Evaluation of Fermented Azolla (*Azolla pinnata*) Extract as an Alternative Nutrient Medium for Spirulina (*Limnospira platensis*) Culture

Mousumi Das[1*], Monira Sultana[1], Md. Amzad Hossain[1], Fazla Rabby Udoy[1], Taslima Akter[1] and SM Rafiquzzaman[2]

ABSTRACT

Limnospira platensis formerly known as *Spirulina platensis* or *Arthrospira platensis* and hereafter referred to as 'spirulina' in this study, is a filamentous cyanobacterium with high nutritional value. It is widely used as a dietary supplement for humans and as a feed supplement for animals. Kosaric medium (KM) is the most widely used commercial medium for *Spirulina* sp. production; however, it incurs high production costs. This study aimed to evaluate fermented *Azolla pinnata* extract medium (FAEM) as a cost-effective alternative medium for spirulina culture. Growth, pigmentation, and protein content of spirulina were evaluated under a partial substitution design in which KM was replaced by FAEM at 25, 50, 75, and 100%, with 100% KM used as the control. Maximum dry cell weight (143.5±11.97 mg·L^{-1}), optical density (1.54±0.08), specific growth rate (0.49±0.03 day^{-1}), chlorophyll *a* (6.54±0.04 μg·mL^{-1}), and β-carotene (2.93±0.04 μg·mL^{-1}) of spirulina were observed in KM, while comparable results were obtained in FAEM25. However, all growth parameters gradually decreased as the FAEM concentration increased. The protein content of spirulina was significantly ($p<0.05$) higher in FAEM25 (65.6%) than in the other FAEM treatments and KM (59.4%). Total nitrogen and organic carbon contents were higher in FAEM (8640 ppm and 0.023%, respectively) compared to KM (5760 ppm and 0.015%, respectively). The medium cost of FAEM25 (0.18 USD·L^{-1}) was lower compared to KM (0.25 USD·L^{-1}). Therefore, FAEM25 can serve as a cost-effective alternative medium for spirulina cultivation, improving protein content while maintaining growth or pigmentation comparable to those obtained in KM.

Keywords: Cost-effective, Fermented *Azolla pinnata*, Growth, *Limnospira platensis*, Protein content

INTRODUCTION

In the 21st century, microalgae are among the most innovative and promising natural sources of nutritious food and functional products. A liquid, oxygenated environment that provides the appropriate quantity of light, CO_2, and nutrients is ideal for the growth of microalgae (Rosenberg *et al*., 2008). Compared to terrestrial plants, microalgae exhibit higher photon conversion efficiency, CO_2 sequestration, growth rate, and biomass production (Malcata, 2011; Rizwan *et al.*, 2018). Important biochemical components, including essential unsaturated fatty acids, amino acids, carbohydrates, pigments, and bioactive compounds, are present in microalgal biomass (Matos *et al*., 2017; Apandi *et al*., 2019).

Limnospira platensis (Gomont, 1892) Roussel *et al*., 2023, formerly known as *Spirulina platensis* or *Arthrospira platensis* and hereafter referred to as 'spirulina' in this study, is a non-toxic

[1]Department of Aquaculture, Faculty of Fisheries, Gazipur Agricultural University, Gazipur, Bangladesh
[2]Department of Fisheries Biology and Aquatic Environment, Faculty of Fisheries, Gazipur Agricultural University, Gazipur, Bangladesh
[*]Corresponding author. E-mail address: mousumi.aqc@gau.edu.bd
Received 29 June 2025 / Accepted 2 April 2026

filamentous aquatic cyanobacterium that has recently been proposed as a sustainable, highly nutrient-rich, and environmentally beneficial microalga (Spinola *et al*., 2024). According to Usharani *et al*. (2012), spirulina displays a combination of traits found across the plant, animal, and bacterial kingdoms, including photosynthetic capability characteristic of plants, prokaryotic cellular organization comparable to bacteria, and glycogen accumulation, as seen in animal cells. Its dry-weight chemical composition is composed of 60–70% proteins, carbohydrates, minerals, and vitamins. Some functional pigments, such as carotenes, phycocyanin, and chlorophyll, as well as essential fatty acids like γ-linolenic acid (GLA) are present in *Spirulina* sp. (Soni e*t al*., 2017). *Spirulina* sp. was granted Generally Recognized as Safe (GRAS) status by the U.S. Food and Drug Administration (FDA) in 1981 for use as a food ingredient (Amin *et al*., 2024). Because of its bioactive qualities, *Spirulina* sp. can improve the quality, stress tolerance, and general health of cultured fish, making it a useful fish feed supplement as well (Kim *et al*., 2013; Ragaza *et al*., 2020).

The most commonly used medium for *Spirulina* sp. cultivation is Kosaric medium (KM). The composition of the medium, however, can affect the nutritional value of *Spirulina* sp. According to Ghofar *et al*. (2019), nutrient costs are considered a major concern, contributing to the high cost of *Spirulina* sp. biomass production. Consequently, a more efficient, affordable, and accessible medium for large-scale production of spirulina must be found, especially in developing nations.

Azolla pinnata, an aquatic fern belonging to the family Salviniaceae, hosts the obligate nitrogen-fixing symbiotic cyanobacterium *Anabaena azollae* (Kodit *et al*., 2023). Compared with conventional feed ingredients such as maize and wheat bran, *A. pinnata* provides a superior supply of essential amino acids and serves as a good protein source, with protein content ranging from 25–30% (Basak *et al*., 2002; Cherryl *et al*., 2014). *Azolla plants* are reported to contain minerals: calcium, magnesium, potassium, phosphorus, iron, and manganese; vitamin A, precursor beta-carotene, and vitamin B12, probiotics, and biopolymers (Pillai *et al*., 2002; Anitha *et al*., 2016). It can be applied as a fertilizer in its fresh form as well as in dried and compost form (Gouri *et al*., 2012).

Fermentation procedures enhance feedstuff palatability while also improving vitamin availability, protein, and fiber quality (Sun *et al*., 2015; Zhang *et al*., 2017). Using beneficial microorganisms such as *Bacillus subtilis*, bacterial fermentation has been shown to have immunomodulatory and anticancer properties. Remarkably, it promotes the viability and proliferation of intestinal lactic acid bacteria (Liu *et al*., 2012). Therefore, the nutritional and functional qualities of *A. pinnata* are enhanced by the fermentation process. Thus, fermented *A. pinnata* extract medium (FAEM) could be a potential organic nutritional source for the growth of spirulina. Therefore, the main objective of the experiment was to evaluate the potential of FAEM as a nutrient source for the growth performance and composition of spirulina and to ascertain the optimal concentration of inexpensive FAEM for its mass production.

MATERIALS AND METHODS

Preparation of fermented Azolla pinnata extract media

To prepare the FAEM, *A. pinnata* was collected from the Bangladesh Agricultural Research Institute (BARI), Gazipur. The collected plant material was washed thoroughly with tap water to remove external debris and then sun-dried for 3 days. The dried *Azolla* was ground and sieved to obtain a fine powder for fermentation.

A *B. subtilis* culture was obtained from a pure stock maintained at the Department of Fisheries Biology and Aquatic Environment, Gazipur Agricultural University. The bacterium was first cultured in nutrient broth for 14 h. Bacterial growth was then monitored spectrophotometrically at 650 nm, and the culture was used for inoculation when the optical density reached 0.87.

The processed *Azolla* powder was sterilized by autoclaving at 121 ℃ for 15 min. For liquid-state fermentation, the sterilized *Azolla* was mixed with water at a ratio of 1:9 (w/v). Then, 5 mL of the prepared *B. subtilis* culture was inoculated into the mixture and mixed thoroughly. The inoculated mixture was incubated at 37 ℃ for 72 h for fermentation. During fermentation, changes associated with bacterial activity were observed, including cell breakdown, peptide bond degradation, increased protein level, decreased fiber content, and increased bacterial colony formation.

To prepare the fermented *A. pinnata* extract medium, 100 g of the fermented material was mixed with 1 L of distilled water and kept at room temperature for 24 h. The mixture was then filtered under vacuum through Whatman filter paper (0.45 µm pore size) to obtain a clear filtrate.

After filtration, the fermented *A. pinnata* extract medium was autoclaved at 121 ℃ for 20 min. Then, 24 h later, it was removed from the autoclave and stored in the refrigerator. After seven days, it was taken out of the refrigerator and allowed to reach the room temperature for 24 h for the spirulina culture.

