Salinity Treatment as Bacterial Control and Its Impact on Growth and Nutritional Value of *Spirulina* (*Arthrospira platensis*) Culture in Open Pond System

Putri Maura Rangkuti, Dwi umi Siswanti and Eko Agus Suyono*

ABSTRACT

Cultivation of *Spirulina* in open ponds is always problematic due to microbial contamination, which results in reduced growth and productivity. Efficient methods are needed, for example changing the salinity of culture media because salinity can act as a limiting factor for many organisms. One simple and low-cost solution may be to to change the salinity of the culture environment, as salinity can act as a limiting factor for many organsims. The purpose of this study was to examine the impact of salinity (10‰–40‰, with salinity of 5‰ as a control) on bacterial contamination, growth and nutritional values of *Spirulina* cultured in an open pond. Bacterial contaminants measured using total plate count method revealed significant reduction of bacteria for the 10‰ treatment at day 5, but by day 10 the bacteria grew back because they were tolerant to the increase in salinity. Growth of Spirulina was enhanced at 10‰ and 20‰ in terms of density, while dry biomass was increased only at 20‰. Protein content was unchanged or even decreased at 20‰–40‰, while lipid content increased with salinity. Chlorophyll *a*, total chlorophyll and phycocyanin tended to decrease with increased salinity. Chlorophyll *b* tended to decrease when salinity increased to 10‰ and 20‰ (but without statistical support), and remained unchanged (relative to 5‰) at 30‰ and 40‰. Carotenoid was increased at levels of 10‰–30‰ but was not different from the control at 40‰. In conclusion, the present study showed that modification of salinity level may not be useful to prevent microbial contamination of *Spirulina* culture in open pond systems.

Keywords: *Arthrospira platensis*, Contamination, Growth, Open ponds, Salinity

INTRODUCTION

Spirulina, also known as *Arthrospira platensis*, is one of the most popular microalgae that has been cultivated for decades and for many industrial purposes due to its nutritional value (Usharani *et al*., 2012). At present, the most common cultivation method for *Spirulina* is using outdoor ponds, since this is more affordable than any other method (Deruyck *et al*., 2019). However, ponds can be easily contaminated by several groups of organisms such as bacteria, fungi and protozoa due to the direct contact with open air (von Alvensleben *et al*., 2013). Microbial contamination results in decreased production and loss of microalgae populations and their final products (Meseck, 2007; Forehead and O'Kelly, 2013).

In recent years, several methods have been employed in an effort to overcome microbial contamination, for instance, the use of physical sterilization or antibiotics. However, these methods require expensive equipment and high doses of chemicals, and hence are not suitable for large-scale cultivation (Deruyck *et al*., 2019). Consequently, an alternative method is needed to overcome the contaminants with higher efficiency.

Department of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
*Corresponding author. E-mail address: eko_suyono@ugm.ac.id
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Contaminant control can be carried out by changing the salinity in salt-tolerant microalgae cultures. This is because salinity can act as a limiting factor for several organisms (Ji *et al*., 2019). This method is preferable since it does not require any high-priced equipment or chemicals, and thus does not increase investment cost. *Spirulina* tolerates a wide salinity range, and even tolerates extreme levels exceeding 88‰ (Almahrouqi *et al*., 2015a). When exposed to high salinity, *Spirulina* increases its cell metabolism and stimulates the accumulation of metabolite compounds such as lipids, carbohydrates, pigments, as well as certain antioxidant compounds that potentially increase the cell's resistance in high salinity (Zeng and Vonshak, 1998; Yılmaz *et al*., 2010; Sujatha and Nagarajan, 2014; Almahrouqi *et al*., 2015a). Additionally, *Spirulina* grows at a salinity range of 5–35‰ with optimum dry weight, biochemical composition and fatty acid profile (Almahrouqi *et al*., 2015b). Due to its beneficial metabolic properties in certain salinity levels, the alteration of this parameter may not only be used for contamination control but can also lead us to obtain better nutritional values from *Spirulina*. Previous research by von Alvensleben *et al*. (2013) showed that the salinity of the culture medium was able to inhibit the growth of contaminants in *Picochlorum atomus* microalgae cultures, but contaminant inhibition by modifying salinity in *Spirulina* has not been widely reported. Increasing salinity to a specific level has also been reported to increase microalgae biomass, which was related to the increased photosynthetic activity (Dhiab *et al*., 2007). The purpose of this study was to examine the responses in microbial contamination and growth rate of *Spirulina* to different levels of salinity in an open pond system and evaluate the impact of salinity on nutritional value of the microalgae.

