SCREENING *SPIRULINA* STRAINS FOR PROTEIN PRODUCTIVITY BASED ON CULTIVATION UNDER PHOTOBIOREACTOR CONDITIONS

A Thesis Submitted to
the Graduate School of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Environmental Engineering

by
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December 2022
İZMİR

ACKNOWLEDGEMENTS

First of all, I would like to acknowledge and give my warmest thanks to thank my advisor Assist. Prof. Dr. Altan ÖZKAN and my co-advisor Assoc. Prof. Dr. Ali Oğuz BÜYÜKKİLECİ for their guidance, advice, and support throughout my thesis.

I would like to thank my thesis defense committee members Assoc. Prof. Dr. Sırma YEĞİN and Assist. Prof. Dr. Mehmet Ali KÜÇÜKER for their valuable recommendations.

I would like to express my special thanks to my fiance Büşra AYVAZ for her endless support and advice. I would like to thank my lab friend Tuğçe SEZGİN and my co-workers İlknur AYRİ, Esin BALCI, Yiğithan KAZANCI, and Begüm TERZİ for their precious help and advice.

I would like to thank the Izmir Institute of Technology Scientific Research Projects Coordinator for their financial support.

Finally, I would like to express my gratitude to my mother Rukiye ULUS, and my grandmother Nevriye ARSLAN for being with me and supporting me throughout my life. I couldn't have made it without them.

ABSTRACT

SCREENING *SPIRULINA* STRAINS FOR PROTEIN PRODUCTIVITY BASED ON CULTIVATION UNDER PHOTOBIOREACTOR CONDITIONS

Spirulina is an industrially produced algae for consumption as a nutritional supplement owing to its exceptionally high protein content. The delivery of the desired metabolite profiles highly depends on selecting the correct strains for growth. In this regard, the information in the literature is limited as the strains cultivated industrially are unknown, and the strains of academic research were mostly locally isolated or procured from local sources.

The current study is the first step of research activities planned to assess the large-scale production potential of *Spirulina* in Izmir. Thus in this work, *Spirulina platensis* 2340, *Spirulina maxima* 84.79, *Spirulina platensis* 85.79, *Spirulina platensis* 86.79, and *Spirulina platensis* 29 were screened for biomass and protein productivity during cultivation under standardized process conditions of a bubble column photobioreactor.

The final biomass concentrations were strain specific and ranged from 1.2 to 1.9 g/L. An inverse relationship existed between the logistic model-based final biomass concentrations and the production rate constants. Thus, the peak productivities were more evenly distributed and ranged between 0.15 to 0.20 g/L-day. SP 29 had the ideal protein content vs. cultivation time profile as it was consistently high and varied in the narrow range of 60 to 64%. Higher protein contents could be reached with the other strains, but they also had higher variations during the growth period. The final protein concentrations varied from 0.4 g/L to 1.4 g/L. The highest peak productivity obtained was about 0.11 g/L-day, which could be obtained by three of the strains. The results clearly show the importance of strain selection for sustaining protein-rich biomass production with *Spirulina*.

ÖZET

SPİRULİNA SUŞLARININ PROTEİN ÜRETKENLİKLERİNİN FOTOBİYOREAKTÖR KOŞULLARINDA KÜLTİVASYONLA KIYASLANMASI

Spirulina, yüksek derecede yüksek protein içeriği sayesinde besin takviyesi olarak tüketim için endüstriyel olarak üretilen bir fotosentetik alg türüdür. Endüstriyel üretim için doğru suş seçimi, istenilen metabolit profillerinin elde edilebilmesi için büyük bir öneme sahiptir. Bu bağlamda, endüstriyel olarak yetiştirilen suşların bilinmemesi ve akademik araştırma suşlarının çoğunlukla yerel olarak izole edilmesi veya yerel kaynaklardan temin edilmesi nedeniyle literatürdeki bilgiler sınırlıdır.

Mevcut çalışma, *Spirulina*'nın İzmir'deki büyük ölçekli üretim potansiyelini değerlendirmek için planlanan araştırma faaliyetlerinin ilk adımıdır. Bu nedenle, bu çalışmada, *Spirulina platensis* 2340, *Spirulina maxima* 84.79, *Spirulina platensis* 85.79, *Spirulina platensis* 86.79, and *Spirulina platensis* 29, bir kabarcık kolon fotobiyoreaktörde standartlaştırılmış işlem koşulları altında yetiştirme sırasında biyokütle ve protein üretkenliği açısından incelenmiştir.

Nihai biyokütle konsantrasyonlarının suşa özgün olduğu ve 1,2 ila 1,9 g/L arasında değiştiği gözlemlenmiştir. Lojistik modele dayalı nihai biyokütle konsantrasyonları ile üretim hızı sabitleri arasında ters bir ilişki tespit edilmiştir. Bundan dolayı, maksimum üretkenlikler daha eşit bir şekilde dağılmış ve 0,15 ile 0,20 g/L-gün arasında değişmiştir. SP 29, protein içeriği diğer suşlara kıyasla daha yüksek ve nispeten az (%60 ile %64 arasında) değişim gösterdiği için, zamana karşı elde edilen protein miktarı suşlar içinde ideal olandır. Diğer suşlarla daha yüksek protein içeriklerine ulaşılabilmesine rağmen, protein içeriğinde ki değişim miktarlarının fazla olduğu gözlemlenmiştir. Nihai protein konsantrasyonları 0,4 g/L ila 1,4 g/L arasında değişkenlik göstermiştir. Elde edilen en yüksek üretkenlik yaklaşık 0.11 g/L-gün olup, büyütülen suşların üçü bu üretkenliğe ulaşabilir. Sonuçlar, *Spirulina* ile protein açısından zengin biyokütle üretimini sürdürmek için suş seçiminin önemini açıkça göstermektedir.

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CHAPTER 1

INTRODUCTION

Microalgae are microorganisms that utilize readily available resources, such as carbon, nitrogen, phosphorus, and light, to grow and biosynthesize commercially valuable organics, including proteins, lipids, and pigments (Brennan and Owende 2010). Both prokaryotic and eukaryotic photosynthetic microorganisms can be classified as microalgae (Chisti 2008). Species and strains belonging to *Spirulina* (*Arthrospira*) genus are among these prokaryotes. They are grouped with bacteria based on cell structure and with microalgae based on photosynthetic capability. *Spirulina* cells have a characteristic spiral shape, with lengths usually ranging from 200 to 300 μm and diameters ranging from 5 to 10 μm. In nature, they dwell in alkaline (pH 9.5) water bodies with high carbonate and bicarbonate contents and under tropical and subtropical climates (Chakchak et al. 2021; Soni et al. 2017). Their growth rates are highly temperature dependent, but their optimum growth temperature usually ranges between 29 to 35°C (Soni et al. 2017).

Spirulina, particularly those that belong to *S. platensis* and *S. maxima* genera, have been produced commercially based on their high nutritional value (Muys et al. 2019; Soni et al. 2017). This production generated a global market of 288 million Euro in 2017, and this volume was expected to grow by 10% annually (Muys et al. 2019). The main reasons for this intense and expanding economic activity are (i) the protein content of *Spirulina*, reaching up to 70% of the dry biomass under appropriate cultivation conditions, (ii) the capability of *Spirulina* to biosynthesize all the essential amino acids, (iii) the presence of other essential nutrients including antioxidants, vitamins, minerals, and unsaturated fatty acids, (iv) high digestibility of *Spirulina* due to its polysaccharide-based cell wall, and (v) the ability of *Spirulina* to grow at high rates suspended in nutrient medium without the requirement of agricultural land (Lupatini et al. 2017; Muys et al. 2019; Soni et al. 2017). Furthermore, *Spirulina* has a lower environmental impact than other animal-based sources and is therefore considered a more sustainable alternative (Ye et al. 2018).

It is also possible to enrich *Spirulina* further with the processes to be carried out during or after production. This way, functional products can be obtained, and the

product's added value can be increased. For example, *Spirulina* biomass can be enriched with iron and used to overcome the iron deficiency in an anemic population. Iron supplements can have potential adverse side effects on the digestive system. Therefore, new forms and methods of iron supplementation have been investigated (Kim et al. 2014). In this regard, forming iron peptide (protein) complexes has been one focus area. The controlled growth conditions and high protein content of *Spirulina* make it an ideal platform for such enrichment activities.

Another advantage of *Spirulina* is that it has a lower environmental impact than animal-derived proteins and therefore is a more sustainable protein source (Ye et al. 2018). In addition to proteins, *Spirulina* is also a good source of vitamins, lipids, fibers, minerals, carbohydrates, and some natural pigments (phycobiliproteins and carotenoids) (Becker 2007). These features have generated a significant amount of commercial interest in the production of *Spirulina* biomass.

Open pond, tubular and flat-plate photobioreactor systems are used for the cultivation of *Spirulina* (Lupatini et al. 2017; Soni et al. 2017; Vonshak, 1997). Cells are grown in suspension in aquatic nutrient media in all these systems. The light delivered to the microalgae serves as the metabolic energy source. Inorganic carbon, nitrogen, and phosphorus required for biosynthesis are provided in dissolved form in the nutrient media. In these reactors, the suspensions are mixed by movement and/or air injection into the suspensions. In doing so, the cells are kept suspended, the CO_2 consumed gets replenished, and the dissolved accumulated gets removed.

One major decision that has to be made before the test of large-scale production of any algae-based products is the selection of the strain to be cultivated. This is especially important for *Spirulina* because (i) it is highly advertised and consumed as a dietary supplement for its exceptionally high protein content, ranging from 60 to 70% of the dry biomass, (ii) the protein content of *Spirulina* varies significantly based on the strain used for cultivation, (iii) likely because it is considered as a commercial secret the information on the strains used for industrial scale production is not available, and (iv) the strains used for academic research are mostly local isolates or obtained from local collection centers out of the reach of the general academic circles (Andrade et al. 2019; Lupatini et al. 2016; Muys et al. 2019). The current work is the first step of a range of research activities planned for the large-scale production of *Spirulina* in Izmir. It thus aims to perform a protein productivity screening with the strains available in algae culture collection centers. For this, five strains belonging to two commercially produced

species of *Spirulina* were cultivated under the standardized process conditions of a bubble column photobioreactor at indoor conditions, and the resulting biomass and protein productivities were compared. Furthermore, possible productivity increase methods were identified for future studies by conducting a mass balance analysis around the photobioreactor inlet aeration and outlet exhaust gas CO_2 concentrations.

CHAPTER 2

LITERATURE REVIEW

Spirulina (Arthrospira) is photosynthetic blue-green microalgae that grow best in the mesophilic temperature range (between 29 to 35C°) and alkaline environments (pH 9.5). It is best known for its high protein content that can reach up to 70% (Chakchak et al. 2021; Soni et al. 2017; Vonshak 1997). Based on these features, *Spirulina* has been cultivated commercially for consumption as a nutrient supplement. *Spirulina* is also rich in pigments, particularly the blue pigment phycocyanin, and thus has been utilized industrially as a source of natural blue food colorant (Marzorati et al. 2020). Other potential applications, such as its use in fish feed formulations, have been intensely researched (Olvera-Novoa et al. 1998).

2.1. Microalgae Cultivation Systems

Microalgae are cultivated in open systems such as unstirred ponds and raceway-type ponds or closed photobioreactors such as tubular and flat plate photobioreactors (Chew et al. 2018). In all these systems, cells are cultivated in suspension in a water-based nutrient media. Light and inorganic carbon has to be delivered to the cells as energy and carbon sources, respectively. Nitrogen and phosphorus salts are added to the cultivation media. The cells are kept suspended during growth to improve their access to light and nutrients. This is achieved through either air flow introduced to the suspensions or through the movement of the suspension in the cultivation system.

2.1.1. Open Ponds

Unstirred Ponds

The most simple open system for growing microalgae is shallow, unstirred ponds. They have been used for the commercial production of a number of microalgae species, such as *Dunaliella salina*, due to low capital and operational costs. Unstirred pools are usually built in natural water formations at a depth of 50 cm to ensure

sufficient light delivery. One major disadvantage of these systems frequently observed is the carbon delivery limited growth conditions created due to the absence of mixing. Other important disadvantages include low rate (diffusion-based) nutrient delivery and light-limited growth conditions that develop particularly with increasing biomass concentration (Chew et al. 2018). Also, as with all the other open systems, the system is vulnerable to contamination. Herbicides and pesticides have been used to solve these issues (Kusmayadi et al. 2020).

Raceway Ponds

Raceway ponds have been used on a large scale to produce microalgae for the last 70 years (Arutselvan et al. 2022). In these systems, microalgae suspensions are grown in open circular ponds with a depth of approximately 30-40 centimeters (Lupatini et al. 2017). During cultivation, the suspension is kept in motion with an impeller-stirrer to prevent settling and homogenize the overall growth environment (Lupatini et al. 2016). These systems offer limited control over the process conditions. For example, contamination risk and evaporative water losses are high (Poddar et al. 2022). Despite these disadvantages, they are the most commonly used platforms for *Spirulina* cultivation. This is mainly due to (i) low initial investment costs, (ii) high operational ease, (iii) maintenance of low hydrodynamic shear rate growth conditions due to the absence of mechanical pumping or aeration, (iv) *Spirulina*'s ability to grow under extreme environmental conditions including high alkalinity (pH>9.5) and salinity minimizing the contamination risks, and (v) easy reactor clean up (Chisti 2007; Lupatini et al. 2017; Soni et al. 2017).

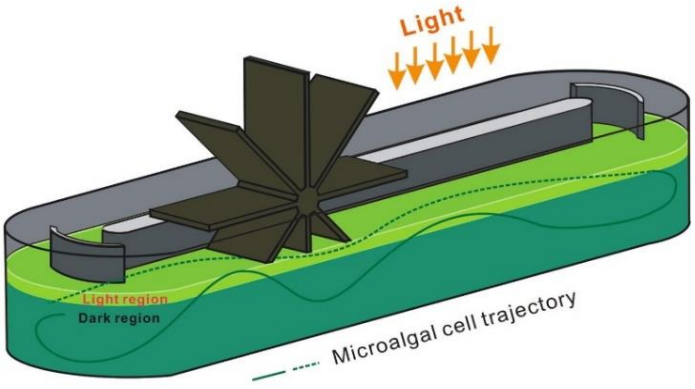

Figure 1. Schematics of raceway pond

(Source: Z. Chen et al. 2016)

2.1.2 Closed Systems

Tubular, flat plate, and column photobioreactors are the most frequently used closed systems. These have been designed to overcome the major limitations of the open systems. This is achieved through the isolation of the cultivation vessel from direct contact with the environment. This isolation aids with the (i) reduction of contamination with microbial and other organic as well as inorganic contaminants, (ii) improvement of CO_2 availability, and (iii) control of the process conditions such as temperature, pH, and water chemistry. Transparent materials such as glass or acrylic have been used as cultivation vessels. Reactor geometries are set to increase the surface area to volume ratios and resultingly improve light availability (Chisti 2007; Davis et al. 2011). The major disadvantage of closed systems is higher construction and operation costs compared to open systems. Species of medium and high value have been cultivated with these systems to improve economics (Gong and Jiang 2011; Lehr and Posten 2009).

Tubular Photobioreactors

Tubular photobioreactors consist of transparent materials of cylindrical shape that have a diameter of around 10 cm. This restriction mainly aims to reduce light path length and thus increase light availability. These reactors can be positioned vertically or horizontally with respect to the ground, or they can be inclined or helical (Carvalho et al. 2006). A homogeneous mechanical pump is used to flow the suspensions in tubes, or an air lifting system is used for concurrent CO_2 transfer to the liquid media (Eriksen 2013). These systems typically suffer from O_2 built-up and CO_2 depletion along the tube lengths. This is why the suspensions are pooled in containers after certain lengths and bubbled with air or CO_2-enriched air to remove the excess O_2 and replenish the consumed CO_2. The design of these reactors is based on a number of important process parameters such as light intensity, maximum tolerable O_2, and CO_2 concentrations, optimum temperature, and biomass concentrations. For example, cells cultivated at high light intensity may experience photooxidative damage when dissolved O_2 built up is not prevented. Excessive CO_2 enrichment may also lower the cultivation pH below the optimum range. Light availability may lower productivity when biomass concentration exceeds certain thresholds (Chew et al. 2018).

