Aquaculture Studies, 24(2), AQUAST1419
http://doi.org/10.4194/AQUAST1419

RESEARCH PAPER

Water Fern *Azolla pinnata* Extract as a Novel Organic Nutrient Source for the Cultivation of Spirulina *Spirulina platensis*

Mst. Sobnom Binta Mofiz[1], Md. Amzad Hossain[1,*], Joya Saha[1], Md. Akibul Hasan Bakky[2], Taslima Akter[1], Mst. Rubia Banu[3]

[1]Department of Aquaculture, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh.
[2]Institute of Marine Sciences, Shantou University, Shantou 515063, China.
[3]Department of Fisheries Management, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh.

How to Cite

Mofiz, M.S.B., Hossain, M.A., Saha, J., Bakky, M.A.H., Akter, T., Banu, M.R. (2024). Water Fern *Azolla pinnata* Extract as a Novel Organic Nutrient Source for the Cultivation of Spirulina *Spirulina platensis*. *Aquaculture Studies*, *24(2), AQUAST1419*. http://doi.org/10.4194/AQUAST1419

Article History
Received 20 March 2023
Accepted 09 August 2023
First Online 21 August 2023

Corresponding Author
Tel.: +8802996695323
E-mail: amzad@bsmrau.edu.bd

Keywords
Azolla extract
Microalgae
Spirulina platensis
Organic medium

Abstract

This study aimed to observe the potentiality of azolla extract as a cost-effective organic medium. An 18-day culture experiment of *Spirulina platensis* was conducted using various percentages of dried azolla extract (DAE, 10-50%) or boiled azolla extract (BAE, 10-40%) in place of inorganic Kosaric medium (control). Growth, pigmentation, minerals and lipids contents of *S. platensis* cultured were recorded. Compared with the control, similar results were obtained in biomass concentration and specific growth rate when up to 40% KM was replaced with 40% DAE or 20% BAE. The mineral and lipid contents of *S. platensis* were unaffected by the replacement of KM with azolla extracts, except that Ca, Zn, and Fe concentrations increased as the replacement amount of KM with DAE and BAE increased. The KM had the highest chlorophyll *a* content. Similar findings were observed for chlorophyll *a* of the *S. platensis* in DAE40 and BAE20. It was concluded that KM could be partially replaced with DAE or BAE as the novel organic media for *S. platensis* cultivation, where DAE is better than BAE.

Introduction

Spirulina is a blue-green microalga that is filamentous and has numerous cells. Because the species are obligate alkaliphiles, they can thrive in tropical and sub-tropical lakes with increased alkalinity, where other creatures have a hard time surviving (Belay, 2008). Cyanobacterial species such as spirulina use their capacity to thrive at extreme pH to escape contamination by different microbes (Touloupakis et al., 2016). Spirulina has all the essential nutrients, containing a healthy balance of all essential elements. It contains high-quality protein, dietary minerals, vitamins, and important fatty acids (Belay, 2008; Hosseini et al., 2013; Sharoba, 2014; Gutierrez-Salmean et al., 2015). Spirulina also contains significant amounts of antioxidants, including flavonoids, phenolics, vitamin E, and several pigments that absorb light, including chlorophylls, carotenoids, phycocyanin, and, to stop the body from being harmed by free radicals; they are all necessary. (Kumar et al., 2005; El-Baky et al., 2008; Chu et al., 2010; Michael et al., 2018). Spirulina has gotten much attention because of its unique nutritional profile, and it is grown in vast amounts for use in pharmaceutical products, feed additives, and food for humans and animals (Habib et al., 2008; Chu et al., 2010; Chen, 2011; Michael et al., 2018). The Food and Agriculture Organization (FAO) of the United Nations has referred to spirulina as a "highly digestible protein product," and spirulina deserves the title of "the food of the future" more than any other food on Earth (Liu et al., 2023; Markou et al., 2023).

However, because the culture media impact the nutrient content and growth of spirulina biomass, one of the limits to producing spirulina on an industrial scale is the need for more cost-effective nutrient sources

(Carvalho et al., 2004; Richmond, 2004). Because of this, the high price of growth media, which is primarily based on the Kosaric medium (KM), limits the widespread production of spirulina around the world (Belay, 2008; Habib et al., 2008; Madkour et al., 2012; Tarko et al., 2012). The KM is costly because it contains analytical-grade chemicals for which spirulina's nutritional requirements can account for up to half of the overall cost of producing biomass (Xia & Murphy, 2016; Zhu et al., 2016). This high production cost necessitates the establishment of an affordable alternative medium capable of providing identical nutrients for the culture of microalgae (Chia et al., 2018). Various researchers have made several efforts to establish a practical and cost-effective growth media capable of producing spirulina biomass (Raoof et al., 2006; Chen, 2011; Gami et al., 2011; Madkour et al., 2012; Kumari et al., 2015). In order to formulate different types of media, readily accessible sources have been used, including wastewater, salt water, and industrial effluents (Leema et al., 2010). As a growing media, crop-based organic substrates such as molasses (Andrade & Costa, 2007), cabbage extracts (Akhtar et al., 2014), and sugarcane bagasse (Pelizer et al., 2015) have also been investigated. Food waste hydrolysates, for example, have been effectively used to culture microalgae and have shown good microalgae development (Pleissner et al., 2013; Lau et al., 2014). The nutrient content of agro-industrial effluents can also be used to assist the production of Spirulina at a lower cost (Phang et al., 2000). Recent studies have reported on the cultivation of spirulina in many types of wastewater which consists including municipal wastewater, swine wastewater and saline wastewater (Lee et al., 2020; Mata et al., 2020)

Azolla is a freshwater fern that floats freely and is among the aquatic plants with the quickest growth rate. Aquatic ecosystems can get an excellent supply of nitrogen from azolla owing to its endosymbiotic blue algae, *Anabaena azollae*, which is able to absorb unlinked nitrogen (N2) from the atmosphere directly. In Southeast Asian countries, it is widely utilized as a bio-fertilizer and green manure for rice cultivation. Spirulina needs nitrogen to flourish, and azolla has a high nitrogen concentration, making it a perfect biofertilizer (Khatun et al., 2019). The azolla is rich in minerals, including calcium, phosphorus, potassium, magnesium, copper, zinc, and other elements. As a result, azolla extract can be a good source of nitrogen and other nutrients, allowing for rapid growth and mass multiplication of *S. platensis.* Organic goods or mediums have recently gained popularity among consumers, though spirulina growers prefer KM. Consequently, this research sought to evaluate the effects of azolla extract as a nutrition source on the growth, mineral and lipid contents, and pigmentation of *S. platensis*.

