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Effect of Variable Salinity and Phosphorus Culture Conditions on Growth and Pigment Content of *Chlorella vulgaris*

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Abstract

Background/Objective: *Chlorella vulgaris*, microalgae belonging to Chlorellaceae family is widely used as food supplement and as feedstock for biodiesel production. Hence, it is needed for clean and cost-effective algal production with a high yield of biomass. This study will determine the algal response under various growth conditions for high growth, biomass yield with high pigment content. **Methods/ Statistical Analysis:** In our study, experiments in controlled conditions with variable NaCl concentration (0.0M, 0.1M, 0.15M, 0.2M and 0.25 M) and variable phosphorus concentration (0.04 g/l, 0.02g/l, 0.01g/l and 0 g/l K_2HPO_4) were conducted to study the response of *Chlorella vulgaris* in respect to growth and pigment components. All experiments were conducted in triplicates and the results were studied statistically by SPSS PASW 18.0 for two ways ANOVA. **Findings:** Under variable phosphorus conditions in respect of days the significant increase in growth was observed between 14th day to 21st day after which stationary trend was observed. Also, when phosphorus concentration was decreased from 0.04g/l to 0.02 g/l, 0.01g/l and 0.0g/l the growth, biomass and pigment composition showed a declining trend. Culture growing at 0.04g/l of K_2HPO_4 concentration in medium showed highest growth (1.281 ± 0.003 d^{-1}, where d is number of days) with variable NaCl concentrations the cell growth was favored at minimum salt concentration and with the increase of NaCl from 0.0M to 0.25M the growth and pigment components showed a decline. In respect of days with variable NaCl, the growth showed a significant increase from zero-day to 21st day thereafter only slight increase was seen on the 28th day. This result goes in consensus with previous findings. Hence, *Chlorella vulgaris* isolated from Rajasthan region is adaptable to grow at low salinity and control phosphorus (0.04 g/l) conditions for high biomass with high pigment content which can be used for high nutritive purposes. **Improvement/Application:** *Chlorella vulgaris* yields high biomass and pigments both at 0.0 Molar salinity and 0.04 g/l phosphorus concentration**.** These conditions were successfully applied to grow *Chlorella vulgaris* in lab conditions to get the highest yield of biomass with high pigment content.

Keywords: *Chlorella vulgaris*, Growth, Pigments, Total chlorophyll, Phosphorus, Carotenoids and Salinity

1. Introduction

Microalgae are fast growing photosynthetic organisms which can be found flourishing in surrounding environment. The understanding of ecophysiology of microalgal species is important for two important reasons: first, to understand the fate of this autotropic plankton in natural ecosystems; secondly to optimise the large scale biomass production[1]. Uses of microalgae are very wide which include food products, biofuels, and many algal species are used as a tool for phytoremediation. One of the first use of microalgal biomass was for aquaculture[1,2]. In recent decades, microalgal research has gained momentum. The enclosed capsules of processed biomass of

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Chlorella vulgaris and *Spirulina platensis* as nutraceuticals are available in large scale and *Haematococcus pluvialis* is widely used for the production of astaxanthin[3]. There are numerous others sorts of algae which are additionally rich source omega-3 unsaturated fats, thus, can be used as a live feed and diet supplement for many. There are some varieties of algae which are rich in iron, potassium, iodine, magnesium and calcium. It also contains the high level of minerals and vitamins, for example, B12 and β-carotene and protein. The nutritional composition of microalgae mainly consists of carbohydrate, proteins, lipids and trace elements. In addition to using algae as nutrition, it can be used actively as fertilizers, animals feed and an agent for pollution control. Algal biomass can be utilized to uproot overwhelming metal contaminants, for example, uranium[4]. There are some varieties of algae which act as a very rich source of organic fertilizer and can be either used as raw or can be mixed with the compost.

Numerous investigations of algal species have demonstrated that microalgae react to the changes in the ecosystem where they are grown[5,6]. The environmental factors like pH, temperature, light intensity and nutritional factors (nitrogen, phosphorus, and iron) affect the growth, lipid yield, pigment composition of the alga. This behaviour response is a biotechnological characteristic for scientific industry and that can be controlled so as to control the algae biochemical composition and development, concentrating on particular mixes and higher productivity[1].

