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Short Communication

Enhancement of Astaxanthin Content in Mixed Culture of *Dunaliella* sp. and *Azospirillum* sp. under Light Intensity Treatment

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Abstract

Dunaliella sp. is a potential natural source of carotenoid pigments such as astaxanthin, β-carotene, and lutein. *Dunaliella* sp. can also accumulate other valuable products such as glycerol and protein. Another species is *Azospirillum* sp., which is known as microalgal growth-promoting bacteria. These bacteria are often cultured with microalgae because they contain indole-3-acetic acid, which can significantly increase the growth of microalgae. This study aimed to examine the pigment content in mixed culture of *Dunaliella* sp. and *Azospirillum* sp. after being treated with different light intensity treatment. In this study, *Dunaliella* sp. were cultivated by mixing with *Azospirillum* sp. under light stress. Two treatments were performed at light stress intensity of 3000 and 6000 lx. Light intensity is widely used as an important parameter in cultivation, which can affect the growth and production of microalgal biomass. In addition, spectrophotometric UV-Vis based measurement was conducted to investigate every single pigment content in all treatments under light stress for eight days. The number of cells, carotenoid pigments, and astaxanthin had increased significantly. Pigments of chlorophyll a and chlorophyll b also significantly increased at lower light treatments. Based on the results, the bacterium *Azospirillum* sp. and high light intensity significantly increased the growth and cell division of microalgae. Therefore, the combination of *Azospirillum* sp. and light stress intensity in microalgae cultivation could increase the growth and pigment of *Dunaliella* sp.

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1. Introduction

Microalgae are marine or freshwater microorganisms consisting of a single eukaryotic cell. They are also considered as unicellular flora representatives with great application potential in various branches of science and technology. At present, microalgae are widely used in various fields because of their ability to synthesize various biologically active substances, grow biomass, and adjust their biochemical composition depending on cultivation conditions (Olasehinde *et al*., 2017). Several species are industrially produced and commercialized for functional food, feed, cosmetic industries, and pharmaceutical markets (Silva *et al*., 2022). Microalgae produce pigments that are focused on three types, namely, chlorophyll, phycobilin, and carotenoids (Elfiza *et al*., 2019).

As a species of microalgae, *Dunaliella salina* is a halotolerant green alga that contains manifold nutrients and possesses a remarkable ability to adapt to the environment (Chen *et al*., 2020). *Dunaliella salina* is also known as the best commercial source of natural β-carotene. In addition, different species of *Dunaliella* can accumulate a significant number of valuable fine chemicals such as carotenoids, glycerol, lipids, vitamins, minerals, and proteins. They also have a great application potential for biotechnological processes such as the expression of foreign proteins and treatment of freshwater (Tafreshi and Shariati, 2009). Carotenoids are used as ingredients in vitamin supplements, cosmetics, health food products, and addictive substances. Carotenoids that have been studied include carotene, lutein, lycopene, zeaxanthin, and astaxanthin (Coates *et al*., 2013). As a type of carotenoid, astaxanthin is a powerful antioxidant, nutritional supplement, and potential therapeutic compound, which shows activities against different ravaging diseases and disorders (Patil *et al*., 2022).

Most microalgae have been cultured as monoculture. In most monoculture systems, lipid accumulation occurs under nutrient-depleted conditions, but the stress conditions that induce increased storage of lipids also inhibit biomass productivity (Hannon *et al*., 2010; Contreras-Angulo *et al*., 2019; Mandal *et al*., 2020). Therefore, current efforts are directed toward developing efficient production strategies that allow rapid cell growth and lipid accumulation during the cultivation of microalgae (Chisti, 2013). A potential production strategy is the co-culturing of microalgae with bacteria.

Azospirillum sp., known as microalgal growth-promoting bacteria, can establish synergistic and mutualistic relationships with microalgae. Ramos-Ibarra *et al*. (2019) stated that the mutualistic interaction of microalgae with *Azospirillum brasilense*, a plant growth-promoting bacterium, improves physiological and biotechnological processes based on microalgae because of their ability to produce several phytohormones as cytokinins, gibberellins, jasmonic acid, brassinosteroids, and auxins, primarily indole-3-acetic acid (IAA).