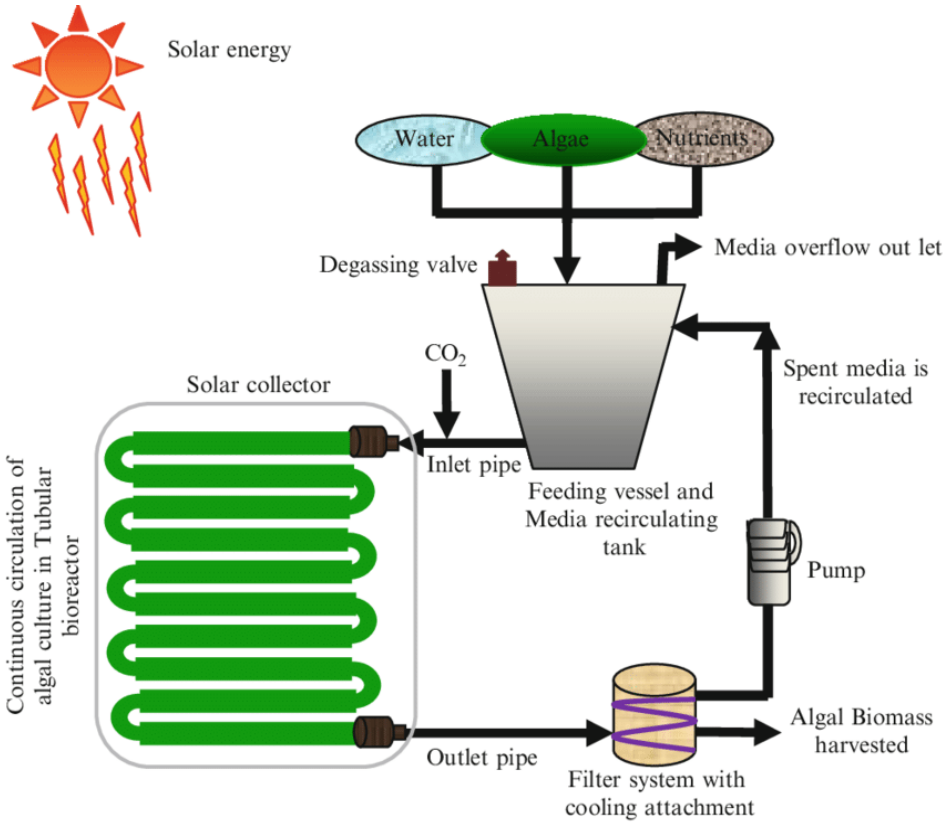

Figure 1. Schematics of a tubular PBR operated at outdoor conditions (Source: Prakash Pandey and Tiwari, 2010)

Flat Panel Photobioreactors

Flat panels are one of the oldest and most typical reactor types. The cells are cultivated within transparent sheets of limited depth to ensure the establishment of optimum light availabilities (Ting et al. 2017). The suspensions are constantly aerated to keep the cells suspended and to increase gas transfer rates. Some typical features of these systems are (i) high illumination area, (ii) low dissolved oxygen concentration, and (iii) low capital and operating costs. The disadvantages include (i) cell adhesion over cultivated surface and reduction of light availability, (ii) excessive heating of the suspensions during daytime due to the high surface area to volume ratio and (iii) high hydrodynamic shear generated within the suspensions due to aeration (Ting et al. 2017; Dudek et al. 2012; Chew et al. 2018).

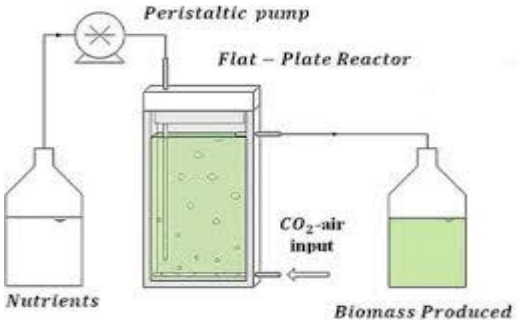

Figure 3. Schematics of a flat-plate PBR
(Source:Egbo, Okoani, and Okoh 2018)

Bubble Column Photobioreactor

In bubble column reactors, algae are cultivated in transparent cylindrical vessels with height-to-diameter ratios of 2 or more. Algal suspensions are constantly aerated during growth by pumping air or air/CO_2 mixtures to the spargers installed at the reactor bottom. It has several advantages, including low investment cost, high surface area/volume ratios, low hydrodynamic shear, and high mass transfer rates (Singh and Sharma 2012; Chew et al. 2018). The disadvantages of this system are that the solubility of CO_2 in water is low and slow, as well as the possibility of cell settling due to the random flow pattern that develops (Lam and Lee 2013; Langley et al. 2012; Chew et al. 2018). CO_2 fixation is vital for growth. For this, it is necessary to increase the residence time of CO_2 in the medium (Guo et al. 2019).

2.2. Media for *Spirulina* Cultivation

Zarrouk media is currently the most preferred medium for growing *Spirulina* since it sustains high biomass production rates and high protein contents (Saranraj and Sivasakthi 2014). While it offers these critical productivity advantages, it also creates potential economic bottlenecks as well since the chemicals used for media preparation (listed in Table 1) can account for up to one-third of the total biomass production cost (Lim et al. 2021).

Table 1. Zarrouk media recipe (ZARROUK 1966)

Ingredients	**Amount added per L of medium (g/L)**
$NaHCO_3$	16.8
$NaNO_3$	2.5
NaCl	1
K_2SO_4	1
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.01
$CaCl_2.2H_2O$	0.004
EDTA	0.008
Distilled Water	999ml
Micronutrients	**1ml**
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.4H_2O$	0.222
Na_2MoO_4	0.0177
$CuSO_4.5H_2O$	0.079

A number of existing autotrophic cultivation media have been tested and also modified for *Spirulina* growth per the aforementioned economic concerns. These include the central food technological research institute (CFTRI) (Salunke et al. 2016), Blue Green (BG-11) (Fanka et al. 2022), Kosaric Medium (Phang et al. 2000), F/2 and Conway (Dineshkumar et al. 2016). BG-11, F/2, and Conway media generated lower biomass productivity than the Zarrouk medium (Dineshkumar et al. 2016).

An alternative method targeted the replacement of high-cost nutrient media chemicals with low-cost and/or waste-based ones. Table 2 summarizes the origin and the properties of the waste-based media tested for *Spirulina*-based protein production and also the productivity outcomes. These studies used domestic and industrial wastewaters, plant-based wastes, urea, and fertilizer as nutrient sources. In general, alternative media generated lower final biomass concentrations when compared with Zarrouk media without resulting in a significant change in the protein contents.

The development of a *Spirulina*-based municipal wastewater treatment plant has been highly appealing because such technology would combine to the production of commercially valuable biomass with the oxygenation of wastewater and the removal of nutrients. Zhai et al. (2017) achieved high nutrient removal efficiencies, in particular for nitrogen and phosphorus, with synthetic municipal wastewater (Zhai et al. 2017). However, the final biomass concentrations were about half of the Zarrouk medium, and the associated protein contents were about 8% less. Inferior performance with wastewater was attributed to nutrient-limited growth conditions, particularly for phosphorus (Zhai et al. 2017).

Municipal wastewater nutrient profile optimization can potentially improve the performance on the biomass production side. However, the level of improvement can also be limited due to variations observed with wastewater parameters, including nutrient concentrations and pH. Thus, waste nutrients with industrial origin have been considered as a more reliable source for production at more standard rates and biomass profiles.

Han et al. (2021) cultivated *Spirulina* in 25, 50, 75, and 100% raw and autoclaved soy sauce wastewater and compared the productivities with the Zarrouk medium's (Han et al. 2021). Final biomass concentrations obtained with raw soy sauce wastewater exceeded those obtained with autoclaved version at all the dilution ratios. This improved performance was attributed to possible synergistic interactions *Spirulina* may have had with the raw wastewater microbiota. Zarrouk medium generated the highest biomass concentration without significantly changing biomass protein content.

Jung et al. (2014) developed a modified Zarrouk medium by utilizing waste oyster shells and soil extracts as micronutrients source (Jung et al. 2014). The biomass profiles generated, including lipids, protein, and carbohydrates, were indifferent to the nutrient source. However, the biomass concentrations, phycocyanin, and chlorophyll contents decreased with respect to the Zarrouk medium-based growth.

Taufiqurrahmi et al. (2017) supplemented the inorganic cultivation medium MSI with rice husk charcoal extract to improve biomass and phycocyanin productivity (Taufiqurrahmi et al. 2017). The supplementation increased the final biomass concentrations by 1.6 times. However, this productivity increase was accompanied by a reduction in phycocyanin contents.

Pereira et al. (2022) developed a brewing residue-based nutrient medium and tested its performance for biomass and protein production at different dilution ratios and

airflow rates (Pereira et al. 2022). The biomass and protein productivities were not enhanced with the aeration rate increase. A final biomass concentration of 0.73 g/L and protein content of 56% was obtained at 75% brewing residue and 1 L/min air flow rate. When the cultivation volume and aeration rates were tripled, keeping the dilution rate constant, the same productivity parameters were 0.65 g/L and 55%, respectively. The authors concluded that brewing process residues could serve as a low-cost *Spirulina* medium mainly because of the high biomass protein contents.

Rahman et al. (2021) developed a fish meal-based medium for *Spirulina platensis*-based protein production at a reduced cost (Rahman et al. 2021). The strain grew with 15, 20 and 25% (volume/volume) fish meal-based medium. An inverse relation was found between the enrichment ratio and the final biomass concentrations. Kosaric medium generated the highest final concentration.

Rangel-Yagui (2004) tested urea as a nonconventional low-cost nitrogen source for enhanced chlorophyll production from *S. platensis* (Rangel-Yagui et al. 2004). Following the optimization of urea and light availability, it was concluded that the optimum urea concentration was 500 mg/L, but the light intensity that maximized the biomass growth, biomass chlorophyll content, and chlorophyll productivity were different. For example, the highest biomass and chlorophyll production rates were obtained at 5600 lx and 1400 lx, respectively.

Madkour et al. (2012) formulated a low-cost cultivation media by using urea or ammonium nitrate as the nitrogen, super phosphate fertilizer as the phosphorus, muriate of potash as the potassium, and commercial-grade sodium bicarbonate as the carbon sources (Madkour et al. 2012). Urea, as the nitrogen source, generated the lowest volumetric biomass production rate as well as the average protein content. Ammonium nitrate increased biomass productivity but largely failed to reach the biomass productivity obtained with Zarrouk media. However, the biomass protein contents were similar in both cases.

Limited natural phosphorus reserves motivated researchers to develop nutrient media formulations from nutrient-rich waste resources such as domestic and urban wastewater. Markou et al. (2019) utilized struvite crystals recovered from anaerobic digestion plants as an alternative phosphorus source for the cultivation of *A. platensis* SAG 21.99 using the Zarrouk medium (Markou et al. 2019). A struvite-P concentration of 86 mg/L resulted in a higher biomass concentration than the control without any change in protein contents. Furthermore, the presence of a range of contaminants,

Table 2. Alternative media tested for *Spirulina*-based protein production

Media	Biomass concentration	Protein content	References
Raw soya sauce wastewater	1.984 g/L	65.57±1.26 %	(Han et al. 2021)
Zarrouk medium	2.451 g/L	66.25±1.97%	
Synthetic municipal wastewater	262.5 mg/L	46.02%	(Zhai et al. 2017)
Zarrouk medium	549 mg/L	54.25%	
Zarrouk medium (utilized with oyster and soil)	~2.2 g/L	50.42±1%	(Jung et al. 2014)
Zarrouk medium	~1.9 g/L	50.72±0.3%	
Rise husk bio charcoal Medium	0.39 g/L	n.a	(Taufiqurrahmi et al. 2017)
MSI	0.24 g/L	n.a	
75% (v/v) Brewing residual (1.8L)	0.73±0.01 g/L	55.58% (w/w)	(Pereira et al. 2022)
75% (v/v) Brewing residual (5.4L)	0.65±0.01 g/L	54.86% (w/w)	
Zarrouk medium (1.8L)	1.01±0.1 g/L	53.41% (w/w)	
15-20-25% Fish meal medium	0.533-0.417-0.378 g/L	n.a	(Rahman et al. 2021)
Kosaric medium	0.689 g/L	n.a	
Standard cultivation medium (with 500 mg/L urea)	1.945 g/L	n.a	(Rangel-Yagui et al. 2004)
Standard cultivation medium	1.878 g/L	n.a	
Zarrouk medium (with 8.33 mM NH_4NO_3)	0.028±0.006 mg/L/day	52.62±0.911%	(Madkour, Kamil, and Nasr 2012)
Zarrouk medium (with 2.94 mM urea)	0.021±0.002 mg/L/day	47.10±6.678%	
Zarrouk medium	0.052±0.005 mg/L/day	52.95±0.53%	
Struvite as a P source (700.8 g/L)	~1.05 g/L	67.7±6.3%	(Markou et al. 2019)
Zarrouk medium	~0.85 g/L	68.3±1.9%	
Tequesquite+ Equivalent N and P+ ½ $NaHCO_3$	2.57 g/L	543.6±32.1 mg/g,dry weight	(Martínez-Jerónimo, Flores-Hernández, and Galindez-Mayer 2017)
Zarrouk medium	2.93 mg/L	523.3±2.5 mg/g,dry weight	

including heavy metals, bisphenol A, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons, was tested and levels detected were lower than the limits dictated by the European Union regulations.

Tequesquite is a naturally occurring mineral highly rich in ions contained in *Spirulina* growth media formulations, including Na^+, K^+, CO_3^{2-}, HCO_3^-, NO_3^- and PO_4^{3-} (Martínez-Jerónimo et al. 2017). Martinez-Jeronimo et al. (2017) tested the biomass and

protein productivities of formulations prepared with tequesquite and had the best performance with media containing nitrogen and phosphorus concentrations equivalent to and bicarbonate concentration half of the Zarrouk medium. The alternative medium's final biomass concentration and productivity were about 2.6 g/L, and 0.24 g/L-day, while the same parameters were equal to 2.9 g/L and 0.27 g/L-day, respectively, with the Zarrouk medium. The protein contents with both media were similar. However, the cost of the alternative medium was approximately 41% of that of the Zarrouk medium.

2.3. Phycocyanin production from the *Spirulina*

In addition to its high nutritional value, *Spirulina* has also been produced at an industrial scale owing to its high phycocyanin content, which usually ranges from 8 to 22% of the dry algal weight (Taufikurahman et al. 2020; Jaouen et al. 1999). Phycocyanin extracted from *Spirulina* biomass is mainly used as a natural blue colorant in the food and cosmetics industries. Food-grade phycocyanin is a high-value bioproduct with a price of around 500 USD per kg (Isabel et al. 2021). The commercial interest at high cost is due to the rarity of blue pigments in nature (Isabel et al. 2021). It also has anticarcinogenic, anti-inflammatory, and antioxidant properties and, thus, potential applications in medical and pharmaceutical industries (Eriksen 2008; Román et al. 2002). Furthermore, it has spectral properties fit for use as a fluorescent tag in biomolecular research and is also commercially produced for this line of use (Eriksen 2008).

Both the United States Food and Drug Administration and European Union have approved the use of phycocyanin as a food colorant. The global phycocyanin market was valued at 155 million USD in 2020. The market value is expected to expand and generate a value of 410 million USD in 2030 (Market 2019). The increase in phycocyanin demand is attributed to the adverse health effects of artificial blue colorants, the uniqueness of blue color in food products and its ability to capture consumer's attention, and the ease of *Spirulina* production (Isabel et al. 2021; Khazi et al. 2018).

