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# Investigating the Impact of Nitrogen Addition in Growing Media using Liquid Organic Fertilizer on Pigment and Protein Content of Spirulina sp.

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## ABSTRACT

*Spirulina sp. is a highly promising microalgae with substantial protein and pigment content, holds great economic potential across various industries. However, the cultivation of Spirulina sp. often faces the challenge of high production costs, particularly in media formulation. To address this issue, the development of more affordable alternative media formulations is crucial. One potential approach involves utilizing a combination of liquid organic fertilizer derived from Canna indica waste and urea for cultivation. This study aims to determine the optimal ratio of the fertilizers mixture that influences protein and pigment synthesis. The study design employed a completely randomized design with seven treatment groups along with the control. The result shows that the treatment 2A (20 mL/L organic fertilizer; 125 ppm urea) demonstrated the highest dry biomass weight. Treatment 2C (22 mL/L organic fertilizer; 150 ppm urea) exhibited the best specific growth rate and carotenoid production, with the carotenoid production significantly differing from the control. Treatment 1A (20 mL/L organic fertilizer; 100 ppm urea) yielded the highest levels of chlorophyll and phycobiliproteins. Treatment 1B (20 mL/L organic fertilizer; 125 ppm urea) resulted in the highest protein production.*

**Keywords*: Alternative Medium, Pigment, Liquid Organic Fertilizer, Nitrogen, Protein, Spirulina sp.***

## 1. INTRODUCTION

Microalgae are microscopic algae that contain chlorophyll and other photosynthetic pigments and exist in water. Microalgae can be found in all sun-exposed habitats [1], spirulina sp. is a type of microalgae that has numerous health advantages for humans. The protein content of Spirulina sp. is considerable, accounting for up to 46-63% of its dry weight. Similar to the protein content of meat, which is 71-76% of its dry weight, and greater than the protein content of soybeans, which is 40% of its dry weight [2]. In addition to containing a high protein content, Spirulina sp. It also contains various pigments, such as chlorophyll, phycocyanin, and beta-carotene that are beneficial in the pharmaceutical industry that is, as additives that provide various effects, such as antiviral, antibacterial, anti-tumor, neuroprotector, and protection on the cardiovascular system. Spirulina sp. pigments are also very valuable economically [3].

The growing substrate has a significant impact because it provides the nutritional components that Spirulina sp. needs to survive. The quantity of biomass production and key compounds in the microalgae will be significantly influenced by the presence of the right nutrients in the growing medium. One of the macro components required to promote the growth of Spirulina sp. is nitrogen. Because, according to study, nitrogen will have an impact on how much pigment is produced by these microalgae [4].

Based on earlier research, Spirulina sp. successfully grown very well in a medium containing organic fertilizer from fermented

Canna indica shoots waste from the Canna indica flour manufacturing industry [5], proteins and pigments, which are rich in Spirulina sp. product content, have not been produced optimally, so optimization is required. One of the optimization processes is to change the nitrogen content of the media, taking into account the significance of these elements' impact on the production of proteins and pigments in microalgae. The use of liquid organic fertilizer (LOF) derived from Canna indica shoots in the culture medium of Spirulina sp. is a positive step because it can lower production costs while also reducing Canna indica shoots waste, allowing the Canna indica flour industry to achieve zero waste. The addition of nitrogen to LOF Spirulina sp. growing media is anticipated to be an alternative fertilizer to promote Spirulina sp. growth, primarily for protein and pigment production. The goal of this research was to determine the best concentration of additional nitrogen in organic fertilizer media derived from canna canopy waste for use as a growing medium for Spirulina sp., thereby boosting protein and pigment production.

## 2. METHOD

### *A. Materials and Tools*

The tools used in this study were 1.5 L bottles, aeration hose, 8W Philips TL Daylight lamp, aerator, Sedgewick Rafter Cell, light microscope, oven, analytical balance, filter unit, desiccator, water bath, UV-

VIS spectrophotometer, 10 mL macropipette, P-1000 micropipette, P-200 micropipette, 10 mL clear tip, 1000 µL blue tip, 200 µL yellow tip.

The materials used in this study were Spirulina sp. culture, Spirulina Medium, sterile distilled water, liquid organic fertilizer (POC) canna canopy, urea fertilizer, Bovine Serum Albumin (BSA), liquid nitrogen, folin-phenol, biuret reagent, NaNO3 0.1 M, aluminum foil.

