

Preliminary Culture Studies of Microalgae Isolated from the Freshwater Tributaries of Carigara bay, Leyte, Philippines

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ABSTRACT

Phytoplankton is the base or the bottom of the aquatic food chain and serve as primary producers. Meanwhile, Carigara bay is one of the most successful fishing lands in the Philippines but there are still scarcity of literatures regarding the bay, connected freshwater resources and biological components of the aquatic environment. Therefore, this study was explored. Sampling was conducted on the 5 coastal towns surrounding the bay. Water samples from the stream, rivers and falls were collected using a 2.5-3L Plexi glass sampler, transferred in 3 1L cap bottle and brought in the laboratory for isolation of phytoplankton. Isolation was done using Binangonan Research Station Pantastico or BRSP medium. Preliminary culture studies were conducted to selected phytoplankton namely Genus *Anabaena*, *Oscillatoria*, *Asterococcus* and *Chlorococcum*. The 4 phytoplankton were preliminary optimized in terms of light intensities, pH and temperature. Cell density in every 2 days was gathered through haemocytometer method and specific growth rate was computed. Mean and standard deviations were used to summarize the data in optimized results for the light intensities, temperature and pH desired by the isolated phytoplankton and data were further subjected to Analysis of Variance (ANOVA). The 4 phytoplankton grow best at 3000 lux, pH 7.27 and 25 °C according to the optimization procedure conducted. It can be also noted that the optimization tests is important to sustain the mass production of phytoplankton.

Key words: Initial, Isolation, Microalgae, Optimization, Tributaries.

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INTRODUCTION

Microalgae or commonly known as phytoplankton, the main focus of this study are polyphyletic assemblage of organisms that are the oldest forms of life on Earth and considered as tiniest plant in the archipelago with sizes ranging from few to many micrometers.^[1-3] From its biological diversity to industrial importance, phytoplankton are now gaining much attention for feed, food and other important products in various fields and

industries yet they are thought of as the most poorly studied group of aquatic organisms.^[4] Noteworthy, some of the organisms which exist already in the commercial market are *Spirulina*, *Chlorella*, *Haematococcus* and *Chaetoceros*.^[4-7] Because of this, industrial microalga is a field that needs to be explored primarily to produce promising high-valued chemicals for nutraceuticals, functional food and living feed and other feed additives.^[8] Additionally, phytoplankton were visualized as the “food for the future” because of its many applications.^[9-11] Historically, the first micro algal organism to be commercialized for food industry was the *Nostoc* sp. over many decades and consumed in China, Taiwan, Japan and other Southeast Asian nation.^[4,12] Carigara Bay, as one of the unexplored areas in the Philippines being the site of the study, is located in the Province of Leyte where it surrounds 5 coastal towns.

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Freshwater tributaries such as streams and rivers of the Carigara bay are present keeping the marine life moist in condition over many months Figure 1.^[13] The bay itself and its freshwater resources are abounding with diversity of organisms but poorly studied as shown by dearth of reports. In fact, the study on the assessment of water quality and initial identification of zooplankton and phytoplankton was solely the existing biological report about Carigara bay.^[14] Noticeably, too, are current publications stating that most of the micro algal cultivation is sold in the market for animal food and pharmaceutical products and the algal biomass is starting to have a high rate of demand for aquaculture industry including fish feed providing an initiative revenue for algae industry even microalgae cultivation is only a few decades old.^[15,16]

Objectives of the Study

Therefore, the objective of this study is to isolate and culture phytoplankton from the freshwater tributaries in terms of light intensity, temperature and pH tests preliminarily which can be used for large-scale production.