The cultivation process conditions such as media type, pH, temperature, light quantity, and quality are critical parameters that control *Spirulina*-based feedstock productivity (Chaiklahan et al. 2011; Madhyastha and Vatsala 2007). Per the nutrient

cost's significant share in total production cost, one area of intense research has been the development of inexpensive cultivation media alternatives or modification of the existing recipes (Table 3). For example, Taufikurahman et al. developed a medium based on anaerobically digested dairy manure wastewater supplemented with $NaHCO_3$ and NaCl and reported similar phycocyanin productivities compared with Zarrouk medium (Taufikurahman et al. 2020). Leema et al. cultivated *Spirulina platensis* using a seawater and freshwater mixture as the base to reduce operating costs. Although the developed medium can indeed serve as a cost-effective medium, the phycocyanin productivities sustained were lower than those with the Zarrouk medium.

Media reuse has also been tested to reduce both nutrient and water costs. Bom et al. reported decreasing final biomass concentration with the increasing number of reuse cycles but achieved an increase in phycocyanin concentrations at single reuse with respect to the cultivation with fresh nutrient medium. In parallel, the peak volumetric phycocyanin production rate of *S. platensis* could be increased by increasing the replacement ratio of the fresh medium with the used medium from *Chlorella sorokiniana* cultivation. These enhancements were attributed to the growth stress experienced by cultures due to nutrient-limited growth conditions developed (Bom et al. 2019; Ho et al. 2018).

Based on phycocyanin's role as a light-harvesting pigment, the research aiming for maximized phycocyanin productivity has focused on optimizing light quality and quantity as growth parameters. Chen et al. cultivated *S. platensis* ATCC29408 at three light intensities, including 750, 1500, and 3000 μmol/m^2-sec, generated with white, red, green, yellow, and blue LEDs. Blue light generated the lowest average phycocyanin content of 12.6%, while all the other light sources resulted in an average value ranging from 14.6 to 15.2%. The highest phycocyanin concentration and production rate were obtained with red and blue light, respectively. Enhanced production rate with blue light was explained by the shift in metabolic pathways resulting in increased synthesis and accumulation of phycocyanin as opposed to other metabolites (Chen et al. 2010).Ho et al. cultivated *S. platensis* at a light intensity of 400 μmol/m^2sec using a fluorescent lamp, white LED, red LED, or blue LED as light sources (Ho et al. 2018). Contrary to the findings of Chen et al., the use of blue and red LEDs clearly inhibited growth and reduced the maximum phycocyanin contents. White LEDs generated the highest biomass concentration, phycocyanin content, and volumetric phycocyanin productivity

of about 8 g/L, 15%, and 140 mg/L-day, respectively. These results indicate high variability in phycocyanin production characteristics across different strains.

The effect of cell disruption methods on the efficiency and purity of phycocyanin extraction is crucial. The best method is the one that can be applied with maximum efficiency and at minimum cost. Some of the commonly used extraction methods are freezing and thawing, homogenization, sonication, and microwaves (Rocha et al. 2021).

During the freezing and thawing process, as the fluid in the cell freezes and thaws, stretching and contraction movements are observed in the cell membrane; as a result, the cell membrane is damaged (Rocha et al. 2021). In study, an increase in the amount of phycocyanin obtained was observed when the process was repeated more than once (Tavanandi et al. 2018). Although phycocyanin extraction and purity yield obtained is higher than the other methods, its applicability for industrial use is limited due to high energy and time requirements (Tavanandi et al. 2018; Eriksen 2008).

Homogenization is a method of cell disruption by creating high shear force. The critical parameters of this method are extraction time and homogenization rate. Increasing the temperature during the process can improve extraction efficiency, but the extraction purity will decrease (Silveira et al. 2007).

Sonication is the disintegration of cells due to acoustic cavitations that develop due to ultrasonic sound waves targeted to the suspensions. Extraction time and power density are the most critical parameters during the process (Rocha et al. 2021). The increase in these two parameters causes an increase in temperature, resulting in phycocyanin loss (Rocha et al. 2021).

During microwave-assisted extraction, microwaves cause the vibration of water and other polar molecules in biomass, causing temperature increase and water evaporation. Evaporated water exerts pressure on the cell walls and causes cell lysis (Hahn et al. 2012). Phycocyanin degradation due to exposure to a high-temperature environment is one of the potential limitations of the method (Aky et al. 2018).

Extracted phycocyanin is graded into different categories according to its purity. Phycocyanin purity is determined by the ratio of A_{620}/A_{280}. A_{620} is the absorbance value of phycocyanobilin at 620 nm, and A_{280} is the absorbance value of aromatic amino acids in all proteins at 280 nm (Eriksen 2008). A_{620}/A_{280} ratio between 0.7 and 3.9 is classified as a food grade, between 3.9 and 4 as a reactive grade, and greater than 4 as an analytical grade (Eriksen 2008). Purification methods can be applied alone or in

combination with other methods to increase the quality of phycocyanin (Kuddus et al. 2013). Some of the methods are precipitation with ammonium sulfate, two-phase aqueous extraction, micro and ultrafiltration, ion exchange chromatography, and chitosan absorption (Zhang and Chen 1999; Chaiklahan et al. 2012; Patil et al. 2006; Chen et al. 2016). One of the big problems in industrial production is that the cost of the purification process can correspond to 80% of the total cost (Júnior and De 2005). Therefore, it is essential to develop more economical, efficient new methods or to optimize the currently used ones.

Table 3. Summary of the results from the phycocyanin production studies

Strain	Medium	Cultivation conditions	Extraction Method	Phycocyanin content	References
Spirulina platensis CS1	Zarrouk medium ($NaHCO_3$ replaced with Na_2CO_3)	3000 lux (Red light), 35°C, pH 10, 16h:8h light:dark	Liquid nitrogen Freezing and thawing Sonication Lysozyme method	110.2 mg/L 101.8 mg/L 82.2 mg/L 101.62 mg/L	(Soundarapandian 2015)
Spirulina platensis	Zarrouk medium ADDMW+16,8 g/L $NaHCO_3$+ 25 g/L NaCl	16h:8h light:dark,4000 lux,3 L/min air flow	Cold maceration	4,91±0,18 (mg/L*day) 3,92±0,64 (mg/L*day)	(Taufikurahman et al. 2020)
Spirulina platensis ATCC29408	Zarrouk medium	30°C, 120 rpm, 5 days, 3000 µmol/m^2s (Red light)	Homogenized with phosphate buffer (pH 7)	0,0633 g/L*day	(H. Chen et al. 2010)
Spirulina LEB-18	Zarrouk medium Zarrouk medium (Recycled one time)	12h:12h light:dark, Open raceway pond	Ultrasonic bath with sodium phosphate buffer (pH 6.9)	2,07 mg/L 2,47 mg/L	(Bom et al. 2019)
Spirulina platensis	Zarrouk medium ($NaHCO_3$ replaced with CO_2)	Outdoor conditions (pH8.5)	Phosphate buffer (pH 7), 4°C, overnight	14% (g/g)	(Mehar et al. 2019)
Spirulina platensis	Zarrouk medium Seawater (2:1 seawater:fresh water)	14h:10h light:dark, 26±1°C, 120rpm, 100 µmol/m^2s	Enzymatic hydrolysis+ shaking bath+Homogenizer	150,65 mg/L 122,66 mg/L	(Leema et al. 2010)
Spirulina platensis	Zarrouk medium 50% Recycled medium from Chlorella sorokiniana Mb-1+ 50% Zarrouk Medium	28-30°C, 400 µmol/m^2s (White light), 2,5%CO_2	Phosphate buffer (pH 7) 4°C, 20h	75.2±3.9 mg/L*day 86.6±3.8 mg/L*day	(Ho et al. 2018)

2.4 Harvesting methods of *Spirulina*

Photobioreactors currently used to grow *Spirulina* hardly generate algal suspensions with biomass concentrations exceeding 0.5 kg/m^3 (Ozkan et al. 2012). The moisture content of the produced biomass has to be lowered below 4% to preserve the nutrient content during storage (Soni et al., 2017). To fulfill this requirement, the suspension output has to go through harvesting and dewatering processes that are usually filtration, centrifugation, sedimentation, or flotation-based (Soni et al., 2017).

Filtration is the most frequently utilized method for the commercial production of *Spirulina* due to its ease of application (Soni er al., 2017). High efficiency with *Spirulina* is mainly due to its morphology; *Spirulina*, a filamentous alga, is relatively larger than other strains with typical lengths and helix diameters ranging from 100 to 3000 µm, and 20 to 150 µm, respectively (Cheng et al. 2018; Wu et al. 2012). This allows the use of inexpensive 380 to 500 mesh screens and the establishment of removal efficiencies of up to 95% (Vonshak 1997). Inclined screens process 2.5 to 9 m^3 of suspension per m^2 of the filter every hour (Vonshak 1997). Vibrating screens can be used to reduce area requirements (Vonshak 1997). The slurry with 8 to 10% dry solids is usually filtered again under a vacuum to increase the solid content to 20 to 30% (Vonshak 1997). Filtration is also advantageous compared with other methods because (i) it does not require the introduction of chemicals, which can contaminate the final product (Singh and Patidar 2018), (ii) it does not result in significant levels of cell lysis (Monte et al. 2018), (iii) it does not require handling of sophisticated instruments (Deconinck et al. 2018). One potential disadvantage is filter clogging, which can be minimized by pore size optimation.

Centrifugation is among the most widely used algae harvesting methods owing to its high efficiency, reliability, and ease of operation (Laamanen et al. 2021; Uduman et al. 2010). However, it is notorious for high energy input, initial investment, and operational expenditure requirements (Soni et al. 2017; Uduman et al. 2010; Panis and Carreon 2016) . Since maintenance of cell integrity is a must for commercial *Spirulina* production, it is critical to keep the shear stress cells experience within a certain threshold.

Sedimentation uses gravity to separate the cells from the aqueous phase (Laamanen et al. 2021). It offers several critical advantages, including simple operation

and low energy consumption (Uduman et al. 2010). The main limitation behind its more widespread use is two folds: the cell size and density control the recovery efficiency and slow process rates (Uduman et al. 2010; Mennaa et al., 2017). Flocculants may need to be added to increase yield depending on the physicochemical properties of the strain harvested (Uduman et al. 2010; Henderson et al. 2008). In such cases, the flocculant's toxicity must be considered during flocculant selection.

Flotation systems concentrate the cells at the liquid surface and collect the algal concentrate (Laamanen et al. 2021). It introduces fine air bubbles to the suspensions either through the reduction of hydrostatic pressure or through injection. Following the attachment of these bubbles to the algae, the cell density decreases, and vertical mobility gets initiated. This method particularly works well with small-sized and low-density particles (Uduman et al. 2010).

2.5 Drying methods for *Spirulina*

The harvested biomass by weight is still 70 to 80% water (Soni et al. 2017; Vonshak 1997). *Spirulina* is edible in this form, but for long-term storage without nutrient loss, the water content has to be lowered to 4% (Soni et al. 2017). Solar drying, spray drying, and freeze drying are the most frequently used methods to remove excess water and to avoid biochemical degradation (Soni et al. 2017; Ma et al. 2019; Silva et al. 2021). The nutrient value of the biomass can also be detrimentally affected based on the method of choice and its operational conditions, particularly the temperature (Ma et al. 2019).

Solar drying is the most widely preferred method per its low investment and energy input requirements (Udayan et al. 2022). Solar energy can be directly or indirectly utilized, i.e., the biomass can be placed under solar irradiance, or unsaturated air previously warmed at solar collectors can be interacted with the biomass (Silva et al. 2021). The drying time has to be carefully monitored and kept below a certain duration to ensure product quality (Soni et al. 2017). The biomass harvesting method and the magnitude of incident solar energy in the area are critical for the viability of this method (Udayan et al. 2022).

Spray drying atomizes the *Spirulina* suspensions within a drying chamber and removes the water contents through a rapid process called flask drying (Vonshak 1997).

The drying usually occurs at low temperatures and is completed within seconds (Vonshak 1997). The end product is in powder form, which negates the need for a grinding process before packaging. It has relatively easy process control and homogeneous product quality (Chen et al. 2015). The main limitations of its more widespread use are high investment and operational energy input requirements (Chen et al. 2015). In addition, protein, phycocyanin, and ß-carotene degradations are potential issues reported with *Spirulina* (Claude et al., 2015; Brennan and Owende 2010).

Freeze-drying is a two-step process: the first freezes the cells and exposes them to a vacuum, and the latter dewaters the algae by heating and sublimation (Kim and Kim 2022). Bioactive metabolites are usually well preserved as the process operates at low temperatures without contact with oxygen (Kim and Kim 2022). These features are mainly exploited for food applications (Chen et al. 2015). The process's high capital and operating costs hinder more widespread use (Soni et al. 2017; Chen et al. 2015).

CHAPTER 3

MATERIALS AND METHODS

3.1. Selection of *Spirulina* Strains for Protein Production Experiments

S. platensis and *S. maxima* are industrially grown species for their high protein contents (Muys et al. 2019; Soni et al. 2017). The strain level information is difficult to obtain because of (i) the commercial secrecy around the production processes and (ii) the reliance on local isolates that are not deposited at culture collections. Thus, the selection of strains of high protein productivity is only possible after screening; the study presented aims to perform this. Five strains from the two species were procured from three major culture collection centers (Table 4). They were cultivated in a laboratory-scale bubble column photobioreactor to represent scaled-up growth conditions under standard process conditions, and the resultant biomass and protein productivity characteristics were assessed.

Table 4. Information on the strains included in the current study

Strain number	**Strain name**	**Abbreviation**
UTEX2340*	*Spirulina platensis*	SP2340
SAG84.79**	*Spirulina maxima*	SM84
SAG85.79	*Spirulina platensis*	SP85
SAG86.79	*Spirulina platensis*	SP86
CCALA29***	*Spirulina platensis*	SP29

* Culture Collection of Algae at the University of Texas at Austin, U.S.A

** Culture Collection of Algae, Germany

*** Culture Collection of Autotrophic Organisms, Czechia

3.2. Cultivation of the Inocula for the Photobioreactor Experiments

The inocula for the photobioreactor experiments were grown in 500 mL flasks. The starter cultures procured from the culture centers were used to initiate the flask cultures. Zarrouk medium was used (Zarrouk 1966) for all the cultivation experiments as it is a standard medium known to support *Spirulina* growth (Vonshak 1997). Sterile techniques were used for the inoculation, and the following subculturings: media (except sodium bicarbonate) and flasks were autoclaved. Before subculturing, sodium bicarbonate was filter-sterilized and added to the medium, and the transfers were performed in a laminar flow cabinet. The strains were grown in a light and temperature-controlled incubator under the process conditions described in Table 4. Photosynthetically active radiation (PAR) intensity reaching flask surfaces was measured with a PAR sensor (Li-COR, Li-250A) and adjusted to 100 $\mu E/m^2$–sec through manipulation of the distance to the light source. The incubator temperature was set to 20℃. The flasks were manually mixed once every day for 10 seconds.

Table 5. Process conditions for the flask cultures

Process parameter	**Value**	**Units**
Cultivation temperature	20	℃
Culture volume	150	mL
Average incident light intensity	100	$\mu m/m^2$-s
Light:dark period	14:10	hour:hour

3.3. PBR Design and Characterization

Flask cultures were used to start growth at a laboratory scale (4 L) bubble column photobioreactor (Figure 4). The protein productivity analyses were performed during this growth. The photobioreactor cultivation was critical to (i) control all the process parameters, including aeration rate, temperature, and light intensity, (ii) ensure representation of growth conditions at an industrial setting, and (iii) have enough suspension volume that will allow the generation of sufficient sample size for subsequent protein content analyses. This reactor type was chosen as it is easy to scale up and has low energy input requirements (Chew et al. 2018).

