International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2015): 6.391

Metabolic Engineering for Production of UV Protective Compounds and Carotenoids from a Hot Spring Cyanophyte Synechocystis Pevalekii

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Abstract: ***The methanolic extract of hot spring isolate S. pevalekii is rich in carotenoid zeaxanthin and exhibits high absorbance in UV A region and shows 5 star rating when incorporated in gel formulation. The objective of present study was to optimise production of UV protective carotenoids in S. pevalekii. It is a known fact that the carotenoid production is enhanced at nutrient deprived condition. Hence various physicochemical parameters like inoculums size, Nitrates and Sulphates were optimised for highest biomass production as well as for production of UV protective carotenoids.***

Keywords: Thermotolerant, Carotenoids, Optimization, Sunscreen

1. Introduction

Various factors are required by algae for the growth which include carbon, nitrogen, phosphorus, iron, and magnesium along with optimum light, temperature and pH conditions. The nutrients deplete as the algae grow in the culture medium at an exponential rate[1]. It is known factor that changing the physicochemical parameters like light intensities and regimen, temperature and nutrient content changes the bioactive production in them [2]. The metabolites like chlorophylls and proteins are produced at a maximum rate when the algae grow exponentially. But lipids, carotenoids and phenolics are produced when stationary phase is achieved and nutrients deplete.

Microalgae are microscopic unicellular organisms capable to convert solar energy to chemical energy via photosynthesis. They are a source of numerous bioactive compounds that can be potentially used for commercial purposes. Microalgae are harnessed to produce a wide range of metabolites such as proteins, lipids, carbohydrates, carotenoids or vitamins for health, food and feed additives, pigments, cosmetics and for energy production [3].

Studies have always proved that the content of carotenoid has always responded to the changes in the media composition. One of the major factors leading to carotenoid accumulation is Nitrogen deprivation. Nitrogen deprivation represents a feasible nutritional stress for carotenoid accumulation [4]. Employing various stress parameters like high salinity, high irradiance with high temperature and nitrogen starvation increased the carotenoid content of *Dunaliella salina*. Though high salinity (3M) decreased the cell growth, it increased the carotenoid production. Nitrogen starvation also had a positive effect on β- Carotene accumulation [5]. The effect of different stress conditions like high salinity and low nitrogen levels in *Spirulina platensis* have been reported for enhanced carotenoid production. These stress conditions lead to cessation of growth, decrease in biomass and increase in carotenoid level [6].

In previous studies, a thermotolerant genera *Synechocystis pevalekii* capable of producing a pigment that had high absorbance at UV region was isolated. The pigment was incorporated in sunscreen formulation, which resulted in five star boot star rating. The present study deals with metabolic engineering or standardization of physicochemical parameters to optimize production of UV protective pigment of *S. pevalekii*.

2. Materials and Methods

Effect of Inoculum Size on the Growth Pattern of Synechocystis. Pevalekii

The experiment was carried out at flask level, in Erlenmeyer flasks containing 100ml of Zarrouk's medium and the culture was incubated at room temperature on a rotary shaker (Orbitek, Orbital Shaker) for a span of 24 days. The biomass interpretation was done at 4 day intervals. Three different inoculum sizes were checked for optimum growth in the Zarrouk's medium with standard media composition. The inoculum sizes used were 0.1g/L, 0.3g/L and 0.5g/L. The biomass produced at various intervals was noted in **table 1**

Effect of Various Nitrate Concentrations on Biomass and Pigment Production.

In Zarrouk's medium Sodium nitrate ($NaNO_3$) is the nitrate source for *S. pevalekii*. Different concentrations of $NaNO_3$ viz., 0.625g/L, 1.25g/L, 2.5g/L and 5g/L were used to study the effect of various nitrate concentrations on biomass and pigment production. The usual concentration used in Zarrouk's medium is 2.5g/L. 0.3g/L of inoculum size was used to inoculate Zarrouk's medium containing variable quantity of sodium nitrate. The experiment was carried out in Erlenmeyer flasks of 250ml capacity for 24 days. The

culture was incubated on a rotary shaker (Orbitek, Orbital Shaker). The amount of total dry biomass was interpreted at every 4 days. At every 4 day interval the biomass was subjected to methanolic extraction and chlorophyll a, b and carotenoid content was noted with the help of **Lichtentaler and Wellburn equation**.