MATERIALS AND METHODS

Sampling sites

Fourteen stations were established at the upper coastal towns adjoining the Carigara Bay. Station 1 was the Carigara Bay at Brgy. Libertad (Capoocan) (11°39'N 124°53'E); Station 2 was a stream at Brgy. Libertad (Capoocan) (11°37'N 124°52'E); Station 3 was the Carigara Bay at Brgy. Visoria (Carigara) (11°30'N 124°68'E); Station 4 was the Lindog River at Brgy. Uyawan (Carigara) (11°27'N 124°66'E); Station 5 was the Bislig River at Brgy Bislig (Carigara) (11°29'N 124°67'E); Station 6 was the Carigara Bay at Brgy. Duka (Barugo) (11°36'N 124°78'E); Station 7 was the Himanglos River at Brgy. Hilaba (Barugo) (11°31'N 124°73'E); Station 8 was the Canomantag River at Brgy. Canomantag (Barugo) (11°30'N 124°71'E); Station 9 was the Carigara Bay at Brgy. Mawod-pawod (San Miguel) (11°19'N 124°51'E); Station 10 was the Caraycaray River at Brgy. Caraycaray (San Miguel) (11°20'N 124°52'E); Station 11 was the Lipasan Falls at Brgy. Pinarigusan (San Miguel) (11°21'N 124°53'E); Station 12 was the Carigara Bay at Brgy. Kalangawan Guti (Babatngon) (11°23'N 124°51'E); Station 13 was the Tula-an Falls at Brgy. Tula-an (Babatngon) (11°24'N 124°52'E); and Station 14 was the Busay Falls at Brgy. Busay (Babatngon) (11°25'N 124°53'E).

Collection of water samples

The water samples were collected on July 2-8, 2018 which was considered as the 1st sampling and on November 23-26, 2018 which was the 2nd sampling. Collection of samples from the freshwater tributaries of the bay, the water samples were collected in integrated manner, thus, from surface, middle and approximately 0.5 meter away from the bottom. Most have depths ranging from 0.75-2m. Depths were measured using the plexi glass sampler attached to a rope with measurement in meters and/or the secchi disk. The 3L water samples from the river/falls were apportioned for the different analyses and this study needed one 1L as a live sample for the isolation and culture studies of phytoplankton.

Identification of Phytoplankton

In order to quantify and identify, isolate and separate phytoplankton, standard protocol on washing and plating techniques were done.^[17,18] For qualitative determination of phytoplankton, several references on algal taxonomy and biodiversity were used such as the following: Taxonomy and Ecology of Algae in Fishponds and Fish pens of Laguna and some Physiological Studies of *Navicula accomoda* Hust.^[17] Taxonomy of the Freshwater Algae of Laguna de Bay and Vicinity;^[19] Illustrations of the Freshwater Plankton of Japan;^[20] Algae of the Western Great Lakes Area;^[21] and How to Know the Freshwater Algae.^[22]

Isolation of Phytoplankton

For the isolation, BRSP medium (Binangonan Research Station Pantastico medium) was used. After growth colonies and establishment of uni-algal cultures, the isolates were maintained in each test tubes containing 10 ml of BRSP media at a temperature of $25 \pm 2^\circ\text{C}$, pH of 7.27 and light intensity of 3,000lx.

Preliminary Culture Studies

Optimization tests were conducted for the 4 selected phytoplankton namely Genera *Asterococcus*, *Chlorococcum*, *Anabaena* and *Osillatoria*. For the light intensity test, the growth of the selected micro algal species was tested at different light intensities: 1500lx, 3000lx, 6000lx and 9000lx. The temperature was kept constant at $25 \pm 2^\circ\text{C}$ and pH of the BRSP medium at 7.27. For the temperature test, 4 different temperatures, 20, 30, 35°C and room temperature, $25 \pm 2^\circ\text{C}$ were assessed for the different selected micro algal species. For the pH test, the pH was manipulated by adding concentrated 11.00M HCl or 6.00M NaOH. pH ranges of 7, 7.27, 8 and 9 were managed to observe the optimum pH of the algal culture. The replicates of cultures were kept

in the known optimum light intensity obtained from the preceding experiment and room temperature of $25 \pm 2^\circ\text{C}$. For each planktonic species isolated, three replicates were made for the experimental set-up; wherein 100ml of BRSP liquid medium was inoculated with 1ml of the uni-algal species.

Evaluation of Algal Growth

A 0.5ml of sample from the different treatments was taken and preserved with 0.1ml Lugol's solution. This was done every 2 days for the duration of the culture period until growth of cells was observed to decline. Cell density (cells/ml) was determined using a Neubauer Germany Hemacytometer. Cells were counted under a Compound light microscope (CH₂O Olympus) and specific growth rate was computed.^[23] The data were subjected to Analysis of Variance (ANOVA).