Analysis of nitrogen, phosphorus and organic carbon content of culture media

The total nitrogen, total phosphorus, and organic carbon content of the culture media were estimated according to AOAC (2007) using the following formulae. Total nitrogen was estimated by the Kjeldahl method. The sample was digested with concentrated sulfuric acid and a catalyst mixture, then distilled and titrated with standard hydrochloric acid. The percentage of nitrogen was calculated using the following formula:

$$\text{Nitrogen (\%)} = \frac{\text{Miliequivalent of nitrogen (0.014)}\times \text{titrant value (mL)}\times\text{Strength of HCl}}{\text{Weight of the sample}} \times 100$$

Phosphorus was determined by colorimetric analysis after digesting the sample using a mixed acid solution. The absorbance of the sample and blank was measured at the appropriate wavelength using a spectrophotometer. The phosphorus concentration was calculated using the formula:

$$\text{Phosphorus (\%)} = (S\text{-}B) \times df \times (1\times10^{-4}) \times f$$

S = Sample absorbance
B = Blank absorbance

$$df = \frac{\text{Digested sample filtration volume in 100 mL}\times\text{50 mL volume}}{\text{0.5 mL plant extract sample}\times\text{10mL aliquot}}\times 100$$

$$f = \frac{\text{Mean std}}{\text{Mean abs}}$$

Organic carbon content was estimated following the Walkley and Black wet oxidation method. The sample was oxidized using potassium dichromate and sulfuric acid, and the unreacted dichromate was titrated with ferrous ammonium sulfate. The organic carbon content was calculated using the equation:

$$\text{Organic carbon (\%)} = \frac{10\times(\text{Blank-Reading})\times 0.003}{\text{Weight of the sample}\times\text{Blank}}\times 100$$

Collection of spirulina

The pure stock culture of spirulina was procured from the Live Food Culture Laboratory of the Department of Aquaculture, Faculty of Fisheries, Gazipur Agricultural University, Gazipur-1706, Bangladesh.

Preparation of Kosaric medium

Table 1 shows the composition of KM. For the preparation of KM, the materials listed in Table 1, nos. 1–8 were weighed using an electronic balance and combined in a 1.0 L conical flask and mixed. Then, 0.5 mL of micronutrient solution was added to that solution. The medium was well mixed, autoclaved at 121 ℃ for 15 min, and chilled for 24 h.

Table 1. Composition of KM for spirulina culture.

Serial No.	Chemicals/compounds	Concentration in stock solution ($g \cdot L^{-1}$)
1	$NaHCO_3$	9.00
2	K_2HPO_4	0.25
3	$NaNO_3$	1.25
4	K_2SO_4	0.50
5	NaCl	0.50
6	$MgSO_4.7H_2O$	0.10
7	$CaCl_2$	0.02
8	$FeSO_4.2H_2O$	0.005
9	Micronutrient solution	0.5 $mL \cdot L^{-1}$

Note: Composition of micronutrient solution ($g \cdot L^{-1}$) showing: i) H_3BO_3: 2.86; ii) $MnCl_2.4H_2O$: 1.81; iii) $ZnSO_4.7H_2O$: 0.22; iv) $CuSO_4.5H_2O$: 0.08; v) MoO_3: 0.01; vi) $CoCl_2.6H_2O$: 0.01

Experimental design of spirulina culture

The growth performance of spirulina was evaluated under a partial substitution design using fermented *Azolla pinnata* extract medium (FAEM) as a replacement for Kosaric medium (KM). Five media treatments were prepared: 100% KM as the control, and four FAEM substitution levels in which KM was replaced by FAEM at 25, 50, 75, and 100%. Accordingly, the media compositions were as follows: FAEM25 (150 mL FAEM + 450 mL KM), FAEM50 (300 mL FAEM + 300 mL KM), FAEM75 (450 mL FAEM + 150 mL KM), and FAEM100 (600 mL FAEM only). The experiment was conducted in a completely randomized design (CRD) with five treatments, each with three replicates, for 22 days.

Culture of spirulina

The 22-day experiment was conducted under a completely randomized design (CRD) comprising five treatments, each replicated three times. Spirulina was cultured in 1 L Erlenmeyer flasks, with the initial inoculation containing a 10% suspension (OD at 620 nm = 0.20) (Habib, 1998). Before initiating spirulina culture, the pH of all media was adjusted to above 9.5 by adding 0.1 N NaOH. The flasks were kept under LED lights in the growth chamber. The light: dark cycle was maintained at 16:8 h during the culture period. Samples were collected from each flask at 4-day intervals to measure optical density (OD), chlorophyll *a*, β-carotene content, and physicochemical properties. Sufficient aeration was provided to each flask by an aerator (Sobo pump, Aquarium pump SB-348A) to maintain optimum dissolved oxygen concentration for the culture of spirulina.

Determination of the physicochemical properties of the culture media

The physicochemical parameters for each treatment were documented, using the method described by Eaton *et al*. (2005). On the sampling day, water temperature (ºC), pH, dissolved oxygen (DO) ($mg \cdot L^{-1}$), and light intensity ($Lux \cdot m^{-2} \cdot s^{-1}$) of each culture treatment were recorded using a thermometer (HQ30d flexi, Hach, USA), electric pH meter (sens IONTM + PH3, Hach, USA), dissolved oxygen meter (HQ40d multi, Hach, USA), and a lux-meter (LX-9621, China), respectively.

Measurement of spirulina cell biomass

The sample from each treatment, containing 15 mL of spirulina suspension, was filtered and weighed. Whatman GF/C filter paper was used, with a 47 mm diameter and a 0.45 µm pore size. The sample was rinsed with 10 mL of acidified water (pH 4) after filtration to remove insoluble salts. Subsequently, the filter papers were placed in a glass petri dish and left overnight at 70 ºC in

the oven. The filter papers were weighed after the petri dish was cooled for 20 min in a desiccator. Cell dry weight was determined using the methodology of Eaton *et al*. (2005).

$$W = \frac{\text{FFW-IFW}}{\text{Amount of sample taken for filtration (mL)}} \times 100$$

Where, W = Cell dry weight in $mg \cdot L^{-1}$;
FFW = Final filter weight in g; and
IFW = Initial filter weight in g

Specific growth rate (SGR)

The specific growth rate (SGR, day^{-1}) of cultured microalgae was calculated using the following equation (Eaton *et al*., 2005):

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

where X_2 is the biomass concentration at the end of the selected time interval, X_1 is the biomass concentration at the beginning of the selected time interval, and t_2-t_1 is the elapsed times in days.

Measurement of optical density

The optical density (OD) of samples collected at different sampling dates was measured at 620 nm using a UV-Vis spectrophotometer (DR 3900). Samples of spirulina from the different treatments were transferred to test tubes and analyzed using a spectrophotometer. The OD of the samples was then recorded at 620 nm.

Estimation of chlorophyll a, and β-carotene

A spirulina sample (10 mL) from each treatment was centrifuged for 10 min at 4,000 rpm. Following centrifugation, the sediment was dissolved in 5 mL of 80% acetone and centrifuged again for 10 min at 4,000 rpm. The supernatant was collected and used to measure chlorophyll *a* and β-carotene. The solutions were analyzed using a spectrophotometer to quantify absorbance at 412, 431, 460, and 480 nm. Distilled water (100%) was used as a blank.

Chlorophyll *a* and β-carotene were calculated according to Eijckelhoff and Dekker (1997) using the following equation.

$$\text{Chlorophyll } a\ (\mu g \cdot mL^{-1}) = -1.709\ A_{412} + 11.970\ A_{431} - 2.998\ A_{460} - 5.708\ A_{480}$$

$$\beta\text{-carotene}\ (\mu g \cdot mL^{-1}) = -0.430\ A_{412} + 0.251\ A_{431} - 4.376\ A_{460} + 13.216\ A_{480}$$

Estimation of protein content of spirulina

The crude protein content of spirulina cultured in FAEM25, FAEM50, FAEM75, FAEM 100, and the control (100% Kosaric medium) was ascertained by measuring total nitrogen content by the standard Micro-Kjeldahl method. Approximately 30 mg of the sample, 1.0 g of catalyst mixture, and 5 mL of concentrated H_2SO_4 were placed in a Kjeldahl tube and digested in a digestion unit (Digestor, Model-DK20) for 90 min to yield a clear solution. The digest was subsequently distilled and titrated concurrently with a fully automated distillation and titration unit (Model UDK152). The distillation and titration unit automatically calculated the protein percentage in the sample using the following formula: Protein (%) = Nitrogen (%) × 6.25 (for plant protein).

