Volume 1 Issue 1 (2022): 1-7
Tropical Marine Environmental Sciences
e-issn 2962-0759

Population Growth and Chlorophyll Content of *Spirulina platensis* Fertilized with *Azolla microphylla*

Muhammad Aliv Kusuma Putra[1*], Irvina Nurrachmi[1], Irwan Effendi[1]

[1]Faculty of Fisheries and Marine, Universitas Riau, Indonesia
Kampus Bina Widya KM. 12,5, Simpang Baru, Kec. Tampan, Kota Pekanbaru, Riau 28293
*malivkusumaputra@gmail.com

Article Info

Received
30 August 2022

Accepted
24 September 2022

Keywords:
Liquid Fertilizer, Macrophites, Microalgae, Organic Fertilizer, Water Fern.

Abstract

Spirulina platensis is a marine microalga that has potential as a natural fish feed and is considered an absolute food supplement to combat malnutrition in some countries. *Azolla microphylla* is a water fern that has been cultivated, contains high protein, grows quickly and is widely found in tropical waters. This study analyzed the effect of liquid fertilizer of *A.microphylla* on population growth and chlorophyll content of *S. platensis.* A completely randomized design (CRD) was used and the treatments were A (3 mL/L), B (5 mL/L) and C (7 mL/L), D (negative control without liquid fertilizer) and E (positive control, addition of 1 ml/L Walne fertilizer). The measurement results showed that the water quality during the study was in good condition, the water temperature ranged from 28-29 C, pH (7-8) and salinity (25 - 26 ppt). The highest population density level of *S. platensis* (321,500 cells/mL) was recorded in treatment B. Then followed by treatment D (270,600 cells/mL), treatment C (260,000 cells/mL), treatment A (220,000 cells/mL) and treatment E (150,000 cells/mL). The highest chlorophyll content was found in treatment C of 0.362 g/L, treatment D (0.302 g/L), treatment A (0.182 g/L), treatment B (0.250 g/L), and treatment E (0.072 g/L).

1. Introduction

Spirulina platensis is a multicellular marine microalga that undergoes photosynthesis, one of the most promising energy sources because it is renewable and neutral to CO_2, one of which is the chlorophyll pigment contained in it. Chlorophyll pigment is the main factor affecting photosynthesis. Has many benefits of considerable attention in the health care and food sectors as a protein and vitamin supplement and has the potential as a good natural food and contains high value bioactive compounds, and heavy metal absorption in the polluted environment. Laboratory-scale natural feed cultures use a lot of Walne and Guillard fertilizers for rearing media. Semi-mass and bulk cultures usually use commercial fertilizers (Urea, TSP, and ZA). The high price of fertilizer is a problem that must be faced in cultivating microalgae, therefore it is necessary to use alternative fertilizers that are easily available, rich in nutrients and relatively inexpensive. (Salunke *et al.*, 2016; Bezerra *et al.*, 2011; Gonçalves *et al.*, 2016; Huesemann *et al.*, 2016; Hanriyani *et al.*, 2019).

Azolla microphylla is a plant that belongs to the aquatic weed and is widely found in tropical waters. Contains macro and micro elements, including N (4.5%), P (0.5-0.9%), K (2-4.5%), Ca (0.4-1%), Mg (0.5-0.6%), Fe (0.06-0.26%), Mn (0.11-0.16%). Azolla is known to be able to symbiotically with the blue-green bacteria *Anabaena azollae* and fix nitrogen directly from the air (Effendi *et al.*, 2019; Effendi *et al.*, 2020). This potential makes Azolla used as green manure both in paddy fields and dry land (Lestari *et al.*, 2019). In optimal conditions, Azolla will grow well with a growth rate of 35% per day. The

nutritional value of Azolla contains high protein content between 24-30%. The content of essential amino acids, especially lysine, was 0.42% higher than the concentrate of corn, bran, and broken rice. Liquid fertilizer made from Azolla is proven to increase the population of this microalgae with a certain concentration due to the content of Nitrogen (N), Phosphorus (P), Iron (Fe), Magnesium (Mg) in *A. microphylla* (Leksono *et al.,* 2017; Albab *et al.,* 2017; Ali *et al.,* 2012).

