Evaluation of the culture of *Spirulina* sp. with Walne nutrient plus vitamin B12, KCl, NPK, ZA CaO and urea

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Abstract. *Spirulina* sp. is a blue green microalga (*Cyanophyceae*) with cells that colonize to form spiral-shaped filaments. *Spirulina* has active ingredients that can function as antioxidants. *Spirulina* can be processed into products that have economic value and various benefits. The aim of this study was to investigate the evaluation of the culture of *Spirulina* sp. both on the laboratory scale and semi-mass scale, using Walne nutrient plus vitamin B12, KCl, NPK, ZA, CaO and Urea. The working method started from the preparation of tools and materials, culture media, stock of fertilizers, sterilization of tools, seed maintenance, measurements of water quality parameter and growth calculations. Culture on a laboratory scale was done with a volume of 2 L of water, while culture on a semi-mass scale was done with a volume of 80 L of water. Culture of *Spirulina* sp. While reached its peak of population on day 9, accounting for 9,065 cells/mL, but then the figure decreased on the following days, while on a semi-mass scale the growth continued to show an increase until day 15 with a density of 23,275 cells/mL and had not experienced any signs of decline in population.

1. Introduction

Microalgae have the ability to utilize inorganic compounds which play a role in wastewater treatment [1,2]. Microalgae are often used in cultivation activities as natural feed, but they are also widely used in aquaculture, health, feed and food industries [3]. *Spirulina* sp. is one type of microalgae which has the potential and is often used as it contains high levels of nutrients such as protein, fatty acids, vitamins, and antioxidants [4]. Several types of microalgae can be used as raw materials for vitamins [5].

*Spirulina* sp. is one of the most frequently researched and developed microalgae as it has great potential. *Spirulina* sp. is a blue-green algae that is classified as cyanobacteria, one-celled and spiral-shaped. Based on its habitat, *Spirulina* sp. can grow well in tropical and subtropical waters, both in freshwater and marine waters [6]. *Spirulina* sp. which can grow optimally at the salinity of 20 ppt in public waters can be found in estuaries. Estuaries have salinities ranging from 20-30 ppt [7,8].

*Spirulina* sp. is classified as a type of plankton that has a higher content of phycocyanin bio-pigments than other phytoplankton [9]. Phycocyanin is a substance that has benefits for health, one of which is to inhibit tumor growth [10]. Fish fed with an additional 0.5-1% of *Spirulina* sp. have shown an increase in the growth rate by 17-25% and a decrease in the death rate by 30-50%. [11] In Common carp *Cyprinus carpio*, it is shown that there has been a reduction in the number of *Aeromonas hydrophila* bacteria in the liver and heart of fish added with *Spirulina* sp. in the feed. This proves that...
Spirulina sp. is also able to stimulate the natural immune system in fish. Due to the important role of Spirulina sp. it is necessary to study how to analyze the production of Spirulina sp. through the culture process using Walne nutrient plus vitamin B12, KCl, NPK, ZA, CaO and Urea.

2. Material and Method

2.1. Sterilization
Sterilization is a process to remove live microbes so as not to interfere with the research process. Sterilization can be done by using disinfectants, chemicals, ultraviolet, sun drying, autoclaves, and ovens. Equipment such as jar lids, aeration hoses, and other equipment that was not heat-resistant could simply be cleaned using alcohol and washed with soap. Equipment such as a petri dish or other heat-resistant device was washed in an autoclave or oven. Equipment that had been washed was rinsed thoroughly with distilled water, and then dried with sterile wet tissue. Equipment such as an aeration hose that was completely dry was steamed in boiling water for ±15 minutes and then allowed to cool and dry before it could be used again.

Equipment such as an Erlenmeyer flask was washed and then dried until no water remained. The mouth of the Erlenmeyer flask was then plugged with sterile cotton and gauze and finally was covered with aluminium foil before sterilized in an autoclave. Meanwhile, seawater sterilization was done using chlorine. The sea water was first filtered with cotton which was placed in a water funnel, and then the filtered sea water was sterilized with 60 ppm of chlorine for 24 hours. Sterile seawater was later stored in an opaque and tightly closed container. Sea water that had been sterilized using chlorine was boiled for 15 minutes, and after ± 24 hours the new sea water could be used [12].

