Determination of the Growth Curve of *Chlorella* sp. under Optimum Conditions in Automated Growth Chambers for Biofuel Production

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ABSTRACT

Microalgae have a great potential for producing biofuels because of their capacity to accumulate high content of lipids. *Chlorella* sp. is the mostly applied microalgae sp. for biofuel production at the industrial level. The current study aimed to determine the optimum conditions for mass culture *Chlorella* sp. and to generate a growth curve to determine growth patterns over time. The samples were collected from *Chlorella* pure cultures available in the laboratory. *Chlorella* colonies were identified morphologically. Liquid cultures were prepared in BG 11 medium, The effects of four different temperatures (20°C, 25°C, 30°C, and 35°C), two light intensities (6000 lux and 2000 lux.) under aerated and non-aerated conditions on the growth of *Chlorella* cultures in 250 mL flasks (n=3) were studied using a custom-designed automated growth chamber using Arduino technology. The culture growth was monitored by determining the cell density (cells/ml) and light absorbance (750 nm) at 0, 96, 192, 288, and 384 hours after inoculation. and After 16 days, cells were harvested (6000 rpm, 5 min, room temperature) and the dry biomass (g/ml) was measured after oven drying at 70 °C. Optimal conditions for the efficient mass culture of *Chlorella* sp. were found as 30 °C temperature, 6000 lux light intensity with aeration conditions. Under those optimum growth conditions, 6L photo-bioreactors were designed. Absorbance and cell density (cells/ml) of *Chlorella* sp. were monitored with time to develop the growth curve of *Chlorella* sp. The growth of isolated *Chlorella* sp. was characterized by an exponential phase from 8.86 to 45.41 hr after inoculation with a specific growth rate of 0.06 hr^{-1} and a doubling time of 11.46 hr. The *Chlorella* growth rate was 0.027 hr^{-1} without optimum conditions, and the doubling time (Tg) was 25.6 hr.

KEYWORDS: *Automated Growth Chambers, Biofuel, Chlorella, Growth Curve, Microalgae, Optimum Conditions*

1 INTRODUCTION

Fossil fuels have played a vital role over the last few decades in domestic, transportation and industrial sectors despite they are less eco-friendly and nonrenewable. It has been estimated that fossil fuels will be depleted in less than 50 years and that will greatly affect the global socio-economy. As a result, renewable energy has grown significantly to meet energy demand by rising population and development across the world. Biofuel is a renewable and long-term energy source that can be used to replace fossil fuels. Biofuel is a kind of fuel that originated from plant oils, animal fats, and

https://doi.org/10.54389/GGAY8887

microorganisms. Biofuels are available in both liquid and gaseous forms. Bio-alcohol, bio-diesel, and bio-oil are examples of liquid biofuels.

Microalgae are unicellular photosynthetic microorganisms found in freshwater and marine habitats that have a high lipid content in their cells and the ability to synthesize biofuels. Each microalgae species has unique growing conditions that must be optimized in order to achieve high yields. Growth chambers with diverse conditions can be applied to determine the optimum growth of microorganisms to find their exponential growth.

Microalgae biomass consists of three key biochemical components, carbohydrates, proteins and lipids or natural. Microalgae possess properties such as rapid growth rate with short generation cycles, short harvesting life, efficient photosynthesis, and contain high lipid content in certain species. They can convert nutrients in a medium or wastewater into biomass and Microalgae are utilized to extract high-value bio-products such as lipids, proteins, carbohydrates, pigments, antioxidants, cosmetics, dyes, pharmaceuticals, functional food, food additives and others. They are useful for CO_2 mitigation, wastewater treatment, biofuel and biofertilizer production as well. There are numerous techniques to transform microalgae into biofuel through transesterification of lipids, fermentation of the algal biomass, anaerobic digestion, and thermochemical conversion.

Chlorella sp. is the most cultivated eukaryotic microalgae which belong to the family *Chlorellaceae*. Various *Chlorella* strains have proved to be acceptable for biodiesel synthesis. *Chlorella* sp. is the widely used species for commercial production of Biodiesel industries in a number of countries such as the USA and China.

