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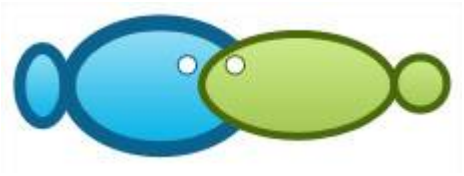

Influence of different culture media and light intensity on the growth and biomass productivity of a newly isolated *Chlorella* sp. UNM-IND1 from Waepella hot spring, South Sulawesi, Indonesia

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Abstract. The *Chlorella* sp. UNM-IND1 is a newly isolated microalga species from Waepella hot spring in Sinjai Regency, South Sulawesi, Indonesia. As a newly isolated species, there is no information available regarding its optimum culture condition for high growth rate and biomass productivity. Therefore, this study aimed to analyze the growth and productivity of the alga under different culture media and light intensity. The alga was cultured under various culture media including F/2, Walne, Jaworski, and NPK+urea at salinity 0% NaCl and incubated at 25°C, the light intensity of 2500 lux with light and dark cycles of 12:12 hours. The best culture media was then used for the light-intensity study. The cultures were grown in NPK+urea media under different light intensities (2500, 3500, and 8000 lux) and incubated at 25°C with light and dark cycles of 12:12 hours. The alga was cultured for 2 weeks. The results showed that the highest growth (0.77 day^{-1}) and biomass productivity (0.13 g L^{-1} day^{-1}) was obtained when the alga was cultured in NPK+urea media. For the light intensity, the highest growth rate (0.94 day^{-1}) was obtained at the highest light intensity (8000 lux). For the biomass productivity, both light intensity of 3500 and 8000 lux have equal biomass productivity of about 0.311 and 0.310 g L^{-1} day^{-1}, respectively. This study indicated that the microalga *Chlorella* sp. UNM-IND1 prefers NPK+urea medium and high light intensity for higher growth rate and biomass productivity.
Key Words: *Chlorella* sp., culture media, extremophiles, hot springs, light intensity.

Introduction. Microalgae are photosynthetic prokaryotic and eukaryotic microorganisms that can be found in almost all aquatic ecosystems (Mata et al 2010) including extreme habitats (Merino et al 2019). Extreme habitat is an environment that has conditions beyond normal limits to support the life and growth of organisms such as an environment with very high temperatures (hot springs), very low temperatures (snow), high salinity (hypersaline ponds/lakes), very high pH or highly acidic (Varshney et al 2015). Microalgae that can live in extreme habitats show that these microalgae have certain mechanisms to adapt to that environment. Several commercially important microalgae are known to live under extreme conditions. For example, *Dunaliella salina* is a green alga that can live at high salinity and light intensity and accumulate beta-carotene and glycerol as a mechanism to survive against high light intensity and salinity (Indrayani 2017). *Spirulina* sp. has a very high protein content of up to 70% and has a selective environment to grow well at very high pH. The selective environment of *D. salina* and *Spirulina* allows these two types of microalgae to be commercially cultured in outdoor conditions without contamination (Avron & Ben-Amotz 1992).

Microalgae have great potential as a source of important biochemical compounds that have very wide applications in the food, feed, cosmetic, nutraceutical, chemical, and pharmaceutical industries, even the biofuel industry (Olaizola 2003).

Exploration of microalgae for any commercial application requires a long process. Species or strain selection is the first and most important step in microalgae bioprospecting activities for commercial applications (Borowitzka 2013). One of the strategies employed to obtain potential strains/species of microalga is the collection and isolation of microalgae species from natural environments, in particular selective or extreme environments. There are several extreme aquatic habitats/environments specifically hot springs in South Sulawesi. Waepella hot spring is one of them. It is a famous recreational area located in Sinjai Regency, South Sulawesi. This habitat has not been studied for microbial diversity. We have successfully isolated several microalgal species from the Waepella hot spring and one of them is *Chlorella* sp. UNM-IND1. This strain is of interest as it grows fast when transferred from agar media into liquid media. Most importantly, this alga does not stick to the culture vessel and it remains suspended in the medium under mixing conditions resulting in fast growth and high biomass productivity. As a newly isolated species, information about the optimal culture conditions for high growth rate and biomass productivity is unknown. Therefore, this study aimed to analyze the growth and biomass productivity of the alga under various culture media and light intensity. This research is expected to be the basis for the development of the local species microalgae for commercial applications.

