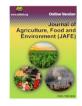


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Research Article

Low-cost spirulina manufacturing technique by using supernatant of digested rotten ladies finger (*Abelmoschus esculentus*)

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A B S T R A C T

The goal of the study was to assess the culture method and spirulina (Spirulina *platensis*) production in the supernatant of three distinct digested rotting ladies fingers, using Kosaric medium (KM) as the control. Two hundred gram rotten ladies finger was allowed to digestion in 4.0 L distilled water using aeration in 5.0 L glass jar. After 30 days, almost colorless supernatant was found, screened through 300 µm net and added with 9 g/l NaHCO₃, 0.20 g/l urea and 0.50 ml/L micro nutrients. Three different concentrations such as 25, 50 and 75% of supernatant with three replications were prepared. Next, three digested rotten ladies finger medium (DRLFM) treatments were infected with 10% spirulina (at OD620 = 0.20) to develop. It was recorded that spiruling grew well at 12^{th} day and then it was found to fall and continued up to 18th days. The cell weight of spirulina attained a maximum of 16.45 ± 0.78 mg/L (dry weight basis) in KM followed by 13.785 ± 0.643 , 10.354 ± 0.233 and 2.738 ± 0.177 mg/L in supernatant of 50, 75 and 25% DRLFM, respectively on the 12th day of culture. Similar trend was also observed in optical density of media, content of chlorophyll <u>a</u> (mg/L), total biomass (mg/L), particular growth rate of spirulina (based on cell weight and chlorophyll a). It could be concluded that mass culture of S. platensis may be done in supernatant of 50% digested rotten ladies finger media.

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Introduction

Bangladesh stands as the world's largest deltaic nation (Mahmuda et al., 2020; Rahman et al., 2021). Additionally, the sub-sector of fisheries and aquaculture is crucial in mitigating the effects of protein deficit (Baroi et al., 2019). Due to Bangladesh's declining natural fisheries resources and growing human population, aquaculture is becoming increasingly important for producing fish (Mahmud et al., 2021; Nasrin et al., 2021; Rahman et al., 2021). According to Mou et al. (2023), the sub-sector of fisheries accounts for 1.24% of overall export revenue, 26.50% of the country's agricultural GDP, and 3.57% of its total GDP. This massive output generates a sizable sum of foreign exchange (Biswas et al., 2021). The use of various algae and chemicals has increased along with the growth of aquaculture in Bangladesh (Uddin et al., 2020). Fish raised for commercial purposes can be fed algae or other natural and supplemental

foods (Tandra et al., 2019). According to Islam et al. (2020), aquaculture has continued to expand globally and is predicted to gradually close the gap in aquatic food products. One of the foods that are highest in nutrients on the earth is blue-green algae (Rasel et al., 2022). The most popular types of blue green algae are Spirulina platensis. Because of its high protein, vitamin, and nutrient contents, these algae are regarded as super foods. In addition to stabilizing carbon dioxide in the water, microalgae are crucial for oxygenation (Rahman et al., 2022). Spirulina is a blue-green, multicellular alga. Their length is between 300 and 500 µm, and they are minuscule (Hossain et al., 2021). Fish that consume microalgae can better absorb nutrients. A variety of fish species' growth performance and survival rate can be enhanced by silica nanoparticles through enhanced digestion, improved nutrient absorption and utilization, and increased feed conversion efficiency (Murshed et al., 2023). Spirulina