Statistical analysis

The data on cell dry weight, optical density, chlorophyll *a*, and β-carotene were analyzed using one-way ANOVA followed by the least significant difference (LSD) at the 5% significant level to identify significant differences between the means. Statistix10 software was used for data analysis.

RESULTS AND DISCUSSION

Nutritional composition of media

The total nitrogen, phosphorus, and organic carbon content in Kosaric medium were 5,760 ppm, 21.21 ppm, and 0.0151%, respectively, while 8,640 ppm, 13.03 ppm, and 0.023% were found in FAEM (Table 2). FAEM contained higher

Table 2. Total nitrogen, phosphorus and organic carbon content of Kosaric and fermented *Azolla pinnata* extract media.

Culture media	Nitrogen (ppm)	Phosphorus (ppm)	Organic carbon (%)
Kosaric medium	5,760	21.21	0.015
Fermented *Azolla pinnata* extract medium	8,640	13.03	0.023

levels of nitrogen and organic carbon, while its phosphorus content was lower than that in the Kosaric medium. The higher nitrogen and organic carbon content in *Azolla* extracts could be attributed to the fermentation process.

Nutrient concentration is a major factor that controls the growth and composition of microalgae (Akbarnezhad *et al*., 2020). The adequate availability of organic carbon, nitrogen, and phosphorus in the FAEM likely contributed to the enhanced growth and biomass production of spirulina. The nitrogen and organic carbon content in FAEM are beneficial for the growth of microalgae, especially spirulina (Khan *et al*., 2018). Nitrogen is a key macronutrient essential for microalgal growth, reproduction, and the synthesis of proteins, lipids, and carbohydrates, and its concentration strongly influences growth and biochemical composition, while nitrogen depletion in the cultivation medium reduces growth (Nyabuto *et al*., 2015; Zarrinmehr *et al*., 2020).

Gorgij *et al*. (2018) reported that variations in nitrogen sources significantly influenced the growth and protein content of spirulina, with higher nitrogen availability generally enhancing biomass production and protein accumulation. All treatments showed an increase in chlorophyll content with increasing nitrogen concentration. Ruliaty *et al*. (2022) reported that nitrogen is vital for *Spirulina* sp. growth. Choosing the right nitrogen concentration and source is important, as it impacts on the metabolic activity and nutritional composition of spirulina (Madkour *et al*., 2012).

Borges *et al*. (2013) discovered that carbon is the most important ingredient required for *Spirulina* sp. development since the cells contain around 50% (ww^{-1}) of this element. Autotrophic growth (ideal for large-scale open agriculture) can be delivered as CO_2, carbonate, or bicarbonate. Bashir *et al*. (2019) reported that in a mixotrophic mode, cells harness energy from both light and organic carbon within the medium. (Perez-Garcia and Bashan, 2015). In mixotrophic systems, where microalgae use both photosynthetically fixed inorganic carbon and external organic carbon, *Spirulina* sp. often exhibits enhanced biomass production, metabolic activity, and overall carbon-processing efficiency compared to purely autotrophic growth. For example, supplementing culture media with simple organic carbon sources such as acetate significantly increased biomass, upregulated carbon metabolism pathways (e.g., Calvin cycle, glycolysis, TCA cycle), and supplied additional energy and carbon skeletons for rapid growth in *Spirulina* sp. cultures under mixotrophic conditions (Li *et al*., 2023). Similar benefits have been observed with more complex organic sources, such as molasses or brewery wastewater, demonstrating spirulina's capacity to assimilate diverse external organic molecules and shift its biochemical profile under mixotrophic conditions (Andrade and Costa, 2007). In the present study, FAEM contained measurable levels of organic carbon, which may have contributed to cellular metabolism and growth performance (Pereira *et al*., 2019). However, it is important to note that mixotrophic growth was not directly evaluated, as specific measurements of organic carbon uptake were not conducted. Therefore, the observed responses may indicate a mixotrophic-like effect rather than confirmed mixotrophic metabolism. This interpretation is speculative and would benefit from further experimental validation.

Table 3. Average physico-chemical parameters in different culture treatments during the investigational period. Data are presented as mean±standard deviation (SD), n = 3.

Treatments	Parameters	Values (on different sampling day)				
		6th	10th	14th	18th	22th
KM	Temperature (°C)	31.43±0.06	32.37±0.28	32.23±0.31	30.57±0.26	30.67±0.12
	DO (mg·L^{-1})	7.17±0.04	7.04±0.02	7.20±0.04	7.68±0.06	7.33±0.09
	pH	10.16±0.01	10.21±0.06	10.24±0.01	10.25±0.01	10.24±0.03
FAEM25	Temperature (°C)	31.43±0.09	32.73±0.07	32.07±0.54	30.73±0.07	30.67±0.13
	DO (mg·L^{-1})	7.51±0.06	7.04±0.02	7.21±0.09	7.65±0.02	7.45±0.04
	pH	10.40±0.03	10.17±0.06	10.18±0.06	10.15±0.06	10.00±0.03
FAEM50	Temperature (°C)	31.67±0.30	32.27±0.13	32.07±0.06	30.50±0.16	30.53±0.06
	DO (mg·L^{-1})	7.46±0.03	7.01±0.09	7.22±0.06	7.67±0.09	7.63±0.04
	pH	10.04±0.01	10.08±0.01	10.11±0.02	10.13±0.06	10.13±0.01
FAEM75	Temperature (°C)	31.40±0.50	32.67±0.13	32.10±0.09	30.30±0.50	30.53±0.16
	DO (mg·L^{-1})	7.16±0.03	7.02±0.02	7.07±0.02	7.30±0.11	7.49±0.07
	pH	9.99±0.09	9.95±0.04	9.98±0.02	10.05±0.04	10.02±0.01
FAEM100	Temperature (°C)	31.47±0.37	32.43±0.07	32.33±0.16	30.57±0.34	31.00±0.10
	DO (mg·L^{-1})	7.16±0.06	7.07±0.03	7.00±0.04	7.33±0.02	7.36±0.04
	pH	9.68±0.04	9.75±0.04	9.76±0.04	9.82±0.06	9.78±0.01

Note: Means within a row did not differ significantly ($p \geq 0.05$).

Physico-chemical parameters

The average values (mean±SD) of the physico-chemical parameters in different treatments of spirulina culture during the experimental period are presented in Table 3. No significant difference ($p>0.05$) was observed in the average physico-chemical parameters among the treatments at each sampling time.

In the present study, the observed ranges of temperature, pH, dissolved oxygen (DO), and light intensity were 26.33±0.16 to 30.3±0.22 °C, 9.67±0.01 to 10.25±0.01, 7.00±0.04 to 7.68±0.06 mg·L^{-1}, and 2180 to 2970 lux·m^{-2}·s^{-1}, respectively, across all treatments. Khatun *et al*. (2019) reported an optimal temperature range of 28.22±0.12 to 31.53±0.12 °C, which is closely aligned with the current findings, though the present study recorded a slightly lower minimum temperature. Rafiqul *et al*. (2005) suggested that pH 9.0 was ideal for spirulina biomass production, whereas the pH values in this study (9.67–10.25) were marginally higher, indicating that the strain used here could tolerate and perform well in a more alkaline environment.

Additionally, the DO range (7.00–7.68 mg·L^{-1}) observed in this study was higher than the optimal range (3.1–5.5 mg·L^{-1}) reported by Jabber (2005). This variation may be due to differences in culture systems, aeration rates, or photosynthetic activity under experimental conditions. Similarly, the light intensity in this study (2,180–2,970 lux·m^{-2}·s^{-1}) slightly exceeded the optimal range (2,000–2,500 lux·m^{-2}·s^{-1}) suggested by Akter *et al*. (2019), which could be attributed to variations in light source, culture density, or nutrient composition of the medium.

Overall, the variations in physico-chemical parameters in the current study, compared with previous studies, suggest that spirulina exhibited a broader tolerance to environmental parameters in the experimental media. However, because no significant differences in the parameters were observed among the treatments, the variations in media composition may not have substantially influenced the overall culture environment of spirulina.