Material and Method. This study was conducted from March to December 2022. The microalgae species used in this study was *Chlorella* sp. UNM-IND1 isolated from Waepella hot spring in Sinjai Regency, South Sulawesi, Indonesia. The alga was isolated using the agar plating technique in Guillard's F/2 agar medium (2% w/v) (Andersen & Kawachi 2005). The alga was maintained in the Agricultural Technology Laboratory, Faculty of Engineering, Universitas Negeri Makassar.

Growth and productivity under different culture media. This experiment aimed to analyze the effect of different culture media on the growth and biomass productivity of *Chlorella* sp. UNM-IND1. The media used were Guillard's F/2, Walne, Jaworski, and NPK+urea. Microalgae were cultured using 300 mL Erlenmeyer containing 150 mL culture in respective media and triplicates. The cultures were incubated at 25±1^{o}C with a light intensity of about 2500 lux and a light and dark cycle of 12 hours:12 hours.

Growth and productivity under different light intensities. This experiment aimed to analyze the effect of different light intensities on the growth and biomass productivity of *Chlorella* sp. UNM-IND1. The microalgae were cultured using NPK+urea medium in 300 mL volume Erlenmeyer containing 150 mL culture. The NPK+urea medium was chosen based on the previous experiment showing that the NPK+urea medium was the best medium for growing *Chlorella* sp. UNM-IND1. The cultures were incubated under different light intensities (2500, 3500, and 8000 lux) at a temperature of 25±1^{o}C and a light and dark cycle of 12 hours:12 hours.

Analytical methods

Specific growth rate (SGR). Calculation of the cell number of the cultures was carried out every two days for 2 weeks using a Neubauer hemocytometer. The growth curve was generated from the cell density data. The calculation of the SGR used the formula proposed by Moheimani et al (2013):

$$SGR = [Ln(N2/N1)/(t2-t1)]$$

where: N2 is the cell density at time t2 and N1 is the cell density at time t1 within the exponential phase.

Dry weight (biomass). Dry weight was determined following the method of Moheimani et al (2013). Briefly, five mL of culture was filtered through pre-weighed and pre-combusted

Whatman GF/C, 25 mm using Millipore filter apparatus. The filters were removed from the apparatus and then dried in an oven at 75°C for 5 hours. The filters were then cooled in a desiccator before weighing. The DW was determined by the following equation:

$$DW\ (g\ L^{-1}) = (\text{weight of filter+algae}) - \text{weight of filter}$$

Biomass productivity. Biomass productivity was determined by the following equation:

$$\text{Biomass productivity}\ (g\ L^{-1}\ d^{-1}) = SGR \times DW$$

Statistical analysis. One-way analysis of variance (ANOVA) was used to analyze significant differences between treatments. To precisely test differences between conditions, the Pairwise multiple comparison procedure (Holm-Sidak Method) was used. All statistical analysis was performed using Sigma-Plot 14 Systat Software Inc., USA.

Results and Discussion

Growth and biomass productivity of Chlorella sp. UNM-IND1 under different growth media. Microalga *Chlorella* sp. UNM-IND1 is a newly isolated alga from Waepella hot springs in Sinjai Regency, South Sulawesi, Indonesia. As a new isolate, there is no information available regarding the best culture medium for high growth rate and biomass productivity. Four different media namely F/2, Walne, Jaworski, and NPK+urea media were chosen for the determination of the best culture media for optimum growth and high productivity. The results of this study showed that the alga could grow well in all types of culture media used. There was no lag phase observed from all cultures following initial inoculation. The initial cell density for all cultures was the same at approximately 100 x 10^4 cells mL^{-1}. Cell density increased exponentially on the second day reaching an average of 471 x 10^4 cells mL^{-1} in the NPK+urea medium, 434 x 10^4 cells mL^{-1} in the F2 medium, 405 x 10^4 cells mL^{-1} in the Walne medium, and 330 x 10^4 cells mL^{-1} in the Jaworski medium. Afterward, the growth of the cultures slowed down before reaching the maximum cell density on day 10^{th}. The highest cell density was obtained from the culture grown in the NPK+urea medium (2214 x 10^4 cells mL^{-1}) and the lowest one was obtained in the Jaworski medium (1262 x 10^4 cells mL^{-1}). All the cultures entered the death phase on day 12 (Figure 1).