MATERIALS AND METHODS

Medium preparation and salinity treatments

Spirulina monoculture obtained from Nogotirto Algae Park (microalgae cultivation at Yogyakarta, Indonesia) was cultivated in regular mass cultivation medium with salinity of 5‰ for 10 days in a 20-L fiberglass culture container without aeration or light control. The initial density of *Spirulina* was 4×10^4 cells·mL^{-1}. Except for NaCl, each treatment used the same culture medium: 5 g·L^{-1} NaCl, 0.05 g·L^{-1} CH_4N_2O, 0.03 g·L^{-1} Nitrogen phosphate, 0.03 g·L^{-1} Kalium fertilizer, 0.15 g·L^{-1} $(NH_4)_2SO_4$, and 0.075 g·L^{-1} Na_2CO_3. The salinity levels used in the study were 5‰ (as control), 10‰, 20‰, 30‰, and 40‰, each with three replications. To adjust salinity, NaCl was added to the culture media and salinity was regularly measured using a refractometer.

Growth measurement

The growth of Spirulina was measured each day for 10 days by both cell density and dry weight. The cell density was measured microscopically with hemocytometer using the "big block" method (Figure 1) (Edhy *et al*., 2003), while the dry weight was measured by weighing 15 mL of dried culture media, obtained by draining with filter paper, washing three times using Aquadest, then drying in the sun for up to four days. The specific growth rate of *Spirulina* was calculated by using the specific growth rate formula by Deruyck *et al*. (2019):

$$\mu = \frac{\ln N_t - \ln N_0}{T_t - T_0}$$

where μ = specific growth rate; N_t = cell density/biomass at observation time; N_0 = initial cell density/biomass; T_t = observation time; and T_0 = initial time.

Microbial contamination assessment

Microbial contamination was evaluated using the total plate count method on day 5 and day 10 of cultivation. The culture samples (100 µL) were aseptically collected and then filtered using sterile filter paper. The filtered samples were then gradually diluted until 10^{-6} L using sterile test tubes. Each diluted sample was inoculated in PCA medium at 28 ºC for 24 h. The total number

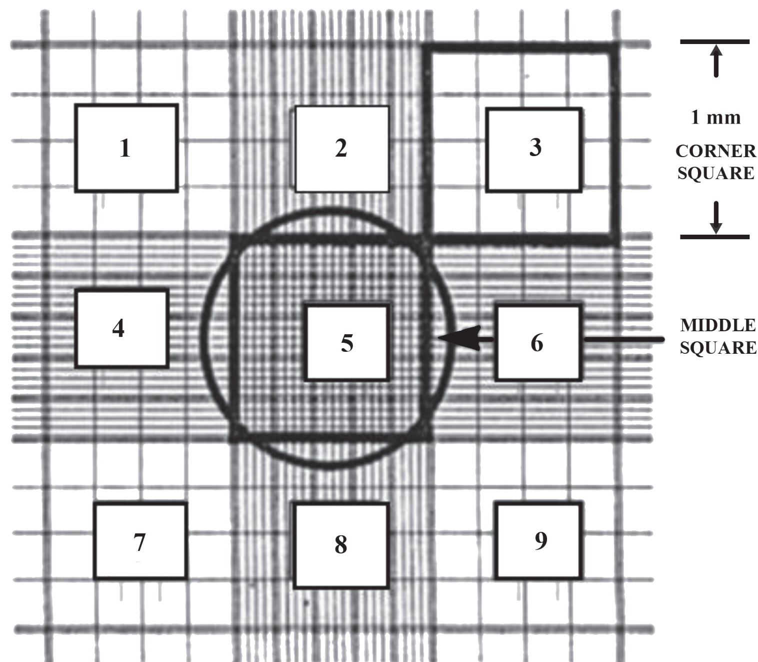

Figure 1. Neubauer-improved hemocytometer schematic (central area has a grid of 5×5 lines).
Note: The number of cells was calculated from field numbers 1, 3, 7, and 9. Each observation field consists of 25 boxes. Cells in fields 2, 4, 5, 6, and 8 were not counted.

of colonies was counted using colony counters and stated as colony-forming units (CFU) (Radji and Manurung, 2010). The percentage reduction in contamination was determined by comparing the number of microbial colonies on day 5 or day 10 with the initial count (day 0).

