

Original Research Paper

Effect of Salinity, pH, Light Intensity on Growth and Lipid Production of Microalgae for Bioenergy Application

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Article history

Received: 10-06-2015

Revised: 27-07-2015

Accepted: 06-10-2015

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Abstract: The crisis of energy producing molecules (fuels) is expected to increase in future, which is currently produced from crude mineral oil. Biodiesel is most reliable, non-toxic, biocompatible liquid fuel that can replace the existing unsustainable sources of energy. Among all the known sources, microalgae display high potential for the production of biodiesel owing to their numerous benefits like higher biomass productivities than plants, no agricultural land requirement, cultivation in waste water and accumulation of 20-50% triacylglycerols. Microalgae biomass and lipid content plays an important role in commercial production of biodiesel. The present work was carried out to develop an axenic culture of a potential microalga *Chlorella sp.* for high biomass and enhanced lipid accumulation. The important growth parameters like pH, light colour, light intensity and photoperiod were studied for better production of *Chlorella* biomass. The effect of salinity on cell growth was also studied and compared with normal Fogg's medium grown cells. The main biomolecules like carbohydrate, protein, lipid and chlorophyll content were also estimated with the help of standard biochemical methods in salt supplemented and without salt Fogg's medium. The cellular lipid content was increased by growing the cells under different salt concentrations. The micro algal strain showed highest growth of 0.822 g L⁻¹ and 1.021g L⁻¹ in Fogg's medium and under 0.2 M NaCl supplemented medium respectively. However, the maximum lipid production of 0.1842 g L⁻¹ was estimated by growing the cells in Fogg's medium including 0.5 M NaCl with slight compromise in cell growth (0.858 g L⁻¹). The lipid content of *Chlorella sp.* was found to be 26.84% as compared to 14% obtained under normal culture condition. Thus, growing *Chlorella sp.* under salt supplemented medium and optimizing light requirement will produce high biomass and oil for biodiesel production.

Keywords: Microalgae, *Chlorella*, Salinity, Light Intensity, Lipid, Biofuel

Introduction

The energy crisis and modern industrial development bring new challenges to our present researchers, leaders and educators to discover an alternative source of energy. Increase in industrial development and population is major cause for continuous increase in carbon dioxide content in earth atmosphere, which ultimately leads to enhanced greenhouse effect (Mata *et al.*, 2010). Global increase in population has increased the pressure on available sources of energy and the efforts of developing sustainable energy from algae biomass as it has the huge potential of becoming major contributor to bio-fuel industry (Ho *et al.*, 2014). The companies should develop the algae market gradually as it

is linked to many services such as fish farming, organic farming, pharmaceuticals and bio-plastics and then final target should be fuel. Algae biomass power is becoming a great success for many industrial applications like bio-fuel because of its incredible growth rate, no competition with food crops, requires less land cover and can be grown easily in waste waters (Aslan and Kapdan, 2006). The fuel extracted from food crops is not efficient, as half of the energy will be wasted in arranging the system for biofuel production (<http://www.altenergy.org>). Therefore microalgae feedstock is better option because cultivating algae is easier than other food crops as it does not need any agricultural land and requires less doubling time (Chisti, 2007). It is a carbon neutral fuel, which does not add to total

carbon emissions as it absorbs same amount of carbon dioxide during its growth phase (Hossain *et al.*, 2008). Although the production of algae is much better than the other raw materials, but due to low lipid productivity, it is still not commercially viable (Nigam *et al.*, 2011).

Algae have the capability to manipulate its growth rate as well as its biochemical composition under varying physiochemical conditions (Al-Qasbi *et al.*, 2012). Researchers have been trying to manipulate the algae biomass by various physiochemical parameters, genetic engineering, light sensitive photo-bioreactors etc. Various stress conditions have been studied with different experimental setups to obtain the desired lipid in high amount for biodiesel production (Sharma *et al.*, 2012; Fan *et al.*, 2014). Moreover an alga has the potential to survive and manipulate the lipid metabolic pathways under varying physiochemical conditions (Mohammed *et al.*, 2013). Stress conditions such as nitrogen deprivation and ferrous sulfate treatment resulted in high lipid generation in *Chlorella Vulgaris* (Guschina and Harwood, 2006). Various physiochemical parameters are known to influence the lipid content of microalgae such as light intensity, pH, salinity, temperature, nitrogen, salinity etc (Behnaz *et al.*, 2013; Yeesang and Cheirsilp, 2011; Liang *et al.*, 2011).