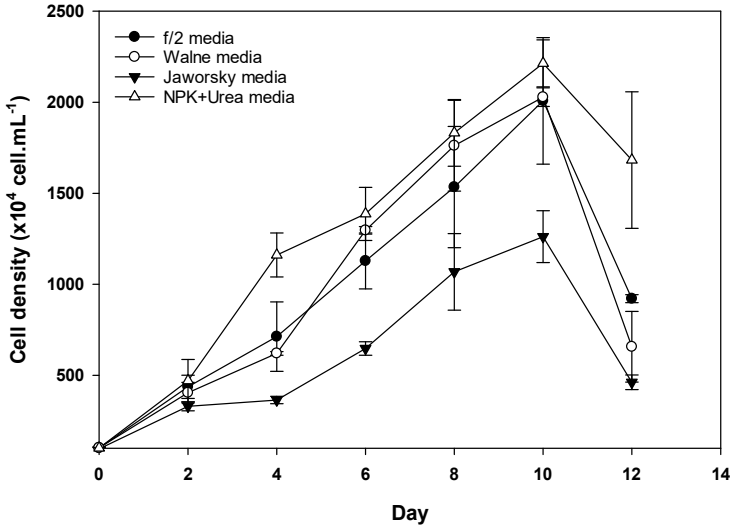

Figure 1. Growth curve of *Chlorella* sp. UNM-IND1 under different culture media.

There was no significant difference in the SGR between different culture media tested (One Way Anova, $p > 0.05$). The SGR of the alga ranged from 0.61 to 0.77 day^{-1}. Cultures grown using NPK+urea medium showed the highest SGR whereas their counterpart grown using Jaworski medium had the lowest SGR (Figure 2).

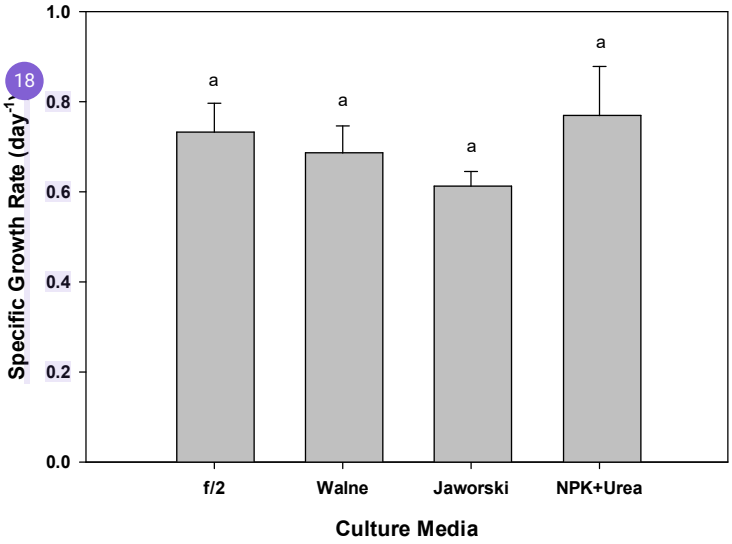

Figure 2. The specific growth rate of *Chlorella* sp. UNM-IND1 under different culture media.

Determination of dry biomass was taken at the exponential phase (day 4) and at the stationary phase (day 10). The biomass yield of all the cultures at the exponential phase was almost the same ranging from 0.053 to 0.067 g L^{-1}. At the stationary phase, the biomass increased to more than two folds in Walne and NPK+urea media. The cultures grown in Walne media had the highest biomass (0.18 g L^{-1}) whereas the ones grown in Jaworski media had the lowest biomass (0.087 g L^{-1}). Statistical analysis showed that there was no significant difference in the biomass of the culture among different culture media used (One Way Anova, $p > 0.05$) (Figure 3).

