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GROWTH KINETICS OF FOUR FRESH WATER ISOLATED MICROALGAE FOR OPTIMAL BIOMASS AND LIPID PRODUCTION USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Microalgae require a wide range of chemical and physical factors for their growth, proliferation, and differentiation. These factors affect the morphology, physiology and metabolic activities of the organism. Therefore, in the present investigation, lipid producing microalgae were isolated and identified, abiotic growth factors such as different autotropic media, pH, nutrients, and media optimization studies were carried out to obtain highest biomass and lipid from microalgae which have the potential for effective biodiesel production. Growth studies were carried out of the isolated algal strains, the optimum temperature for all the experiments was kept at 25 ± 1 ^{o}C and light intensity of 1.2 ± 0.2 klux was maintained. Four strains with highest percentage wise Biomass : Lipid ratio were considered for studies, these strains are Chlorella vulgaris AS-3, Chlorella pyrenoidosa AS-6, Scenedesmus dimorphus AS-13, all 3 strains were isolated from unkal lake and Scenedesmus quadricauda AS-18 was isolated from Rayanaal lake. Out of the four isolated strains Chlorella vulgaris (AS-3) showed a significant increase in lipid content by 3 %. The organism was found to grow well in the optimized BG-11 media with the pH of 6.5 and culture age of 6 weeks and concentration of sodium nitrate, FAC, potassium bicarbonate and magnesium sulphate of 2, 0.24, 0.3 and 0.8 g/L respectively. The data obtained by the by the above growth studies can be used for mass culturing of the organism in invivo conditions in open ponds and furthers studies can be done using different media compositions. Hence, AS-3 was found to be an ideal candidate for biodiesel production and further characterization of the microalgae was carried out.

KEYWORDS: *Biomass, Lipid, Microalgae, Optimisation, Chlorella Vulgaris AS-3*

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INTRODUCTION

Microalgae, which grow in aquatic environments, are simple microscopic heterotrophic and/or autotrophic photosynthetic organisms, ranging from unicellular to multi-cellular in form. In contrast to aquatic plants, microalgae do not have real embryos, roots, stems or leaves. They are able to use water, sunlight, and CO_2 to synthesize biomass through photosynthesis (Ozkurt, 2009). The synthetic biomass can then further be converted into biodiesel, fertilizer and other useful products. More than 40,000 different species of microalgae have been identified (Fuentes-Grunewald *et al.,* 2009), and most of them have a high content of lipids, accounting for between 20 and 50% of their total biomass (Chisti, 2007). Members of green algae are among the most common microalgae, especially in freshwater (Neenan, 1986). Approximately

8,000 species are estimated to be in existence (Sheehan *et al.,* 1998). The simple morphology of the unicellular auto sporic green algae always leads to taxonomical difficulties. The first monograph of algae belonging to Chlorophyceae was put forth by Fott and Novakova, (1969), based on morphological features, established the nomenclatural types, but also showed the phenotypic plasticity of the investigated species. Microalgal growth, biomass, and lipid production are mainly controlled by light, temperature, available carbon dioxide, pH and nutrients (Tzovenis *et al.,* 1997; Zhu *et al.,* 1997). The biomass content of microalgae can be affected by such factors as growth rate, environmental conditions and life cycle (Richmond, 1986). The overall reaction process can be summarized as follows: $6CO_2$+ $12H_2O$ + photons = $C_6H_{12}O_6$ + $6O_2$ + $6H_2O$, Apart from sunlight and CO_2, water, nitrogen, and phosphorus are the three major inputs for algae growth. Major nutrients such as N and P alone contribute to about10 to 20% of algae biomass (Benemann, 1996), As well as macro-ingredients including N, P, Mg, Na, Ca, and K, micro-ingredients like Mo, Mn, B, Co, Fe and Zn are also required. In general, the growth of microalgae goes through four phases, lag phase, exponential phase, stationary phase, and lysis phase. Specific growth conditions, which vary between microalgae species, are required in order to successfully, cultivate microalgae. Various culture media have been developed for isolation and cultivation of microalgae. Some of them are modifications formulated after a detailed study on the nutrient requirement of the organism. Different autotrophic media has a different effect on the growth of various alga species (Scott *et al.,* 2010). Likewise, various autotrophic media supporting the growth of a wide range of microalgae can be formulated by carefully manipulating major nutrients. Therefore media composition and type are vital and most important, media has to be inclusive of essential components that are found in the natural environment to support and not exclude the growth of isolates of interest. Factors that influence microalgae growth include (Mata *et al.,* 2010) abiotic factors such as light intensity, temperature, O_2, CO_2, pH, salinity, nutrients (N, P, K etc) and toxins; biotic factors such as bacteria, fungi, viruses and competition for abiotic matters with other microalgae species; operational factors such as mixing and stirring degree, width and depth, dilution rate, harvest frequency and addition of bicarbonate. Therefore, in the present investigation, lipid producing microalgae were isolated and identified abiotic growth factors such as different autotropic media, pH, culture age, nutrients, and media optimization studies were carried out to obtain highest biomass and lipid from microalgae which have the potential for effective biodiesel production.

MATERIALS AND METHODS

Isolation and Screening of Microalgae for Biomass and Lipid Content

The algae which were isolated and identified from the water samples of different lakes of Dharwad district, they were further segregated in terms of lipid production.

Isolation and Identification

Isolation and enumeration of algae were done as per the methods described by Welch, (1948), Adoni *et al.,* (1985) and Agarker *et al.,* (1994). Identification was done by consulting the monographs by Philipose, (1967), Gandhi, (1998) and Prescott, (1998). Lipid producing algae were recognized by the list of algae as per Mata *et al.,* (2010).

Biomass Estimation

The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight gram per liter.

Lipid Extraction and Estimation

Extraction of lipid was done following the protocol of Bligh and Dyer, (1959) and Lee *et al.,* (1998).

Nile Red Staining

Microalgal cells (0.5 ml) were collected by centrifugation at 1,500 rpm for 10 min and washed with saline solution (0.5 ml) several times. After the collected cells were re-suspended in the same solution (0.5 ml), the Nile red solution was added to cell suspensions (1:100 v/v) and incubated for 10 min. After washing once, stained microalgal cells were observed by fluorescent microscopy (Lee *et al.,* 1998).

Growth Kinetics of the Isolated Microalgae

In the present investigation growth studies were carried out of the isolated algal strains, the optimum temperature for all the experiments was kept at 25 ± 1°C and light intensity of 1.2 ± 0.2 klux was maintained. Such experimental conditions were optimized by Ye *et al.,* (2012) while experimenting with *Chlorella* and *Scenedesmus* sp. Aeration was provided using a 300 PSI 12 -volt air compressor, with a controlled air flow of 0.2 kg/hr/flask. The carbon-di-oxide content of the air was not measured. Effect of different autotrophic media, lake water, media pH, culture age, different nitrogen sources and different levels of sodium nitrate and ferric ammonium citrate on biomass and lipid production of isolated microalgae was studied. Optimized levels of ferrous ammonium citrate (FAC) and sodium nitrate concentration on biomass and lipid production of isolated microalgae was studied.