Materials and Methods

Experimental Design

For 18 days, the experiment was carried out in the Live Food Culture Laboratory of the Department of Aquaculture at Bangabandhu Sheikh Mujibur Rahman Agricultural University using a completely randomized design. As shown in Table 1, dried or boiled azolla extracts were employed in place of the control medium, i.e., Kosaric medium (KM), at varied concentrations.

Preparation of Azolla Extract

Azolla (*Azolla pinnata*) was obtained from the Bangladesh Rice Research Institute in Gazipur for this investigation. The plants were rinsed with water to make them free of unwanted impurities. Then the plants were spread on blotting paper to remove excess water. To prepar*e* azolla powder, 1.0 kg of fresh azolla was dried in a hot air oven for 24 hours at 70 ºC. After that, it was ground to powder by using a kitchen grinder. To prepare dried azolla extract (DAE), azolla powder was dissolved in 2.0 L distilled water and kept for 72 hours for extraction. For preparing boiled azolla extract (BAE), 1.0 kg of fresh azolla was boiled with 2.0 L of deionized water for 30 minutes and cooled. To eliminate big particles and indigenous germs, the DAE and BAE were filtered using glass microfiber filters (934H, Whatman, USA). Then each solution was diluted to 4.0 L using deionized water and kept in a refrigerator at 4°C, which was taken as 100% concentration of the respective solution. The chemical components of azolla are presented in Table 2.

Table 1. Design of the experiment of *S. platensis* cultivation in different media

Culture medium	Culture media combination (%)	Culture media combination (ml)	
		KM	Azolla extract
KM	KM (100%)	400	-
DAE10	KM (90%) + DAE (10%)	360	40
DAE20	KM (80%) + DAE (20%)	320	80
DAE30	KM (70%) + DAE (30%)	280	120
DAE40	KM (60%) + DAE (40%)	240	160
DAE50	KM (50%) + DAE (50%)	200	200
BAE10	KM (90%) + BAE (10%)	360	40
BAE20	KM (80%) + BAE (20%)	320	80
BAE30	KM (70%) + BAE (30%)	280	120
BAE40	KM (60%) + BAE (40%)	240	160

KM = Kosaric medium, DAE= Dried azolla extract, BAE= Boiled azolla extract.

Preparation of Kosaric Medium

Kosaric medium (KM) was prepared from analytical grade chemicals collected from a local supplier (Sigma-Aldrich, USA). The following chemicals were dissolved in distilled water (in g L^{-1}): $NaHCO_3$ 9.00, $NaNO_3$ 1.25, K_2HPO_4 0.25, K_2SO_4 0.50, NaCl 0.50, $MgSO_4 \cdot 7H_2O$ 0.10, $CaCl_2$ 0.02, $FeSO_4 \cdot 2H_2O$ 0.005, $CaCl_2$ 0.02 and micronutrient solution of 0.5 mL was added. Micronutrient solution was prepared previously as (g L^{-1}): H_3BO_3 2.86, $MnCl_2 \cdot 4H_2O$ 1.81, $ZnSO_4 \cdot 7H_2O$ 0.22, $CuSO_4 \cdot 5H_2O$ 0.08, $CoCl_2 \cdot 6H_2O$ 0.01, MoO_3 0.01. The ingredients were weighed with an electronic balance and placed in a 1.0 L conical flask for the preparation of KM. Following that, 0.5 mL of the micronutrient solution was pipetted into the flask, and distilled water was added to reach the final volume of 1.0 L. The medium was thoroughly mixed and sterilized in an autoclave at 121°C for 15 minutes with moist heat, cooled, and then stored.

Determination of Nutrient Composition

The method EPA 200.8 was used to determine nutritional elements using inductively coupled plasma mass spectrometry (ICP-MS) (EPA, 1994). Based on the previously stated process, the materials were decomposed utilizing microwave-assisted acid digestion (Julshamn et al., 2007). The digested samples were then diluted in deionized water to a volume of 25 mL. ICP-MS (Thermo Scientific, Waltham, USA) was used to determine the nutrient element concentrations in KM, DAE, and BAE, which are shown in Table 3.

Experimental Culture of *S. platensis*

The pure stock of *S. platensis* was maintained in Kosaric medium (KM) in the Live Food Culture Laboratory of Bangabandhu Sheikh Mujibur Rahman Agricultural University's Department of Aquaculture. For this research, *S. platensis* was obtained from that stock. *S. platensis* stock growth was observed every other day, and purity was examined under a microscope. Before the culture initiation of *S. platensis*, depending on the pH condition of the culture medium, either 0.1 N HCL or 0.1 NaOH was added to adjust the pH to 9.0. In 30 conical flasks (500 mL size), the experimental cultivation of *S. platensis* was carried out, each containing 400 mL medium for 10 treatments with 3 replications (Table 1). *S. platensis* was used to create a culture with a 10% suspension in each flask (OD at 620 nm = 0.20) (Habib, 1998). The flasks were maintained in a light-to-dark (12h:12h) cycle with fluorescent lighting (TFC, FL-40, and SD/38-day light), and electric aquarium air pumps (RS-628A) were used to aerate them continuously.

Estimation of the Biomass of *S. platensis*

A filter paper (Whatman GF/C, 0.45 m mesh size, 47 mm diameter) was used to filter 10 mL of *the S. platensis* sample. To eliminate insoluble salts, the sample was rinsed with 20 mL of acidified water (pH = 4.0) during filtering. The filter paper was then placed in a glass Petri dish and kept in the oven overnight at 70°C. The Petri dish was set in desiccators for 20 minutes to cool, and then the filter paper was weighed (FFW).

Table 2. The chemical constitution of azolla

Nutrient	% dry matter
Dry matter	4.82
Organic matter	82.32
Crude protein	21.95
Ether extract	4.79
Total ash	16.90
Crude fiber	14.35
Nitrogen free extract	41.81

Table 3 Nutrient compositions of KM, DAE, and BAE

Element	KM	DAE	BAE
N (g L^{-1})	0.36	0.79	0.75
P (g L^{-1})	0.12	0.20	0.15
K (g L^{-1})	0.37	0.12	0.15
Na (g L^{-1})	3.91	0.17	0.27
Fe (mg L^{-1})	1.47	7.76	7.46
Mg (mg L^{-1})	9.87	40.4	41.1
Ca (mg L^{-1})	7.21	140.3	136.3
B (mg L^{-1})	0.23	LoD	LoD
Mn (mg L^{-1})	0.25	2.75	2.05
Zn (µg L^{-1})	25.01	1090.19	1029.18
Cu (µg L^{-1})	10.18	250.08	196.70
Co (µg L^{-1})	1.24	LoD	LoD
Mo (µg L^{-1})	3.33	LoD	LoD

LoD= Limit of detection; KM = Kosaric medium, DAE= Dried azolla extract, BAE= Boiled azolla extract.