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Total protein analysis

The total protein content of *Spirulina* in each salinity treatment was analyzed after 10 days of treatment using Kjeldahl method as described by Rosaini *et al*. (2015). The harvested biomass of *Spirulina* was dried and destructed using pure sulfuric acid (H_2SO_4) with selenium as the catalyst and then heated until the mixture turned clear. The study used 1 g of dry biomass only for protein analysis in three replications. Aquadest was added to the mixture to reach a total volume of 100 mL, and this was distilled using a distillation flask with the addition of 30% NaOH solution. The steam from the boiling water carried the vapor of the volatiles to a condenser, where both were cooled and condensed into the liquid state. The condensed vapor was then collected into an Erlenmeyer flask containing 5 mL of boric acid (1 N) and 2 drops of methyl red. The mixture was then titrated using HCl (0.02 N) until the mixture turned red. The volume of HCl for titration was then used for the total N calculation using the formula:

$$\%N = V\ HCl \times N\ HCl \times 14.008 \times 100\%$$

where %N = percentage of nitrogen; V HCl = volume of HCl used for titration; N HCl = HCl normality.

The percentage of N is measured as a parameter of total protein because element N is the main constituent of amino acids, while a chain of amino acids is a protein.

Lipid content analysis

The total lipid content was analyzed after 10 days of treatment using the soxhletation methods described by Laenggeng and Nurdin (2017). One to two grams of dried biomass was homogenized using mortar and pestle, then placed in a thimble made of filter paper (A in the equation below). The thimble was then placed into the soxhletation apparatus with a weighted lipid flask (B) and extracted for 3 h using 15 mL of diethyl ether as a solvent. The lipid flask was then dried in an oven and weighed (C). The total lipid content was then calculated using the following formula:

$$\text{Total lipid (\%)} = \frac{(C-B)}{A} \times 100\%$$

where A = sample weight; B = initial lipid flask weight; C = final lipid flask weight.

Pigment analysis

Pigmentation was analyzed following Ilavarasi *et al*. (2012) after 10 days of treatment, whereby 30 mg of dry biomass was homogenized with 10 mL of 99.98% methanol using mortar and pestle and subsequently incubated in a water bath at 50 °C for 30 min. The aliquot was then centrifuged at 402 g for 10 min. The supernatant was transferred by pipetting into a cuvette for pigment analysis. The pigment contents of the sample were determined with the spectrophotometric method at several wavelengths. The wavelengths and formulas for pigmentation analysis were from Lichtenthaler and Buschmann (2001) and Marrez *et al*. (2013) as mentioned below (where A = absorbance):

$$\text{Chlorophyll } a\ (\text{mg}\cdot\text{L}^{-1}) = (16.72 \times A_{665.2})-(9.16 \times A_{652.4})$$

$$\text{Chlorophyll } b\ (\text{mg}\cdot\text{L}^{-1}) = (34.09 \times A_{652.4})-(15.28 \times A_{665.2})$$

$$\text{Carotenoids } (\text{mg}\cdot\text{L}^{-1}) = (1000\ A_{470}-1.63\ \text{Chlorophyll } a-104.96\ \text{Chlorophyll } b)/221$$

$$\text{Phycocyanin } (\text{mg}\cdot\text{L}^{-1}) = (A_{620}-0.474A_{652})/5.34$$

Data analysis

The data were analyzed using ANOVA followed by Duncan's multiple range test; the results were considered significant if $p<0.05$. Data analysis was performed using the IBM SPSS application version 23.

RESULTS

Growth rate of Spirulina

The growth pattern of *Spirulina* in the salinity treatments of 5‰, 10‰, 20‰, 30‰, and 40‰ is presented in Figure 2. The results show that both cell density (Figure 2a) and dry biomass (Figure 2b) had similar responses to salinity, in which the 10‰ salinity treatment had the highest values for cell density and dry biomass, and both parameters gradually decreased at higher levels of salinity. The cell density produced from the 10‰ and 20‰ salinity treatments (9.82±3.27 cells·mL^{-1} ×10^4 and 8.90±3.27 cells·mL^{-1}×10^4) was higher than the control (6.18±2.55 cells·mL^{-1}×10^4). However, at higher salinity (30‰ and 40‰), cell density declined to a level similar to the control. Similar results were observed for dry biomass, which was significantly higher than the control at 10‰ salinity (1.56±0.12 g·mL^{-1}) and gradually declined at higher salinity, so that the density was not significant different from the control (1.10±0.36 g·mL^{-1}).