Effect of Media Constituents on *Chlorella Vulgaris* (AS-3) Using Response Surface Methodology (RSM)

Influence of media constituents on *Chlorella vulgaris* (AS-3) using Response surface methodology. All the experiments were carried in 250 ml Erlenmeyer flasks containing 100 ml BG-11 medium with varying concentrations of sodium nitrate, ferrous ammonium citrate (FAC), potassium bicarbonate and magnesium sulphate. The culture flasks were incubated for 6 weeks at 25 ± 1°C temperatures with 1.2 ± 0.2 klux with continuous light with aeration.

Experimental Design and Analysis of Data

The experimental design employed was a 4 factors (5 levels of each variable), 27 run Box-Behnken with 3 control points, second order orthogonal design with 1 replications at the centre points, in coded levels of variables (1, -1, 0, 2, -2) (Akhnazarova and Kafarov, 1982). The four independent variables for growth were concentrations of A: sodium nitrate, B: ferrous ammonium citrate (FAC), C: potassium bicarbonate and D: magnesium sulphate. The yield of biomass and lipids in the culture was approximated by a second degree polynomial (Eq.1) with linear, quadratic and interaction effects (in the coded level of variables) using the method of least squares (Little and Hills, 1978).

Eq.1 $Y_{ijk} = b_0 + \sum_{n=0}^{n} bi\ xi + \sum_{i=1}^{n}\sum_{j=1}^{n} b\ ij\ \ xi\ xj + \Sigma$ ijk

The number of variables, denoted by n and i, j and k, are integers. The coefficients of the polynomials are represented by bo, bi, and bij and Îijk is the random error; when i < j, bij represents the interaction effects of the variables xi and xj. The response surface graphs were obtained from the regression equations in the actual level of variables, keeping the response function on the Z axis with X and Y axes representing the two independent variables while keeping the other (third) variable constant at their center (corresponding to 0 level in coded level) points.

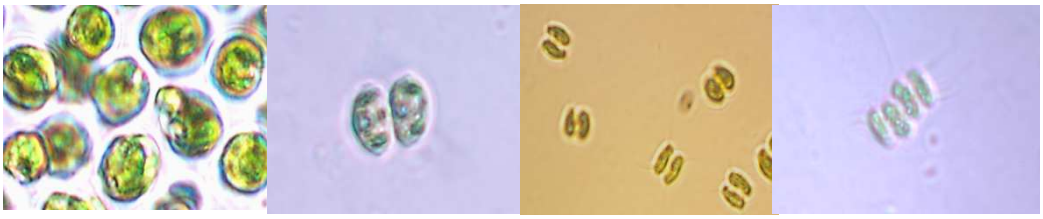
Statistical Analysis

One way Annova test was performed for the statistical analysis using SPSS software. Posthoc = Tukey Alpha P-value ≤ 0.05.

RESULTS

Isolation and Identification of Microalgae from Lake

Out of all 20 lipid producing strains, four strains with highest percentage wise Biomass : Lipid ratio i.e. more than 30% were considered for further growth studies, these strains are *Chlorella vulgaris*(AS-3), *Chlorella pyrenoidosa*(AS-6), *Scenedesmus dimorphis*(AS-13),(Figure.1) these strains were isolated from Unkal lake (L-7) and*Scenedesmus quadricauda* (AS-18), isolated from Rayanaal lake (L-9).The highest percentage of lipids 36.36 % was obtained in algal strain AS-3, followed by 32.91 %in AS-6, 31.01 % in AS-18 and 30.55 % in AS-13. Bright field Microscopic Images of all four isolated strains was taken.



(a) (b) (c) (d)

Figure 1: Bright Field Microscopy Images of Algal Strains with High Biomass and Lipid Content (100X) (a) Chlorella Vulgaris (AS-3), (b) Chlorella Pyrenoidosa (AS-6), (c) Scenedesmus Dimorphus (AS-13) and (d) Scenedesmus Quadricauda (AS-18)

Nile Red Staining of Microalgae Cells

All the results were positive for the presence of lipid granules which were observed in orange or yellow color. Lipid granules in AS-3 were present throughout the cytosol; in AS-6 and AS-13 lipid granules were epicentric. In AS-18 the lipid granules were found to be close to the inner cell wall membrane (Figure.2).

(a) (b) (c) (d)

Figure 2: Nile Red Staining Images of Algal Strains (100X) (a) Chlorella Vulgaris (AS-3), (b) Chlorella Pyrenoidosa (AS-6), (c) Scenedesmus Dimorphus (AS-13) and (d) Scenedesmus Quadricauda (AS-18)

Influence of Autotrophic Media on Biomass and Lipid Production

In the present investigation, Influence of different autotrophic media Chu-13, BG-11, and BBM on biomass and lipid production of the isolated microalgae was studied with using three different conditions Continuous light (CL), 16:8 light and dark cycles (16:8) and Continuous light with aeration (CL+A)**.** Out of the three freshwater algae, media tested, BG-11 media and Continuous light with aeration (CL+A) as growth condition showed good efficiency towards the production of biomass and lipid production in AS-3, AS-6, and AS-18, Were as AS-13 showed good lipid production in

BG-11 media and 16:8 growth condition. AS-3, AS-6, AS-13, and AS-18 showed good lipid production with BG-11 media and Continuous light with aeration (CL+A) as growth condition. (Table 1 and 2).

Table 1: Effect of Different Autotrophic Media on Biomass Content of Isolated Microalgae

Biomass in G/L				
Organism	**Media**	**Continuous Light**	**16: 8 Light and Dark Cycles**	**Continuous Light with Aeration**
***Chlorella vulgaris* (AS-3)**	CHU-13	1.22 ± 0.008*	1.76 ± 0.005*	1.90 ± 0.03*
	BG-11	1.60 ± 0.05*	2.21 ± 0.005*	2.32 ± 0.005*
	BBM	0.81 ± 0.012*	1.26 ± 0.003*	1.42 ± 0.008*
***Chlorella pyrenoidosa* (AS-6)**	CHU-13	0.67 ± 0.008*	0.84 ± 0.008*	0.98 ± 0.003*
	BG-11	1.06 ± 0.03*	1.58 ± 0.005*	1.65 ± 0.003*
	BBM	0.52 ± 0.005*	0.64 ± 0.005*	1.46 ± 0.005*
***Scenedesmus dimorphus* (AS-13)**	CHU-13	0.64 ± 0.003*	0.78 ± 0.011*	0.96 ± 0.003*
	BG-11	1.16 ± 0.03*	1.90 ± 0.05*	1.85 ± 0.003*
	BBM	0.40 ± 0.005*	0.51 ± 0.003*	0.88 ± 0.005*
***Scenedesmus quadricauda* (AS-18)**	CHU-13	0.72 ± 0.005*	0.89 ± 0.008*	0.98 ± 0.006*
	BG-11	0.97 ± 0.005*	1.32 ± 0.003*	1.40 ± 0.0057*
	BBM	0.53 ±0.005*	0.70 ±0.003*	0.97 ±0.003*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Table 2: Effect of Different Autotrophic Media on Lipid Content of Isolated Microalgae