Chlorella vulgaris is surely the most studied unicellular microorganism belonging to group Chlorophyta. This alga has its commercial importance as a sources of protein, vitamins, essential amino acids and fatty acid. Chlorella is one of the richest source of chlorophyll which is broadly utilized as a health nourishment and food supplement, and in addition in the pharmaceutical and cosmetics industry[7]. It has been delivered monetarily in a few nations for its utilization as nutraceutical food and medicinal reason because of its significant substance especially pigments and proteins[7]. This microalga has a standout amongst the most amazing chlorophyll substance found in nature[8].

This study focus on the assessment of the effect of the variable range of NaCl and phosphorus on biomass yield, growth, and chlorophyll content of *Chlorella vulgaris*.

2. Materials and Methods

2.1 Material

The pure culture of experimental alga (*Chlorella vulgaris*) was collected from Banasthali Vidyapith. The experiment was carried out in the laboratory of Biotechnology, Department of Bioscience and Biotechnology, Banasthali University, Rajasthan, India.

2.2 Medium and Cultivation Condition

This experiment used 2000 ml Erlenmeyer flask which contained 1000 ml BG11 medium at pH 7.0 to grow the green microalga *Chlorella vulgaris*. The culture room was at 25 ± 2 °C with light intensity of 75 µmol photon m^{-2} s^{-1} under a photoperiod of 14: 10 h without sparging with air or CO_2. This was regarded as control culture. To avoid clumping of cultures and help the growth, culture were shaken gently thrice a day. Three different sets were used for each medium.

2.3 Experimental Setup

Four variable phosphorus treatments (K_2HPO_4 as phosphorus source at 0.04 g/l, 0.02 g/l, 0.01 g/l and 0.0g/l respectively), each with a set of triplicates, were carried out to study the effect of variable concentration of phosphorus on the growth and pigments of *Chlorella vulgaris*. Another set of five salinity treatments (0.0 M, 0.1M, 0.15M, 0.2M and 0.25M), each with a set of triplicates, were carried out to study the effect of salinity on growth and pigments of *Chlorella vulgaris*. Cultures for evaluation were matured in 150 ml Erlenmeyer flasks with 50 ml BG11 medium, pH was set at 7.0. All the developing cultures were grown in a culture room with the constant room temperature and other parameters as specified above.

2.4 Growth Analysis and Dry Cell Weight Measurement

Algal growth was measured by taking absorbance and biomass. Absorbance was measured on regular intervals (7, 14, 21 and 28 days respectively) by recording the changes in optical density at 663nm with a UV/ vis spectrophotometer (Systronic Pvt Ltd). Gravimetrical method[9] was used in the experiment to determine the dry cell weight of biomass. Centrifuge was used to extract the biomass from the known culture quantity. The extracted culture was then dried at 80°C to remove the moisture content.

Below formula was used to calculate the Biomass productivity (mg l^{-1} d^{-1}) using below formula:

$$\text{Biomass productivity} = \frac{X1 - X0(\text{mg L} - 1)}{\text{number of days}} \quad (1)$$

(Where X1 = final concentration of biomass; X0 = initial concentration of biomass)

2.5 Pigment Estimation (Arnon 1949)

Pigment (total chlorophyll, chlorophyll a, chlorophyll b and carotenoids) contents in *Chlorella vulgaris* were estimated by using Arnon's method[10]. 10 ml of culture cells were harvested. They are then homogenized in 3 ml of ice-cold 80% acetone incubated overnight at 4°C. Cell culture and acetone mixture were transferred to a centrifuge tube for centrifugation at 3500 rpm for 10 mins. 1 ml of the supernatant was taken in a cuvette and the absorbance was measured at 645 nm, 663 nm, 632 nm and 480 nm.