The growth of *S. platensis* really needs nutrients, both macronutrients and micronutrients so that these microalgae can photosynthesize well and have high chlorophyll content. Nitrogen and phosphorus are basic components in the formation of proteins for the growth and development of these microalgae. The components that affect the formation of chlorophyll are N, P, Mg and Fe. One way to meet nutritional needs for good growth is to provide fertilizers such as Walne or agricultural technical fertilizers that contain the components needed for these phytoplankton to grow well (Barus *et al.,* 2018; Lestari *et al.,* 2019). The use of *A. microphylla* as an alternative fertilizer is possible, due to the presence of components that can support the growth and development of this microalgae (Soni *et al.,* 2019; Effendi *et al.,* 2021; Nainggolan *et al.,* 2018). This study aimed to analyze the effect of fertilizer application made from *A.microphylla* on population growth and chlorophyll content in *S.platensis*

2. Methodology

2.1. Time, Place and Materials

This research was conducted in April - May 2021 at the Marine Microbiology Laboratory, Faculty of Fisheries and Marine, Riau University. Pure culture of *S. platensis* obtained from the natural feed laboratory of BBPBAP Jepara. Central Java, Indonesia. *A. mycrophylla* is a collection of our laboratory. Walne fertilizer (Na_2EDTA 45g, NaH_2PO_4, H_2O 20g, $FeCl_3.6H_2O$ 1.5g, H_3BO_3 33.6 g, $MnCl_2$ 0.36g, $NaNO_3$ 100g) as a source of nutrients, distilled water and absolute methanol was purchased from commercial sellers. Seawater as a growth medium was collected from Padang Beach, West Sumatra, Indonesia.

2.2. Research Design

The research design used in this study was a completely randomized design (CRD). Consists of 5 treatments with 3 repetitions. Consists of A (3 mL/L), B (5 mL/L) and C (7 mL/L), D (negative control without fertilizer application) and E (positive control, addition of 1 mL/L Walne fertilizer). The research data were statistically analyzed using one way ANOVA and continued with the LSD (Least Significant Different) test.

2.3. Liquid Fertilizer Preparation and Growing Media of *S. platensis*

A. microphylla washed with clean water repeatedly to remove dirt that sticks. Then dried in an outdoor system under the hot sun for 3 days. The dried Azolla is then ground into powder and then dissolved in distilled water (1:4) for 3-4 weeks and stirred every day. After soaking then squeezed so that the liquid in it can come out. The supenatant extracted from *A.microphylla* was filtered until there was no remaining pulp. This liquid fertilizer was then sterilized using an autoclave (121 ^oC for 15 minutes), placed in a sterile glass container and closed to avoid contamination.

The growing medium used was seawater mixed with fresh water. The water temperature ranged from 28-29 ^oC, pH (7-8), and salinity (25 - 26 ppt) as much as 1000 ml. A total of 1 L was put in a 2 L capacity container and then a solution of Azolla liquid fertilizer was added according to the concentration of each treatment. The media was aerated to supply air into the growing media for *S. platensis*.

2.4. Growth of *S. platensis*

Pure cultures of *S. platensis* were put into culture media with a density of 100,000 cells/mL. Number of the microalgae seeds required for inoculation measured by using the following formula:

$$V_1 = \frac{N_2 \times V_2}{N_1}$$

where:

V1 = Volume of initial stocking seeds (mL)
V2 = Volume of culture medium used (mL)
N1 = Density of seeds/stock used (cells/mL)
N2 = desired density of *S. platensis* seedlings (cells/mL)

The population density calculation was carried out every two days after the initial inoculating of the seeds. Calculations were performed using the Sedgwick Rafter Counting Cell (SRC). The sample was dripped as much

as 1 mL above the SRC using a dropper, then observed using a microscope with a magnification of 100x. The growth of *S. platensis* was calculated based on one sinusoid/wave. The density calculation is carried out using the formula:

$$N=\frac{1000}{3,14\left(d/2\right)^2}\times n$$

where:

N = Density of *S. platensis* (cell/mL)
D = Field of view diameter (mm)
N Average number of *S. platensis* per field of view (cell/mL)

2.5. Chlorophyll content of *S. platensis*

A volume of 10 ml of *S. platensis* culture was placed into centrifuge tube and then centrifuged at 3000 rpm for 15 minutes. The centrifuged supernatant was discarded and the *S. platensis* pellet at the bottom of the tube was extracted with 10 ml of absolute methanol, then disrupted with a homogenizer and incubated at 70 ^{o}C for 2 minutes. The resulting mixture was centrifuged at 3000 rpm for 5 minutes. The obtained filtrate was measured its absorbance using a spectrophotometer at a wavelength of 665 nm. Chlorophyll content is calculated by the following formula:

Chlorophyll µg/L = absorbance coefficient × A_{665}

where:

A_{665} = Read the absorbance of the filtrate at a wavelength of 665 nm
Chlorophyll µg/L = Chlorophyll content of *S.platensis* (µg/L)
Absorbance coefficient = 1.69.

3. Result

3.1. Population of *S. platensis*

There were differences in the population density of *S. platensis* in each treatment at 14 days. The highest population was in treatment B (321,500 cells/mL), and the lowest one in treatment E (negative control) with only 150,000 cells/mL. In treatment A, the highest population density reached 220,000 cells/mL, while in treatment C (260,000 cells/mL) and in treatment D (positive control) the highest density reached 270,600 cells/mL (Table 1). Based on the ANOVA test at the 6th and 12th days there was a significant difference ($p<0.05$) of each treatment.

Table 1. Population density of *S. platensis* (×10^3 cell/mL)

Cultivation Period (day)	Experimental treatments				
	3 ml/L (A)	5 ml/L (B)	7ml/L (C)	Positive control (D)	Negative control (E)
0	100	100	100	100	100
1	121.3	126.1	121.4	126.7	120
3	150	189.2	155	192	145
6	175	275.5	200	248	150
3	180	290	233.5	270.6	135
12	220	321	260	261	125
14	170	245	220	230	109

3.2. Population of *S. platensis*

Measurement of chlorophyll content was carried out at 1, 3, 6, 9, 12 and 14 days. The chlorophyll content ranges from 0.010 to 0.362. The highest chlorophyll content was found in treatment C (7 mL/L) which was 0.362 g/L. Treatment B (5 mL/L) was 0.310 g/L, treatment D (positive control) was 0.302 g/L, treatment A (3 ml/L) was 0.182 g/L and the chlorophyll content in treatment E (negative control) was 0.072 g/L (Table 2).

Table 2. Chlorophyll content of *S. platensis*

Cultivation Period (day)	Experimental treatments				
	3 ml/L (A)	5 ml/L (B)	7ml/L (C)	Positive control (D)	Negative control (E)
1	0.011	0.016	0.020	0.022	0.010
3	0.051	0.059	0.080	0.062	0.046
6	0.093	0.147	0.241	0.204	0.058
9	0.125	0.223	0.304	0.302	0.072
12	0.182	0.250	0.362	0.291	0.051
14	0.101	0.310	0.322	0.261	0.022

4. Discussion

4.1. Population growth of *S. platensis*

Has been used as a fertilizer for several plant species. For example, for Chinese kale (Barus *et al.*, 2018), rice plant (Setiawati *et al.*, 2017; Sajjad *et al.*, 2021) several kinds of agricultural crops (Lestari *et al.*, 2019; Widiastuti *et al.*, 2016). The results of measurements for each treatment during the study showed that the total population density of *S. platensis* increased differently due to different types of fertilizers and their dosages. The nutritional content of *S. platensis* is obtained from the fertilizer, the higher the fertilizer applied the effect on the nutrient content (Belay, 2008). The results of the ANOVA test obtained a significant value of $p < 0.05$, which means that there is a significant difference in the application of liquid organic fertilizer fermented from *A. microphylla* to the population density of the microalgae.

