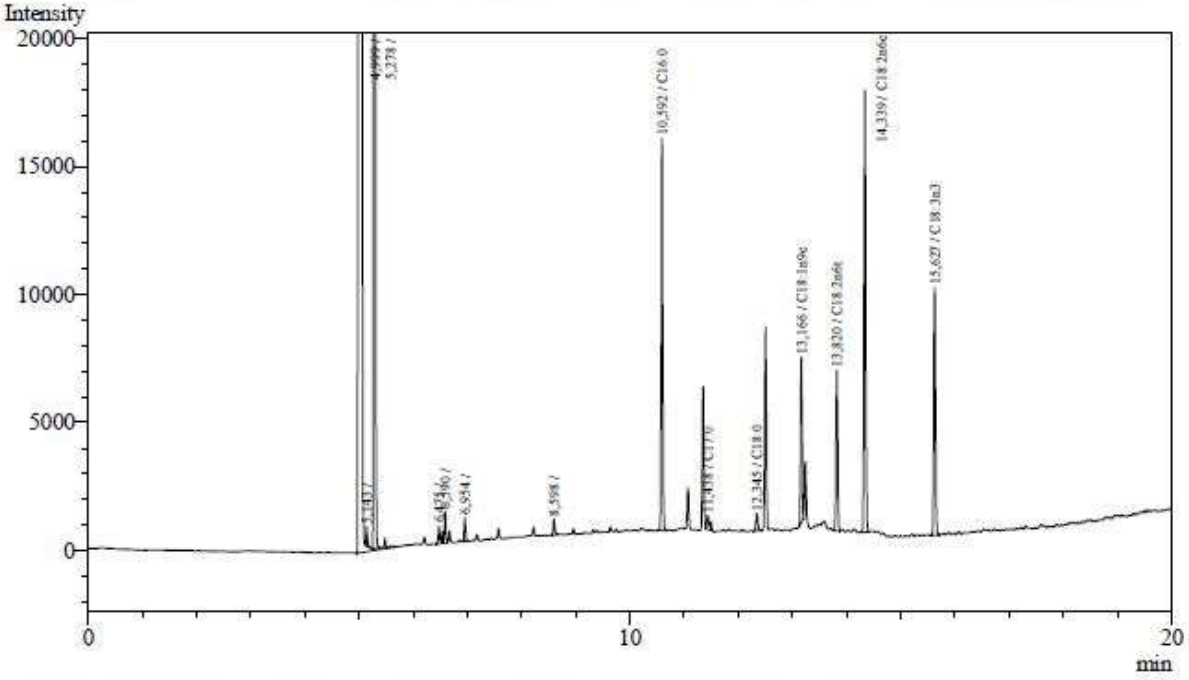


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	5,000	68001919	48536508	0,000		S		
2	5,144	2111	1321	0,000		T		
3	5,279	133332	86854	0,000		T		
4	6,476	1136	629	0,000				
5	6,591	1973	1143	0,000		V		
6	6,956	1536	881	0,000				
7	8,600	1270	598	0,000				
8	10,595	41702	20932	0,000	ppm		1	C16:0
9	11,441	1304	519	0,000	ppm		2	C17:0
10	12,348	2797	1191	0,000	ppm		3	C18:0
11	13,168	18477	9165	0,000	ppm		4	C18:1n9c
12	13,821	12149	5709	0,000	ppm		5	C18:2n6t
13	14,341	48037	22519	0,000	ppm		6	C18:2n6c
14	15,628	18886	8651	0,000	ppm		7	C18:3n3
15	22,090	1533	305	0,000		V		
16	22,575	1639	421	0,000		V		
17	22,631	1211	387	0,000		V		
Total		68291012	48697733					

Figure B.5. Gas chromatography report for PBR D.

Analysis Date & Time : 30.08.2020 21:06:46
User Name : Admin
Vial# : 15
Sample Name : SampleE_FAME
Sample ID : UNK-0005
Sample Type : Unknown
Injection Volume : 1,00
ISTD Amount :

Data Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Data\30082020\Sample_FAME_E.gcd
Method Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Methods\FAME-SupelcoMethod_.gcm