Effect of FAEM media on cell dry weight, optical density and specific growth rate

Figure 1 depicts the average cell dry weight of spirulina under various treatments during the trial period. After 6 days of incubation, there was a significant ($p<0.05$) increase in cell dry weight among the treatments. In the exponential phase, the maximal cell dry weight in the KM medium (control) reached 143.5±11.97 mg·L^{-1}, whereas FAEM 25 attained 139±11.79 mg·L^{-1}, and there was no significant difference ($p>0.05$). The mean cell dry weight of spirulina in FAEM50, FAEM75, and FAEM100 was 131.5±11.47, 86.5±9.30, and 59±7.68 mg·L^{-1}, respectively, and decreased gradually with increasing FAEM composition. A significant difference ($p<0.05$) was observed among these treatments compared with the control and FAEM25.

In the present study, KM and FAEM25 concentrations were optimum for the growth performance of spirulina. Mofiz *et al*. (2023) reported that Kosaric medium can be partially substituted with azolla extract up to 40% dried azolla extract or 20% boiled azolla extract without significant effects on the growth, biomass yield, mineral composition, lipid content, or chlorophyll *a* level of spirulina, highlighting azolla extract, particularly the dried form, as a cost-effective organic culture medium. Similarly, Korsa *et al*. (2024) found that application of *Azolla microphylla* based fertilizer at an optimal concentration of 5 mL·L^{-1} significantly enhanced the population density (321,500 cells·mL^{-1}) and chlorophyll content of spirulina. According to El-Sheekh *et al*. (2012), nutrient availability, particularly nitrogen and carbon, affects the proliferation and biochemical composition of microalgal cells. The potentiality of the *Azolla* plant as nutrient source is further explained by Richmond (2003) and Nyabuto *et al*. (2015) who found that fermented *A. pinnata* extract has an adequate amount of carbon, nitrogen, and phosphorus that participate in numerous metabolic processes, including energy transmission, membrane synthesis (phospholipids), nucleic acids biosynthesis, signal transduction, energy conversion, and photosynthesis of microalgae. The present study showed that higher

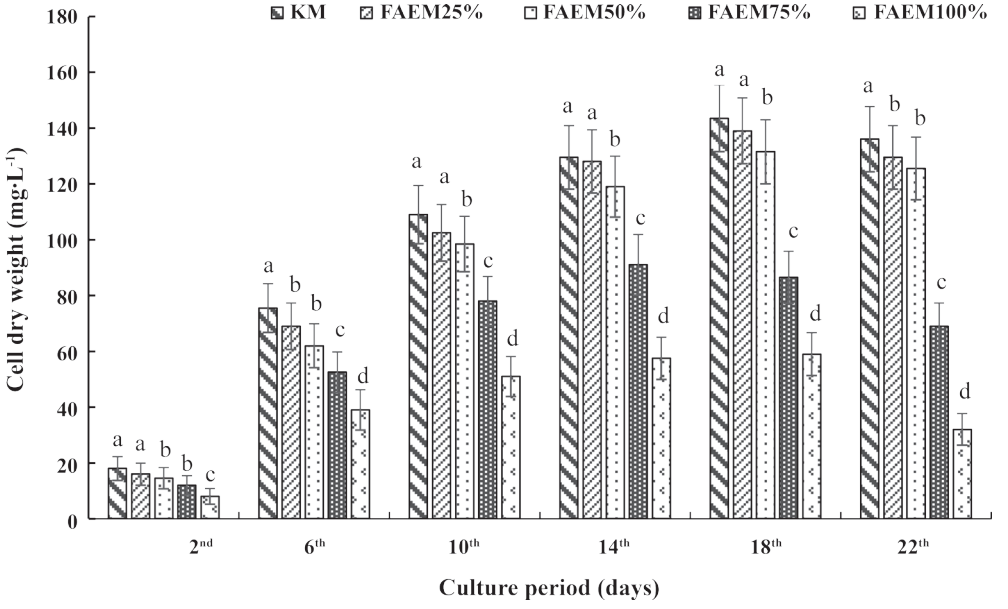


Figure 1. Mean cell dry weight of spirulina cultured in Kosaric medium (KM) and different substitution levels of fermented *Azolla pinnata* extract medium (FAEM) over the culture period. Error bars represent ±SD (n = 3) under different treatments over the culture period. Different lowercase letters above the bars indicate significant difference ($p<0.05$) among treatments at the same sampling time.

concentration of FAEM (FAEM50, FAEM75, and FAEM100) gradually decreased the cell weight of spirulina. This result may be explained by the dark color of FAEM at higher concentrations, which reduced light penetration and led to a sharp decline in microalgae growth (Yeesang and Cheirsilp, 2014). Furthermore, high organic matter in highly concentrated FAEM may have affected the medium's turbidity and oxygen balance, resulting in poor growth conditions (Kebede and Ahlgren, 1996). Imbalances in nutrient availability at high FAEM levels, such as excess organic carbon and inadequate bioavailable nitrogen, may disturb the C:N ratio required for optimum protein and biomass synthesis (Markou and Nerantzis, 2013). Such metabolic stress not only inhibits photosynthesis but may also divert energy to maintenance pathways rather than biomass production, explaining the reduced cell growth reported at high FAEM concentrations. In addition, lower nutrient levels in high concentrations of FAEM could be responsible for reduced cell growth in *Spirulina* sp.

Optical density (OD_{620}) of spirulina increased until the exponential phase (18th day). The maximum optical density (OD_{620}) across treatments was observed on the 18th day, as shown in Figure 2. The highest OD_{620} (1.54) was recorded in KM, followed by 1.46 in FAEM25, and the lowest (0.64) was found in FAEM100.

According to Michael *et al*. (2019), *Spirulina* sp. showed the best growth under low-cost conditions in a medium supplemented with commercial NPK10-20-20 fertilizer, with higher chlorophyll content (0.99±0.02%), dry weight (0.75±0.01 g·100 mL^{-1}), and optical density (1.74–2.06). In this study, several factors may have contributed to the higher optical density in KM (control), as it contained an optimal nutrient level. FAEM25 showed an optical density that was significantly similar to that of control treatment. Optical density has a positive correlation with mean cell weight. Since KM and FAEM25 showed a higher mean cell weight of spirulina, their optical density also increased. These results indicate that the lowest optical density at higher FAEM levels may be due to the low nutrient concentration in the media. Another explanation is that elevated FAEM concentrations can suppress photosynthesis

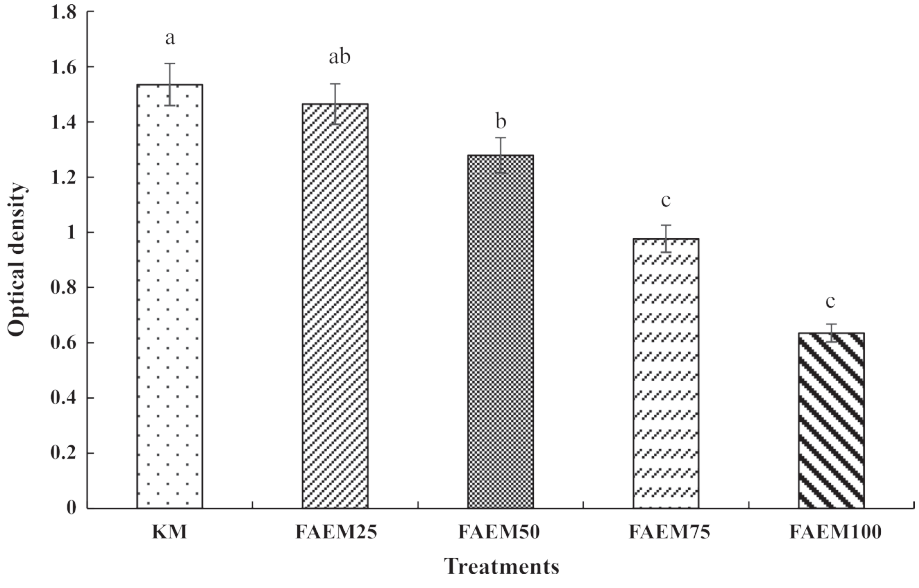


Figure 2. Mean optical density of spirulina cultured in Kosaric medium (KM) and different substitution levels of fermented *Azolla pinnata* extract medium (FAEM) on the 18th day of culture. Error bars represent ±SD (n = 3). Different lowercase letters above the bars indicate significant differences ($p<0.05$) among treatments.

at higher levels. Spirulina could not grow in a medium containing excessive organic matter, as this would alter turbidity and cause a dark color (Cheunbarn and Peerapornpisol, 2010). Hadiyanto *et al*. (2012) also reported that the dark hue caused by wastewater reduced light intensity in the medium, thereby hindering photosynthesis in *Spirulina* sp.