2.2. Preparation of Water in the Culture Media
Preparation of water in the culture media was carried out for seawater because the salinity of seawater could vary every day, so it was necessary to increase or decrease the salinity according to the desired salinity. The formula used to calculate the required salinity was from [12].

2.3. The Culture Stage of Spirulina sp.
The next stage was the culture stage of Spirulina sp., done by putting the plankton seeds into the media. The next stage was adding 2.945 mL of water with a salinity of 20 ppt (obtained from 3000 mL of seawater – 55 mL) in 9 glass jars that would be used for culture containers [12]. Aeration hoses, lights, and water heaters were set to the desired temperature (25°C, 30°C and 35°C). Vitamins were later added to each of the Walne fertilizer media as much as 3 mL, and vitamin B12 was added as much as 3 mL. The last stage was the addition of Spirulina sp. as much as 55 mL in each medium. The growth of Spirulina sp. was later observed every day.

2.4. Laboratory
Culture was carried out using a transparent plastic bottle with a volume of 1.5 L. The culture container was filled with mineral water that had been previously prepared, and then was added with fertilizer. The compositions of the fertilizer were dissolved by adding hot water to the fertilizer so that they could dissolve well. Filling of water and fertilizer as the media was done first before inoculation of Spirulina sp. into the media. The results of this scale culture were then cultured further into 2 L using a container in the form of a jar [13].

2.5. Intermediate
Intermediate scale culture activities were carried out using an aquarium with dimensions of 25 cm x 25 cm x 45 cm. The inoculants used for this culture were Spirulina sp. with 16 L scale. In the first step,
the aquarium was filled with 17 L of sterilized water and then added with fertilizer until it dissolved completely. Ready culture media could be added with inoculants from a laboratory scale as much as 3 L or 15% of the total culture per aquarium [13].

2.6. Mass Culture
Marg et al. described a method using a seawater-based culture medium with a pH ranging from 6.5–8 [14]. The medium also contained 1.2–3% w/v sodium bicarbonate, 0.1–0.3% w/v nitrogen concentration, 0.1–0.3% w/v phosphorus content, and 0.1–0.3% w/v potassium content. The culture method began with growing Spirulina sp. on an agar slant medium at a temperature of 25–35°C, light radiation of 1000–2000 lux, and the length of irradiation of 12-16 hours per day for 25–40 days. The culture was then transferred to a seawater-based culture medium. Cultivation of the culture was carried out at a temperature of 25–35 °C with light radiation of 2000–3000 lux and the length of irradiation of 12–16 hours per day, generally for 6-12 days. The culture was then transferred to an open cement line containing a seawater-based culture medium. The running culture had to be stirred at a rate of 20–25 cm/second using pedal wheels at a temperature of 20–25 °C and light radiation of 3000–4500 lux.

2.7. Measurements of water quality parameter
Parameters measured included temperature, pH, and salinity. Spirulina sp. can grow in the liquid media under alkaline conditions at a pH range between 8.5–11.0, while the optimum pH for the growth of Spirulina sp. ranges from 9.0 to 10.0 [15]. These microalgae can grow well in the liquid media under alkaline conditions. The growth of Spirulina sp. is influenced by the optimum temperature factor in the range of 35-37 °C, but can tolerate relatively low temperatures at night [15.3].