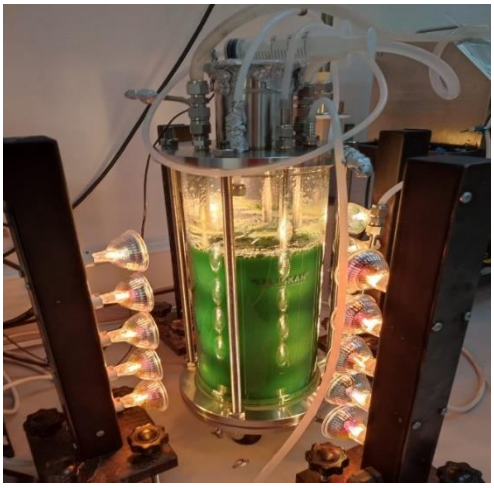

Figure 4. Image of the bubble column photobioreactor used for the current study.

The photobioreactor had three major pieces: a cylindrical glass vessel of 13 cm diameter and stainless-steel top and bottom plates that housed the cylinder. The top plate had the ports for the sampling line, temperature measurement port, air exhaust, and heat exchanger coil inlet and outlet. The bottom plate had the aeration line connection on one side and four 1 mm diameter-sized holes that introduced the air to the suspensions on the other. The photobioreactor was fully autoclavable, and all the connections were made with leak-free Swagelok-type connectors. Thus, the cultures could be cultivated axenically once the photobioreactor was autoclaved.

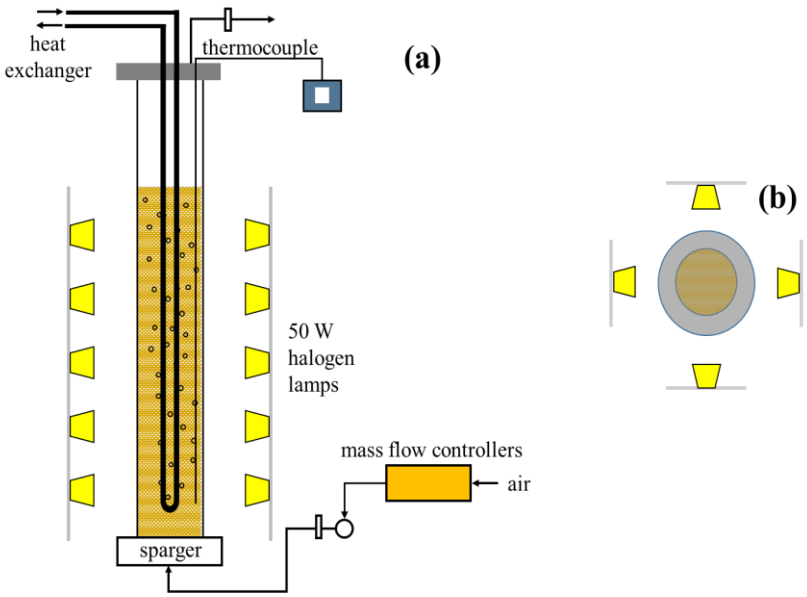

Figure 5. Schematics of photobioreactor system from (a) side, and (b) top view

3.3.1. Aeration System

Spirulina suspensions were constantly aerated during growth to mix the cultures, replenish the depleted CO_2 and avoid oxygen accumulation. A mass flow controller was used to maintain a constant aeration gas flow rate of 2 L/min. The air was filter sterilized with a HEPA filter of 0.22 µm pore size and humidified before being pumped to the suspensions through the openings mentioned above. The aeration system's mass transfer coefficient (k_{La}) was measured using Van't Riet's method, and a k_{La} value of 12.5±0.3 hr^{-1} was found for oxygen at a gas flow rate of 2 L/min.

3.3.2. Illumination System

An illumination system with a controlled light intensity output was used for the experiments. The system consisted of 20 halogen light bulbs that were grouped into four and placed vertically in columns. The bulbs were placed homogeneously across the length of the working volume of the reactor. The columns were placed on four sides of the reactor perpendicular to the cylinder surface. The distance between the bulb surface and the cylinder was 10 cm. A dimmer switch was integrated into the circuit to control the light output.

The photosynthetically active radiation (PAR) intensity vs. dimmer setting relation was calibrated through a series of measurements with a PAR sensor (Li-COR, Li-250A). For this, (i) the glass cylinder surface was divided into squares with a side length of 1.25 cm, and (ii) the PAR intensities were measured at the center of these squares using the PAR sensor at four dimmer settings. This calibration allowed the set of the average intensity up to about 500 µE/m^2-sec. The controlled delivery of light was critical for avoiding light inhibition and also for the correct representation of the growth conditions.

3.3.3. Temperature Control System

A temperature control coil and a temperature measurement port were inserted into the cultivation vessel through the upper plate using leak-free connectors. A refrigerated circulator pumped temperature-regulated water through the coil tube. The

resulting cultivation temperature was constantly monitored with a thermocouple inserted into the measurement port. This setup mainly served to remove the excess heat from the halogen light bulbs and maintain a constant cultivation temperature of 30°C.

3.4. Two-stage Photobioreactor Cultivation Process

The strains were cultivated according to a two-stage cultivation protocol (Table 6). The two stages only differed in terms of the average incident light intensity delivered to the suspensions: the average intensity of the 1st and the 2nd stages were 50 and 250 µm/m^2-s, respectively. The transition to the second stage was triggered when the daily optical density readings at 750 nm (OD 750nm) exceeded 0.5. This protocol was designed to avoid photoinhibition that was observed with some of the strains.

Table 6. Process conditions during the two-stage photobioreactor cultivation

Process parameter	Value	Units
Cultivation temperature	30	°C
Initial culture volume	4.0	L
Aeration gas flow rate	2000	mL/min
CO_2 partial pressure aeration gas	≈ 380	ppm
Initial OD 750nm, Stage I	0.06	(-)
Initial OD 750nm, Stage II	>0.5	(-)
Average incident light intensity, Stage I	50	µm/m^2-sec
Average incident light intensity, Stage II	250	µm/m^2-sec

Flask cultures were washed with fresh Zarrouk's media and added to the reactor to start the first growth stages at an initial OD 750 nm of 0.06. The cumulative volume of the culture was 4 liters. Since the optimum growth temperature of *Spirulina* usually ranges between 29 to 35°C, the cultivation temperature was set to 30°C (Muys et al. 2019; Soni et al. 2017). An air compressor and and a mass flow controller were used to pump air to the suspensions through the diffuser at the bottom plate at a flow rate of 2 L/min.

Daily samples were taken from the sampling port and analyzed only for OD 750 nm during the first stage. As already discussed, the second stage was initiated when the measured values exceeded the set threshold of 0.5. In terms of process conditions, these stages were differentiated with the increase in incident light intensity to 250 µm/m^2-sec. In terms of sampling protocols, the main differentiation was the increase in sample

volumes and the associated biomass concentration and protein content analyses performed.

3.4.1. Optical Density of Diatom Suspensions

The changes in biomass concentration were estimated by daily optical density (OD) measurements made at a wavelength of 750 nm using a 1 cm cuvette. When the measured OD values exceeded the linear range, the samples were diluted serially with nanopure water before measurement. The change in optical density values as a function of cultivation time is presented in Appendix A1. A correlation factor was established between OD 750 nm and biomass concentration for each strain based on these results, as presented in Appendix A1.

3.4.2. Biomass Concentration Analysis

The cells were harvested with vacuum filtration for biomass analysis. Samples of definite volume (V_{sample}) were filtered using preweighed Whatman No 2 filter paper (m_i) and washed with nanopure water to remove the remaining nutrient medium and impurities. The filters were dried at 80ºC until constant weight (m_f). The biomass concentration was calculated according to:

$$X_{biomass} = \frac{m_f - m_i}{V_{sample}} \tag{3.2}$$

where $X_{biomass}$ is the biomass concentration (in g/L). The ash contents of the biomass samples from all strains were assayed by combustion in a muffle oven (Magma Therm, MT Series) at 550 ℃ for 8 hours. The ash content was calculated according to the following relation:

$$F_{ash} = \frac{m_{remain}}{m_{algae}} \tag{3.3}$$

where F_{ash} is the ash content, m_{remain} is the mass of solids remaining after combustion, and m_{algae} is the mass of the algae combusted. Since the ash contents measured with all

the strains were less than 10%, the biomass concentrations were reported according to Equation 3.2.

3.4.3 Total Protein Content Analysis

The algae cakes remaining over the filters were washed with nanopure water and transferred to aluminum plates. The samples were dried at 80℃ for 12 hours and homogenized with mortar and pestle. The carbon, hydrogen, nitrogen, and sulfur contents of the dried samples were analyzed with an organic elemental analyzer (Flash 2000, Thermo Scientific). The detection oven temperature was 65ºC, and the furnace temperature was 950ºC. The carrier gas and reference gas flow rates were set to 140 mL/min, and 100 mL/min, respectively. The oxygen flow rate was 250 mL/min. 2,5-Bis(5-tert-butyl-2-benzoxazolyl) thiophene (($C_{26}H_{26}N_2O_2S$) was used as the calibration standard. The calibration measurements were made at the beginning of each run and repeated five times. CHN analysis were performed at Çankırı Karatekin University Central Research Laboratory. The crude protein content of the biomass was estimated from the nitrogen contents by:

$$Y_{protein} = 6.25 * Y_N \qquad (3.3)$$

where $Y_{protein}$ and Y_N are the protein and nitrogen contents of the biomass (% dry weight), respectively. The conversion factor of 6.25 was adopted from the literature on *Spirulina*-based protein production (Jones 1941; Costa et al. 2022; Zhou et al. 2022).

3.4.4 Morphological Characterization of the Spirulina strains

The strain morphology was characterized based on an image-based technique (Vonshak 1997). The cells were imaged using a light microscope (Nikon Eclipse Ci-L Plus) with a 10X objective lens. ImageJ image analysis software was used to measure the helix pitches and cell lengths of the flask cultures manually. At least 100 cells were analyzed to estimate average values for each strain.

CHAPTER 4

RESULTS AND DISCUSSION

The biomass concentration, protein concentration, and biomass carbon concentration vs. (photobioreactor) cultivation time profiles obtained with the strains were sigmoidal. Thus, the logistic model described by Equation 4.1 was fitted to the results with nonlinear, least squares regression with the Marquardt method on Statgraphics Centurion Software. This facilitated a more accurate comparison of the strain performances (Ozkan and Rorrer 2017b; 2017a). The model used is defined as:

$$C_{P-i}(t) = \frac{C_{0,P-i} e^{k_{P-i}(t)}}{1 + \frac{C_{0,P-i}}{C_{f,P-i}}(e^{k_{P-i}(t)} - 1)} \tag{4.1}$$

where $C_{P\text{-}i}$ is the product concentration at time t, i.e., biomass concentration (g/L), protein concentration (g/L), or biomass carbon concentration. $C_{0,P\text{-}i}$ is the measured initial product concentration, $C_{f,P\text{-}i}$ and $k_{P\text{-}i}$ are the final product concentration, and logistic model rate constants estimated in units of g/L and hr^{-1}, respectively. The peak productivities were quantified by using the estimated parameters:

$$R_{P-i} = \frac{k_{P-i}\, C_{f,P-i}}{4} \tag{4.2}$$

where $R_{P\text{-}i}$ is the peak productivity in g/L-hour (Ozkan and Rorrer 2017b; 2017a).

The only exception to the applicability of the logistic model was with the protein and biomass carbon concentration profiles of SP 85. In these cases, the final concentrations ($C_{f,protein}$ and $C_{f,bio\text{-}carbon}$) reported are the concentrations measured at the end of the cultivation period (Table 7), and the peak productivities ($R_{P,protein,}$ and $R_{P,bio\text{-}carbon}$) reported are linear regression based.

4.1. Biomass Concentration

Figure 7 presents the change in biomass concentrations during the strains' photobioreactor growth. The final biomass concentrations ($C_{f\ biomass}$) varied significantly across the strains (Table 7). For example, $C_{f\ biomass}$ of SP 86 and SP 29 were around 1.2 g/L and 1.4 g/L, respectively, and those of SP 2340 and SP 85 were about 1.9 g/L. Furthermore, the strains with lower $C_{f\ biomass}$ values had higher biomass production rate constants ($k_{P\text{-}biomass}$), indicating they reached their respective $C_{f\ biomass}$ values sooner. This resulted in a more balanced distribution in the peak biomass productivities ($R_{P,biomass}$), as shown in Figure 6. SM 84 and SP 29 generated the highest $R_{P,biomass}$ values of around 0.20 g/L-day, and those of the three other strains were about 0.15 g/L-day. Thus, in cases where the harvesting's contribution dominates the cost of production, cultivation of species of high $C_{f\ biomass}$, namely SP 2340 and SP 85, can be advantageous. Similarly, when the production costs are mostly photobioreactor-based, selection of species of higher $R_{P,biomass}$, in the current study SM 84 and SP 29, can make more financial sense.

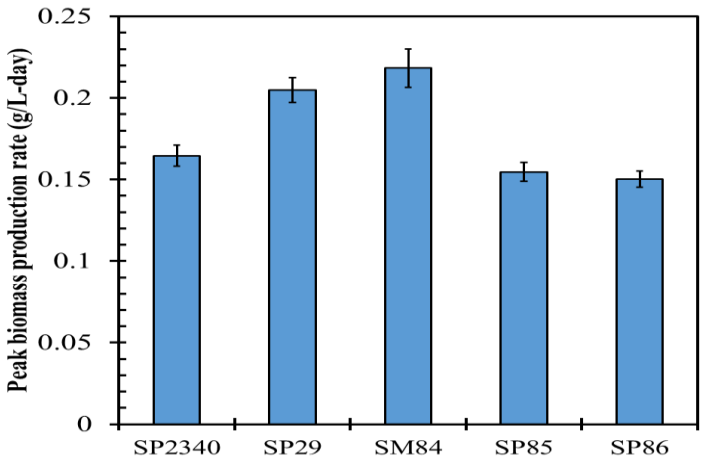

Figure 6. Comparison of peak biomass production rates obtained with *Spirulina* strains during photobioreactor cultivation. All errors are 1.0 S.E.

These results agree well with those available in the literature. For example, Zhu et al. reported maximum biomass concentrations of about 1.2 g/L with three other *Spirulina* strains cultivated with Zarrouk medium in a columnar photobioreactor aerated

with air (Zhu et al. 2021). Furthermore, parallel to current results, volumetric biomass productivities were around 0.15 g/L-day. Andrade et al. reported maximum biomass productivities and concentrations of about 0.18 g/L and 2.3 g/L, respectively, during cultivation in raceway ponds using Zarrouk medium under outdoor conditions with *Spirulina* sp. (Andrade et al. 2019). Finally, peak biomass productivities ranging from 0.12 to 0.14 g/L-day and maximum biomass concentrations ranging from 1.1 to 1.3 g/L were reported when *Spirulina* was grown in nutrient media other than Zarrouk's (Ragaza et al. 2020).

