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EFFECT OF NITROGEN, PHOSPHORUS CONCENTRATIONS, PH AND SALINITY RANGES ON GROWTH, BIOMASS AND LIPID ACCUMULATION OF *CHLORELLA VULGARIS*

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Keywords:

Chlorella vulgaris, Microalgae, Growth, Biomass, Lipids, Nitrogen, Phosphorus, pH, Salinity, Biomass.

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ABSTRACT: Microalgal like *Chlorella 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 concentration of both phosphorus and nitrogen where decreased in medium. At the same time, the effect of variation in pH and saline ranges on growth, biomass and lipid content of *C.vulgaris* were also evaluated. The growth, biomass and lipid content showed a significant difference due to the variation in pH and saline range. The growth and lipid content was maximum at control range i.e pH 7 with respect to 6, 8 and 9. On the other hand we could see that with the increase of salinity from 0.1M to 0.25 M the growth decreased whereas the lipid content showed an increasing trend.

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 [1, 2, 3], and food for aquaculture, pharmaceutical products, and biofuel productions [3, 4].

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There will a substantial change in quality and quantity of lipids within the cells and they vary as a result of changes in growth conditions (light, temperature etc.) or nutrient media characteristics (concentration of Nitrogen, Phosphorus and Iron) [5, 6, 3]. Biodiesel which is now widely accepted as the alternative to standard diesel and Algae is one of the promising source for biodiesel production [7]. 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 [7, 8].

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 [9, 7]. There are quite a few species of microalgae which have high content of oil and can be manipulated to produce more oil [10, 7]. 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 [11, 12]. Nitrogen deficiency in algal culture enhances the biosynthesis and accumulation of lipids [13, 3, 14, 15, 16, 12] and triglycerides [17, 18, 12]. The limitation of nitrogen can be considered as an efficient environmental pressure to enhance lipid accumulation [19, 20, 21]. Other effects of nitrogen reduction are Carbon dioxide fixation, decrease in oxygen evolution, Chlorophyll content and tissue production [22, 23, 12].

Other than Nitrogen, Phosphorus is important component required for normal growth and development of algal cells [11, 12]. It is studied that 1% of dry weight of algae is constituted by phosphorus [25, 12]. 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 [23, 12].

The research has been done on many algal species for biodiesel production. *Chlorella* was selected as test organism as it has great potential to be a resource for biodiesel production due to its fast growth and easy cultivation. However, lipid content in *Chlorella* under general growth conditions is 14-30% by weight of dry biomass [4, 5]. Certain culture conditions could result in higher quantities of lipids to find if these could promote the biomass productivity or lipid accumulation under lab conditions, the effect of nutrients and other growth parameters such as pH values and salinity ranges *viz.* on growth and lipid content in marine strain *Chlorella vulgaris* should be investigated comprehensively to further validate this study.

This study is conducted to investigate the growth response, biomass and lipid content of *C. vulagris* by varying the concentration of Nitrate $NaNO_3$, Phosphorus K_2HPO_4, pH and salinity ranges.

MATERIAL AND METHODS:

Culture 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 ± 2º 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: 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.

Optimization of medium on different Nitrogen, Phosphorus, pH and Salinity 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 1.5 g/l, 0.75 g/l and 0.375 g/l and 0.0 g/l respectively and $NaNO_3$ replaced by NaCl), and another set of triplicates for different phosphorus concentration (K_2HPO_4as phosphorus source at 0.04 g/l, 0.02 g/l, 0.01 g/l and 0.0g/l respectively and K_2HPO_4replaced by KCl) the pH was set at 7.0.

At the same time two set of triplicates of cultures were also grown on optimized medium at various ranges of pH (6.0–9.0 at an interval of 1.0) and salinity ranges (0.1M–0.25M at an interval of 0.05, for saline cultures the pH was set at 7.0). All cultures were kept in a culture room at 25 ± 2º 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.

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.

Dry cell weight measurement: Dry cell weight (dcw) was determined by gravimetrical method [26]. 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.

Extraction and Estimation of lipid from algal biomass:

Extraction of lipid was done following the protocol of Bligh and Dyer (1959) [24]. 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.