3. Results and Discussion
3.1. Laboratory-scale water quality
The availability of adequate nutrients and sufficient sunlight are also important factors which support the growth of these microalgae. Salinity of water affects the osmotic pressure of the waters. The higher the osmotic pressure, the higher the salinity of the waters [16]; [17]. For multicellular aquatic organisms, the osmotic pressure of cells is directly related to the absorption of nutrients for their metabolism [18,19].

| Day | Temperature in the morning (°C) | Temperature in the evening (°C) | pH | Salinity |
|-----|---------------------------------|---------------------------------|-----|----------|
| 1   | 26                              | 27                              | 7   | 32       |
| 2   | 26                              | 27                              | 7   | 32       |
| 3   | 25                              | 26                              | 7   | 32       |
| 4   | 26                              | 27                              | 7   | 32       |
| 5   | 26                              | 27                              | 7   | 32       |
| 6   | 25                              | 27                              | 7   | 33       |
| 7   | 26                              | 27                              | 6   | 33       |
| 8   | 26                              | 27                              | 6   | 33       |

Cyanobacteria (including Spirulina species) can live in mesohaline waters [16]. Prambodo et al. stated that Spirulina platensis is a photoautotrophic organism that requires light to carry out photosynthesis [20]. During the research, the color of the culture was getting darker and darker. This indicates that there is a good photosynthesis process for the growth of Spirulina sp.
3.2. Intermediate scale water quality

Table 2. Results of the measurements of intermediate scale water quality

| Day | Temperature in the morning (°C) | Temperature in the evening (°C) | pH | Salinity |
|-----|---------------------------------|---------------------------------|-----|----------|
| 1   | 25                              | 26                              | 7   | 32       |
| 2   | 25                              | 26                              | 7   | 32       |
| 3   | 25                              | 26                              | 7   | 32       |
| 4   | 25.5                            | 26                              | 7   | 32       |
| 5   | 25                              | 27                              | 7   | 32       |
| 6   | 24.5                            | 25                              | 7   | 33       |
| 7   | 25                              | 26                              | 6   | 33       |
| 8   | 25                              | 26                              | 6   | 33       |
| 9   | 25                              | 25.5                            | 7   | 33       |
| 10  | 25                              | 24.5                            | 7   | 33       |
| 11  | 24                              | 25                              | 7   | 33       |
| 12  | 25                              | 26                              | 7   | 34       |
| 13  | 25.5                            | 26                              | 7   | 34       |
| 14  | 25                              | 26                              | 7   | 34       |
| 15  | 25                              | 25.5                            | 7   | 34       |

3.3. Harvesting

Harvesting on the laboratory scale of this *Spirulina sp.* culture was carried out on day 8 during the death phase when the density of *Spirulina sp.* experienced a decrease. The results of this culture were then used as inoculants for the semi-mass scale of *Spirulina sp.* culture. In the study by Suminto, harvesting was done when *Spirulina sp.* was in the final exponential phase ± on day 7 [16]. Harvesting could be done using a 25-micron plankton net. Cultures that had reached peak population were deposited first by turning off aeration. Following this, *Spirulina sp.* was filtered using a plankton net, and then weighed to determine the resulting biomass.

In the intermediate scale of *Spirulina sp.* culture, harvesting was not done because when the density was observed under a microscope, *Spirulina sp.* had not shown a decline stage. The results of the harvesting of intermediate-scale plankton were usually stocked into a tilapia hatchery or a medium for *Daphnia sp.*

Growth of *Spirulina sp.*

Data of the daily population density for *Spirulina sp.* on the laboratory scale culture are presented in the table 3.

Table 3. The results of growth measurements of *Spirulina sp.* for 8 days

| Day | Density (Cell/ml) |
|-----|-------------------|
| 1   | 3.520             |
| 2   | 4.875             |
| 3   | 5.025             |
| 4   | 7.780             |
| 5   | 8.975             |
| 6   | 9.065             |
| 7   | 5.095             |
| 8   | 4.745             |
Figure 1. Growth of *Spirulina* sp. for 8 days

The highest density of *Spirulina* sp. in the laboratory scale culture was obtained on day 6, accounting for 9.065 cells/mL. On day 2 of observation of pure culture, the cell density became 4.875 cells/mL and continued to rise until day 6 into 9.065 cells/mL. According to Hariyati, a significant increase indicates that plankton is in the exponential phase [21]. Reproductive ability of *Spirulina* sp. in this phase is supported by nutrients, pH, temperature, and light intensity. The peak phase of laboratory scale culture was noted on day 6 with a density of 9.065 cells/mL.