Before filtering, the filter paper was weighed after being dried in an oven for 24 hours at 70°C. (IFW). The biomass weight of *S. platensis* in dry form was obtained using the formula below (Clesceri et al., 1989):

$$\text{Biomass concentration (g L} - 1) = \frac{(\text{FFW} - \text{IFW})\ (\text{g})}{\text{Quantity of the sample used for filtering (mL)}} \times 1000$$

Where IFW- initial filter paper weight; FFW- final filter paper weight

Chlorophyll *a* Concentration Estimation

An electric filtration unit was used to filter a 10 mL *S. platensis* sample using filter paper (Whatman GF/C, 0.45 m mesh size, 47 mm diameter). This filtered sample, along with the filter paper, was placed in a test tube, crushed with a glass rod, and blended with 10 mL of 100% redistilled acetone. After that, to prevent light contact, the test tube was wrapped in foil paper and refrigerated for the night. The refrigerated sample was then homogenized for 2 minutes before being centrifuged for 10 minutes at 4000 rpm. The supernatant was separated after centrifugation and used to determine the amount of chlorophyll *a* in the sample. A UV spectrophotometer was used to determine the optical densities of the sample at 664 nm, 647 nm, and 630 nm (DR 6000, Hach, USA). Simultaneously, a blank with 100 percent acetone was run. The following formula was used to compute the chlorophyll *a* content (Clesceri et al., 1989):

Chlorophyll *a* (mg L^{-1}) = 11.85 (OD 664) - 1.54 (OD 647) - 0.08 (OD 630)

Determination of Specific Growth Rate

The specific growth rate (SGR, day^{-1}) of cultured *S. platensis* was calculated using the equation below (Clesceri et al., 1989).

$$\text{SGR } (\mu\ day^{-1}) = \ln (X_1 - X_2) / t_2 - t_1$$

Where,

X_1 = Biomass concentration at the end of the selected time interval, X_2 = Biomass concentration at the beginning of specified time interval, and $t_2 - t_1$= amount of time that has passed since the chosen time interval.

Statistical analysis

One-way analysis of variance (ANOVA) was used to statistically evaluate the data in Statistix 10 (Analytical Software, Tallahassee, USA), with significance determined using the package's least significant difference (LSD) option. The Shapiro-Wilk and Levene tests were used to determine normality and homogeneity, respectively. The significance level was established at $p<0.05$.

Results

Biomass Production

Figure 1 shows the variation in biomass concentration throughout 18 days of culture. The statistical differences in biomass concentration at the end of 18 days of cultivation are presented in Figure 2. Initially, there were no differences in biomass

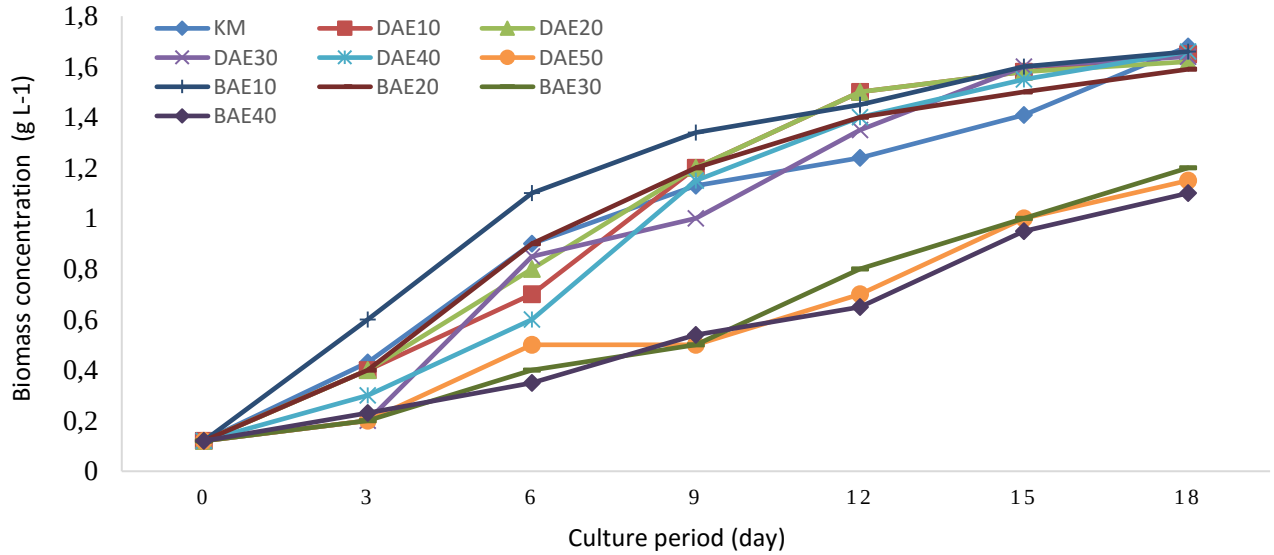

Figure 1. Growth curve of *S. platensis* cultivated in different culture media. KM = Kosaric medium, DAE= Dried azolla extract, BAE=Boiled azolla extract.

concentration between treatments; however, after 3 days of incubation, there was a considerable rise in biomass concentration and variation among treatments. In the control KM medium, the maximal biomass concentration reached (1.66 g L^{-1}) after 18 days of culture. The biomass concentration did not decrease when KM was replaced with DAE at a 40% replacement level; however, the biomass concentration fell when the replacement level was increased to 50%. On the other hand, only 20% of KM could be replaced with BAE without impacting biomass concentration.

Specific Growth Rate

The specific growth rate (SGR) of *S. platensis* in KM after 18 days of cultivation was 0.143 µ Day^{-1} (Figure 3), while SGR values for DAE10, DAE20, DAE30, and DAE40 were 0.141, 0.139, 0.138, and 0.141 µ day^{-1}, respectively indicating that up to 40% KM can be replaced with DAE without affecting the SGR. Replacing KM at a 50% level with DAE decreased the SGR value (0.128 µ Day^{-1}). In the case of BAE, SGR values were 0.138 and 0.140 µ day^{-1} in BAE10 and BAE20, respectively, and were similar to that of KM. On the other hand, a further increase in the replacement level of KM with BAE, i.e., BAE30 (SGR 0.132 µ day^{-1}) and BAE 40 (SGR 0.129 µ day^{-1}), significantly decreased the SGR value.