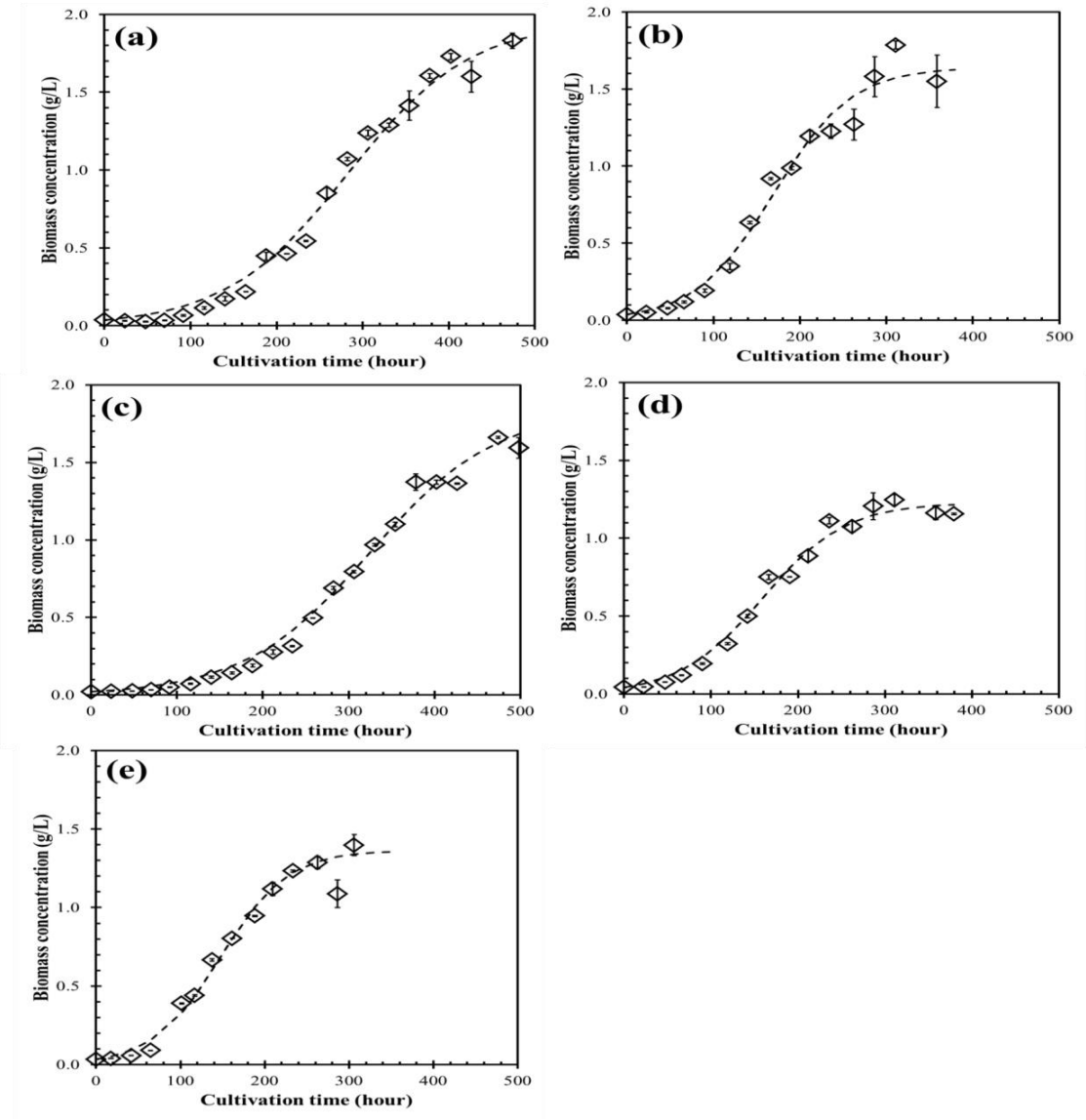

Figure 7. Comparison of biomass concentrations during photobioreactor cultivation of (a) *Spirulina platensis* 2340, (b) *Spirulina maxima* 84.79, (c) *Spirulina platensis* 85.79, (d) *Spirulina platensis* 86.79, and (e) *Spirulina platensis* 29

(Dashed lines present to the logistic model. Each data point represents the mean±1.0 S.E. of triplicate measurements.)

4.2. Biomass Protein Content

Figure 8 presents the change in biomass protein contents (% dry weight) of the strains during their photobioreactor growth. These ratios were estimated based on the nitrogen contents of the biomass samples determined based on CHN analyses and a nitrogen content to biomass protein content conversion factor of 6.25 frequently used for *Spirulina*. Due to sample size limitations, a single protein content value is reported for the first growth stages, where OD 750 nm was less than 0.5. This value was equal to the average protein contents measured during the second stage. Furthermore, some of the samples were combined, at equal mass ratios, before the CHN analyses due to the minimum sample size requirement of the elemental analyzer used. These samples are denoted as a range of cultivation time (i.e., 164.3 hr to 188.2 hr for SP 2340) instead of a single time point.

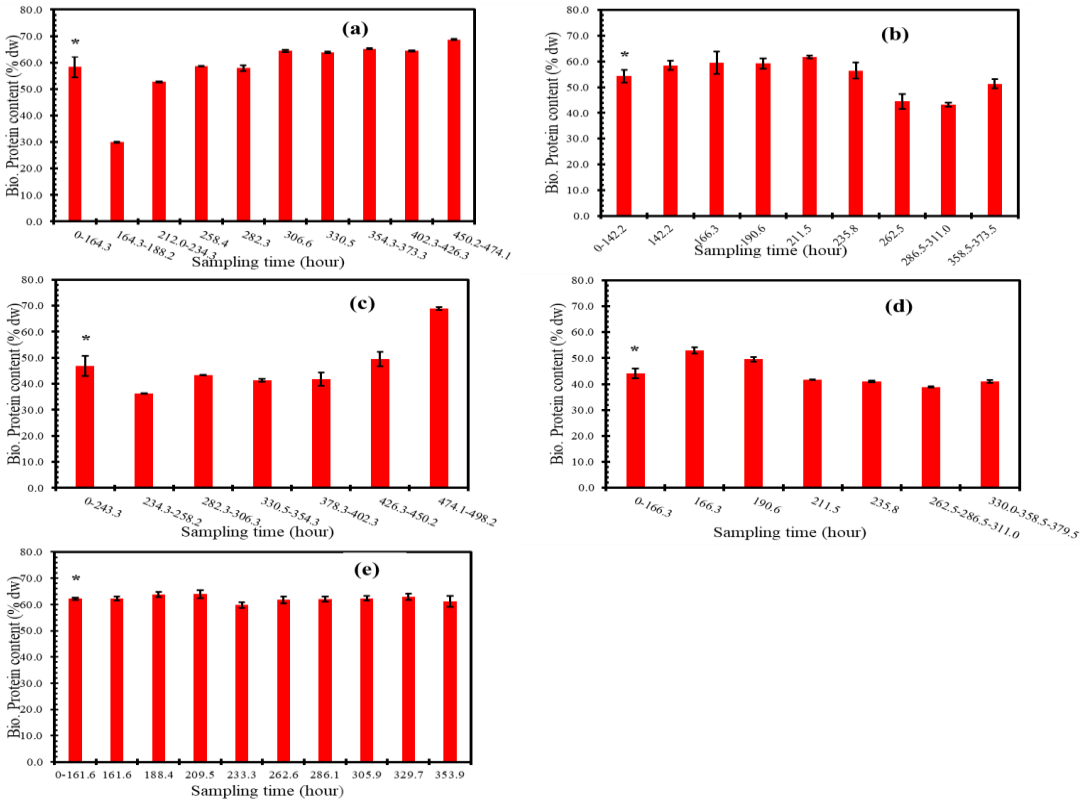

Figure 8. Comparison of biomass protein contents during photobioreactor cultivation of (a) *Spirulina platensis* 2340, (b) *Spirulina maxima* 84.79, (c) *Spirulina*

platensis 85.79, (d) *Spirulina platensis* 86.79, and (e) *Spirulina platensis* 29 (Each data point represents the mean±1.0 S.E. of triplicate measurements. Data points marked with * are based on the average of protein contents measured with the respective strain during the second growth stage.)

SP 29 had the ideal protein content profile for commercial-scale production. The reason behind is twofold: (i) its protein content was high, higher than 60% at all points tested, and (ii) this ratio changes within a very narrow range of 60 to 64% during the whole cultivation duration regardless of the growth stages. The protein content of SP 2340 increased from about 30 to 65% as the culture progressed into the stationary phase. Similarly, the protein ratio of SP 85 was about 35% at the onset of 2nd growth stage and reached the highest recorded value of 70% towards the end of the growth period. In contrast, the protein contents of SM 84 and SP 86 declined during the same transition, from 60% down to 50% and from 50 down to 40%, respectively. This reduction can be explained by increased lipid biosynthesis in algae in the stationary growth phases (Hussain et al. 2020). All the measured ratios are within the protein content ranges reported for *Spirulina* cultivated at the laboratory scale (Muys et al. 2019).

Table 7. Logistic model-based biomass productivity parameters obtained during photobioreactor cultivation of the *Spirulina* strains. (All errors are 1.0 S.E.)

Parameter & units	**SP2340**	**SM84**	**SP85**	**SP86**	**SP29**
$C_{f\,biomass}$ (g/L)	1.95±0.06	1.64±0.06	1.84±0.06	1.23±0.03	1.36±0.04
$k_{P\text{-}biomass}$ (hr^{-1})	$1.40\cdot10^{-2}\pm3\cdot10^{-4}$	$2.22\cdot10^{-2}\pm8\cdot10^{-4}$	$1.36\cdot10^{-2}\pm2\cdot10^{-4}$	$2.10\cdot10^{-2}\pm6\cdot10^{-4}$	$2.50\cdot10^{-2}\pm6\cdot10^{-4}$
$C_{f\,protein}$ (g/L)	1.37±0.07	0.78±0.03	1.14±0.01*	0.48±0.02	0.82±0.04
$k_{P\text{-}protein}$ (hr^{-1})	$1.36\cdot10^{-2}\pm4\cdot10^{-4}$	$2.50\cdot10^{-2}\pm9\cdot10^{-4}$	-	$2.29\cdot10^{-2}\pm1\cdot10^{-3}$	$2.56\cdot10^{-2}\pm6\cdot10^{-4}$
$C_{f\,bio\text{-}carbon}$ (g/L)	0.88±0.03	0.71±0.02	0.75±0.01*	0.51±0.01	0.60±0.02
$k_{P\text{-}bio\text{-}carbon}$ (hr^{-1})	$1.39\cdot10^{-2}\pm3\cdot10^{-4}$	$2.23\cdot10^{-2}\pm6\cdot10^{-4}$	-	$2.09\cdot10^{-2}\pm7\cdot10^{-4}$	$2.55\cdot10^{-2}\pm7\cdot10^{-4}$

The results show the importance of the strain and harvesting time selection for sustaining protein-rich biomass productivity at an industrial scale. Considering that *Spirulina* is usually advertised and produced for its exceptionally high protein content

exceeding 60%, the use of strains SM 84 and SP 86 is not preferable (Andrade et al. 2019; Lupatini et al. 2016). The use of SP 2340 and SP 29 is advantageous in this regard. SP 29 has the added benefit of containing high protein content during the log phase as well, which can be particularly critical when continuous production methods are to be implemented.

4.3. Protein Productivity

Figure presents the change in protein concentration with respect to cultivation time for all the strains. The final protein concentrations ($C_{f\ protein}$) and protein production rate constants varied ($k_{P\text{-}protein}$) significantly across the strains. The photobioreactor growth of SP 2340 generated the highest $C_{f\ protein}$ value of 1.37 g/L and the lowest $k_{P\text{-}protein}$ value of $1.36 \cdot 10^{-2}$ hr^{-1}. SM 84 and SP 29 had the highest $k_{P\text{-}protein}$ values and $C_{f\ protein}$ values of about $2.5 \cdot 10^{-2}$ hr^{-1} and 0.80 g/L, respectively (Table 7). The final protein concentration measured with SP 85 was about 1.1 g/L. The peak productivities ($R_{P,protein}$) of SP 2340, SM 84, and SP 29 were about 0.11 g/L-day, which was higher than the other two strain's $R_{P,protein}$ values (Figure 9). In accordance with these results, Prates et al. reported a peak protein productivity of about 0.11 g/L-day when *Spirulina* sp. LEB 18 was cultivated using Zarrouk's medium and artificial illumination at a bubble column photobioreactor.

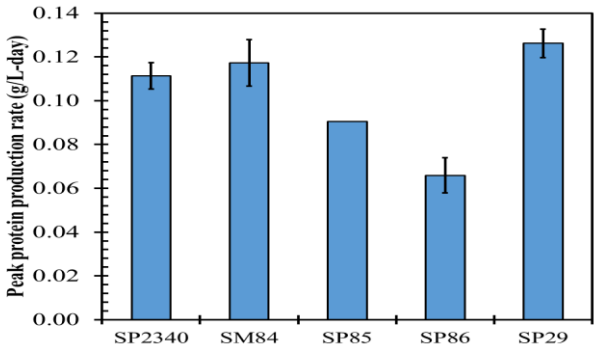

Figure 9. Comparison of peak protein production rates obtained with *Spirulina* strains during photobioreactor cultivation. All errors are 1.0 S.E.

From the commercial production point of view, SP 2340 and SP 85 are the strain of choice to minimize the harvesting costs per unit mass of protein production. When photobioreactor construction and maintenance costs are more of a concern, the growth

of SP 2340, SM 84, and SP 29 can be more advantageous per their higher peak volumetric productivities. For these strains, a semi-batch growth system can be used to keep the culture at the biomass concentration ranges that generate peak productivity. SP 29 can be the ideal strain for such a production scheme when its consistently high protein content is considered.

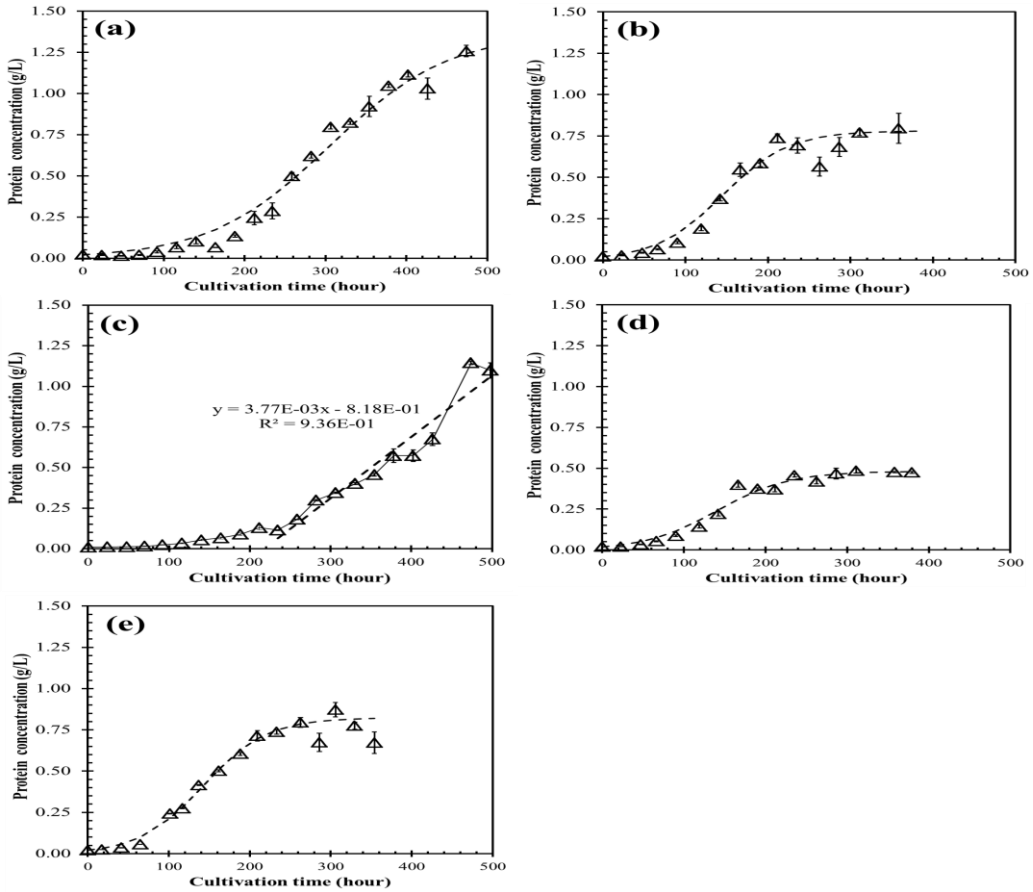

Figure 10. Comparison of protein concentrations during photobioreactor cultivation of (a) *Spirulina platensis* 2340, (b) *Spirulina maxima* 84.79, (c) *Spirulina platensis* 85.79, (d) *Spirulina platensis* 86.79, and (e) *Spirulina platensis* 29. Dashed lines present the best fit to the logistic model. All error bars represent propagated errors based on 1.0 S.E. of the fitted parameters.