The specific growth rate (SGR) of spirulina in distinct treatments is presented in Figure 3. An upward trend in SGR of spirulina was observed during the exponential phase (on the 18th day). In the exponential phase, the specific growth rate (SGR) of spirulina in KM and FAEM25 was significantly higher than that in the other treatments. SGR in FAEM50, FAEM75, and FAEM100 was 0.41±0.02, 0.29±0.01, and 0.173±0.01 day^{-1}, respectively, which was significantly different compared with KM and FAEM25.

Toyub *et al*. (2011) reported SGR values of 0.41–0.50 day^{-1} for spirulina cultured in papaya skin powder medium and 0.41–0.49 day^{-1} in papaya leaf ash-based medium. These findings support the potential of plant-based organic media for sustaining spirulina growth. In the present study, FAEM25 produced SGR values comparable to KM, indicating that FAEM can serve as an alternative nutrient source for spirulina culture. However, higher FAEM proportions reduced SGR, which may be attributed to increased turbidity and the resulting limitation of light penetration.

In this study, the protein content of spirulina ranged from 49.47 to 59.79% of dry weight, which is comparable to the 58.2% reported in *Spirulina* sp. by Alvarenga *et al*. (2011). The relatively high protein content observed in the present study may be related to nitrogen availability in the culture medium, which supplies more nitrogen for protein synthesis. Similarly, Piorreck *et al*. (1984) also observed that increased nitrogen in the growth medium led to higher biomass and protein levels in spirulina.

Effect of FAEM on chlorophyll a and β-carotene content of spirulina

The mean chlorophyll a and β-carotene contents of spirulina in different treatments on the 18th day are presented in Figure 4. The chlorophyll *a* concentration of spirulina increased

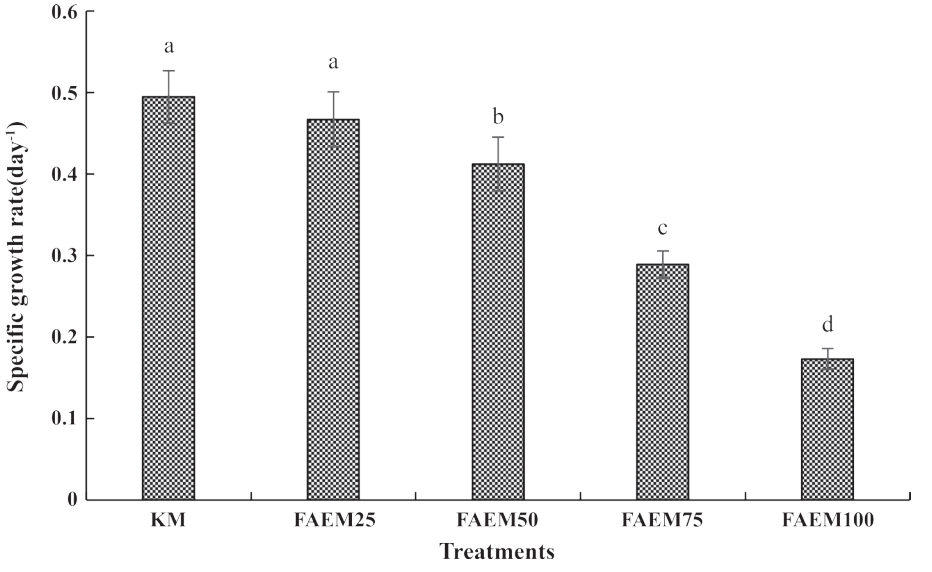


Figure 3. Mean specific growth rate (SGR) of spirulina cultured in Kosaric medium (KM) and different substitution levels of fermented *Azolla pinnata* extract medium (FAEM) on the 18th day of culture. Different lowercase letters above the data points indicate significant differences ($p<0.05$) among treatments.

until the 18th day, after which it dropped. The KM used as a control had the highest chlorophyll *a* concentration (6.54±0.04 μg·mL^{-1}). Similar findings were noted for chlorophyll *a* of spirulina in FAEM25 (6.20±0.13 μg·mL^{-1}), which was not significantly different from the control. On the other hand, higher concentrations of fermented *Azolla pinnata* extract in FAEM50 (5.73±0.17 μg·mL^{-1}), FAEM75 (5.28±0.19 μg·mL^{-1}) and FAEM100 (4.47±0.07 μg·mL^{-1}) decreased the chlorophyll *a* content. There was a significant difference ($p<0.05$) among the treatments FAEM50, FAEM75 and FAEM100 compared with KM and FAEM25 on the 18th day of culture. Piorreck *et al*. (1984) stated that the medium composition controls the chlorophyll concentration of spirulina. Since nitrogen is an essential component of the chlorophyll molecule, the kind and quantity of nitrogen available may matter. Changes in nitrogen and other nutrient concentrations in higher FAEM may decrease the chlorophyll *a* concentration.

In the current study, the highest chlorophyll *a* content was found in KM and FAEM25. Normally, as biomass concentration increases, chlorophyll concentration increases as well. Given the clear correlation between optical density and cell weight, chlorophyll content also increased across treatments. Conversely, the higher FAEM levels showed a lower chlorophyll *a* concentration because the medium was darker and had lower light penetration. The two most crucial elements for spirulina pigment formation under mixotrophic conditions are light and an organic carbon source; however, light cannot penetrate a highly concentrated medium (Mirhosseini *et al*., 2022). Similarly, Mirhosseini *et al*. (2022) tested different concentrations of sugarcane molasses (0, 0.5, 1.0, and 1.5 g·L^{-1}) as an affordable carbon source for *Arthrospira maxima* and found that limited light penetration during culture prevented the highest pigment accumulation required for photosynthesis. In both studies, photosynthetic pigment levels decreased as the concentration of the organic medium increased. This interpretation is speculative and would benefit from further experimental validation. The results of the previous study were quite similar to those of the current investigation. In this experiment, media containing FAEM25 supported pigment accumulation, indicating that the physiological activity of spirulina was affected by nutrient concentration and light penetration.

An upward trend in β-carotene of spirulina was observed up to the exponential phase (18th day), after which it declined. The highest β-carotene

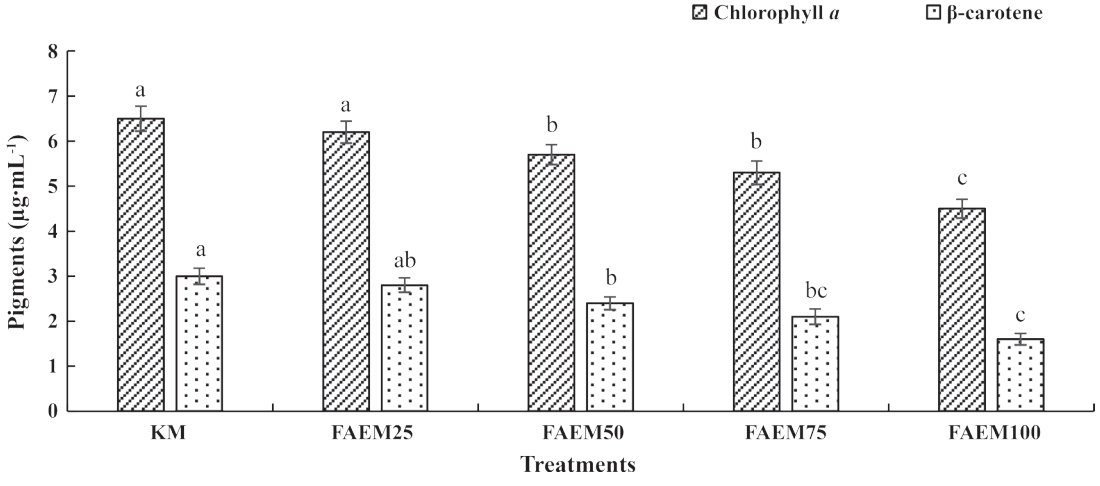


Figure 4. Pigments (Chlorophyll *a*, and β-carotene) (μg·mL^{-1}) content of spirulina in various treatments on the 18th day of culture. Bars represent mean (n = 3) and error bars represent ±SD. Different lowercase letters above indicate significant differences ($p<0.05$) among mean chlorophyll *a*, while differences among mean β-carotene were denoted with different uppercase letters.

content was recorded in KM (2.93±0.04 μg·mL^{-1}), followed by FAEM25 (2.77±0.12 μg·mL^{-1}), which was not significantly different from the control ($p \geq 0.05$). In contrast, FAEM50, FAEM75, and FAEM100 showed significantly lower β-carotene contents than KM and FAEM25 ($p < 0.05$). This variation may be attributed to differences in the nutrient composition, medium coloration, and light penetration into the culture media.

