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Short Communication

Effect of Aquaculture Wastewater and Zarrouk in Increasing Biomass, Protein, and Carotenoids Levels of *Spirulina platensis*

Anisa Millah Taqiyyah[1], Yenny Risjani[2]*, Asep Awaludin Prihanto[3], Uun Yanuhar[4], and Mohammad Fadjar[5]

[1]Department of Aquaculture, Faculty of Fisheries and Marine Science, Brawijaya University, Malang. Indonesia
[2]Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Brawijaya University, Malang. Indonesia
[3]Department of Fisheries Product Technology, Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang. Indonesia
[4]Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Brawijaya University, Malang. Indonesia
[5]Department of Aquaculture, Faculty of Fisheries and Marine Science, Brawijaya University, Malang. Indonesia

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*) Corresponding author:
E-mail: risjani@ub.ac.id

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Abstract

Increased productivity of *Spirulina* sp. in the form of high protein, carotenoids, and biomass content can be achieved by improving its nutrient supply. Inorganic fertilizers are nutrient sources, which are generally used in the culture of this organism on laboratory and industrial scale, but there are several drawbacks, including their high costs and limited availability. Several studies have also reported the use of zarrouk fertilizer as a standard culture medium for *Spirulina platensis*. Therefore, this study aims to determine the effect and the best concentration of fish culture wastewater treatment in *Spirulina platensis* culture using biomass, protein content, and carotenoid pigments as indicators. A two-factorial completely randomized designs (CRD) was used in this study, where the factors include the dose of organic waste and Zarrouk fertilizer. The microalgae samples, namely *S. platensis* were cultured using fresh water. This research consists of two factors. The first factor is the dose of organic waste, and the second factor is the dose of Zarrouk fertilizer. The wastewater treatment consisted of 0, 2, 4, and 6 ml/L, while Zarrouk dosages include 0, 0.5, and 1 ml/L. The best results were obtained from the sample treated with 6 ml/L aquaculture wastewater without the addition of Zarrouk. Furthermore, this treatment gave biomass production of 0.781 g/L, 50.441% protein, and 1.246 mg/L carotenoids. Based on the results, *S. platensis* culture can be carried out using fish culture wastewater without the addition of Zarrouk fertilizer.

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1. Introduction

Spirulina is a blue-green microalgae (Soni *et al*., 2017) that is rich in essential amino acids, functional lipids, complex carbohydrates, vitamins, and minerals (Ricigliano and Simone-Finstrom, 2020). It also contains high amounts of micro and macronutrients; hence, the microalgae can be used in health care as well as the food industry as a protein and vitamin supplement (Soni *et al*., 2019), thereby increasing its commercial interest (Salunke *et al*., 2016). *S. platensis* cultures are often carried out with analytical fertilizers, such as Zarrouk, but they are relatively expensive and have limited availability (Sopandi *et al*., 2020). To overcome this problem, other nutrient sources that are more economical, can produce high biomass as well as increase the productivity of *S. platensis* are needed. Furthermore, Zarrouk fertilizer is a standard medium for *S. platensis* because it contains completed and balanced macro and micro elements for the survival and biochemical content of the *S. platensis*. A previous study revealed that the carbon, nitrogen, and protein content of *S. platensis* decreased in a combination of Zarrouk and Urea media (Al Mahrouqi *et al*., 2022).