Light is the main energy source for microalgae, and the intensity and wavelength of light influence cell metabolism and biomass composition (Katam *et al*., 2022). Light can increase the ATP produced during photosynthesis; the increase in ATP will trigger a faster metabolic rate and affect the metabolism of carotenoids in algal cells (Peri *et al*., 2009). There have been many studies on microalgae culture with various light intensity treatments, but mixed culture of *Dunaliella* sp. and *Azospirillum* sp. and light stress treatment to get robust pigment content in microalgae has never been done before. Thus, this study was conducted to examine the pigment production and study the symbiotic consortia by morphological imaging in mixed cultures of *Dunaliella* sp. and *Azospirillum* sp. under various light stress intensities.

2. Materials and Methods

2.1 Experimental Design

Dunaliella sp. were obtained from BBPBAP Jepara, Central Java, Indonesia, and *Azospirillum* sp. was obtained from the Faculty of Agricultural, Universitas Gadjah Mada. *Dunaliella* sp. starter was cultivated in Bold's Basal Medium. *Azospirillum* sp. starter was cultivated in BTB-2 medium for 16 hours (Choix *et al*., 2012). Then mixed culture of *Dunaliella* sp. and *Azospirillum* sp. was cultivated with synthetic growth medium in 500 mL bottles. The initial culture of *Dunaliella* sp. was 10^6 cell/mL, and the initial culture of *Azospirillum* sp. was 10^9 CFU/mL. All experiments were performed at 22°C ± 2°C with pH maintained at 7–8 with light stress intensities of 3000 and 6000 lx for eight days in triplicate. Every morning, the cells were harvested for parameter measurement. The microalgal cell abundance and morphology were determined using a Neubauer hemocytometer under a light microscope (Olympus CX2li, Tokyo, Japan) and coupled with Optilab. For all treatments, alcohol was added as a diluting factor, and at the mixed culture cell observation, methylene blue was added to observe bacteria under a microscope.

2.2 Pigment Analysis

The pigment content was analyzed by using a UV-Vis spectrophotometer (Genetis 10 UV) in accordance with the method of Pruvost *et al.* (2011) with absorption spectra in the range of 400–750 nm. Chlorophyll a, chlorophyll b, and carotenoid were extracted by taking 2 mL of sample, centrifuged at 12,000 rpm for five minutes which was added with 1.25 mL of methanol and then incubated at 4°C overnight in the dark. Astaxanthin content was measured using the method of Boussiba *et al.* (1992) with slight modifications.

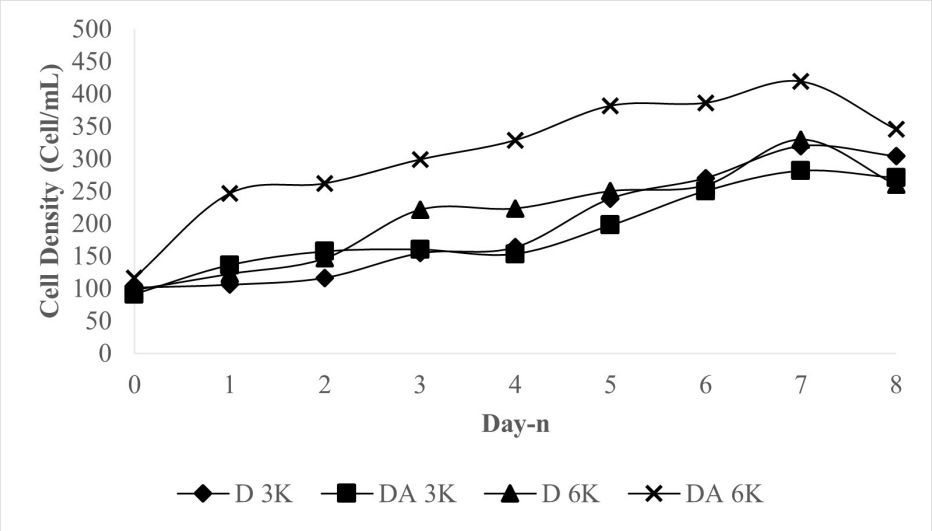


Figure 1. Number of cells × 10^6 (cell/mL) in a single culture of *Dunaliella* sp. and mixed culture of *Dunaliella* sp. and *Azospirillum* sp. with light intensities of 3000 and 6000 lx (Amelia, 2018).