Carotenoids and chlorophyll are closely related pigments, and this study also found a similar pattern in their accumulation. β-carotene and chlorophyll *a* were highest in FAEM25. Micronutrients such as C, N, P, K, Zn, and others that enhanced chlorophyll synthesis may contribute to increased β-carotene levels. Using LEDs with a 70% red and 30% blue composition, Lima *et al*. (2018) observed that the highest concentration of β-carotene ranged from 5.45 μg·mL^{-1} to 6.91 μg·mL^{-1} while examining the impact of spectral light quality on pigment concentrations in *Spirulina* sp. In line with this, FAEM25's higher performance in the current investigation was most likely due to a balance between appropriate nutrient supply and light penetration, which promoted efficient photosynthetic activity while triggering moderate photoprotective responses. Under some situations, β-carotene synthesis may increase to guard against reactive oxygen species produced during photosynthesis (Lamers *et al*., 2010). Higher FAEM concentrations may limit light penetration and create suboptimal redox conditions, thereby inhibiting chlorophyll and β-carotene production. This study indicates that FAEM25 could provide adequate nutrients and sufficient light penetration to support greater accumulation of the pigment β-carotene than the other FAEM proportions.

Protein content

The protein content of spirulina cultured under different culture media for 22 days is shown in Figure 5. The protein content of spirulina in KM, FAEM25, FAEM50, FAEM75, and FAEM100 were 59.4, 65.6, 63.2, 61.4, and 62.5%, respectively. The highest protein content in spirulina was observed in FAEM25 medium. Protein content was higher in FAEM than in the control medium, which could be due to the higher nitrogen content in FAEM media. FAEM media also act as a probiotic, which may potentially improve the biochemical composition of *Spirulina* sp.

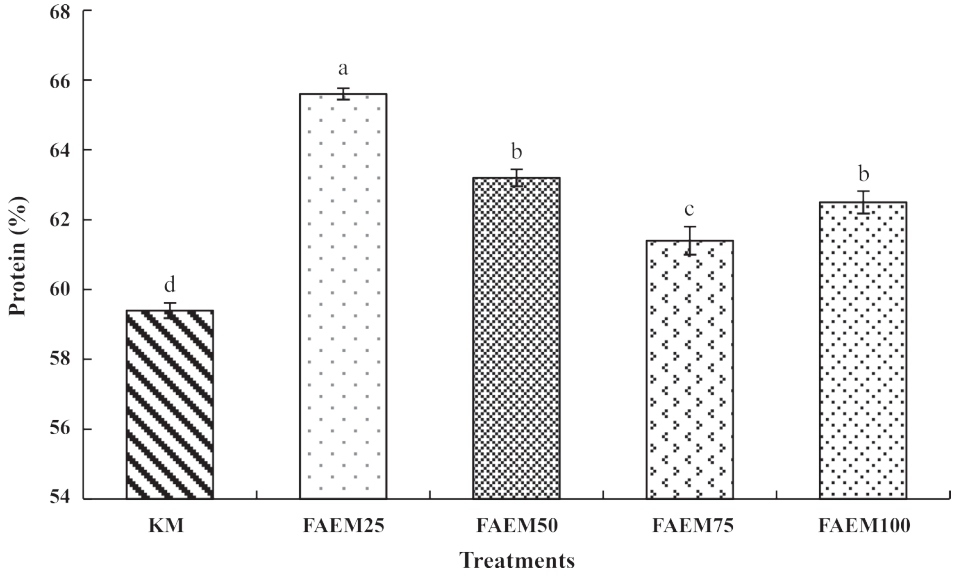


Figure 5. Mean protein content of spirulina cultured in Kosaric medium (KM) and different substitution levels of fermented *Azolla pinnata* extract medium (FAEM) after 22 days of culture. Error bars represent ±SD (n = 3). Different lowercase letters above the bars indicate significant differences ($p < 0.05$) among treatments.

The protein content observed in the present study is consistent with previous reports on *Spirulina* sp. Alvarenga *et al*. (2011) reported a protein content of 58.2%, while Marrez *et al*. (2014) found high protein content in spirulina grown in BG-11 and modified BG-11 media, most likely due to the high nitrogen concentration in those media. Similarly, Piorreck *et al*. (1984) reported that increasing nitrogen levels in the nutrient medium enhanced both biomass and protein content in spirulina. Therefore, the higher protein content observed in FAEM25 and the other FAEM treatments compared with KM may be associated with the higher nitrogen content of FAEM.

Cost analysis

The approximate cost to prepare 1.0 L of KM, FAEM25, FAEM50, FAEM75, and FAEM100 was 0.25, 0.18, 0.12, 0.061, and 0 USD, respectively. In addition, the approximate cost of one kg of dry spirulina production in the exponential period on the 18th day in each medium is provided in Table 4. It is important to note that the present cost analysis pertains solely to partial raw material expenses and explicitly excludes costs associated with labor, energy consumption, and culture medium preparation under laboratory-scale experimental conditions. Accordingly, it does not represent a comprehensive techno-economic assessment of commercial-scale *Spirulina* sp. production. Notably, the maximum cost of spirulina culture was observed in the KM medium, with lower costs as the FAEM concentration increased.

The major cost in small-scale *Spirulina* sp. production is the growth medium (Ahsan *et al*., 2008). Raoof *et al*. (2006) reported that preparing 1,000 L of Kosaric medium would cost Rs. 3,659 (USD$ 79.5) in India and noted that the production cost of Kosaric medium is similarly high in other countries. In this study, the cost of spirulina culture was higher in KM than in FAEM25, FAEM50, FAEM75, and FAEM100 treatments, and the protein content was higher in FAEM25, FAEM50, FAEM75, and FAEM100 than in KM. Therefore, FAEM may reduce the cost of spirulina culture.

Table 4. Estimated partial raw materials cost of spirulina media preparation (USD·L^{-1}) and culture media cost per kg dry biomass of spirulina (USD·kg^{-1} dry biomass) under different treatments.

Treatments	Cost (USD·L^{-1})			Cost USD·kg^{-1} dry biomass of spirulina
	KM	FAEM	Total	
KM	0.25	0	0.25	1,742.16
FAEM25	0.18	0	0.18	1,294.96
FAEM50	0.12	0	0.12	912.55
FAEM75	0.061	0	0.061	705.20
FAEM100	0	0	0	0

CONCLUSIONS

This study was conducted to evaluate the potential of FAEM as an alternative nutrient source for *Spirulina* sp. culture. The results demonstrated that FAEM contains a high concentration of nitrogen and organic carbon, indicating its suitability as a supplementary organic nutrient for microalgal growth. The incorporation of FAEM into the culture medium supplementation levels. Specifically, the addition of 25% FAEM in combination with standard KM medium resulted in growth performance, pigment production, and biomass yield comparable to those achieved with the standard medium alone. Furthermore, *Spirulina* sp. cultivated under these conditions exhibited high protein content, suggesting that FAEM supplementation improved the biomass quality. These findings highlight the potential of

that can reduce the overall culture cost of spirulina while maintaining comparable production efficiency and nutritional quality. However, the study also revealed that higher concentrations of FAEM negatively affected the growth rate, pigment content, and other physiological parameters of spirulina, likely due to nutrient imbalance or the presence of inhibitory compounds. Thus, supplementing standard KM with 25% FAEM is recommended as an effective strategy to maximize productivity and product quality while minimizing production costs.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Aquaculture, Gazipur Agricultural University for the laboratory and technical support.

LITERATURE CITED

Abdul-Adel, E., M.M. Saleh and J.M. Salman. 2019. Production of photosynthesis pigments by Spirulina platensis under different NaCl concentrations. **Plant Archive** 19(2): 3254–3258.

Ahsan, M., B. Habib, M. Parvin, T.C. Huntington and M.R Hasan. 2008. **A Review on Culture, Production and Use of *Spirulina* as Food for Humans and Feeds for Domestic Animals.** Food and Agriculture Organization of the United Nations, Rome, Italy. 33 pp.

Akbarnezhad, M., M.S. Mehrgan, A. Kamali and M.J. Baboli. 2020. Effects of microelements (Fe, Cu, Zn) on growth and pigment contents of *Arthrospira (Spirulina) platensis*. **Iranian Journal of Fisheries Sciences** 19(2): 653–668. DOI: 10.22092/ijfs.2019.120614.

