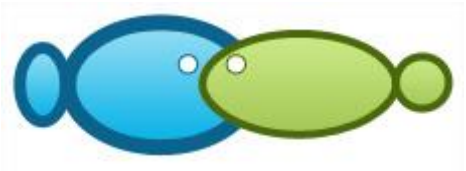

Advancements in culture media: enhancing *Spirulina platensis* (*S. platensis*) production and nutrition qualities through Zarrouk medium optimization

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Abstract. *Spirulina platensis* (*S. platensis*) is a blue-green microalgae with a high potential for producing quality biomass. The growth of *S. platensis* culture is significantly influenced by the composition of the culture medium, especially the levels of nitrate and phosphate, which play an essential role in chlorophyll production. The ratio of the nutrients nitrogen (N) and phosphorus (P), as well as the pH of the media, are the main factors in determining the growth rate, nutritional composition, and quality of the biomass produced. An incorrect N:P ratio and pH can inhibit growth and reduce nutrient content, so optimal values are needed to support the highest quality of *S. platensis*. This research aims to identify the influence and determine the best N:P ratio and pH values to obtain optimal growth patterns, nutritional content, and amino acid fractions in *S. platensis*. The method used was experimental in two stages: first, variations in the N:P ratio, and second, variations in pH, using a Completely Randomized Design (CRD) with five treatments and four replications. The results showed that variations in the N:P ratio had a real influence on the quantity and quality of *S. platensis* cells, with an N:P ratio of 4:1 producing the best growth, water quality, protein content, and amino acid fractions. Additionally, variations in the pH of the culture media also affect the quantity and quality of *S. platensis*, where pH 9.0 produces the highest cell quality, protein content, and amino acid fractions. These results show the importance of setting culture media to optimize the yield of high-quality Spirulina biomass.
Key Words: nutrition, pH, N:P ratio, *S. platensis*, Zarrouk medium.

Introduction. *Spirulina platensis* is a microalga from the *Cyanophyta* group or blue-green algae which is widely used as a natural food source in the aquaculture and nutrition industry (Jung et al 2019; AlFadhly et al 2022a; Gogna et al 2023). This microalga is known for its very high protein content, around 50% of dry weight, and nitrogen content of 7-10% (Shanthi et al 2021; Mousavi et al 2022). It also contains essential nutrients such as carbohydrates, fats, vitamins, minerals, antioxidants, and bioactive pigments such as chlorophyll, phycobiliprotein, and carotene (Widawati et al 2022). This diverse nutritional content makes it ideal as a natural feed in aquaculture, especially for fish and shrimp larvae, where the use of *S. platensis* has been proven to increase the growth and health of these organisms (Al-Zayat 2019; Alagawany et al 2021; Trevi et al 2023).

In hatcheries, the availability of natural food such as *S. platensis* is critical because it can produce high-quality seeds, especially at the larval stage, which has specific nutritional requirements and appropriate particle sizes (Chilmawati et al 2020). Until now, natural food such as *S. platensis* is still difficult to replace with artificial food because of its more complete nutritional content and ability to meet the biological needs of larvae (Chen et al 2024; Ahmad & Ashraf 2023; Villaró et al 2023; Siti 2024). *S. platensis* production on a large scale requires proper optimization of culture media for optimal growth and

nutritional quality (Ragaza et al 2020; Gómez et al 2021; Thevarajah et al 2022; Li et al 2024). The use of Zarrouk media, which is rich in nutrients such as nitrogen (N) and phosphorus (P), has become a practical approach because these two elements are essential in supporting biochemical processes in *S. platensis* cells, such as the synthesis of chlorophyll pigments, which are crucial for capturing light energy and enhancing photosynthetic activity, which drives the production of energy-rich compounds like glucose and other organic molecules necessary for growth and metabolism (Richmond, 2004). Apart from that, the potential of hydrogen (pH) value plays a vital role in improving culture media, which can increase CO_2 availability through the formation of dissolved bicarbonate (Yin et al 2019; Gatimel et al 2020; Zhu et al 2020; Adamu et al 2023), as the growth of *S. platensis*, but unstable or extreme pH can disrupt the activity of enzymes and inhibit photosynthesis and cell growth (Sharma et al 2020; Muhammad et al 2021; Tariq et al 2023) which can reduce the production and nutritional value of *S. platensis* cells.