has also high nutritional content; people have been using it as food since ancient times. Spirulina has been proven to contain up to 70% protein, large amounts of fatty acids (18-22%), essential amino acids, minerals, vitamins (especially B₁₂), antioxidants, pigments (phycobili proteins and carotenoids), and polysaccharides. Moreover, bioactive substances found in spirulina include carotenes, necessary fatty acids, minerals, vitamins, and other pigments with antioxidant qualities (Vonshak, 1997). In addition to its function in reducing nitrite and nitric oxide synthase to lessen liver lipid peroxidation, its high ß-carotene content allows it to scavenge reactive oxygen species (ROS) molecules, such as peroxyl, alkoxyl, and hydroxyl radicals. Without adding extra calories or fat, spirulina offers all the nutrients that are necessary (Habib et al., 2021; Rasel et al., 2022). As a result, spirulina production for commercial purposes has drawn attention from all over the world for usage in pharmaceuticals, animal feed, and human food supplements (Madkour et al., 2012). The mass culture of spirulina, which grows more quickly than most other plants (Shay 1993), offers hope for addressing the world's protein scarcity and food crises. Humans have traditionally consumed spirulina because it is safe and nutrient-dense. It seems to have a great deal of potential for growth, particularly when grown on a local scale for environmental mitigation, livelihood development, and nutritional enhancement (Habib et al., 2008). It is receiving more and more attention for the development of possible medications as well as the food-related elements (Quoc and Pascaud, 1996). This alga is being investigated extensively, both for its purported therapeutic benefits and for nutritional reasons. A vast array of nutritious vegetables is being grown in Bangladesh. It would be beneficial financially if these nutrient-rich vegetable wastes were employed as a source of nutrients for spirulina cultures. Research attempted to advance a less expensive medium by using readily accessible vegetable wastes, like the rotten ladies finger, which contain essential minerals and nutrition and may be useful for the growth of Spirulina platensis (Akhtar et al., 2012). The goal was to grow Spirulina at home. Mineral-rich, including N, P, K, and other elements, palm oil mill effluent (POME) is ideal for use as media and nutrients in plants and algae (Effendi et al., 2021; Rahman et al., 2022). Spirulina is utilized as a feed element in aquaculture to enhance development, the physiological reactions of fish species, feed efficiency, and carcass quality to illness (Habib et al., 2021; Habib et al., 2022). Spirulina is a large, fast-growing cyanobacterium (0.5 mm), it can be employed as an unusual ingredient in algal feed and could provide fish culture with an alternate source of protein (Rasel et al., 2022). Ladies fingers are a valuable vegetable that might deteriorate and turn to trash. Due to its rapid growth and lack of competition with food crops for resources, spirulina can be utilized to create biofuel. A significant amount of ladies finger waste is produced annually in the country, where 30-40% of the population (Abelmoschus esculentus) rots. This percentage is rising daily. The goal of this study is to determine how well Spirulina platensis grows in the supernatant of digested rotten ladies fingers (Abelmoschus esculentus).

Materials and methods

Site of experimentation

The experiment was carried out for a period of three months from in Live Food Aquaculture Laboratory, Department of

Aquaculture, Bangladesh Agricultural University (BAU), Mymensingh-2202, Bangladesh.

Experimental design

Supernatant of digested rotten ladies finger medium (DRLFM) was used to produce *Spirulina platensis* in laboratory condition. The experiment had four (1 to 4) treatments. The first three treatments contained 25, 50, 75% DRLF and the last one contained KM and regarded as control medium. Each treatment had 3 replications. The composition of KM described in Table 2.

Culture of microalgae

An assortment of decaying *ladies finger*

The rotten ladies finger was chosen for performing the research work for *Spirulina platensis* culture. The rotten ladies finger was collected from Kamal Ranjit (KR) Market, Bangladesh Agricultural University, Mymensingh-2202.

Preserving authentic stock culture of Spirulina platensis

KM (Zarrouk's, 1996) was used to maintain the pure stock culture of *Spirulina platensis* in laboratory condition. At every alternative day growth of *Spirulina platensis* was monitored and examined with a microscope to verify its purity, giving the Bold and Wynne (1978) keys as a guide.

Table	1.	Concentration	of	DRLFM	(Digested	Rotten
Ladies	Fi	nger Medium) fo	or S	pirulina Pl	latensis.	

Sl. No.	Rotten ladies finger	Concentration/dilution	
	ingredients	of RLFM (%)	
1.	Digested Rotten Ladies	25	
	Finger medium (DRLFM)	23	
2.	Digested Rotten Ladies	50	
	Finger medium (DRLFM)	30	
3.	Digested Rotten Ladies	75	
	Finger medium (DRLFM)	15	

In order to prepare the supernatant of decaying ladies finger, the collected samples were first digested by aerating them into a 5 liter volumetric flask under 3 liters of distilled water. After several days, when the concentrations of juice become less dense, 1 litre of distilled water was added on the flask. The total amount of rotten ladies finger was 250g. It should be focused that the concentration of rotten ladies finger of 250g/4L was maintained during digestion. Ladies finger digestion took 30 days to complete, and the supernatant was removed from the flask and filtered through plankton net. Then the digested rotten ladies finger was diluted using distilled water following the above concentration (Table 1). Each treatment had three replications. After that, the medium was thoroughly combined and sterilized for 15 minutes at 121°C in a high pressure bumping water autoclave. After autoclaving the media were kept for 3 days to be sure about any contamination free before starting the culture of microalgae.