RESULTS

Microalgae were isolated in all the freshwater stations established in the sampling sites. Most of the isolates are under the Phylum Chlorophyta and Cyanophyta and only 2 genera for Phylum Bacillariophyta. Six (6) isolated phytoplankton were screened for their large-scale potential but only 4 survived. The chosen 4 phytoplankton belong to only two (2) phyla namely Chlorophyta commonly known as green algae and Cyanophyta commonly known as blue-green algae. Bacillariophyta (diatoms) and Dinophyta (brown algae) were not considered in the study because they are known for their difficulty to be cultured in the laboratory and maintain their growth conditions in a short period of time. The 4 phytoplankton are as follows:

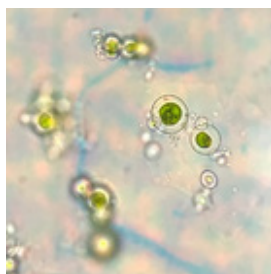


Plate 1: Genus *Asterococcus*.

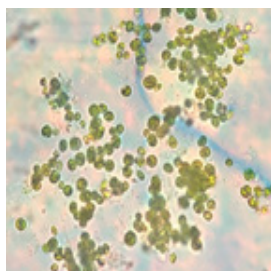


Plate 2: Genus *Chlorococcum*.

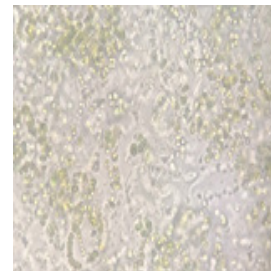


Plate 3: Genus *Anabaena*.

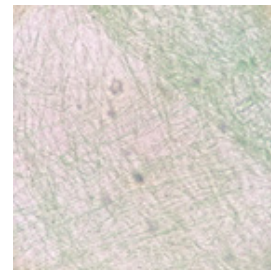


Plate 4: Genus *Oscillatoria*.

These organisms were subjected to preliminary culture studies as presented in the following figures. The phytoplankton were optimized by light intensity, temperature and pH tests. Means and its standard error (SEM) were used to summarize all the gathered data from the experiment. Two-factor analysis of variance (2-way ANOVA) was used to determine the effect of different conditions on the specific growth rate of different phytoplankton (*Asterococcus*, *Chlorococcum*, *Anabaena* and *Oscillatoria*). Figure 2 shows the specific growth rate of phytoplankton under various light intensities. All of the 4 organisms grow best in 3000lux.

Statistical analyses were conducted to determine the effect of different light intensities (1500, 3000, 6000 and 9000 lux) on the specific growth rate of different microalgae (*Asterococcus*, *Chlorococcum*, *Anabaena* and *Oscillatoria*). Table 1 explains that the three highest specific growth rates happened to *Asterococcus* when it was subjected to 3000 and 6000 lux of light intensity and *Chlorococcum* when subjected to 3000 lux of light intensity, followed by *Asterococcus* microalgae when subjected to 1500 lux of light intensity. All the rest have lower specific growth rates. Moreover, Table 2 noted that there was a significant interaction between microalgae and light intensity ($F = 2.269$, $p=0.043$), indicating that the specific growth rate of the different types of microalgae depend on light intensity.

Furthermore, Figure 3 summarizes the specific growth rate of the 4 microalgae under various temperatures. The figure displays that most of the phytoplankton have the highest cell density at 25°C and lowest at 35°C .

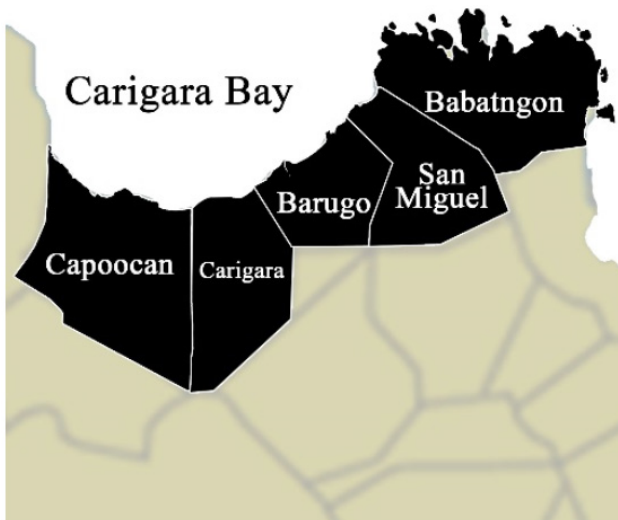


Figure 1: Sampling stations at 5 coastal towns surrounding the Carigara bay and its freshwater resources.