In Sri Lanka, fossil fuels are used as the major source of energy, especially as the only source of liquid fuel, and their prices directly influence on the country's socio-economy and development. Sri Lanka imported 1,094,586 MT of crude oil from January to December 2021, while other petroleum imports were 2173168.037 MT, for a total of Rs. 287,310,530,708 spent (Central Bank of Sri Lanka, 2022). In 2022, the country is facing a severe economic recession and the importation of petroleum fuel has been identified as a great dependency of the country which affects on the well-being of its population. Therefore, it is imperative to investigate environmentally benign, renewable, and low-cost alternative fuels in order to achieve sustainable socio-economic development of the country. Microalgae species have been isolated and identified in various environments of Sri Lanka (Sandani *et al.*, 2020) and few studies have been conducted to identify the potential of some species including *Chlorella* species for biodiesel production. In this research, the key endeavour was to measure optimum growth conditions and growth curve of *Chlorella* sp. in order to apply for industrial scale cultivation and lipid extraction for biofuel production by using automated growth chambers.

2 METHODOLOGY

2.1 Designing a Novel Growth Chamber for Microalgae Production

A novel Growth Chamber was designed to incubate liquid cultures and culture plates of microalgae and cyanobacteria in 470mm x 395mm x 355mm sealed Rigifoam boxes under controlled conditions of light intensity, temperature and aeration using Arduino technology (will be patented). The boxes were illuminated with 12V white LED light strips, The temperature within the growing chamber was controlled with the installation of a cooling system (Thermoelectric Peltier TEC1-12706 Cooler Kit). Air was supplied with BOYU U-9900 Air Pump with a filter system to prevent contaminations. Light intensity and temperature were continuously monitored using a photometer (Brannan light meter) and temperature sensors (Adafruit DHT11 sensor). The effect of light intensity, temperature and aeration can be measured. The data was received by the Arduino board and transferred into ESP 8266 board. Transferred data were saved in the cloud through the internet (Figure 6).

2.2 Determination of Optimum Light Intensity, Temperature and Aeration

Four identical growth chambers with 20, 25, 30 and 35 °C were constructed as described in the above section. Each chamber was separated in the middle using a Rigifoam partition creating two identical sub-chambers. In the two sub-chambers, two light intensities (6000 lux and 2000 lux) were adjusted and six cultures of *Chlorella* sp. in 250 mL conical flasks were placed. Among these flasks, three flasks

were supplied with filtered air and the rest was kept without aeration. A control was set up by keeping a 250 mL *Chlorella* sp. culture under room temperature, ambient light and non-aeration conditions. Flasks were shaken twice a day. The culture growth was monitored by determining the cell density and cell biomass at 0, 96, 192, 288, and 384 hours after inoculation.

2.3 Determination of Absorbance and Cell Density

The absorbance of the liquid cultures was determined at 750 nm wavelength by using a spectrophotometer (Jenway 6305) at 0, 96, 192, 288, and 384 hours. A calibration curve was developed between the absorbance and cell density using a serial dilution up to 10^{-6}. Cell density was determined using the Neubauer hematocytometer (Marienfeld 0642110) counting method.

2.4 Harvesting and Determination of the Biomass

After 16 days, microalgal biomass was extracted from cultures by centrifuging at 6000 rpm for 5 minutes at room temperature. The cell pellet was separated using a micropipette and dried in an oven at 70 ^{0}C for 3 hours to obtain dry matter.

2.5 Determination of the Growth Curve of *Chlorella*

In a 2 L Conical Flask, 1800 ml of growth medium (BG-11) was prepared and 200 ml of inoculum was added. The culture was kept under optimal growth conditions determined from the previous experiment (30 ^{0}C, 6000 lux, and aerated). A Neubauer hematocytometer was used to assess initial cell density, and a UV/Vis spectrophotometer at 750 nm wavelength was used to assess absorbance (Griffiths *et al.*, 2011). Cell density and absorbance were monitored continuously at appropriate time intervals in triplicates taken from the sample. The growth curve was plotted between the natural logarithm of cell density vs time and the exponential growth period was determined. The specific growth rate (μ) was calculated using the formula,

$$\mu = \frac{lnN_1 - lnN_0}{t_1 - t_0} \quad (1)$$

Where N_1 and N_0 are the numbers of cells at times t_1 and t_0. Doubling time (T_g) was calculated using the following formula,

$$T_g = \times \ln(2) \times \mu^{-1} \quad (2)$$

2.6 Statistical Analysis

Effects of the studied factors on the growth of *Chlorella* sp. were determined using the General Linear Models procedure using SPSS Statistical software (version 26.0). Regression Analysis was conducted to determine the exponential growth curve.

