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Growth Performance of *Chlorella spp.* in Different Water Conditions as Culture Media

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ABSTRACT

Chlorella is a spherical, single cell green algae belonging to phylum Chlorophyta. It multiplies rapidly, requiring only carbon dioxide, water, sunlight, and a small amount of minerals to reproduce. This study was conducted to determine the growth performance of Chlorella spp. in different water conditions as culture media. Chlorella was cultured using empty glass bottles provided with aeration to have a steady supply of oxygen. There were three treatments with three replications in a Randomized Complete Block Design (RCBD) which include sterilized seawater as Treatment 1 (T1); non-sterilized seawater as Treatment 2 (T2); and tilapia pond water as Treatment 3 (T3). All the culture media were fertilized with ammonium, urea, and phosphate after the inoculation of cells. Counting of Chlorella cells was done daily and the different water parameters were monitored twice a day. Results revealed that there is significant difference among the growth of Chlorella in three treatments (Fc= 7.09; Ft = 3.3) using ANOVA at 5% level of significance. On further analysis through DMRT, results revealed that T1 (sterilized seawater) and T3 (Tilapia pond water) have no significant difference from T2 (non-sterilized seawater). Result of the study shows that tilapia pond water can be a potential medium for Chlorella production for the possible supply of Chlorella for other industrial endeavors.

INTRODUCTION

The conventional system of fish culture depends largely on the availability of natural fish food organisms like plankton and a favorable environmental condition. Brown et al., (1997), stated that microalgae are at the base of aquaculture food chain and are generally acknowledged for their nutritional value. Their nutritional values can provide a high quality nutritional package for different stages of aquaculture animals (Banerjee et al., 2010; Khatoon et al., 2009, 2012). Nutritional composition of algae is well documented and it mainly contains proteins, carbohydrates, lipids and trace nutrients, including vitamins, antioxidants, and trace elements. These algal components have the characteristics to be a natural supplement in human and animal feed to replace synthetic components. At present microalgae are widely used as one of the most important food sources for different groups of commercially important aquatic organisms in both freshwater and marine aquaculture (Duerr et al., 1998). Algae can be used as human food supplements and pharmaceuticals (Pulz and Grass, 2004; Apt et al., 1999) and have been suggested as a very good candidate for fuel production (Shenk, 2008).

One of the microalgae that have been found with good nutritional properties are the green algae, *Chlorella sp*. (Natrah et al., 2000; Goh Jr. et al., 2009; Goh et al., 2010). Like other plants, Chlorella requires a few things to grow and multiply: sunlight, water, carbon dioxide and nutrients. Once all of these requirements have been met, Chlorella will make use of the

photosynthetic process to rapidly multiply. To guarantee high rates of growth in Chlorella operations, providing the algae with basic nutrients are needed. Algae needs nitrogen, phosphorus and potassium for proper cell development and function, wherein, all these nutrients are already present in substantial amount in seawater and it was also found out by Figueredo and Giani, (2005), that the presence of fish (tilapia) increases the availability of nitrogen and phosphorus which can lead to the growth of microalgae. Many species of Chlorella have the ability to grow photosynthetically or in the absence of light by taking up organic materials directly from the medium (Shah et al., 2003).

In pond culture, Chlorella spp. can be a high quality food organism that can be used to produce high quality fish or fry with lower investment cost of production. Also, the presence of *Chlorella spp.* in shrimp culture can control luminous bacteria (*Vibrio harveyi*) that can solve or reduce losses in shrimp production. It is also used as ingredients for some pharmaceutical high quality products such as cosmetics and vitamins.

Thus, this study was conducted to determine the growth performance of *Chlorella spp*. in different water conditions using seawater, sterilized seawater, and tilapia pond water as culture media which may contain nutrient materials that can support the growth of *Chlorella spp.* for various uses.

MATERIALS AND METHODS

Materials

The materials used in the study were: Chlorella starter culture, empty glass bottle with at least 750 – 1000 ml capacity, air pumps (aerators), pipette, vial, microscope, haemacytometer, refractometer, pH meter, thermometer, hand tally counter, filter bag, fertilizer and log/record book.