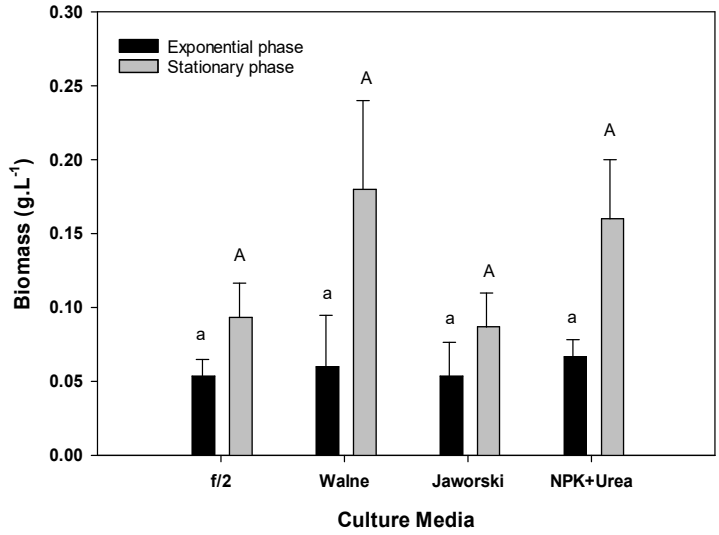

Figure 3. Biomass yield of *Chlorella* sp.UNM-IND1 under different culture media.

From the SGR and the biomass yield data, biomass productivity was calculated. Alga cultured in Walne and NPK+urea media had the same biomass productivity of about 0.124 g L^{-1} d^{-1}, and the lowest biomass productivity was obtained in Jaworski media (0.053 g L^{-1} d^{-1}). The statistical analysis showed that there was no significant difference in the biomass productivity of the microalga under different media (One Way Anova, $p > 0.05$) (Figure 4).

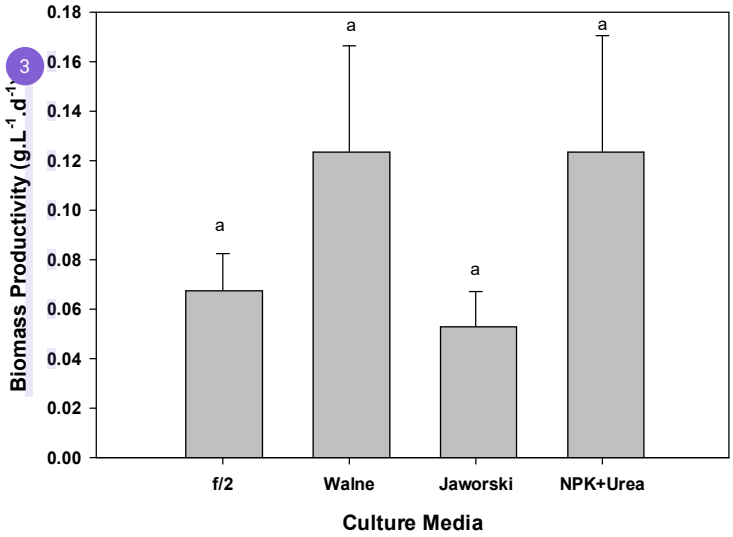

Figure 4. Biomass productivity of *Chlorella* sp. UNM-IND1 under different culture media.

Microalgae require nutrients to make up biomass through photosynthesis and the nutrient requirements of algae varied naturally. As with the higher plants, algae require macronutrients such as nitrogen, phosphorous, and carbon as well as micronutrients and vitamins (Markou et al 2014; Khan et al 2018). Various culture media are available for microalgae, each of which has a different composition and concentration. To find out the best culture medium for the growth and productivity of the newly isolated microalgae *Chlorella* sp. UNM-IND1, the alga was grown on various culture media including F/2, Walne, Jaworski, and agricultural fertilizers NPK+urea.