Bacterial Reduction

The microbial reduction in the five salinity treatments is presented in Figure 3. On day 5 of cultivation, there was a reduction in the number of microbes in all treatments. On day 5 in the control (5‰), the number of microbes (67.5×10^{4}±0.5 CFU) decreased sharply compared to day 0. Likewise, in treatments 10 ‰, 20‰, 30‰, and 40‰, microbes decreased compared to day 0. On day 5, 10‰ salinity reduced bacteria by the greatest amount among treatments, but the number of bacteria increased again by day 10.

Total protein content

The total protein content of *Spirulina* in the five salinity treatments is presented in Figure 4a. The results show that the mean total protein content of *Spirulina* cultured at 10‰ (62.94±6.91%) was

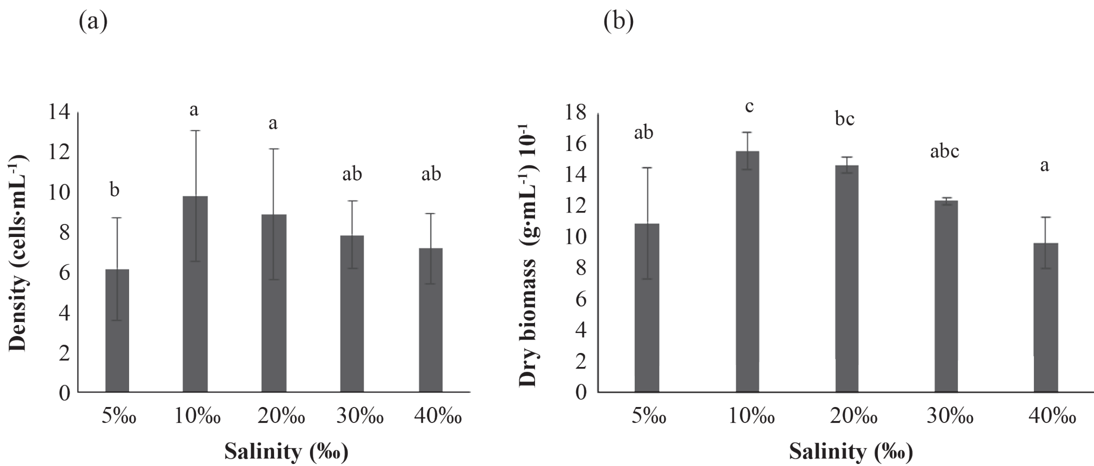

Figure 2. Cell density (a) and dry biomass (b) of *Spirulina* cultured in different salinities over 10 days of cultivation. Different lowercase letters above bars indicate significant ($p<0.05$) difference between means, and error bars represent SD.

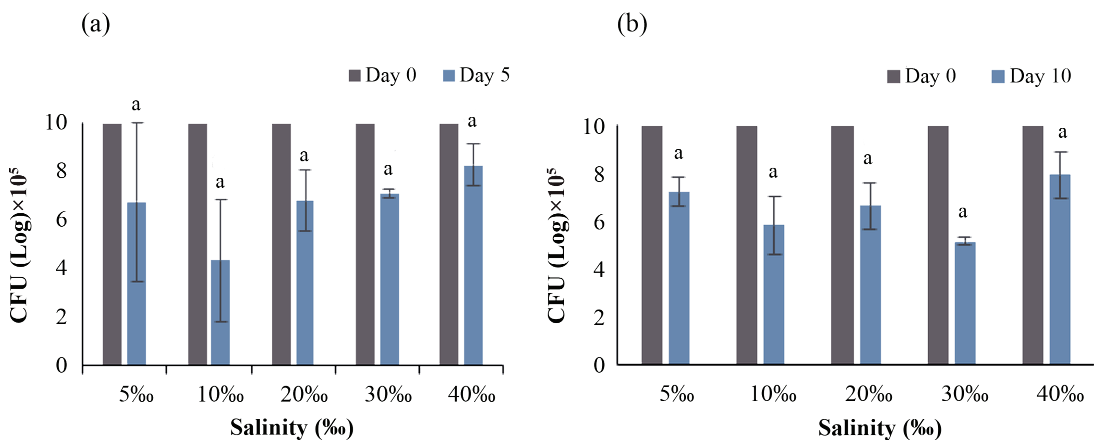

Figure 3. The microbial contamination of *Spirulina* mass culture at 5 days (a) and 10 days (b) of cultivation in different levels of salinity. Different lowercase letters indicate significant ($p<0.05$) difference between treatments.

higher than the control (56.39±1.47%), although the difference was not supported by the statistical test ($p>0.05$). However, at higher salinity (20‰–40‰), protein gradually decreased, and all these treatments were significantly ($p<0.05$) lower in total protein than the control.