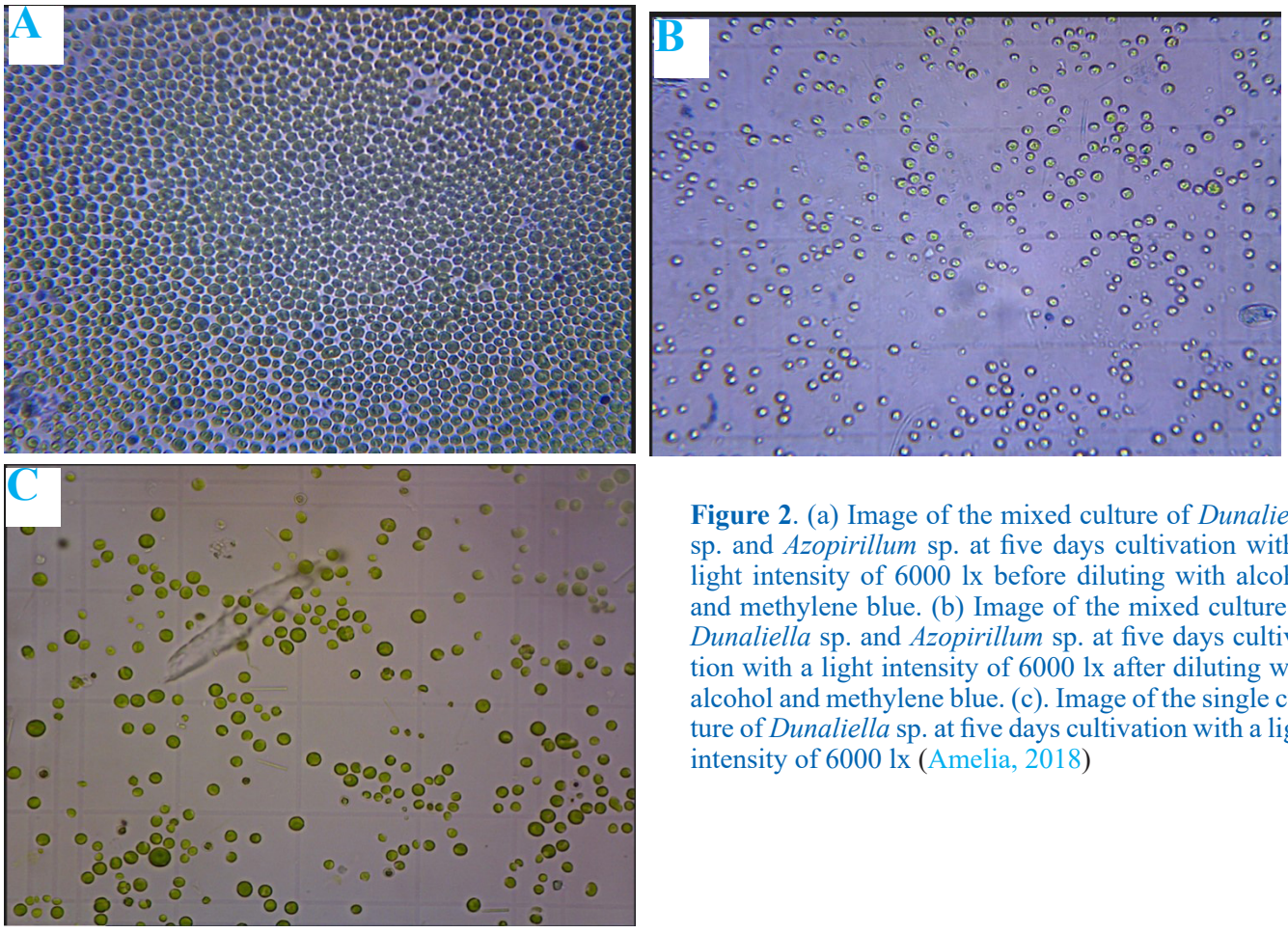


Figure 2. (a) Image of the mixed culture of *Dunaliella* sp. and *Azopirillum* sp. at five days cultivation with a light intensity of 6000 lx before diluting with alcohol and methylene blue. (b) Image of the mixed culture of *Dunaliella* sp. and *Azopirillum* sp. at five days cultivation with a light intensity of 6000 lx after diluting with alcohol and methylene blue. (c). Image of the single culture of *Dunaliella* sp. at five days cultivation with a light intensity of 6000 lx (Amelia, 2018)

3. Results and Discussion

3.1 Cell Growth

Understanding the life cycle and phases of microalgae is important to increase productivity (Suyono *et al*., 2016). During culture, several phases are observed, namely, the lag phase, exponential phase, and death phase. On day seven, the number of cells in the mixed culture of *Dunaliella* sp. and *Azospirillum* sp. with a light intensity of 6000 lx was 419. 25 × 10^6 cell/mL (Figure 1). In mixed culture with a light intensity of 3000 lx, the highest number of cells was 281. 55 × 10^6 cells/mL. At the beginning of its growth, the number of cells in the mixed culture with a light intensity of 3000 lx exceeded the single culture of *Dunaliella* sp. with a light intensity of 3000 and 6000 lx, but growth decreased and then increased again (Amelia, 2018). The number of cells in *Dunaliella* sp. continuously increased in the consortium culture, which was due to IAA (auxin) found in *Azospirillum* sp., thereby leading to constant cell division. According to Bashan and de-Bashan (2010), auxins are responsible for the division, extension, and differentiation of plant cells and tissues.

Based on the graph, the lowest number of cells was found in the single culture of *Dunaliella* sp. with a light intensity of 3000 lx. Its growth does not always exceed other treatments. The peak growth in single culture *Dunaliella* sp. was obtained on the seventh day, and then it entered the death phase on the following day (Amelia, 2018). Furthermore, the production of microalgal biomass is affected by various factors such as temperature, oxygen, pH, and light.