Several previous studies have shown that inappropriate N:P ratios and pH can inhibit growth and reduce the nutritional quality of *S. platensis* (Lim et al 2021; AlFadhly et al 2022b), ultimately impacting production efficiency (Richmond, 2004). Although Zarrouk's medium is widely used, further research is needed to determine the optimal N:P ratio and pH to sustainably increase cell production efficiency and nutritional quality in *S. platensis*. Therefore, this study aims to determine the most effective combination of N:P ratio and pH in enhancing the biomass production and nutritional content of *S. platensis*. The results of this research are expected to improve the efficiency of *S. platensis* production, support the sustainability of the aquaculture industry, and contribute to various other applications that require high-quality natural nutrient sources, including aquaculture fisheries and functional food sectors.

Material and Method

Description of the study sites. This research was conducted at the Natural Feed Laboratory, Faculty of Fisheries and Marine Sciences, Diponegoro University, from September 15 to September 30, 2023.

Preparation of tools and materials. The tools were sterilized using hot steam in an autoclave at 121°C under 1 atm pressure for 20 minutes. The culture containers were sterilized by washing with liquid soap, soaking in chlorine for 24 hours, and then rinsing and drying. The seawater was sterilized with a 60 ppm chlorine solution for 24 hours and neutralized with a 20 ppm sodium thiosulfate (Isnansetyo & Kurniastuti 1995).

Preparation of media culture. The Zarrouk media used for culturing *S. platensis* has the following composition per liter:

Table 1

Composition of *S. platensis* culture media

No	*Compotition*	*Requirements for 1 Liter*
1	NaCl	1 g
2	$CaCl_2$	0.04 g
3	$NaNO_3$	2.5 g
4	$FeSO_4$	0.01 g
5	EDTA	0.08 g
6	K_2SO_4	1 g
7	$MgSO_4$	0.2 g
8	$NaHCO_3$	16.8 g
9	K_2HPO_4	0.5 g
10	Urea	0.96 g
11	Triple Superphosphate	1.23 g
12	KOH	1.4 g

Source: Chilmawati et al 2024.

Culture media was made by mixing the Zarrouk media composition and A5 micronutrients into 1 Liter of seawater. It was then sterilized using an autoclave at 121°C, 1 atm pressure for 20 minutes. Then, the media was conditioned according to the N:P ratio in each treatment. The nitrogen used comes from $NaNO_3$, and the phosphorus comes from NaH_2PO_4.

Research design. This research uses two levels. The first level aims to study the influence and determine the N: P waste on Zarrouk culture media. The second stage is to study the influence and determine the media pH on Zarrouk media with the best N:P ratio due to the previous stage.

The experimental design used in the first stage is a Completely Randomized Design (RAL) with five treatments and four repetitions. The treatment used in this level I research is the culture of *S. platensis* in media with:

Treatment A: N:P ratio = 1:1
Treatment B: N:P ratio = 4:1
Treatment C: N:P ratio = 8:1
Treatment D: N:P ratio = 12:1
Treatment E: N:P ratio = 16:1

The experimental design used in stage II is a Completely Randomized Design (RAL) with five treatments and four repetitions. The treatment used in this level II research is the culture of *S. platensis* in media with:

Treatment A: The pH of the culture medium is 7.5
Treatment B: culture media pH 8.0
Treatment C: culture media pH 8.5
Treatment D: pH of the culture medium 9.0
Treatment E: Culture medium pH 9.5

This research refers to Rasdi & Qin's (2014), study of the effect of the N:P ratio on the growth and chemical composition of *Nannochloropsis oculata* and *Tisochrysis lutea*. Zarrouk media is used as a basic formula for the N:P ratio for other manipulations. The N:P ratio in other culture media is manipulated by reducing and adding N and P to obtain the N:P ratio studied.