Lipid in G/L				
Organism	**Media**	**Continuous Light**	**16: 8 Light and Dark Cycles**	**Continuous Light with Aeration**
***Chlorella vulgaris* (AS-3)**	CHU-13	0.40 ± 0.01*	0.64 ± 0.008*	0.69 ± 0.003*
	BG-11	0.53 ± 0.06	0.83 ± 0.03*	0.84 ± 0.015*
	BBM	0.27 ± 0.012*	0.43 ± 0.008*	0.46 ± 0.03*
***Chlorella pyrenoidosa* (AS-6)**	CHU-13	0.25 ± 0.011*	0.26 ± 0.005*	0.31 ± 0.005*
	BG-11	0.33 ± 0.008*	0.52 ± 0.0011*	0.52 ± 0.008*
	BBM	0.11 ± 0.006*	0.17 ± 0.008*	0.21 ± 0.008*
***Scenedesmus dimorphus* (AS-13)**	CHU-13	0.18 ± 0.005*	0.21 ± 0.003*	0.25 ± 0.008*
	BG-11	0.33 ± 0.005*	0.53 ± 0.008*	0.56 ± 0.005*
	BBM	0.09 ± 0.006*	0.13 ± 0.003*	0.23 ± 0.005*
***Scenedesmus quadricauda* (AS-18)**	CHU-13	0.20 ± 0.005*	0.26 ± 0.005*	0.30 ±0.003*
	BG-11	0.31 ± 0.005*	0.39 ± 0.008*	0.42 ± 0.003*
	BBM	0.15 ± 0.003	0.16 ± 0.012*	0.27 ± 0.003*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Influence of Different of Ph Levels

Influence of different pH levels (6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) on biomass and lipid production of the isolated microalgae was studied. In the present investigation high amount of biomass was obtained in AS-3 and AS-18 with the media pH of 6.5, were as in AS-6 and AS-13 with pH 7. Low biomass was observed in all the four strains with the alkaline pH of 8- 8.5. High lipid content was observed in all the four isolated strains AS-3, AS-6, AS-13, and AS-18, when the media pH was adjusted to 6.5 and lowest lipid content was found with the media pH at 8.5.(Table.3 and 4).

Table 3: Effect of Different of Ph Levels on Biomass Content of Isolated Microalgae

Organism	Biomass in G/L					
	Ph 6	Ph 6.5	Ph 7	Ph 7.5	Ph 8	Ph 8.5
Chlorella vulgaris (AS-3)	2.20 ±0.057*	2.34 ± 0.012*	2.32 ± 0.012*	1.76 ±0.03*	1.88 ±0.005*	1.73 ±0.008*
Chlorella pyrenoidosa (AS-6)	1.50 ±0.057*	1.6 ± 0.057*	1.64 ± 0.01*	1.43 ±0.008*	1.42 ±0.011*	1.37 ± 0.005*
Scenedesmus dimorphus (AS-13)	1.83 ±0.057*	1.83 ± 0.033*	1.86 ± 0.012*	1.79 ±0.008*	1.68 ±0.008*	1.72 ± 0.006*
Scenedesmus quadricauda (AS-18)	1.34± 0.008*	1.43 ± 0.008*	1.37 ± 0.037*	0.93 ±0.005*	0.85 ± 0.005*	0.77 ± 0.005*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Table 4: Effect of Different of Ph Levels on Lipid Content of Isolated Microalgae

Organism	Lipid in G/L					
	Ph 6	Ph 6.5	Ph 7	Ph 7.5	Ph 8	Ph 8.5
Chlorella vulgaris (AS-3)	0.82 ± 0.005*	0.86 ± 0.014*	0.85 ± 0.012*	0.66 ± 0.005*	0.62 ± 0.005*	0.62 ± 0.015*
Chlorella pyrenoidosa (AS-6)	0.52 ± 0.005*	0.55 ± 0.005*	0.53 ± 0.008*	0.46 ± 0.008*	0.45 ± 0.005*	0.35 ± 0.014*
Scenedesmus dimorphus (AS-13)	0.53 ± 0.008*	0.56 ± 0.011*	0.54 ± 0.012*	0.52 ± 0.014*	0.40 ± 0.006*	0.32 ± 0.008*
Scenedesmus quadricauda (AS-18)	0.42 ± 0.012*	0.45 ± 0.003*	0.43 ± 0.005*	0.27 ± 0.005*	0.26 ± 0.014*	0.22 ± 0.011*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Influence of Culture Age

Influence of Culture age on biomass and lipid productivity of the isolated microalgae was studied for a period of 7 weeks. Highest biomass and lipid was produced during the 6th week of culturing in all the four strains AS-3, AS-6, AS-13, and AS-18. The biomass and lipid content was found to be constant or declined during the estimation after the 7th week of culturing.(Table. 5 and 6).

Table 5: Effect of Culture Age on Biomass Content of Isolated Microalgae

Organism	Biomass in G/L						
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Chlorella vulgaris (AS-3)	0.48 ± 0.008*	0.71 ± 0.005*	0.85 ± 0.011*	1.00 ± 0.057	1.82 ± 0.015*	2.32 ± 0.008*	2.00 ± 0.008*
Chlorella pyrenoidosa (AS-6)	0.32 ± 0.003*	0.79 ± 0.008*	0.88 ± 0.013*	0.95 ± 0.008*	1.26 ± 0.005*	1.65 ± 0.012*	1.62 ± 0.057*
Scenedesmus dimorphus (AS-13)	0.43 ± 0.005*	0.92 ± 0.005*	1.00 ± 0.066*	1.52 ± 0.005*	1.77 ± 0.012*	1.86 ± 0.008*	1.80 ± 0.06*
Scenedesmus quadricauda (AS-18)	0.59 ± 0.008*	0.68 ± 0.005*	0.93 ± 0.005*	1.15 ± 0.012*	1.20 ± 0.015*	1.40 ± 0.005*	1.35 ± 0.008*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Table 6: Effect of Culture Age on Lipid Content of Isolated Microalgae

Organism	Lipid in G/L						
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
***Chlorella vulgaris* (AS-3)**	0.12 ± 0.014*	0.21 ± 0.005*	0.33 ± 0.015*	0.33 ± 0.012*	0.65 ± 0.005*	0.86 ± 0.008*	0.81 ± 0.008*
***Chlorella pyrenoidosa* (AS-6)**	0.07 ± 0.005*	0.23 ± 0.014*	0.28 ± 0.005*	0.32 ± 0.015*	0.41 ± 0.005*	0.54 ± 0.005*	0.52 ± 0.008*
***Scenedesmus dimorphus* (AS-13)**	0.11 ± 0.005*	0.23 ± 0.005*	0.27 ± 0.003*	0.45 ± 0.005*	0.51 ± 0.005*	0.55 ± 0.003*	0.54 ± 0.005*
***Scenedesmus quadricauda* (AS-18)**	0.11 ± 0.014*	0.22 ± 0.005*	0.25 ± 0.005*	0.31 ± 0.003*	0.35 ± 0.008*	0.41 ± 0.012*	0.38 ± 0.003*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Influence of Different Nitrogen Sources

Influence of different nitrogen sources (sodium nitrate, potassium nitrate, ammonium nitrate, calcium nitrate and urea) on biomass and production of isolated microalgae was studied. In the present investigation, by using sodium nitrate as nitrogen source, good biomass was produced in all the four strains AS-3, AS-6, AS-13 and AS-18 followed by potassium nitrate and calcium nitrate. Poor results for biomass production were observed when ammonium nitrate was used as nitrogen source. By using sodium nitrate as nitrogen source good lipid was produced in all the four strains AS-3, AS-6, AS-13 and AS-18 followed by ammonium nitrate in AS-6, potassium nitrate in AS-3. Poor results for lipid production were observed when calcium nitrate was used as nitrogen source.(Table.7 and 8).