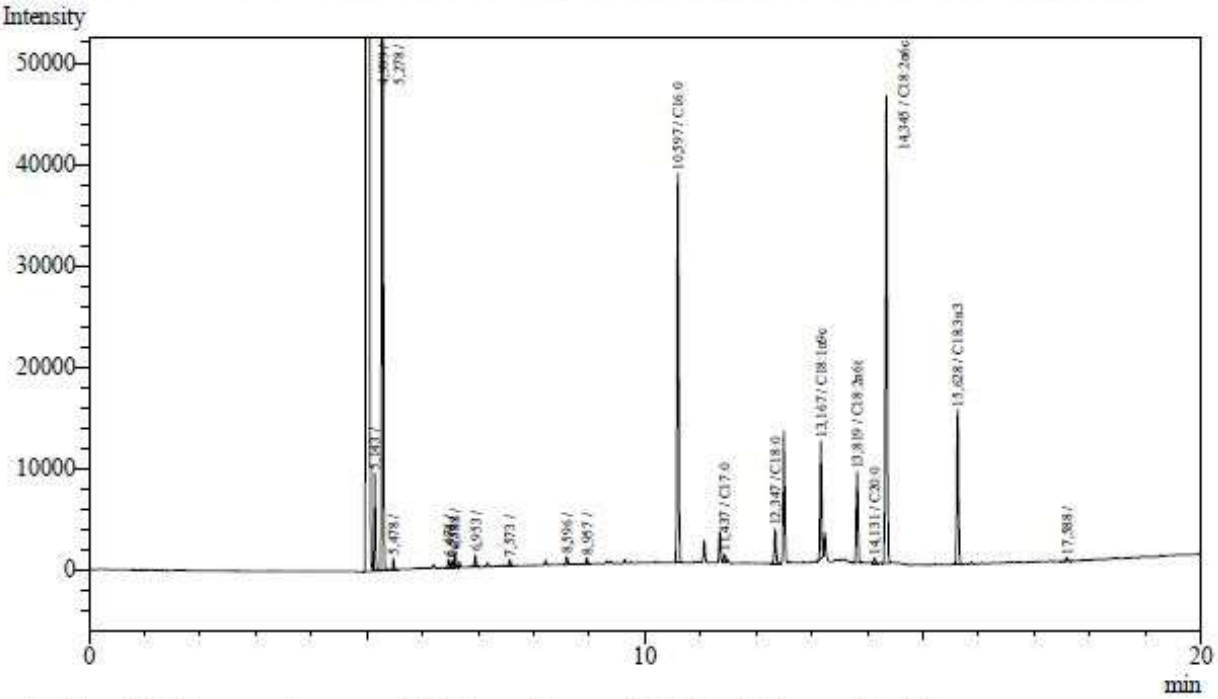


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	4,999	67168625	47051830	0,000		S		
2	5,143	1169	758	0,000		T		
3	5,278	303137	197807	0,000		T		
4	6,475	1371	694	0,000		V		
5	6,590	2155	1215	0,000		V		
6	6,954	1570	900	0,000				
7	8,598	1227	605	0,000				
8	10,592	30114	15209	0,000	ppm		1	C16:0
9	11,438	1284	532	0,000	ppm		2	C17:0
10	12,345	1681	674	0,000	ppm		3	C18:0
11	13,166	12864	6316	0,000	ppm		4	C18:1n9c
12	13,820	13481	6217	0,000	ppm		5	C18:2n6t
13	14,339	36473	17194	0,000	ppm	V	6	C18:2n6c
14	15,627	21142	9632	0,000	ppm		7	C18:3n3
15	22,089	1341	260	0,000		V		
16	22,563	1560	400	0,000		V		
17	22,619	1155	356	0,000		V		
18	25,126	1060	51	0,000		V		
Total		67601409	47310650					

Figure B.6. Gas chromatography report for PBR E.

Analysis Date & Time : 30.08.2020 21:40:24
User Name : Admin
Vial# : 16
Sample Name : SampleF_FAME
Sample ID : UNK-0006
Sample Type : Unknown
Injection Volume : 1,00
ISTD Amount :

Data Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Data\30082020\Sample_FAME_F.gcd
Method Name : C:\GCsolution\Data\FAME Analysis\12082020-SLB-IL\Methods\FAME-SupelcoMethod_.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	4,999	67669212	47858380	0,000		S		
2	5,143	14024	9373	0,000		T		
3	5,278	174382	114565	0,000		T		
4	5,478	1647	1129	0,000		T		
5	6,474	1530	777	0,000				
6	6,536	1131	682	0,000		V		
7	6,588	2437	1416	0,000		V		
8	6,953	2001	1165	0,000		V		
9	7,573	1430	601	0,000		V		
10	8,596	1634	773	0,000				
11	8,957	1081	583	0,000				
12	10,597	76003	38148	0,000	ppm		1	C16:0
13	11,437	2080	772	0,000	ppm		2	C17:0
14	12,347	7213	3356	0,000	ppm		3	C18:0
15	13,167	23317	11477	0,000	ppm		4	C18:1n9c
16	13,819	19215	8775	0,000	ppm		5	C18:2n6t
17	14,131	1117	478	0,000	ppm	V	6	C20:0
18	14,345	98854	45650	0,000	ppm		7	C18:2n6c
19	15,628	33229	15178	0,000	ppm	V	8	C18:3n3
20	17,588	1099	354	0,000				
21	22,089	1141	306	0,000		V		
22	22,548	1627	433	0,000		V		
23	22,622	2007	366	0,000		V		
24	25,121	1195	50	0,000				
Total		68138606	48114787					

Figure B.7. Gas chromatography report for PBR F.