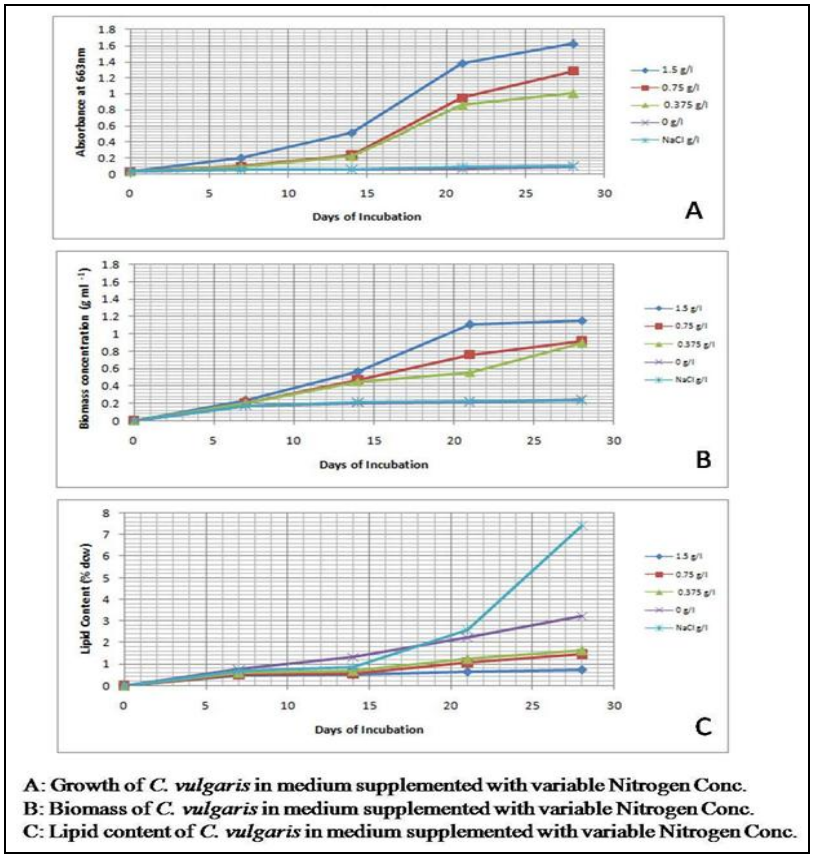
Statistical Analysis:

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.

RESULT AND OBSERVATION:

Effect of Nitrogen on Growth and Biomass:

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



A: Growth of *C. vulgaris* in medium supplemented with variable Nitrogen Conc.
B: Biomass of *C. vulgaris* in medium supplemented with variable Nitrogen Conc.
C: Lipid content of *C. vulgaris* in medium supplemented with variable Nitrogen Conc.

FIG. 1: EFFECT OF VARIABLE NITROGEN CONCENTRATION ON GROWTH, BIOMASS AND LIPID CONTENT OF *CHLORELLA VULGARIS*

The growth was significantly affected; maximum algal density was observed in control culture (1.5 g/l $NaNO_3$). On day 28th 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 NaNO3, 0.0g/l $NaNO_3$ and $NaNO_3$ replaced by NaCl. An increasing trend was observed from zero days to 21st day after which a stationary phase was attained on 28th day.

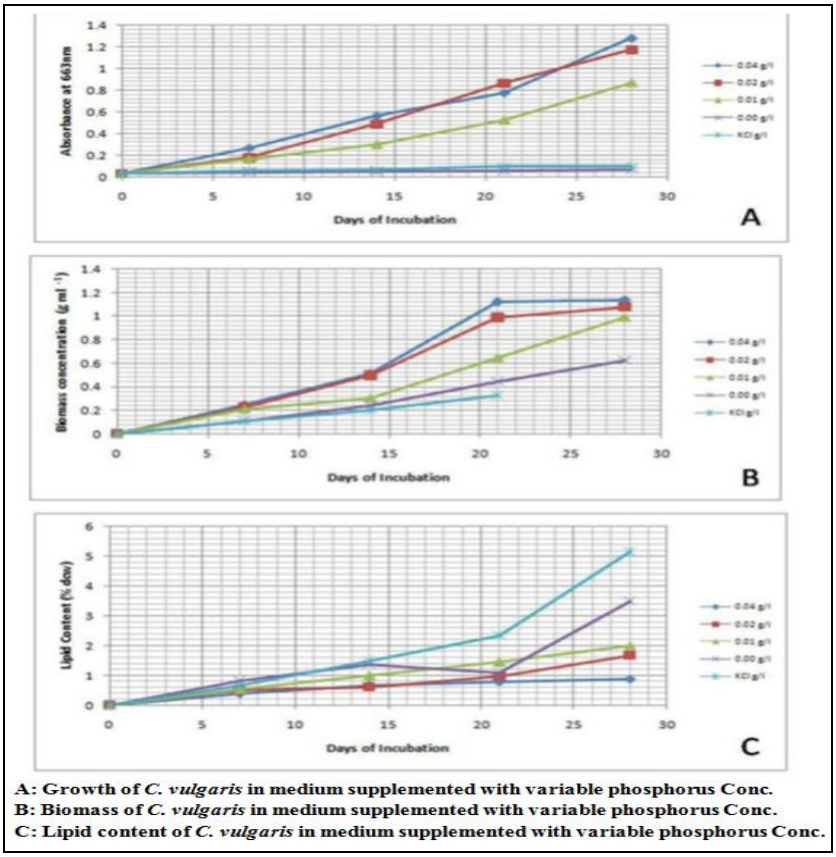
The biomass yield also showed a similar trend like growth. This is depicted in **Fig.1 (B).** The maximum biomass with respect to zero days showed highest increase on day 28th with 115 fold increases in control culture, minimum biomass was observed in 0.0g/l $NaNO_3$ medium and medium with $NaNO_3$ replaced by NaCl with only 25 and 23 fold increase respectively from zero days to 28th day.