The present study focused on the enhancement of growth and lipid accumulation of *Chlorella sp.* The effect of high salinity, light intensity, photoperiod, pH, on growth and lipid production were determined.

Materials and Methods

Microalgae and Growth Medium

The microalga strain used for this study is freshwater green algae *Chlorella sp.*, which was purchased from National Chemical Laboratory, Pune and grown photo-autotrophically in Fogg's medium (Rai *et al.*, 2013). The medium was sterilized before inoculation.

Cell Growth and Biomass Estimation

The microalga strain was cultured in 500 mL Fogg's medium contained in cotton stoppered 11 Erlenmeyer flasks at temperature $28 \pm 1^\circ\text{C}$ with 24 h fluorescent illumination (40 watt, white tube light). The cultures were subjected to intermittent shaking in orbital shaker at 150 rpm, $28 \pm 1^\circ\text{C}$ and sub cultured at every 15 days. A preliminary experiment was conducted to study the cell growth of the microalga strain without any stress. Cell growth was monitored by measuring the optical density at 660 nm by UV/visible spectrophotometer (Shimadzu UV-1650) at every third day. Algal biomass was collected by centrifuging the algal cultures at 8000 rpm for 10 min on the 15th day of inoculation. To obtain the dried algal biomass, the supernatant was discarded and the pellets

were oven dried at 60°C . The total biomass was then determined gravimetrically and expressed in g L^{-1} .

Effect of Levels of Salinity on Algae Growth

The effect of a range of salinity levels (0M, 0.2M, 0.5M, 0.8M, 1.1M) on growth rate of *Chlorella sp.* was carried out in order to know its salinity tolerance and to examine if this inducing stress increases the lipid content. All cultures were initiated with an O.D. of about 0.1.

Estimation of Biochemical Components

Lipid

The lipid was extracted through method Bligh and Dyer (1959). A mixture of 2 mL methanol and 1 mL chloroform was made and added to 1 g algal biomass. It was kept for 24 h at room temperature to dissolve the lipids properly. The mixture was centrifuged at 3000 rpm for 10 min. Supernatant was separated 2 mL of chloroform was again added to the pellets and shaken properly. It was again centrifuged at 3000 rpm for 5 min and supernatant was separated. After adding 2 mL of 1% KCL to the supernatant, two layers were formed. Lower layer was pipette out and weighed:

$$\text{Lipid Content (\%)} = \text{wt. of lipid (g)} \times 100 / \text{wt. of culture (g)}$$

Protein Estimation

The crude protein was determined by method (Lowry *et al.*, 1951). The absorbance of the sample was checked and the concentration was determined using standard curve:

$$\text{Total Protein Content} = \text{wt. of protein (from BSA curve)} \times 100 / \text{dry cell mass (g)}$$

Carbohydrate Measurement

The content of carbohydrate is estimated by the modified method of 3, 5-Dinitrosalicylic acid calorimetrically using 100 mg of dry algal powder (Miao *et al.*, 2004). The carbohydrate content was estimated using DNS reagent and optical density of the sample was determined against the blank at 540 nm in a UV-visible spectrophotometer:

$$\text{Carbohydrate Content (\%)} = \text{wt. of carbohydrate (from Glucose standard curve)} \times 100 / \text{dry cell mass (g)}$$

Chlorophyll Estimation

A known aliquot of algal sample was centrifuged for 5 min and supernatant was discarded. The pellet was resuspended in the same amount of methanol and shaken thoroughly. The tubes were kept in hot water bath for 30 min and centrifuged. The absorbance was measured at 665 nm (A_{665}) and Chlorophyll a was estimated according to equation (Mackinney, 1941):

$$\text{Chlorophyll } a \text{ (mg/L)} = 13.42 \times A_{665}$$

Effect of Different Colour of Light on Algae Growth

Experiments were designed in different flasks to see the effect of colors (red, green, yellow and white) on algae growth. The different colored tube lights were provided as the photosynthesis process varies for different colors. Biomass production and lipid accumulation were estimated at each wavelength.