The number of *Dunaliella* sp. is very dense, possibly because IAA is derived from the bacterium *Azospirillum* sp., and a high light intensity induces rapid cell division. In mixed culture, with light intensity, 6000 lx cells are round and small. The number of cells is vast, affecting the counting chamber on the Hemacytometer, which is not visible, and the cells are difficult to count (Figure 2a). Furthermore, after the mixed culture was given alcohol and methylene blue (Figure 2b), the bacteria were stained with blue, and then the cells could be counted. Other cell morphology was also observed in single culture *Dunaliella* sp. with a light intensity of 6000 lx (Figure 2c). Therefore, in *Dunaliella* sp., the morphology is round and small, but the cell density does not exceed the mixed culture. This study provides experimental evidence that microalgae in a stable culture interact well with a population of bacteria *Azospirillum* sp., and light has also been shown to induce cell division.

3.2 Chlorophyll and Carotenoid Content

Microalgae obtain energy for metabolism and reproduction from chlorophyll, a natural pigment abundant in microalgae to support the oxygenic process of photosynthesis (da Silva and Lambordi, 2020). The difference is that chlorophyll-a is the most crucial pigment for photosynthesis in microalgae because it converts photons into chemical energy (Prawira-Atmaja *et al*., 2018). Chlorophyll b and other accessory pigments are mainly involved in light harvesting in LHCs and are expected to improve light-harvesting efficiency (Nick *et al*., 2013; Chen, 2014; Voitsekhovskaja and Tyutereva, 2015). The highest chlorophyll content was obtained from a single culture of *Dunaliella* sp. with a light intensity of 3000 lx on the fourth day (Figure 3a). In the mixed culture of *Dunaliella* sp. and *Azospirillum* sp., chlorophyll a increased consistently, and the highest concentration was obtained from mixed culture with light intensity 3000 lx on the eighth day (Figure 3b).