4.4. Assessment of CO_2 Replete Growth Conditions

The CO_2 consumption rate of the *Spirulina* cultures during photobioreactor cultivation can be calculated based on mass balance around the photobioreactor inlet aeration and outlet exhaust gas CO_2 concentrations according to the following equation:

$$X_C(t) = \frac{v_0\, P}{RT\, V_L} \int_0^t \left(Y_{CO_2,in} - Y_{CO_2,out}\right) dt \qquad (4.1)$$

where X_C is the carbon consumption or biomass carbon production per unit volume of suspension (mmol/L), v_0 is the volumetric aeration gas flow rate into the suspension (L/hr), P is the system pressure (1.0 atm), R is the ideal gas constant (L.atm/mol.K), T is the temperature (303 K), $Y_{CO_2,in}$ and $Y_{CO_2,out}$ are the mole fraction of CO_2 in the inlet and exhaust gas streams, respectively (Ozkan and Rorrer 2017a).

The carbon transfer rate to the culture, as described by Equation 4.1, is one of the main limitations on algal biomass productivity, particularly for the high rate closed photobioreactor systems (Fu et al. 2019). Thus, to fully characterize and accurately compare the biomass and protein productivities of the strains, the establishment of growth under CO_2-replete conditions is critical.

One way to assess the existence of CO_2-limited growth conditions is to compare the CO_2 delivery rate to that of consumption. The highest CO_2 delivery rate from the gas to the liquid phase can be estimated when all the CO_2 in the air introduced to the suspension is fully captured, i.e., $Y_{CO_2,out}$ = 0 ppm. This correlates to a maximum CO_2 transfer rate of 0.46 mmol/L-hr at an inlet CO_2 concentration ($Y_{CO_2,in}$) of 380 ppm, and also to a rate of 11.0 mmol/L-day (0.132 g/L-day) when the whole (both light and dark periods) cultivation period and to 6.4 mmol/L-day (0.077 g/L-day) when only light (photosynthetically active) period are considered (Fu et al. 2007).

The CO_2 consumption rates can be approximated by the combination of results from the biomass productivities and the associated carbon content analyses. Biomass carbon concentration can be calculated with the following relation:

$$C_{bio-carbon}(t) = C_{biomass}(t) * Y_C \qquad (3.3)$$

where $C_{bio\text{-}carbon}$ is the biomass carbon concentration at time t (g/L), $C_{biomass}$ is biomass concentration at time t (g/L), and Y_C is the carbon content of the biomass at time t determined according to CHN analysis. Y_C of the biomass samples is presented in Appendix A. The carbon contents did not change between strains, except for SP 85 and growth stages; they were between 40% to 45%. These ratios are in accordance with the literature values on algal carbon contents in general and on *Spirulina* carbon contents in particular (da Rosa et al. 2016; Chisti 2007; Zhu et al. 2021). The only exception was

SP 85, whose carbon ratio ranged from about 29% to 45% within the measurement duration.

Figure 11 presents the change in biomass carbon concentrations of the strains as a function of cultivation time. Except for SP 85, biomass carbon concentrations were sigmoidal in shape and thus could be analyzed with the logistic model. SP 2340 generated the highest final biomass carbon concentration of ($C_{f\ bio\text{-}carbon}$) 0.88 g/L and the lowest production rate constant ($k_{P\text{-}bio\text{-}carbon}$). In contrast, SP 29 had $C_{f\ bio\text{-}carbon}$ of 0.60 g/L and the highest $k_{P\text{-}bio\text{-}carbon}$ across the strains.

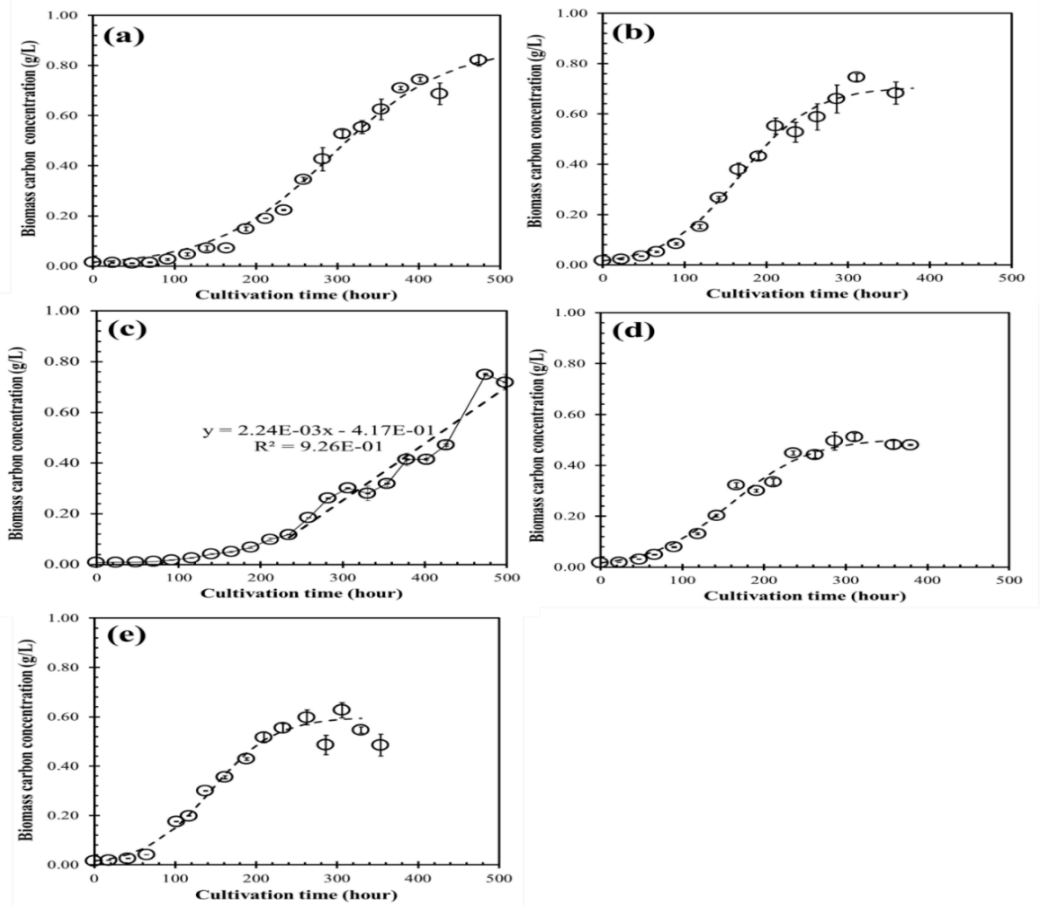

Figure 11. Comparison of biomass carbon concentrations during photobioreactor cultivation of (a) *Spirulina platensis* 2340, (b) *Spirulina maxima* 84.79, (c) *Spirulina platensis* 85.79, (d) *Spirulina platensis* 86.79, and (e) *Spirulina platensis* 29 (Dashed lines present the best fit to the logistic model. Each data point represents the mean±1.0 S.E. of triplicate measurements)

Figure 12 compares the peak biomass carbon productivities ($R_{peak\text{-}C}$) to the maximum daily CO_2 transfer rate (CO_2-TR-max) that could be obtained from the

aeration gas at $Y_{CO_2,out}$ of 0 ppm when 14-hour light periods are taken into account. $R_{peak\text{-}C}$ obtained with SM 84, and SP 29 exceeded the threshold. The source of the unaccounted nongaseous carbon may have been the HCO_3^- (aq) available in the nutrient media, as some algal species are known for their ability to use HCO_3^- for photosynthesis (Chi et al. 2011). Furthermore, the CO_2-TR-max was within the uncertainty range of SP 2340's peak biomass carbon production. The CO_2 transfer rate from the gas phase to the liquid phase must exceed the peak biomass carbon productivities to ensure the establishment of CO_2-replete growth at all times during growth.

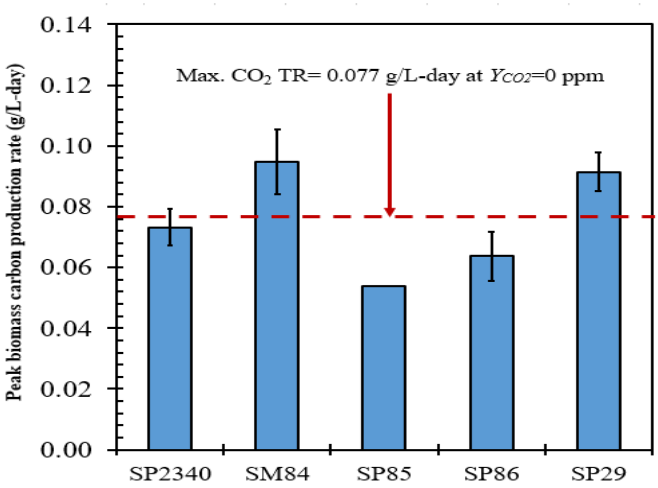

Figure 12. Comparison of peak biomass carbon production rates obtained with *Spirulina* strains during photobioreactor cultivation. (The production rate of SP 85 is based on linear regression of the data, as described in Figure 7.)

Thus, the biomass productivities of at least some strains could be enhanced and compared more accurately at increased CO_2 transfer rates. This necessitates the cultivation of the strains at elevated aeration gas CO_2 concentrations or increased aeration gas flow rates. Indeed, increases in biomass production rates and final biomass concentrations have been reported with *Spirulina* when the aeration gas CO_2 concentration was increased from 0.0380% to 15% (Zhu et al. 2021).

4.5. Morphology of strains

Spirulina cells are marked with their characteristic spiral shape. However, following certain exposures, such as UV or temperature, or cultivation medium alterations, they may lose this shape and turn to a linear form (Zapata et al. 2021). Although not entirely clear, this linearization is considered to be mutation-based and permanent (Ali and Saleh 2012; Zhi and Zhao 2005; Vonshak 1997). This morphological property of the strains, along with trichome length, and helix pitch distances, are essential for designing and operating the harvesting systems (Ali and Saleh 2012; Cheng et al. 2018).

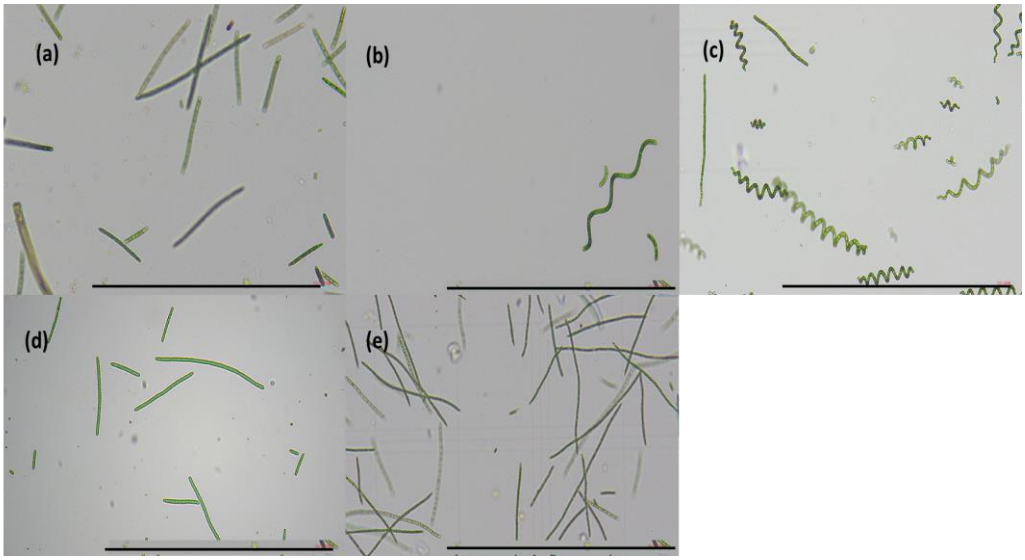

Figure 13. Microscopic images of (a) *Spirulina platensis* CCALA29, (b) *Spirulina maxima* 84.79, (c) *Spirulina platensis* 85.79, (d) *Spirulina platensis* 86.79, and (e) *Spirulina platensis* 2340 strains

The spiral form of some strains has been reported to have higher biomass and protein productivity (Zhi and Zhao 2005). Due to these reasons, a light microscopy-based characterization was performed with the inoculum cultures (Table 8). SM 84 and SP 85 were the only strains that had cells of spiral morphology. SM 84 had the longest average cell length and the helix pitch. A direct correlation between these morphologies and biomass or protein productivity characteristics could not be found.

Table 8. Morphologic characteristics of the *Spirulina* strains.

Strains	**Helical/total (%)**	**Cell length (µm)**	**1.0 S.E.**	**Helix pitch (µm)**	**1.0 S.E.**
SP2340	0	183	8.48 (n=129)		
SM84	85	312	25.02 (n= 120)	99	3.22 (n= 120)
SP85	85	167	7.83 (n=129)	30	0.76 (n=129)
SP86	0	138	6.40 (n= 158)		
SP29	0	146	4.33 (n= 174)		

CHAPTER 5

CONCLUSION

Spirulina maxima and *Spirulina platensis* have been in high demand as dietary supplements for their unique nutritional properties, particularly for their high protein contents exceeding 60% of the dry biomass weight. On the supply side, the production of biomass with the desired nutritional profile is only possible through the correct selection of strains to be cultivated. Strain-level information on industrially produced *Spirulina* is not available, likely because it is a commercial secret. Furthermore, the strains used for academic research have mostly been either isolated from the local environment or procured from national algae collection centers.

The current work is the first step of a range of research activities planned to test *Spirulina*'s large-scale production in Izmir, and for that, it aims to screen *Spirulina* strains obtained from algae collection centers for protein productivity. Five strains belonging to the two species have been cultivated under the standardized process conditions of a bubble column reactor at indoor conditions. The resulting biomass and protein productivities were measured as a function of cultivation time, and productivity characteristics were compared between the strains. Possible methods of further productivity increase were identified by conducting a mass balance analysis around the photobioreactor gas inlet and outlet CO_2 concentrations.

The final biomass concentrations of the strains varied from about 1.2 to 1.9 g/L across the strains. The strains of lower final biomass concentration had higher production rate constants. This resulted in a more even distribution of peak production rates, ranging between 0.15 to 0.20 g/L-day. These final concentrations and productivities were similar to those reported in the literature and thus could be considered promising. Because there were no strains that combined high productivity with high final yield, the strain selection for commercial production has to be picked based on the comparison of the harvesting and photobioreactor installation and maintenance costs.

SP 29 had the ideal protein content profile during photobioreactor growth: the protein ratio of the biomass was relatively high and varied within a very narrow range of 60 to 64%. This opens up the possibility of using continuous growth systems for this

strain. The protein content of the rest of the strains changed between the log and stationary phases of growth. For example, the ratio of SP 2340 and SP 85 increased from about 30 to 35% to about 65 to 75% during the stationary phase. On the contrary, the protein content of SM 84 and SP 86 reduced during the same period. The results clearly indicate the importance of strain as well as cultivation method and harvesting time selection for sustaining protein-rich biomass productivity at an industrial scale.

The protein concentrations were highly strain-dependent: the final protein concentrations obtained ranged from 0.4 g/L to 1.4 g/L. The strains of higher final protein concentrations had lower production rate constant. This resulted in a more homogeneous distribution in peak productivities. Three strains, namely SP 2340, SM 84, and SP 29, had peak biomass production rates of around 0.11 g/L-day, which was superior to those of the other two strains.

The CO_2 availability of the cultures was assessed for future improvement of the strain productivities. The maximum carbon transfer rate to the suspensions was estimated under the assumption that all the CO_2 available in the aeration gas was delivered to the suspensions. This rate was compared with the peak biomass carbon production rates determined based on elemental analysis of the biomass samples. The two strains' peak biomass carbon production rates exceeded the maximum CO_2 transfer rate. This indicates the development of CO_2-limited growth conditions during the growth of these strains. Thus, enhancement in biomass productivity should be expected with the enrichment of CO_2 content in the aeration gas or with an increased aeration gas flow rate for these two strains. Future studies are planned to test the validity of these expectations.

