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Differential Nitrogen Source Utilization Drives Growth Dynamics of *Chlorella* spp. in Walne Medium

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ABSTRACT

Microalgae, particularly *Chlorella* spp., play a crucial role as primary producers and natural feed in aquaculture systems, making their efficient cultivation especially important in response to the rising global demand for sustainable protein sources. This study aims to analyze the effect of different nitrogen sources, namely $NaNO_3$, KNO_3, and urea ($(NH_2)_2CO$), on cell density and specific growth rate of *Chlorella* spp. A laboratory-based experimental design with a completely randomized design was applied using three treatments with replications. Data were collected through daily observation of cell density using a haemocytometer over a 14-day culture period and analyzed descriptively through growth curves and specific growth rate calculations. The results showed that all treatments exhibited similar growth patterns consisting of lag, exponential, stationary, and decline phases, with peak growth occurring on the ninth day. However, $NaNO_3$ produced the highest cell density (4.6×10^7 cells mL^{-1}) and growth rate ($0.173\ day^{-1}$), followed by urea and KNO_3. These findings indicate that *Chlorella* spp. can adapt to different nitrogen forms, although nitrate remains the most efficient source. Importantly, urea demonstrated comparable performance, suggesting its potential as a cost-effective alternative in microalgae cultivation. This study contributes theoretically to understanding nitrogen utilization in microalgae and practically offers insights for optimizing culture media to improve efficiency in aquaculture applications.

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1. INTRODUCTION

Phytoplankton are a fundamental component of aquatic ecosystems, acting as primary producers in the aquatic food chain. The presence of phytoplankton, particularly *Chlorella* spp., is crucial for the success of fish farming because it serves as natural food for fish larvae, shrimp, and other aquatic organisms (Ratnawati, 2022). Globally, the increasing demand for food, particularly animal protein sources from the fisheries sector, is driving the development of efficient and sustainable aquaculture technologies (FAO, 2020). Nationally and locally, microalgae cultivation is a strategic solution for providing high-quality natural food due to its high nutritional value and rapid growth (García et al., 2020). However, the success of *Chlorella* spp. cultivation is highly dependent on nutrient availability, particularly nitrogen, which plays a role in protein and chlorophyll formation (Li et al., 2019). Nitrogen in culture media is generally provided in various forms, such as $NaNO_3$, KNO_3, and urea ($(NH_2)_2CO_2$), each influencing metabolic pathways differently (Yuniarti et al., 2023). These differences in nitrogen forms have the potential to affect microalgae density and growth rate, making further study crucial for efficiency and production optimization (Zhang et al., 2019).

Based on this background, the problem formulation in this study focuses on examining the effects of different nitrogen sources, namely $NaNO_3$, KNO_3, and $(NH_2)_2CO$, on the growth of *Chlorella* spp. This study specifically aims to analyze the growth pattern of *Chlorella* spp. density under each nitrogen source treatment, to evaluate the specific growth rate of *Chlorella* spp. in each treatment, and to determine the conditions under which different nitrogen sources provide optimal responses for microalgae growth. Previous research has demonstrated that nitrogen is a key nutrient influencing phytoplankton growth. It plays a crucial role in the synthesis of proteins, enzymes, and chlorophyll, which are essential for photosynthesis and cell division. In microalgae cultures, nitrogen is commonly available in the form of nitrate, ammonium, or organic compounds such as urea, each exhibiting different absorption efficiencies and metabolic pathways.

Several studies have shown that the use of $NaNO_3$ as a nitrogen source provides stable results in supporting the growth of *Chlorella* spp., because the nitrate form is easily absorbed and directly utilized in cell metabolism (Markou et al., 2017). On the other hand, KNO_3 is also often used as an alternative nitrogen source, although its effectiveness can be affected by environmental conditions and the composition of the culture medium (Gour et al., 2018). Urea ($(NH_2)_2CO$) as an organic nitrogen source is known to have good potential because it can be converted into ammonia which is then utilized by microalgae cells (Yaakob et al., 2021). However, previous research has shown that different nitrogen sources do not always significantly impact microalgae growth. This is due to other factors influencing growth, such as light intensity, temperature, pH, and the availability of other nutrients in the culture medium (Sajjadi et al., 2018).