At the beginning of the growth, the proportion of chlorophyll a and b was small because microalgal cells are just starting to adapt to the culture environment (Figure 3 and Figure 4). The highest chlorophyll b content in a single culture was obtained at a light intensity of 3000 lx (Figure 4a). At the same light intensity (3000 lx), in the mixed culture of *Dunaliella* sp. and *Azospirillum* sp., the highest chlorophyll content was 27.99 g/mL after eight days of cultivation. Mixed culture with a light intensity of 6000 lx experienced the highest chlorophyll b content on the fourth day of cultivation, and the number of cells decreased the next day (Figure 4b) (Amelia, 2018).

The content of chlorophyll a and b in the mixed culture was higher than that in the single culture probably because the bacteria *Azospirillum* sp. contained in the mixed culture could significantly increase the quantity of photosynthetic pigments such as chlorophyll a and b. In addition, *Azospirillum* sp. contained in the consortium culture can increase the absorption of NO_3^+, NH_4^+, K^+, Fe^+, and some micronutrients. This absorption of minerals and micronutrients can increase cell growth, thereby increasing pigment production (Bashan and de-Bashan, 2010).

Another factor that affects the level of pigment in culture is light. In general, excessive light can cause cellular photodamage. In this study, the content of chlorophyll a and b was higher in cultures with light intensities of 3000 lx than those with light intensities of 6000 lx. The microalga grown in low light would use energy cells to catch the light that is more efficient; therefore, the chloroplast was accordingly expanded. This adaptation is often described as a light shade adaptation. These adaptations could improve the chlorophyll content 2–10 times (Darley, 1982; Ferreira *et al*., 2015).

Carotenoids are one of the most important pigments. They perform several functions in microalgae: involved in light harvesting, but also contribute to stabilizing the structure and aid in the role of photosynthetic complexes-beside quenching chlorophyll triplet states, scavenging reactive oxygen species, and dissipating excess energy (Demming and Adams, 2002). This study found that the best carotenoid content was obtained in a mixed culture of *Dunaliella* sp. and *Azospirillum* sp.

with a light intensity of 6000 lx on the eighth day of cultivation (Figure 5b). Although carotenoid pigments increase daily in a mixed culture of *Dunaliella* sp. and *Azospirillum* sp. with a light intensity of 3000 lx, the concentration still does not exceed the mixed culture with light intensity 6000 lx (Amelia, 2018).