Calculations as per Arnon equation[10] (1949):

Total Chlorophyll (mg/l) = 20.2 (Abs at 645) + 8.02(Abs at 663) (2)

Chlorophyll A (mg/l) = 12.7(Abs at 663) – 2.69(Abs at 645) (3)

Chlorophyll B (mg/l) = 22.9(Abs at 645) – 4.68 (Abs at 663) (4)

Carotenoid (mg/l) = Abs at 480 * 200 (5)

2.6 Statistical Analysis

The experiments were carried out in triplicates and the statistical analysis was done by SPSS PASW 18.0 for two ways ANOVA and Tukey's test.

3. Results and Discussion

3.1 Effect of Phosphorus on Growth and Biomass

The variable phosphorus had a significant effect on the growth rate of *Chlorella vulgaris*. During the total days of experiment run, it was observed that growth of the algae increased steadily (Figure 1). The maximum growth (1.281 ± 0.003 d^{-1}, where d is number of days) with respect to phosphorus concentration was observed in control culture (0.04g/l of K_2HPO_4), on day 28th there was an approximate 5fold increase from zero days in control

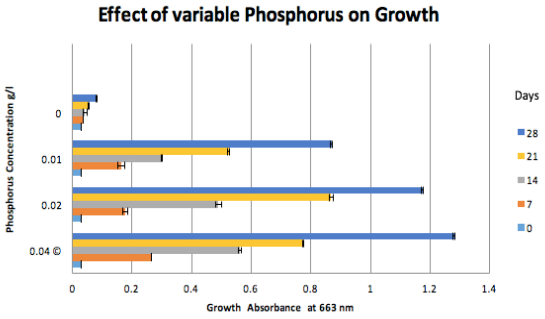

Figure 1. Effect of variable phosphorus concentration on growth of *Chlorella vulgaris*. (Absorbance at 663 nm; 0.04g/l is control).

culture. Minimum growth was observed in phosphorus deficient culture (0.081 ± 0.001 d^{-1}, where d is number of days). Biomass productivity in the control culture (0.04 g/l K_2HPO_4) was the highest (86.42 mg l^{-1}) and maximum biomass productivity was observed on day 21st. It has been reported that unfavourable conditions like deficiency of minerals (Nitrogen and phosphorus) and nutrients in many unicellular green algae impacts growth and biomass and promotes biosynthesis of lipids. A variety of biochemical structure is seen in algae and it relies on which supplement is restricted and to what degree[11].

The two major macronutrients for development and metabolism system of algal cells are nitrate and phosphates. Nitrogen is a significant component for the development of protein and nucleic acid while phosphorus is a critical piece for DNA and RNA. Limitation and lack of them moves the metabolic pathway of microorganism[11]. Phosphorus is a critical segment required for ordinary development and improvement of algal cells[11,12]. It is studied that 1% of dry weight of green growth is constituted by phosphorus[11,13]. Culture with limitation of phosphorus displays fall in the combination and recovery of medium in the Calvin –Benson cycle and an important decrease in the light usage used for carbon dioxide fixation[11,14].

3.2 Effect of Salinity on Growth

The effect of variable NaCl concentration was studied and it was observed that during days of cultivation the growth increased significantly. Maximum growth (1.384 ± 0.003 d^{-1}) was observed in the control culture (0.0 M) on day 21st after which a decline was observed. The growth curve of *Chlorella vulgaris* with respect to variable saline ranges is shown in Figure 2. There was approximate 6folds increase from zero days to 21st day in control culture. We

can conclude that growth differs significantly ($P < 0.05$). Tukey's post-hoc analysis suggests that control culture showed maximum growth and it decreased as salinity in the medium was increased from 0.1M to 0.25M. In all other mediums with variable (0.1M - 0.25M) salinity range, the increasing trend of growth was observed from zero days to 28st day. Lowest growth was observed in culture with 0.25M salinity (0.507 ± 0.009 d^{-1}), an increase of approximate 2 fold was observed with respect to zero days on day 28th. The highest biomass productivity of 54.28 mg l^{-1} was observed in control culture (0.0 M) on day 14th, the biomass productivity decreased as the NaCl concentration was increased in the medium.

Varieties in salinity impact the development of marine microalgae despite the fact that these organisms are tolerant to any fluctuations in salinity. As indicated by[15], flexibility to salinity varies amongst different algae and are categorised as halophilic and halotolerant in the presence of salinity. Reducing salinity is also one of a kind ways to increase the biomass productivity in marine microalgae. The variable salinity on starch digestion system demonstrates its species-particular and development condition-subordinate nature[16]. High saline content has been accounted to restrain the development, lipid and triacylglyceride accumulation of *Dunaliella sp*[17].