The limitations of this study lie in the limited number of studies that simultaneously compare the effects of three nitrogen sources ($NaNO_3$, KNO_3, and urea) on two main growth parameters, namely cell density and specific growth rate in a controlled culture system. In addition, there are still limited studies that examine the effectiveness of alternative nitrogen sources (such as technical KNO_3 and urea) compared to $NaNO_3$ as a standard in Walne media, especially in the context of microalgae production efficiency (Arora et al., 2020; Singh et al., 2019). This study aims to analyze the effect of different nitrogen sources on the growth of *Chlorella* spp., both in terms of cell density and specific growth rate. Furthermore, this study also aims to identify the most effective nitrogen source for supporting microalgae growth in Walne medium (Xin et al., 2017).

The novelty of this study lies in its comparative approach, which simultaneously integrates two key growth parameters, density and specific growth rate, within a single controlled experimental design. In addition, this study offers contextual value by evaluating the use of more economical alternative nitrogen sources as substitutes for $NaNO_3$ in *Chlorella* spp. cultures. This research is expected to contribute both theoretically and practically. From a theoretical perspective, it enriches the understanding of microalgal growth dynamics in response to different nitrogen sources and provides a more comprehensive insight into the metabolic flexibility of *Chlorella* spp. Practically, the results of this study can serve as a reference for developing more efficient and

economical microalgae culture techniques, particularly in the aquaculture industry. The use of alternative nitrogen sources such as urea and KNO_3 has the potential to reduce production costs without compromising the quality of the culture. Furthermore, this research can also serve as a basis for further research on optimizing microalgae culture media on a larger scale.

2. MATERIALS AND METHOD

2.1 Research Design

This study used a laboratory experimental approach with a completely randomized design (CRD) consisting of three nitrogen source treatments: $NaNO_3$ as a control, technical KNO_3, and $(NH_2)_2CO_2$ (urea). This approach was chosen because it allows for controlled testing of the effect of the independent variable (nitrogen source) on the dependent variables, namely the density and growth rate of Chlorella spp. The experimental design involved repetition of each treatment to increase the validity of the results. This method is relevant because the research focuses on the biological responses of microalgae under strictly controlled environmental conditions, allowing for clear causality (Kumar et al., 2018).

2.2 Location and Participants

The research was conducted in the microalgae culture laboratory at the Ambon Marine Aquaculture Center, Maluku Province, Indonesian Institute of Sciences, which has controlled phytoplankton culture facilities. The research object was the microalgae *Chlorella* spp. cultured in Walne medium. This organism was selected based on its role as an important natural food in fisheries cultivation, its rapid growth rate, and its ease of cultivation. The experimental units consisted of culture vessels (Erlenmeyer flasks), each containing media with a different treatment. Each treatment was replicated three times to ensure data consistency. The number of experimental units was deemed adequate because it met the replication principle in biological experiments, thus optimally representing the growth conditions of *Chlorella* spp.

2.3 Data Collection Techniques

The data collected in this study included cell density and specific growth rate of *Chlorella* spp. Data collection was carried out using the following techniques. First, direct observation (laboratory observation), cell density was counted every 24 hours using a hemocytometer under a microscope. Observations were made consistently for 14 days to determine growth patterns. Second, data documentation, the calculated data is recorded in a daily table which is then used to create growth graphs and specific growth rates. Finally, mathematical calculations, the specific growth rate was calculated using the microalgae growth formula based on changes in cell density over time.

Data validity was maintained through method triangulation, which involves comparing direct observation results with mathematical calculations. Furthermore, replication was performed for each treatment to increase data reliability. From the research ethics aspect, all procedures were carried out according to laboratory standards, maintaining the sterility of the media, and ensuring that there was no contamination that could affect the research results.

2.4 Research Procedures

The research was conducted through several systematic stages as follows. Preparation of tools and materials: through includes sterilization of equipment, preparation of Walne media, and preparation of nitrogen sources according to treatment. Making culture media: Walne media was prepared with a complete nutrient composition, then modified in the nitrogen source section according to the treatment ($NaNO_3$, KNO_3, and urea). Inoculation of *Chlorella* spp.: Microalgae seeds were introduced into each culture vessel with the same volume to ensure initial uniformity. Culture maintenance: the culture was maintained for 14 days under controlled environmental conditions, such as light intensity, temperature, and aeration. Daily observations: Cell density was calculated daily, then used to determine the specific growth rate. Data processing: the data obtained is arranged in tables and graphs to facilitate interpretation of the results. This procedure

is carried out sequentially to ensure that each stage runs systematically and produces accurate data.