Preparation of Culture Vessel

Empty glass bottles with 750 - 1000 ml capacity were used as culture vessel. The bottles were cleaned with soap and zonrox in washing, and sterilized to avoid bacteria that can hinder and compete the growth of *Chlorella spp.* during culture. Each culture vessel was provided with one unit air pump to prevent the stratification of cells, provide gas and heat transfer, light intermittency, dispersion of dissolved materials and inhibition of the adherence of cells to the walls of the culture vessels. The experimental set-up was provided with fluorescent lamp for illumination purposes and much more for light requirement of *Chlorella* for growth and multiplication.

Experimental Set-up

This study used the experimental method with three treatments and three replicates, in a Randomized Complete Block Design (RCBD). The experimental design of the study is shown in

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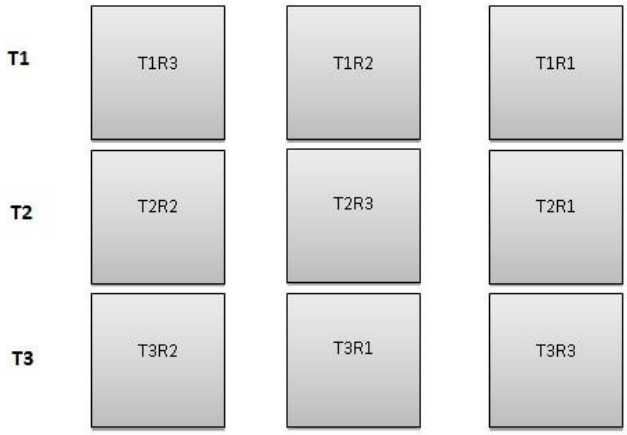
Figure 1.



Legend: T- Treatment; R- Replicates

Figure 1.The Experimental Design of the study

Preparation of Culture Media

The culture medium for *Chlorella* was prepared as follows:

Treatment I (T1), sterilized seawater was used. The seawater source was taken at the Carmen cove approximately 20 meters away from the study site. The seawater was sterilized by boiling for 30 minutes at 100˚C to kill the microorganisms present. After boiling, the water was allowed to cool at room temperature before inoculated with *Chlorella* cells.

Treatment II (T2,) unsterilized seawater was used. The seawater source used for this treatment was at the Carmen cove approximately 20 meters away from the study site.

Treatment III (T3,) tilapia pond water was obtained from tilapia pond with existing tilapia culture. The water was filtered using filter bag so that it will be free from filth or debris before using in the experiment.

All the culture media were fertilized with inorganic fertilizers like Ammonium, Phosphate, and Urea using the common concentration applied in rearing a hatchery, as shown in Table 1.

Table 1. Fertilizers applied in Chlorella *spp.* culture media

Fertilizers	Concentration (ppm)	Water volume (ml)	Quantity (mg)
Ammonium	20	700	14
Phosphate	10	700	7
Urea	10	700	7

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Collection of Inoculum

Chlorella spp. cells used in the experiment were taken from Oversea Hatchery located at Bakay, Tulay, Minglanilla and Cebu. Transportation of Chlorella was done after it was placed in a plastic bag in box provided with ice. Upon reaching the study site, it was placed in a clean styrofoam provided with aerator. Then, Chlorella cells were counted using haemacytometer to have an approximately the same number of cells for inoculation for seeding purposes.

Inoculation of Chlorella Cells

Inoculation of Chlorella was done early in the morning. The three treatments were inoculated with a desired initial density of 1,500,000 cells/ml in each medium using the formula adopted by the Oversea Hatchery in Minglanilla, Cebu in inoculating Chlorella to the different culture media.

Monitoring the Parameters of the Culture Media

Monitoring of the water parameters such as temperature, salinity, and pH were done twice a day at 8:00 A.M, and 3:00 P.M using thermometer, refractometer, and pH meter respectively. The data were recorded accordingly.

Sampling

Sampling was done daily to monitor the population density of Chlorella throughout the duration of the experiment. During sampling, pipette, vial, haemocytometer, and microscope were used. Using pipette, 1 ml sample in each treatment replicates were collected and placed into vials. The vials containing the sample were placed in a refrigerator in order for the Chlorella cells to stop multiplying before these were counted. The one (1) ml sample from every bottle was placed into the haemacytometer evenly allowing the cells to settle for 5 minutes (Baptist, 1993). Then, the haemacytometer containing the sample was placed under the microscope for and calculated using the formula by Guillard, (1973).

Treatment of Data

The data gathered was treated using ANOVA at 5% level of significance, and was further tested with DMRT if there is significant difference of their treatment means of the samples.