This study found that the alga could grow well on all culture media tested and there was no lag phase observed following initial inoculation. The culture experienced the highest growth rate in the first two days because the cells undergo rapid division due to the availability of nutrients and abundance of light to support their growth. Afterward, the growth rate slowed down and reached the maximum cell density on day 10. The slow growth was caused by the decrease in nutrients consumed by the actively growing cells so they are not sufficient to support cell growth and division (Van Vooren et al 2012; An et al 2020; Yaakob et al 2021). On day 12, all cultures entered the death phase. The death phase is marked by a decrease in the cell densities due to cells death which is caused by various factors including nutrients deprivation (Zachleder et al 2014; Vítová et al 2015), limited light received by the cells (Borowitzka 2016), limited CO_2 (Cho et al 2011; Park et al 2011) and possibility of metabolic waste build up in the culture medium. The death phase is also marked by changes in the color of the culture, the formation of foam on the surface of the culture media, and clumps of algal cells that settle to the bottom of the culture media.

The results of this study indicate that the microalgae *Chlorella* sp. UNM-IND1 can grow well on all types of media tested with the same growth curve pattern. The fundamental difference between the four types of media used is the N source where F/2, Walne and Jaworski media use $NaNO_3$ as N source while agricultural fertilizers use NPK and urea as N sources. Although there were no significant differences in the SGR, biomass and biomass productivity, the highest SGR and biomass productivity were obtained in the cultures using media enriched with NPK+urea agricultural fertilizers. The preferential uptake of urea by the microalgae cell is probably due the physiologycal capacity of urea uptake by the microalgae. In addition, the conversion of urea to ammonium is more energetically beneficial because the hydrolysis of urea results in the formation of 2 ammonium molecules (Finlay et al 2010; Donald et al 2011) and carbon dioxide (CO_2) as a side product that will be utilized by cells in the process of photosynthesis (Glibert et al 2014). The results of this study are in line with the results of a study conducted by Erratt (2017) who examined the preferential use of different N sources (NO_3, NH_4 and urea) from 3 species of microalgae namely *Dolichospermum flos-*

aquae, *Microcystis aeruginosa* and *Synechococcus* sp. He found that urea was the preferred N source for all three types of microalgae. Similarly, Indrayani (2017) also found that the microalgae *Amphora* sp. MUR258 can utilize and assimilate different N sources (ammonium, nitrate and urea). However, urea seems to be the preferable niotrogen source.

The microalga isolate *Chlorella* sp. UNM-IND1 achieved higher SGR and biomass productivities in NPK+urea media. The ability of the microalga to grow well on NPK+urea media is advantageous considering that NPK+urea is an agricultural fertilizer that is easy to obtain and it is cheaper compared to that of laboratory-grade mediums such as F/2, Walne, and Jaworski. The urea fertilizer is not only cheaper but also has a higher N content so that the application rate is lower, the solubility of urea is higher and the use of urea fertilizer is safer because it does not explode so it is safer during transportation and storage (Glibert et al 2006; Paerl et al 2016).

Growth and biomass productivity of Chlorella sp. UNM-IND1 under different light intensities. Light is a source of energy for the process of photosynthesis where the quality and quantity of light are the main factors that control the growth and productivity of microalgae culture (Pulz & Scheibenbogen 1998; Masojídek et al 2011). Nonetheless, excessive light can reduce productivity due to photoinhibition and photodamage (Borowitzka 2016). The effect of different light intensities on the growth and biomass productivity of the newly isolated microalga *Chlorella* sp. UNM-IND1 was studied. The study showed that the alga could grow well in all light intensity tested. The growth curve of all cultures showed a similar pattern. The cultures could adapt well following inoculation as no lag phase was observed. All the cultures grew fast in the first four days reaching the highest cell density on day 6 before entering the death phase on day 8 marked by a gradual decrease in the cell density (Figure 5).