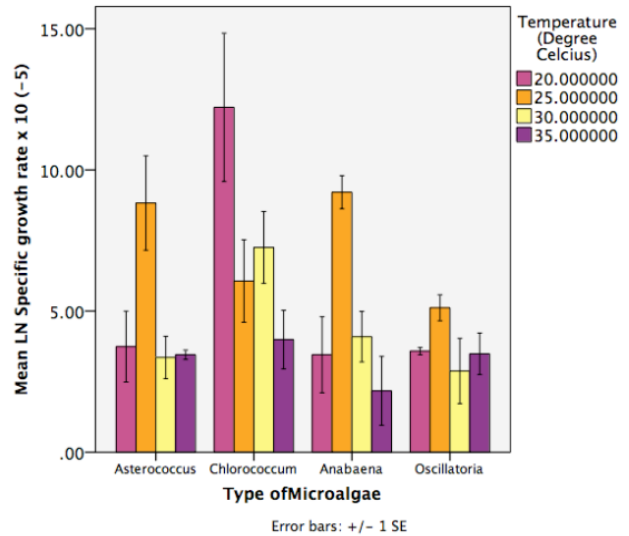


Figure 3: Specific Growth Rate of Microalgae under various temperatures.

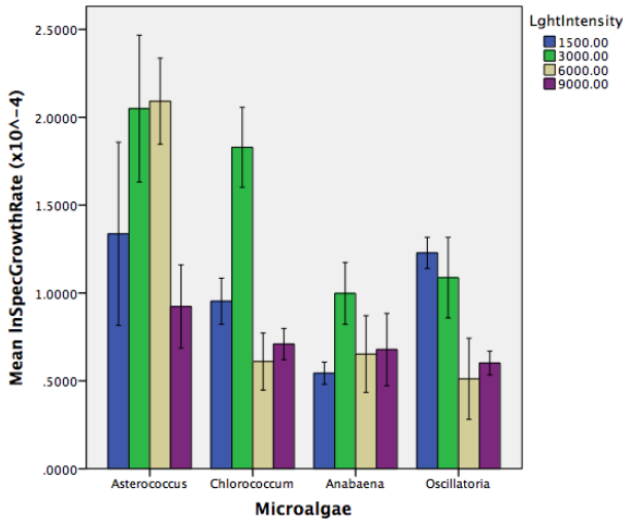


Figure 2: Specific Growth Rate of Microalgae under various light Intensities.

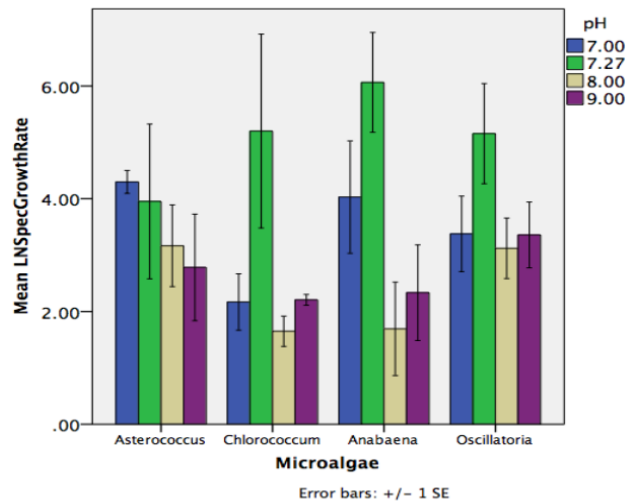


Figure 4: Specific Growth Rate of Microalgae under various pH.