Table 2. Composition of Kosaric medium (Modified after Zarrouk, 1996) for Spirulina platensis culture.

Sl. No.	Chemicals/compounds	Concentration in stock solution g/L	
1	NaHCO ₃	9.0	
2	K ₂ HPO ₄	0.250	
3	NaNO ₃	1.250	
4	K_2SO_4	0.50	
5	NaCl	0.50	
6	MgSO ₄ 7H2O	0.10	
7	CaCl ₂	0.02	
8	FeSO ₄ .2H ₂ O	0.005	
9	A5 micronutrient solution	0.5ml/L	
	a) A ₅ micronutrient solution	g/L	
	i) HBO4	2.86	
	ii) MnCl ₂ .4H ₂ O	1.81	
	iii) ZnSO ₄ .7H ₂ O	0.22	
	iv) CuSO4.7H ₂ O	0.08	
	v) MoO ₃	0.01	
	vi) CoCl ₂ . 6H ₂ O	0.01	

Preparation of control medium

In order to create the Control medium, the chemicals listed in Table 2 (numbers 1 through 8) were weighed using an electric balance and placed into a 1L conical flask. After pipetting 0.5 ml of the micronutrient solution into the flask, distilled water was added to create the volume 1 L. The preparation of RLFM involved a number of steps, including mixing, autoclaving, and cooling.

Spirulina platensis cultivation experimental design

Three types of media (25, 50 and 75%) viz., rotten ladies finger media (RLFM) and Kosaric Medium (KM) were used to culture Spirulina platensis. Inoculum Spirulina platensis was collected from the stock culture. Experimental design is presented in Table 3.

Table 3. Three different doses of supernatant of digested rotten ladies finger (DRLF) through dilution to culture Spirulina.

Types of medium	Treatments	Replications	Amounts (DRLFM) (ml/L)	Duration of culture (days)
Supernatant	1	3	25	
of DRLF	2	3	50	18
	3	3	75	
Kosaric Medium (KM)	4	3	-	18

Culture of Spirulina platensis in supernatant of DRLFM and KM

Spirulina platensis microalgae were grown in 1.0 L volumetric flasks using four treatments: three from the supernatant of DRLFM at three different concentrations (25, 50, and 75%) and one KM as a control. Each treatment had three replications. To create a culture with 10% spirulina suspension (optical density at 620 nm = 0.20), spirulina was put into each culture flask (Habib, 1998). 20 milliliters of spirulina suspension are needed to achieve the necessary density. In the Live Food Culture Laboratory, all of the flasks were maintained in a light-dark (12h:12h) environment under fluorescent lamps. These culture flasks was continuously aerated using electric aquarium aerator.



Each flask was subjected to eight sub-samplings (15 ml vial) on alternate days in order to measure the dry cell weight, spirulina's chlorophyll a content, and culture media properties. Every glassware that was utilized in the experiment was overnight sterilized using dry heat at 70°C.

Estimation of Spirulina platensis cell weight and chlorophyll a

A sample of 20 milliliters of spirulina suspension was filtered through sartorius filter paper with a 0.45 um mesh size and a 47 mm diameter in order to estimate the weight of the cells. The filter sheets were weighed before being filtered after being dried at 70°C for 24 or all night. To get rid of insoluble salts, the filtered samples underwent three rounds of washing. Subsequently, the filter papers were placed in a glass dish and allowed to cool in an oven set at 70°C for the entire night. Before the filter papers were weighed, the petri dish was placed in a desiccator for 20 minutes. According to Clesceri et al. (1989), the dry weight of the algae on the filter paper was calculated using the equation below.

Dry weight (mg/L), $W = \frac{FFW - IFW}{Amount of sample taken for filtration (ml)} X100$ Where,

W = Cell dry weight in mg/L;

FFW = Final filter paper weight in g; and

IFW = Initial filter paper weight in g.