### *B. Production of Liquid Organic Fertilizer*

The method for making LOF refers to research by Pardosi et al.[6]. The materials used to make LOF are Canna indica shoots, brown sugar, EM4 for plants, and clean water. As much as 3.6 kg of Canna indica shoots finely chopped and then put into a bucket. 9.1 grams of brown sugar, 36 mL of EM4 for plants, and 5,472 L of water are added to a bucket filled with Canna indica shoots. The mixture will then be fermented for 14 days in the dark, at 30-35°C, and given a circulation system using an aerator. Fertilizer that has been fermented will be filtered to take only the liquid fertilizer and then sterilized using an autoclave.

### *C.* Spirulina *sp. Propagation*

*Spirulina* sp. culture plants are first propagated to have sufficient cell density, namely 10 x $10^4$ units/mL [5]. Spirulina medium is used to propagate cultures [7, 8, 9]. *Spirulina* sp. was propagated in 5 liter containers and then cultivated under lighting circumstances of 2500 lux from light, with a light duration of 12:12 light to dark, room temperature 20°C, and aeration. This propagation culture was produced for 7 days.

### *D. Treatment Culture of* Spirulina *sp.*

The media used in the treatment culture were liquid organic fertilizer media from canna canopy waste and urea fertilizer. The concentration of urea fertilizer used in this study will be given based on the research of Amanatin & Nurhidayati [10], namely with concentrations of 100, 125 and 150 ppm [10]. The combination of LOF and urea concentrations is presented in Table 1. The combination of LOF and urea concentrations is presented in Table 1. The culture will be grown under the light conditions of a Philips 8W TL Daylight lamp with a light emission of ±2500 lux, a duration of 12:12 hours in the dark, given aeration with an aerator, and cultivated until it entered the death phase.

Table 1. Fertilizer Media Concentration In Growing Media

| **Treatment** | ***Canna indica* LOF Concentration (mL/L)** | **Urea Addition Concentration (ppm)** |
|---|---|---|
| Control | 18 | 0 |
| 1.A | 20 | 100 |
| 1.B | 20 | 125 |
| 1.C | 20 | 150 |
| 2.A | 22 | 100 |
| 2.B | 22 | 125 |
| 2.C | 22 | 150 |

### *E. Sample Extraction*

Sample extraction was carried out by filtering 10 mL of *Spirulina* sp. using filter unit to take only the solids. Then, the sample will be

dried briefly in the air at room temperature. The sample will be crushed using liquid nitrogen. The extracted samples are used for pigment and protein assays.

*F. Growth Analysis*

Growth was observed every 2 days for 16 days. The growth parameters to be measured were the growth rate and weight of *Spirulina* sp. dry biomass. Specific growth rate (μ) is analyzed by the following formula [11]:

$$K' = \frac{ln\left(\frac{N_{t_2}}{N_{t_1}}\right)}{t_2 - t_1}$$

$$\mu = \frac{K'}{ln\, 2}$$

K' = divisions time$^{-1}$
μ = specific growth rate (g/L/day)
$N_1$ = dry biomass at $t_1$ (g/L)
$N_2$ = dry biomass at $t_2$ (g/L)
$t_1$ = time 1 (day)
$t_2$ = time 2 (day)

The dry weight of *Spirulina* sp. is determined by the following formula [11]:
Dry Weight (DW) = (weight of filter paper + algae) - (weight of filter paper).

*G. Analysis of Chlorophyll and Carotenoid Content*

A total of 10 mL of *Spirulina* sp. culture was collected and extracted. The centrifuge container was filled with 10 mL of absolute methanol. The pellet-containing tube was wrapped in aluminum foil and put in a 70°C water bath for 10 minutes. The supernatant was obtained by vortexeing the material and then filtering it. A spectrophotometer will be used to detect the supernatant at 480 and 665 nm. Chlorophyll content of *Spirulina* sp. was calculated using method by Ritchie [12], whereas its carotenoid content was determined using method by Strickland dan Parsons [13].
Chlorophyll-a (μg/mL) = 12, 9447 × $A_{665}$
Carotenoid (μg/mL) = 4 × $A_{480.}$