Statistical analyses were conducted to determine the effect of temperature (20, 30, 35 and 25 degree Celsius) on the specific growth rate of different microalgae (*Asterococcus*, *Chlorococcum*, *Anabaena* and *Oscillatoria*). Table 3 shows that the highest specific growth rates happened to *Chlorococcum* microalgae when it was subjected to 20°C of temperature (mean = 12.22 ± 2.63 °C). *Anabaena* and *Asterococcus* microalgae (both at 25 degrees) come second and third with mean = 9.21 ± 0.59 °C and 8.83 ± 1.67 °C, respectively. *Anabaena* at 35°C showed the lowest specific growth rate (2.17 ± 1.22 °C). Furthermore, Table 4 explains that the two-factor analysis of variance showed that there was a significant interaction between microalgae and temperature (F

= 4.045, $p=0.002$), indicating that the specific growth rate of the different types of microalgae depends on temperature.

Lastly, Figure 4 summarizes the specific growth rate of microalgae at various pH. The figure concludes that the microalgae had the highest cell density at pH 7.0.

Statistical analyses were conducted to determine the effect of pH (7, 7.27, 8 and 9) on the specific growth rate of different microalgae (*Asterococcus*, *Chlorococcum*, *Anabaena* and *Oscillatoria*). Table 5 demonstrates that the highest specific growth rates happened to *Anabaena* microalgae at a pH of 7.27 (mean = 6.06 ± 0.89) while *Chlorococcum* at pH = 8 showed the lowest specific growth rate (1.65 ± 0.29 °C). Furthermore, Table 6 shows that

Table 1: Statistical Analysis on the Specific Growth Rate of Microalgae ($\times 10^{-4}$) under various light intensities.

| Microalgae | Light Intensity (lux) | | | |
|---------------------|-----------------------|-----------------|-----------------|-----------------|
| | 1500 | 3000 | 6000 | 9000 |
| <i>Asterococcus</i> | 1.34 \pm 0.52 | 2.05 \pm 0.42 | 2.09 \pm 0.24 | 0.92 \pm 0.24 |
| <i>Chlorococcum</i> | 0.95 \pm 0.13 | 1.83 \pm 0.23 | 0.61 \pm 0.16 | 0.71 \pm 0.09 |
| <i>Anabaena</i> | 0.54 \pm 0.06 | 1.00 \pm 0.18 | 0.65 \pm 0.22 | 0.68 \pm 0.21 |
| <i>Oscillatoria</i> | 1.23 \pm 0.09 | 1.09 \pm 0.23 | 0.51 \pm 0.23 | 0.60 \pm 0.07 |

Values expressed as mean \pm SEM, n = 3.

Table 2: Two-factor Analysis of Variance for the Specific Growth Rate of Microalgae under various light intensities.

| Source of Variation | F stat | p - value |
|----------------------------|--------|-----------|
| Microalgae | 10.583 | < 0.001 |
| Light Intensity | 7.194 | 0.001 |
| Microalgae*Light Intensity | 2.269 | 0.043 |

Table 3: Specific Growth Rate of Microalgae ($\times 10^{-5}$) under various temperatures.

| Microalgae | Temperature ($^{\circ}$ C) | | | |
|---------------------|-----------------------------|-----------------|-----------------|-----------------|
| | 20 | 25 | 30 | 35 |
| <i>Asterococcus</i> | 3.74 \pm 1.25 | 8.83 \pm 1.67 | 3.36 \pm 0.75 | 3.45 \pm 0.17 |
| <i>Chlorococcum</i> | 12.22 \pm 2.63 | 6.06 \pm 1.46 | 7.25 \pm 1.27 | 3.99 \pm 1.04 |
| <i>Anabaena</i> | 3.45 \pm 1.35 | 9.21 \pm 0.59 | 4.09 \pm 0.90 | 2.17 \pm 1.22 |
| <i>Oscillatoria</i> | 3.58 \pm 0.13 | 5.12 \pm 0.46 | 2.88 \pm 1.16 | 3.49 \pm 0.74 |

Values expressed as mean \pm SEM, n = 3.

