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Synergistic Influence of Environmental Factors for Increased Energy Production by Microalga

Ms. Vijetna Singh* and Ms. Alka Thakur**

**M. Tech. Research Scholar, Department of Electrical Engineering,*
Sri Satya Sai University of Technology and Medical Sciences, Sehore (Madhya Pradesh), INDIA
***Associate Professor, Department of Electrical Engineering,*
Sri Satya Sai University of Technology and Medical Sciences, Sehore (Madhya Pradesh), INDIA

(Corresponding author: Vijetna Singh)
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ABSTRACT: Microalgal like *C. vulgaris*, being a unicellular green algae, is been widely studied for nitrogen and phosphorus removal and as potential feedstock for biodiesel production. This not only saves water from eutrophication but also becomes a good source of biodiesel. Hence, if two approaches are combined we can overcome the major challenge of both eutrophication and energy crisis. In the present study we have examined the effect of decreasing Nitrogen and phosphorus concentration in medium on growth, biomass and lipid content of *C. vulgaris*. Significant decrease in growth and biomass was observed with the decrease of nitrogen and phosphorus concentration in the medium from (1.5g/l to 0.0g/l) and (0.04g/l to 0.0g/l) respectively. Whereas the lipid accumulation showed reverse trend of increase when the

Keywords: Biomass, *Chlorella vulgaris*, Carbon dioxide concentration of both phosphorus and nitrogen where decreased in medium, Growth, Lipids, Microalgae, Nitrogen, Phosphorus.

I. INTRODUCTION

Microalgae are unicellular photosynthetic organisms which use light energy and carbon dioxide for production of biomass. The biomass produced can be used for various application, some are extraction of high rate value foods (such as γ-linolenic, arachidonic acid, eicosapentaenoic and docosahexaenoic acids (K.H.M. Cardozo *et al*; I. Valencia *et al*; and A. Converti *et al*), and food for aquaculture, pharmaceutical products, and biofuel productions (P. Spolaore *et al* and A. Converti *et al*). There will a substantial change in quality and quantity of lipids within the cells can vary as a result of changes in growth conditions (light, temperature etc) or nutrient media characteristics (concentration of Nitrogen, phosphorus and Iron) (A.M. Illman *et al* and Z.Y. Liu *et al* and A. Converti *et al*).

Biodiesel which is now widely accepted as the alternative to standard diesel and Algae is one of the promising source for biodiesel production (Nigam *et al.* 2011). Triacylglycerol (TAG's) are the sustainable feedstock for biodiesel production, photosynthetic microorganisms that convert water, sunlight and CO_2 to sugars from which macromolecules such as lipids and TAG's can be obtained (Singh and Gu 2010 and Nigam *et al.,* 2011).

Intensive research and development on several aspects is needed for commercial production of biodiesel. The optimization of selected strains is required for biomass production and lipid profile (Pruvost *et al* 2011 and Nigam *et al* 2011). There are quite a few species of microalgae have high content of oil and can be manipulated to produce more oil (Gao 2010 and Nigam *et al* 2011). Many researchers have proved that lipid tend to accumulate in nutrient deficient (nitrogen and phosphorus) conditions.

Nitrogen is an essential constituent of all structural and functional proteins in the algal cells and accounts for 7% -20 % of cell dry weight (Hu 2004, Murthy *et al* 2013). Nitrogen deficiency in algal culture enhances the biosynthesis and accumulation of lipids (Thompson 1996, Converti *et al* 2009, Shifrin *et al* 1981, Wang *et al* 2009, Demirbas 2010, Murthy *et al* 2013) and triglycerides (Takagi *et al* 2000, Stephenson *et al* 2010, Murthy *et al* 2013). The limitation of nitrogen can be considered as an efficient environmental pressure to enhance lipid accumulation (Goldberg and Cohen, 2006; Rodolfi *et al*., 2009 and Xin *et al*., 2010). Other effects of nitrogen reduction are Carbon dioxide fixation, decrease in oxygen evolution, Chlorophyll content and tissue production (Kolber *et al* 1988,