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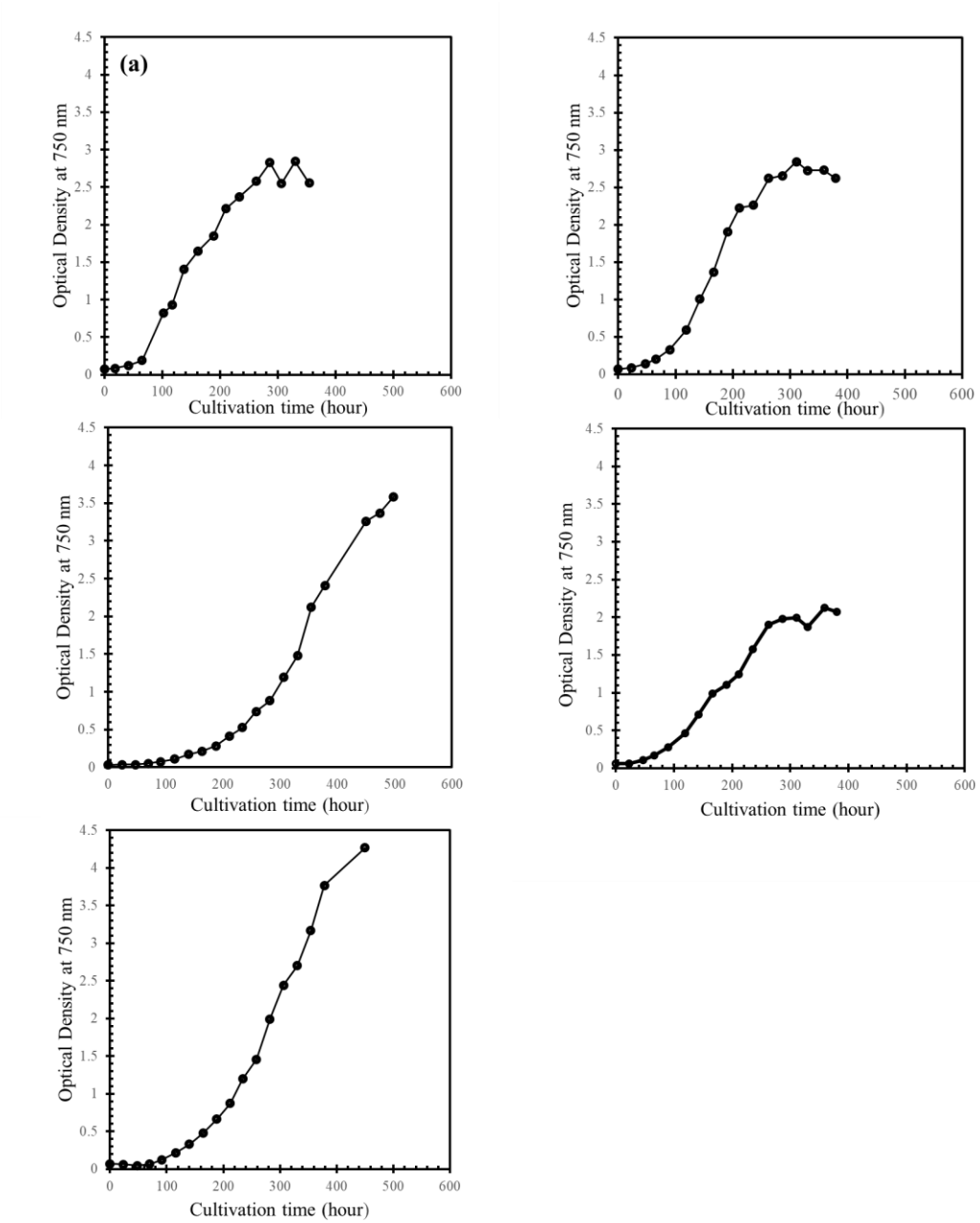
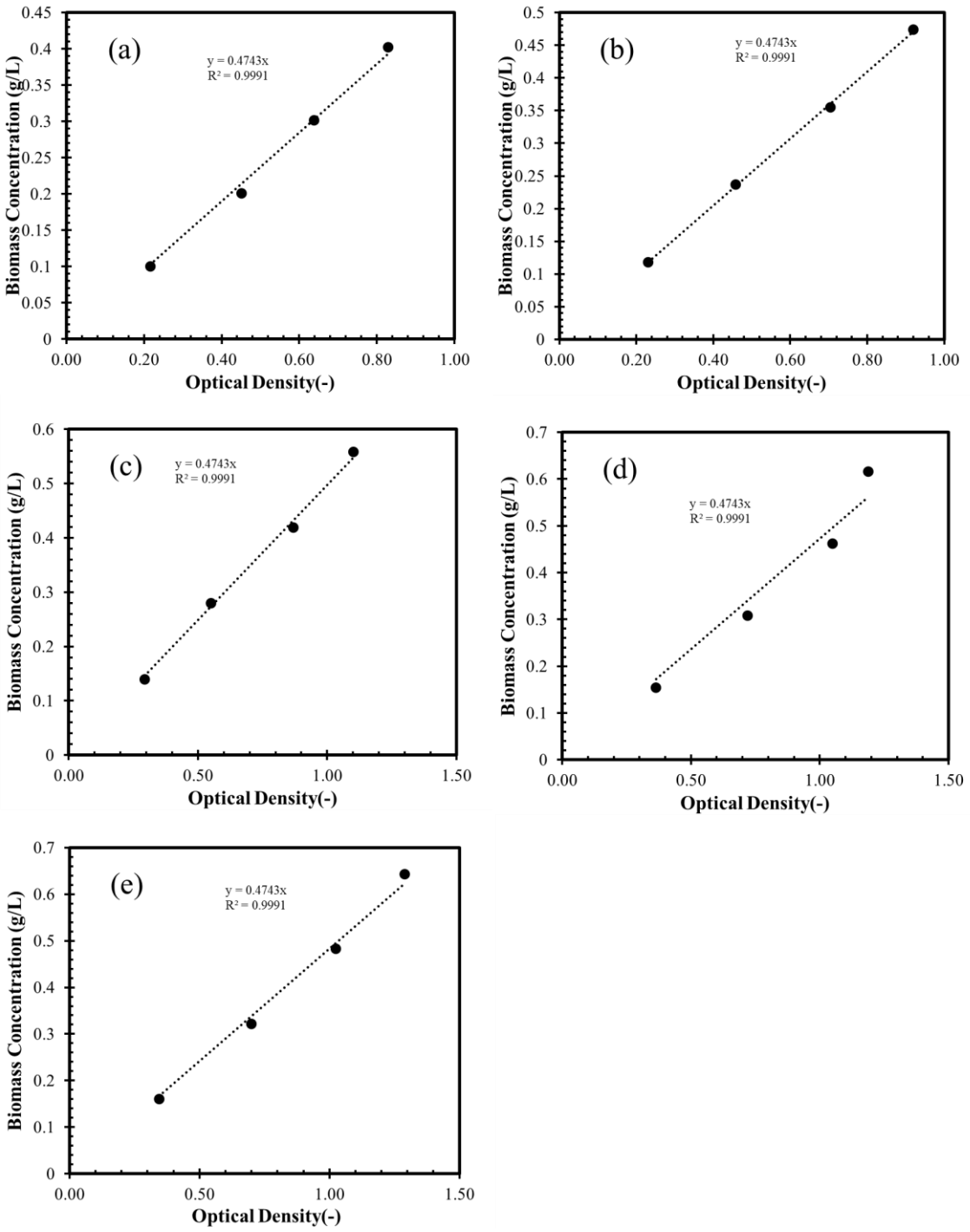
APPENDIX A

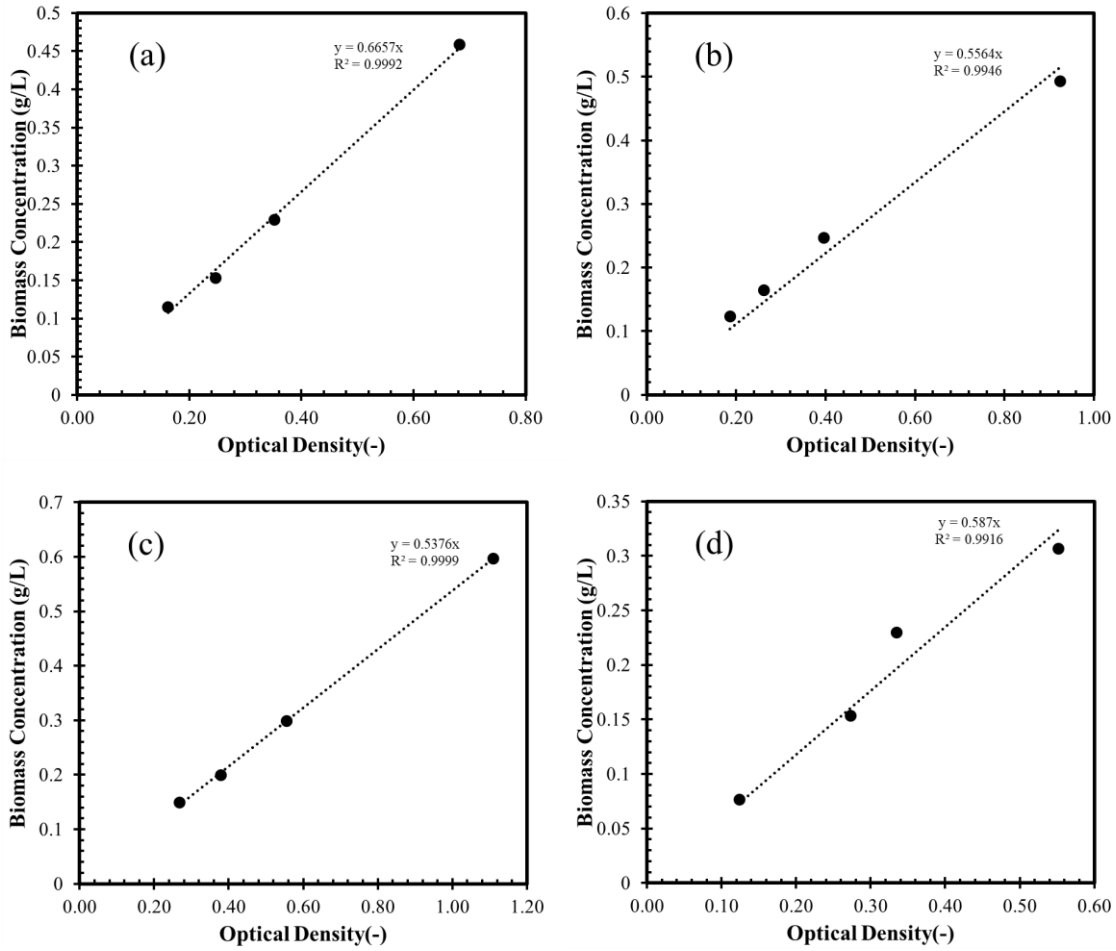
A1. Optical Densities at 750 nm vs. cultivation time for (a) *Spirulina maxima* CCALA29, (b) *Spirulina maxima* 84.79, (c) *Spirulina maxima* 85.79, (d) *Spirulina maxima* 86.79, and (e) *Spirulina platensis* 2340 strains



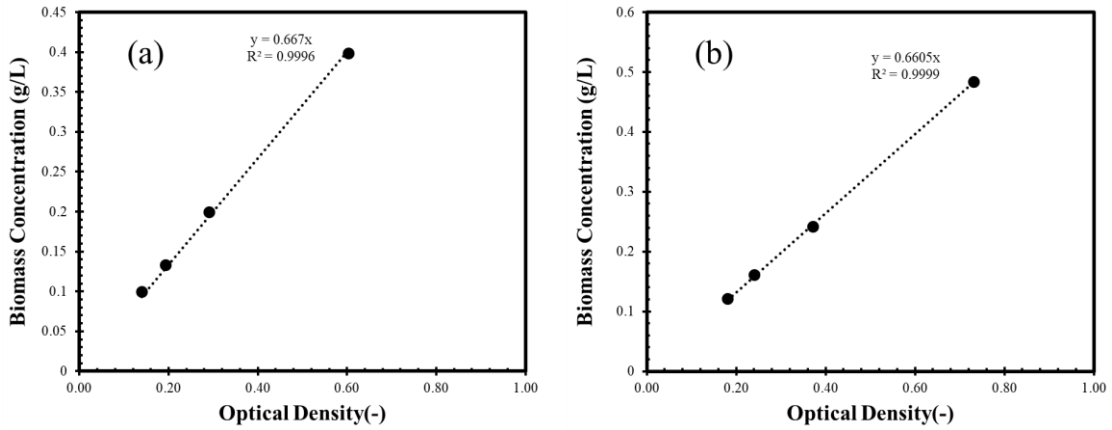
A2. Calibration curve between optical density and biomass concentration for *Spirulina maxima* CCALA29, (a) 161.6, (b) 188.43, (c)209.52, (d) 233.33, and (e) 262.63 hour



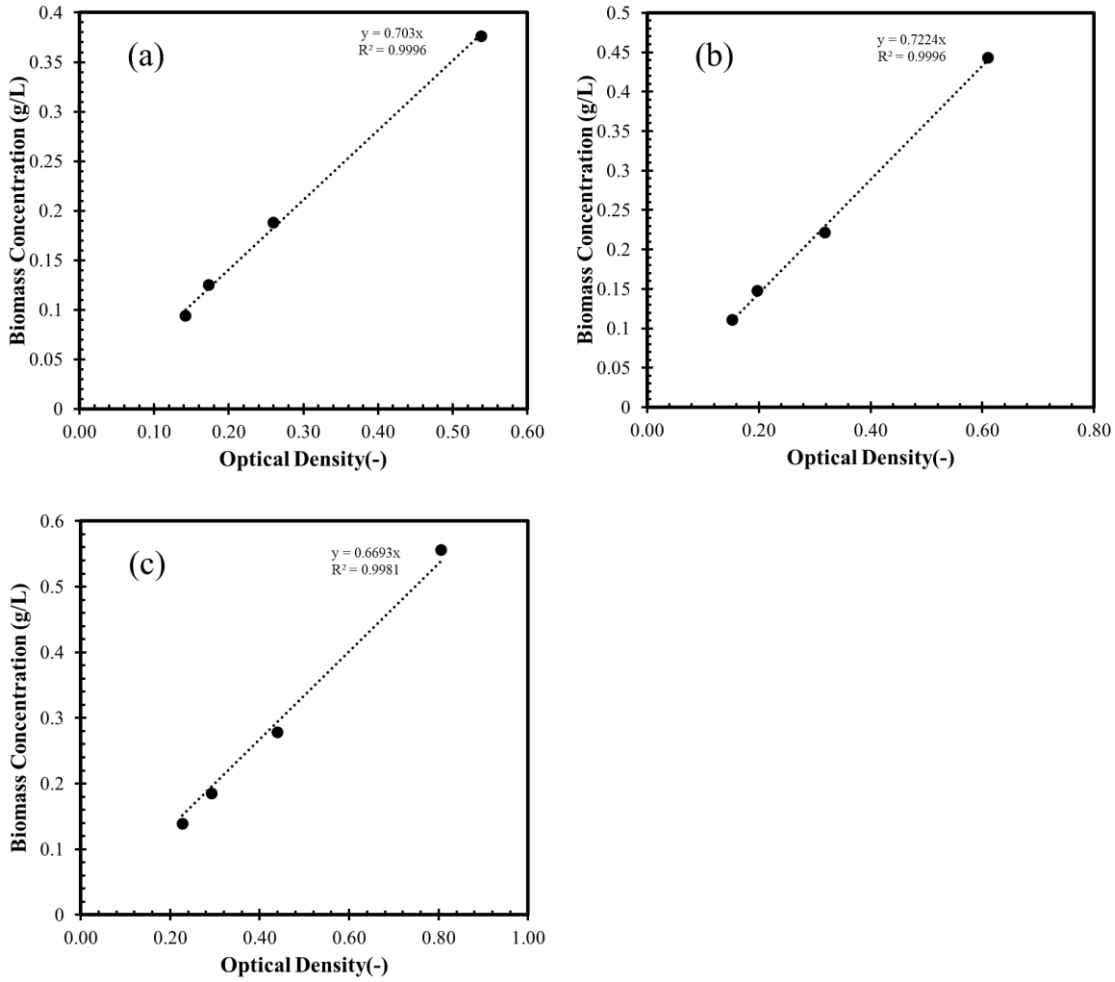
A3. Calibration curve between optical density and biomass concentration for *Spirulina maxima* 84.79 , (a) 166.3, (b) 190.6, (c)211.5, (d) 235.8 hour