Optimization of Light Intensity Needed for Algae Growth

The effect of different light intensities on algae growth and lipid production was measured by carrying out the experiment at different light intensities viz., 2100, 2700 and 3300 lux. The light intensity was measured by using Luxmetre (MEXTECH LX 1010B).

Growth of Algae at Various pH

The algal cultures were grown in Fogg's growth media with varying pH-5, 6, 7, 8 in order to determine the optimum pH that supports maximum growth and lipid accumulation. The culture was initiated with 0.1 OD. The pH was maintained using 1N NaCl and 1N HCl.

Effect of Light: Dark Period on Algae Growth

The effect of photoperiod on growth and lipid content of *Chlorella sp.* was determined by subjecting the samples to three different photoperiods for 15 days: 24 h light, 16 h light: 8 h dark and 8 h light: 16 h dark. The culture was initiated with 0.1 OD.

Statistical Analysis

To validate the results, all the experiments were performed in triplicates. Further, they have been repeated twice to ensure the reproducibility of the results and the average values are listed.

Results and Discussion

Time Course Study of Microalga Growth Subjected to no Stress Vs Salinity

Microalga growth follows the different stages viz. lag, log, stationary and death phase of growth curve like in an ideal microbial population. The exponential phase was reached maximum in 360 h (15 days). The

stationary phase starts after 360 h of growth and ends at nearly 500 h, which is the optimum time to harvest the cells and extract lipid (Fig. 1). The microalga cultures supplied with 0.2 M showed better growth than 0.5 M NaCl and reached the maximum biomass production at around 360 h as given by normal Fogg's grown cells.

Growth and Lipid Accumulation at Different Salt Concentration

In order to know the microalga's salinity tolerance, *Chlorella sp.* was subjected to a range to salinity gradient. The algal cells can tolerate a lower NaCl concentration and thus it grows in the normal way but with an increase in NaCl concentration chlorophyll degradation takes place which leads to cell death (Table 1). Thus, optimum NaCl concentration is required for proper growth of the microalga, above or below which there is a decrement in growth rate (Ruangsomboon, 2012; Takagi *et al.*, 2006). The microalga showed maximum biomass of 1.021 g L⁻¹ when subjected to 0.2 M NaCl concentration. This finding was in accordance to an earlier report by (Salama *et al.*, 2013; Mohan and Devi, 2014). Studies on photosynthesis indicate that there is a requirement of chloride ion for the production of adenosine triphosphate and flavin mononucleotide by Hill reaction. Interestingly, Eyster *et al.* (1958) showed that autotrophically grown *Chlorella sp* require more chloride ion. Another finding by Talukdar *et al.*, 2012 suggested the presence of salt tolerating enzymes in microalga which accounts for their growth under salinity induced conditions.

A negative trend in the growth rate of the microalga was observed when the NaCl concentration was further elevated to 0.5 M. This decrement in growth is due to the accumulation of reactive oxygen species (Kalita *et al.*, 2011). However, studies of Asulabh *et al.* (2012; Kan *et al.*, 2012) indicated that there is a positive relationship between oxidative stress and the triglyceride content. The microalga species showed highest lipid production of 0.1842 g L⁻¹ at 0.5M and the lipid accumulation observed around 21.4% i.e., 1.6 times higher than the cells grown in normal Fogg's media. This is at par with the findings of Dujjanutat and Kaewkannetra (2011) which stated that oxidative stress significantly induces lipid accumulation in freshwater species.