Table 7: Effect of Different Nitrogen Sources on Biomass Content of Isolated Microalgae

Organism	Biomass in G/L				
	Calcium Nitrate	Potassium Nitrate	Sodium Nitrate	Urea	Ammonium Nitrate
***Chlorella vulgaris* (AS-3)**	1.75 ± 0.005*	1.72 ±0.057*	2.32 ± 0.008*	1.80 ± 0.008*	0.68 ±0.005*
***Chlorella pyrenoidosa* (AS-6)**	0.85 ±0.005*	0.97 ±0.003*	1.63 ± 0.013*	1.30 ±0.057*	0.56 ±0.011*
***Scenedesmus dimorphus* (AS-13)**	0.67 ±0.005*	0.74 ±0.011*	1.86 ± 0.014*	1.54 ± 0.015*	0.41 ±0.008*
***Scenedesmus quadricauda* (AS-18)**	1.12 ±0.014*	0.71 ±0.006*	1.33 ± 0.066	1.12 ± 0.011*	0.32 ±0.006*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Table 8: Effect of Different Nitrogen Sources on Lipid Content of Isolated Microalgae

Organism	Lipid in G/L				
	Calcium Nitrate	Potassium Nitrate	Sodium Nitrate	Urea	Ammonium Nitrate
***Chlorella vulgaris* (AS-3)**	0.58 ± 0.005*	0.52 ± 0.005*	0.84 ± 0.015*	0.63 ± 0.006*	0.15 ± 0.003*
***Chlorella pyrenoidosa* (AS-6)**	0.27 ± 0.005*	0.31 ± 0.003*	0.53 ± 0.008*	0.41 ± 0.008*	0.10 ± 0.005*
***Scenedesmus dimorphus* (AS-13)**	0.15 ± 0.003*	0.18 ± 0.005*	0.56 ± 0.012*	0.40 ±0.003*	0.07 ± 0.003*
***Scenedesmus quadricauda* (AS-18)**	0.32 ± 0.005*	0.22 ± 0.015*	0.42 ± 0.008*	0.26 ± 0.011*	0.07 ± 0.005*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Influence of Different Levels of Sodium Nitrate

Influence of different levels of sodium nitrate (1, 1.5, 2, 2.5 and 3 g/L) on biomass and lipid production of isolated microalgae was studied. Significantly highest biomass was obtained with the sodium nitrate concentration of 2 g/L in AS-3, AS-6 and AS-13, were as good biomass was obtained in AS-18 with the sodium nitrate concentration of 1.5 g/L. Significantly highest lipid was obtained with the sodium nitrate concentration of 2 g/L in all four isolated algae AS-3, AS-6, AS-13, and AS-18. Low biomass and lipid were obtained with the sodium nitrate concentration of 1 g/L.(Table.9 and 10).

Table 9: Effect of Different Levels of Sodium Nitrate Concentration on Biomass Content of Isolated Microalgae

Organism	Concentration of Sodium Nitrate				
	1 G/L	1.5 G/L	2 G/L	2.5 G/L	3 G/L
***Chlorella vulgaris* (AS-3)**	1.54 ± 0.012*	2.31 ± 0.008*	2.34 ± 0.015*	1.85 ± 0.005*	1.72 ± 0.006*
***Chlorella pyrenoidosa* (AS-6)**	0.73 ± 0.033*	1.65 ± 0.008*	1.67 ± 0.01*	1.12 ± 0.014*	0.82 ± 0.012*
***Scenedesmus dimorphus* (AS-13)**	0.97 ± 0.006*	1.85 ± 0.003*	1.86 ± 0.008*	1.24 ± 0.011*	1.33 ± 0.01*
***Scenedesmus quadricauda* (AS-18)**	0.73 ± 0.008*	1.42 ± 0.011*	1.41 ± 0.005*	1.27 ± 0.003*	1.36 ± 0.012*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Table 10: Effect of Different Levels of Sodium Nitrate Concentration on Lipid Content of Isolated Microalgae

Organism	Concentration of Sodium Nitrate				
	1 G/L	1.5 G/L	2 G/L	2.5 G/L	3 G/L
***Chlorella vulgaris* (AS-3)**	0.48 ± 0.005*	0.85 ± 0.005*	0.86 ± 0.006*	0.66 ± 0.008*	0.52 ± 0.008*
***Chlorella pyrenoidosa* (AS-6)**	0.23 ± 0.005*	0.54 ± 0.011*	0.56 ± 0.011*	0.33 ± 0.008*	0.22 ± 0.012*
***Scenedesmus dimorphus* (AS-13)**	0.27 ± 0.003*	0.53 ± 0.013*	0.67 ± 0.091	0.35 ± 0.003*	0.31 ± 0.006*
***Scenedesmus quadricauda* (AS-18)**	0.15 ± 0.005*	0.42 ± 0.008*	0.45 ± 0.015*	0.32 ± 0.008*	0.33 ± 0.01*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Influence of Different Levels of Ferrous Ammonium Citrate

Influence of different levels of ferrous ammonium citrate (FAC) (0.03, 0.06, 0.12, 0.24 and 0.48 g/L) on biomass and lipid production of isolated microalgae was studied. Significantly highest biomass and lipid were obtained with FAC concentration of 0.24 g/L in AS-3, AS-6 and AS-18 and with FAC conc^{n} of 0.48 g/L in AS-13. (Table.11 and 12).