Another alternative fertilizer that can be used as culture media *Spirulina* sp. is organic waste obtained from various activities, including the food industry (El-Kassas *et al*., 2015), agriculture (Taufiqurrahmi *et al*., 2016), tofu industry (Hadiyanto *et al*., 2019), paper (Setiawan *et al*., 2019), and herbal medicine (Hadiyanto *et al*., 2019). Furthermore, the wastewater can serve as a source of nutrition due to its nitrate and phosphate content (Taufiqurrahmi *et al*., 2016). These findings are consistent with Wuang *et al*. (2016) that the specific growth rate of *Spirulina* increased in the culture media. A previous study also revealed that the biomass of the microalgae increased in domestic wastewater with 80% nitrate and 93% phosphate uptake (Zhou *et al*., 2017). Nogueira *et al*., (2018) revealed that the cell density of *S. platensis* could increase in fish farming wastewater media from $17x10^4$cells/ml to $40x10^4$cells/ml. Cardoso *et al*. (2020) showed that the best treatment to produce the highest biomass, carbohydrates, lipids, and carotenoids in Spirulina was a combination of this media and 25% Zarrouk. This finding is in line with Cardoso *et al*. (2021a), where it improved the uptake of sulphate (94%), phosphate (94%), bromine (97%), and Chemical Oxygen Demand (COD) (90%). Cardoso *et al*. (2021b) also revealed that the pigments, namely chlorophyll-a, chlorophyll-b, total carotenoids, and phycocyanin of *Spirulina* increased in aquaculture wastewater media with the addition of Zarrouk. *Spirulina* cultured in industrial wastewater media had an NH_4 and nitrogen absorption of 94% and 82%, respectively (Han *et al*., 2021). Furthermore, Ashour *et al*. (2021) stated that the microalgae were able to utilize NH_4 to form biomass. Wongsnansilp and Phinrub (2022) showed that *Spirulina* grown in fish farming wastewater media has a higher cell density compared to others cultured in purified domestic medium. A study by Napolitano *et al*. (2022) revealed that *S. platensis* cultured on aquaculture wastewater media had a lower impact on the environment compared to others on a standard medium.

Previous studies on the use of wastewater for *S. platensis* culture focused on its effect on the microalgae's biomass. Discussion on the effect of wastewater as a source of nutrition, such as protein and carotenoids requires novelty studies due to the presence of various factors affecting the nutritional content of the waste. *S. platensis* requires media with a balanced nutrient content to obtain optimal biomass, protein, and carotenoid production. The nitrates used by this organism also need to be in an analytical and technical form for maximum absorption. Based on previous findings, there are limited studies on the use of aquaculture wastewater as a source of nutrients for *S. platensis*. Therefore, this research aims to determine the effect of fish culture wastewater on the biomass, protein, and carotenoids of *S. platensis*. The results are expected to reduce the use of analytical fertilizer without affecting the biochemical content of the microalgae as well as reduce the cost and increase the productivity of the culture.

2. Materials and Methods

2.1 Material

S. platensis used in this study was obtained from PSAL UB, and it was related to *Arthrospira* sp. taken from seawater. The study used fish culture wastewater and Zarrouk. The fish culture wastewater was obtained from a catfish rearing pond (*Clarias* sp.) measuring 1×2×1 m^3 with a density of 100 fish with a size of 10-15 cm. Furthermore, the samples were fed twice a day with floating pellets, containing 25-27% protein, 5% fat, 6% fiber, and 12% water content. The wastewater was treated with 1 ml/L of Probiotic Effective Microorganisms (EM4) for one week before it was added to the culture medium and sterilized using an autoclave. The wastewater used in this study contains nitrate, phosphate, and potassium at a concentration of 1.006, 0.804, and 0.672 mg/L, respectively. Rahman *et al*. (2022) revealed that a nitrate content of 1 ppm can increase the biomass of *Spirulina*. Additionally,

the Zarrouk fertilizer used was selected based on the recommendation of Madkour *et al.* (2012).