Total lipid content

Total lipid content of *Spirulina* significantly increased in the 10‰ treatment (0.00135±0.00017 $mg \cdot g^{-1}$ dw) as compared to the control (0.0127± 0.0004 $mg \cdot g^{-1}$ dw) (Figure 4b). However, unlike total protein, total lipid content significantly ($p<0.05$) increased at the higher salinities of 20‰–40‰ (0.0139±0.0003 $mg \cdot g^{-1}$ dw to 0.0143±0.0002 $mg \cdot g^{-1}$ dw; $p>0.05$).

Pigmentation

The chlorophyll, carotenoid, and phycocyanin contents of *Spirulina* in the five salinity treatments are presented in Figure 5. The results show that the chlorophyll *a* (Figure 5a) was slightly lower ($p>0.05$) in 10‰ salinity (14.60±3.66 $mg \cdot g^{-1}$ dw) compared to control (17.38±1.43 $mg \cdot g^{-1}$ dw). Higher salinities resulted in further decreases in chlorophyll *a*, and the differences were significant ($p<0.05$) for 20‰ (9.91±1.45 $mg \cdot g^{-1}$ dw), 30‰ (8.93±0.14 $mg \cdot g^{-1}$ dw), and 40‰ (7.55±1.82 $mg \cdot g^{-1}$ dw). In contrast, the 10‰ and 20‰ treatments significantly ($p<0.05$) reduced the chlorophyll *b* content. The salinities of 30‰ and 40‰ resulted in chlorophyll *b* content to levels similar (not significantly different) to the control (Figure 5b).

When total chlorophyll (Figure 5c) content was considered, it was found that increasing salinity from 5‰ to 10‰ caused a slight decrease of this parameter as compared to the control, but without statistical support ($p>0.05$). At higher salinity, significant decreases ($p = 0.03$) of total chlorophyll content was observed (11.56±1.68 $mg \cdot g^{-1}$ dw, 11.34 ±0.38 $mg \cdot g^{-1}$ dw and 10.34±2.14 $mg \cdot g^{-1}$ dw in 20‰, 30‰, and 40‰, respectively) as compared to the control (19.89±1.04 $mg \cdot g^{-1}$ dw).

The carotenoid content significantly increased when salinity was raised to 10‰ (7.84± 0.50 $mg \cdot L^{-1}$), 20‰ (8.28±0.36 $mg \cdot L^{-1}$) or 30‰ (7.66±0.60 $mg \cdot L^{-1}$). However, at the highest salinity (40‰) carotenoid content (6.72±0.42 $mg \cdot L^{-1}$) was at a level similar to the control (6.26±0.68 $mg \cdot L^{-1}$) (Figure 5d). Additionally, the phycocyanin content was highest at 10‰ (0.0064±0.0012 $mg \cdot L^{-1}$), which was not different from the control, and gradually decreased at higher salinity. The lowest phycocyanin content was 0.0021±0.0002 $mg \cdot L^{-1}$ at 40‰. Significant differences in phycocyanin content were detected between low (10‰) and high (20‰–40‰) salinity treatments ($p<0.05$) (Figure 5e).

