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Effects of Glucose and Lime Levels on the Growth and Water Quality of *Chlorella ellipsoidea* Cultivation

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ABSTRACT

This study aimed to investigate the effects of glucose and lime concentrations in the culture medium on growth of *Chlorella ellipsoidea* and water quality during the cultivation. The experiment included seven treatment groups: a control group (0.5 g/L glucose, 0.4 g/L lime) and groups with reduced glucose concentrations (0.4, 0.3, 0.2, 0.1 g/L) and lime concentrations (0.2, 0.1 g/L). Cultivation lasted for 15 days, with algal growth assessed by cell counting, and water quality parameters such as alkalinity, pH, and ammonia levels analyzed daily. The results showed that the G20 (0.1 g/L glucose) group achieved highest cell density of $7.15\pm0.92 \times 10^7$ cells/mL on day 10, which was not significantly different ($p>0.05$) from the G40, G60, and L50 groups, but higher than the control and other treatment groups. Furthermore, alkalinity in the G20 group remained within the range of 130-187 mg/L, which is suitable for cultivation and subsequent use as feed for *Moina* sp. The pH values of all groups remained consistent, ranging from 7.2-8.8 throughout the experiment. Total ammonia levels remained below 1 mg/L during the first 9 days, increasing in the later period, with no significant differences between the treatment groups. In conclusion, reducing the glucose concentration to 0.1 g/L in the culture medium promoted maximum growth of *C. ellipsoidea*, while maintaining water quality at an optimal level. This approach offers potential for developing cost-effective algal culture media and scaling up cultivation for commercial production.

HIGHLIGHTS

1. Study explored nutrient formula for *Chlorella ellipsoidea* and measured water quality.
2. G20 formula (0.1 g/L glucose) gave highest growth, safe water quality for *Moina* sp.
3. Reduced algae culture costs, created suitable environment for *Moina* sp. cultivation.

1. INTRODUCTION

Unicellular microalgae such as *Chlorella* sp. are recognized as valuable sources of nutrients, bioactive compounds, and renewable biomass for various applications (Chew et al., 2017; Singh and Patidar, 2018). *Chlorella* sp. is rich in proteins, essential amino acids, vitamins, pigments like chlorophyll and carotenoids, and polyunsaturated fatty acids beneficial for human and animal nutrition (Barka and Blecker, 2016; Kong et al., 2024; Kamolrat et al., 2024). Its superior nutritional profile, ease of cultivation, and ability to accumulate high biomass make *Chlorella* sp. a promising feedstock for live feed production in the aquaculture industry (Muller-Feuga et al., 2003). *Chlorella* sp. cells possess spherical morphology, exist as single cells without mucilage and setae (Safi et al., 2014; Kim et al., 2018), making them highly suitable as feed for aquatic animal larvae or as a nutritional source for zooplankton, which are commonly used as feed in larval aquaculture and ornamental fish cultivation (Lan et al., 2022; Joshua et al., 2024). Among the important cultivated zooplankton species,

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Moina sp. is particularly prevalent. *Moina* sp. is a crucial live feed organism for aquatic animal larviculture and ornamental fish rearing. As a member of Subphylum Crustacea within the Order Cladocera, it represents an essential zooplankton in natural food web systems. Its significance stems from its appropriate body size matching the mouth dimensions of fish larvae and small ornamental fish species (Gogoi et al., 2016). The organism exhibits high reproductive rates, filter-feeding behavior, and possesses rich nutritional composition, including essential amino acids, highly unsaturated fatty acids, and vitamins (Rottmann et al., 2003; Shidik et al., 2021; Rasdi et al., 2020; Suhaimi et al., 2022; Wang et al., 2022). These characteristics have made it widely adopted in aquaculture operations. Consequently, efficient mass production of *Moina* sp. is vital to meet the increasing demand for quality live feeds in aquaculture hatcheries.