*H. Analysis of Phycobiliproteins Content*

The extracted *Spirulina* sp. was then ground with 0.1 M NaNO3, pH 6.8. Samples will be frozen and thawed repeatedly to increase extraction capability. *Spirulina* sp. extract then filtered to obtain the supernatant. Samples were measured at wavelengths of 650 nm, 620 nm, and 565 nm. Phycobiliprotein concentration will be measured based on the following formula [14]:
C-Phycoerythrin = 0.00251 $A_{650}$ - 0.0321 $A_{620}$ + 0.0787 $A_{565}$
C-Phycocyanin = -0.0911 $A_{650}$ + 0.166 $A_{620}$
Allophycocyanin = 0.159 $A_{650}$ - 0.0410 $A_{620}$

*I. Analysis of Protein Content*

Analysis of protein content was based on the Lowry method [15], modified by Dorsey *et al.* [16], the analysis was started by preparing standard proteins using Bovine Serum Albumin with concentrations of 0, 50, 100, 150, 200, 250, 300, and 350 μg. The sample used in protein measurement is a sample that has been extracted. The sample was added 1 mL of biuret reagent and homogenized, then transferred into a 10 mL centrifuge tube. Biuret reagent was added again as much as 1 mL and homogenized. A total of 3 mL of biuret reagent was added to the centrifugation tube. After adding the Biuret reagent, the tube containing the protein sample will be left for 20 minutes. After that, 0.5 mL of folin-phenol reagent was added, then vortexed. Samples were kept for 10 minutes. The sample was then filtered using filter paper to separate the liquid and solid phases, then tested calorimetrically using spectrophotometer with an absorbance of 660 nm. The results of the absorbance readings of protein standards were used to determine the protein content of the sample from the standard curve. Calculation of the percentage of protein based on the following formula [15]:

$$Protein\ content\ (mg.L^{-1}) = \frac{Protein\ value\ from\ standard\ curve}{Volume\ of\ digested\ material \times Culture\ volume}$$

*J. Statistical Analysis*

Data processing was carried out using SPSS 16.0 with the One-Way Anova program with a 95% confidence interval.

## 3. RESULT AND DISCUSSIONS

A. Growth Analysis

Based on the research conducted, each growth phase occurs with a different duration between treatments (Figure 1). The lag phase in the control and 1C treatment groups occurred on day 0 to day 2, this can be seen from the growth chart which decreased in the first 2 days. The lag

phase in the other treatment groups occurred only on day 0, meaning that the lag phase occurred in less than two days and immediately entered the logarithmic phase, marked by an upward graph in the first two days. The logarithmic phase in the control group only lasted 6 days starting on the 2nd day, whereas the treatment group had a longer logarithmic phase, which was between 9 to 15 days, which was possible from the first day of culture to the 14th and 16th days. Treatment groups 1A and 1B had a longer duration of the logarithmic phase compared to the other treatment groups. The death phase in the control group occurred faster than the treatment group, namely on the 14th day. Most of the treatment groups experienced a death phase on the 16th day, except for treatment groups 1A and 1B which were still in the logarithmic phase.

The duration of the lag phase is affected by the complexity of the nutrition. Generally, microalgae experience a longer lag phase when grown on media with nitrogen limitation [17], it is believed that the lengthier lag period in the control group results from the media's lower nitrogen content when compared to the treatment group. A longer lag period, however, could happen for a variety of reasons. One of the elements that can influence how long the lag phase lasts is the capacity of microalgae to adapt to a new environment [18].

Based on the graph shown in Figure 1., Spirulina sp. cultivation in LOF media with the addition of urea on average continued to gain dry weight until day 14 and began to decline on day 16. However, in contrast to Spirulina sp. cultivation in the control group (K) that was not given the addition of urea, dry weight loss occurs faster, that is, on the 14th day. The use of urea as a source of nitrogen can prolong the logarithmic phase and increase the dry weight of Spirulina, so that its growth age can be longer than the use of LOF medium only [19], the highest amount of dry biomass produced by Spirulina sp. at treatment 2A on day 14, which was 1.12 g/L.

Based on statistical tests, the weight of dry biomass between treatments had a noticeable difference on day 0 and day 6. Significant differences on day 0 were shown by the control group over the entire treatment group, as well as 1B against 2A and 2B. Significant differences on day 6 were shown by the control group against 1C.