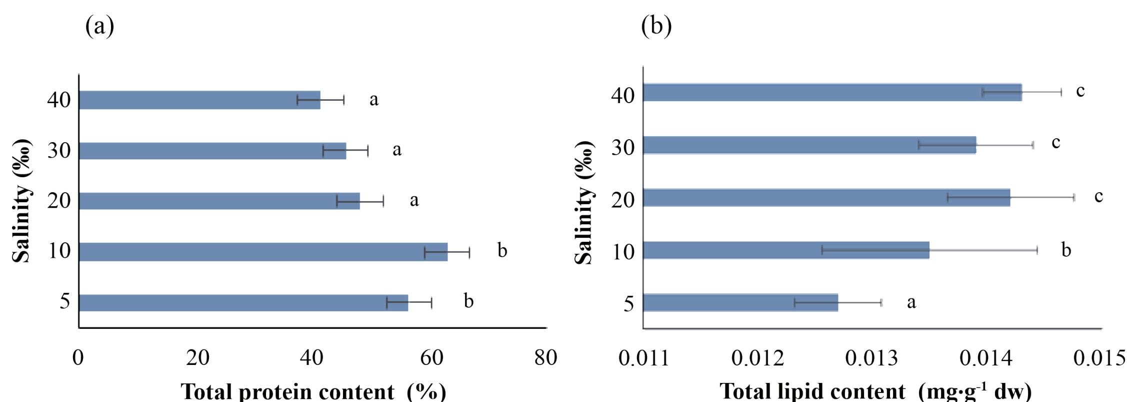

Figure 4. Total protein content (a) and total lipid content (b) of *Spirulina* cultured in control salinity (5‰) and salinity treatments of 10‰, 20‰, 30‰, and 40‰. Different lowercase letters indicate significant ($p<0.05$) difference between means and error bars represent SD.