However, the cultivation of Chlorella algae as feed for *Moina* sp. often faces challenges in controlling water quality in the culture system, particularly the increase in alkalinity, pH, and accumulation of ammonia to levels that can significantly impact the survival and growth of *Moina* sp. (Benider et al., 2002; Yang et al., 2012; Shidik et al., 2021). Alkalinity levels above 200 mg/L can significantly reduce the survival rate of *Moina* sp. juveniles (Benider et al., 2002; Rottmann et al., 2003). The optimal pH for *Moina* sp. growth is between 7.0 and 8.0. This pH range supports the best growth and reproduction of *Moina* sp. If the pH is too low or too high, the production rates significantly decrease (Yuslan et al., 2021; Rottmann et al., 2003), and the optimal ammonia level for the growth of *Moina mongolica* should not exceed 2.63 mg (NH_3-N)/L. This level is lower than the harmful threshold for aquatic insect larvae (4.3 mg (NH_3-N)/L) but higher than the optimal level for *Daphnia magna* (0.66 mg (NH_3-N)/L) (He et al., 2001).

The main components in algal culture media contributing to increased alkalinity and pH are carbon sources like molasses or ammonium hydroxide and the alkalizing agent lime ($CaCO_3$) (Ilavarasi et al., 2011). Glucose, used as the primary carbon source, and lime are crucial in maintaining water quality in closed photobioreactor systems or indoor cultivation, enhancing water transparency for light absorption (Thewaratmaneekun et al., 2006). Monitoring ammonia levels in algal culture water is essential before introducing *Moina* sp. into the pond to prevent nutrient accumulation and high concentration (American Public Health Association, 2005). While previous studies have optimized algal culture media for general purposes (American Public Health Association, 2005; Thewaratmaneekun et al., 2006; Ilavarasi et al., 2011), research specifically investigating suitable glucose and lime levels for *Chlorella* sp. cultivation aimed at producing feed for *Moina* sp. is lacking. Maintaining optimal water quality parameters is crucial for high survival and growth rates of *Moina* sp. when using algae as feed. The economic and environmental importance of producing high-quality and high-quantity *Moina* sp. in the aquaculture industry is significant. By improving *Moina* sp. production, reliance on natural live feeds can be reduced, preserving natural resources and enhancing farm productivity. Furthermore, reducing the impact on natural ecosystems from harvesting wild live feeds can have positive environmental outcomes.

This study aims to develop practical *Chlorella ellipsoidea* cultivation processes that can be applied in the industry by determining the appropriate levels of glucose and lime for optimal *C. ellipsoidea* growth while maintaining suitable water quality for subsequent use as feed for *Moina* sp. Additionally, advancements in photobioreactor designs and mixotrophic cultivation strategies could improve Chlorella cultivation for live feed purposes (Acién Fernández et al., 2019; Yu et al., 2023). Enhancing this process will increase the efficiency of live feed production in aquaculture farms, benefiting both the economy and the environment.

2. METHODOLOGY

2.1 Algal strain and culture conditions

C. ellipsoidea strain TISTR 8261 used in this study was isolated from local natural water bodies using plankton net sampling (Figure 1). Unialgal culture was established through single-cell isolations by micropipette washing technique (Stein, 1973). The isolated cells were transferred to illuminated test tubes containing Bold's basal medium for growth. After approximately 4 weeks, the culture was scaled up in Erlenmeyer flasks until sufficient cell density was achieved for experimentation. The algae were maintained under aseptic conditions to prevent contamination (Joseph and Ajithkumar, 2015) in a culture room equipped with fluorescent lighting at 3,000 lux and temperature controlled at 25±2°C.

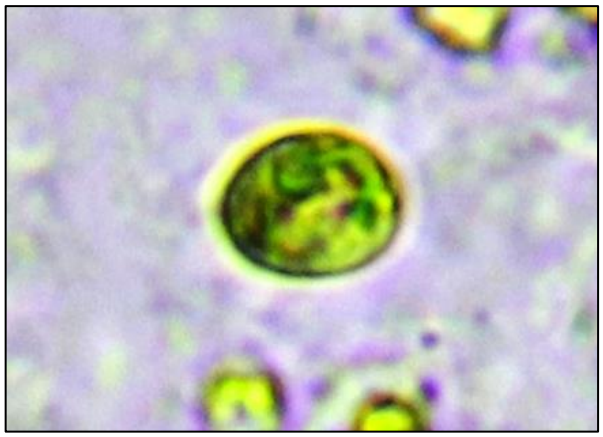

Figure 1. Microscopic view of *C. ellipsoidea* TISTR 8261 cells in culture at 400× magnification