Barsanti and Gualtieri *et al* 2005, Murthy *et al* 2013). Other than Nitrogen, Phosphorus is important component required for normal growth and development of algal cells (Hu 2004, Murthy *et al* 2013). It is studied that 1% of dry weight of algae is constituted by phosphorus (Borchardt and Azad 1968, Murthy *et al* 2013). Effects of phosphorus limitation include a reduction in the synthesis and regeneration of substrates in the Calvin –benson cycle and a consequential reduction in the rate of light utilization required for carbon dioxide fixation (Barsanti and Gualtieri *et al* 2005, Murthy *et al* 2013. This study is conducted to investigate the growth response, biomass and lipid content of *C. vulagris* by varying the concentration of Nitrate $NaNO_3$ and Phosphorus K_2HPO_4.

II. MATERIAL AND METHODS

A. Organism and growth conditions

Established cultures of the green microalgae *C. vulgaris* were grown in 150 ml Erlenmeyer flasks containing 50 ml BG11 medium at pH 7.0 in a culture room at 25 ± 2° C under a photoperiod of 14 : 10 h at light intensity of 75 µmol photonm^{-2} s^{-1} PAR without sparging with air or CO_2.

The mineral salt medium composition per liter of distilled water was as follows:

Macronutrients: 1.5g $NaNO_3$, 0.04g K_2HPO_4, 0.075g $MgSO_4.7H_2O$, 0.036g $CaCl_2.2H_2O$, 0.006g Citric Acid, 0.006g Ferric Ammonium Citrate, 0.001g EDTA (Disodium magnesium salt) and 0.02g Na_2CO_3.

Micronutrients (A5 Solution) 1.0ml/liter : 2.286mg H_3BO_3, 1.81mg $MnCl_2.4H_2O$, 0.222mg $ZnSO_4.7H_2O$, 0.039mg $Na_2MoO_4.2H_2O$, 0.079mg $CuSO_4.5H_2O$, 0.0494mg $Co(No_3)_2.6H_2O$. pH was set between 7.0-7.5. The cultures were hand shaken two or three times daily to avoid sticking. This was referred to as control culture.

B. Optimization of medium on different Nitrogen and Phosphorus ranges

The one set of triplicates of cultures for evaluation were grown in 150 ml Erlenmeyer flasks containing 50 ml BG11 medium on different Nitrogen concentrations ($NaNO_3$ as nitrogen source at 1.5g/l, 0.75g/l and 0.375g/l and 0.0g/l respectively and $NaNO_3$ replaced by NaCl), and another set of triplicates for different phosphorus concentration (K_2HPO_4 as phosphorus source at 0.04g/l, 0.02g/l, 0.01g/l and 0.0g/l respectively and K_2HPO_4 replaced by KCl) the pH was set at 7.0 and were kept in a culture room at 25 ± 2° C under a photoperiod of 14 : 10 h at light intensity of 75 µmol photonm^{-2} s^{-1} PAR without sparging with air or CO_2.

C. Growth Analysis

Algal growth was measured on regular intervals (7, 14, 21 and 28 day respectively) by recording the changes in optical density at 663nm with a spectrophotometer.

D. Dry weight measurement (Rai et al.1991)

Dry cell weight (dcw) was determined gravimetrically. A known volume of algal culture was centrifuged at 5000 rpm for 15 min and the harvested biomass was dried at 80°C to constant weight.

E. Extraction and Estimation of lipid from algal biomass

Extraction of lipid was done following the protocol of Bligh and Dyer (1959). To a 15 ml glass vial containing a known amount of algal biomass, 2 ml methanol and 1 ml chloroform were added and kept for 24 h at room temperature. The mixture was agitated in a vortex for 2 min, and 1 ml of chloroform was again added and the mixture shaken vigorously for 1 min; 1.8 ml of distilled water was added and the mixture was agitated in a vortex again for 2 min. The layers were separated by centrifugation for 10 min at 2000 rpm. The lower layer was filtered through Whatman No. 1 filter paper into a previously weighed clean vial (*W*1). Evaporation was carried on in a water bath and the residue was further dried a 104ºC for 30 min. The weight of the vial was again recorded (*W*2). Lipid content was calculated by subtracting *W*1 from *W*2, and was expressed as % dcw.