Table 11: Effect of Different Levels of Ferrous Ammonium Citrate (FAC) Concentration on Biomass Content of Isolated Microalgae

Organism	Concentration of Ferrous Ammonium Citrate (FAC)				
	0.03 G/L	0.06 G/L	0.12 G/L	0.24 G/L	0.48 G/L
***Chlorella vulgaris* (AS-3)**	1.52± 0.008*	2.33± 0.014*	2.37± 0.003*	2.46± 0.057*	2.30± 0.008*
***Chlorella pyrenoidosa* (AS-6)**	0.72± 0.012*	1.72± 0.015*	1.77± 0.008*	1.65± 0.008*	1.84± 0.005*
***Scenedesmus dimorphus* (AS-13)**	0.85± 0.005*	1.86± 0.033*	2.13± 0.088*	2.11± 0.06	2.16± 0.066
***Scenedesmus quadricauda* (AS-18)**	0.54± 0.023*	1.43± 0.012*	1.45± 0.012*	1.53± 0.057	1.50± 0.005*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Table 12: Effect of Different Levels of Ferrous Ammonium Citrate (FAC) Concentration on Lipid Content of Isolated Microalgae

Organism	Concentration of Ferrous Ammonium Citrate (FAC)				
	0.03 G/L	0.06 G/L	0.12 G/L	0.24 G/L	0.48 G/L
***Chlorella vulgaris* (AS-3)**	0.55± 0.005*	0.88± 0.008*	0.94± 0.033*	0.96± 0.006*	0.90± 0.014*
***Chlorella pyrenoidosa* (AS-6)**	0.18± 0.005*	0.55± 0.005*	0.56± 0.012*	0.52± 0.008*	0.57± 0.012*
***Scenedesmus dimorphus*(AS-13)**	0.15± 0.005*	0.57± 0.006*	0.62± 0.011*	0.51± 0.006*	0.64± 0.012*
***Scenedesmus quadricauda* (AS-18)**	0.05± 0.005*	0.45± 0.005*	0.46± 0.008*	0.50± 0.003*	0.48± 0.0033*

Values are Mean ± SEM of 5 Readings, *Significant P ≤ 0.05

Effect of Optimized Levels of Media Components on Biomass and Lipid Production of *chlorella* Vulgaris (AS-3) Using Response Surface Methodology (RSM)

Influence of optimized levels of ferrous ammonium citrate (FAC) (0.24 g/L) and sodium nitrate (2 g/L) on biomass and lipid production of isolated microalgae was studied. A gradual increase in the biomass and lipid content was observed in all the four isolated microalgae. Significant results were obtained with AS-3, where the initial lipid percentage was 36.36 and after the growth and optimization studies, the percentage of lipid increased to 39.43. An increase of 3 % lipid content was observed. The other three strains should minimum or no increase in lipid content.

Optimized levels of sodium nitrate, ferrous ammonium citrate (FAC), potassium bicarbonate and magnesium sulphate on biomass and lipid of *Chlorella vulgaris* AS-3 was also studied. A significant increase in biomass content was observed with the increase in carbonate concentration and constant concentration of sodium nitrate at 2 g/L. the biomass was increased from 2.46 to 2.55 g/L after optimization studies. FAC and magnesium sulphate concentration played in important role in lipid production, the concentration of both the parameters was found to be reciprocal to each other. No significant increase in lipid content was seen after the optimization studies.(Table.13)

In the present investigation, high levels of bicarbonate and nitrate in the range of experimental variables are desirable but their individual effects depend on the concentrations of FAC and sulphate, respectively. Orthogonal experimental design in the coded and actual level of variables with biomass and lipid content of *Chlorella vulgaris* (AS-3) is depicted in table.14. ANOVA table for the yield of biomass of *Chlorella vulgaris* (AS-3) in the Coded level of variables is depicted in table.15. Where as table.16 show the ANOVA for the yield of lipid of *Chlorella vulgaris* (AS-3) in a coded level of variables. The regression equation for the response function of biomass and lipid yields of Chlorella *vulgaris* (AS-3) in the actual level of variables is depicted in table.17.The optimum medium conditions of sodium nitrate, ferrous ammonium citrate (FAC), potassium bicarbonate and magnesium sulphate concentration were found to be, 2.00, 0.02, 0.02 and 0.08 g/L respectively for biomass yield of 2.55 g/L. whereas in the control BG-11 medium yielded only 2.47 g/L of biomass. A similar observation was done by Dayanand *et al.,*(2010) while working with *Botryococcus sp.* and Deng *et al.,*(2011) while working with *Chlamydomonas and Chlorella* species. Banerjee *et al.,*(2002) reported a noticeable increase in the amount of hydrocarbon production in the presence of excess phosphate. However, there are no reports on the interaction effects of phosphate and nitrate or sulphate and citrate. In the present study, no significant increase was observed in terms of lipid concentration. Graph.1 to 8 shows the Biomass and lipid content of *Chlorella vulgaris* (AS-3) as a function of sodium nitrate and potassium bicarbonate, potassium bicarbonate and ferrous ammonium sulphate (FAC), sodium nitrate and ferrous ammonium sulphate (FAC), ferrous ammonium sulphate (FAC) and magnesium sulphate.

Table 13: Influence of Optimized Levels of Ferrous Ammonium Citrate (FAC) Concentration and Sodium Nitrate on Biomass and Lipid Content of Isolated Microalgae

S.No.	Organism	Biomass in G/L	Lipid in G/L	Lipid in %
1	*Chlorella vulgaris* (AS-3)	2.46± 0.005*	0.97± 0.014*	39.43
2	*Chlorella pyrenoidosa* (AS-6)	1.65± 0.005*	0.55± 0.005*	33.33
3	*Scenedesmus dimorphus* (AS-13)	2.16± 0.088	0.66± 0.008*	30.55
4	*Scenedesmus quadricauda* (AS-18)	1.54± 0.006*	0.50± 0.003*	32.46

Note: 1.Optimized Level of Ferrous Ammonium Citrate (FAC) Used Was 0.24 G/L

2. Optimized Level of Sodium Nitrate Used Was 2 G/L

3. Values Are Mean ± SEM of 5 Readings, *Significant $P \leq 0.05$

Table 14: Orthogonal Experimental Design in Coded and Actual Level of Variables with Biomass and Lipid Content of Chlorella Vulgaris (AS-3)

Run Order	Std Order	Centre Points	Coded Level of 'A'	Actual Level of 'A'	Coded Level of 'B'	Actual Level of 'B'	Coded Level of 'C'	Actual Level of 'C'	Coded Level of 'D'	Actual Level of 'D'	Biomass in g/L	Lipid in g/L
1	10	1	-1	3	0	0.255	0	0.03	-1	0.05	2.45	0.93
2	23	1	0	2	0	0.03	0	0.03	0	0.1	2.47	0.97
3	17	1	-1	1	0	0.255	0	0.01	1	0.075	2.44	0.92
4	15	1	-1	2	0	0.03	-1	0.05	0	0.075	2.46	0.96
5	4	1	0	3	0	0.48	1	0.03	1	0.075	2.45	0.95
6	3	1	1	1	0	0.48	1	0.03	0	0.075	2.46	0.96
7	12	1	0	3	1	0.255	-1	0.03	0	0.1	2.44	0.92
8	22	1	0	2	0	0.48	0	0.03	0	0.05	2.46	0.96
9	24	1	-1	2	-1	0.48	0	0.03	0	0.1	2.48	0.96
10	1	1	0	1	-1	0.03	0	0.03	1	0.075	2.44	0.93
11	21	1	1	2	0	0.03	0	0.03	1	0.05	2.47	0.97
12	8	1	-1	2	1	0.255	0	0.05	0	0.1	2.48	0.96
13	11	1	1	1	0	0.255	-1	0.03	0	0.1	2.43	0.93
14	9	1	0	1	0	0.255	0	0.03	0	0.05	2.41	0.9
15	18	1	0	3	1	0.255	0	0.01	1	0.075	2.43	0.92
16	16	1	1	2	-1	0.48	0	0.05	0	0.075	2.47	0.96
17	25	0	0	2	-1	0.255	-1	0.03	0	0.075	2.48	0.95
18	27	0	0	2	1	0.255	0	0.03	-1	0.075	2.49	0.96
19	6	1	1	2	0	0.255	0	0.05	-1	0.05	2.49	0.96
20	19	1	0	1	1	0.255	1	0.05	0	0.075	2.43	0.94
21	20	1	0	3	0	0.255	-1	0.05	-1	0.075	2.42	0.92
22	13	1	1	2	1	0.03	0	0.01	0	0.075	2.46	0.95
23	2	1	0	3	-1	0.03	1	0.03	0	0.075	2.43	0.91
24	7	1	0	2	0	0.255	-1	0.01	1	0.1	2.47	0.97
25	5	1	0	2	-1	0.255	0	0.01	-1	0.05	2.48	0.96
26	26	0	0	2	0	0.255	1	0.03	-1	0.075	2.49	0.98
27	14	1	-1	2	0	0.48	1	0.01	0	0.075	2.47	0.97