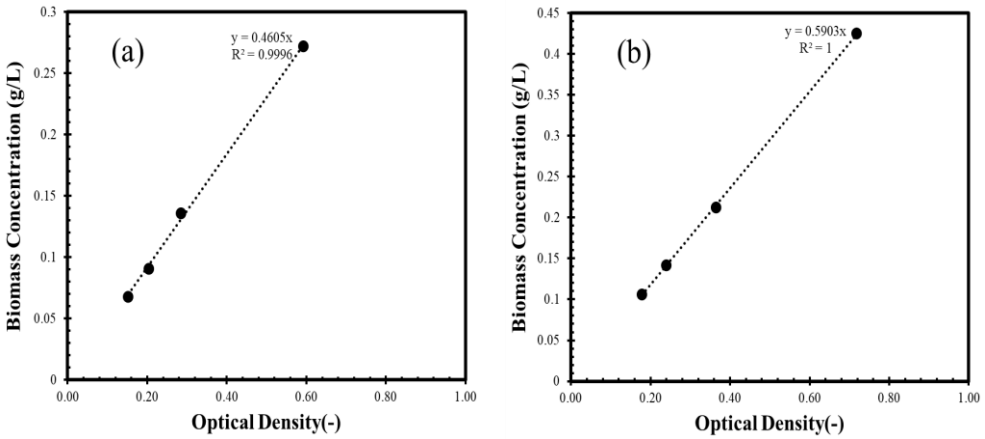
A4. Calibration curve between optical density and biomass concentration for *Spirulina maxima* 85.79 , (a) 306.3, (b) 330.5 hour



A5. Calibration curve between optical density and biomass concentration for *Spirulina maxima* 86.79, (a) 190.6, (b) 211.5, (c) 235.8 hour



A5. Calibration curve between optical density and biomass concentration for *Spirulina platensis* 2340, (a) 234.3, (b) 258.4 hour



A6. Results from the CHN analysis of the strains

Sample Code	%N	%C	%H	%S
STD-BBOT001	6.51	72.52	6.09	7.44
STD-BBOT002	6.51	72.52	6.09	7.44
STD-BBOT003	6.51	72.52	6.09	7.44
STD-BBOT004	6.51	72.52	6.09	7.44
STD-BBOT005	6.51	72.52	6.09	7.44
Average	6.51	72.52	6.09	7.44
S.E.	0.00	0.00	0.00	0.00
***Spirulina maxima* CCALA29**				
Cultivation time= 161.6 hour	%N	%C	%H	%S
SM29/1	9.89	45.21	6.54	0.34
SM29/2	9.82	44.63	6.57	0.30
SM29/3	10.20	42.53	6.60	
Average	9.97	44.12	6.57	0.21
S.E.	0.12	0.82	0.02	0.02
Cultivation time= 188.4 hour	%N	%C	%H	%S
SM29/1	10.42	45.59	6.63	0.21
SM29/2	10.27	46.00	6.56	0.25
SM29/3	9.92	44.27	6.21	0.22
Average	10.21	45.29	6.47	0.22
S.E.	0.15	0.52	0.13	0.01
Cultivation time= 209.5 hour	%N	%C	%H	%S
SM29/1	9.94	44.67	6.63	0.19
SM29/2	10.04	46.10	6.61	0.23
SM29/3	10.70	47.81	6.54	
Average	10.23	46.19	6.59	0.14
S.E.	0.24	0.91	0.03	0.02
Cultivation time= 233.3 hour	%N	%C	%H	%S
SM29/1	9.56	44.63	6.65	0.26
SM29/2	9.85	47.60	6.57	0.22
SM29/3	9.28	42.69	6.69	
Average	9.56	44.97	6.64	0.16
S.E.	0.17	1.43	0.03	0.02
Cultivation time= 262.6 hour	%N	%C	%H	%S
SM29/1	9.61	45.12	6.49	0.22
SM29/2	9.71	44.08	6.29	0.16
SM29/3	10.31	50.07	6.54	
Average	9.88	46.42	6.44	0.13
S.E.	0.22	1.85	0.08	0.02

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A2. (cont.)

Cultivation time= 286.1 hour	%N	%C	%H	%S
SM29/1	10.06	44.86	6.56	0.13
SM29/2	10.08	44.81	6.30	0.16
SM29/3	9.63	44.36	6.27	
Average	9.92	44.67	6.38	0.10
S.E.	0.15	0.16	0.09	0.01
Cultivation time= 305.9 hour	%N	%C	%H	%S
SM29/1	10.04	44.55	6.35	0.10
SM29/2	10.17	44.20	6.39	0.08
SM29/3	9.71	45.77	6.35	
Average	9.98	44.84	6.36	0.06
S.E.	0.14	0.48	0.01	0.01
Cultivation time= 329.7 hour	%N	%C	%H	%S
SM29/1	10.11	43.67	6.38	0.11
SM29/2	10.35	44.88	6.30	0.10
SM29/3	9.74	44.39	6.57	0.00
Average	10.07	44.31	6.41	0.07
S.E.	0.18	0.35	0.08	0.04
Cultivation time= 353.9 hour	%N	%C	%H	%S
SM29/1	10.21	44.87	6.21	
SM29/2	10.00	44.23	6.34	
SM29/3	9.13	43.09	6.66	
Average	9.78	44.07	6.40	
S.E.	0.33	0.52	0.14	
***Spirulina maxima* 84.79**				
Cultivation time= 142.2 hour	%N	%C	%H	%S
SM84/1	9.82	43.39	6.14	
SM84/2	9.43	41.15	6.28	
SM84/3	8.82	41.88	6.39	
Average	9.35	42.14	6.27	
S.E.	0.29	0.66	0.07	
Cultivation time= 166.3 hour	%N	%C	%H	%S
SM84/1	10.09	43.92	6.32	0.43
SM84/2	8.13	36.02	5.12	0.30
SM84/3	10.31	44.08	6.50	0.30
Average	9.51	41.34	5.98	0.34
S.E.	0.69	2.66	0.43	0.04

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A2. (cont.)

Cultivation time= 190.6 hour	%N	%C	%H	%S
SM84/1	10.09	46.05	6.21	4.52
SM84/2	9.07	41.94	9.10	
SM84/3	9.27	43.12	6.99	
Average	9.48	43.70	7.43	1.51
S.E.	0.31	1.22	0.86	
Cultivation time= 211.5 hour	%N	%C	%H	%S
SM84/1	9.79	44.59	6.32	0.11
SM84/2	9.77	43.44	6.12	0.09
SM84/3	10.07	50.72	6.76	
Average	9.88	46.25	6.40	0.07
S.E.	0.10	2.26	0.19	0.01
Cultivation time= 235.8 hour	%N	%C	%H	%S
SM84/1	8.81	40.54	6.22	0.13
SM84/2	10.00	48.60	6.40	0.10
SM84/3	8.29	39.88	7.65	
Average	9.03	43.01	6.76	0.08
S.E.	0.51	2.80	0.45	0.01
Cultivation time= 262.5 hour	%N	%C	%H	%S
SM84/1	8.03	49.81	6.80	
SM84/2	6.77	45.29	9.18	
SM84/3	6.55	43.93	7.68	
Average	7.12	46.34	7.89	
S.E.	0.46	1.78	0.70	
Cultivation time= (286.5-311.0 hour)	%N	%C	%H	%S
SM84/1	7.13	41.90	8.67	
SM84/2	6.69	42.62	7.99	
SM84/3	6.92	40.69	8.66	
Average	6.91	41.73	8.44	
S.E.	0.13	0.56	0.23	
Cultivation time= (358.5-379.5 hour)	%N	%C	%H	%S
SM84/1	8.22	45.87	6.72	
SM84/2	7.76	40.75	8.92	
SM84/3	8.69	47.28	9.11	
Average	8.22	44.63	8.25	
S.E.	0.27	1.98	0.77	

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A2. (cont.)

***Spirulina maxima* 86.79**				
Cultivation time= 166.3 hour	%N	%C	%H	%S
SM86/1	8.16	41.27	8.90	
SM86/2	8.41	43.21	9.46	
SM86/3	8.86	44.35	9.37	
Average	8.48	42.95	9.24	
S.E.	0.21	0.90	0.17	
Cultivation time= 190.6 hour	%N	%C	%H	%S
SM86/1	8.04	41.15	9.13	
SM86/2	7.67	38.74	9.07	
SM86/3	8.09	39.82	8.87	
Average	7.93	39.90	9.03	
S.E.	0.13	0.70	0.08	
Cultivation time= 211.50 hour	%N	%C	%H	%S
SM86/1	6.65	37.67	5.54	0.40
SM86/2	6.69	37.85	5.61	0.38
SM86/3	6.67	37.97	5.59	0.41
Average	6.67	37.83	5.58	0.40
S.E.	0.01	0.09	0.02	0.01
Cultivation time= 235.8 hour	%N	%C	%H	%S
SM86/1	6.60	40.37	5.83	0.39
SM86/2	6.43	40.33	5.80	0.36
SM86/3	6.61	40.27	5.82	0.34
Average	6.55	40.32	5.82	0.36
S.E.	0.06	0.03	0.01	0.01
Cultivation time= (262.5-286.5-311.0) hour	%N	%C	%H	%S
SM86/1	6.16	41.23	5.87	0.29
SM86/2	6.21	41.04	5.78	0.29
SM86/3	6.27	41.13	5.78	0.26
Average	6.21	41.13	5.81	0.28
S.E.	0.03	0.06	0.03	0.01
Cultivation time= (330.0-358.5-379.5) hour	%N	%C	%H	%S
SM86/1	6.40	41.70	5.97	0.30
SM86/2	6.62	41.37	5.78	0.33
SM86/3	6.65	41.51	5.82	0.28
Average	6.56	41.53	5.85	0.30
S.E.	0.08	0.10	0.06	0.01

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A2. (cont.)

***Spirulina maxima* 85.79**				
Cultivation time= (234.3-258.2) hour	%N	%C	%H	%S
SM85/1	5.82	37.21	5.48	0.17
SM85/2	5.82	37.21	5.33	0.18
SM85/3	5.79	37.29	5.35	0.17
Average	5.81	37.24	5.39	0.17
S.E.	0.01	0.03	0.05	0.00
Cultivation time= (282.3-306.3) hour	%N	%C	%H	%S
SM85/1	6.96	37.73	5.60	0.29
SM85/2	6.93	37.84	5.52	0.21
SM85/3	6.89	37.94	5.67	0.35
Average	6.93	37.84	5.60	0.28
S.E.	0.02	0.06	0.04	0.04
Cultivation time= (330.5-354.3) hour	%N	%C	%H	%S
SM85/1	6.78	27.18	9.17	
SM85/2	6.64	28.91	7.36	
SM85/3	6.47	30.85	7.14	
Average	6.63	28.98	7.89	
S.E.	0.09	1.06	0.64	
Cultivation time= (378.3-402.3) hour	%N	%C	%H	%S
SM85/1	6.33	29.30	8.10	
SM85/2	7.48	32.94	8.35	
SM85/3	6.22	28.29	6.89	
Average	6.68	30.17	7.78	
S.E.	0.40	1.41	0.45	
Cultivation time= (426.3-450.2) hour	%N	%C	%H	%S
SM85/1	8.19	34.86	7.93	
SM85/2	8.53	36.19	8.82	
SM85/3	7.05	32.58	7.15	
Average	7.92	34.54	7.97	
S.E.	0.45	1.05	0.48	
Cultivation time= (474.1-498.2) hour	%N	%C	%H	%S
SM85/1	10.85	44.46	6.32	0.41
SM85/2	11.14	45.68	6.42	0.35
SM85/3	11.08	45.19	6.38	0.39
Average	11.03	45.11	6.37	0.39
S.E.	0.09	0.36	0.03	0.02

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A2. (cont.)

***Spirulina platensis* 2340**				
Cultivation time= (164.3-188.2) hour	%N	%C	%H	%S
SP2340/1	4.75	33.54	4.91	0.32
SP2340/2	4.88	33.36	5.15	0.34
SP2340/3	4.73	32.38	4.85	0.41
Average	4.79	33.09	4.97	0.36
S.E.	0.05	0.36	0.09	0.03
Cultivation time= (212.0-234.3) hour	%N	%C	%H	%S
SP2340/1	8.36	40.93	5.86	0.55
SP2340/2	8.41	41.08	5.82	0.59
SP2340/3	8.52	41.11	5.94	0.49
Average	8.43	41.04	5.87	0.54
S.E.	0.05	0.06	0.03	0.03
Cultivation time= 258.4 hour	%N	%C	%H	%S
SP2340/1	9.35	40.69	5.67	0.67
SP2340/2	9.44	40.77	5.83	0.67
SP2340/3	9.36	40.43	5.86	0.56
Average	9.39	40.63	5.79	0.63
S.E.	0.03	0.10	0.06	0.04
Cultivation time= 282.3 hour	%N	%C	%H	%S
SP2340/1	9.45	40.88	5.80	0.67
SP2340/2	9.40	40.59	5.75	0.67
SP2340/3	8.94	38.10	5.16	0.50
Average	9.27	39.86	5.57	0.62
S.E.	0.16	0.88	0.21	0.06
Cultivation time= 306.3 hour	%N	%C	%H	%S
SP2340/1	10.39	42.95	6.05	0.66
SP2340/2	10.15	42.62	6.05	0.71
SP2340/3	10.37	42.30	5.93	0.62
Average	10.30	42.62	6.01	0.66
S.E.	0.08	0.19	0.04	0.03
Cultivation time= 330.5 hour	%N	%C	%H	%S
SP2340/1	10.14	42.95	5.96	0.78
SP2340/2	10.27	43.03	6.10	0.79
SP2340/3	10.24	43.24	6.05	0.78
Average	10.22	43.07	6.04	0.79
S.E.	0.04	0.09	0.04	0.00

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A2. (cont.)

Cultivation time= (354.3-378.3) hour	%N	%C	%H	%S
SP2340/1	10.47	44.26	6.19	0.76
SP2340/2	10.35	44.33	6.20	0.75
SP2340/3	10.48	44.03	6.13	0.76
Average	10.43	44.21	6.17	0.76
S.E.	0.04	0.09	0.02	0.00
Cultivation time= (402.3-426.3) hour	%N	%C	%H	%S
SP2340/1	10.31	43.12	6.22	0.64
SP2340/2	10.38	42.96	6.15	0.67
SP2340/3	10.22	42.81	6.13	0.68
Average	10.30	42.96	6.17	0.67
S.E.	0.05	0.09	0.03	0.01
Cultivation time= (450.2-474.1) hour	%N	%C	%H	%S
SP2340/1	11.06	45.02	6.49	0.62
SP2340/2	11.00	45.13	6.43	0.61
SP2340/3	10.91	44.49	6.14	0.61
Average	10.99	44.88	6.35	0.61
S.E.	0.04	0.20	0.11	0.00

A7. Change of the nitrogen content of strain (a) *Spirulina platensis* 2340 , (b) *Spirulina maxima* 84.79, (c) *Spirulina maxima* 85.79, (d) *Spirulina maxima* 86.79, and (e) *Spirulina maxima* CCALA29