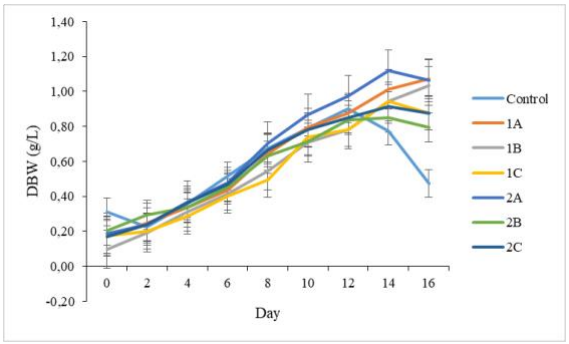


Figure 1. Growth Curve of Spirulina sp.

The specific growth rate of each group has varying values because the duration of the logarithmic phase varies. The 2C treatment group had the best specific growth rate value of 0.211 g/L/day (Table 2). Based on statistical tests, there was no significant difference in specific growth rates between control and treatment.

Table 2. Dry Biomass Weight and Specific Growth Rate of *Spirulina* sp.

| Treatment | Day | DBW (g/L) | SGR (g/L/day)* |
|---|---|---|---|
| Control | 12 | 0.90 ± 0.23 | 0.130 ± 0.05[a] |
| 1A | 16 | 1.07 ± 0.14 | 0.139 ± 0.01[a] |
| 1B | 16 | 1.03 ± 0.47 | 0.168 ± 0.07[a] |
| 1C | 14 | 0.94 ± 0.33 | 0.171 ± 0.05[a] |
| 2A | 14 | 1.12 ± 0.67 | 0.164 ± 0.03[a] |
| 2B | 14 | 0.85 ± 0.45 | 0.164 ± 0.03[a] |
| 2C | 14 | 0.91 ± 0.46 | 0.211 ± 0.09[a] |

* The same letter indicates no significant differences

B. Pigment Content

Chlorophyll and Carotenoids Measurement of chlorophyll pigment content began when Spirulina sp. was at the peak of the logarithmic phase, which is on the 12th to the 14th day. The type of chlorophyll calculated is chlorophyll a. Chlorophyll-a is the main and only photosynthetic pigment possessed by Cyanophyta such as Spirulina sp. [20]. The highest chlorophyll production was shown in the 1A treatment (LOF 20 mL/L; Urea 100 ppm) and 2C (LOF 22 mL/L; Urea 150 ppm/L), but both peak on different days. The 2C treatment reached its highest value on day 12 with a value of 1.586 µg/mL, while treatment 1A reached its

highest value on day 14 with a value of 1.643 µg/mL. Based on the statistical tests conducted, the 2C treatment group that had the highest achievement on day 12 did not differ significantly from the control and the entire treatment group on day 14 did not differ significantly from the control.

Nitrogen is a fundamental component of chlorophyll formation, thus chlorophyll production depends on the amount and type of nitrogen used in the medium [21], chlorophyll production in the 1A treatment continued to increase from day 12 to day 14. The production of the pigment chlorophyll is generally produced in the logarithmic phase, while in the stationary phase Spirulina produces other metabolites [22], when compared to controls that did not use urea as an additional nitrogen source, chlorophyll production was lower. Chlorophyll production in the control group also decreased on day 14.

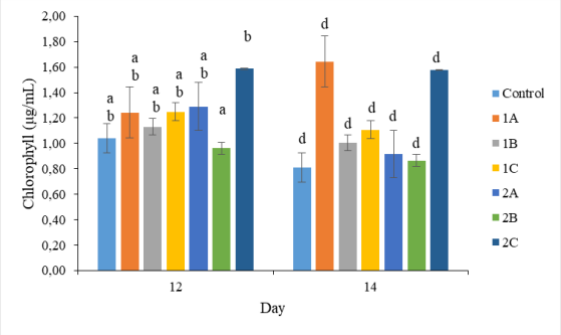


Figure 2. Chlorophyll content of Spirulina sp.
* The same letter indicates no significant differences.

Measurement of carotenoid pigments is carried out at a time when Spirulina sp. is at the peak of the logarithmic phase on the 12th to the 14th day. Based on the results obtained, the highest carotenoid content was produced by the 2C treatment group on day 12, which was 0.401 µg/mL. Based on the results obtained from statistical tests, the 2C treatment group had significant differences against the Control group on day 12, while on day 14 there was no significant difference between treatments (Figure 3).