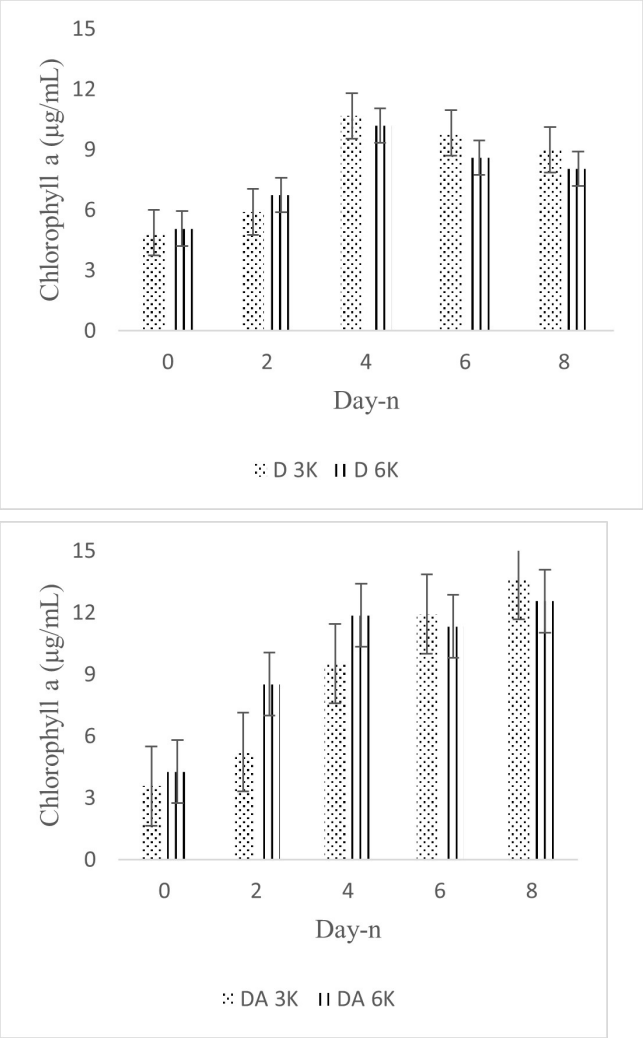


Figure 3. (a) Chlorophyll a content in a single culture of Dunaliella sp. with light intensities of 3000 and 6000 lx. (b) Chlorophyll a content in a mixed culture of Dunaliella sp. and Azospiriilum sp. with light intensities of 3000 and 6000 lx (Amelia, 2018).