Statistical Analysis: All the experiments were performed in triplicates, the results were analyzed statistically by SPSS PASW 18.0 for two ways Anova and Tuckey's test.

III. RESULT AND DISCUSSION

A. Effect of Nitrogen deficiency and limitation on Growth and Biomass

The time course of *C. vulgaris* culture growth under the effect of reduction of $NaNO_3$ concentration is shown in figure 1.

The concentration of nitrate $NaNO_3$ was reduced to half and quarter of standard medium whereas in one medium $NaNO_3$ was deficient and in another $NaNo_3$ was replaced by NaCl.

The growth was significantly affected; maximum algal density was observed in control culture (1.5 g/l $NaNO_3$). On day 28^{th} 54 fold increase from zero days was observed in control culture. The growth showed decrease as $NaNO_3$ in medium was reduced from 0.75 g/l $NaNO_3$, 0.375 g/l $NaNO_3$, 0.0g/l $NaNO_3$ and $NaNO_3$ replaced by NaCl.

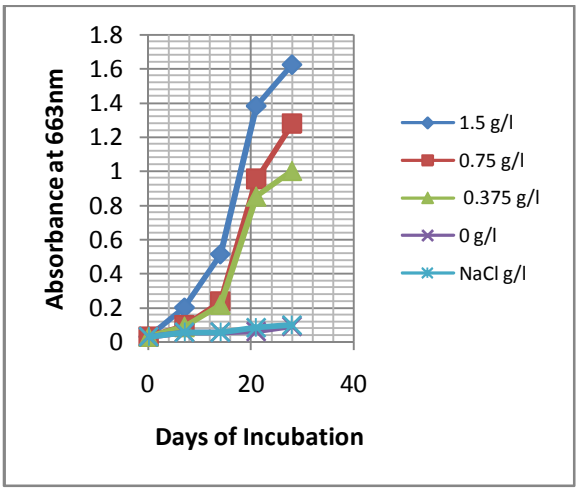

Fig. 1. Growth of *C. vulgaris* in medium supplemented with variable nitrogen Concentration.

An increasing trend was observed from zero days to 21st day after which a stationary phase was attained on 28th day.

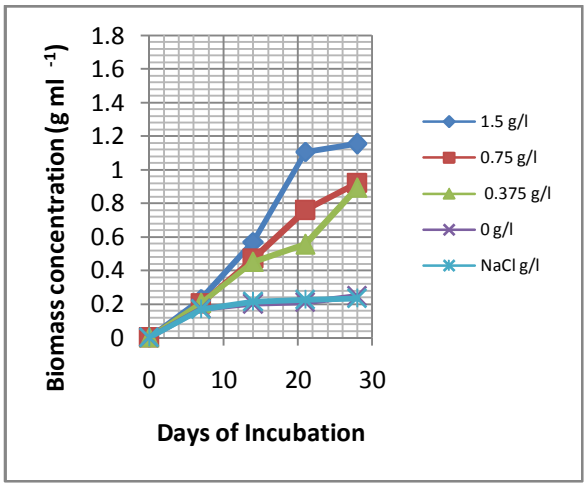

Fig. 2. Biomass of *C. vulagris* in medium supplemented with variable nitrogen Concentration.

The biomass yield also showed a similar trend like growth. This is depicted in figure 2. The maximum biomass with respect to zero days showed highest increase on day 28th with 115 fold increases in control culture, minimum biomass was observed in 0.0g/l $NaNO_3$ medium and medium with $NaNO_3$ replaced by NaCl with only 25 and 23 fold increase respectively from zero days to 28th day.