Calculating the Spirulina's Chlorophyll a content

The amount of chlorophyll a in the Spirulina platensis samples was assessed after they were collected at various intervals. Filter sheets were used in an electric filtration device to filter 10 milliliters of S. platensis sample. These filtered samples were placed in test tubes with filter paper, powdered with a glass rod, and then combined with 10 milliliters of 100% redistilled acetone. To prevent light from coming into touch with the test tubes, foil papers were wrapped around each one. Overnight, the test tubes that had been wrapped were stored in an LMS Laboratory Refrigerator. The chilled samples were then centrifuged for 10 minutes at 4000 rpm after being homogenized for 2 minutes. The supernatant was separated and taken for chlorophyll analysis after centrifugation. The samples' optical densities were calculated at 664 nm. Using a UV spectrophotometer to measure wavelengths of 647 nm and 630 nm (Clesceri et al., 1989). Simultaneously, a blank using 100% acetone was run. Clesceri et al. (1989) provided the following formula for calculating the concentration of chlorophyll a.

Chlorophyll a (mg/L) = 11.85 (OD 664) - 1.54 (OD 647) -0.08 (OD 630)

Total biomass of spirulina

Vonshak and Richmond (1988) provided the following formula, which was used to compute total biomass: Total biomass = Chlorophyll $a \times 67$

Specific growth rate (SGR) based on total biomass of spirulina, chlorophyll <u>a</u> content, and dry weight (Clesceri *et al.*, 1989)

Specific growth rate (μ/day) of cultured spirulina on the basis of dry weight

SGR (μ/day) = In (X₁-X₂)/t₁-t₂

Where, $X_1 = Dry$ weight of biomass concentration of the last of selected time interval:

 X_2 = Dry weight biomass concentration at beginning of selected time interval;

And t_1 - t_2 = Time that has passed between a chosen time of day.

Based on chlorophyll a, the specific growth rate (μ/day) of cultured spirulina

SGR (μ/day) = In (X₁-X₂)/t₁-t₂

Where, X_1 = Chlorophyll <u>a</u> at the conclusion of the chosen time frame

 X_2 = Chlorophyll <u>a</u> at the starting of selected time interval;

And $t_1 - t_2 =$ Time that has passed between a chosen time of day.

Based on total biomass, the specific growth rate (μ/day) of cultured spirulina

SGR $(\mu/day) = In (X_1 - X_2)/t_1 - t_2$

Where, X_1 = Total biomass at the conclusion of the chosen time frame;

 X_2 = Total biomass at the start of selected time interval;

and t_1 - t_2 = Time that has passed between a chosen time of day.

Statistical analysis

In order to determine the dry cell weight, proximate composition of spirulina, total biomass, chlorophyll a, and specific growth rates for each of the four treatments, one method of analysis was used. ANOVA, or analysis of variance and their noteworthy variations employing the Duncan's New Multiple Range (DNMR) test after Turkey's test (Zar, 1984).

Results

Measures of spirulina growth

Optical density of media contained spirulina

Spirulina was present in the medium's optical density (OD) was 0.034 ± 0.002 on the first day and tended to increase up to 8th day (0.285 ± 0.085) and then decreased gradually at the end of the experiment when cultured in supernatant of 25% DRLF (Fig. 1). It was reached at peak on 12th day (2.207 ± 0.103) and then decreased up to 18th day (1.322 ± 0.096) when spirulina cultured in supernatant of 50% DRLF (Fig. 1). In the medium with 75% DRLF supernatant, the greatest value (1.324 ± 0.062) was discovered on the 12th day of the experiment and continued to decline until the 18th day. It was recorded (2.665 ± 0.114) in Kosaric medium on 10^{th} day and then went down up to 18^{th} day (0.956 ± 0.093) of experiment (Fig. 1).

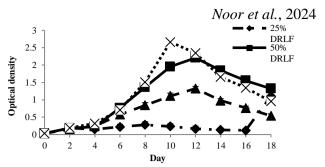


Figure 1. The optical density of three distinct digested rotten ladies fingers and KM were found to contain *S. platensis* at mean values. Standard errors are shown by vertical bars.

Spirulina's weight in cells of spirulina

Cell weight of spirulina increased from initial day (0.0022 \pm 0 mg/L) up to 12th day (2.738 \pm 0.177 mg/L) of culture in 25% DRLF and then dropped to 18th day (1.678 \pm 0.135 mg/L) of experiment (Fig. 2). Highest cell weight of spirulina was found to be 13.785 \pm 0.643 mg/L when grown in medium of 50% DRLF on 12th day of culture and then went down up to 18th day (Fig. 2). Cell weight of spirulina increased from initial day (0.0022 \pm 0 mg/L) up to 12th day (10.354 \pm 0.233 mg/L) of culture in 75% DRLFM which followed definite trend of growth (Fig. 2). Highest cell weight of KM contained spirulina was 16.452 \pm 0.78 mg/L on 10th day (6.782 \pm 0.28 of experiment (Fig. 2).