Salinity stress influences heavily to the different natural and biologically grown systems for the development and improvement of microalgae. Salinity stress can prompt in the expansion of lipid substance in microalgae because of its important role in bringing the changes in the metabolic system of fatty acid[18]. In our experiments, the growth rate and biomass decreased as the NaCl concentration in the growth medium increased and hence highest growth was observed in 0.0 M culture and lowest was observed in the culture grown in 0.25 M salinity. This could be associated with the fact that *Chlorella vulgaris* was unable to adapt at higher saline ranges. Cultivation with different salinity ranges although showed similar time course i.e growth increased from 0 to 21st day and declined on day 28[19]. They also demonstrated the reason for the reduced growth at high level of salinity. That was due to the fact that the photosynthic rate decreases in marine microalgae at high salinity. All the previous study on the reaction of different species of microalgae to check the response of salinity on growth has demonstrated *Dunaliella salina* grows more in lower salinity and less on higher salinity level[20,21]. This study also demonstrated that most species of microalgae prefers non saline environment to show higher growth[17,22].

3.3 Effect of variable salinity and variable phosphorus on Pigments

3.3.1 Chlorophyll a

Variable NaCl had the significant effect on chlorophyll a content. The chlorophyll a content showed a marked increase from zero days to 21st day, and then changed slightly in next 7 days. The algae growing at 0.0 M NaCl had the highest chlorophyll a value (0.537 ± 0.008 mg l^{-1}) (Figure. 3). Approximate 2 fold increase was observed in chlorophyll a value at the end of 28 days with respect to day zero. Minimum chlorophyll a was observed in algae growing at 0.25M (0.394 ± 0.022 mg l^{-1}).

In the variable phosphorus experimental set, Chlorophyll a content showed an increasing trend from zero days to 28th day in all culture mediums whereas, a decrease was seen with the decrease in K_2HPO_4 supplied for growth of *Chlorella vulgaris*. Maximum amount of chlorophyll a was recorded on 0.04 g/l phosphorous

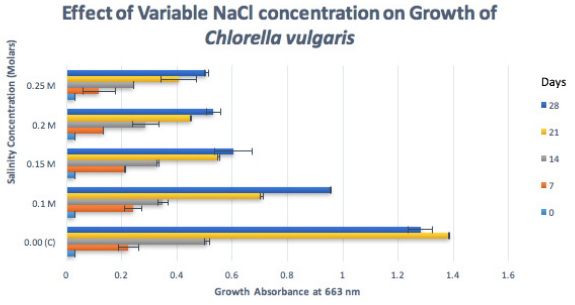

Figure 2. Effect of variable NaCl (salinity) concentration on growth of *Chlorella vulgaris*. (Absorbance at 663nm; 0.0M is control culture).

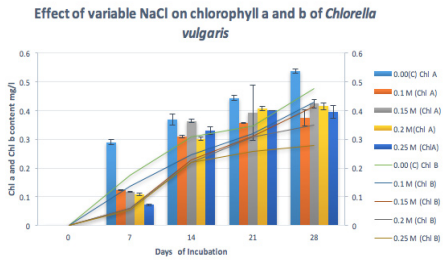

Figure 3. Effect of variable NaCl (salinity) concentration on Chlorophyll a and b content of Chlorella vulgaris. (Chl a - Chlorophyll a, Chl b – Chlorophyll b).

(1.676 ± 0.083 mg l^{-1}) while it gradually declined on 0.02 g l^{-1} (1.143 ± 0.014 mg l^{-1}) > 0.01 g/l (0.513 ± 0.020 mg l^{-1}), and 0.0 g l^{-1} (0.241 ± 0.011 mg l^{-1}) on 28 days (Figure 4).