2.2 *C. ellipsoidea* culture medium preparation

The basal culture medium consisted of (g/L): urea (CH_4N_2O) 0.6, phosphorus pentoxide (P_2O_5) 0.2, and potassium oxide (K_2O) 0.2, with carbon sources from glucose ($C_6H_{12}O_6$) and lime ($CaCO_3$). Previous studies on *Chlorella* sp. cultivation have reported glucose concentrations ranging from 0.5-2.5 g/L in culture media. In this study, the culture medium was optimized with initial concentrations of glucose at 0.5 g/L and lime at 0.4 g/L. The experiment was divided into seven treatment groups with varying levels of glucose and lime (Figure 2).

The experiment was designed as a Completely Randomized Design (CRD) with three replications per treatment (n=21). Nutrient solutions were prepared by dissolving each component in 1,000 mL Erlenmeyer flasks and made up to a final volume of 750 mL with distilled water. Each flask was equipped with air inlet and outlet tubes and sealed with cotton plugs. Initial *C. ellipsoidea* inoculum with cell density of 10^6 cells/mL or higher was added at 20 mL/L to each experimental group, resulting in an initial *C. ellipsoidea* concentration of 2.1×10^5 cells/mL. *C. ellipsoidea* cultures were maintained in a controlled environment room at 25±2°C, illuminated by fluorescent lamps providing 3,000 lux intensity with a 24:0 hour light: dark cycle.