Table 1. Effect of NaCl concentration on cell growth and lipid accumulation

NaCl concentration	Biomass production (g/L)	Lipid production (g/L)	Lipid content (%)
0.0M	0.822±0.043	0.1108±0.005	13.47
0.2M	1.021±0.070	0.1525±0.017	15.00
0.5M	0.858±0.009	0.1842±0.068	21.40
0.8M	0.108±0.036	0.008±0.0050	7.40
1.1M	0.016±0.021	0.0005±0.007	3.12

Data are expressed as mean ± SD, n=3

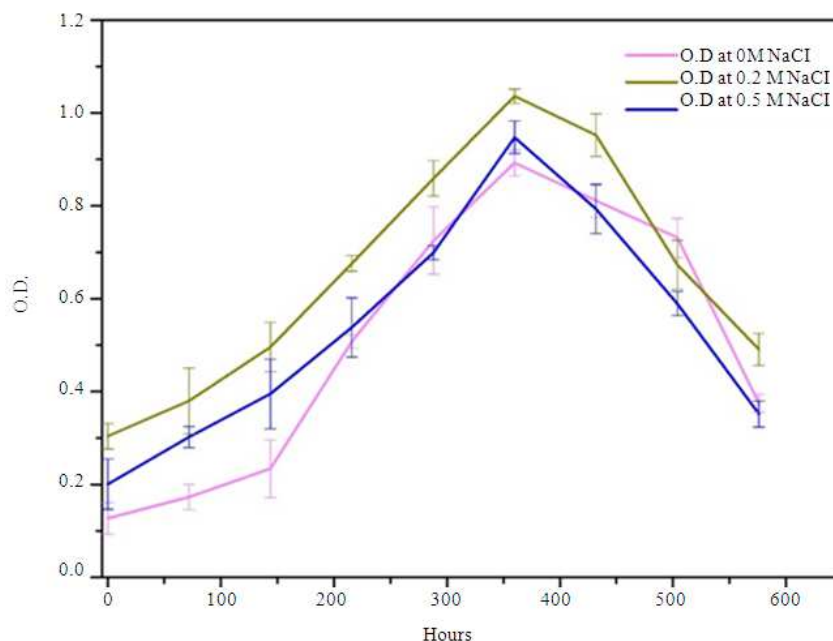


Fig. 1. Growth of *Chlorella sp.* under normal Fogg's medium and with salt (0.2M and 0.5 M NaCl)

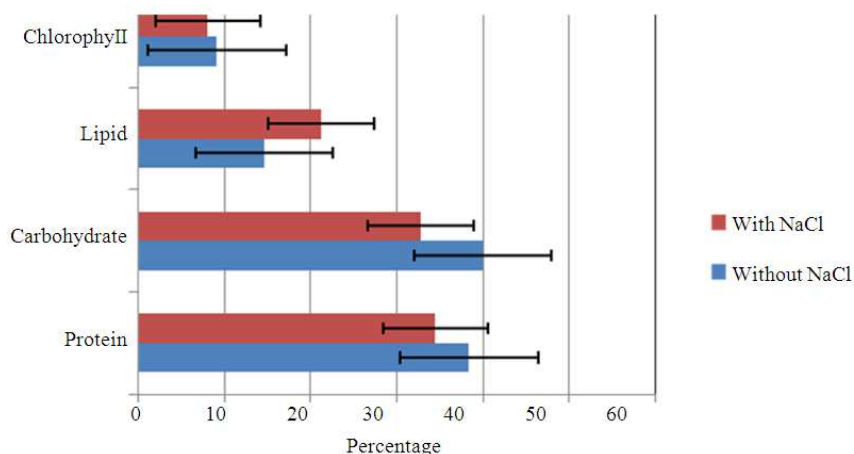


Fig. 2. Estimation of biochemical components of *Chlorella sp.* under salt supplemented and without salt culture medium

The basic biochemical estimation for *Chlorella sp.* i.e., protein, carbohydrate, lipid and chlorophyll content were determined with and without salt supplementation (Fig. 2). Protein content was 38.46% and carbohydrate content was 40.06% obtained in the cells grown under normal Fogg's medium. Total Chlorophyll and lipid were also calculated as 9.17 and 14.64% respectively. Lipid accumulation of cells grown under salt was increased to 21.24% and carbohydrate was decreased to 32.80%. The chlorophyll content and protein contents were not much changed and recorded 8.15 and 34.57% respectively. The possible explanation for lipid enhancement is the metabolic pathway of algae is triggered towards lipid accumulation by the synthesis of acetyl Co A rather than the formation of hexose. In the present study, the comparison of

biochemical contents of *Chlorella sp.* is first time done with and without NaCl supplemented medium.