The production of carotenoids depends on the availability of carbon, in contrast to chlorophyll which depends on the presence of nitrogen. Carotenoids can still be produced even if they live in an environment that is experiencing nitrogen deficiency. Carotenoids do not need too much nitrogen for their synthesis and rather need Acetyl-CoA as a precursor that promotes their production. [23]. El-Baky conducted research by growing Spirulina in environments with different nitrogen concentrations. Based on the results of the study, carotenoids are produced better when in an environment that experiences nitrogen deficiency than environments with optimum or excess nitrogen amounts [24], urea is a combination compound of carbon and nitrogen, thus the higher the amount of urea used, the higher the amount of carbon available to support the production of carotenoids [25, 26].

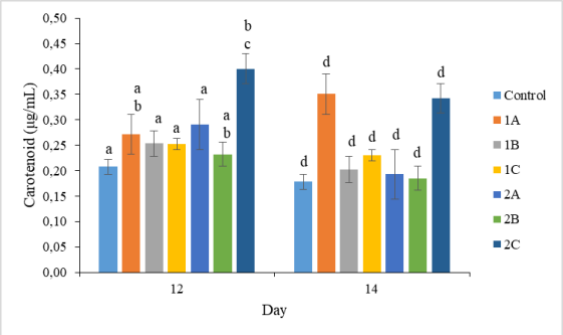


Figure 3. Carotenoid content of Spirulina sp.
* The same letter indicates no significant differences.

Phycobiliproteins the phytobiliprotein pigments calculated in this study include phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC) obtained at the peak of the logarithmic phase. Based on the calculation results, each phycobiliprotein pigment was produced the highest by the 1A treatment group on day 14. The PE, PC, and APC content of the 1A treatment group was 0.0038 mg/mL, 0.0053 mg/mL, and 0.0083 mg/mL respectively (Figure 4, 5, and 6). The most widely produced pigments are allo-phycocyanin pigments, which are phycobiliproteins that are absorbed at wavelengths of 598, 629, and 650 nm, and develop at wavelengths of 660 nm [14]. On the 12th day of treatment 2A had the highest phycobiliprotein content but did not differ significantly from control. Significant differences were seen in groups 1A and 2B, that is, the groups with the lowest content.

The production of phytobiliproteins depends on the presence of assimilable nitrogen. Nitrogen in the medium will strongly support the production of phytobiliproteins in Spirulina to a certain extent of concentration. If it has reached the maximum absorption limit, excess nitrogen can no longer be tolerated and will

become toxic [27], phycobiliproteins are quite sensitive to the presence of nitrogen. Most Cyanobacteria, including Spirulina sp., show that the production of phytobiliproteins becomes inhibited when in a medium with too high nitrogen content [28], urea will be degraded into ammonia, a type of nitrogen that will be absorbed by Spirulina sp. Excess ammonia can damage cells, thus inhibiting the formation of pigment [29], based on the results obtained in this study, phytobiliprotein production was not good in the treatment group other than treatment 1A. Although the amount of phytobiliprotein has increased, the number of increases is not as good as the treatment group 1A.

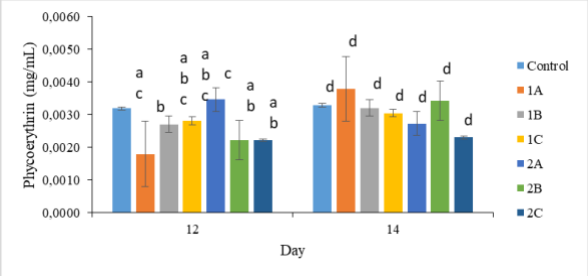


Figure 4 Phycoerythrin content of Spirulina sp.
* The same letter indicates no significant differences.

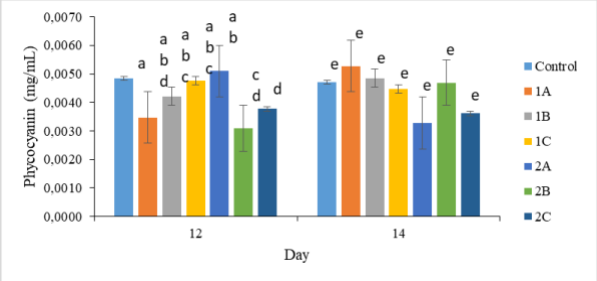


Figure 5 Phycocyanin content of Spirulina sp.
* The same letter indicates no significant differences.