Inoculation sel S. platensis. The inoculation process involves planting *S. platensis* seeds with a cell density of 5×10^4 cells/mL or 4.7 log cells/ml. According to Suminto (2005), inoculation of *S. platensis* with an initial density of 50.000 cells/mL provides an optimal starting point for growth, ensuring sufficient cell availability to utilize nutrients in the culture medium effectively while minimizing the risk of overcrowding that could hinder growth.

S. platensis culture. The culture was carried out twice with a volume of 5 liters. The first culture was on a laboratory scale for 14 days to obtain growth patterns, and the second culture was on a mass scale of 100 liters to obtain nutritional content test samples. The culture technique begins with preparing sterilized tools and materials. The second step is to put 4 liters of sterile seawater into each jar container, then put the culture media into the jar at a dose of 1 mL/1 liter of seawater. Then, the inoculant *S. platensis* seeds with a density of 10 cells/l are put into a 400 mL jar. The ratio of inoculant to sterile seawater media used is 100 mL of inoculant/1 Liter of seawater (Rasdi & Qin, 2014). Aeration is given to each jar to meet the oxygen supply in the culture medium. The salinity used in *S. platensis* culture is 15-30 ppt (Widawati et al 2022).

The same thing was done in the second stage, where *S. plantensis* was cultured on a laboratory scale according to the treatment in the second stage by applying the best N:P ratio from the first research stage on culture media with different pH. For laboratory scale cultures, cell density is calculated by taking a 1 mL sample using a pipette and placing it on a hemocytometer, then observing it under a microscope with 100x magnification. Cell density calculations were carried out every day for 14 days of culture. The culture process is transparent light green in the initial culture period and changes to dark green. The dark green indicates that the cells have experienced the highest peak density.

Data analysis

Cell density measurements. *S. platensis* cell density during the observation was calculated using the following formula (Suminto 2005):

$$P = N \times 10^4 \text{ sel/ml}$$

Where:
P = cell density (cells/ml)
N = number of cells in 1 hemocytometer field of view Lag phase time.

Lag phase time. Calculation of the lag phase time of *S. platensis* is done by calculating linear regression during the exponential phase (Suminto & Hirayama 1996) with the formula:

$$Y = A + B$$

Where:
Y = logarithm of cell density
A = lag phase time (days)
B = constant

The lag phase time (A) is calculated by Y = initial culture density; this formula is then used to calculate A to be:

$$Y = Ak + B$$

Where:
Y = logarithm of cell density on day 0
A = phase lag time
K = growth rate
B = linear regression calculation constant during the exponential phase

Specific growth rate. Calculation of the specific growth rate of *S. platensis* is calculated using the k value in regression (Fogg, 1965) with the formula:

$$k = \frac{\log(Wt - W0)}{\Delta t} \frac{\log(Wt - W0)}{\Delta t}$$

Where: k = specific growth rate
W_t = cell density in the late exponential phase (log cells/ml)
W_0 = cell density in the early exponential phase (log cells/ml)
Δ_t = time difference between the final exponential phase and the initial exponential phase (days)

Water quality measurements. Water quality measurements in *S. platensis* culture containers were carried out three times during the research, namely on day 1, day 7, and day 14 of culture. Water quality is measured using a pH meter, spectrophotometer, and thermometer. The variables that are measured are temperature, salinity, and pH.

Protein determination. Samples originating from culture results harvested in the stationary phase were sedimented, and 500 mL of the sediment was taken and put into a sample container. Samples were tested for crude protein content using the semi-micro Kjeldahl method and amino acid fraction testing using the by-difference method (Fiaz et al 2021; Hoxha et al 2022).