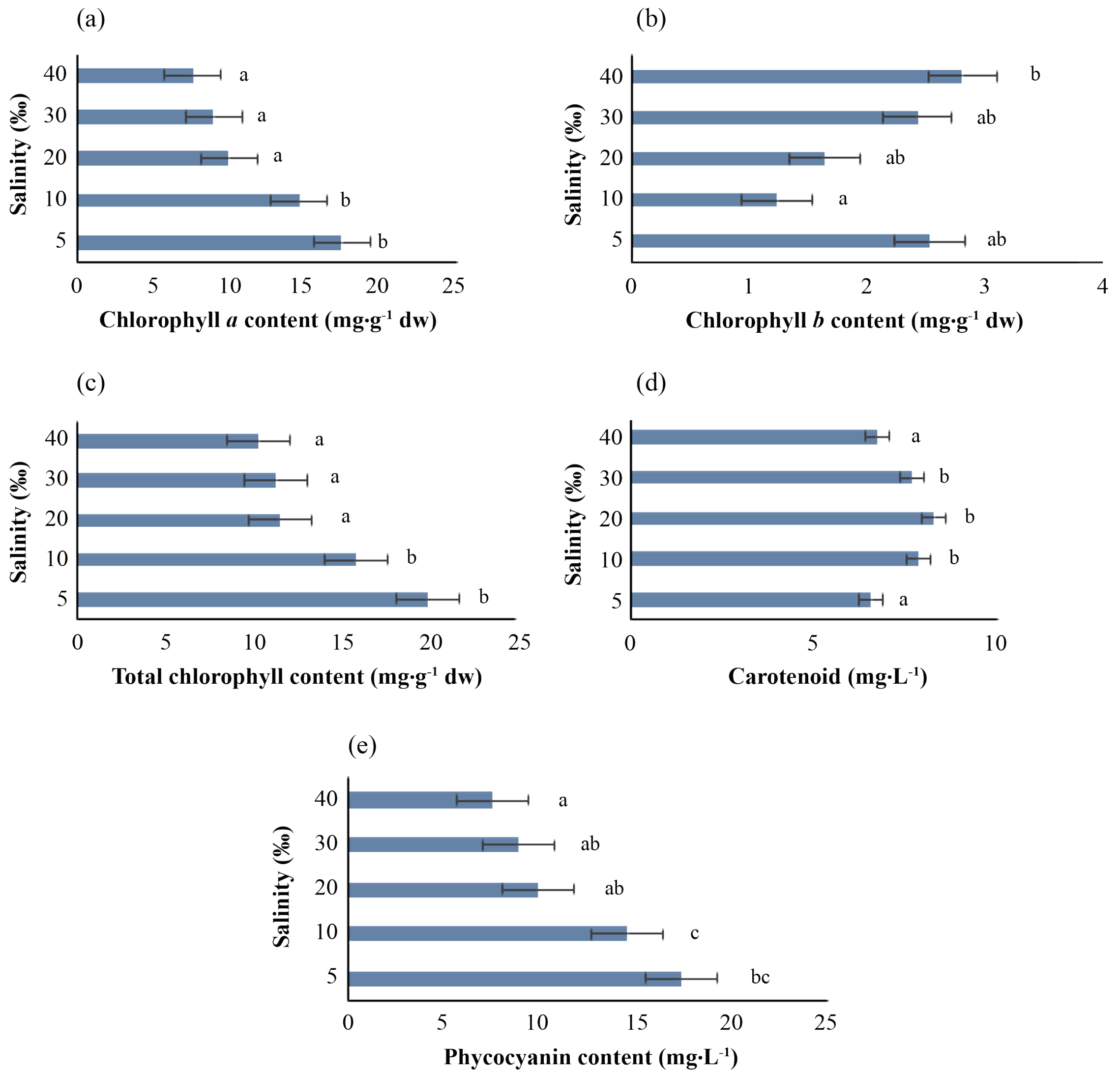

Figure 5. Chlorophyll *a* (a); chlorophyll *b* (b); total chlorophyll (c); carotenoid (d) and phycocyanin (e) contents of *Spirulina* cultured in salinity treatments of 5‰, 10‰, 20‰, 30‰, and 40‰. Different lowercase letters indicate significant ($p<0.05$) difference between means and error bars represent SD.

DISCUSSION

Growth rate of Spirulina

Our results indicated that the growth of *Spirulina* was stimulated in moderate levels of salinity (10–30‰). We found that both cell density and dry biomass had a nearly similar growth pattern, in which the 10‰ salinity treatment had the highest growth rate in both cell density and dry biomass. This finding corresponds well with a previous study, in which the optimum salinity to promote the growth of *Spirulina* was observed at 13‰ (Ravelonandro *et al*., 2011). At the optimum level of salinity, NaCl can stimulate the activity of the photosystem of the thylakoid membrane and its photosynthetic activity, and thus increase the metabolism and growth of *Spirulina* (Dhiab *et al*., 2007). On the other hand, the reduction of growth rates at higher levels of salinity can be caused by

higher sodium chloride toxicity associated with increased salinity (Munns, 2002; Singla and Garg, 2005). According to Almahrouqi *et al*. (2015a) and Rangkuti (2021), even though *Spirulina platensis* can tolerate a wide range in salinity, high salinity is known as the main growth inhibitor; it potentially inhibits the electron transport activity in photosynthesis reactions, thereby lowering metabolism and growth rate.

Microbial reduction

The effectiveness of the salinity treatments in lowering the number of microbes was assessed at day 5 and day 10. The results indicated that at day 5 of cultivation, the moderate level of salinity (10‰) showed the highest microbial reduction. Levels of salinity lower than this (such as in the control) can be tolerated by a wide group of organisms, and therefore the contaminant removal is less effective. On the other hand, at higher salinity some microbial species may thrive since the conditions are more favorable to salt-tolerant organisms due to the selection against more salt-sensitive competitors (Rath *et al*., 2019). These findings strengthen the statement from Abdul-Adel *et al*. (2019) that the sensitivity of microbial reduction to salinity may vary depending on genetic and environmental conditions that may affect their ability to survive in high salinity levels. Based on these findings, salinity and time are essential to reduce the microbes in microalga culture (Rangkuti, 2021).

Our study also showed that the salinity of 10‰ has the potential to reduce bacteria in microalga cultures. This finding concurs with previous research from Zhu *et al*. (2016) showing that cultivating microalgae in a saline environment can inhibit contaminants and invasive organisms. Given the differences due to genetics and local environmental conditions, however, it is essential to determine the optimal salinity range and time to reduce microbes without damaging the microalgae cells (Abdul-Adel *et al*., 2019).

The reduction of microbial number in all salinity treatments may have occurred since salinity acts as a growth-limiting factor in several organisms (Ji *et al*., 2019), including microbes. Previous research has shown that salinity can reduce microbial activity and biomass, and alter the structure of microbial communities. Salinity affects the decrease in microbial biomass caused by osmotic pressure. This has an impact on drying and cell lysis (Yan *et al*., 2015).

Total protein and lipid content

Our results are congruent with a previous study by Almahrouqi *et al*. (2015a) stating that *Spirulina* cultivated in 15‰ salinity showed the highest level of protein compared to control and higher levels of salinity. The moderate level of salinity may have yielded the highest total protein because the absorption of various minerals that are essential for cell metabolism, including protein synthesis, are regulated by Na^+ and Cl^- ions (Esna-Ashari and Gholami, 2010). However, excessive concentrations of Na^+ and Cl^- inhibits the absorption of various important nutrients (Ca, K, N and Mg) (Abdallah *et al*., 2016) through competitive interactions (Stoeva and Kaymakanova, 2008), thus affecting cell metabolism and protein synthesis. The reduction of protein produced in higher levels of salinity has also been reported by Mutawie (2015). This reduction may occur due to cell energy being largely utilized for carbohydrate and lipid metabolism to overcome the osmotic stress (Ravelonandro *et al*., 2011), thus lowering protein synthesis.