Akter, T., M.I. Haque, M. Das and M.A. Hossain. 2019. Growth performance analysis of Spirulina platensis production by substituting K2SO4-K of kosaric medium with MOP-K. **Bangladesh Journal of Botany** 48(3): 529–535. DOI: 10.3329/bjb.v48i3.47914.

Alvarenga, R.R., P.B. Rodrigues Cantarelli, M.G. Zangeronimo, J.W.D. Silva Júnior and L.J. Pereira. 2011. Energy values and chemical composition of spirulina (*Spirulina platensis*) evaluated with broilers. **Revista Brasileira de Zootecnia** 40: 992–996. DOI: 10.1590/S1516-35982011000500008.

Amin, M., A. Haq, A. Shahid, R. Boopathy and A. Syafiuddin. 2024. ***Spirulina* as a food of the future.** In: Pharmaceutical and Nutraceutical Potential of Cyanobacteria (eds. M.A. Mehmood, P. Verma, M.P. Shah and M.J. Betenbaugh), pp. 53–83. Springer, Dordrecht, Netherlands.

Andrade, M.R. and J.A. Costa. 2007. Mixotrophic cultivation of microalga Spirulina platensis using molasses as organic substrate. **Aquaculture** 264(1–4): 130–134. DOI: 10.1016/j.aquaculture.2006.11.021.

Anitha, K.C., Y.B. Rajeshwari, S.B. Prasanna and S.J. Shilpa. 2016. Nutritive evaluation of *Azolla* as livestock feed. **Journal of Experimental Biology and Agricultural Sciences** 4: 670–674. DOI: 10.18006/2016.4(Issue6).670.674.

Association of Official Analytical Chemists (AOAC). 2007. **Official Methods of Analysis,** 18th ed. Gaithersburg, Maryland, USA. 1094 pp.

Apandi, N.M., R.M. Mohamed, A. Al-Gheethiand and A.H.M. Kassim. 2019. Microalgal biomass production through phycoremediation of fresh market wastewater and potential applications as aquaculture feeds. **Environmental Science and Pollution Research** 26: 3226–3242. DOI: 10.1007/s11356-018-3937-3.

Basak, B., M.A.H. Pramanik, M.S. Rahman, S.U. Tarafdar and B.C. Roy. 2002. Azolla (*Azolla pinnata*) as a feed ingredient in broiler ration. **International Journal of Poultry Science** 1: 29–34. DOI: 10.3923/ijps.2002.29.34.

Bashir, K.M.I., S. Mansoor, N.R. Kim, F.R. Grohmann, A.A. Shah and M.G. Cho. 2019. Effect of organic carbon sources and environmental factors on cell growth and lipid content of *Pavlova lutheri*. **Annals of Microbiology** 69(4): 353–368. DOI: 10.1007/s13213-018-1423-2.

Borges, J.A., G.M.D. Rosa, L.H.D. Meza, A.A. Henrard, M.D.R. Souza and J.A.V. Costa. 2013. *Spirulina* sp. LEB-18 culture using effluent from the anaerobic digestion. **Brazilian Journal of Chemical Engineering** 30: 277–288. DOI: 10.1590/S0104-66322013000200006.

Cherryl, D.M., R.M.V. Prasad, R.S. Jagadeeswara, P. Jayalaxmi and S.D. Kumar. 2014. A study on the nutritive value of *Azolla pinnata*. **Livestock Research International** 2(1): 13–15.

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

Eaton, A.D., L.S. Clesceri, E.W. Rice and A.E. Greenberg. 2005. **Standard methods for the examination of water and wastewater,** 21st ed. American Public Health Association (APHA) Press, Washington, D.C., USA. 1368 pp.

Eijckelhoff, C. and J.P. Dekker. 1997. A routine method to determine the chlorophyll *a*, pheophytin a and β-carotene contents of isolated photosystem II reaction center complexes. **Photosynthesis Research** 52(1): 69–73. DOI: 10.1023/A:1005834006985.

El-Sheekh, M.M., M.Y. Bedaiwy, M.Y. Osman and M.M. Ismail. 2012. Mixotrophic and heterotrophic growth of some microalgae using extract of fungal-treated wheat bran. **International Journal of Recycling of Organic Waste in Agriculture** 1: 1–9. DOI: 10.1186/2251-7715-1-12.

Ghofar, H.S.A., M.J. Jahromi, F.N.M. Ikhsan and A.A. Samsudin. 2019. The effects of varying dilution levels of wastewater on the cultivation of *Spirulina* sp. **Malaysian Journal of Animal Science** 22(1): 25–33.

Gorgij, J.M., M. Yahyavei, K. Rohani-Ghadikolaei and A.R. Salarzadeh. 2018. The effect of different Nitrogen sources on growth rate and protein content of *Spirulina platensis*. **Iranian Fisheries Science Research Institute** 26(7): 57–65.

Gomont, M.A. 1893. Monographie Des Oscillariées: (Nostacacées Homocystées). **Annales des Sciences Naturelles, Botanique Série** 7 16: 91–264.

Gouri, M.D., J.S. Sanganal, C.R. Gopinath and C.M. Kalibavi. 2012. Importance of azolla as a sustainable feed for livestock and poultry-A review. **Agricultural Reviews** 33(2): 93–103.

Habib, M.A.B. 1998. **Culture of selected microalgae in rubber and palm oil effluents and their use in the production of enriched rotifers.** Doctoral Thesis, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. 532 pp.

Jabber, M.A. 2005. **Culture and growth performance of *Spirulina platensis* in different concentrations of sesame meal media.** Master Thesis, Bangladesh Agricultural University, Mymensingh, Bangladesh. 75 pp.

Kebede, E. and G. Ahlgren. 1996. Optimum growth conditions and light utilization efficiency of *Spirulina platensis* (= *Arthrospira fusiformis*) (Cyanophyta) from Lake Chitu, Ethiopia. **Hydrobiologia** 332(2): 99–109. DOI: 10.1007/BF00016689.

Khan, A.I., M.A.B. Habib, M.S. Hossain and M.I. Miah. 2018. Culture of *Chlorella vulgaris* in press mud media as sugar mill waste. **International Journal of Fisheries and Aquatic Research** 3(2): 41–45.

Khatun, S., M.A. Hossain, T. Akter, M.R. Banu and A.Q.M.R. Kawser. 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. DOI: 10.3329/aba.v23i1.51472.

Kim, S.S., S. Rahimnejad, K.W. Kim and K.J. Lee. 2013. Partial replacement of fish meal with *Spirulina pacifica* in diets for parrot fish (*Oplegnathus fasciatus*). **Turkish Journal of Fisheries and Aquatic Sciences** 13(2): 197–204. DOI: 10.1038/s41598-018-32956-4.

Kodit, N.I.A., Q.Q.A.M. Razemin and A.L. Noh. 2023. Taxonomic significance of morphological structure in Azolla. **Revelation and Science 1** (Special Issue: Plant Science 1): 101–113.

Korsa, G., D. Alemu and A. Ayele. 2024. Azolla plant production and their potential applications. **International Journal of Agronomy** 2024(1): 1–12.

Lamers, P.P., C.C. Van De Laak, P.S. Kaasenbrood, J. Lorier, M. Janssen, R.C. De Vos and R.H. Wijffels. 2010. Carotenoid and fatty acid metabolism in light-stressed *Dunaliella salina*. **Biotechnology and Bioengineering** 106(4): 638–648. DOI: 10.1002/bit.22725.

Li, P., Z. Hu, Q. Yin and C. Song. 2023. Improving the growth of Spirulina in CO_2 absorption and microalgae conversion (CAMC) system through mixotrophic cultivation: Reveal of metabolomics. **Science of the Total Environment** 858: 159920. DOI: 10.1016/j.scitotenv.2022.159920.

Lima, G.M., P.C. Teixeira, C.M. Teixeira, D. Filócomo and L.C. Lage. 2018. Influence of spectral light quality on the pigment concentrations and biomass productivity of *Arthrospira platensis*. **Algal Research** 31: 157–166. DOI: 10.1016/j.algal.2018.02.012.

Liu, C.H., C.H. Chiu, S.W. Wang and W. Cheng. 2012. Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. **Fish Shellfish Immunology** 33(4): 699–706. DOI: 10.1016/j.fsi.2012.06.012.

Madkour, F.F., K.E.W. Kamil and H.S. Nasr. 2012. Production and nutritive value of *Spirulina platensis* in reduced cost media. **The Egyptian Journal of Aquatic Research** 38(1): 51–57. DOI: 10.1016/j.ejar.2012.09.003.