A: Sodium Nitrate, B: Ferrous Ammonium Citrate (FAC), C: Potassium Bicarbonate and D: Magnesium sulphate

Table 15: ANOVA Table for the Yield of Biomass of Chlorella Vulgaris (AS-3) in Coded Level of Variables

Source of Variation	Coefficient	SE Coefficient	P-Value
Constant	2.382692	0.028856462	**0.0000**
A	0.065416	0.030034758	**0.0470**
B	-0.002083	0.030034758	0.9457*
C	0.027916	0.030034758	0.3684*
D	-0.002916	0.030034758	0.9240*
AB	-0.001875	0.036784915	0.9601*
AC	0.010625	0.036784915	0.7769*
AD	0.000625	0.036784915	0.9867*
BC	0.000625	0.036784915	0.9867*
BD	-0.000625	0.036784915	0.9867*
CD	0.000625	0.036784915	0.9867*
ABCD	-0.000625	0.036784915	0.9867*

Table 16

	Durbin-Watson Test for Autocorrelation in Residuals	
	DW Statistic	2.027
	P-Value Positive Autocorrelation	0.5441
	P-Value Negative Autocorrelation	0.4803

*** Significant at P ≤ 0.05, A: Sodium Nitrate, B: Ferrous Ammonium Citrate (FAC), C: Potassium Bicarbonate and D: Magnesium Sulphate**

Table 17: ANOVA Table for the Yield of Lipid of Chlorella Vulgaris (AS-3) in Coded Level of Variables

Source of Variation	Coefficient	SE Coefficient	P-Value
Constant	0.9633	0.008164966	**0.0000**
A	-0.0025	0.004082483	0.5517*
B	0.0058	0.004082483	0.1786*
C	0.0008	0.004082483	0.8417*
D	0.0025	0.004082483	0.5517*
AB	0.0025	0.007071068	0.7298*
AC	-0.005	0.007071068	0.4930*
AD	-0.01	0.007071068	0.1827*
BC	-0.005	0.007071068	0.4930*
BD	6.4763	0.007071068	1.0000
CD	-0.0025	0.007071068	0.7298*

Table 18

	Durbin-Watson Test for Autocorrelation in Residuals	
	DW Statistic	1.258
	P-Value Positive Autocorrelation	0.0314
	P-Value Negative Autocorrelation	0.9922

*** Significant at P ≤ 0.05, A: Sodium Nitrate, B: Ferrous Ammonium Citrate (FAC), C: Potassium Bicarbonate and D: Magnesium Sulphate**

Table 19 Regression Equation for the Response Function of Biomass and Lipid Yields of Chlorella Vulgaris (AS-3) in the Actual Level of Variables

Parameter	RSM Regression Model
Biomass yield	(2.382692308) + (0.065416667) * A: sodium nitrate + (-0.002083333) * B: FAC + (0.027916667) * C: potassium bicarbonate + (-0.002916667) * D: magnesium sulphate + (-0.001875) * AB + (-0.010625) * AC + (0.000625) * AD + (0.000625) * BC + (-0.000625) * BD + (0.000625) * CD + (-0.000625) * ABCD
Lipid yield	(0.963333333) + (-0.0025) * A: sodium nitrate + (0.005833333) * B: FAC + (0.000833333) * C: potassium bicarbonate + (0.0025) * D: magnesium sulphate + (0.0025) * AB + (-0.005) * AC + (-0.01) * AD + (-0.005) * BC + (6.4763E-19) * BD + (-0.0025) * CD + (-0.035416667) * AA + (0.004583333) * BB + (-0.002916667) * CC + (-0.002916667) * DD

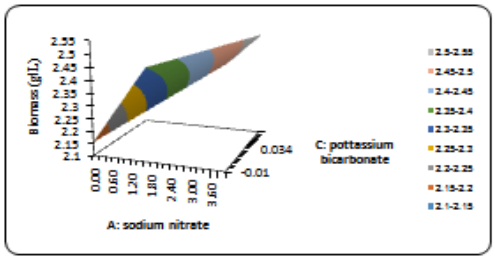


Figure 3: Biomass Content of *Chlorella* Vulgaris (AS-3) as a Function of Sodium Nitrate and Potassium Bicarbonate

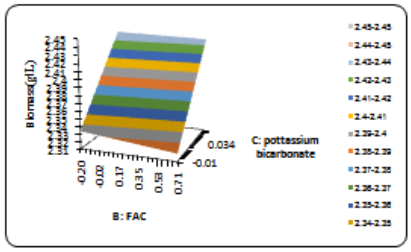


Figure 4: Biomass Content of *Chlorella* Vulgaris (AS-3) as a Function of Potassium Bicarbonate and Ferrous Ammonium Sulphate (FAC)

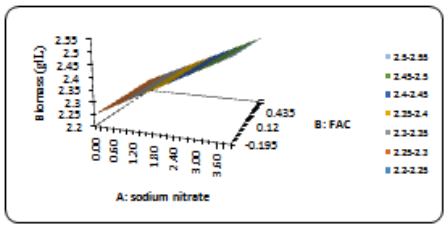


Figure 5: Biomass Content of *Chlorella* Vulgaris (AS-3) as a Function of Sodium Nitrate and Ferrous Ammonium Sulphate (FAC)

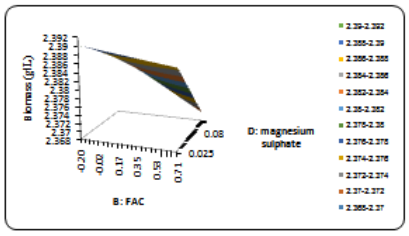


Figure 6: Biomass Content of Chlorella Vulgaris (AS-3) as a Function of Ferrous Ammonium Sulphate (FAC) and Magnesium Sulphate

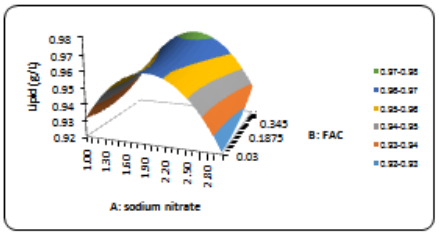


Figure 7: Lipid Content of Chlorella Vulgaris (AS-3) as a Function of Sodium Nitrate and Ferrous Ammonium Citrate (FAC)