3.3.2 Chlorophyll b

The chlorophyll b content increased significantly from zero days to 28th day in variable NaCl. The culture growing at 0.0 M NaCl had highest chlorophyll b value (0.478 ± 0.014 mg l^{-1}) (Figure 3). Approximate 4fold increase was observed in chlorophyll b value at the end of 28 days. The culture growing at 0.25 M reported minimum chlorophyll b content (0.278 ± 0.015 mg l^{-1}) on 28th days.

In variable phosphorus concentration, Chlorophyll b content showed a similar trend as Chlorophyll a and showed an increasing trend from zero days to 28th day in all culture mediums whereas, a decrease was seen with the decrease in K_2HPO_4 supplied for growth of *C. vulgaris* (Figure 4). Maximum amount of chlorophyll b was recorded on day 28th in control culture (0.04 g l^{-1}) (0.279 ± 0.004 mg l^{-1}) while it gradually declined on 0.02 g l^{-1} (0.200 ± 0.011 mg l^{-1}) > 0.01 g l^{-1} (0.129 ± 0.013 mg l^{-1}) > 0.0 g l^{-1} (0.087 ± 0.029 mg l^{-1}).

3.3.3 Total chlorophyll

Total Chlorophyll contents were also observed in algae culture of *Chlorella vulgaris*. In variable NaCl, the total chlorophyll showed an increasing trend from zero days to 28th day. The maximum amount of total chlorophyll contents were observed in control conditions 0.0 M salinity (0.730 ± 0.013 mg l^{-1}). With the increasing salinity, the total chlorophyll contents showed a decrease (Figure 5).

In variable phosphorus concentration maximum amount of total chlorophyll contents was found on day 28th in control culture (0.04 g/l) (1.202 ± 0.055 mg l^{-1}) which were declined on 0.02 g/l (0.898 ± 0.011 mg l^{-1}) > 0.01 g/l (0.438 ± 0.013 mg l^{-1}) and 0.0 g/l (0.259 ± 0.018 mg l^{-1}). As per days of incubation there was increasing trend from zero days to 28th days (Figure 6).

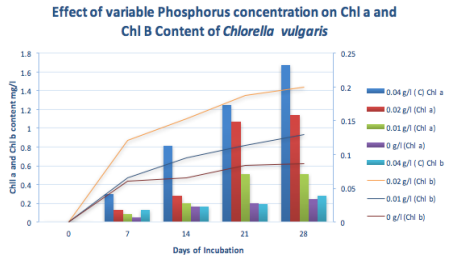

Figure 4. Effect of variable phosphorus concentration on Chlorophyll a and b content of *Chlorella vulgaris*.

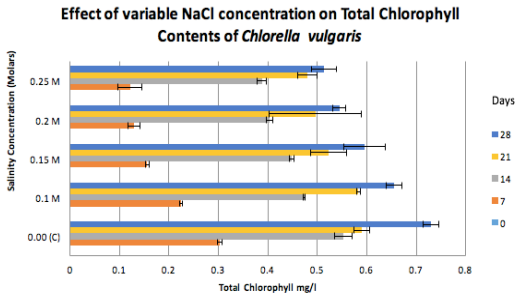

Figure 5. Effect of variable NaCl (salinity) concentration on Total Chlorophyll of *Chlorella vulgaris*.

3.3.4 Carotenoids

Cultures growing in variable NaCl showed an increase from zero day to 28th day. Maximum contents of carotenoid were observed 0.0M salinity (0.178 ± 0.008) followed by 0.1M (0.042 ± 0.003), 0.15M (0.041 ± 0.002), 0.2M (0.036 ± 0.001) and minimum in 0.25M salinity (0.032 ± 0.002) (Figure 7).

With variable phosphorus concentration carotenoid content showed increase from zero days to 28th days (Figure 8). Maximum contents of Carotenoid were observed on day 28th in culture with 0.02 g/l phosphorous (0.099 ± 0.003 mg l^{-1}) followed by control culture (0.04 g/l K_2HPO_4) (0.076 ± 0.011 mg l^{-1}), 0.0 g/l (0.065 ± 0.001 mg l^{-1}) and minimum in 0.01 g/l (0.054 ± 0.001 mg l^{-1}).