B. Effect on Lipid Content

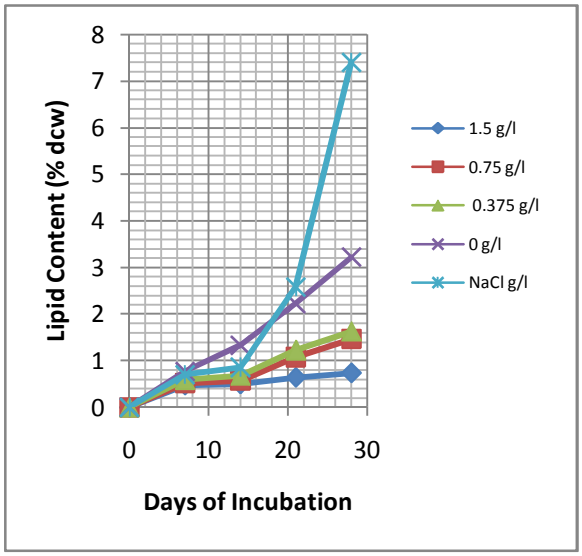

Fig. 3. Lipid Content of *C. vulagris* in medium supplemented with variable Nitrogen concentration.

An additional investigation was carried out to study the effect of reduction and limitation of $NaNO_3$ concentration in medium on Lipid pool of *C. vulgaris*. This is depicted in figure 3. The results showed a very significant ($P < 0.05$) increase in lipid content with decrease in $NaNO_3$ concentration. The culture growing in 0.375 g/l $NaNO_3$ showed 2 fold increases in lipid content with respect to control culture (1.5 g/l $NaNO_3$). The culture growing in 0.0g.l $NaNO_3$ medium resulted in 3 fold increase in lipid pool, whereas the most significant increase of 10 folds was observed in culture growing in medium in which $NaNO_3$ is replaced by NaCl.

C. Effect of Phosphorus deficiency and limitation on Growth and Biomass

Figure 4 shows the result of experiments conducted at varied phosphorus limitation and deficiency resulted in significant ($P<0.05$) increase in growth, biomass and lipid content. The growth significantly increased from zero day to 21st day on day 28th stationary phase was attained maximum growth was observed in control culture (0.04 g/l K_2HPO_4) with 42 fold increase from zero day.

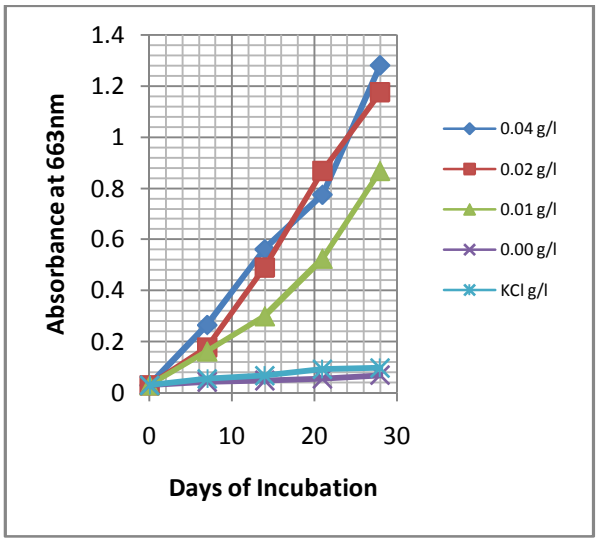

Fig. 4. Growth of *C. vulagris* in medium supplemented with variable Phosphorus Concentration.

On day 28th minimum growth was observed in 0.0 g/l K_2HPO_4 and medium with K_2HPO_4 replaced by KCl with only 2 fold and 3 fold increase respectively from zero days.

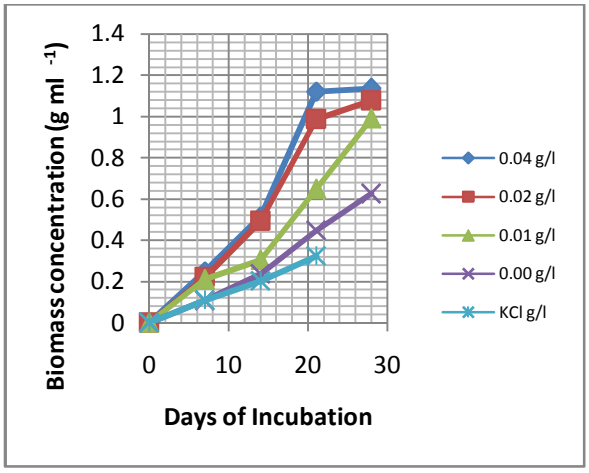

Fig. 5. Biomass of *C. vulagris* in medium supplemented with variable Phosphorus Concentrate.