Optimization of Light Intensities and Colour of Light on Cell Growth and Lipid Accumulation

Cells grown under red coloured light showed best growth among all coloured lights used in the experiment. Yellow and white light also had good effect on cell growth while green light showed minimum cell growth. The highest lipid obtained was 0.0921g L⁻¹ when algae were grown under red light. The lipid obtained was 0.0761 g L⁻¹ and 0.0813 g L⁻¹ for yellow and white (control) light respectively. The lowest lipid obtained was 0.0210g/L when algae were grown under green light (Table 2).

Table 2. Effect of different light colours on growth and lipid production

Color	Dry cell weight (g/L)	Lipid production (g/L)	Lipid content (%)
White (Control)	0.5526	0.0713	12.90
Red	0.6996	0.0921	13.10
Yellow	0.5992	0.0761	12.70
Green	0.2894	0.0210	7.25

Table 3. Effect of various light intensities on growth and lipid production

Light intensity (lux)	Dry weight (g/L)	Lipid (g/L)	Lipid content (%)
2100	0.5426	0.0741	13.60
2700	1.0500	0.2042	19.44
3300	0.6743	0.0943	13.90

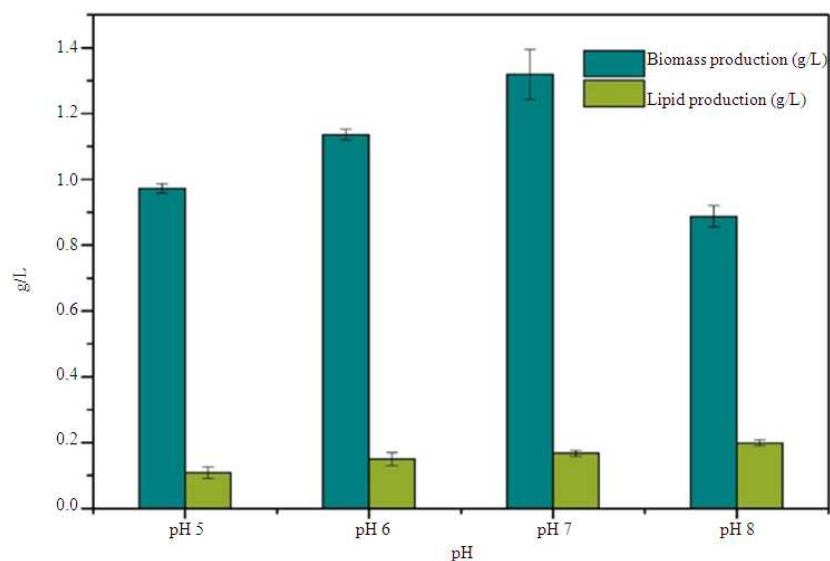


Fig. 3. Biomass and lipid production under different pH (5, 6, 7 and 8)