These microalgae can be grown in different media even in waste media. This biota grows by utilizing sugar as a carbon source, and protein hydrolyzate as a nitrogen source. The organic materials needed for the growth of these microalgae are abundant in plant-derived wastes such as tapioca waste, latex waste, and oil palm. Based on research, it is known that Spirulina has been successfully used as a biofilter in cow farm liquid waste. Cow farm liquid waste contains organic material that is used by these biota as food ingredients, especially nitrate (NO3). Nitrate is the main form of nitrogen in natural waters and is the main nutrient in algae growth (Danesi *et al.*, 2002; Mutia *et al.*, 2021).

From the results of this increase in population density, it can be stated that during the population growth period *S. platensis* went through several phases, namely from day 0 to day 4, this microalgae experienced a lag phase, namely adapting to a new environment. The lag phase or adaptation phase is a phase when the cell density does not change, but the cell size in that phase increases. Photosynthesis is still actively taking place and organisms undergo metabolism but cell division has not yet occurred so that the density has not increased. Then following on the 5th day to the 12th day, *S. platensis* experienced an exponential phase, namely the microalgae experienced constant division so that they experienced cell density and experienced an increase in the amount of density, this is in accordance with what was stated by the exponential phase marked by an increase in density. *S. platensis* cells until they reach their maximum density. The fastest doubling time is usually reached during the exponential phase, that is, the growth phase when cells divide rapidly and steadily.

The increase in the microalgae population was only up to the 12th day, after that it decreased until the 14th day. In this case *S. platensis* experienced a stationary phase, which began to decrease compared to the exponential phase where the density growth rate was smaller than the death rate, the decrease in growth was caused by reduced nutrients in the medium and greater competition for nutrients, as well as living space (Natalia *et al.*, 2019; Mutia *et al.*, 2021). Population density growth of *S. platensis* can be compared in each treatment. The density of *S. platensis* increased from day 0 to day 12, after which the total population density of this microalgae decreased again on day 14, this can be seen in Figure 1.

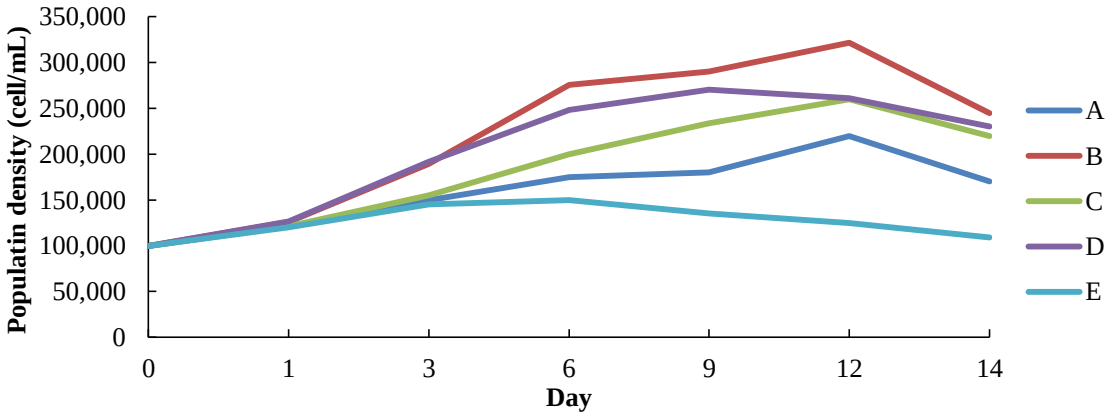

Figure 1. Population density of *S. platensis*

4.2. Population growth of *S. platensis*

The growth and biomass yield of Spirulina depends on nutrients availability, pH, light, and temperature. Media composition and its cost are challenging factors for the viable mass cultivation of cyanobacteria. Two different growth media, such as Zarrouk media and modified media, were used to cultivate Spirulina. Zarrouk media served as standard media for cultivation of this microalgae (Soni *et al.*, 2019). In this study, the highest chlorophyll content was found in treatment C but was not matched by a high population density (Figure 2). The same thing happened in the study that cultured phytoplankton using modified touge extract media. Optimal concentration results were obtained for the highest chlorophyll content of 6%. While the optimal concentration for the highest population is 4% (Adharani *et al.*, 2017). Sari (2017) stated that the highest chlorophyll content was at the highest concentration but was not followed by a high population, because the higher the concentration of fertilizer application, the lower the effectiveness of nutrient utilization. It is suspected that when given a high concentration of fertilizer, the growth of *S. platensis* was inhibited in carrying out cell division which caused the population not to increase, but the excess remaining nutrients were utilized for the development process by producing chlorophyll. High fertilizer concentration results in high chlorophyll content.