Figure 5 showed increase of biomass from zero days to 21st day and stationary phase was attained on 28th day with no further increase in biomass. Maximum biomass yield of 113 fold with respect to zero days was observed in control (0.04g/l K_2HPO_4)and it reduced as phosphorus concentration was decreased. Minimum yield of 41 fold with respect to zero days was observed in medium in which K_2HPO_4 was replaced by KCl.

As shown in figure 6, Lipid content in *C. vulgaris* showed significant increase with number of days of incubation.

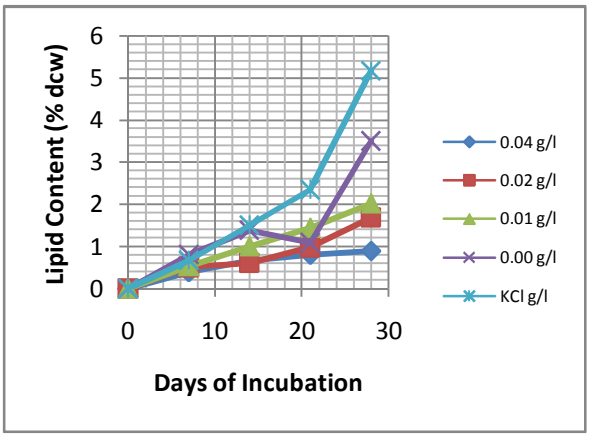

Fig. 6. Lipid variation of *C. vulagris* in medium supplemented with variable Phosphorus Concentrate.

Lipid pool increased from zero days to 28th day. As K_2HPO_4 concentration was decreased the lipid content increased. Minimum lipid content was observed in control culture (0.04 g/l K_2HPO_4). Maximum lipid yield was observed in culture deficient with K_2HPO_4(0.0g/l K_2HPO_4) with 4 fold increase from control culture and in culture where K_2HPO_4 was replaced by KCl with 6 fold increase from control culture.

IV. CONCLUSION

It has been reported that unfavorable conditions like deficiency of minerals (Nitrogen and phosphorus) nutrition in many unicellular green algae impacts growth and biomass and promotes biosynthesis of lipids. Variation of biochemical composition is observed in algae and it depends upon which nutrient is limited and to what degree (Murthy *et al* 2013).

The two major macronutrients for growth and metabolism of algal cells are nitrate and phosphates. Nitrogen is major element for formation of protein and nucleic acid whereas phosphorus is important part of backbone of DNA and RNA. Limitation and deficiency of these key nutrients shifts the metabolic pathway of microorganism (Murthy *et al* 2013).

The experiment conducted to study growth, Biomass and lipid pool of *C. vulagris* with variable nitrogen and phosphorus in medium showed that with decreasing nitrogen and phosphorus growth and biomass decreased but on the other hand we observed the enhanced biosynthesis and accumulation of lipids and TAGs(Thompson 1996, Converti *et al* 2009, Shifrin *et al* 1981, Wang *et al* 2009, Demirbas 2010, Takagi *et al* 2000, Stephenson *et al* 2010, Fogg 1956 and Murthy *et al* 2013) and reduction of protein content (Morris *et al* 1974, Kilham *et al* 1997, Fogg 1956, Heraud *et al* 2005 and Murthy *et al* 2013).

One of the reasons stated by N Malik *et al.;*2012 is that under nitrogen limitation NADPH consumption decreases due to unavailability of nitrogen pool thus there is excess NADPH in cells(Lee *et al* 2001, Mallik *et al* 2011). This result in an increase in pool of acetyl CoA which could not enter TCA cycle due to high concentration of NADPH which inhibit the enzyme citrate synthase (Doi *et al* 1990, Mallik *et al* 2011). Acetyl CoA might then be converted into mallonyl CoA catalyzed by acetyl CoA corboxylase (Accase) the central carbon donor for fatty acid synthesis (Ohlrogge and Browse 1995, Mallik *et al* 2011).

Phosphorus limitation also leads to accumulation of lipids (Murthy *et al* 2013).Still further investigations are required to know how phosphorus limitation works to enhance lipid pool.

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