Table 4: Two-factor Analysis of Variance for the Specific Growth Rate of Microalgae under various temperatures.

| Source of Variation | F stat | p - value |
|------------------------|--------|-----------|
| Microalgae | 6.567 | 0.001 |
| Temperature | 8.327 | <0.01 |
| Microalgae*Temperature | 4.045 | 0.002 |

Table 5: Specific Growth Rate of Microalgae ($\times 10^{-5}$) under various pH.

| Microalgae | pH | | | |
|---------------------|-----------------|-----------------|-----------------|-----------------|
| | 7 | 7.27 | 8 | 9 |
| <i>Asterococcus</i> | 4.30 \pm 0.20 | 3.95 \pm 1.37 | 3.17 \pm 0.72 | 2.78 \pm 0.94 |
| <i>Chlorococcum</i> | 2.17 \pm 0.50 | 5.20 \pm 1.72 | 1.65 \pm 0.29 | 2.21 \pm 0.10 |
| <i>Anabaena</i> | 4.03 \pm 1.00 | 6.06 \pm 0.89 | 1.69 \pm 0.83 | 2.33 \pm 0.85 |
| <i>Oscillatoria</i> | 3.38 \pm 0.67 | 5.16 \pm 0.89 | 3.12 \pm 0.54 | 3.36 \pm 0.58 |

Values expressed as mean \pm SEM, n = 3.

Table 6: Two-factor Analysis of Variance for the Specific Growth Rate of Microalgae under various pH.

| Source of Variation | F stat | p - value |
|---------------------|--------|-----------|
| Microalgae | 0.943 | 0.432 |
| pH | 8.016 | <0.01 |
| Microalgae*pH | 0.895 | 0.541 |

according to the two-factor analysis of variance, there was no significant interaction between microalgae and pH ($F = 0.895$, $p=0.541$), indicating that the specific growth rate of the different types of microalgae does not depend on pH. This means that the different types of microalgae will grow regardless of the value of pH of the water they are in.

DISCUSSION

Phytoplankton are gaining success as source of many industrially important applications because of its amazing growth rate, no competition at all, less land cover, minimal disposal and the ability to grow very fast and easy.^[24] Thus, microalgae feedstock is necessary to optimize its culture conditions for maximum production. The good thing for phytoplankton is that it has the capacity to manipulate its growth rate despite the various changes in its physiological aspects such as light intensities, pH and temperature.^[25] Light intensity plays an important growth parameter for micro algal cells. It stimulates the photo-oxidation process in phytoplankton which contributes to its growth and development. In general, the green algae reach its optimum growth rate at light intensity of 15 to 150 μmol of photons $\text{m}^{-2}\text{s}^{-1}$ or equivalent to 1,000-10,000 lux.^[25,26] As such, *Spirulina platensis* was observed to have an optimum light intensity at 3,500 lux. The experiment lasted for 25 days and showed significant difference in growth pattern where maximum biomass concentration (as dry weight) is 0.73 $\text{g}\cdot\text{L}^{-1}$.^[27] In contrast, *Anabaena ambigua* was noted to have best growth performance when lighted with 7,000 lux significantly at the 10th day compared to 1,500 lux and 4,000 lux. The observation ended at day 18.^[28] In addition, *Nannochloropsis oculata* showed that at 5,000 lux, the organism achieved the highest specific growth rate of 0.236 day⁻¹ with the shortest generation time of 2.9 days. This was followed by 3,000 and 7,000 lux which produced lower growth rate. The longer lag phase at 7,000 lux was due to the culture's difficulty to adapt on the high light intensity at low culture density as well.^[29] On the other hand, temperature is also one of the most important conditions in culturing phytoplankton. *Nannochloropsis salina* cultures grown in Erlenmeyer flasks reached their maximum specific growth rates at 26°C, with no growth seen above 35°C.^[30] Specifically, the optimal tested growth temperature was 26.3°C with the specific growth rate declining to less than 30% at 32.5°C. Green algae species such as *Chlorella*, *Spirogyra*, *Chlamydomonas*, *Botryococcus*, *Scenedesmus*, *Neochloris*, *Haematococcus*, *Nannochloropsis* were observed to have an optimum temperature ranging from 20-30°C.^[31] In