2.2 Method

2.2.1 Experimental design

This study used a factorial Completely Randomized Design (CRD) with 12 different treatments and three replications. The microalgae culture of *S. plantensis* was carried out using fresh water. Furthermore, this study consists of two factors, where the first was the dose of organic wastewater, namely 0 ml/L, 2 ml/L, 4 ml/L, and 6 ml/L. The second factor used was the dosage of Zarrouk fertilizer, which consisted of 0 ml/L, 0.5, ml/L, and 1 ml/L. In this experiment, the treatment contain a combination of aquaculture wastewater and Zarrouk, hence, a total of 12 was obtained. The treatment consisted of 0 aww + 0 Zarrouk (control negative), 0 aww + 0.5 ml/L Zarrouk, 0 aww + 1 ml/L Zarrouk, 2 ml/L aww + 0 Zarrouk, 2 ml/L aww + 0.5 ml/L Zarrouk, 2 ml/L aww + 1 ml/L Zarrouk, 4 ml/L aww + 0 Zarrouk, 4 ml/L aww + 0.5 ml/L Zarrouk, 4 ml/L aww + 1 ml/L Zarrouk, 6 ml/L aww + 0 Zarrouk, 6 ml/L aww + 0.5 ml/L Zarrouk, 6 ml/L aww + 1 ml/L Zarrouk. The Zarrouk treatments were indicated by the color difference on the bar graph, where the green, blue, and yellow colors represent concentrations of 0, 0.5, and 1 ml/l, respectively (Figure 2).

2.2.2 Cell growth

The density of *Spirulina* sp. was calculated using the cell concentration calculation method. Furthermore, the number of *Spirulina* sp. unit cells based on the spiral-shaped filament (helix), where one *Spirulina* unit is one wavelength consisting of one valley and one hill (Buwono and Nurhasanah, 2018). The tools used in this study include a Hemocytometer 0.1 mm and a microscope. Calculation of cell density based on the formula proposed by Armanda (2013):

$$\text{Number of cells (cells/ml)} = \frac{n}{\text{Amount of fields of view}} \times 25 \times 10^4 \quad \ldots\text{Eq (1)}$$

The specific growth rate was calculated using the following formula:

$$\mu = \frac{\ln Nt - \ln No}{t} \quad \ldots\text{Eq (2)}$$

where:

μ = specific growth rate (day^{-1})

Nt = population density at t (cells/ml)

No = initial population density (cells/ml)

t = time (days)

2.2.3 Biochemical test

The analysis carried out in this study includes tests of biomass, protein, and carotenoids. The *Spirulina* biomass test was performed based on the method proposed by Janssen *et al.* (1999). Furthermore, the measurement of biomass was carried out using GF/C filter paper which was heated in the oven at 105°C for two hours and weighed to determine its weight (A). A total of twenty-five milliliters of the sample was then filtered using a filter paper that had been oven-dried as well as a vacuum pump. The filter paper was dried for two hours at 105°C using an oven. It was then placed in a desiccator for 30-60 minutes, and then weighed to determine the weight (B). The biomass was calculated using the formula below:

$$\text{Dry weight/Biomass (g/L)} = \frac{(B-A) \times 1000}{V} \quad \ldots\text{Eq (3)}$$

where:

A = weight of filter paper (gr)

B = weight of filter paper + algae (gr)

V = volume (mL)

The protein test was carried out using the Lowry method with a chemical for detecting phenolic groups, such as the Folin-Ciocalteu reagent, as proposed by Lowry *et al.* (1951). The process started with the addition of 0.5 mL of 1 N NaOH into 0.5 mL microalgae suspension, followed by heating at 90°C for 10 minutes in a water bath and cooling. A total of 2.5 mL of reagent D was added to each tube containing microalgae suspension, and then homogenized until it was evenly distributed (vortex). Subsequently, 0.5 mL of Folin-Ciocalteau reagent was added to the mixture and homogenized until it was evenly distributed (vortex). The solution was then allowed to stand for 30 minutes at room temperature and the absorption was measured using a spectrophotometer with a wavelength of 750 nm. Calculation of protein content was carried out using the formula below (Antika *et al.*, 2021):

$$\%\text{Protein} = \frac{\frac{(OD-a)}{b}}{\text{Biomass} \times 1000} 100\% \quad \ldots\text{Eq (4)}$$

where:

OD = result of a spectrophotometer with a wavelength

of 750 nm
A = intercept in the regression equation of Bovine Serum Albumin (BSA) solution
B = slope in the regression equation of Bovine Serum Albumin (BSA) solution