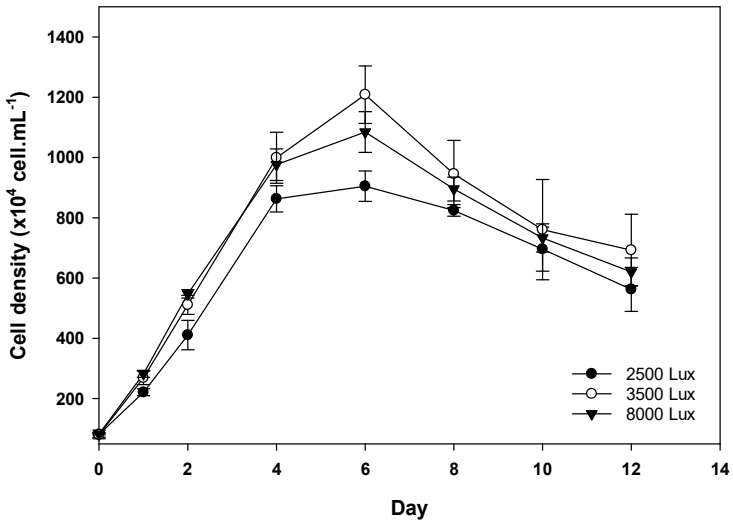

Figure 5. Growth curve of *Chlorella* sp. UNM-IND1 under different light intensity.

There was no significant difference in the SGR of the cultures grown under different light intensities (One Way Anova, $p > 0.05$). The SGR ranged from 0.81 to 0.94 day^{-1}. Cultures grown at the highest light intensity (8000 lux) had the highest SGR whereas the ones grown at 2500 lux had the lowest SGR (Figure 6).

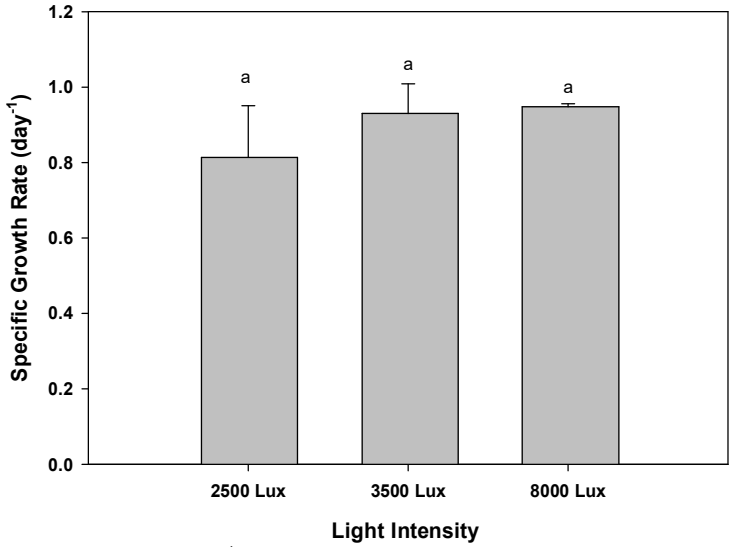

Figure 6. Specific growth rate (day^{-1}) of *Chlorella* sp. UNM-IND1 under different light intensity.

Microalgae cell biomass was calculated to determine the dry weight of biomass during the cultivation process. Microalgae samples used were taken on the 4th and 6th days. The highest biomass on day 4th was obtained from the cultures using a light intensity of 3500 lux with an average biomass of 0.29 g L^{-1}, and the lowest biomass value was 0.16 g L^{-1} obtained at a light intensity of 2500 lux. On day 6, the highest biomass was obtained at a light intensity of 3500 lux of 0.33 g L^{-1} and the lowest at a light intensity of 2500 lux at 0.23 g L^{-1}. The statistical analysis showed that different light intensities had a significant effect on the cell biomass of *Chlorella* sp. UNM-IND1 ($p < 0.05$) (Figure 7).