Amid the research, it was observed that most extreme chlorophyll substance was seen on lower salinity. So

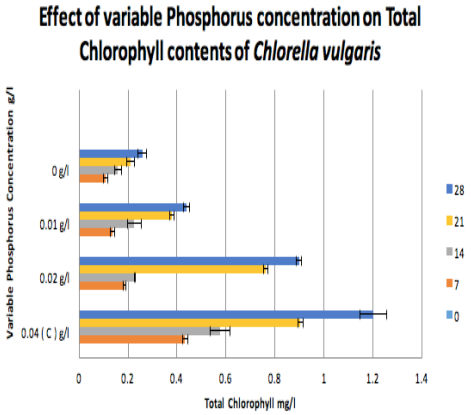

Figure 6. Effect of variable Phosphorus concentration on Total Chlorophyll of *Chlorella vulgaris*.

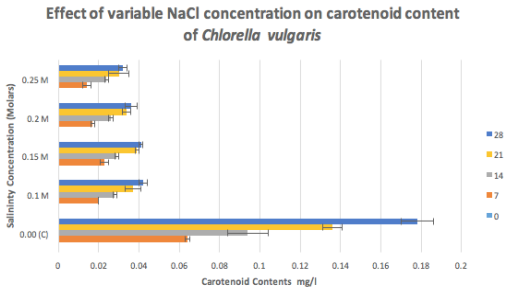

Figure 7. Effect of variable NaCl (salinity) concentration on Carotenoid content of *Chlorella vulgaris.*

also, to this,[23] observed that growth of marine *Chlorella saccharophila* straightly diminished with expanding salinities from 40 to 60 %. In consonance, prior studies have reported that the development of green growth with higher saline concentrations had lower chlorophyll substance[24,25]. It has likewise been accounted for that chlorophyll is essential focus to sodium toxicity quality constraining net rate of osmosis, bringing about lessened photosynthesis and reduced development[26].

Phosphorus is one of the important nutrient which helps in the normal growth of algae. Phosphate is required as an important nutrient to form the biomolecules like nitric acid, phospholipids and proteins[27]. Many researchers have concluded that apart from nitrogen, phosphorus blocks the natural growth of Algae. Approximate phosphorus constitutes 1% of Algae's dry weight. It transfers energy through ATP and this is one of the most important aspect of phosphorus in algae[28]. The most immediate impact of phosphorus limitation is the reduced light utilization for carbon fixation, this is because it restricts the environment in which organism grows in Calvin – Benson cycle declines with limited phosphorus[29].

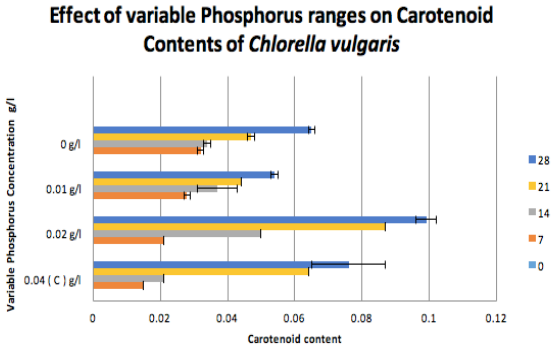

Figure 8. Effect of variable Phosphorus concentration on carotenoid content of *Chlorella vulgaris*.

Lack of phosphorus in the culture reduces the production of chlorophyll a and proteins. This increase the relative sugar level in algae. Lack of phosphate has been observed to bring about a collection of astaxanthin and a general lessening in cell development[30]. It is also observed that lack of phosphorus in other species like to *Selenastrum minutum* decreases the rate of respiration in algal cell[31].

4. Conclusion

The experiments performed confirmed that the growth of green alga *Chlorella vulgaris* showed significant increase/decrease with variable salinity and phosphorus concentration. With higher salinity the growth and pigments of *Chlorella vulgaris* decreased, therefore, for the yield of higher pigments and biomass the NaCl concentration should be kept to a minimum (0.0 Molar to 0.1 Molar). Phosphorus concentration of 0.04 g l^{-1} in culture medium favoured the pigment accumulation, high growth and biomass. Decrease in phosphorus concentration from 0.04 g l^{-1} to 0.00 g l^{-1} showed decline in growth and pigment value.

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