RESULTS AND DISCUSSION

The study include the culture of *Chlorella spp*. using different culture media such as sterilized seawater as Treatment 1(T1); unsterilized seawater as Treatment 2(T2); and tilapia pond water as Treatment 3(T3) as substrate.

Growth Rate of Chlorella spp. in different Culture Media

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The daily growth rate of *Chlorella spp*. in different substrates in terms of their cell density (millions) per ml are shown in Table 2.

Table 2. Cell density (millions) per ml of *Chlorella spp.* in 20 days culture period

Sampling (Day)	Treatments Tre at m e nt 1	Tre at m e nt 2	Tre at m e nt 3	Ave rage St d. Error
0	1.5	1.5	1.6	1.5	0.1
1	1.7	2.0	2.0	1.9	0.2
2	2.2	3.3	3.3	2.9	0.7
3	3.1	4.6	4.4	4.0	0.8
4	4.9	6.6	6.9	6.1	1.1
5	6.5	10.1	10.5	9.1	2.2
6	9.9	13.3	13.4	12.2	2.0
7	14.3	15.6	16.5	15.5	1.1
8	17.0	17.2	20.1	18.1	1.8
9	19.4	17.7	20.2	19.1	1.3
10	20.7	16.8	19.6	19.0	2.0
11	18.1	14.1	22.0	18.1	3.9
12	19.1	16.3	21.6	19.0	2.6
13	20.8	20.9	21.0	20.9	0.1
14	21.4	18.3	16.8	18.8	2.4
15	21.7	14.3	17.8	17.9	3.7
16	24.0	14.2	17.7	18.6	5.0
17	19.6	12.4	14.7	15.6	3.7
18	19.0	15.7	23.4	19.4	3.8
19	20.9	10.4	20.7	17.3	6.0
20	18.2	8.7	20.8	15.9	6.4
Total	**302.5**	**252.8**	**313.3**	**289.5**	**32.3**
Mean	**15.1**	**12.6**	**15.7**	**14.5**	**1.6**

Legend: T1=Sterilized seawater T2=Unsterilized seawater T3=Tilapia pond water

As shown in Table 2, cultured *Chlorella spp.* had an average of 1.5 million cells per ml during inoculation (Day 0) and increase in number of cells throughout the culture period was observed. Tilapia pond water (T3) got the highest mean cell density of 15.7cells/ml among the three treatments in the 20 days culture. Although all the culture media were applied with fertilizers in equal amount (ammonium, urea, phosphate), however, this higher result was attributed to the available nutrients from the tilapia waste that were available in T3 compared with the two other treatments. Based on the study of Figueredo and Giani, (2005) the presence of fish in the pond water increases the nitrogen, and phosphorus which can lead to the favorable growth of microalgae. In sterilized seawater (T1), it had produced a cell density of 15.1million cells/ml which is only a little bit lower compared to Tilapia pond water (T3) due to the absence of contaminants that compete the microalgae in assimilating the nutrients applied in the culture medium. Sterilized seawater (T1) had undergone sterilization process which ensures total inactivation of microbial life and prevents contamination of unwanted organisms and desirable chemicals. While non-sterilized seawater got the lowest number of cells at the end of the experiment due to the presence of contaminants that compete in assimilating the nutrients needed for the cultured algae.

The cultured algae during the 20 days culture period had undergone the different growth

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pattern. Growth patterns of Chlorella spp. in different culture media are presented in Figure 2.