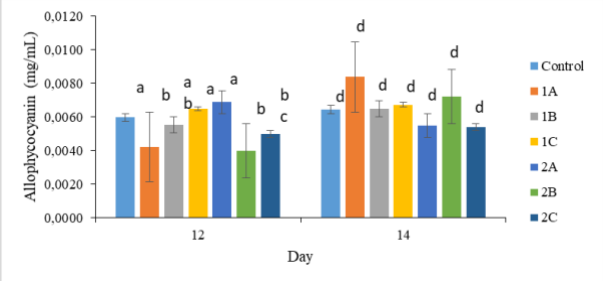


Figure 6 Allophycocyanin content of Spirulina sp.
* The same letter indicates no significant differences.

B. Protein Content

Measurement of proteins is carried out at a time when the growth of Spirulina sp. enters the final logarithmic phase close to the stationary phase, that is, on the 10th, 12th, and 14th days. The highest protein content value was 1.44 mg/L or equivalent to 0.18% of dry weight in sample 1B with a LOF media composition of 20 mL/L Canna indica waste and 125 ppm urea. These results showed that the presence of nitrogenous elements from the POC of cannabis and urea waste played a very important role in protein production in Spirulina sp. Protein production at treatment 1B, on the 12th day, was 130% higher than control and significantly different (Figure 7).

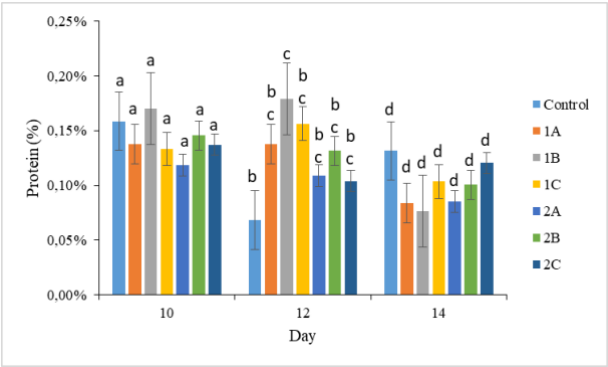


Figure 7 Protein content of Spirulina sp.
*The same letter indicates no significant differences.

High biomass production doesn't always align with high protein production either. Research conducted by Pandey & Tiwari compared the production of Spirulina sp. protein grown in Zarrouk media at different pH conditions, light intensity, and aeration. [30], the result obtained is the highest protein production produced by cultures with low biomass production. This can happen because the pH changes due to the periodic addition of bicarbonate. The resulting dry biomass and protein were 0.68 g/500 mL and 58.5%, respectively. The results of the study conducted by Pandey & Tiwari can support the results obtained in this study, namely factors that affect protein production not only nutrients but also environmental factors, such as pH [30].

Generally, when cultivating microalgae, pH changes occur when entering the exponential phase and occur uniformly. Changes in pH can also be triggered by aeration factors. Aeration is used to meet the needs of CO2 and also to stir Spirulina sp. thus that nutrients and light can be

perfectly distributed [31], however, an aeration rate that is too high can convert pH levels into acids due to an increase in the rate of CO¬2 fixation. An increase in the rate of CO¬2 fixation leads to reduced activity of extracellular enzymes that can interfere with photosynthesis and the formation of biomass and its metabolites [32], during the study, neither pH nor aeration was measured periodically, however it is likely that changes in pH and aeration flow rate have an impact on protein formation.

## C.CONCLUSION

The use of liquid organic fertilizer of Canna indica waste and urea in Spirulina sp. cultivation has an influence on the production of biomass, protein, and pigment. The best dry biomass weight was generated at 2A treatment and did not differ significantly from control. The specific growth rate and pigment production of carotenoids obtained the best results when cultivated at 2C treatment, but only carotenoid production showed significant differences to the controls. The highest production of chlorophyll and phytobiliprotein pigments was obtained at treatment 1A and did not differ significantly from control. The highest protein content was produced by the 1B treatment and did not differ significantly from the control.

More research is needed to determine the concentration of the combination of Canna indica waste liquid organic fertilizer and urea. LOF used in the cultivation of Spirulina sp. and periodically measuring pH, aeration, and lighting measurements. A good understanding of the methods used is very necessary to minimize errors.

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