Statistical analysis. Based on observations during research on *S. platensis*, growth pattern data was obtained, which included phase lag time, growth rate, maximum cell density, and final density. The results were then analyzed descriptively with statistical tests and presented in graphical form. The data that has been obtained is then analyzed statistically using normality, homogeneity, and additivity tests. Regular, homogeneous, and additive data to be continued with the analysis of variance (ANOVA). The ANOVA test results showed significant differences, and the least significant difference test (BNT) was carried out.

Results

Differences in N:P ratios in culture media S. platensis. Using varying N:P ratios produces different *S. platensis* cell growth patterns. Based on daily cell density data, a graph was obtained showing the differences in growth patterns in Table 2 and Figure 1. Statistical tests on cell density data showed that the data was distributed normally, homogeneously, and additively.

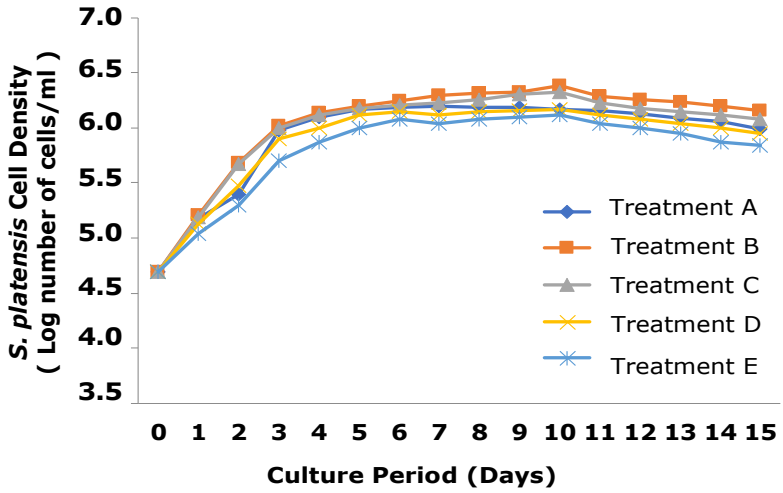

Figure 1. *S. platensis* growth patterns with different N:P ratio treatments.

The results showed that variations in the N:P ratio treatment made a difference in the growth pattern variables of *S. platensis*. Treatment B produced the shortest adaptation time (0.34±0.15 days) and the highest specific growth rate (0.38±0.05 per day), accompanied by the shortest stationary phase length (4.75±0.50 days) and maximum cell density (6.38 log number of cells/ml) and highest final density (6.15 log number of cells/ml). In contrast, treatments D and E, with longer adaptation times (0.31±0.06 days for D and 0.62±0.11 days for E), showed lower maximum and final cell densities. Treatment C showed a relatively high specific growth rate (0.41±0.00 per day) with the same length of stationary phase (7.00±0.00 days) and achieved a relatively high maximum cell density (6.32 log number of cells/ml). The details in Table 2 show that variations in the N:P ratio affect the duration of adaptation, specific growth rate, length of stationary phase, and cell density, where treatment B provides the most optimal results for the growth of *S. platensis*.

Table 2

Variable values for *S. platensis* cell growth patterns in each treatment

Treatment N:P Rasio	*Growth Pattern Variables*				
	Duration of adaptation time (day)	*Specific growth rate (day)*	*Stationary phase length (day)*	*Maximum cell density (Log number of cells/ml)*	*Final research density (Log Number of cells/ml)*
A	0.52±0.33^{c}	0.31±0.01^{c}	4.00±0.00^{c}	6.19±0.00^{c}	5.85±0.00^{c}
B	0.34±0.15^{a}	0.38±0.05^{a}	4.75±0.50^{a}	6.38±0.00^{a}	6.15±0.00^{a}
C	0.62±0.11^{b}	0.41±0.00^{b}	7.00±0.00^{b}	6.32±0.00^{b}	6.08±0.00^{b}
D	0.31±0.06^{d}	0.35±0.01^{d}	7.00±0.00^{d}	6.17±0.00^{d}	5.96±0.00^{d}
E	0.62±0.11^{e}	0.26±0.01^{e}	7.00±0.00^{e}	6.12±0.00^{e}	5.85±0.00^{e}

Differences in pH of the media in culture S. platensis. Table 3 and Figure 2 present the growth pattern of *S. platensis*. To see the effect of different N:P ratios and pH on cell growth, the cell density of *S. platensis* was observed and calculated every day for 14 days. The cells were observed under a microscope at 100x magnification.