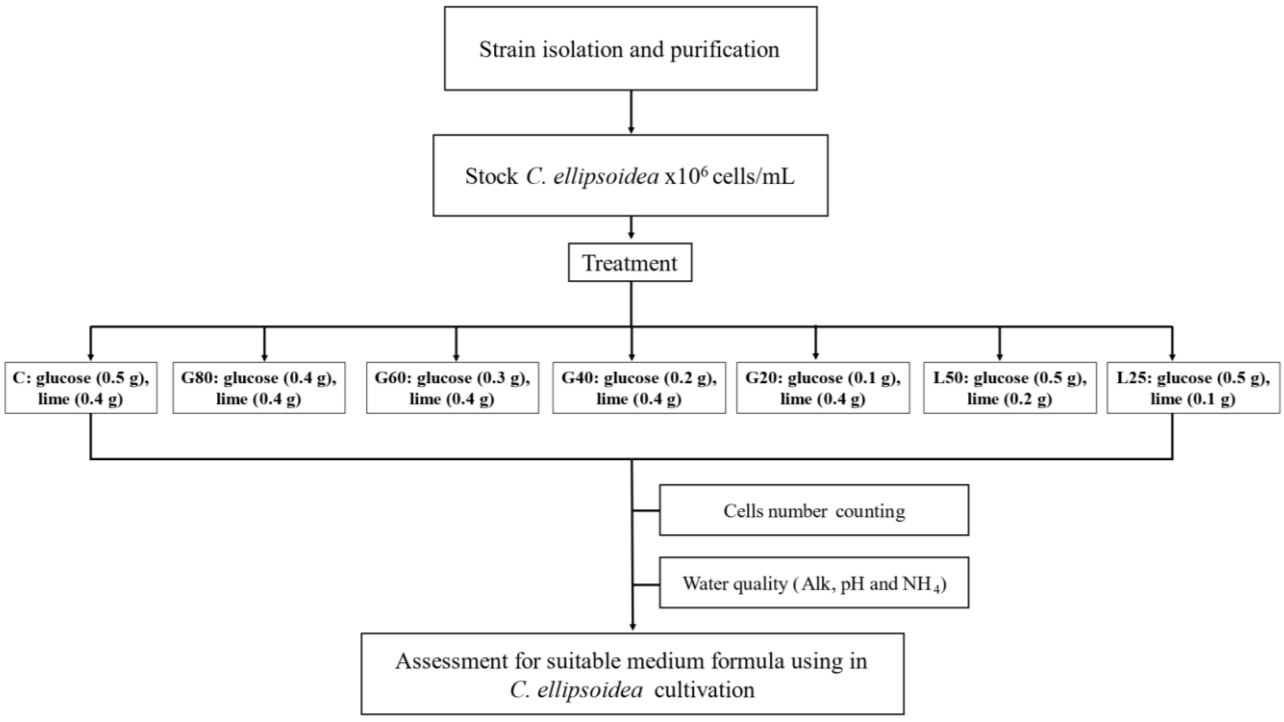

Figure 2. Flowchart of *C. ellipsoidea* culture system in the laboratory

2.3 Sampling and analysis

C. ellipsoidea cells samples (5 mL) were collected daily at 11:00 am to study growth rates through cell counting, from the initial inoculation until *C. ellipsoidea* reached death phase. Cell counting was performed using a hemocytometer (1/10 mm; BOECO; Germany) under a microscope at 400x magnification by randomly counting five small squares as described by Absher (1973). The total algal cells per milliliter were calculated using the following formula:

$$\text{Total cells/mL} = \frac{\text{grid } (1+2+3+4+5)}{5} \times 0.25 \times 10^6$$

Water quality parameters were monitored daily at 11:00 am throughout the culturing period, where alkalinity was measured by titration with standardized HCl acid and reported as mg/L $CaCO_3$ equivalent based on standardized $NaHCO_3$ solution, pH was determined using a digital pH meter (resolution 0.01), and Total ammonia nitrogen (TAN) was analyzed using the Phenate method involving sample digestion with alkaline hypochlorite and sodium nitroprusside oxidizing solution, followed by absorbance measurement at 640 nm wavelength against an ammonium chloride standard curve and reported as mg/L of nitrogen.

2.4 Statistical analysis

Algal growth was analyzed for statistical differences using one-way analysis of variance (ANOVA), with mean comparisons between treatment groups performed using Duncan's new multiple range test. The relationship between glucose and lime concentrations and algal growth was evaluated using Pearson's correlation analysis. A 95% confidence level ($p<0.05$) was set for accepting statistical significance. The SPSS software version 22 was used for statistical computations and analyses.

3. RESULTS

3.1 Growth rate of *C. ellipsoidea*

Glucose level in the culture medium significantly affected the growth rate of *C. ellipsoidea*. The group with an 80% reduction in glucose (G20) exhibited the highest growth in both the lag and log phases. During the initial 3-day lag phase and on day 6 of the log phase (Figure 3), the G20 group showed notably higher cell densities compared to other groups. On day 10, when the algae reached maximum cell density, the G20 group attained 7.15×10^7 cells/mL, while the control and other glucose reduction groups ranged from $2.70\text{-}5.47\times10^7$ cells/mL. Statistical analysis revealed no significant difference between the G20 group and the G40, G60, and L50 groups.