3. RESULTS AND DISCUSSION

3.1 Effect of Light Intensity, Temperature and Aeration on Biomass Growth

Direct counting of cells is a relatively accurate procedure to determine the cell concentration and growth of a microalgae culture. However, it is a time intensive procedure. Turbidimetric methods, e.g., optical density, are very practical and simple methods to apply. Therefore, the cell concentrations were determined using a calibration curve. Aside from that, biomass weight was assessed at the end of the experiment to determine the cell growth of the cultures. The effect of studied factors, temperature, light intensity, and aeration and their interactions were significant on the cell density at different time periods. Table 1 shows the significance of the effects of factors at 384 hr after inoculation. Cell proliferation and metabolite production are both influenced by temperature. According to Carlsson and

Bowles, (2007), the optimal temperature for the growth of *Chlorella sp.* is 30-35 ℃. Figures 2 and 3 indicate the impact of temperature on microalgae cell density and biomass with different light intensities and aeration conditions. Maximum cell density and biomass yield was found at 30 ℃ followed by that at 35 ℃. The results show that cooler temperatures slow down the growth rate of *Chlorella* sp. There was a significant difference between the cell growth under two light intensities i.e., 6000 lux light intensity resulted in higher cell density and biomass than that by 2000 lux. (Figures 2 and 3) According to Cheirsilp and Torpee (2012), light intensity below 5000 lux is appropriate for the growth and biomass production of *Chlorella* sp. When compared with aerated cultures, non-aerated cultures showed significantly lower cell density and biomass.

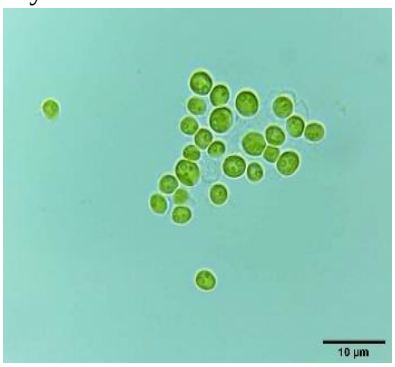

Figure 1. Microscopic view of *Chlorella* sp. (1000x magnification)

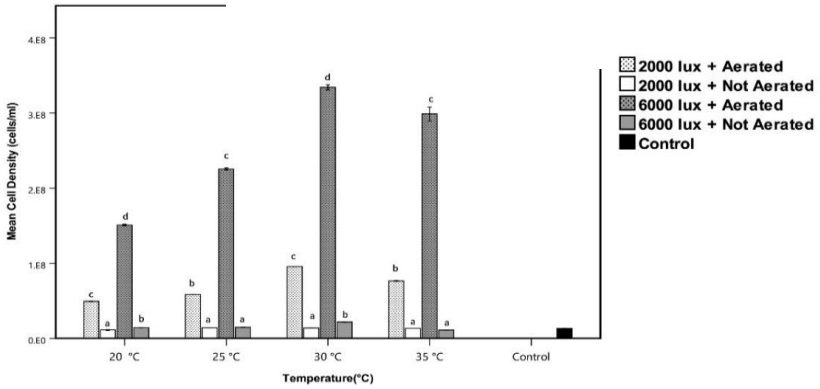

Figure 2. Effect of different temperatures, light intensities and aeration on mean cell density of *Chlorella* sp.

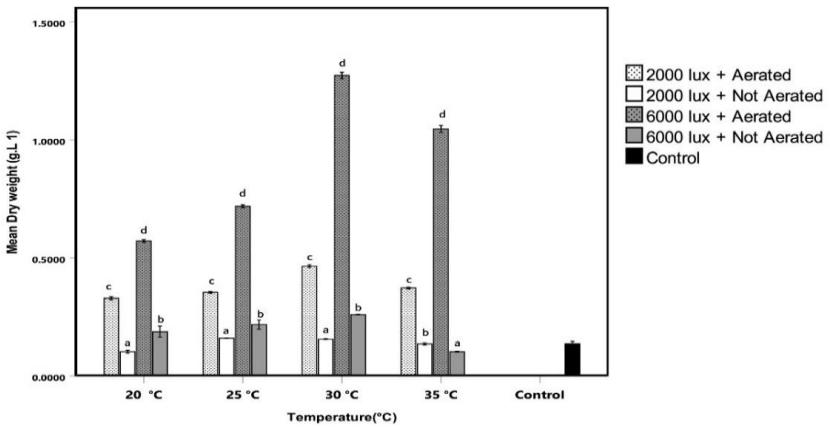

Figure 3. Effect of different temperatures, light intensities and aeration on mean dry weight of *Chlorella* sp.