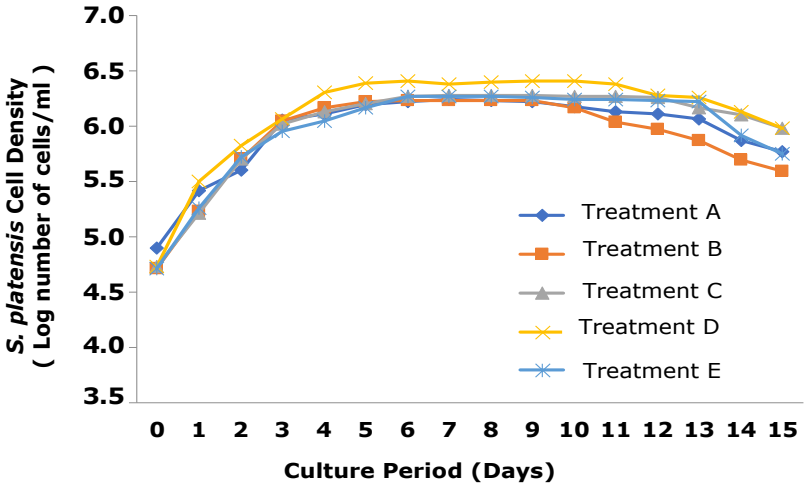

Figure 2. The growth pattern of *S. platensis* with different pH treatments.

The results depicted in Figure 2 show that the cell density of *S. platensis* at the beginning (inoculation) had the same density, namely 4.7 log cells/ml. Each treatment showed increased cell density from the first day of inoculation. All treatments provided an adaptation time of less than 1 day. The most extended stationary phase was obtained at a pH of 9.0, reaching 7 days. In contrast, treatment with media at a pH of 7.5 resulted in the shortest stationary phase, namely 4 days. *S. platensis* growth data, including lag phase time, specific growth rate, maximum cell density, and final density during the study, can be seen in Table 3.

Table 3

S. platensis growth includes lag phase time, specific growth rate, stationary phase length, maximum cell density, and final density

Treatments pH	*Growth rates of S. platensis*				
	Lag phase time (days)	*Specific growth rate (cells/days)*	*Length of stationary phase (days)*	*Maximum cell density (log cells/ml)*	*Final density (log cells/ml)*
7.5	0.52±0.33^{d}	0.29±0.01^{d}	4.00±0.00^{d}	6.20±0.00^{d}	5.75±0.01^{d}
8.0	0.34±0.15^{c}	0.38±0.05^{c}	4.75±0.50^{c}	6.22±0.00^{c}	5.58±0.03^{c}
8.5	0.31±0.11^{b}	0.40±0.00^{b}	7.00±0.00^{b}	6.26±0.00^{b}	5.96±0.01^{b}
9.0	0.62±0.06^{a}	0.32±0.03^{a}	7.00±0.00^{a}	6.39±0.00^{a}	5.96±0.01^{a}
9.5	0.31±0.11^{b}	0.46±0.01^{b}	7.00±0.00^{b}	6.27±0.00^{b}	5.75±0.01^{b}