The carotenoid content in *Spirulina* sp. was assessed using a Spectrophotometer based on the method proposed by Vo and Tran (2014) method. A total of 1 mL of the microalgae culture sample was centrifuged at 1000 rpm for five minutes. Subsequently, 3 ml of ethanol and 1.5 ml of diethyl ether were added to the sample precipitate obtained, followed by homogenization. A total of 2 ml of distilled water and 4 ml of diethyl ether were added to the sample, and then centrifuged at 1000 rpm for five minutes. The diethyl ether layer formed was measured using a UV-vis spectrophotometer with a wavelength of 450 nm. Calculation of carotenoid content is carried out using the formula:

$$\text{Carotenoid } (\mu g/mL) = 25.2 \times A450 \quad \ldots \text{Eq (5)}$$

2.3 Data Analysis

This study used a Factorial Completely Randomized Design (CRD), hence, ANOVA and Least Significant Difference (LSD) tests were needed for the interpretation of the results. The ANOVA test was carried out to determine the effect of treatment as a source of nutrition on the culture media. The results showed that there was a significant difference, thereby, an LSD test was required to determine the treatment with the smallest difference. ANOVA and LSD tests were carried out using the 2013 version of the Microsoft Excel application.

3. Results and Discussion

The adaptation phase of cell growth occurred on the first and second days of culture. This is in line with Prabhath *et al.* (2022) that the fastest and longest lag phase was observed on the 2nd and 8th day, respectively (Figure 1). Furthermore, the lag phase occurred because *S. platensis* has adjusted to the culture media. This made the abundance of microalgae occur in small quantities at the beginning of this growth stage (Piu *et al.*, 2022). The observation results showed that cells experienced a log or exponential phase from day 3 to day 6, which was characterized by an increase in density. Cells in this phase nutrients in the media to carry out metabolism and biosynthesis until peak growth was achieved (Piu *et al.*, 2022). The stationary stage was observed on the 7th day, and the growth phase of *S. platensis* can enter different stationary phases depending on the consumption of nutrients in the log stage. This is in line with Nader *et al.* (2022) that an increase in photosynthetic activity led to increased consumption of nutrients. This condition made the growth phase of the cell enter the stationary stage earlier than usual. Cells in the stationary phase often experience a growth rate that tends to reach zero, due to nutrient depletion in the culture medium. The population of the cultured sample decreased on the 8th day, indicating the death of the microalgae. This finding is consistent with Piu *et al.* (2022) and Fakhri *et al.* (2020) that the death phase was marked by a decrease in density. The decrease in was influenced by the availability of nutrients in the culture media. In this study, there was no additional nourishment in the culture media, but an accumulation of metabolites was observed. This caused the rate of cell death to be greater than the growth. The phase with the highest density can indicate the specific growth rate of the cells (Duarte *et al.*, 2020).

Furthermore, the highest specific growth rate of 0.528 day^{-1} was obtained in *S. platensis* culture treated with 6 ml/L of aquaculture wastewater (Figure 2). The letter notation on the graph shows the LSD test results, where one alphabet shows a significant difference. The LSD test also showed that the treatment consisting of 6 ml/L had a significant effect. High specific growth rates indicate that there is an improvement in the carrying capacity of the media for microalgae (Aulia *et al.*, 2021). Zarrinmehr *et al.* (2020) stated that an increase in the rates was influenced by nitrogen concentration in the media as well as microalgae species. The presence of ammonia nitrogen is also an influential factor because it is preferred by cyanobacteria microalgae. A previous study revealed the compound was preferred because its absorption and assimilation require less energy compared to nitrate (Markou *et al.*, 2019).