Table 1. Effects of factors and factor interactions on cell density after 384 hr after inoculation

Source	Type III Sum of Squares	df	Mean Square	p
Temperature (°C)	$2.4x10^{16}$	3	$8.1x10^{15}$	0.000
Light (lux)	$1.0x10^{17}$	1	$1.0x10^{17}$	0.000
Aeration	$2.5x10^{17}$	1	$2.5x10^{17}$	0.000
Temperature * Light	$8.7x10^{15}$	3	$2.9x10^{15}$	0.000
Temperature * Aeration	$2.1x10^{16}$	3	$7.2x10^{15}$	0.000
Light * Aeration	$9.7x10^{16}$	1	$9.7x10^{16}$	0.000
Temperature * Light *Aeration	$8.5x10^{15}$	3	$2.8x10^{15}$	0.000

df- Degrees of freedom, F- F distribution, p- Probability value. Each value is the mean of three replicates

All of the aerated treatments outperformed the control treatment, which was maintained at room temperature and exposed to ambient light without aeration. When compared to aerated treatments, non-aerated treatments showed lower cell density and biomass. Aside from the 384-hour time interval, there were statistically significant differences between the conditions at the 96-hour, 192-hour, and 288-hour time intervals (data not shown). Figures 2 and 3 indicate statistically significant differences between treatments, with the aerated condition recording the maximum cell density and dry weight production at 30°C temperature and 6000 lux light intensity, which were then used to determine the growth curve of *Chlorella* sp.

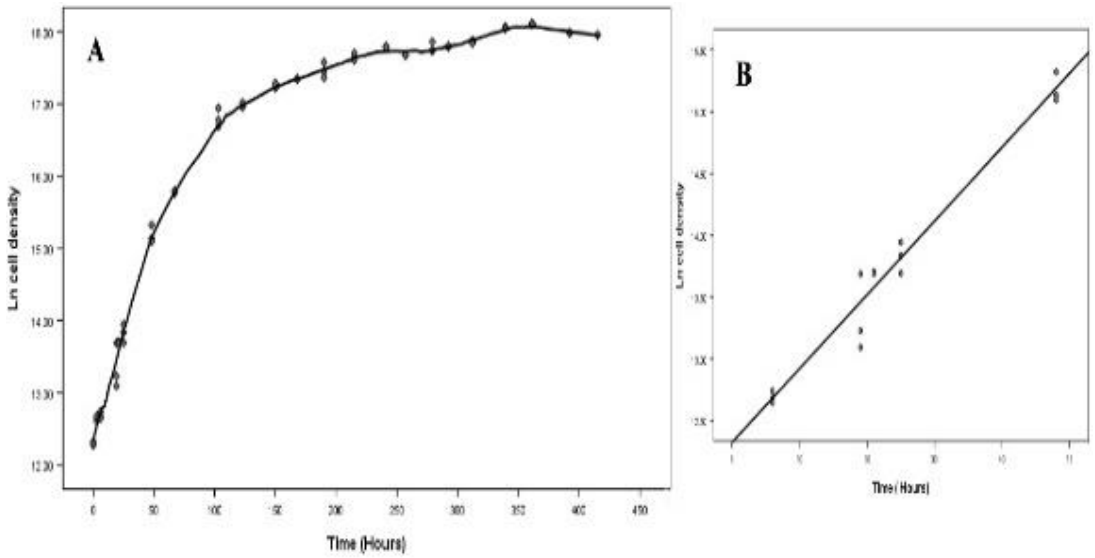

Figure 4. The growth curve of *Chlorella sp.* under 30°C temperature, 6000 lux light intensity with aeration conditions.

A: Growth curve of *Chlorella* sp. as ln cell density vs time (h), B: Exponential phase of the growth curve

Growth Curve of *Chlorella* sp.

Microalgae can grow rapidly if given enough nutrients and the right conditions. The availability of nutrients, temperature, light intensity, photoperiod, aeration, and pH all have a direct impact on algal growth. The growth rates of the microalgal species vary under similar environmental conditions. Figure 4 shows the growth curve of *Chlorella* sp. cultured in BG-11 medium in a 2L conical flask and aerated at 30 °C for 19 days under a 16/8 photo-period in 6000 lux light intensity and Figure 4 b shows the exponential phase of the growth curve which was obtained between 8.86 hr and 45.41 hr. According to the results, the specific growth rate (μ) of *Chlorella* sp. was 0.06 hr^{-1} and the doubling time (T_g) was 11.46 hr. A similar specific growth rate was obtained by Doucha *et al.* 2012 for the production of high-density *Chlorella* culture grown in fermenters. The *Chlorella* growth rate was 0.027 hr^{-1} without optimum conditions, and the doubling time (Tg) was 25.6 hr. The temperature of four growth chambers were maintained accurately (Figure 5) and it can be confirmed that all the *Chlorella* cultures were maintained at provided temperature.