Effect of Various Sulphate Concentration on Biomass Production.

Zarrouk's medium was supplemented with various concentrations of potassium sulphate viz., 1g/L, 2g/L, 3g/L and 4g/L. 0.3g/L of inoculum size was used to inoculate Zarrouk's medium containing variable quantity of Potassium sulphate. These experiments were carried in Erlenmeyer flasks. 100ml of Zarrouk's medium was inoculated with 0.3g/L of culture and were incubated for 24days on a rotary shaker (Orbitek, Orbital Shaker). The amount of total dry biomass was interpreted at every 4 days. At every 4 day interval the biomass was subjected to methanolic extraction and chlorophyll a, b and carotenoid content was noted with the help of **Lichtentaler and Wellburn equation**.

Spectroscopic Determination of the Methanolic Extract.

The UV – VIS absorption spectra of the methanolic fraction was recorded on (Thermofisher Scientific, Genesys 10S UV-VIS Spectrophotometer) with a path length of 1cm. The methanolic fractions were scanned from 200nm to 400nm. The values of Chlorophyll a. chlorophyll b and total carotenoids were calculated by **Lichtentaler and Wellburn equation.**

Calculations of Chlorophyll A, B and Total Carotenoids.

Chlorophyll a, Chlorophyll b and total carotenoids were calculated according to the Lichtentaler and Wellburn equation. The equations specific for methanolic extract were used. The equations are as follows:

Chlorophyll a: 15.65 (A_{666}) – 7.34 (A_{653}) [7]

Chlorophyll b: 27.05 (A_{653}) – 11.21 (A_{666}) [7]

Total carotenoids: 1000 (A_{470}) – 3.27 (chlorophyll a) – 4.04 (chlorophyll b)/ 229 [8].

3. Results and Discussion

Effect of Inoculum Size on the Growth Pattern of Synechocystis Pevalekii

The metabolism of certain algae is highly affected by the inoculum size [9]. The inoculum sizes used were 0.1g/L, 0.3g/L and 0.5g/L. The biomass produced at various intervals was noted in table 3.1. The effect of various inoculums on growth of *Synechocystis pevalekii* is graphically represented in **figure 1**

In case of 0.1g/L inoculum, the lag phase was observed up to the 8th day **(Fig 1)**. Initially the biomass was 0.11g/L. As seen in **table 1**, on the 4th day there was a negligible increase in biomass to 0.12g/L and on the 8th day it increased to 0.20g/L. From the 8th day onwards the culture was in the log phase and on the 12th day the biomass considerably increased from 0.20g/L to 0.40g/L. On the 16th day it was 0.49g/L. The biomass still increased on the 20th and the 24th day as well. The biomass was found to be 0.55g/L on the 20th day and 0.60g/L on the 24th day. In this inoculum size of 0.1g/L the stationary phase was not achieved even on the 24th day. A higher biomass was observed on the 24th day (0.60g/L).

Table 3.1: Effect of various inoculums on biomass production of Synechocystis pevalekii at various time intervals from zero to 24 days

Days	0.1g/L	0.3g/L	0.5g/L
0	0.11	0.30	0.53
4	0.12	0.35	0.58
8	0.20	0.36	0.63
12	0.40	0.56	0.63
16	0.49	0.64	0.59
20	0.55	0.59	0.58
24	0.60	0.52	0.59

When 0.3g/L of inoculum size was used, a small lag phase was also observed till the 8th day **(Fig 1)**. Initially the biomass used was 0.30g/L that slightly increased to 0.35g/L on the 4th day and on the 8th day also there was a negligible increase to 0.36g/L. There after the log phase was achieved. On the 12th day the biomass was substantially increased to 0.56g/L on the 16th day the highest biomass 0.64g/L was observed. There after stationary phase was achieved. On the 20th day the biomass decreased a little bit to 0.59g/L and on the 24th day it further decreased to 0.52g/L.

At 0.5g/L inoculum there was no lag phase observed **(Fig 1)**. The stationary phase was observed very early i.e. on the 8th day. Initially the biomass was 0.53g/Lwhich increased to 0.58g/L on the 4th day. 0.63g/L biomass was observed on the 8th day, which marked the stationary phases of the culture. Thereafter a little decline in the biomass to 0.63g/L on the 12th day and 0.59g/L on the 16th day was observed. The 20th day showed a biomass of 0.58g/L and on the 24th day the biomass was found to be 0.59g/L. It may be possible that due to a higher inoculum size added, there is a faster depletion of nutrients.