Analysis of the data on biomass test showed significantly different results, but the 5% LSD test on several treatments indicated insignificance. The treatment with the highest biomass production was 6 ml/L of wastewater, and it showed significantly different results (Figure 3). The amount of *S. platensis* biomass has a positive correlation with the availability of nutrients in the culture media, and this was in line with a study by Holanda *et al.* (2020), high nutrient content has an impact on higher biomass production. This is due to the greater absorption of nutrients by *S. platensis*. Nogueira *et al.* (2018) also stated that its production increases

along with the nitrogen concentration in the culture media. The formation of spirulina cells was influenced by the initial phosphate content in the culture medium. According to Depraetere *et al.* (2015), *S. platensis* growing under minimal phosphate conditions changes its metabolism to survive. The type of metabolism observed indicates a limitation of protein production and an increase in the accumulation of carbohydrates and lipids.

The highest protein was obtained in the treatment of 6 ml/L of aquaculture (Figure 4). Furthermore, the LSD test showed that it significantly affected the protein content in *S. platensis*. The results showed that the amount of protein increased along with the availability of nutrients in the media but decreased at a certain concentration. Shanthi *et al.* (2021) revealed that the reduction was caused by increased nutrient levels, which were unsuitable for microalgae protein synthesis. The protein content in cells increases with nitrogen concentration up to a certain concentration (Markou and Georgakakis, 2011). This macronutrient was formed from nitrate, nitrite, and ammonium nitrogen absorbed by spirulina through the GS-GOGAT pathway (Sanz-Luque *et al.*, 2015). The BGLN1 enzyme contains an N-terminus, which is important for maintaining protein stability in microalgae (Dong *et al.*, 2021). Asiimwe *et al.* (2022) stated that the N terminus plays a role in protein translocation, structure, and metabolic stability.

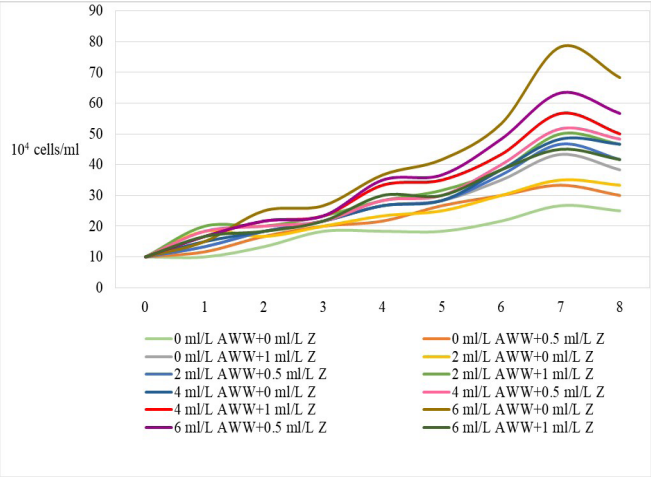

Figure 1. Graph of the cell density (10^4 cells/ml) of S. platensis cultured in aquaculture wastewater for 8 days. Aquaculture Wastewater (AWW). Zarrouk (Z).

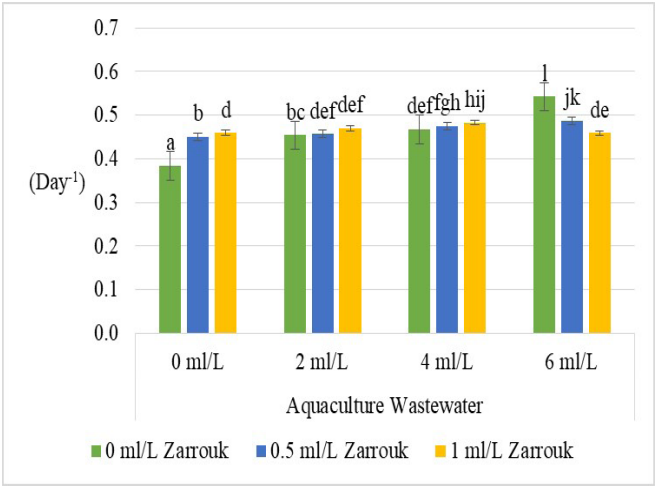

Figure 2. Graph of specific growth rate (Day^{-1}) of *S. platensis* cultured in aquaculture wastewater media for 8 days. The notation consisting of one letter shows the effect of the treatment on the specific growth rate, which is significantly different. Notations a, b, d, and l show significantly different treatment results on the LSD test. def, bc, fgh, hij, jk, and de had no significant difference.