2.5 Data Analysis Techniques

Data analysis was carried out using a descriptive quantitative approach by interpreting the cell density values and specific growth rates obtained from the observations. The analysis stages included the following: data reduction, in which daily data on density and growth rate were summarized in tabular form; data presentation, in which the data were visualized in graphs to observe growth patterns in each treatment; interpretation, in which growth patterns were analyzed based on the microalgae growth phases (lag, exponential, stationary, and death); and comparison between treatments, which was conducted to determine the effectiveness of each nitrogen source on the growth of *Chlorella* spp.

The validity of qualitative data is maintained through the principles of credibility (consistency of results between repetitions), dependability (reliability of procedures), and confirmability (conformity of data with observation results). With this approach, the analysis not only describes growth patterns, but also provides an in-depth understanding of the influence of nitrogen sources on the growth dynamics of *Chlorella* spp.

3. RESULTS AND DISCUSSION

3.1 Density level of *Chlorella* spp

The pattern of changes in the average density level of *Chlorella* spp during the 14-day culture period in three different nitrogen source treatments, namely $NaNO_3$ (control), KNO_3, and $(NH_2)_2CO$, as can be seen on Figure 1.

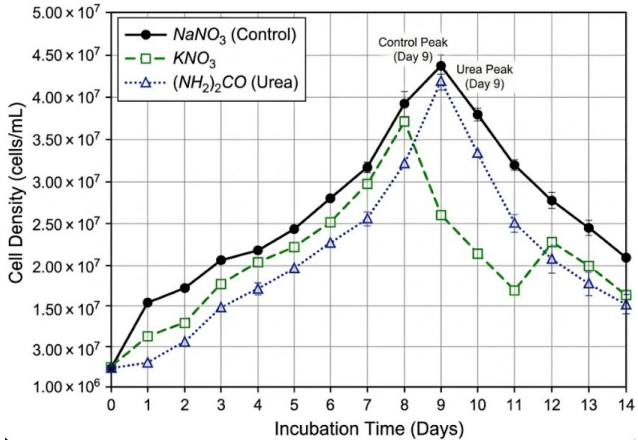

Figure 1. Graph of the average density level of *Chlorella* spp in the three treatments.

Based on the cell density graph, the growth of *Chlorella* spp showed a consistent pattern in the three nitrogen source treatments ($NaNO_3$, KNO_3, and $(NH_2)_2CO$). In the initial phase (day 0 to day 2), cell density was relatively low and increased gradually. In $NaNO_3$ treatment, density increased from 4.08×10^6 cells/mL (day 0) to 1.06×10^7 cells/mL (day 2). In KNO_3, the density increased from 6.72×10^6 to 1.1×10^7 cells/mL, while in urea from 7.44×10^6 to 9.94×10^6 cells/mL. Entering the rapid growth phase (day 3 to day 8), there was a significant increase in all treatments. On day 5, the density reached 2.45×10^7 cells/mL ($NaNO_3$), 2.18×10^7 cells/mL (KNO_3), and 2.5×10^7 cells/mL ($(NH_2)_2CO$). The increase continued until day 8 with almost the same values between $NaNO_3$ and KNO_3, namely 3.79×10^7 cells/mL, and urea at 3.77×10^7 cells/mL.

The peak density occurred on day 9 for all treatments. The $NaNO_3$ treatment showed the highest value of 4.6×10^7 cells/mL, followed by urea at 4.4×10^7 cells/mL, and KNO_3 at 3.3×10^7 cells/mL. After that, there was a gradual decrease in density until day 14. At the end of the observation, the cell density decreased to 1.9×10^7 cells/mL ($NaNO_3$), 1.65×10^7 cells/mL (KNO_3),

and 1.47×10^7 cells/mL ($(NH_2)_2CO$). The growth patterns obtained indicate that *Chlorella* spp. undergoes four distinct growth phases. The lag phase (days 0–2) is characterized by a slow increase as cells adapt to the new environment. The exponential phase (days 3–8) is characterized by a significant increase in density due to active cell division. The stationary phase occurs around day 8 to day 9, when the density reaches its maximum value, particularly in the $NaNO_3$ treatment of 4.6×10^7 cells/mL.