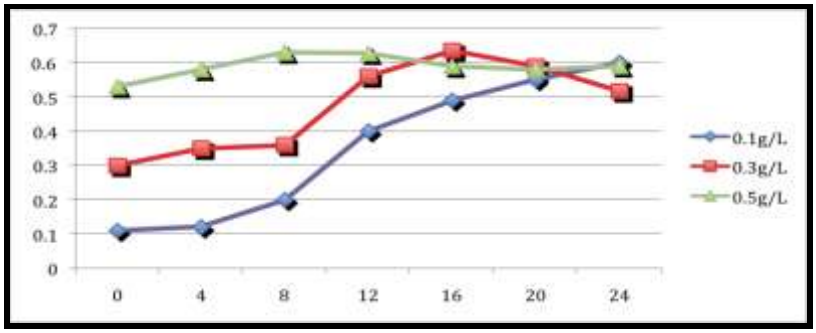

Figure 1: Effect of Various inoculum sizes on growth pattern of S. pevalekii

In a study based on *Prymnesium parvum*, golden alga strains, UTEX 2797 and UTEX 995, it was found that the growth rate of the culture decreased with an increase in the inoculum size, suggesting that this inverse variation was a result of accumulation of harmful toxins produced by the alga at higher inoculum density [10].

Hence for further experiments 0.3g/L inoculum size was selected which shows the beginning of the log phase on the 8th day producing highest biomass of 0.64g/L on the 16thday.

Effect Of Various Nitrate Concentrations on Biomass Production

Nitrate is a crucial component of the growth medium that determines the cell growth and biochemical composition of microalgae [11]. In Zarrouk's medium Sodium nitrate ($NaNO_3$) is used for optimization of nitrate concentration for biomass and carotenoid production. Different concentrations of $NaNO_3$ viz., 0.625g/L, 1.25g/L, 2.5g/L and 5g/L were used. The effect of various concentration of $NaNO_3$ on biomass production is interpreted in **Fig 2 and fig 3.**

When 0.625g/L of $NaNO_3$ was supplied the average biomass produced on the 4th day was 0.3g/L, which increased to 0.42g/L on the 8th day. On the 16th day it was 0.52g/L and on the 20th day it increased to 0.58g/L. On the 24th day 0.6g/L biomass was observed. Thus, when 0.625g/L of nitrate is supplied, the culture grew continuously up to 24 days. There was no stationary phase **(Fig 2 and 3)**. It is also represented in a bar graph representation.

When the amount of $NaNO_3$ supplemented was 1.25g/L on the 4th day the biomass observed was 0.31g/L, which increased to 0.39g/L on the 8th day. On the 12th day it was 0.46g/L. On the 16th day the biomass increased to 0.51g/L. On the 20th day there was negligible increase to 0.52g/L. This is well interpreted in **figure 2** where the stationary phase appears on the 16th day itself and thereafter there was a negligible increase in the biomass.

Similar observations were made when the culture was supplemented by 2.5g/L of sodium nitrate. After the 16th day there was negligible growth and the stationary phase was achieved. The highest biomass of 0.53g/L was observed on the 20th day.

When the algae was supplemented with 5g/L of sodium nitrate it did not contribute in biomass production. The highest biomass was only 0.49g/L in 24 days of culture.

On comparing all the concentrations it seems that the highest biomass content obtained was 0.60g/L on the 24th day, when the sodium nitrate concentration was 0.625g/L, whereas the highest biomass count in the case of 1.25g/L and 2.5g/L of Sodium nitrate reached to around 0.52g/L and 0.53g/L respectively. The highest concentration of sodium nitrate 5g/L, did not support the biomass production. **Hence it can be interpreted that the lowest concentrations of sodium nitrate i.e. 0.625g/L, proved to be beneficial for growth. It produced the highest biomass and cultures remained in log phase throughout the 24 days of the experiment.**