Effect of Nitrogen on Lipid Content:

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 **Fig. 1 (C).** 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.

Effect of Phosphoruson growth and biomass:

Fig.2(A) 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. 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.



A: Growth of *C. vulgaris* in medium supplemented with variable phosphorus Conc.
B: Biomass of *C. vulgaris* in medium supplemented with variable phosphorus Conc.
C: Lipid content of *C. vulgaris* in medium supplemented with variable phosphorus Conc.

FIG.2: EFFECT OF VARIABLE PHOSPHORUS CONCENTRATION ON GROWTH, BIOMASS AND LIPID CONTENT OF *CHLORELLA VULGARIS*

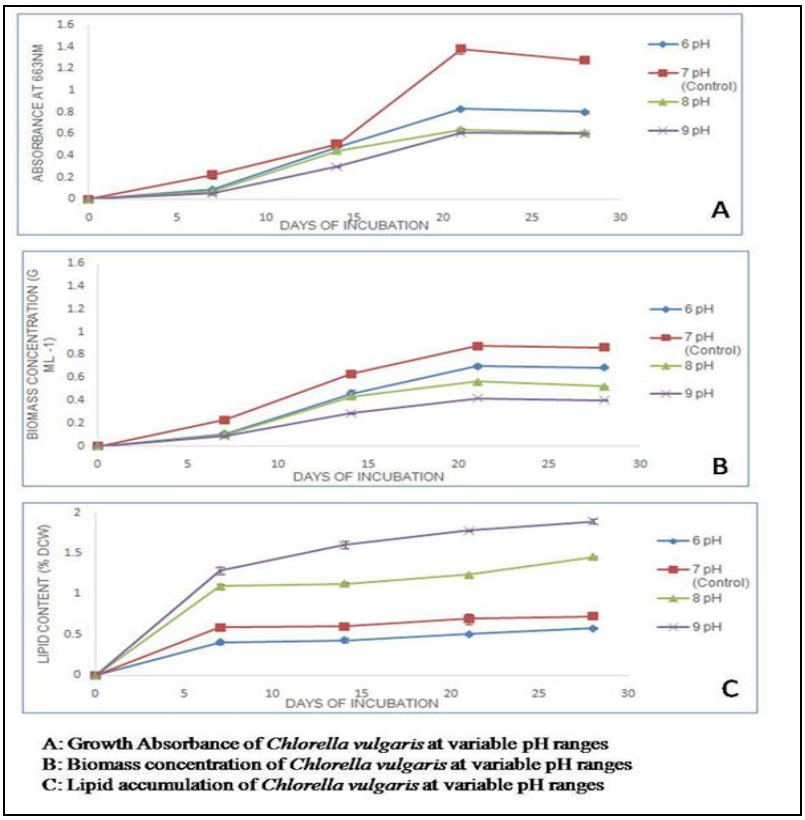
Fig.2 (B) showed increase of biomass from zero days to 21st day and stationary phase was attained on 28th day with no further increase in biomass. Maximum biomass yield of 113 fold with respect to zero days was observed in control (0.04g/l K_2HPO_4) and it reduced as phosphorus concentration was decreased. Minimum yield of 41 fold with respect to zero days was observed in medium in which K_2HPO_4 was replaced by KCl.