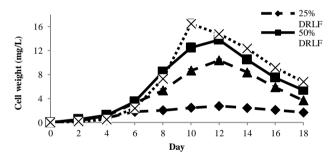


Figure 2. Mean values of cell weight (mg/L) of *S. platensis* grown in supernatant of three digested rotten ladies finger, and Kosaric medium. Vertical bars represent standard errors.

Chlorophyll <u>a</u> of spirulina

Chlorophyll <u>a</u> of spirulina increased from first day (0.0015 ± 0 mg/L) up to 12th day (2.121 ± 0.087 mg/L) of culture of 25% DRLFM and then dropped to 18th day (1.221± 0.077 mg/L) of experiment (Fig. 3). However, chlorophyll <u>a</u> of spirulina cultured in 50% DRLFM was reached at peak on 12th day (11.425 ± 0.46 mg/L) and then went down up to 18th day of experiment (Fig. 3). Chlorophyll <u>a</u> of spirulina grown in 75% DRLFM was higher (8.564 ± 0.332mg/L) on 12th day than other day and then dropped to 18th day (2.235±0.432 mg/L) of experiment (Fig. 3). The highest chlorophyll <u>a</u> content of spirulina grown in KM was 14.86 ± 0.462 g/L on the tenth day of the experiment, and it dropped until the eighteenth day (5.232 ± 0.326 mg/L) (Fig. 3).



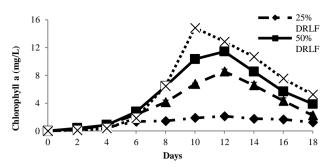


Figure 3. S. platensis cultured in the supernatant of three distinct DRLFM and KM was measured for mean amounts of chlorophyll <u>a</u> (mg/L). Standard errors are shown by vertical bars.

Total spirulina biomass

On the twelfth day of growth, compared to the previous days of the experiment, a higher total biomass (mg/L) of spirulina produced in all the media was observed (Fig. 4). The highest total biomass of spirulina grown in the culture of 50% DRLFM was recorded 765.475 \pm 30.12 mg/L on 12th day which was decreased up to 18th day (259.223 \pm 42.68 mg/L) of culture during the experiment (Fig. 4). Once more, the total biomass of spirulina grown in a 75% DRLFM culture grew from the experiment's first day (0.1005 \pm mg/L) to day 12th (573.788 \pm 24.2 mg/L), and subsequently dropped until day 18 (149.745 \pm 21.25 mg/L) (Fig. 4). The highest total biomass of spirulina cultured in Kosaric medium was found to be 995.352 \pm 26.96 mg/L on 10th day and then dropped to 18th day (350.544 \pm 20.64 mg/L) during the experiment (Fig. 4).

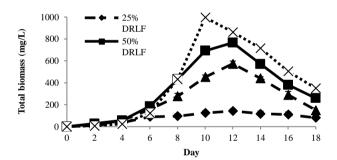


Figure 4. The average total biomass (mg/L) of *S. platensis* cultured in KM and the supernatant of DRLFM was measured. Standard errors are shown by vertical bars.

Comparison of spirulina growth factors Optical density of media contained spirulina

Spirulina (*S. platensis*) included in KM had an optical density that was noticeably (P <0.05) greater than that of supernatant of 50% DRLF followed by 75 and 25% DRLF (Table 4). There were significant (P < 0.05) differences among the optical density of media of 50, 75 and 25% DRLF during the research.

Cell weight

Spirulina (*S. platensis*) cultivated on Kosaric media was shown to have the highest cell weight (mg/L) (Table 4). Table 4 shows a substantial (P < 0.05) difference in cell weight between spirulina grown in Kosaric media and that cultured in the supernatant of 50% DRLF medium, followed by 25% and 75% DRLF. There were significant (P > 0.01) differences between the spirulina cells cultured on 50, 25, and 75% DRLF medium.

Chlorophyll a of spirulina

Spirulina cultivated in KM had significantly (P <0.05) more chlorophyll <u>a</u> (mg/L) than spirulina cultured in 50% DRLFM supernatant followed by 75 and 25% DRLF media (Table 4). There were significant differences among the chlorophyll <u>a</u> of spirulina grown in supernatant of 50, 75 and 25% DRLFM during the research.