The next phase was the stationary phase, in which the growth of *Spirulina* sp. tended to be stable and decrease gradually [21]. On day 7, culture of *Spirulina* sp. decreased in number to 5.095 cells/mL. During the observation on day 8 of *Spirulina* sp. culture, there was a rapid decline in number, accounting for 4.745 cells/mL, entering the death phase which was thought to be influenced by several factors such as decreased nutrition. As a consequence, on day 8, *Spirulina* sp. Had to be harvested so that the density obtained was not too low.

Table 4. The results of growth measurements of *Spirulina* sp. for 15 days

| Day | Density (cell/ml) |
|-----|-------------------|
| 1   | 2.175             |
| 2   | 2.875             |
| 3   | 3.375             |
| 4   | 4.540             |
| 5   | 5.125             |
| 6   | 6.350             |
| 7   | 10.375            |
| 8   | 11.120            |
| 9   | 11.895            |
| 10  | 13.340            |
| 11  | 14.245            |
| 12  | 15.975            |
| 13  | 16.550            |
| 14  | 18.810            |
| 15  | 23.275            |

Daily population density data for *Spirulina* sp. on semi-mass scale culture is presented in the table 4. Based on the results of the cell counting of *Spirulina* sp. in semi-mass culture, the inoculant density
was 9.065 cells/mL. The highest density of *Spirulina sp.* in semi-mass scale culture was still unknown because until day 15 *Spirulina sp.* had not yet experienced a stationary phase or a death phase. On the first day of measurement, the density obtained was 2.175 cells/mL. During this time, *Spirulina sp.* showed the adaptation phase (lag phase). According to Hariyati [21], the lag phase is the stage of *Spirulina sp.* to adapt to its new environment, so the increase in cells is still low. Afterwards, through observation on the second day of pure culture, it was noticed that the cell density became 2.875 cells/mL, and in the observation on the third day of semi-mass scale culture, it reached 3.375 cells/mL. On the fourth day, the growth continued to increase until reaching 4.540 cells/mL and rose even higher into 6.350 cells/mL on the fifth day. Then, on the sixth day the number of cultures reached 8.480 cells/mL. When the culture of *Spirulina sp.* entered one week, the number inclined more into 10.375 cells/mL and continued to rise until reaching 23.275 cells/mL on the fifteenth day. According to Hariyati [21], a significant increase indicates that plankton is in an exponential phase. Reproductive ability of *Spirulina sp.* in this stage was supported by nutrients, pH, temperature, and light intensity. The exponential phase of laboratory scale culture was noted on day 15 with a density of 23.275 cells/mL.

![Figure 2. Growth of Spirulina sp. for 15 days](image)

According to Suantika and Hendrawandi [5], the exponential phase has the ability to multiply cells, and these rapid cell activities make cell growth to reach its maximum point, yet it has a short adaptation time during the culture process. The death phase usually occurs on day 14 or 15 until the end of maintenance because cell death is greater than its growth. Hariyati [21] explained that the decrease in growth is caused by reduced nutrients, less light intensity, greater competition for nutrients, living space, and light.

### 4. Conclusion

The culture process of *Spirulina sp.* started from the sterilization of tools, preparation of culture media, application of fertilizers, stock of inoculants, maintenance, and harvesting. Culture on a laboratory scale used a plastic jar with a volume of 2 L of water, while in a semi-mass scale it used a tank with a volume of 80 L of water. The culture of *Spirulina sp.* reached its peak population on day 6, accounting for 9.065 cells/mL, but then experienced a decrease on the following days, while in the semi-mass scale the culture of *Spirulina sp.* still continued to show an increase until day 15 with a density of 23.275 cells/mL without showing any indication for a decline in population.

### 5. References

[1]  Sari L A., Pursetyo K T, Arsad S, Masithah E D, Setiawan E, and Affandi M 2019 *Pol Res.* 38, S27-S32.
6. Acknowledgment
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