The increase of lipid content in higher levels of salinity has been previously reported by Rafiqul *et al*. (2003) and Ji *et al*. (2018). Microalgae use various strategies to overcome salinity stress, one of which is to accumulate lipids. A weakness of algal cells is that their rigid cell walls provide limited ability to adjust cell volume. Therefore, the accumulation of organic matter becomes very important to maintain osmoregulation and balance the osmotic pressure between the extracellular fluid and the cytosol. These organic substances are characterized by small organic molecules with a neutral charge and low toxicity at high concentrations. Lipids are produced as high-energy storage compounds that are synthesized when microalgae encounter unfavorable

environmental conditions. This specialized response of microalgae has been studied recently by scientists with the aim of lipid exploitation for biofuel and biodiesel industries (Suyono *et al*., 2016; Shetty *et al*., 2019).

Pigment content

The reduction in chlorophyll content of *Spirulina* in higher levels of salinity has been reported previously by Sharma *et al*. (2014). The high concentration of NaCl can decrease the growth of cells and inhibit the biosynthesis of chlorophyll *a*. On the other hand, the chlorophyll *b* content varied with no significant differences among salinity treatments, since chlorophyll *b* tends to have less sensitivity to salinity than chlorophyll *a*, which is more salt sensitive (Lutts *et al*., 1996).

Additionally, the results show that the total chlorophyll content was significantly decreased in higher salinity. This in in line with data from the study by Abdul-Adel *et al*. (2019). The reduction of chlorophyll content can be caused by the reduction of the photosynthetic rate due to high osmotic stress and accumulation of toxic ions (Moradi and Ismail, 2007). The results of the present study are in agreement with previous studies showing that chlorophyll is the main target of the salt toxicity (Abdul-Adel *et al*., 2019).

The results of this study also support research presented by Pisal and Lele (2005), who mentioned that higher levels of salinity were able to increase carotene production of microalgae. The increase of carotene production in high salinity is one of the cell's responses for protection against salt toxicity. The reduction of photosynthetic activity due to salt toxicity may be associated with specific ion deficiencies, increased reactive oxygen species (ROS) and osmotic stress, which can affect the biochemistry and physiology of microalgae (Suyono *et al*., 2015). Pigments (e.g., carotenoids) act as antioxidants to protect microalgae from ROS and osmotic stress caused by high salt concentrations, thereby increasing cell resistance to salt toxicity (Suyono *et al*., 2015; Shetty *et al*., 2019).

The increase of phycocyanin content at low salinity and decrease at higher salinity has also been reported by Abdul-Adel *et al*. (2019) and Abd El-Baky (2003). Phycocyanin is one of the phycobiliproteins that are involved in the antioxidative mechanisms of *Spirulina*. In moderate salinity, the phycocyanin production is stimulated to regulate the antioxidative mechanism in salt toxicity. In higher levels of salinity, the regulation of oxygen in phycobiliproteins is disrupted, thus inhibiting the electron transport process in photosystem II. As a result, the phycobilliosome is disrupted, thus reducing the phycocyanin levels in *Spirulina* (Abdul-Adel *et al*., 2019).

CONCLUSION

The experimental results revealed significant reduction of bacteria for *Spirulina* pond culture with 10‰ salinity at day 5, but by day 10 the bacteria had increased again, perhaps because they were tolerant to the increase in salinity. Growth of *Spirulina* was enhanced at 10‰ and 20‰ in terms of density, while dry biomass was increased only at 20‰. Protein content was unchanged or decreased at 20‰–40‰, while lipid content increased with salinity. Chlorophyll *a*, total chlorophyll and phycocyanin tended to decrease with increasing salinity. Chlorophyll *b* tended to decrease when salinity increased to 10‰ and 20‰ and remained unchanged (relative to 5‰) at 30‰ and 40‰. Carotenoid was increased in treatments of 10‰–30‰, but was not different from the control at 40‰. Although modification of salinity can improve some production parameters in open pond systems for *Sprirulina*, the reduction in microbial contamination is only temporary, and thus this does not appear to be an effective solution to the problem.

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