The differences in density values between treatments indicate that nitrogen source affects growth rate, but not drastically. $NaNO_3$ as a control produced the highest density, indicating that nitrate in this form is more optimally utilized by cells. Urea ($(NH_2)_2CO$) showed performance close to $NaNO_3$, especially at the peak phase (4.4×10^7 cells/mL), indicating that organic nitrogen can also be utilized effectively. In contrast, KNO_3 showed a lower density at its peak (3.3×10^7 cells/mL), although in the previous phase it was comparable to the other treatments. This indicates a possible difference in the efficiency of nitrogen uptake or metabolism of KNO_3 compared to the other forms. The decrease in density after 9th day indicates a death phase, which is likely caused by nutrient limitation, metabolite accumulation, or intercellular competition in the culture medium.

Theoretically, these results indicate that nitrogen is an important factor in the growth of *Chlorella* spp., but different forms of nitrogen produce relatively similar growth responses. This suggests that *Chlorella* spp. has the ability to physiologically adapt to various nitrogen sources (Rasdi & Qin, 2018; Wang et al., 2019). Practically, these results have important implications for microalgae cultivation. Urea ($(NH_2)_2CO_2$) can be an effective alternative to $NaNO_3$ because it produces nearly equivalent densities at the peak phase (Khalili et al., 2020). This has the potential to reduce mass-scale production costs because urea is generally more economical and readily available. Meanwhile, the use of KNO_3 can still be used, but it may be less than optimal if the primary goal is to achieve maximum density (Sharma et al., 2021).

Therefore, the choice of nitrogen source in *Chlorella* spp. cultures can be tailored to production objectives, both for cost efficiency and biomass maximization. The results of this study indicate that differences in nitrogen sources did not significantly impact the growth patterns of *Chlorella* spp. This is evident from the similarity of the growth curve patterns and peak time (day 9th) across all treatments (Ferreira et al., 2019).

This similarity indicates that environmental factors and culture medium conditions play as important a role as the type of nutrient used. Despite differences in maximum values, overall, all three treatments supported microalgae growth well. Thus, this study confirms that varying nitrogen sources has a greater impact on the level of growth optimization, rather than on the basic growth capacity of *Chlorella* spp. This reinforces the understanding that microalgae have the flexibility to utilize various forms of nitrogen as long as their nutritional needs are met.

3.2 Specific growth rate of *Chlorella* spp.

The pattern of changes in the specific growth rate of *Chlorella* spp during a 14-day culture period in three nitrogen source treatments, namely $NaNO_3$, KNO_3, and $(NH_2)_2CO$ (Figure 2).

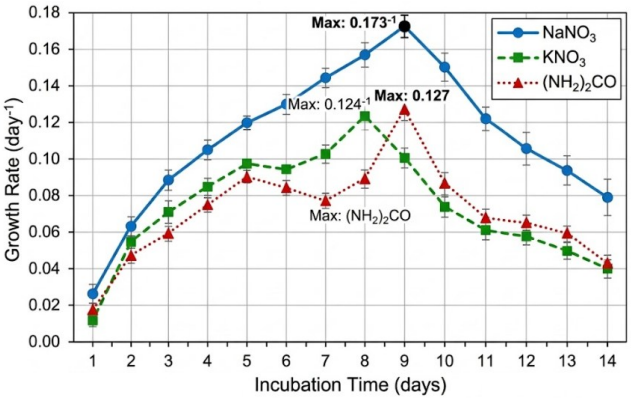

Figure 2. Graph of specific growth rate of *Chlorella* spp.

Based on the growth rate graph, it can be seen that the specific growth rate of *Chlorella* spp experienced an increasing pattern, reached a peak, then decreased in the three treatments ($NaNO_3$, KNO_3, and $(NH_2)_2CO$). In the initial phase (day 1 to day 3), the growth rate was still relatively low. The $NaNO_3$ treatment showed an increase from 0.038/day to 0.098/day. In KNO_3, it increased from 0.011/day to 0.050/day, while in $(NH_2)_2CO_2$, it increased from 0.040/day to 0.054/day. This indicates the cell adaptation phase to the culture environment.