Chlorophyll *a*

The mean chlorophyll *a* content of *S. platensis* in various treatments after 18 days of cultivation is presented in Figure 4. The KM employed as a control had the greatest chlorophyll *a* content (21.60 mg L^{-1}). Similar results were observed for chlorophyll *a* of the *S. platensis* in DAE 40 (21.58 mg L^{-1}) and BAE20 (20.95 mg L^{-1}), which, statistically, did not differ from the control. However, higher levels of KM replacement with DAE (DAE50 18.51 mg L^{-1}) or BAE (BAE30 and BAE40, 17.25 and 15.26 mg L^{-1}, respectively) reduced levels of chlorophyll *a.*

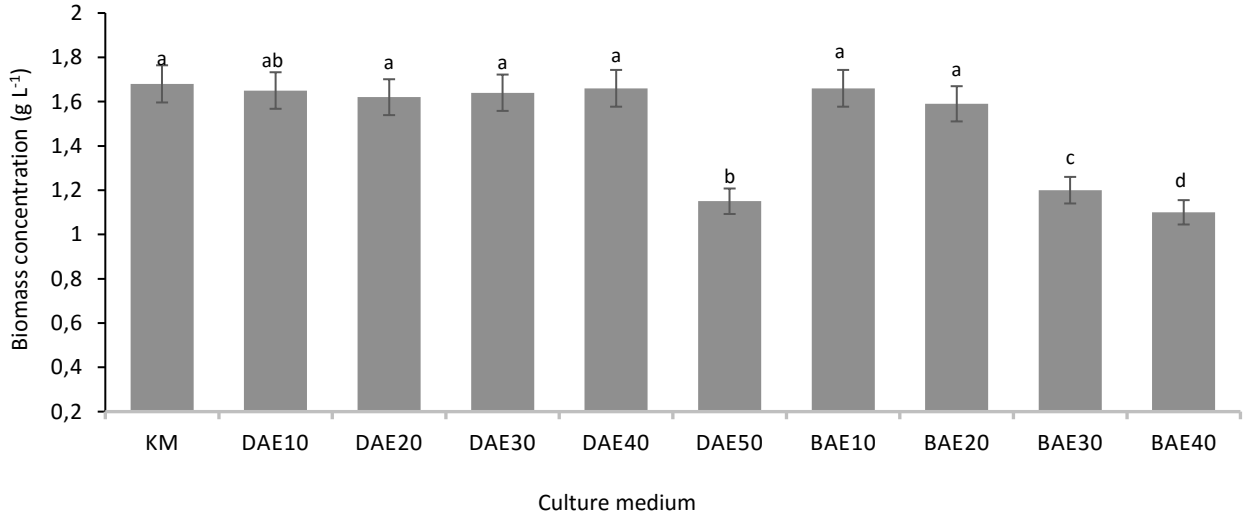

Figure 2. Biomass concentration (g L^{-1}) of *S. platensis* under different treatments after 18 days of culture. Means with separate letters differ significantly (P<0.05). KM = Kosaric medium, DAE= Dried azolla extract, BAE= Boiled azolla extract.

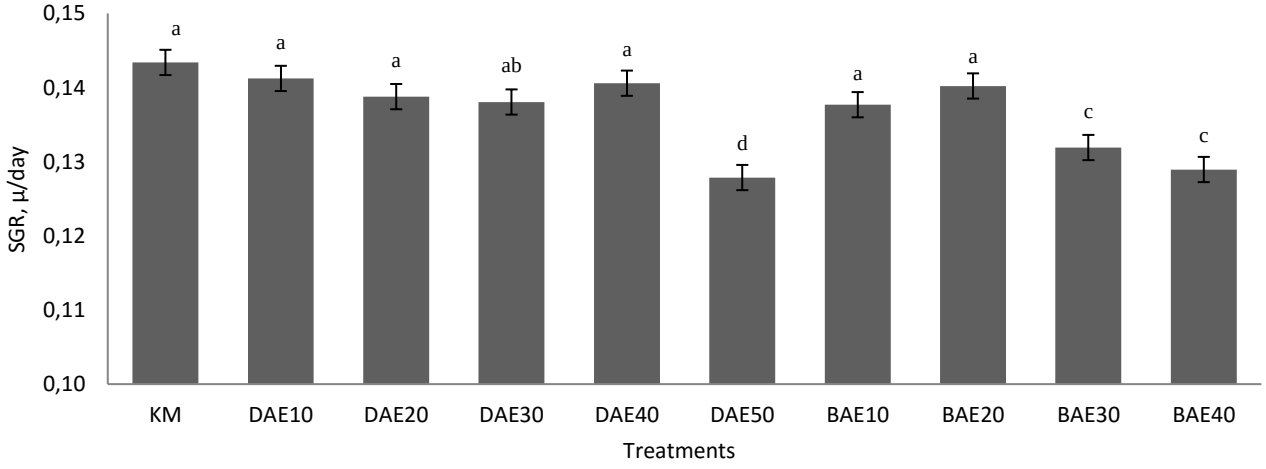

Figure 3. Specific growth rate (SGR, µday^{-1}) of *S. platensis* in different treatments after 18 days of culture. Means with separate letters differ significantly (P<0.05). KM = Kosaric medium, DAE= Dried azolla extract, BAE= Boiled azolla extract.

Lipid and Mineral Contents of *S. platensis*

The mineral and lipid content (dry matter basis) in *S. platensis* grown in different media are represented in Table 4. Except for calcium (Ca), zinc (Zn), and iron (Fe), the replacement of KM with DAE or BAE had little effect on the mineral content of *S. platensis*. The Zn concentration in *S. platensis* elevated with the increase of the replacement level of KM with DAE or BAE may be because the Zn contents of DAE and BAE were a few times higher than that in KM. Similarly, Ca and Fe contents elevated in *S. platensis* with the BAE and DAE replacement level, as these elements were also higher in BAE and DAE than KM. Although Mn and Mg were higher in BAE and DAE, and Na was lower, these differences were not reflected in the mineral contents of *S. platensis*. The lipid contents of *S. platensis* were not affected by different culture media.