Protein content. Cultivation of *S. platensis* in culture media with different pHs has shown variations in cell protein content, reflecting the microalgae cells' quality. The analysis showed that treatment with pH 7.5 (treatment A) produced a protein content of 61.57%, while at pH 8.0 (treatment B), the protein content increased to 62.17%. Furthermore, at pH 8.5 (treatment C), the protein content increased again to 67.68%, followed by treatment D at pH 9.0, which reached the highest protein content of 67.85%. Even though pH 9.5 (treatment E) also shows a high protein content, namely 67.42%, pH 9.0 remains the most optimal treatment for increasing the protein content of *S. platensis*. Previous research by Pandey et al. (2010) supports this finding, where the optimum protein and chlorophyll content in *S. platensis* was recorded at 64.3±0.11% in cultures with pH 9. This

shows that pH nine is an ideal growth condition for cell protein accumulation. The pH factor influences the solubility and bioavailability of nutrients in the culture medium, affecting cell metabolism. The increase in protein levels in media with higher pH can be attributed to optimizing photosynthesis and metabolic processes, which contributes to increased productivity of *S. platensis* cells. The high protein content in *S. platensis* significantly impacts various aspects, including growth, reproductive productivity, and survival of microalgae. Nutritional adequacy, which depends on protein content, is an essential factor in determining the quality and yield of cultivation. Research by Kandathil-Radhakrishnan et al. (2020) and Rasdi et al. (2020) shows that good nutritional quality supports growth and increases production efficiency and resistance to unfavorable environmental conditions. Therefore, setting the correct pH in the culture medium is essential in cultivating *S. platensis* to achieve optimal results.

Amino acid fraction of S. platensis. The analysis of amino acid fractions from *S. platensis* cultivated in various media pH treatments showed diversity in the types and amounts of amino acids. There are 18 amino acid fractions identified, with varying concentrations of each amino acid. For example, L-Phenylalanine was 21,723.60 to 40,068.28 g/kg, and L-Proline was 20,827.83 to 23,178.26 mg/kg. Other amino acids, L-Serine and L-Threonine 23,331.54–36,237.17 mg/kg and 23,838.80–37,163.03 mg/kg, and the highest concentration was found in L-glutamic acid 60,666.79 to 71,825.06 mg/kg. This diversity indicates that pH conditions in the culture medium influence the synthesis and accumulation of amino acids in *S. platensis* cells. A balanced supply of essential amino acids supports optimal microalgae growth. The essential amino acids required by *S. platensis* include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Sanz et al 2019; Khadka, 2021). By meeting the needs of these essential amino acids, microalgae can increase growth efficiency and productivity. In this context, a sufficiently high fraction of amino acids, especially in optimal pH treatment, supports the physiological functions necessary for protein synthesis and cell metabolism. An exemplary amino acid balance contributes to healthy growth and the sustainability and productivity of *S. platensis* cultivation (Spínola et al 2022; Uzlasir et al 2022;2023). The availability of essential amino acids influences metabolic processes, biomass development, and resistance to environmental stress. Therefore, proper pH regulation in the culture medium is critical in achieving optimal amino acid balance and increasing crop yield and nutritional quality of *S. platensis*.

Discussion. The research showed that all treatments applied to *S. platensis* resulted in an adaptation time of less than one day. Analysis of variance showed that washing *S. platensis* cells had a significant effect ($p<0.01$) on adaptation time. The Specific Growth Rate (LPS) of *S. platensis* also showed a significant influence ($p<0.01$) from variations in the N:P ratio, where treatment without cell washing only reached the stationary phase for 4 days. On the other hand, washing the cells two to three times resulted in the most extended stationary phase, reaching 7 days, compared to washing in phase I, which was only 5 days. In cultures with an N:P ratio of 8, the stationary phase reached 4 days, with analysis of variance confirming that variations in the N:P ratio had a significant effect ($p<0.01$) on the length of the stationary phase.