Entering the active growth phase (day 4 to day 8), there was a significant increase in all treatments. On day 5, the growth rate reached 0.128/day ($NaNO_3$), 0.084/day (KNO_3), and 0.087/day ($(NH_2)_2CO$). The increase continued until the 8th day with values of 0.159/day ($NaNO_3$), 0.124/day (KNO_3), and 0.116/day ($(NH_2)_2CO$). The peak growth rate occurred on ninth day. The highest value was achieved by the $NaNO_3$ treatment at 0.173/day, followed by $(NH_2)_2CO_2$ at 0.127/day, and KNO_3 at 0.114/day. After that, the growth rate experienced a gradual decrease until day 14. At the end of the observation, the growth rate values were 0.110/day ($NaNO_3$), 0.064/day (KNO_3), and 0.049/day ($(NH_2)_2CO_2$).

The specific growth rate patterns obtained showed a close relationship with cell density dynamics. The initial phase with low growth rates reflects the lag phase, where cells are still physiologically adapting to the culture medium. The exponential phase is characterized by a significant increase in growth rate, particularly on days 4 to 8. During this phase, cells undergo maximum division due to sufficient nutrient availability and optimal environmental conditions. The highest growth rate in the $NaNO_3$ treatment (0.173/day) indicates that this nitrogen source is the most efficient in supporting cell metabolism.

The $(NH_2)_2CO$ treatment showed a relatively high growth rate, approaching that of $NaNO_3$, especially at the peak phase (0.127/day). This indicates that nitrogen in the form of urea can be utilized well by *Chlorella* spp., although it may require a conversion process before being used in metabolism (Tan et al., 2020; Widjaja et al., 2018). In contrast, KNO_3 showed a lower growth rate than the other treatments. However, the growth pattern followed the same trend, indicating that KNO_3 can still support growth, but with lower efficiency (Rizwan et al., 2018). The decline in growth rate after day 9 indicates that the culture is entering the stationary phase, leading to the death phase. This is likely due to nutrient depletion, increased cell density leading to competition, and the accumulation of metabolic waste in the medium (Liang et al., 2019).

Theoretically, these results suggest that specific growth rate is an important indicator in evaluating the efficiency of nutrient utilization by microalgae. A higher growth rate indicates the cells' ability to optimally utilize nutrients for division and metabolism (Xin et al., 2017). From a practical perspective, these results indicate that $NaNO_3$ is the most optimal nitrogen source for increasing the growth rate of *Chlorella* spp. However, $(NH_2)_2CO$ also shows considerable potential as an alternative because it produces a relatively high growth rate (Arora et al., 2020).

Using urea as a nitrogen source can be a more economical option for mass production, especially considering cost efficiency and material availability. Meanwhile, using KNO_3 can be considered as an alternative, although the results are not as optimal as the other two nitrogen sources. When compared with the cell density data, the specific growth rate pattern shows strong consistency. The peak growth rate on day 9 coincides with the peak cell density in the previous graph. This indicates that the increase in growth rate directly contributes to the increase in cell number in the culture. Furthermore, the decline in growth rate after day 9 was also accompanied by a decrease in cell density. This confirms that the specific growth rate can be used as an early indicator to predict the population decline phase (Sajjadi et al., 2018). Overall, all three treatments showed similar patterns, thus concluding that differences in nitrogen sources did not alter growth patterns but did affect growth rates. $NaNO_3$ gave the best results, followed by $(NH_2)_2CO$, and finally KNO_3.

4. CONCLUSION

This study shows that different nitrogen sources ($NaNO_3$, KNO_3, and $(NH_2)_2CO$) have an effect on the growth of *Chlorella* spp, both in terms of cell density and specific growth rate. The growth

patterns produced in the three treatments were relatively similar, namely through lag, exponential, stationary, and decline phases, with peak growth occurring on day 9. However, quantitatively, $NaNO_3$ produced the highest density and growth rate, followed by urea ($(NH_2)_2CO$), and then KNO_3. These findings confirm that, despite their different nitrogen forms, *Chlorella* spp. exhibits the ability to adapt to various nitrogen sources. Practically, urea can be an efficient and economical alternative to $NaNO_3$ in microalgae cultures without significantly reducing productivity. This research contributes to the optimization of microalgae culture media, particularly in terms of nutrient utilization efficiency. However, limitations lie in the laboratory scale and limited environmental variables. Therefore, further research is recommended to test nitrogen source combinations and their application on a larger production scale and under more diverse environmental conditions.

AUTHORS CONTRIBUTION

FPP and ETA designed and conducted the research, AK analyzed and interpreted the data, and all authors contributed to writing the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest and take full responsibility for the content of the article, including any implications of AI-generated art.

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