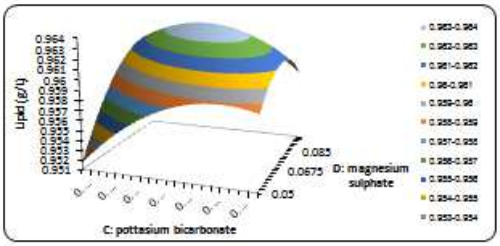


Figure 8: Lipid Content of *Chlorella* Vulgaris (AS-3) as a Function of Potassium Bicarbonate and Magnesium Sulphate

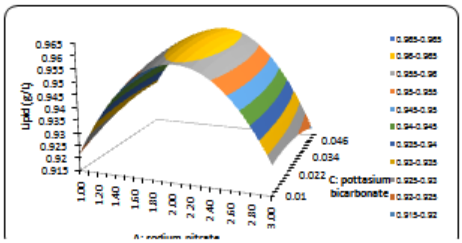


Figure 9: Lipid Content of Chlorella Vulgaris (AS-3) as a Function of Sodium Nitrate and Potassium Bicarbonate

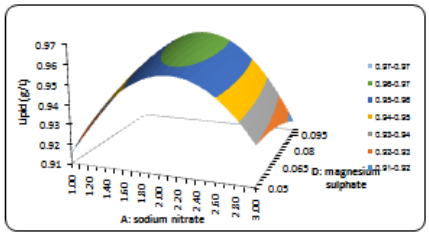


Figure 10: Lipid Content of Chlorella Vulgaris (AS-3) As a Function of Sodium Nitrate and Magnesium Sulphate

DISCUSSIONS

Various culture media have been developed for isolation and cultivation of microalgae. Some of them are modifications formulated after a detailed study on the nutrient requirement of the organism. Different autotrophic media has a different influence on the growth of various alga species (Scott *et al.,* 2010). Likewise, various autotrophic media supporting the growth of a wide range of microalgae can be formulated by carefully manipulating major nutrients. Therefore media composition and type are vital and most important, media has to be inclusive of essential components that are found in the natural environment to support and not exclude the growth of isolates of interest. Biomass and oil production in *B. braunii, Chlorella* and *Scenedesmus species* has been investigated in a number of studies using different media such as Z8, BG11, BBM and CHU 13. Rani *et al.,* (2011) reported very high biomass and lipid content, with the growth of *B. braunii, Chlorella* and *Scenedesmus* in blue-green algae (BG-11) medium. Apart from BG-11, other media have been used for microalgae cultivation. *B. braunii* (SKU: AC-1006 strain) has been cultured in BBM and its modified form (BBM-3N) with higher biomass and lipid production in BBM-3N compared to BBM by Velichkova *et al.,* (2012). Cheng *et al.,* (2011) Suggested that CHU 13 medium was better for *Scenedesmus abundans* growth than either BG-11 or BBM media. Another study conducted by Ambati and Aswathanarayana, (2010) compared the growth of *C.vulgaris* in four autotrophic media (CHU13, Z8, BBM, and BG-11) at 25˚C and found that the highest biomass was produced in the BG11 medium. Reports on other microalgae *Ankistrodesmus falcatus* have shown better growth in BBM compared to BG-11 medium Kalita, *et al.,* (2011). In the present investigation different autotrophic media were tested to check efficient biomass and lipid content, out of the three freshwater algae media tested, BG-11 showed good efficiency towards the high production of biomass and lipid, and among the four tested strains, *Chlorella vulgaris* (AS-3) showed significant results other three strains. *B. braunii* 765 strain was observed to have good growth in BG-11 at 25˚C under the continuous light by Ge*et al.,* (2011). Algal species are more tolerant of the broad range of pH values. Lam and Lee, (2012). Green microalgae can be found in many different pH environments; a limited number of species are able to grow and photosynthesize under very low pH. The most studied species of green algae isolated from acidic environments is *Chlamydomonas acidophila*. Earlier studies were conducted by Lam and Lee, (2012), cultivated *Chlorella vulgaris* in media with pH values of 3, 4, 5, 6,

7, 8 and 9, and came to the conclusion that there was no great difference in the growth characteristics of the algae. Of course, the tolerance ability is species-dependent. In the present investigation, *Chlorella vulgaris* (AS-3), showed significant high biomass and lipid at ph 6.5, the optimum pH levels of all the strains tested were between the range of 6 to 7.5, the biomass and lipid content was found to be decreased at alkaline pH of 8.5. Similar results were obtained by Garcia *et al.,* (2000), Zhu, (2010) and Munir *et al.,* (2015).

Studies on culture age and its pattern of bioaccumulation of nutrients help us to understand the time at which good biomass and lipid can be extracted from a particular algal species. Earlier studies have suggested that the age and previous history of a culture of algae used to seed a new culture may exert an effect on the daughter cultures for a considerable length of time (Mast and Pace, 1938) and even on the grand-daughter cultures (Pratt, 1940). Hence to obtain good biomass and lipid the culture age of the starter culture also is to be considered and formulated. Hence, in the present investigation, 3-week old culture is been used as inoculum for all growth experiments carried out. Fábregas *et al.,* (1989) reported that the lipid content of *D.tertiolecta* increased, during the 6^{th} week of culturing using BBM media. Munir *et al.,* (2015) Observed high growth in terms of fresh weight with 3.23 ± 0.022 g and 3.16 ± 0.021 g fresh weight respectively with *Spirogyrasp.* and *Oedogoniumsp.*, during the 4^{th} week of culturing. Von Dach, (1942) worked on a colorless flagellate, *Astasia klebsii* and obtained high biomass on the 7^{th} week of culturing. Ge*et al.,* (2011) *cultivated B. braunii* 765 strain in BG-11 media and found high biomass and lipid content during the 6^{th} week of culturing. In the present investigation, it was observed that the biomass of the cells also decreased as the age of the cultures increased. *Chlorella vulgaris* (AS-3) showed significant results in terms of biomass and lipids during the 6^{th} week of culturing, in comparison with other three isolated strains. High biomass and lipid content were found in all the four algal strains AS-3, AS-6, AS-13, and AS-18 during the 6^{th} week of culturing. Similar observations were done by Fábregas *et al.,* (1989), Ge *et al.,* (2011) and Munir *et al.,* (2015).