The highest maximum cell density in the stationary phase came from cultures using an N:P ratio of 8. The analysis of variance showed a significant effect of *S. platensis* cell washing on maximum cell density. The difference in the N:P ratio also had a significant effect ($p<0.01$) on the final density. Varying nitrate and phosphate concentrations influence cell abundance, implicating increased biomass and nutrient utilization (Andersen et al 2020; Ziganshina et al 2022). However, excessively high doses of nitrate and phosphate can reduce biomass (Amini et al 2019), while unbalanced nitrate application can inhibit growth (Araujo et al 2020; Gonzalez-Camejo et al 2020). With appropriate nitrogen and phosphorus and supportive environmental conditions, optimal photosynthesis processes can be achieved, increasing growth rates and high biomass concentrations (Mutia et al 2021). Therefore, meeting the appropriate needs for nitrogen and phosphorus is crucial because a deficiency can reduce cell protein content and disrupt protein synthesis.

This suggests that careful nutritional management is critical to increasing the productivity of *S. platensis* in culture and ensuring success in its biotechnological applications.

S. platensis has four phases in its growth cycle: lag phase (adaptation phase), exponential phase, stationary phase, and death phase. The growth of *S. platensis* is influenced by various factors, including culture media that provide nutrients and environmental conditions such as temperature, light, and pH. Based on the research results, the growth of *S. platensis* showed a significant increase in optimal pH. All treatments given resulted in an adaptation duration of less than one day, where the adaptation phase usually took place between days 0 and 1 of culture (Nurani et al 2012). In short, this lag phase is caused by the inoculant seeds' ability to adapt quickly to the new culture medium. Washed microalgae experience a short adaptation time, so they can achieve a higher specific growth rate by optimally utilizing the nutrients in the culture media (Andreas et al 2014). During the research, the specific growth rate of *S. platensis* showed optimal conditions when it entered the exponential phase, namely the phase where cell density increases rapidly due to intensive cell division. Analysis of the variance of the specific growth rate of *S. platensis* showed that pH levels in the culture media had a significant effect ($P<0.01$) on the growth rate, with the highest yield at pH 9.5 of 0.46±0.01 cells/day. This supports the findings of Pandey et al. (2010), who stated that *S. platensis* grows optimally at high alkalinity levels, especially in the pH range of 9-9.5. The alkalinity level of the culture medium plays a vital role in the physiological conditions and nutrient availability of *S. platensis* cells, where the solubility of carbon and mineral sources in the medium is determined by pH (Sun et al 2019; Ilesanmi et al 2020). Thus, choosing the proper pH dramatically determines the success of this microalgae growth.

Microalgae cells reach maximum density in the stationary phase with a stable growth rate. Based on the analysis of variance, the duration of the stationary phase of *S. platensis* was significantly influenced ($P<0.01$) by pH variations in the culture medium. The most extended stationary phase, namely seven days, is achieved at pH 9.0, while pH 7.5 produces the shortest stationary phase, namely four days. The increase in the *S. platensis* cell population is directly proportional to the increase in biomass (Saad et al 2023), and the highest biomass concentration of 4.9 mg/mL was achieved at pH 9.0 (El-Monem et al 2021). After the stationary phase, the increase in cell density can be influenced by the ideal conditions of the media and the quality of the seeds. *S. platensis* cell seeds that have been washed can reduce contaminants, such as other microalgae and bacteria, thereby allowing *S. platensis* to absorb nutrients optimally (Chilmawati et al 2023). Therefore, pH conditions in the culture media are crucial in supporting the sustainable growth and production of *S. platensis*.

Conclusions. This study demonstrated that N:P ratio variations significantly affect the quantity and quality of *S. platensis* cells. Specifically, Treatment B (N:P ratio = 4:1) in the culture medium promoted optimal growth patterns and favorable water quality conditions. In addition, protein levels and amino acid fractions in *S. platensis* cells also reached the highest values at this ratio. This quality improvement correlates with better growth conditions, reflecting the importance of nutritional balance in microalgae cultures. Apart from the N:P ratio, the pH of the culture medium also plays a crucial role, where a pH of 9.0 provides the best cell quantity and quality results. These findings emphasize that proper regulation of the N:P ratio and pH of the culture medium is the key to increasing the productivity and quality of *S. platensis*, which has implications for the development of commercial applications of this microalgae. This is important for a more efficient and sustainable cultivation strategy in the future.

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