Discussion

Biomass Concentration

It was depicted in Figure 1 because the experiment lasted just 18 days, and no degradation phase was seen. Nutritional circumstances considerably impacted the biomass concentration at the conclusion of cultivation. Similar nutritional profiles in DAE and BAE likely explain the supporting impact of azolla extract in biomass production. Phosphorus (P) and Nitrogen (N) are the most vital macronutrients for the growth of microalgae, where N is required for protein and amino acid biosynthesis. P, on the other hand, is mainly required for energy transmission, membrane synthesis (phospholipids), and nucleic acids (DNA and RNA) biosynthesis (Richmond, 2004). The availability of N is critical since it is a fundamental component in producing large amounts of microalgae biomass (Wu and Hsieh, 2009). The N from BAE and DAE was sufficient to support *S. platensis* growth (Table 3). By producing several structural and functional components necessary for microalgae's healthy growth and development, P, a macronutrient, plays a crucial role in cellular metabolic processes (Richmond, 2004). Azolla appeared to be a good source of P (Table 3), as evidenced by Setiawati et al. (2018), who found that both fresh and compost-powdered azolla supplied a considerable quantity of accessible P in soil. Iron (Fe) is also essential for microalgal growth because it is required for various metabolic pathways and chlorophyll production (Yun et

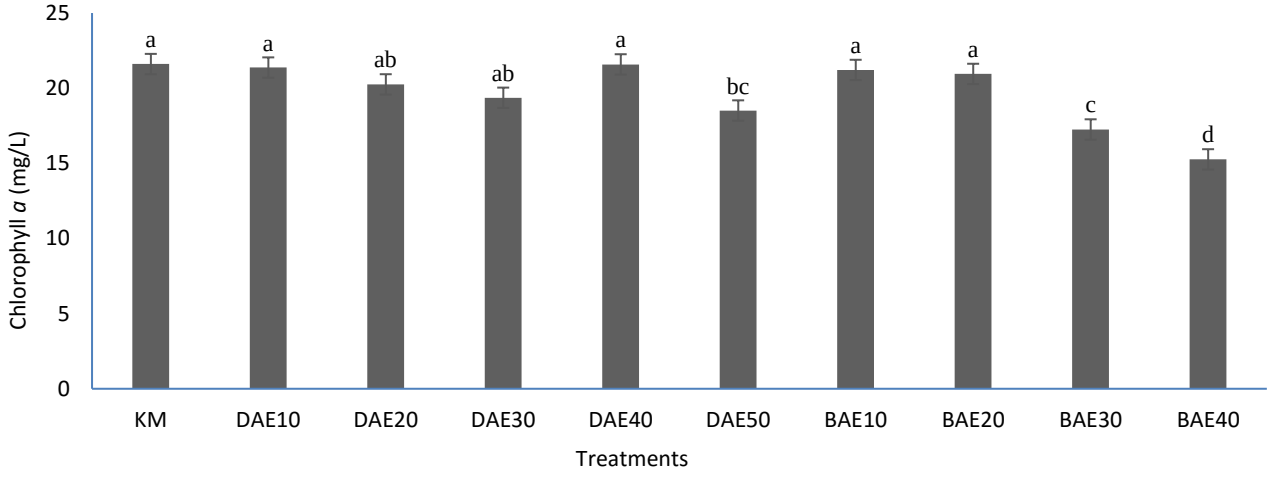

Figure 4. Chlorophyll *a* content (mg L^{-1}) of *S. platensis* in different treatments after 18 days of culture. Means with separate letters differ significantly ($P<0.05$). KM = Kosaric medium, DAE= Dried azolla extract, BAE= Boiled azolla extract.

Table 4. Minerals and lipid contents of *S. platensis* (dry matter basis) cultured for 18 days with different replacement levels of KM with DAE and BAE

Culture media	Minerals and lipid contents of S. platensis (dry matter basis)							
	K (mg/g)	Na (mg/g)	Ca (mg/g)	Mg (mg/g)	Fe (µg/g)	Mn (µg/g)	Zn (µg/g)	Lipid (%)
KM	13.50	11.56	1.05	1.46	153.2	95.3	112.9	8.39
DAE10	14.02	11.45	1.10	1.31	147.1	96.9	113.4	8.23
DAE20	13.02	12.30	1.01	1.43	140.4	105.4	108.0	8.49
DAE30	13.50	10.95	1.53	1.29	168.7	101.3	105.3	8.19
DAE40	13.67	11.56	1.73	1.38	245.6	105.8	199.1	8.41
DAE50	14.56	11.89	1.88	1.31	254.3	100.4	205.8	8.23
BAE10	14.70	12.01	1.38	1.28	123.7	100.6	100.2	8.76
BAE20	13.34	12.03	1.99	1.39	185.6	98.7	198.3	8.54
BAE30	13.23	11.45	1.97	1.43	245.8	96.3	297.9	8.45
BAE40	13.45	11.34	2.09	1.47	233.9	107.3	260.4	8.65

al., 2014). Compared to KM's iron level (1.47 mg L^{-1}), both DAE and BAE had higher iron contents (8.89 and 7.46 mg L^{-1}, respectively), which helped the growth of *S. platensis*.

Various researchers that worked with bovine wastes, vermicompost, digested rotten potato, digested rotten apple, swine wastes, and chicken wastes as nutrients in the medium for microalgae cultivation proved that organic sources of nutrients could support microalgae growth (Abdelhay et al., 2019; Habib et al., 2019; Mezzomo et al., 2010; Sopandi et al., 2020). Industrial wastes are also considered as possible alternatives. Molasses, a by-product from the sugar industry, was found to be an effective medium (Andrade & Costa, 2007). Unused waste cabbage leaf extracts were a nutritious and cheap alternative medium for spirulina culture, especially at the rural levels (Akhtar et al., 2014). Another study noted better *Spirulina* sp. biomass production using a goat manure medium (Sopandi et al., 2020). Cheunbarn and Peerapornpisal (2010) found that Spirulina sp. grew abundantly in a medium containing 10% anaerobically processed swine wastewater effluent. According to Khatun et al. (2019), banana leaf extract could be used efficiently as a potential nutrition source for *S. Platensis* culture. In addition, Jain and Singh (2013) examined the different concentrations of cow dung ash medium (CDAM) for the growth of *S. platensis* biomass production in lab conditions, where the ash from cow dung supplies the culture media with nutrients, lowering the expense of cultivation. The relatively better performance of DAE compared to BAE in this study may be due to the better extraction of nutrients (Table 3). According to Li et al. (2020), Zn plays an important role in microalgae cell growth and play a role as an enzyme cofactor for carbonic anhydrase, superoxide dismutase (SOD), and ribonucleic acid polymerase. The Zn content in DAE was 1090.2 Zn (µg L^{-1}), whereas, in BAE, it was 1029.2 (µg L^{-1}). However, azolla extract could not fully support growth in DAE50, BAE30, and BAE 40, where biomass concentration dropped, possibly due to a lack of micronutrients such as B, Co, and Mo in azolla extracts. Even though DAE and BAE have higher levels of most nutrients, the KM could not be replaced with more than 50% DAE or 20% BAE, which could be owing to the absence or scarcity of some nutrients in DAE and BAE (Table 3). Another reason could be that BAE and DAE inhibited photosynthesis because darker colors reduced light penetration at higher replacement levels. In the same concentration level, BAE was darker than DAE. Light intensity plays a vital role in microalgae cell production (Baidya et al., 2021). Again, according to Cheunbarn and Peerapornpisol (2010), *S. platensis* couldn't grow in a medium with a lot of organic matter since it would change the dark color and turbidity. Hadiyanto et al. (2012) also confirmed that the dark color generated by the presence of wastewater reduced light intensity in the medium, impairing photosynthesis. Overall, the present study suggests that up to 40% and 20% KM can be replaced with DAE and BAE, respectively, without compromising the biomass concentration of *S. platensis.* Overall, the current research reveals that DAE and BAE can replace up to 40% and 20% of KM, respectively, without reducing the biomass concentration of *S. platensis*.