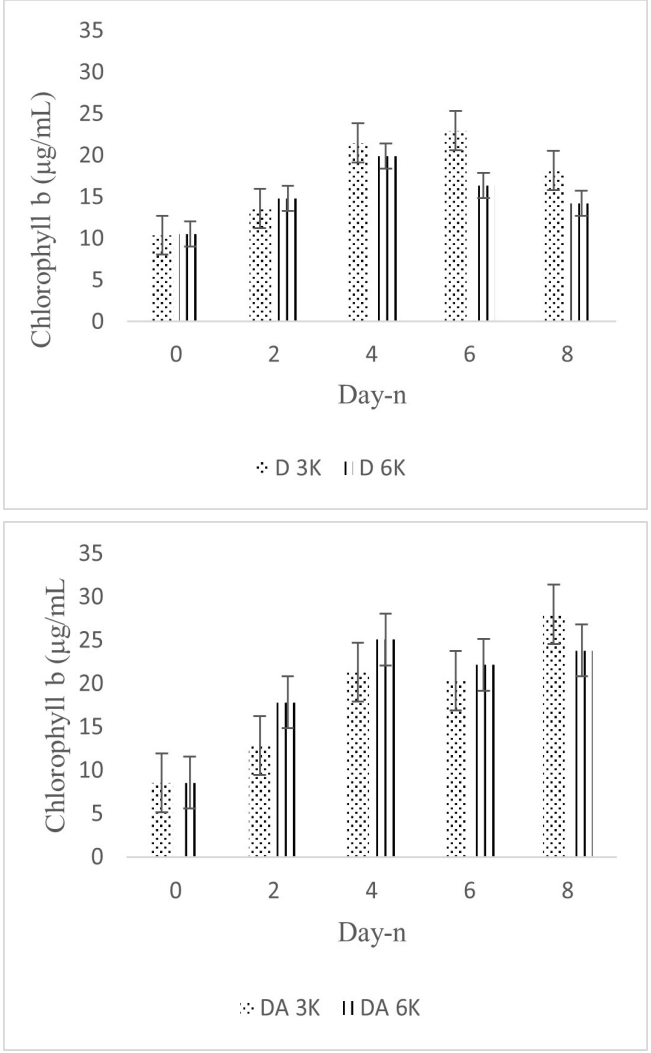


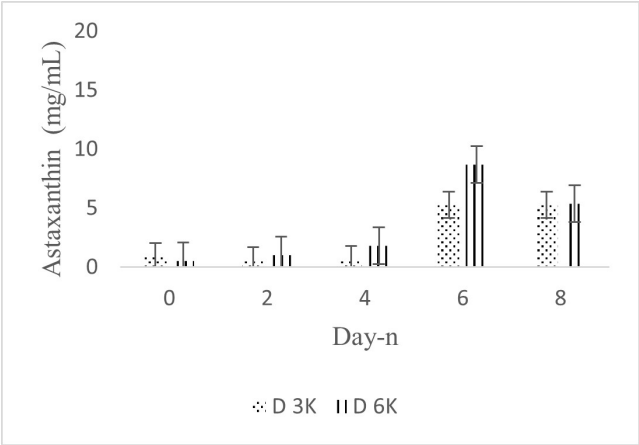
Figure 4. (a) Chlorophyll b content in a single culture of Dunaliella sp. with light intensities of 3000 and 6000 lx. (b) Chlorophyll b content in a mixed culture of Dunaliella sp. and Azospiriilum sp. with light intensities of 3000 and 6000 lx (Amelia, 2018).

Guedes *et al.* (2010) also reported that an increase in light intensity will be followed by an increase in the content of cells in the chlorophyll, but long exposure to high light intensity will rapidly decrease chlorophyll pigment. Meanwhile, the content of carotenoids, which serves as protective pigments, will increase. The decrease in chlorophyll content under high light intensity is related to the amount of light energy required to increase the number of microalgal cells; thus, a lower cellular chlorophyll content is required. In this study, the addition of *Azospirillum* sp. into the culture of *Dunaliella* sp. could be an advantage for *Dunaliella* sp. because *Azospirillum* can build defense pigments from environmental stress. As reported by Cassán *et al.* (2001), the inoculation of *Azospirillum* bacteria (plant stress homeo-regulating bacteria) in mixed culture can significantly increase photosynthetic and photoprotective pigments to help cells continuously photosynthesized under unfavorable light conditions. On the contrary, *Azospirillum* in a mixed culture could regulate *Dunaliella* cell homeostasis under abiotic stress.

3.3 Astaxanthin Content

An increase in astaxanthin content was observed in a single culture of *Dunaliella* sp. with a light intensity of 3000 and 6000 lx (Figure 6a). The highest astaxanthin content was obtained from a single culture

of *Dunaliella* sp. with a light intensity of 6000 lx on the sixth day after cultivation. From another image of the mixed culture of *Dunaliella* sp. and *Azospirillum* sp., the high intensity of light (6000 lx) causes microalgal cells to make a defense and maintain their cells to keep growing by producing astaxanthin (Figure 6b). The highest astaxanthin content was obtained with a value of 13.54 mg/mL. During the lag and log phases, the proportion of astaxanthin in a single culture of *Dunaliella* sp. and mixed cultures of *Dunaliella* sp. and *Azospirillum* sp. is few. This result may be due to the treatment in the form of light intensity and the addition of *Azospirillum* sp., which causes cell metabolism to synthesize more other carotenoid pigments or other biomass such as carbohydrates, proteins, and lipids (Amelia, 2018).



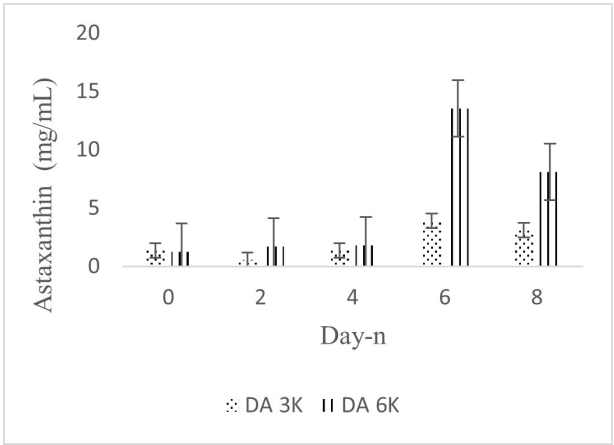
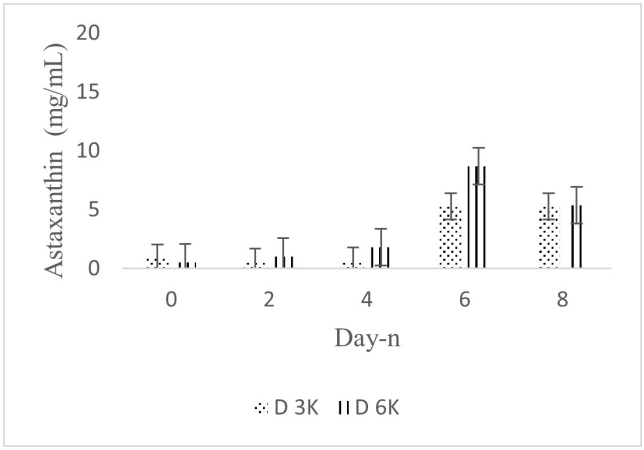