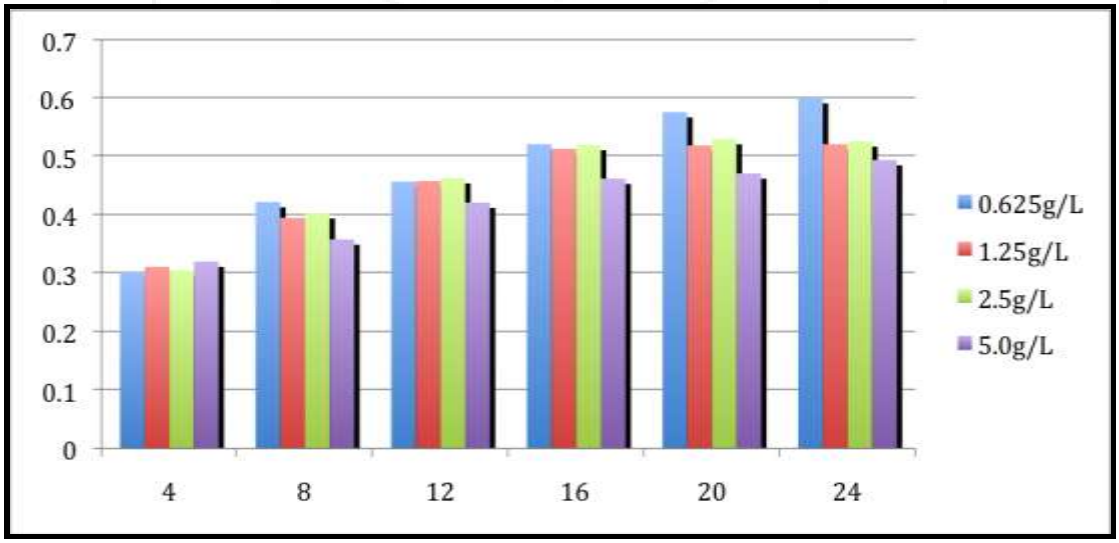

Figure 2: Consolidated growth pattern in all the concentrations of sodium nitrate (Column graph representation)

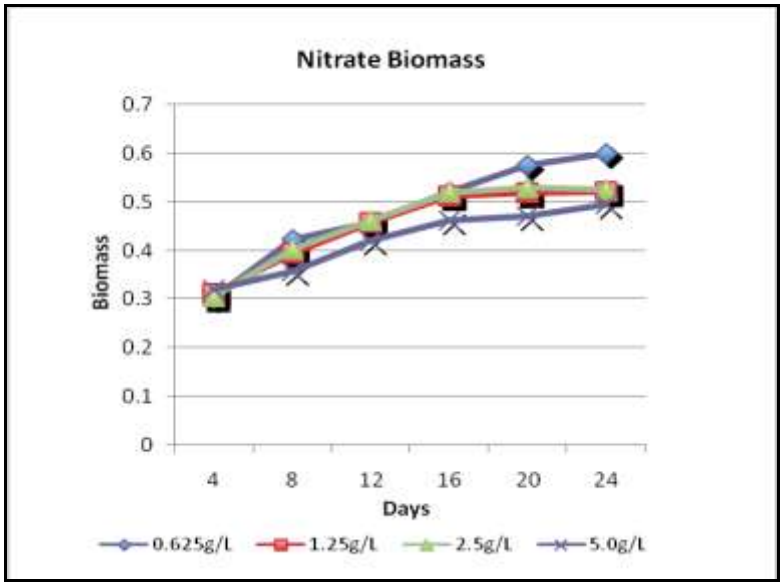

Figure 3: Consolidated growth pattern in all the concentrations of sodium nitrate (Line graph representation)

Effect of Various Sodium Nitrate Concentration on Chlorophyll A.

Earlier, chlorophyll was regarded as a tool to measure growth of algae [12]. Here also nitrate concentration conducive for biomass production was indicating high chlorophyll a content. At the most effective concentration 0.625g/L of Sodium nitrate which was responsible for highest biomass on the 20th day the chlorophyll a content was also as high 34.36µg/ml.

As seen in the **table 2** when 0.625g/L Sodium nitrate was supplemented the chlorophyll a concentration on the 4th day was 21.89µg/ml, 21.90µg/ml on the 8th day and 30.62µg/ml on the 12th day. On the 16th day it had shown a further increase to 33.08µg/ml. The chlorophyll a content on the 20th day was 34.37µg/ml however the chlorophyll a content showed a decline to 24.23µg/ml on the 24th day. **Fig 4** shows the correlation between chlorophyll and biomass content in 0.625g/L. **Table 2** gives the values for biomass, Chlorophyll a, b, carotenoids and mean UV absorbance at 0.625g/L of Sodium nitrate.