Specific Growth Rate

Compared to biomass concentration, specific growth rate (SGR) is a more significant metric with practical value in evaluating the scale-up potential of microalgae biomass production. Microalgal growth can be aided by adding a variety of nutritional sources, resulting in a larger biomass yield.

The overall SGR values in this experiment were consistent with those found by Andrade and Costa (2007), who employed molasses as a substrate for *S. platensis* culture and had the maximum specific growth rate of 0.147 µ day^{-1} at 0.75 g L^{-1} molasses content. The SGR ranged from 0.41 to 0.49 µ day^{-1} when various quantities of jackfruit seed powder, added with urea, and banana leaf ash were used to cultivate *S. platensis* by Toyub et al. (2005). However, in the present study, the reduced SGR values seen when a large percentage of azolla extract was added to the media could be due to the impurities in the medium obstructing the SGR of the *S. platensis*.

Chlorophyll *a*

Figure 4 shows that photosynthetic activity following chlorophyll pigment synthesis was not impeded at replacement levels of 40% and 20% KM with DAE and BAE, respectively. It looked to be of similar magnitude to the one observed in control KM. On the other hand, a higher level of KM replacement with azolla extracts, as in DAE50, BAE30, and BAE40, negatively affected the chlorophyll *a* contents of *S. platensis*. The range of chlorophyll *a* in the present study coincided with Jain and Singh (2013), who reported a value of 23.0 mg L^{-1}, when employing cow dung ash as a substitute for synthetic media. Like the present study, organic substrates such as digested potato or molasses also supported the chlorophyll *a* concentration of *S. platensis* (Habib, 2008; Dineshkumar et al., 2015).

Finally, the data show that a 40% and 20% of synthetic Kosaric media can be replaced with organic nutrients source DAE and BAE, respectively, without affecting biomass concentration, SGR value, nutrient contents, and the amount of chlorophyll *a* in *S. platensis*.

Mineral and Lipid Contents of *S. platensis*

The mineral contents in *S. platensis* were rich in the present study, especially Fe, Ca, and Zn, in replacing KM with DAE or BAE (Table 4). The Fe concentration in *S. platensis* elevated nearly 1.6 times higher with DAE 40, DAE 50, or BAE 30, BAE 40 than KM, which may be

because the Fe contents of DAE and BAE were a few times higher than that in KM. Marrez et al. (2014) observed that high Fe content in *S. platensis* corresponded to high Fe content in the culture medium. Moreover, Ca and Zn contents in *S. platensis* were twice greater with the BAE20, BAE 30, and BAE40 and nearly twice with DAE 50 and DAE 40 replacements than in KM. This result may be because the Ca and Zn contents of BAE and DAE were higher than KM. These values were greater than that (155.8 and 8.4 mg/100 g DW, respectively) obtained by Marrez et al. (2014). Previously, it was proved by Madkour et al. (2012) that a positive correlation exists between the contents of macronutrients in spirulina and the medium. As a result, the source and culture conditions or media influence the mineral content of *S. platensis*. Although Mn was higher in BAE and DAE, and Na was lower, it was not reflected in the mineral contents of *S. platensis*. The lipid contents of *S. platensis* were not affected by different culture media. Ungsethaphand et al. (2009) concluded that the blue-green algae could not show any significant transformation in the lipid composition with the medium nutrient source alteration. Recently, Sopandi et al. (2020) observed no significant difference in the lipid content of *S. platensis* biomass in different media at the early stage of cultivation.

Conclusions

This study showed that azolla extract could significantly support *S. platensis* growth and accumulation of chlorophyll a in *S. platensis*. Using azolla extract to cultivate *S. platensis* culture would be feasible because azolla is rapidly growing and can be produced at a very low cost. In contrast, DAE would be a better option than BAE.

Ethical Statement

Animal or human subjects were not used in this research. Consequently, protocol approval was not required.

Funding Information

Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh

Author Contribution

Each author contributed equally to this work. MAH and MRB assisted with the preparation of the manuscript, guided technical instructions, and participated in the study design. The study was conducted, the data were evaluated, and the manuscript was written by MSBM. The research's data were gathered by MAHB, who also assisted with the statistical analysis. TA conceptualized, co-supervised, and gave the study a direction. All authors have reviewed the manuscript and approved its publication.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Acknowledgements

The Department of Aquaculture at the Bangladesh Agricultural University in Mymensingh, Bangladesh, and the Bangladesh Rice Research Institute in Gazipur, Bangladesh, who provided the authors with azolla seeds and stock cultures of *S. platensis*, respectively, are gratefully acknowledged.

References

Abdelhay, R., Moustafa, Y., & EL-Metwaly Essa, E. (2019). The use of vermicompost in cultivation and production of *Spirulina platensis*. *Egyptian Journal for Aquaculture*, *9*(3), 1-11.

Akhtar, N., Ahmed, M.M., Hossain, A., Jahan, M.A.A., & Akhter, H. (2014). Growth response of *Spirulina platensis* (nordst) geitler, in cabbage extract and anti-bacterial activities in different culture media. *Bangladesh Journal of Scientific and Industrial Research 49*(4), 205–210.