Figure 5. Carotenoid content in a single culture of *Dunaliella* sp. with light intensities of 3000 and 6000 lx. (b) Carotenoid content in a mixed culture of *Dunaliella* sp. and *Azospiriilum* sp. with light intensities of 3000 and 6000 lx (Amelia, 2018).

Cazzaniga *et al.* (2022) reported that microalgal growth under high light can lead to photo-oxidative damage and decrease the rate of photosynthesis. Photoinhibition can be reduced by the engineered accumulation



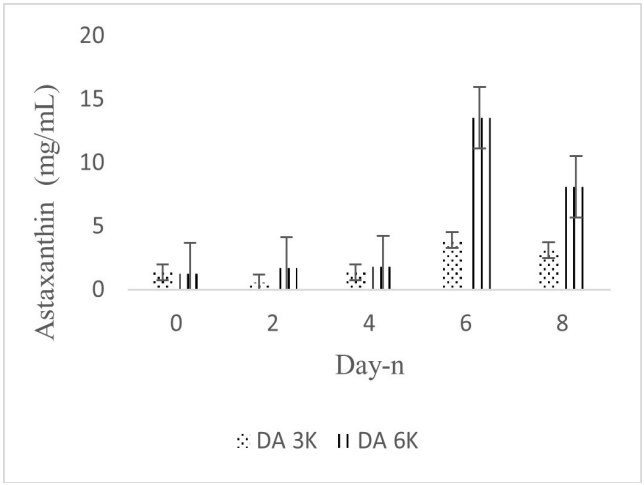


Figure 6. Astaxanthin content in a single culture of Dunaliella sp. with light intensities of 3000 and 6000 lx. (b) Astaxanthin content in a mixed culture of Dunaliella sp. and Azospiriilum sp. with light intensities of 3000 and 6000 lx (Amelia, 2018).

of astaxanthin in *Chlamydomonas reinhardtii* with high light tolerance. High light causes photo-oxidative stress in microalgae, which causes pigment bleaching, lipid oxidation, and a decrease in photosynthetic efficiency. In addition, according to Bashan and de-Bashan (2010), *Azospirillum* inoculated in microalgae culture will significantly increase not only the quantity of photosynthetic pigments chlorophyll a and b, but also photoprotective pigments such as violaxanthin, zeaxanthin, antheroxanthin, lutein, neoxanthin, and carotene to help microalgae perform photosynthesis under unfavorable light conditions.

4. Conclusion

Differences in light intensity and inoculation of *Azospirillum* sp. could increase the cell count and pigment content of mixed cultures. High light intensity in a mixed culture of *Dunaliella* sp. and *Azospirillum* sp. could increase the number of cells and the content of photosynthetic pigments such as astaxanthin and carotenoids compared with the high content of chlorophyll a

and b at low light intensity. Furthermore, transcriptomic and metabolomic analyses could be applied to study the interaction between microalgae and bacteria in mixed culture microalgae in the future.

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Authors' Contributions

The contribution of each author is as follows, EAS; provided the main ideas and finalized the manuscript. RA; collected the data, wrote the manuscript, revised the data, and designed the figures. WRA; analyzed data and revised the manuscript draft. All the authors discussed, read, and contributed to the final manuscript.

Conflict of Interest

The authors declare that this study was conducted without any commercial or financial relationship that could be construed as a potential conflict of interest.

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