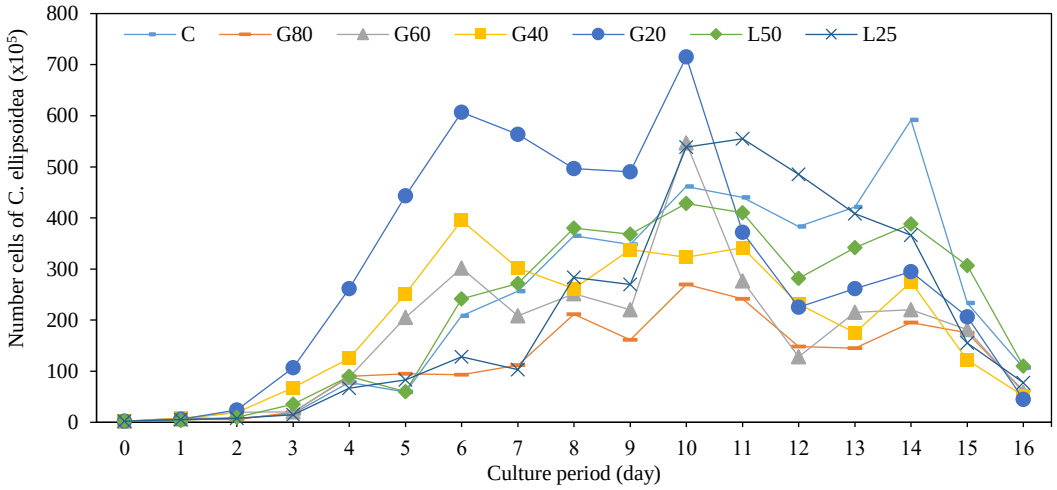

Figure 3. Growth rates of algae under different treatment levels

3.2 Water quality of *C. ellipsoidea* cultivation

Glucose level also influenced water quality during cultivation. The G20 group maintained alkalinity levels between 130-187 mg/L, suitable for *Moina* sp. feed, especially during maximum algal growth from days 6-10. In contrast, other treatment groups exhibited alkalinity levels exceeding 200 mg/L (Table 1). All treatment groups showed pH fluctuations within the range of 7.2-8.8 throughout the 15-day cultivation period (Table 2), which is safe for *Moina* sp. cultivation.

Total ammonia levels gradually increased over time in all groups but remained below 1 mg/L for the first 9 days before rising further, without significant differences between the treatment's groups (Figure 4).

Table 1. Alkalinity (mg/L as $CaCO_3$) during 15-day *C. ellipsoidea* cultivation with different glucose and lime levels

Treatment/day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
C	153	221	221[a]	221[a]	221[a]	221[a]	221[a]	221	221	221	221	221	221	210	221[a]
G80	142	221	221[a]	221[a]	221[a]	221[a]	221[a]	221	221	221	221	221	221	221	221[a]
G60	164	221	221[a]	221[a]	221[a]	221[a]	221[a]	221	221	221	221	221	221	215	221[a]
G40	130	215	221[a]	221[a]	221[a]	221[a]	221[a]	221	221	221	221	221	221	221	221[a]
G20	102	193	130.33[b]	147.33[b]	187[b]	153[b]	153[b]	210	198	198	210	221	221	210	175.67[ab]
L50	153	221	221[a]	221 [a]	221 [a]	221 [a]	221 [a]	221	221	221	221	204	210	210	187[ab]
L25	147	221	221[a]	221 [a]	221 [a]	221 [a]	221 [a]	221	221	221	221	221	198	176	148[b]

Means within rows with different letters indicate significant differences ($p<0.05$) between treatment groups.

Table 2. pH values during 15-day *C. ellipsoidea* cultivation with different glucose and lime levels

Treatment/day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
C	7.3	8.5	8.1	8.5	8.6	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.3	8.5	8.2
G80	7.7	8.3	7.8	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.2
G60	7.2	8.7	8.2	8.5	8.6	8.5	8.8	8.8	8.3	8.5	8.5	8.3	8.5	8.5	8.3
G40	7.7	8.3	8.2	8.5	8.6	8.5	8.5	8.5	8.3	8.5	8.5	8.5	8.5	8.5	8.5
G20	8.0	8.2	8.0	8.0	8.5	8.3	8.0	8.2	8.3	8.2	8.3	8.5	8.5	8.3	8.2
L50	7.8	8.7	8.0	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.3	8.3	8.5	8.2
L25	7.3	8.5	8.5	8.5	8.7	8.5	8.5	8.5	8.3	8.3	8.3	8.0	8.2	8.0	7.8

No significant differences ($p>0.05$) in mean pH values were observed between treatment groups on any given day.