Effect of Phosphorus on Lipid Content:

Furthermore, investigation was also carried out to study the effect of reduction and limitation of phosphorus concentration in medium on Lipid pool of *C.vulgaris*. As shown in **Fig. 2 (C),** Lipid content in *C.vulgaris* showed significant increase with number of days of incubation. 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.

Effect of varied pH:

The Growth curve of *C.vulgaris* is shown in **Fig. 3 (A),** the maximum algal density was statistically significant ($P < 0.05$) in control culture (pH 7) with respect to pH 6, 8 and 9. In all the cultures with variable pH marked increase from 7th day to 21st day was observed and decline in algal density was seen on day 28. The control culture with pH 7 showed 39.9% increase from culture with pH 6, 53% increase from culture set on pH 8 and 56% increase from culture set on pH 9 on day 21. Day 28 exhibited a marked declining trend. In control culture (pH 7) Algal density exhibited increase of 83% from 7day to 21 day. Experiments were performed in triplicates under optimized conditions to determine the biomass yield, presented in **Fig.3 (B),** the most significant increase in biomass yield to 87.5 % was obtained in control culture on day 21. As compared to pH 6,8and 9 control culture showed statistically significant ($P < 0.05$) biomass yield. pH 7 and pH 6 showed difference of 53.7% whereas pH 8 and pH 9 in comparison to control culture showed 57.4% and 62.8% difference respectively on day 7. The biomass yield kept on increasing till day 21st after that no further increase was observed.



A: Growth Absorbance of *Chlorella vulgaris* at variable pH ranges
B: Biomass concentration of *Chlorella vulgaris* at variable pH ranges
C: Lipid accumulation of *Chlorella vulgaris* at variable pH ranges

FIG.3: EFFECT OF VARIABLE pH RANGES ON GROWTH, BIOMASS AND LIPID CONTENT OF *CHLORELLA VULGARIS*

Effect of pH on Lipid Accumulation:

Experiments conducted at varied pH to study lipid accumulation in *C.vulgaris* resulted in increase/decrease in lipid yield with respect to control (pH 7) shown in **Fig.3(C**). pH 6 showed decrease in lipid pool with respect to pH 7. Lipid yield showed increase of 124 folds and 178 folds respectively in pH 8 and pH 9 on day 21 and decline was observed on day 28.

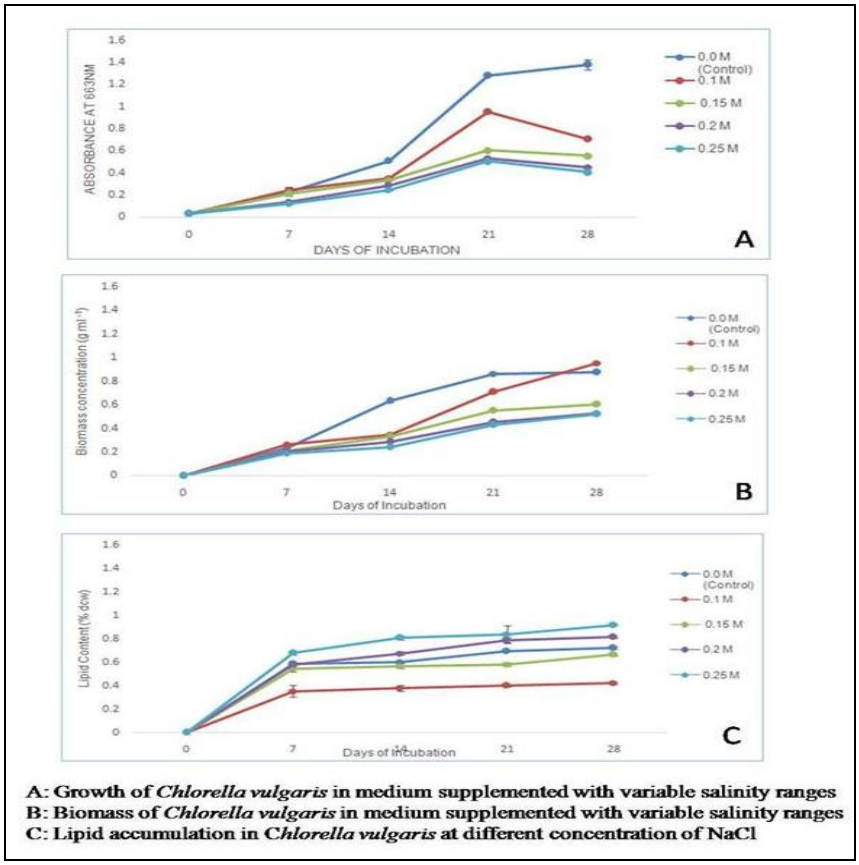
Effect of Salinity on Growth:

The growth curve of *C. vulgaris* with respect to variable saline ranges is shown in **Fig.4 (A).** 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 medium was increased from 0.1M to 0.25M. The 46.1 fold increase was observed on day 21st in the control culture with respect to zero days. In all the mediums with variable salinity range the increasing trend of growth was observed from zero day to 21st day after which decline was observed on 28th day. Lowest growth was observed in culture with 0.25M salinity, an increase of 16.9 fold was observed with respect to zero day on day 21st. The biomass also statistically significant ($P < 0.05$) showed similar trend as shown by the growth *i.e* highest biomass was observed in control culture and minimum in culture growing in 0.25 M saline medium as shown in **Fig. 4 (B).** The increasing trend was observed from zero days to 28th day. Highest 87 fold increase was observed in control culture on day 28th. 0.25 M saline culture showed only 52 fold increase with respect to zero days.

Effect on Lipid Content:

The lipid accumulation in *C. vulgaris* at different saline ranges was studied in triplicates under optimized conditions and it differed significantly ($P < 0.05$). The highest lipid yield was observed in culture growing in 0.25 M saline medium and lowest was observed in control culture (0.0M saline medium) as shown in **Fig.4 (C).**

The yield increased from zero days to 28th day. On 2th day 91 fold increase was observed in 0.25 M saline culture with respect to 81 fold, 72 fold, 66 fold and 42 fold increase in 0.2M, 0.15 M, 0.1 M and control culture i.e 0.0 M respectively.



A: Growth of *Chlorella vulgaris* in medium supplemented with variable salinity ranges
B: Biomass of *Chlorella vulgaris* in medium supplemented with variable salinity ranges
C: Lipid accumulation in *Chlorella vulgaris* at different concentration of NaCl

FIG. 4: EFFECT OF VARIABLE SALINITY RANGES ON GROWTH, BIOMASS AND LIPID CONTENT OF *CHLORELLA VULGARIS*

DISCUSSION: 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 [12].

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 [12].

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 [13, 3, 14, 15, 16, 17, 18, 28, 12] and reduction of protein content [29, 30, 28, 31 12]. One of the reasons stated by [32] is that under nitrogen limitation NADPH consumption decreases due to unavailability of nitrogen pool thus there is excess NADPH in cells [32, 33]. 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 [32, 34]. Acetyl CoA might then be converted into mallonyl CoA catalyzed by acetyl CoA corboxylase (Accase) the central carbon donor for fatty acid synthesis [32, 35]. Phosphorus limitation also leads to accumulation of lipids [12]. Still further investigations are required to know how phosphorus limitation works to enhance lipid pool.
The sudden change in growth conditions and stresses like deficiency of nutrients or high salinity in some algae has significant impact on growth biomass and lipid yield.

The experiments conducted to study growth of *C.vulgaris* with variable pH showed that maximum growth occurs at control pH 7 as the pH was increased to 8 and 9 the decrease in algal density was observed which can be correlated to the fact that higher pH limits carbon availability from CO_2, hence algal growth is suppressed [36, 37, 12]. The biomass extracted from all variable pH ranges also showed highest biomass at control pH7.

This is directly proportional to algal growth. Decreased algal growth observed at alkaline pH is due to the fact that the mother cell, cell wall flexibility increases which in turn prevents its rupture and inhibits autospore release thus increasing the time for cell cycle completion [38, 12]. In terms of days both growth and biomass had shown increasing trend from 0 to 21 day after which the declining trend was observed.

The lipid accumulation at pH 9 was highest and as the pH was decreased the lipid accumulation also showed decrease because alkaline pH indirectly results in increase in triglyceride accumulation [12, 38].

Exposing algae to lower or higher salinity levels than their natural levels can change growth rate and alter composition. 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 0.0M culture and lowest was observed in culture grown in 0.25 M salinity. This could be associated with the fact that *C.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 21day and declined on day 28. Higher lipid content was however observed in the medium with highest NaCl concentration ie at 0.25 M.

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