Malcata, F.X. 2011. Microalgae and biofuels: a promising partnership? **Trends in Biotechnology** 29(11): 542–549. DOI: 10.1016/j.tibtech.2011.05.005.

Markou, G. and E. Nerantzis. 2013. Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions. **Biotechnology Advances** 31(8): 1532–1542. DOI: 10.1016/j.biotechadv.2013.07.011.

Marrez, D.A., M.M. Naguib, Y.Y. Sultan, J.Y. Daw and A.M. Higazy. 2014. Evaluation of chemical composition for *Spirulina platensis* in different culture media. **Research Journal of Pharmaceutical, Biological and Chemical Sciences** 5(4): 1161–1171.

Matos, J.C., N.M. Cardoso, K. Bandarra and C. Afonso. 2017. Microalgae as healthy ingredients for functional food: A review. **Food and Function** 8(8): 2672–2685. DOI: 10.1039/C7FO00409E.

Michael, A., M.S. Kyewalyanga and J.V. Lugomela. 2019. Biomass and nutritive value of *Spirulina (Arthrospira fusiformis)* cultivated in a cost-effective medium. **Annals of Microbiology** 69: 1387–1395. DOI: 10.1007/s13213-019-01520-4.

Mirhosseini, N., R. Davarnejad, A. Hallajisani, E. Cano-Europa and O. Tavakoli. 2022. Sugarcane molasses as a cost-effective carbon source on *Arthrospira maxima* growth by taguchi technique. **International Journal of Engineering** 35(3): 510–521. DOI: 10.5829/IJE.2022.35.03C.03.

Mofiz, M.S.B., M.A. Hossain, J. Saha, M.A.H. Bakky, T. Akter and M.R. Banu. 2023. Water fern *Azolla pinnata* extract as a novel organic nutrient source for the cultivation of spirulina *Spirulina platensis*. **Aquaculture Studies** 24(2): AQUAST 1419. DOI: DOI: 10.4194/AQUAST 1419.

Nyabuto, D.K., A.M. Mariga and G.W. Kibue. 2015. Growth performance and biochemical analysis of the genus *Spirulina* under different physical and chemical environmental factors. **African Journal of Agricultural Research** 36: 3614–3624. DOI: 10.5897/AJAR2015.10210.

Pereira, M.I., B.M. Chagas, R. Sassi, G.F. Medeiros, E.M. Aguiar, L.H. Borba, E.P.E. Silva, J.C.A. Neto and A.H Rangel. 2019. Mixotrophic cultivation of *Spirulina platensis* in dairy wastewater: Effects on the production of biomass, biochemical composition and antioxidant capacity. **PloS one** 14(10): e0224294. DOI: 10.1371/journal.pone.0224294.

Perez-Garcia, O. and Y. Bashan. 2015. Microalgal heterotrophic and mixotrophic culturing for bio-refining: from metabolic routes to techno-economics. **Algal Biorefineries: Products and Refinery Design** 2: 61–131. DOI: 10.1007/978-3-319-20200-6_3.

Pillai, P.K., S. Premalatha and S. Rajamony. 2002. Azolla a sustainable feed substitute for livestock. **Leisa India** 4(1): 15–17.

Piorreck, M., K.H. Baasch and P. Pohl. 1984. Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. **Phytochemistry** 23(2): 207–216. DOI: 10.1016/S0031-9422(00) 80304-0.

Rafiqul, I.M., K.C.A. Jalal and M.Z. Alam. 2005. Environmental factors for optimization of Spirulina biomass in laboratory culture. **Biotechnology** 4(1): 19–28. DOI: 10.3923/ biotech.2005.19.22.

Ragaza, J.A., M.S. Hossain, K.A. Meiler, S.F. Velasquez and V. Kumar. 2020. A review on Spirulina: alternative media for cultivation and nutritive value as an aquafeed. **Reviews in Aquaculture** 12(4): 2371–2395. DOI: 10.1111/raq.12439.

Rahman, M.H. 2021. Smart production of Spirulina (*Spirulina platensis*) using supernatant of digested rotten potato (*Solanum tuberosum*). **Journal of Agriculture, Food and Environment** 2(1): 62–69. DOI: 10.47440/ JAFE.2021.2111.

Raoof, B., B.D. Kaushik and R. Prasanna. 2006. Formulation of a low-cost medium for mass production of *Spirulina*. **Biomass and bioenergy** 30(6): 537–542. DOI: 10. 1016/j.biombioe.2005.09.006.

Richmond, A. 2003. **Biological principles of mass cultivation.** In: Handbook of Microalgal Culture: Biotechnology and Applied Phycology (ed. A. Richmond), pp. 125–177. Blackwell Publishing, Oxford, UK. DOI: 10.1002/9780470995280.ch8.

Rizwan, M., G. Mujtaba, S.E.L.K. Memon and N. Rashid. 2018. Exploring the potential of microalgae for new biotechnology applications and beyond: A review. **Renewable and Sustainable Energy Reviews** 92: 394–404. DOI: 10.1016/j. rser.2018.04.034.

Rosenberg, J.N., G.A. Oyler, L. Wilkinson and M.J. Betenbaugh. 2008. A green light for engineered algae: Redirecting metabolism to fuel a biotechnology revolution. **Current Opinion in Biotechnology** 19(5): 430–436. DOI: 10.1016/j.copbio.2008.07.008.

Roussel, T., S. Halary, C. Duval, B. Piquet, J.P. Cadoret, L. Vernès, C. Bernard and B. Marie. 2023. Monospecific renaming within the cyanobacterial genus *Limnospira* (Spirulina) and consequences for food authorization. **Journal of applied microbiology** 134(8): 1–15. DOI: 10.1093/jambio/lxad159.

Ruliaty, L., I.R. Amalia, R.I. Sari and R. Aulia. 2022. Different Nitrogen Sources to Improve the Quality of *Spirulina platensis* Culture in Mass Scale. **IOP Conference Series: Earth and Environmental Science** 1118: 012015. DOI: 10.1088/1755-1315/ 1118/1/012015.

Soni, R.A., K. Sudhakar and R.S. Rana. 2017. Spirulina–From growth to nutritional product: A review. **Trends in Food Science and Technology** 69: 157–171.

Spinola, M.P., A.R. Mendes and J.A. Prates. 2024. Chemical composition, bioactivities, and applications of Spirulina (*Limnospira platensis*) in food, feed, and medicine. **Foods** 13(22): 3656. DOI: 10.3390/foods 13223656.

Sun, H., J.W. Tang, X.H. Yao, Y.F. Wu, X. Wang, Y. Liu and B. Lou. 2015. Partial substitution of fish meal with fermented cottonseed meal in juvenile Black Sea bream (*Acanthopagrus schlegelii*) diets. **Aquaculture** 446: 30–36. DOI: 10.1016/ j.aquaculture.2015.04.020.

Toyub, M.A., M.Z. Uddin, M.I. Miah and M.A.B. Habib. 2011. Growth performance and nutritional analysis of *Spirulina platensis* in different concentrations of papaya skin powder media. **Bangladesh Journal of Scientific and Industrial Research** 46(3): 333–338. DOI: 10.3329/bjsir.v46i3.9039.

Usharani, G., P. Saranraj and D. Kanchana. 2012. Spirulina cultivation: A review. **International Journal of Pharmaceutical and Biological Archives** 3(6): 1327–1341.

Yeesang, C. and B. Cheirsilp. 2014. Effect of nitrogen, light and organic carbon sources on growth and lipid production of microalgae for biodiesel production. **Journal of Chemical Technology and Biotechnology** 89(6): 936–945.

Zarrinmehr, M.J., O. Farhadian, F.P. Heyrati, J. Keramat, E. Koutra, M. Kornaros and E. Daneshvar. 2020. Effect of nitrogen concentration on the growth rate and biochemical composition of the microalga, Isochrysis galbana. **The Egyptian Journal of Aquatic Research** 46(2): 153–158. DOI: 10.1016/j.ejar.2019.11.003.

Zhang, D., Y. Zhang, B. Liu, Y. Jiang, Q. Zhou and J. Wang. 2017. Effect of replacing fish meal with fermented mushroom bran hydrolysate on the growth, digestive enzyme activity, and antioxidant capacity of allogynogenetic crucian carp (*Carassius auratus gibelio*). **Turkish Journal of Fisheries and Aquatic Sciences** 17(5): 1039–1048. DOI: 10.4194/1303-2712-v17_5_20.