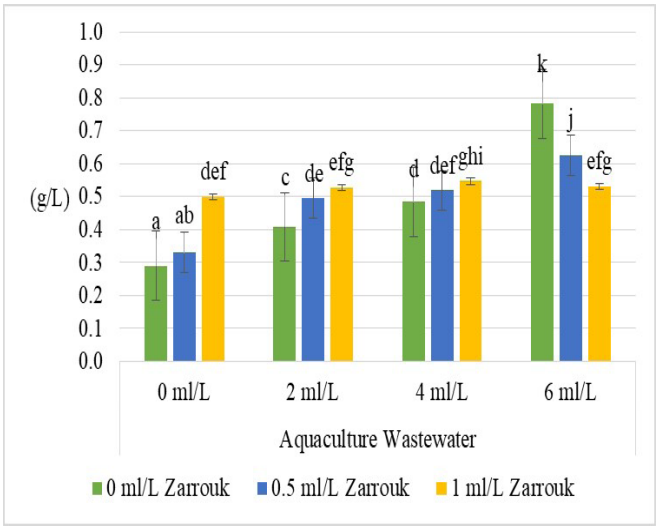

Figure 3. Graph of dry biomass (g/L) of *S. platensis* cultured in aquaculture wastewater media for 8 days. The notation consisting of one letter indicates a significantly different effect of treatment on *S. platensis* biomass. Notations a, c, d, j, and k show significantly different treatment results on the LSD test. ab, de, def, efg, and ghi revealed that there was no significant difference in treatment.

The carotenoid content test showed that the highest results were obtained in the treatment of 6 ml/L of aquaculture wastewater (Figure 5). This finding was supported by the LSD test that the treatment was significantly different. The carotenoid content in *S. platensis* cells increased along with the nitrate level. Apart from being influenced by the nutrient content in the media, the carotenoid was also affected by the different cultivation conditions as well as the microalgae species used (dos Santos *et al.*, 2019). Several studies stated that

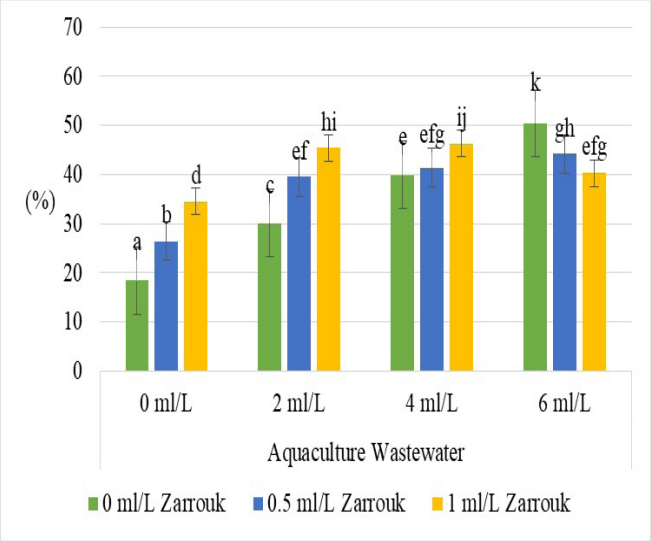

Figure 4. Graph of protein (%) of S. platensis cultured in aquaculture wastewater media for 8 days. The notation consisting of one letter indicates a significantly different effect of treatment on S. platensis protein. A, b, c, d, e, and k show significantly different results on the LSD test. ef, efg, gh, hi, and ij revealed that was no significant difference in treatment.