The components contained in Azolla liquid fertilizer include N (4.5%), P (0.5-0.9%), K (2-4.5%), Ca (0.4-1%), Mg (0.5-0.6%), Fe (0.06-0.26%), and Mn (0.11-0.16%). Nitrogen is an important element for the growth of plankton, forming protein and chlorophyll. In addition to nitrogen, phosphorus is a part of fertility support (Dianursanti *et al.*, 2017; Adharani *et al.*, 2017; Adams *et al.*, 2005). Meanwhile, Cu is needed for lignin synthesis and carbohydrate or protein metabolism. Zn is needed for energy production, protein synthesis and growth. Mn is required for photosynthesis and protein metabolism. Nitrogen and phosphorus are basic components in the formation of proteins for the growth and development of microalgae, while the components that affect the formation of chlorophyll in these microalgae are N, P, Mg, and Fe. The growth and formation of chlorophyll in *S.platensis* is not only influenced by the nutritional content but also by environmental conditions in the rearing media. (Soni *et al.*, 2019; Schroder *et al.*, 2010).

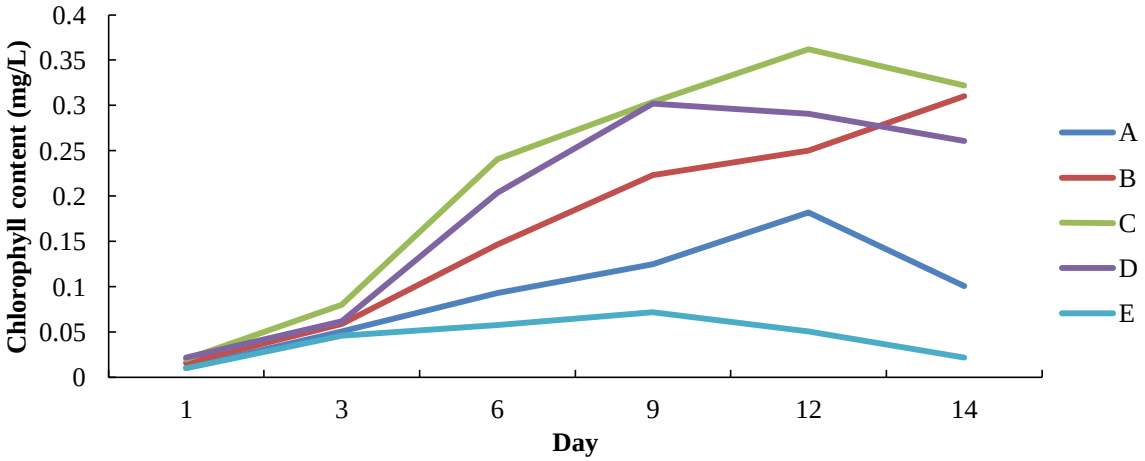

Figure 2. Chlorophyll content of *S. platensis*

5. Conclusion

The application of fertilizer made from *A. microphylla* had a positive effect on population density and chlorophyll content in *S.platensis*. The optimal concentration of Azolla-based fertilizer was 5 mL/L with a density of 321,500 cells/mL. While the optimal concentration for the chlorophyll content of *S.platenis* was 7 mL/L with chlorophyll content reaching to 0.362 g/L

REFERENSI

Abdel-Tawwab, M & H.S. Hamed. (2020). Antagonistic effects of dietary guava (*Psidium guajava)* leaves extract on growth, hemato-biochemical, and immunity response of cypermethrin-

intoxicated Nile tilapia, *Oreochromis niloticus*, fingerlings. *Aquaculture*, 529,735668

Adams, M. (2005). Superfood for optimum health: *Chlorella* and *Spirulina*. New York: Truth Publishing International Ltd. Page 26.