Andrade, M. R., & Costa, J. A. (2007). Mixotrophic cultivation of microalga *Spirulina platensis* using molasses as organic substrate. *Aquaculture*, *264*(1-4), 130-134.

Baidya, A., Akter, T., Islam, M. R., Shah, A. A., Hossain, M. A., Salam, M. A., & Paul, S. I. (2021). Effect of different wavelengths of LED light on the growth, chlorophyll, β-carotene content and proximate composition of *Chlorella ellipsoidea*. *Heliyon*, 7(12), e08525.

Belay, A. (2007). *Spirulina (Arthrospira)*: production and quality assurance. In: Gershwin ME, Belay A (eds) *Spirulina* in Human Nutrition and Health. CRC Press, London, Boca Raton, pp 1–26

Carvalho, J. C. M., Francisco, F. R., Almeida, K. A., Sato, S., & Converti, A. (2004). Cultivation of *Arthrospira (spirulina) platensis* (cyanophyceae) by fed-batch addition of ammonium chloride at exponentially increasing feeding rates. *Journal of Phycology*, *40*(3), 589-597.

Chen, Y. C. (2011). The effect of shifts in medium types on the growth and morphology of *Spirulina platensis* (*Arthrospira platensis*). *Journal of Marine Science and Technology*, *19*(5), 13.

Cheunbarn, S., & Peerapornpisal, Y. (2010). Cultivation of Spirulina platensis using anaerobically swine wastewater treatment effluent. *International Journal of Agriculture & Biology*, *12*(4), 586-590.

Chia, S. R., Chew, K. W., Show, P. L., Yap, Y. J., Ong, H. C., Ling, T. C., & Chang, J. S. (2018). Analysis of economic and environmental aspects of microalgae biorefinery for biofuels production: a review. *Biotechnology journal*, *13*(6), 1700618.

Chu, W. L., Lim, Y. W., Radhakrishnan, A. K., & Lim, P. E. (2010). Protective effect of aqueous extract from *Spirulina*

platensis against cell death induced by free radicals. *BMC complementary and alternative medicine*, *10*(1), 1-8.

Clesceri, L. S., Greenberg, A. E., & Eaton, A. D. (1989). Standard Methods for the Examination of Wastewater, 17th ed. American Public Health Association, Washington DC.

Dineshkumar, R., Umamageswari, P., Jayasingam, P., & Sampathkumar, P. (2015). Enhance the growth of *Spirulina platensis* using molasses as organic additives. *World Journal of Pharmaceutical Research*, *4*(6), 1057-1066.

El-Baky, H. H., El Baz, F. K., & El-Baroty, G. S. (2008). Characterization of nutraceutical compounds in blue green alga *Spirulina maxima*. *Journal of Medicinal Plants Research*, *2*(10), 292-300.

EPA US (1994) Method 200.8: Determination of Trace Elements in Waters and Wastes by Inductively Coupled Plasma-Mass Spectrometry. Revision 5.4. Cincinnati, OH

Gami, B., Naik, A., & Patel, B. (2011). Cultivation of *Spirulina* species in different liquid media. *Journal of Algal Biomass Utilization*, *2*(3), 15-26.

Gutiérrez-Salmeán, G., Fabila-Castillo, L., & Chamorro-Cevallos, G. (2015). Nutritional and toxicological aspects of *Spirulina (Arthrospira)*. *Nutricion hospitalaria*, *32*(1), 34-40.

Habib, M. A. B. (1998). Culture of selected microalgae in rubber and palm oil mill effluents and their use in the production of enriched Rotifer. Doctoral Dissertation, Universiti Putra Malaysia, Selangor DE, Malaysia.

Habib, M. A. B. (2019). Growth performance and estimation of single celled protein in spirulina (*Spirulina platensis*) cultured in supernatant of digested rotten apple. *Bangladesh Journal of Fisheries Research,* 18(1-2), 65-84.

Habib, B., Parvin, M., Huntington, T. C., & Hasan, M. R. (2008). A review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals. FAO Fisheries and Aquaculture Circular No 1034. Rome, FAO pp 33.

Habib, M. A. B., Munni, M. S., & Ferdous, Z. (2019). Culture and production of spirulina (*Spirulina platensis*) in supernatant of digested rotten potato. *Bangladesh Journal of Fisheries*, *31*(1), 55-64.

Hadiyanto, M. M. A. N., & Hartanto, G. D. (2012). Enhancement of biomass production from *Spirulina* sp. cultivated in POME medium. In Proceedings of the International Conference on Chemical and Material Engineering, Semarang Indonesia, pp 1-6.

Hossein, S. M., Khosravi-Darani, K., & Mozafari, M. R. (2013). Nutritional and medical applications of *Spirulina* microalga. *Mini-Rev. Med. Chem*, *13*, 1231-1237.

Hsieh, C. H., & Wu, W. T. (2009). Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. *Bioresource Technology*, *100*(17), 3921-3926.

Jain, S., & Singh, S. G. (2013). Low-cost medium formulation using cow dung ash for the cultivation of Cyanobacterium: *Spirulina (Arthrospira) platensis*. *Emirates Journal of Food and Agriculture*, 682-691.

Julshamn, K., Maage, A., Norli, H. S., Grobecker, K. H., Jorhem, L., Fecher, P., (2007). Determination of arsenic, cadmium, mercury, and lead by inductively coupled plasma/mass spectrometry in foods after pressure digestion: NMKL interlaboratory study. *Journal of AOAC International*, *90*(3), 844-856.

Khatun, S., Hossain, M. A., Akter, T., Banu, M. R., & Kawser, A. Q. M. R. (2019). Replacement of Sodium Bicarbonate and Micronutrients in Kosaric Medium with Banana Leaf Ash Extract for Culture of *Spirulina Platensis*. *Annals of Bangladesh Agriculture*, *23*(1), 37-47.

Kumar, M., Sharma, M. K., & Kumar, A. (2005). *Spirulina fusiformis*: a food supplement against mercury induced hepatic toxicity. *Journal of health science*, *51*(4), 424-430.

Kumari, A., Pathak, A. K., & Guria, C. (2015). Cost-effective cultivation of *Spirulina platensis* using NPK fertilizer. *Agricultural research*, *4*, 261-271.

Lau, K. Y., Pleissner, D., & Lin, C. S. K. (2014). Recycling of food waste as nutrients in *Chlorella vulgaris* cultivation. *Bioresource Technology*, *170*, 144-151.