Total spirulina biomass

Total biomass (mg/L) of spirulina cultured in KM was significantly (P < 0.05) higher than that of spirulina grown in supernatant of 50% DRLFM followed by 75 and 25% DRLFM (Table 4). There were significant differences discovered in the spirulina cultivated in the supernatant of 50,75, and 25% DRLFM overall biomass.

Table 4. Comparing total biomass, chlorophyll <u>a</u>, and cell weight of *S. platensis* grown in supernatant of 25, 50, 75% DRLFM and KM throughout the culture period.

Parameters	T ₁ (25% DRLF)	T ₂ (50% DRLF)	T ₃ (75% DRLF)	T ₄ (KM)
Optical density	0.263 ±0.096	1.146 ± 0.296	0.657±0.232	1.174 ± 15.552
Cell weight (mg/L)	1.631 ± 0.251	6.317±1.581	4.694±1.310	6.977±87.738
Chlorophyll <u>a</u> (mg/L)	1.208 ± 0.135	5.025±1.153	3.598±0.941	5.993±63.039
Total biomass	80.892±0.283	336.672±1.952	241.069±1.734	401.554±116.205

* Chlorophyll <u>a</u> x 67 equals total biomass (Vonshak and Richmond, 1988). At the 5% probability level, figures in common letters do not differ appreciably.

Discussion

With the exception of KM, *S. platensis* was observed to grow more efficiently in the supernatant of 50% DRLFM than in that of 25% and 75% DRLFM. This variance may result from the content and nutrient concentrations of different media. *S. platensis* exhibited the best growing performance in controlled KM. It might have occurred as a result of the nutrients' appropriateness and availability for the species' growth. <u>Hossain (2005)</u> reported a maximum cell growth 724.50 mg/L (dry wt. basis) in KM followed by 410.90, 521.00 and 691.50 mg/L in 60, 80 and 100% UWM, respectively on the 8th day of culture. In 4.0 g/L digested poultry waste, <u>Satter (2017)</u> found that the cell weight and chlorophyll a content of *S. platensis* were significantly (P < 0.05) greater than in other media where temperature, aeration, and light intensity were important factors in the culture system. On the other hand 25% and 75% DRLFM showed lower growth performance of *S. platensis* in relation



to 50% DRLFM which may be due to suitable nutrient concentration and dilution in the medium. These results bear some resemblance to the current findings. The current findings and those of Khan (2003) and Habib (1998) are essentially comparable. In the present study, the highest total biomass of spirulina grown in the culture of 25, 50 and 75% of DRLFM was 142.107 ± 19.91 mg/L,765.475 ± 30.12 mg/L and 573.788 \pm 24.2 mg/L on 12th day, respectively. The chlorophyll a content of inoculated S. platensis was 0.0015 mg/L which attained a high content of 14.856 mg/L which cultured in KM in 10th day and 11.425 mg/L in 50% DRLFM at the 12th day of culture. The findings of Phang et al. (2000), Habib et al. (2003), and Satter (2017) are mostly consistent with these findings. When compared to KM on the 12th day of culture, the supernatant of 50% digested rotting ladies fingers displayed the largest optical density, which is consistent with the findings of (Habib et al. 1997, 2003, Satter 2017). Satter (2017) found that the supernatant of 4 g/L digested poultry waste (DPW) for culture of S. platensis, gave higher growth performances than other two different media but it was little bid lower than the growth of spirulina in KM. However, when cultivated in KM and the digested rotting ladies finger supernatant, it shown nearly identical growth characteristics. Phosphate-phosphorus supply has been thought to be crucial for plankton formation in cultured media. Phosphate-phosphorus was found to be higher on the first day of culture and to be at its lowest on the tenth. The first day had the lowest optical density, and the twelfth day had the highest.

Conclusion

In 50% digested rotting ladies finger media, Spirulina progressed more quickly than in other concentrations. Owing to its superior nutritional status, it may be fed to fish and shrimp. The advantages of cultivating *Spirulina platensis* for both health and economic reasons need to be made more widely known. Fish meal can be substituted with spirulina as an ingredient in fish feed. Lab-scale cycles must be accelerated for commercialization in order to increase yields and productivity. Public- private partnerships ought to take the lead in terms of national industrial production. It is a more enticing power source because it is less polluting and beneficial to the environment. The findings of this research will reduce the feed production cost and it will open a new era for aquaculture.

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