Limitation of algal photosynthetic growth in nature is very often caused by limiting access of nutrients, in particular, the major nutrients nitrogen (N), phosphorus (P) and sulfur (S) (Durmaz, 2007). N is essential for proteins, each amino acid building block contains at least one atom of this major element. In green algae, N is mostly taken up in the form of ammonium (NH_4 +) or nitrate (NO_3−). Lipid content has been reported under both nutrient-replete and deficient growth conditions by Shifrin and Chisholm, (1981) and Roessler, (1990). Neenan, *et al.,* (1986) stated that different nitrogen sources influence the growth rate and biomass production of various algal species. Hence in the present investigation, five different nitrogen sources were used for good biomass and lipid production. Lin and Lin, (2011) reported that the microalgae fed with a combination of urea and sodium nitrate had the highest ash-free dry biomass content with a yield of 4.15 g/. Sodium nitrate can serve as a nitrogen source for many algae, including dinoflagellates, *Chlorella,* and *Chlamydomonas* (Solomon and Glibert, 2008). Li *et al.,* (2013) investigated sodium nitrate as an optimal N source for the heterotrophic cultivation of *C. sorokiniana.* In the present investigation, five different nitrogen sources were used against four isolated strains AS-3, AS-6, AS-13, and AS-18. Significant Results were obtained while using sodium nitrate as nitrogen source good biomass was produced in all the four strains, followed by urea. No much deference in biomass content was observed between sodium nitrate and urea. Similar results were obtained by Grobbelaar, (2004), Solomon *et al.,* (2010) and Li *et al.,* (2013). Similar observations were also made by Sarada *et al.,* (2002) for the green algae *Haematococcus pluvialis*.

Sodium nitrate, as dissolved organic nitrogen, can also be assimilated by algae cells. The concentration of Sodium nitrate is directly proportional to the algal biomass, but an excess amount of nitrates in culture media can inhibit algal growth and lipid production. Illman *et al.,* (2000) found that the nitrogen concentration reduction in the medium increases

the lipid content in all five investigated *Chlorella* strains, among which *C.emersonii*, *C.minutissima,* and *C. vulgaris* gained an increase in lipid content of respectively, 63 %, 56 % and 40 % biomass by dry weight. Macedo and Alegre, (2001) demonstrated that the *Spirulina* lipid content increased approximately 3 times with the nitrogen and temperature decrease. In the present investigation on different levels of sodium nitrate on biomass and lipid production of isolated microalgae**.** Significant results were obtained in all four tested strains, which showed maximum biomass and lipid production at the sodium nitrate concentration of 2 g/L. At the concentration of 1 g/L biomass and lipid was to be lowest in *Chlorella vulgaris* (AS-3), Scenedesmus *dimorphus*(AS-13) and *Scenedesmus quadricauda*(AS-18) and 3 g/L sodium nitrate concentration showed lowest biomass and lipid production in *Chlorella pyrenoidosa* AS-6. Similar results were obtained by Converti *et al.*, (2009) and Tornabene *et al.*, (1983) with *nannochloris sp* and *neochloris oleoabandans* respectively.

Iron is vital for most living organisms. Hence, in the present study iron is used in the form of ferrous ammonium citrate (FAC). In the present investigation, different levels of ferrous ammonium citrate (FAC) on biomass and lipid production of isolated microalgae was analyzed with four isolated strains. Maximum biomass and lipid production were seen at the highest concentration of ferrous ammonium citrate (FAC) used i.e. 0.24 g/L in AS-3, AS-6, and AS-18. Significant results were obtained with *Chlorella vulgaris* (AS-3) with the highest biomass, the biomass and lipid production of all the four strains at FAC concentration of 0.03 g/L was found to be lowest. Similar results were obtained by sung *et al.,* (1998) who cultivated *chlorella sp* KR- with a similar concentration of FAC and by Liu *et al.*, (2006) in case of *chlorella vulgaris* and by Marchetti *et al.,* (2012) with diatom *T. tricornutum.*

Effect of optimized levels of ferrous ammonium citrate (FAC) and sodium nitrate on biomass and lipid production of four isolated strains were studied with the concentration of ferrous ammonium citrate (FAC) at 0.48 g/L and sodium nitrate at 1.5 g/L. Among the various interactions, the positive effect of ferrous ammonium citrate (FAC) x sodium nitrate concentration. There was a significant interaction between the effects of FAC on biomass yield is dependent on the level of nitrate used. The results suggest that the growth of all four strains was optimized to a higher extent. *Chlorella vulgaris* AS-3 showed the significantly, highest biomass and lipid content among all the tested strains. Banerjee *et al.,*(2002) reported a noticeable increase in the amount of hydrocarbon production in the presence of excess iron and nitrates. However, there are no reports on the interaction effects of ferrous ammonium citrate (FAC) and sodium nitrate.

Chlorella sp. was reported to have high adaptability in three types of growth conditions, i.e. mixotrophic, heterotrophic and autotrophic. In the present investigation, media optimization studies were carried out to check optimum levels of nutrients such as sodium nitrate, FAC, potassium bicarbonate and magnesium sulphate. Chen *et al.,* (2010) and Yeh *et al.,* (2010), reported biomass productivity of *Chlorella vulgaris* increased with increasing carbon source followed by slight decrease when the excess of carbon source was supplied. The optimum value achieved in this case however, is higher than the optimum value achieved by other researchers. Yeh *et al.,* (2010) and Chen *et al.,* (2010) studies show that the optimum sodium bicarbonate concentration for biomass productivity of *Chlorella vulgaris*AS-3 was 1.2g/L and 1.5g/L respectively and further increment will lead to biomass productivity reduction. In the present investigation, significant results were obtained in terms of biomass,at 2 g/L concentration sodium nitrate highest biomass was obtained and were as increase in sodium nitrate concentration decreased the biomass content. Increase in the levels of potassium bi -carbonate increased the biomass content. The results also proved that sodium nitrate concentration is reciprocal to that of potassium bi -carbonate concentration. The results obtained in this research exceed and contradict with Yeh *et al.,* (2010) and Chen *et al.,* (2010) research.

In the present investigation, high levels of bicarbonate and nitrate in the range of experimental variables are desirable but their individual effects depend on the concentrations of FAC and sulphate, respectively. The optimum medium conditions of sodium nitrate, ferrous ammonium citrate (FAC), potassium bicarbonate and magnesium sulphate concentration were found to be, 2.00, 0.02, 0.02 and 0.08 g/L respectively for biomass yield of 2.55 g/L. whereas in the control BG-11 medium yielded only 2.47 g/L of biomass. A similar observation was done by Dayanand *et al.,*(2010) while working with *Botryococcus sp.* and Deng *et al.,*(2011) while working with *Chlamydomonas and Chlorella* species. Banerjee *et al.,*(2002) reported a noticeable increase in the amount of hydrocarbon production in the presence of excess phosphate. However, there are no reports on the interaction effects of phosphate and nitrate or sulphate and citrate. In the present study, no significant increase was observed in terms of lipid concentration.

CONCLUSIONS

Therefore, by the present investigation it can be concluded that Out of the four isolated strains *Chlorella vulgaris* (AS-3), *Chlorella pyrenoidosa*(AS-6),Scenedesmus *dimorphus*(AS-13)and *Scenedesmus quadricauda* (AS-18), AS-3 showed a significant increase in lipid content by 3%. The organism was found to grow well in the optimized BG-11 media with the pH of 6.5 and culture age of 6 weeks and concentration of sodium nitrate, FAC, potassium bicarbonate and magnesium sulphate of 2, 0.24, 0.3 and 0.8 g/L respectively. The data obtained by the by the above growth studies can be used for mass culturing of the organism in *invivo* conditions in open ponds and furthers studies can be done using different media compositions. Hence, AS-3 was found to be an ideal candidate for biodiesel production and further characterization of the microalgae was carried out.

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