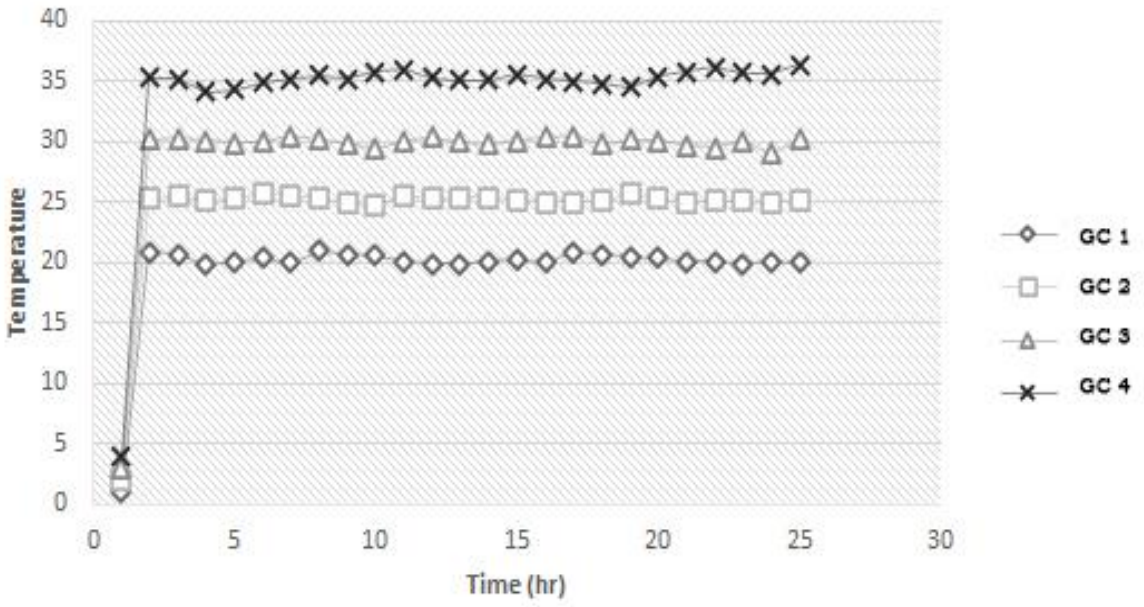

Figure 5. Optimization of temperature in growth chambers
GC 1 - Growth chamber 1, GC 2 - Growth chamber 2, GC 3 - Growth chamber 3, GC 4 - Growth chamber 4

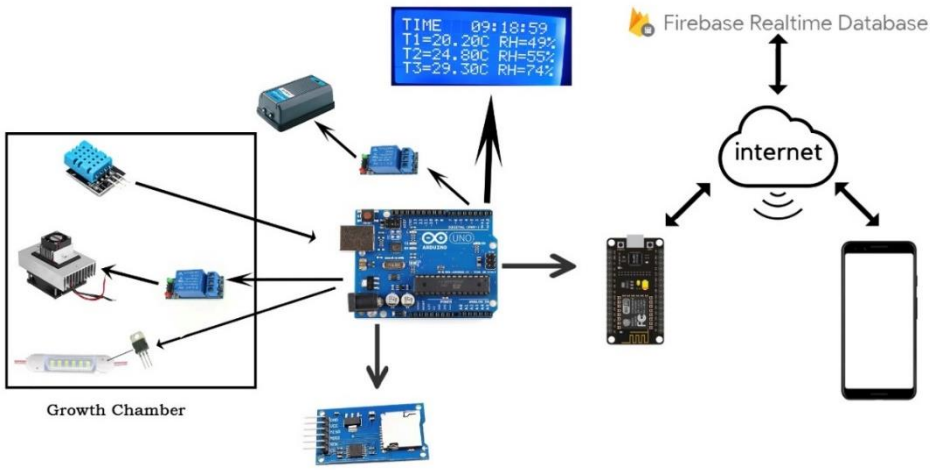

Figure 6. Diagram of Real-Time Monitoring System

4. CONCLUSION(S)

In this study, *Chlorella* species were successfully isolated from wastewater ponds and pure cultures were prepared. Optimum conditions for the efficient mass culture of *Chlorella* were found as 30°C temperature, and 6000 lux light intensity with aeration conditions. Under the optimum growth conditions, the growth of *Chlorella* entered the exponential phase and was found between 8.86 to 45.41 hr after inoculation. The isolated *Chlorella* sp. was characterized with a specific growth rate of 0.06 hr^{-1} and a doubling time of 11.46 hr. These findings are useful for the mass culture of *Chlorella* sp. at the industrial scale for biofuel production

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