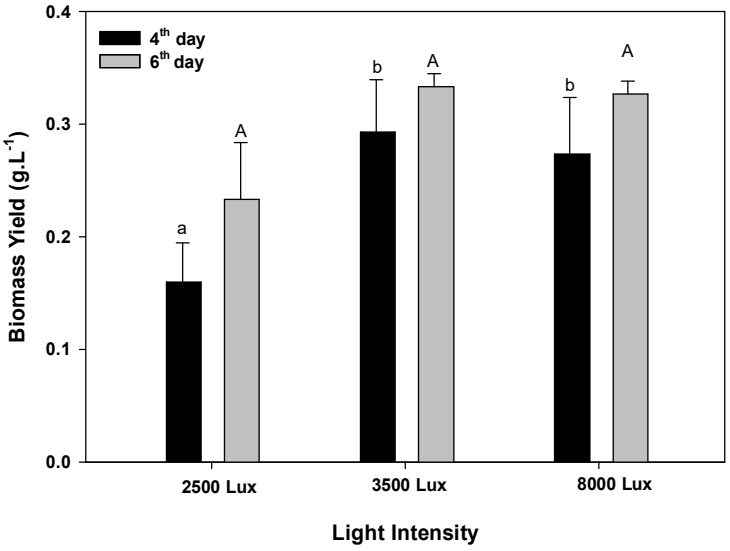

Figure 7. Biomass yield of *Chlorella* sp. UNM-IND1 under different light intensity.

There was a significant difference between the biomass productivity of the cultures grown under different light intensities ($p < 0.05$). The cultures grown at the highest light intensity of 8000 lux and 3500 lux had almos the same biomass productivity of 0.310 and 0.311 g L^{-1} day^{-1}, respectively and the lowest biomass productivity of 0.18 g L^{-1} day^{-1} was obtained at a light intensity of 2500 lux (Figure 8).

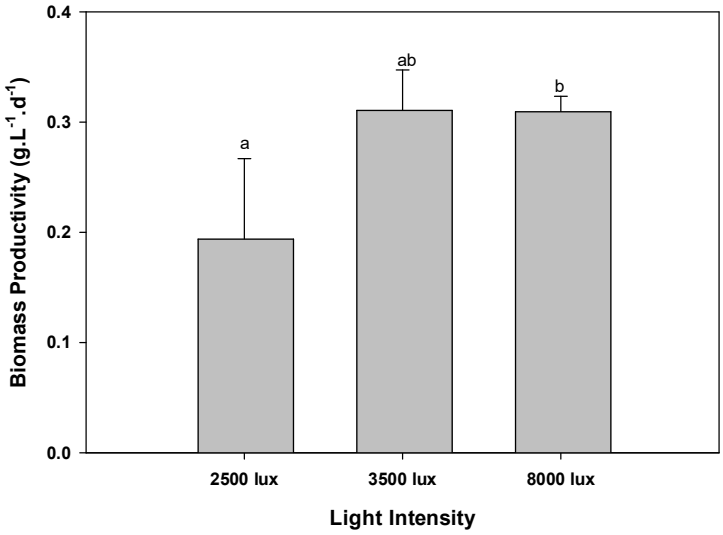

Figure 8. Biomass productivity of *Chlorella* sp. UNM-IND1 under different light intensity.

Microalgae growth is divided into several phases, namely the lag phase, exponential phase, stationary phase, and death phase. During the cultivation period of the microalga *Chlorella* sp. UNM-IND1, almost all phases could be observed except for the lag phase. The cell density of all cultures increased exponentially following initial inoculation from the initial cell density of about 80 x 10^4 cell mL^{-1} on day 0 up to about 282 x 10^4 cells mL^{-1} on day 1. The possible reason for the absence of the lag phase was due to the ability of the alga to quickly adapt to the new environment. The adaptation phase may occur only moments after inoculation, then microalgae can adapt rapidly and grow exponentially within a day as the culture conditions and the media used for inoculum preparation were the same as for the experimental conditions except for the light intensity. According to Wang et al (2012), the adaptation phase will take place more quickly or will not be seen if there are no differences in environmental conditions and nutrients in starter cultivation with experimental media cultivation. After exponential growth in the first two days, the cell density continued to increase at a slower rate reaching maximum cell density on day 6 up to 1208 x 10^4 cells mL^{-1} before entering the death phase on day 8 indicated by a decrease in the cell density.