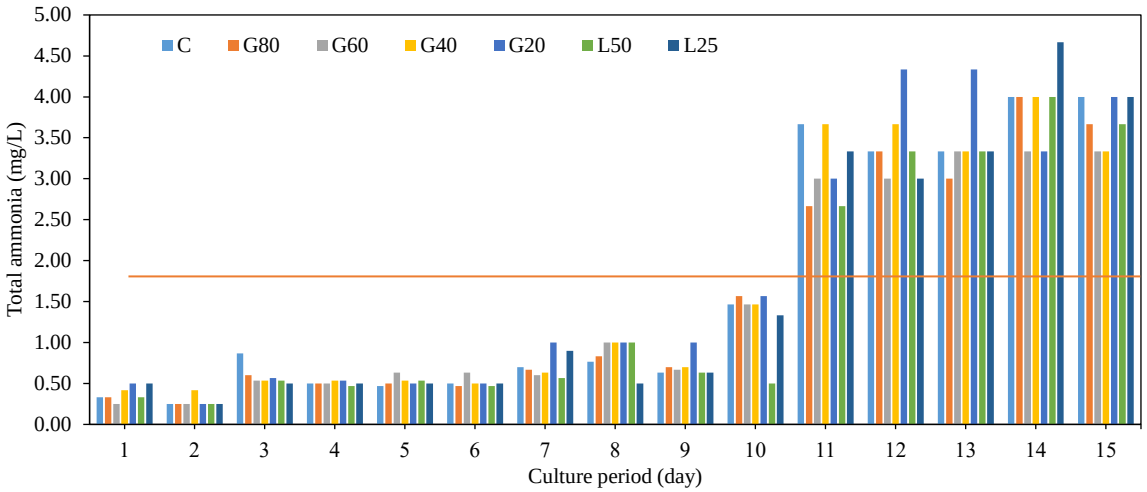

Figure 4. Changes in ammonia nitrogen levels during 15-day *C. ellipsoidea* cultivation under different media formulations

4. DISCUSSION

The study demonstrated that glucose level in the culture medium plays a crucial role in promoting the growth of *C. ellipsoidea*. Reducing the glucose level to 80% (0.1 g/L) led to the highest cell yield during both the lag and log phases of cultivation. According to literature reviews, glucose has been used as a component in Chlorella culture media, with optimal concentrations typically ranging from 0.5-2.5 g/L, depending on species and cultivation objectives (Heredia-Arroyo et al., 2011; Yeh and Chang, 2012; Kong et al., 2013). However, this study demonstrates that glucose concentration can be reduced while maintaining the growth rate of *C. ellipsoidea*. This effect may be attributed to the algae's ability to enhance carbon utilization efficiency through the carbon concentrating mechanism (CCM) under low carbon conditions, as described by Raven et al. (2017). The CCM process increases carbon dioxide uptake efficiency, which is crucial for microalgae to convert CO_2 into biomass and bioproducts (Chew et al., 2017; Kong et al., 2024). Additionally, lowering the glucose level helped maintain suitable water quality, particularly alkalinity, which is critical for controlling pH within an appropriate range for both algal growth and subsequent use as *Moina* sp. feed.

Although this study used a closed culture system, which tends to exacerbate alkalinity issues due to compound accumulation (Wang et al., 2014), the G20 group maintained alkalinity levels between 130-187 mg/L. This range aligns with the optimal alkalinity for aquatic organisms in aquaculture, typically 60-150 mg/L as $CaCO_3$ (Wurts and Durborow, 1992). The findings correspond with Ramaraj et al. (2015), who recommended an alkalinity range of 75-225 mg/L for *Chlorella* sp. cultivation. However, crustaceans may require higher alkalinity levels for body and resting eggs (ephippia) composition formation, molting processes, and osmoregulation (Stevenson, 1985; Shapiera et al., 2011). Nevertheless, excessive alkalinity levels (above 420 mg/L as $CaCO_3$) can adversely affect for *Moina* sp. (Bogart et al., 2016). Higher glucose levels led to a rapid increase in alkalinity, a common issue in mixotrophic cultivation systems using both organic carbon and light (Wang et al., 2014). Maintaining optimal alkalinity is crucial for ensuring high biomass quality, as demonstrated for *Artemia* sp. cultivation in salt ponds (Anh et al., 2009). No significant differences in pH were observed between the treatment groups, with values ranging from 7.2-8.8 throughout the experiment, which are appropriate for both algal cultivation and subsequent use as *Moina* sp. feed (Dhert, 1996). Therefore, pH control may not be a primary concern in developing this algal production system.