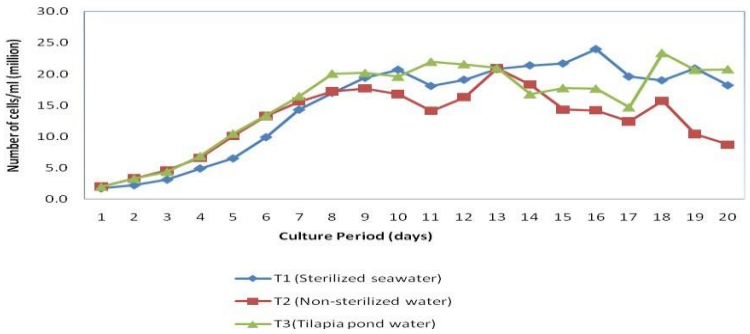

Figure 2. Growth Patterns of *Chlorella spp*. in Different Culture Media

The growth patterns reflected in Figure 2 shows that all the three treatments exhibit growth and had reached the declining phase at the end of the experiment. The growth of Chlorella was already increasing even at lag phase. This implies the instant adoption of the cells with the nutrients available in the medium as observed in the growth pattern (Figure 2). According to Spencer, (1954) the length of the lag phase is least when the inoculum is in its exponential phase. This phase readily occurs when the cells were able to adjust to the medium. In sterilized seawater (T1) exponential growth phase was found from 2nd to 10th day, whereas the non-sterilized seawater (T2) and tilapia pond water (T3) were found from 2nd to 9th day of culture. The stationary growth phase of sterilized seawater (T1) was extended until 19th day. This could be due to the absence of nutrient competitors like zooplanktons that might cause the cultured algae to decline its growth. In contrast, the other two treatments (T2 and T3) had an earlier stationary phase reaching only on the 18th day. During the stationary growth phase of the three treatments, non-sterilized seawater (T2) and Tilapia pond water (T3) show great fluctuation on their daily growth rate compared to sterilized seawater (T1).Fluctuation on their daily growth rate of all the treatments could have been due to nutrient depletion, decreasing light penetration due to overcrowding of cells, and water parameters changes such as salinity and pH. According to Adenan et al., 2013, the optimum salinity and temperature of a growing microalgae are 25ppt and 25 ^{o}C, respectively. The pH should be at the range between 6.5-7.0 (Wang, 2010).While in the study, the pH became acidic, as shown in Table 3.Microalgae is highly dependent on the environmental conditions like salinity, pH, temperature, light as well as nutrition (Banerjee et al., 2011; Liang et al., 2009; Jiand Sherrel, 2008).The average water parameters of *Chlorella spp.* in the three culture medium are shown in (Table 3).

Table 3. Average salinity (ppt), temperature (˚C), and pH of the culture

Water parameters	Treatment 1	Treatment 2	Treatment 3
Salinity (ppt)	36.5	30.7	23.5
Temperature(°C)	27.9	27.8	27.7
pH	5.5	5.4	5.5

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Based on FAO, (1996) to obtain dynamic growth, the optimum temperature is generally 20°C-24°C but most common cultured species can tolerate up to 27°C and the optimum water pH in most cultured algal species ranges between 7 and 9.Therefore, the growth of *Chlorella spp.* might have been affected with the water salinity and pH but not in temperature.

Culture medium that shows the highest *Chlorella spp*. cells produced

Results of the study showed the culture medium that has the highest number of *Chlorella spp.* cells within the 20 day culture period as shown in Table 2 and Figure 2. Data obtained were treated statistically using Analysis of Variance (ANOVA) at 5% level. Result showed that there is significant difference among the three treatments (Fc=7.09; Ft= 3.3). As further examined using Duncan's Multiple Range Test (DMRT), result showed that T1 (sterilized seawater) and T3 (tilapia pond water) have no significant difference from each other. While T2 (non-sterilized seawater) is significantly different from T1 and T3.The similarity of the results between T1and T3 can be attributed to the effect of sterilization in the former which removed the available contaminant that may compete with the nutrients for growth and reproduction. Sterilization prevents contamination of unwanted organisms and eliminates undesirable chemicals (Probert and Klaas,1999) that may affect growth of the cells. Whereas the latter (T3) which is the Tilapia pond water, produces high cell density despite the presence of contaminants in the water. The higher growth of Chlorella cells in T3 is attributed to the amount of nutrients available due to the tilapia culture waste as add on fertilizers to medium. As stated by Figueredo and Giani, (2005), the presence of fish (tilapia) increases the availability of nitrogen and phosphorus which can enhance the growth of microalgae. Moreover, the presence of a wide variety of nitrogen sources, such as ammonia, nitrate, nitrite and urea can be good nutrients used for growing microalgae (Becker, 1994).

CONCLUSION

The study concluded that *Chlorella spp.* showed a higher growth in culture medium that used the water direct from tilapia culture pond. The best growth could have been due to the additional nutrients from tilapia culture that gives additional fertilizers to boast *Chlorella spp.* production. Culturing *Chlorella spp.* in tilapia pond is an additional productivity of the fish pond other than just the culture of tilapia.

RECOMMENDATION

Based on findings, it is recommended that the optimum growth parameters for the algal requirements should be provided within the lag phase to ensure the maximum production of Chlorella cells until the stationary phase.

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