specific, *Chlorella* and *Scenedesmus* were evaluated for the best temperature suited to their growth and development and found out that the optimum temperature ranged from 25-27.5°C.^[32] Also, *Spirulina platensis* was experimented for the optimum light intensity and temperature conditions where *S. platensis* grew and performed well in temperature ranging from 20-35°C. The phytoplankton died after 25 days of experimentation. At 40°C, the said microalgae did not show any exponential growth and no pigmentation at all which means there is no support of growth to the organism. Furthermore, the chlorophyll, lipid and protein contents of the said organism drop down.^[27,33] Moreover, 35°C optimum condition in temperature was evaluated to some phytoplankton namely *Synechococcus* sp., *Arthrocnemum africanum*, *Anabaena ambigua* and *Nostoc muscorum*.^[28] Furthermore, *Chaetoceros calcitrans* and *Chlorella* sp. were investigated for their optimum salinity and temperature where these 2 organisms are its best at 25-30°C with maximum cell growth at 10th day of 2.1×10^6 cells/ml significantly at 5% and 25°C with maximum cell growth at day 10 of 2.9×10^6 cells/ml but not significant at all respectively. *Chlorella* sp. was studied alone for its optimum temperature of 25°C.^[34] Recently, *Nannochloropsis gaditana* was optimized for its culture medium and temperature for omega-3 fatty acid production. It was noted that it has the best specific growth rate at 25°C.^[35] Another green algae was studied alone, *Scenedesmus* sp. was evaluated for its optimum growth conditions such as temperature and pH. It was found out that it can tolerate temperature from 25°C up to 30°C.^[36] Moreover, *Spirogyra* sp. *Oedogonium* sp. and *Chlorella* sp. were optimized for their growth conditions namely temperature and pH. The 3 organisms showed the highest algal growth in temperature conditions ranging from 24-28°C.^[37] In addition, *Spirogyra* sp. alone was assessed for its optimum growth conditions namely temperature, pH and salinity. The said phytoplankton grew best at 25°C and showed the highest biomass, lipid and chlorophyll contents at the said temperature.^[38] More recent, cyanophytes such as *Lyngbya* sp. and *Oscillatoria* sp., can withstand the temperature of 23-25°C.^[39] Furthermore, pH are susceptible to varying conditions. Microalgae production must be kept below pH 8 by the addition of carbon dioxide.^[39] Cyanophyte, *Anabaena ambigua* was observed to have the highest growth rate at pH 7 or neutral and noted that as the pH increases, the growth of the said microalgae decreases.^[28] Lately, specific chlorophyte, *Scenedesmus* sp. was perceived for its optimum concentration for pH and found out that it can tolerate from pH 7-8.^[36] Also, *Spirogyra* sp., *Oedogonium* sp. and *Chlorella* sp. were observed to have the best result with pH 7.5.^[37] In specific, *Spirogyra* sp.

was assessed to perform very well in pH 7 or neutral and yielded the highest biomass, lipid and chlorophyll contents.^[38] In addition, *Nannochloropsis salina* showed the highest growth rate at pH 8 and the observation of the microalgae and counting of their cell densities lasted for 21 days. Recently, with respect to blue-green algae, *Lyngbya* sp. and *Oscillatoria* sp. were noted to have an optimum pH at 9.0.^[39] In general, pH 7-8 was found valuable and conducive to increase micro algal production.

CONCLUSION AND RECOMMENDATION

Carigara bay is composed of multi-gear fishery system that makes the water body a successful fishing land in the Philippines. Fourteen (14) stations were established at the upper coastal towns adjoining the Carigara Bay. Isolation was done using Binangonan Research Station Pantastico medium or BRSP medium. Preliminary culture studies were conducted to selected phytoplankton namely *Genus Anabaena*, *Oscillatoria*, *Asterococcus* and *Chlorococcum*. The 4 microalgae were optimized in terms of light intensities, pH and temperature. Cell density in every two (2) days was gathered through haemocytometer method, specific growth rates was computed and data were further subjected to Analysis of Variance (ANOVA). The 4 phytoplankton grow best at 3000 lux, pH 7.27 and 25°C according to the optimization procedure conducted. It can be also noted that the optimization tests can be used as a guide for mass production of these organisms.

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CONFLICT OF INTEREST

Declaring no conflict of Interest.

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