Adharani, N., Citra. S.C., Hidayat, N.B., Susanto, A.H., Saputra, A. (2017). Modification of bean sprout and urea media to *Spirulina platensis* culture. Proceeding The 1st IBSC: Towards the Extended Use of Basic Science For Enhancing Health, Environment, Energy and Biotechnology. Food Technology. 107-109.

Albab, M. (2017) *Pengaruh konsentrasi pupuk cair Azolla pinnata terhadap kepadatan populasi dan kandungan protein Spirulina platensis. Skripsi.* Fakultas Pertanian-Peternakan Universitas Muhammadiyah Malang, 2017.

Ali, S.K., Saleh, A.M. (2012). *Spirulina platensis*- An Overview. *Internasional Journal of Pharmacy and Pharmaceuntical Sciences*. 4 (3) : 9-15.

Barus, W.A., Utami, S., Azmi, P.A. (2018). Effect of Azolla Bokashi and Liquid Organic Fertilizer of Goat Manure on the Growth and Production of Chinese Kale (*Brassica oleracea* L.). *Indonesian Journal of Agricultural Research* 01 (01): 78 – 86.

Belay, A. (2008). Spirulina (*Spirulina* sp.) Production and Quality Assurance, in Gershwin, M.E and Belay A (Eds.). *Spirulina in Human Nutrition and Health*, *CRC Press*, California, 2-26.

Bezerra, P., Montoya, R., Ortiz, E.Y., Sunao, S., Perego Patrizia, Carvalho, J.C.M., Attilio, A. (2011). Effects of light intensity and dilution rate on the semicontinuous cultivation of arthrospira (*Spirulina*) *platensis*. A kinetic monod-type approach. Bioresour. *Technol*. 102, 3215–3219.

Danesi, E.D.G., Rangel-Yagui, C.D.O., De Carvalho, J.C.M., Sato, S. (2002). An investigation of the effect of replacing nitrate by urea in the growth and production of chlorophyll by *Spirulina platensis*. *Biomass Bioenergy*, 23(4): 261–269.

Dianursanti, D., Zarahmaida, T., Indraputri, C.M. (2018). Optimization growth of *Spirulina platensis* in bean sprouts extract medium with urea fertilizer for phycocyanin production as antioxidant. Proceedings of the International Symposium of Biomedical Engineering (ISBE) 2017 - Bali, Indonesia (25–26 July 2017). doi:10.1063/1.5023961.

Effendi, I., Nurrachmi, N., Tarigan, A.N.F. (2021). Growth of Microalgae *Spirulina platensis* on Media Containing Palm Oil Mill Effluent. IOP Conf. Series: Earth and Environmental Science 695 (2021) 012039. doi:10.1088/1755-1315/695/1/012039.

Effendi I, Pranata A, Feliatra F (2019) The Effect of sun light intensity on the growth of *Azolla hidrophylla* and its Symbiont *Anabeana azollae* in brackish water. Journal of Physics: Conference Series 1351 012096. IOP Publishing. doi:10.1088/1742-6596/1351/1/012096.

Effendi, I., Saputra, E., Tanjung, A., Elizal. (2020). Adaptation of *Azolla hidrophylla* to Brackish Water Ecosystem. International Conference on Environment and Technology. IOP Conf. Series: Earth and Environmental Science 469 012024. IOP Publishing. doi:10.1088/1755-1315/469/1/012024.

Gonçalves, A.L., Rodrigues, C.M., Pires, J.C.M., Simões, M. (2016). The effect of increasing CO_2 concentrations on its capture, biomass production and wastewater bioremediation by microalgae and cyanobacteria. *Algal Res.* 14, 127–136.

Hanryani, P., Efriyeldi., Effendi, I. (2019). The effect of different light colours on the biomass growth of *Spirulina platensis. Asian Journal of Aquatic Science*. 2(2): 132-137.