The SGR of the cultures grown under different light intensities showed no significant differences ranging from 0.81 to 0.94 day^{-1} indicating that the alga could adapt well in response to variation of the light intensity. This is in line with the growth curve in Figure 3 as no lag phase was observed following inoculation of the cells under different light intensities showing that the cells could adapt well to changing light intensity. Although there was no significant difference in the growth rate of the alga under different light intensities, the growth rate of the alga increased with light intensity up to a certain level of light intensity. In this study, the growth rate of the alga increased when the light intensity was increased from 2500 to 3500 lux. However, further increases in the light intensity from 3500 to 8000 lux did not significantly increase the growth rate of the alga. This is in line with general findings that the growth rate of the alga will increase at increasing light intensity up to a certain species-dependent level (Bialevich et al 2022) and further increase in light intensity above the saturation point will lead to photoinhibition (Difusa et al 2015). SGR of the *Chlorella* sp. UNM-IND1 in this study was higher than in other similar studies. For example, Ievina & Romagnoli (2020) reported the highest growth rate of *Chlorella vulgaris* (0.552 day^{-1}) was achieved at a light intensity of 100 µmol m^{-2} s^{-1}. The highest light intensity in this study was 8000 lux which is equal to about 112 µmol photon m^{-2} s^{-1}. Variation in the SGR of the same species of *Chlorella* sp. is not only strain-specific but also the culture conditions including the range of the light intensity tested (Indrayani et al 2020).

The biomass yields and biomass productivity of the *Chlorella* sp. increased with light intensity. However, increasing the light intensity from 3500 lux to 8000 lux did not

significantly increase both the biomass yield and biomass productivity of the alga. The biomass yield of the cultures grown at 3500 lux and 8000 lux was nearly the same at around 0.33 and 3.2 g L^{-1}, respectively. Similarly, the biomass productivity of both light intensity 3500 and 8000 lux was nearly the same at about 0.31 g L^{-1} d^{-1}. The biomass yield of *Chlorella* sp. found in this study is comparable with other studies. Nzayisenga et al (2020) reported the biomass yield of *Chlorella vulgaris* after 8 days of cultivation at three different light intensities of 50, 150, and 300 µE m^{-2} s^{-1} was 0.4, 0.6, and 0.7 g L^{-1}, respectively. Increasing light intensity from 150 to 300 µE m^{-2} s^{-1} did not significantly increase the biomass yield. Khalili et al (2015) studied the influence of different light intensities of 50, 80, and 110 µmol m^{-2} s^{-1} on the growth of *C. vulgaris*. They found that the optimum light intensity for higher biomass production was obtained at a light intensity of 80 µmol m^{-2} s^{-1}. Biomass production was lower at low light intensity (2500 lux) because low light intensity causes a decrease in the rate of photosynthesis resulting in low macromolecule biosynthesis due to the suboptimal synthesis of glucose in the photosynthesis process (Nielsen & Nielsen 2017). Similarly, Parsons & Chapman (2000) stated that if the light intensity is low, it will cause the supply of crude material produced in the photosynthesis process to decrease. The decrease in the rate of photosynthesis will also have an impact on the lower amount of biomass produced (Borowitzka 2016). This study suggests that higher light intensity resulted in higher biomass productivity of the alga. The ability of microalgae to grow well at high light intensity will be beneficial especially if the microalgae will be cultivated in open pond systems outdoors where the intensity of sunlight is far higher above the currently tested light intensity (Indrayani et al 2019). Therefore, outdoor culture trials are certainly needed to further study the light intensity tolerance of the alga species under real outdoor conditions.

Conclusions. The *Chlorella* sp. UNM-IND1 could grow well in all culture media tested. However, for mass cultivation of the alga, NPK+urea medium is the best option due to its low price and easily accessible in the market. For the light intensity, the highest growth and biomass productivity was obtained at the highest light intensity 8000 lux. This study indicated that the *Chlorella* sp. UNM-IND1 is suitable and more economical for mass cultivation outdoors for any commercial application due to its high growth rate and biomass productivity when grown under high light intensity and when using NPK+urea medium.

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Conflict of interest. The authors declare that there is no conflict of interest.

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