Juneja et al. (2013) proposed an optimal pH range of 6.5-9.0 for *C. ellipsoidea* cultivation, indicating that the observed pH levels were acceptable for algal growth. Achieving an optimal pH range depends on various factors such as algal species, carbon and nitrogen sources, light intensity, and temperature. Ammonia levels, another critical factor affecting *Moina* sp. cultivation, remained below 1 mg/L for the first 9 days before increasing later, which resulted from an inverse relationship with *C. ellipsoidea* growth rate. Specifically, during days 1-9, *C. ellipsoidea* utilized ammonia for growth until reaching the stationary phase. When nutrients were depleted, *C. ellipsoidea* entered the death phase. As

C. ellipsoidea mortality increased in the system, ammonia levels consequently rose after day 10 of cultivation. However, no significant differences were found between treatment groups, possibly due to the urea concentration in the culture medium at 0.6 g/L being at an appropriate level for efficient biomass production without affecting ammonia accumulation in *C. ellipsoidea* cultivation. Studies have shown that when *C. ellipsoidea* use urea as a nitrogen source for efficient biomass production, ammonia levels can be effectively controlled (Yu et al., 2023).

Additionally, the balance between nitrogen release and uptake is crucial to prevent toxic ammonia buildup, which is essential for the growth of *Chlorella* sp. (Anyanwu et al., 2022). The observed ammonia levels are considered safe for *Moina* sp. cultivation during the first 9 days, as levels between 5-10 mg/L can adversely affect *Moina* sp. growth and survival (Collos and Harrison, 2014). In large-scale or mass culture outdoor production, agricultural fertilizers such as urea or ammonium sulfate are commonly used (Liao, 1983) to reduce production costs compared to laboratory-scale culture media. While urea is effective for ammonia control, residual ammonia in large-scale systems with high pH (due to dense algal populations, especially during daytime) (Tucker and D'Abramo, 2008) may cause toxicity from unionized ammonia (NH_3) to *Moina* sp. Therefore, large-scale cultivation systems should implement regular water exchange or fresh water addition to reduce ammonia toxicity and organic matter, or utilize aeration systems to convert nitrogen into safer forms. Additionally, aeration can enhance water flea production by improving fecundity and size (Ovie and Ovie, 2004).

The adjustment of lime, serving as a source of calcium and alkalinity, also impacted algal growth. Using 50% of the standard lime level (L50 group) provided comparable cell yields to the G20 group while maintaining better alkalinity control compared to the standard medium formulation. This differs from González-Garcinuño et al. (2014), who reported that calcium levels significantly influence growth rates and lipid accumulation in several Chlorophyta species, including *C. ellipsoidea*. Although a clear relationship between lime levels and algal growth was not observed in this study, calcium remains necessary for maintaining water quality balance in the culture system. Further investigations into the specific roles of calcium, particularly in different algal strains with varying calcium requirements, may be warranted.

In summary, reducing the glucose and lime levels from the standard culture medium formulation for *C. ellipsoidea* cultivation in a closed laboratory system can promote algal growth rates comparable to or better than the standard formulation while maintaining water quality parameters at suitable levels for subsequent use as *Moina* sp. feed. This approach may contribute to developing an efficient culture medium for producing Chlorella algae as a live feed for aquatic animal cultivation, while also reducing the cost of raw materials. Further studies in commercial-scale production systems and with various culture media formulations should be conducted to validate and optimize this approach for practical applications.

5. CONCLUSION

This study demonstrated that reducing the glucose level in the culture medium had a more pronounced effect on promoting the growth of *C. ellipsoidea* compared to reducing the lime level. The G20 group exhibited the highest growth rates and maximum cell density of 7.15×10^7 cells/mL on day 10. The 0.1 g/L glucose level maintained alkalinity within the optimal range of 130-187 mg/L, suitable for *Moina* sp. feed. Lime levels did not significantly affect alkalinity, as both the L50 and L25 groups exhibited alkalinity levels exceeding 200 mg/L. No significant differences in pH and ammonia levels were observed among the groups. Reducing glucose to 80% proved most effective for growth while maintaining suitable water quality. Further investigations in commercial-scale production systems are needed to validate these findings for practical applications.

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DECLARATION OF COMPETING INTEREST

The authors declare no conflict of interest.

AUTHORE CONTRIBUTION

Conceptualization, R.Y. and N.K.; Methodology, N.K. and P.S.; Formal Analysis, R.Y. and N.K.; Investigation, N.K. and R.Y.; Resources, P.S.; Data Curation, N.K.; Writing - Original Draft Preparation, R.Y. and N.K.; Writing - Review and Editing, R.Y., P.S. and N.K.; Visualization, N.K.; Supervision, N.K.; Project Administration, N.K.

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