Lee, S.J., Park, S., Noh, W., Yeom, D.H., Kim, S., Kim, D.W., & Kim, J. (2020). Regeneration of nitrate and phosphate from toilet wastewater using waste alumina adsorbent for cultivation of *Spirulina platensis. Environmental Engineering Research, 25 (3),* 393–399.

Leema, J.M., Kirubagaran, R., Vinithkumar, N.V., Dheenan, P.S., & Karthikayulu, S. (2010). High value pigment production from *Arthrospira* (Spirulina) *platensis* cultures in seawater. *Bioresource Technology, 101*, 9221–9227.

Li, X., Yang, C., Zeng, G., Wu, S., Lin, Y., Zhou, Q., & Zhong, Y. (2020). Nutrient removal from swine wastewater with growing microalgae at various zinc concentrations. *Algal Research*, *46*, 101804.

Liu, Y., Wang, Y., Cao, L., Huang, Z., Zhou, Y., Fan, R., & Li, C. (2023). Preparation and Characterization of Intracellular and Exopolysaccharides during Cycle Cultivation of *Spirulina platensis*. *Foods*, *12*(5), 1067.

Madkour, F. F., Kamil, A. E. W., & Nasr, H. S. (2012). Production and nutritive value of *Spirulina platensis* in reduced cost media. *The Egyptian Journal of Aquatic Research*, *38*(1), 51-57.

Markou, G., Kougia, E., Arapoglou, D., Chentir, I., Andreou, V., & Tzovenis, I. (2023). Production of *Arthrospira platensis*: Effects on Growth and Biochemical Composition of Long-Term Acclimatization at Different Salinities. *Bioengineering*, *10*(2), 233.

Marrez, D. A., Naguib, M. M., Sultan, Y. Y., Daw, Z. Y., & Higazy, A. M. (2014). Evaluation of chemical composition for *Spirulina platensis* in different culture media. *Research journal of pharmaceutical, biological and chemical sciences*, *5*(4), 1161-1171.

Mata, S.N., Santos, T.D.S., Cardoso, L.G., Andrade, B.B., Duarte, J.H., Costa, J.A.V., Souza, C. O. de, & Druzian, J.I. (2020). Spirulina sp. LEB 18 cultivation in a raceway type bioreactor using wastewater from desalination process: production of carbohydrate-rich biomass. *Bioresource Technology, 311*, 123495.

Mezzomo, N., Saggiorato, A. G., Siebert, R., Tatsch, P. O., Lago, M. C., Hemkemeier, M., & Colla, L. M. (2010). Cultivation of microalgae *Spirulina platensis (Arthrospira platensis)* from biological treatment of swine wastewater. *Food Science and Technology*, *30*, 173-178.

Michael, A., Kyewalyanga, M. S., Mtolera, M. S., & Lugomela, C. V. (2018). Antioxidants activity of the cyanobacterium, *Arthrospira (Spirulina) fusiformis* cultivated in a low-cost medium. *African Journal of Food Science*, *12*(8), 188-195.

Pelizer, L.H., de Carvalho, J.C.M., & de Oliveira Moraes, I. (2015). Protein production by *Arthrospira* (Spirulina) *platensis* in solid state cultivation using sugarcane bagasse as support. *Biotechnology Reports, 5*, 70–76.

Phang, S. M., Miah, M. S., Yeoh, B. G., & Hashim, M. A. (2000). Spirulina cultivation in digested sago starch factory wastewater. *Journal of Applied Phycology*, *12*, 395-400.

Pleissner, D., Lam, W. C., Sun, Z., & Lin, C. S. K. (2013). Food waste as nutrient source in heterotrophic microalgae cultivation. *Bioresource Technology*, *137*, 139-146.

Raoof, B., Kaushik, B. D., & Prasanna, R. (2006). Formulation of a low-cost medium for mass production of *Spirulina*. *Biomass and bioenergy*, *30*(6), 537-542.

Richmond, A. (2004). Biological principles of mass cultivation. Handbook of microalgal culture: Biotechnology and applied phycology, Blackwell Publishing Ltd, pp 125-177.

Setiawati, M. R., Damayani, M., Herdiyantoro, D., Suryatmana, P., Anggraini, D., & Khumairah, F. H. (2018). The application dosage of Azolla pinnata in fresh and powder form as organic fertilizer on soil chemical properties, growth and yield of rice plant. In *AIP conference proceedings* (Vol. 1927, No. 1, p. 030017). AIP Publishing LLC.

Sharoba, A. M. (2014). Nutritional value of *Spirulina* and its use in the preparation of some complementary baby food formulas. *Journal of Food and Dairy Sciences*, *5*(8), 517-538.

Sopandi, T., Rohmah, S., & Agustina, S. A. T. (2020). Biomass and nutrient composition of *Spirulina platensis* grown in goat manure media. *Asian Journal of Agriculture & Biology*, *8*(2), 158-167.

Tarko, T., Duda-Chodak, A., & Kobus, M. (2012). Influence of growth medium composition on synthesis of bioactive compounds and antioxidant properties of selected strains of *Arthrospira cyanobacteria*. *Czech Journal of Food Sciences*, *30*(3), 258-267.

Touloupakis, E., Cicchi, B., Benavides, A. M. S., & Torzillo, G. (2016). Effect of high pH on growth of *Synechocystis* sp. PCC 6803 cultures and their contamination by golden algae (*Poterioochromonas* sp.). *Applied microbiology and biotechnology*, *100*, 1333-1341.

Toyub, M. A., Rahman, M. M., Miah, M. I., & Habib, M. A. B. (2005). Growth performance of *Spirulina platensis* in three different concentrations of banana leaf ash with added jackfruit seed powder and urea. *Journal of the Bangladesh Agricultural University*, *3*, 303-308.

Ungsethaphand, T., Peerapornpisal, Y., & Whangchai, N. (2009). Production of *Spirulina platensis* using dry chicken manure supplemented with urea and sodium bicarbonate. *Maejo International Journal of Science and Technology*, *3*(3), 379-387.

Xia, A., & Murphy, J. D. (2016). Microalgal cultivation in treating liquid digestate from biogas systems. *Trends in Biotechnology*, *34*(4), 264-275.

Yun, K. O. N. G., Pei, Z. O. U., Li-ming, S. O. N. G., Zi, W. A. N. G., Jiao-qin, Q. I., Liang, Z. H. U., & Xiang-yang, X. U. (2014). Effects of iron on the algae growth and microcystin synthesis: A review. *China Journal of Applied Ecology*, *25*(5), 1533-1540.