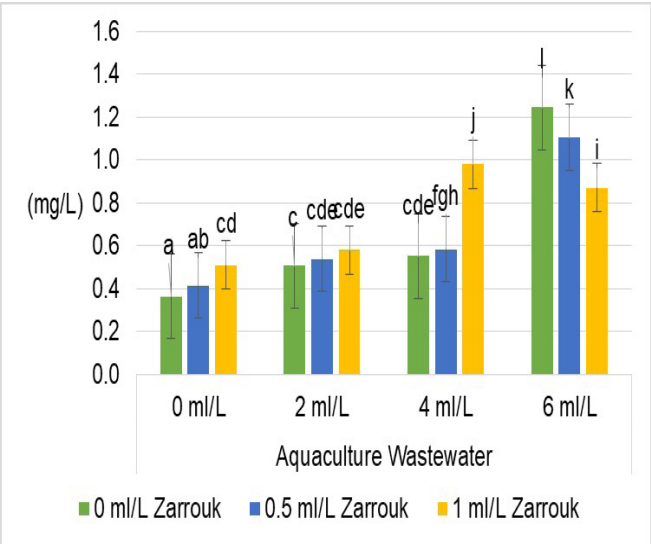

Figure 5. Graph of carotenoids (mg/L) of *S. platensis* cultured in aquaculture wastewater media for 8 days. The notation consisting of one letter indicates that the effect of treatment on the carotenoid *S. platensis* is significantly different. Notations a, c, i, j, k, and l show significantly different results on the LSD test. ab, cd, cde, and fgh revealed that there was no significant difference in treatment.

nitrogen-limited conditions in culture media can cause an increase in its content up to six times, and vice versa. This finding is in line with Menegol *et al.* (2017) that metabolic changes due to nitrogen limitation can cause enzymatic imbalances inducing a reduction in chlorophyll and carotenoid production. Phosphate in culture media can affect the level of carotenoid content in *Spirulina*. This is consistent with research by Satchasataporn *et al.*, (2022) that differences in phosphate content in the media have an impact on the expression of the crtB and crtP genes. The crtB gene encodes phytoene synthase, which initiates the carotenoid pigment biosynthesis process. The biosynthesis is initiated by the combination of two molecules of geranyl pyrophosphate with phytoene synthase as an enzyme. Phytoene molecules are converted into lycopene through isomerization and desaturation processes. A previous study revealed that the phytoene desaturase was encoded by the crtP gene.

The results of ANOVA showed that the aquaculture treatment of wastewater, Zarrouk, and the interaction of wastewater and Zarrouk had an effect on the test parameters. Even so, the test parameters for the Zarrouk treatment showed lower results compared to wastewater treatment. This could be due to the presence of nutrients in Zarrouk which can reduce protein production. One of the nutrients contained in Zarrouk is $NaHCO_3$. The results of Martins *et al.*, (2014) revealed that the highest biopolymer content was obtained in the 8.4 g/L $NaHCO_3$ treatment. High carbon levels are necessary to stimulate biopolymer synthesis, but excess carbon can also inhibit biopolymer production. Pratiwi *et al.*, (2020) revealed that the 5 g/L $NaHCO_3$ treatment on growth media for the microalgae *Spirulina* sp. gives the highest power density results.

4. Conclusion

Aquaculture wastewater can be used as a source of nutrients in *S. platensis* cultivation media without the addition of inorganic fertilizers. Furthermore, the best concentration to obtain *S. platensis* with the highest biomass, protein, and carotenoids was 6 ml/L wastewater. For further research it is better equipped with measurements of nitrate, nitrite, and ammonium absorption because *S. platensis* can utilize nitrogen in the form of nitrate, nitrite, and ammonium.

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Authors' Contributions

The contributions of each author are as follows, AMT; data collected, scripts compiled, and drawings designed. YR, AWP, UY, and MF; developed the main conceptual ideas, provided suggestions, and critical revisions to make this article easy to understand. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they cooperated and have no competing interests.

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