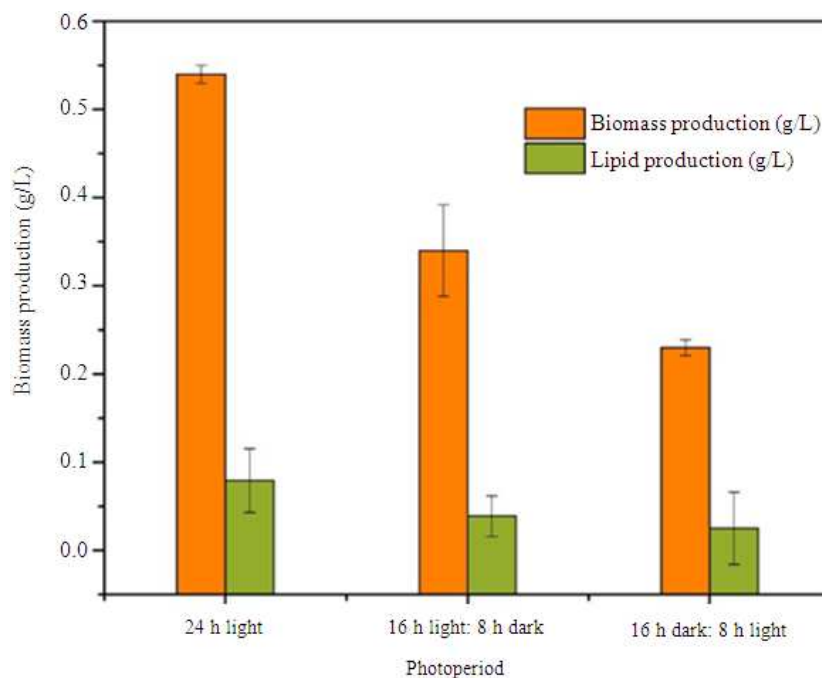


Fig. 4. Biomass and lipid production under different Light: Dark period

Light intensity also has much effect on the growth of *Chlorella sp.* The maximum growth rate increased only up to a certain limit of light intensity i.e., 2700 lux. Maximum biomass was obtained i.e., 1.050 g L⁻¹ at 2700 light intensity whereas when the culture was grown under 3300 lux the biomass was decreased to 0.6743 g L⁻¹ (Table 3). The highest lipid obtained was 0.2042 g L⁻¹ when the flask was kept under 2700 lux and the total lipid accumulation was achieved 19.4% that is more than the lipid obtained when grown at other intensities (Table 3). The experiments were run without adding salt and at pH of 7.0.

Cell Growth and Lipid Accumulation under Various pH

The actual mechanism of lipid accumulation due to pH induced stress is not yet known. But a few reports like that of Guckert and Cooksey (1990) on *Chlorella* CHLOR-1 predicted that alkaline pH reduces the cell release from autospore thereby inducing lipid accumulation. This finding was further supported by the morphological observations by Gardner *et al.* (2011) and also by Shah *et al.* (2013). Similar results were recorded in present experiment, where *Chlorella sp.* showed maximum lipid production of 0.1995 g L⁻¹ with lipid accumulation of 23% at pH 8 (Fig. 3).

Effect of Light: Dark Period on Algal Growth and Lipid Accumulation

Though, photosynthesis and cell photo-acclimatization are both light driven, the response for photoperiod is different for different microalgal species. In our present study, *Chlorella sp.* showed highest biomass and lipid production of 0.54 g L⁻¹ and 0.0791 g L⁻¹ respectively when exposed to 24 h light as shown in Fig. 4. Haiying *et al.* (2011) have reported similar result for the microalgal species *Chlorella minutissima* and other algal species also supports our results viz. *S. dimorphus* and *S. quadricauda* (Goswami and Kalita, 2011), *P. lutheri* (Shah *et al.*, 2013) and *S. obliquus* and *B. braunii* (Krzemińska *et al.*, 2014).

The range of all influential parameters were studied and it was found that the *Chlorella sp.* showed highest growth of 1.175 g L⁻¹ and lipid content of 26.84% at 24 h photoperiod, pH 8.0 and 0.5 M NaCl concentration, keeping light intensity of 2700 lux. There is approximately two times increase in lipid content of *Chlorella sp.* after keeping all the optimized condition with salt supplemented medium.

Conclusion

The present work was carried out to maintain an axenic culture of the microalga *Chlorella sp.* for high biomass and enhanced lipid accumulation. The

microalgal strain *Chlorella sp.* was chosen because of their faster growth rate. The maximum lipid production of 0.1842 g L⁻¹ was achieved by growing the cells in Fogg's medium including 0.5 M NaCl with slight compromise in cell growth (0.858 g L⁻¹). Light intensity also has high impact on lipid accumulation of *Chlorella* cells. After optimizing pH, light intensity and photoperiod of the algal culture, the cellular lipid content of 26.8% for *Chlorella sp.* was obtained as compared to 14% obtained under normal culture condition. It was therefore recommended that further experiments will be carried out to characterize the lipid produced from the *Chlorella sp.* for its applications in biofuels industries.

Acknowledgment

This work was financially supported by a project grant (Ref. No. DST/TSG/AF/2009/101) from Department of Science and Technology, Govt. of India, New-Delhi. Authors are grateful for financial support.

Author's Contributions

Monika Prakash Rai: Mentor and Principal Investigator on the current funded project.

Trishnamoni Gautom: Handled the dissertation work on *Chlorella* growth and biodiesel production.

Nikunj Sharma: Conducting experiments on the effect of color and light on Algae biomass and lipid.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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