Huesemann, M., Crowe, B., Waller, P., Chavis, A., Hobbs, S., Edmundson, S., Wigmosta, M. (2016). A validated model to predict microalgae growth in outdoor pond cultures subjected to fluctuating light intensities and water temperatures. *Algal Res.* 13, 195–206.

Leksono, A.W., Dian, M., Indah, A.Y. (2017). Penggunaan Pupuk Cair Hasil Fermentasi dari Azolla pinnata Terhadap Kepadatan Sel *Spirulina* sp. *Jurnal Ilmu-ilmu Perikanan dan Budidaya Perairan*. 12(1): 56-65.

Lestari, S.U., Mutryarny, E., Susi, N. (2019). *Azolla mycrophylla* fertilizer for sustainable agriculture: compost and liquid fertilizer applications. *International Journal of Scientific & Technology Research*, 8(07):542-547.

Mutia, S., Nedi, S., Elizal. (2021). Effect of nitrate and phospate concentration on *Spirulina platensis* with indoor scale. *Asian Journal of Aquatic Sciences*, 4 (1): 29-35.

Nainggolan, J.G.M., Tanjung, A., Effendi, I. (2018). Pertumbuhan biomassa *Spirulina platensis* dengan pemberian nutrisi yang berbeda dengan skala indoor dan semi out door. *Asian Journal of Aquatic Science*. 2(1): 22-28

Natalia N. Amin B, Effendi I (2019) Effect of addition of different nitrate concentration on *Spirulina platensis* biomass with semi outdoor system. *Asian Journal of Aquatic Sciences*, 2(2): 127-131.

Sajjad, H., Al-Bdairi, J., Jawad, K.A. (2021). The effect of biofertilizer of Azolla, phosphate and nitrogen fertilizers on some growth traits of rice. IOP Conf. Series: Earth and Environmental Science 735 (2021) 012064. doi:10.1088/1755-1315/735/1/012064.

Salunke, K.J., Magar, S.A., Joshi, R.R., Wadikar, M.S. (2016). Comparative study on the growth of *Spirulina platensis* on different culture media. *Biosci. Discov.* 7 (1): 90–92.

Sari, H.S., Endang, D.M. (2012). Effect of azolla pinnata fertilizers on chlorophyll content of *Spirulina platensis*. Universitas Airlangga.

Schroder, J.J., Chordell, D., Smith, A.L., Rosemarin, A. (2010). Suistainable Use of Phosphorus. *Plant Research International*, Part of Wageningen Ur. Business Unit Agrosystem.

Setiawati, M.R., Damayani, M., Herdiyantoro, D., Suryatmana, P., Anggraini, D., Khumairah, F.H. (2017) The Application dosage of *Azolla pinnata* in fresh and powder form as organic fertilizer on soil chemical properties, growth and yield of rice plant. The 1st International Conference and Exhibition on Powder Technology Indonesia (ICePTi) 2017.

Soni, R.A., Sudhakar, K., Rana, R.S. (2019). Comparative study on the growth performance of *Spirulina platensis* on modifying culture media. Energy Reports 5 (2019); 327-336.

Soni, R.A., Sudhakar, K., Rana, R. (2016). Biophotovoltaics and biohydrogen through artificial photosynthesis: an overview. *Int. J. Environ. Sustainable Dev.* 15 (3).

Sudhakar, K., Premalatha, M. (2015). Characterization of microalgal biomass through FTIR/TGA/CHN analysis: application to scenedesmus sp. *Energy Sour. A* 37 (18): 1–8. http://dx.doi.org/10.1080/15567036.2013.825661.

Sudhakar, K., Premalatha, M. (2012). Micro-algal technology for sustainable energy production: state of the art. *J. Sustainable Energy Environ*. 3: 59–62.

Widiastuti, D.P., Davis, J.G., Gafur, S. (2016). Azolla fertilizer as an alternative N source for red spinach production on alluvial and peat soils in West Kalimantan, Indonesia. International Nitrogen Initiative Conference, Solutions to improve nitrogen use efficiency for the world, 4 – 8. December 2016, Melbourne, Australia