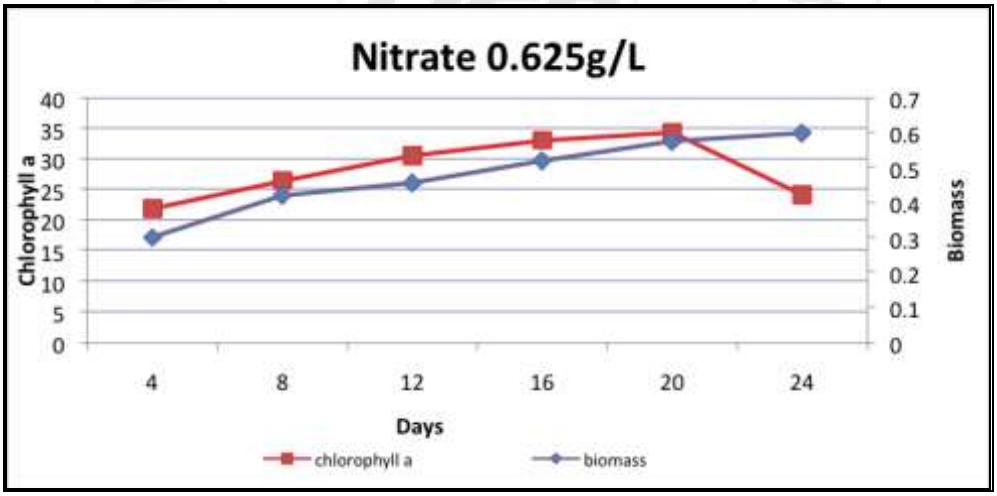

Figure 4: Correlation between chlorophyll a and biomass content in 0.625g/L of Sodium nitrate

Table 2: Values for biomass, Chlorophyll a, b, carotenoids and mean UV absorbance in 0.625g/L of sodium nitrate

Days	Biomass (g/L)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Carotenoids (µg/ml)	Mean Absorbance
4	0.30	21.89	2.38	6.48	1.08
8	0.42	26.50	4.79	5.9	1.35
12	0.46	30.62	11.26	6.69	1.75
16	0.52	33.08	24.43	7.61	2.60
20	0.58	34.37	37.69	10.25	3.02
24	0.60	24.23	53.86	10.99	3.19

At 1.25g/L of sodium nitrate the stationary phase was achieved on the 16th day and thereafter there was negligible growth in biomass on the 20th and the 24th day. At this concentration the highest chlorophyll a content was observed on the 16th day. 31.04µg/ml and thereafter there was little decline in amount of chlorophyll a. It was 29.16µg/ml on the 20th day and 29.47µg/ml on the 24th day **(Table 3)**.

Table 3: Values for biomass, Chlorophyll a, b, carotenoids and mean UV absorbance when S. pevalekii was supplemented with in 1.25g/L of sodium nitrate

Days	Biomass (g/L)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Carotenoids (µg/ml)	Mean Absorbance
4	0.31	11.69	1.98	3.08	0.59
8	0.39	22.08	5.05	5.28	1.15
12	0.46	29.19	8.07	5.84	1.55
16	0.51	31.04	36.10	9.09	2.90
20	0.52	29.16	41.96	9.76	3.02
24	0.52	29.47	51.54	11.04	3.12

Similar results were obtained at 2.5g/L of sodium nitrate. At this concentration also, the stationary phase was achieved on the 16th day. The highest chlorophyll a content 30.41µg/ml was observed on the 16th day. Thereafter chlorophyll a content decreased to 26.45µg/ml on the 20th day. **Fig 5** shows the correlation between chlorophyll and biomass content in 2.5g/L **(Table 4)**

Table 4: gives the values for biomass, Chlorophyll a, b, carotenoids and mean UV absorbance in 2.5g/L of sodium nitrate

Days	Biomass (g/L)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Carotenoids (µg/ml)	Mean Absorbance
4	0.30	7.36	5.42	2.99	0.55
8	0.40	31.18	10.03	6.67	1.79
12	0.46	25.04	18.58	6.2	1.91
16	0.52	30.42	33.24	8.7	2.71
20	0.53	26.46	47.93	12.03	3.10
24	0.53	28.13	45.53	11.72	3.03

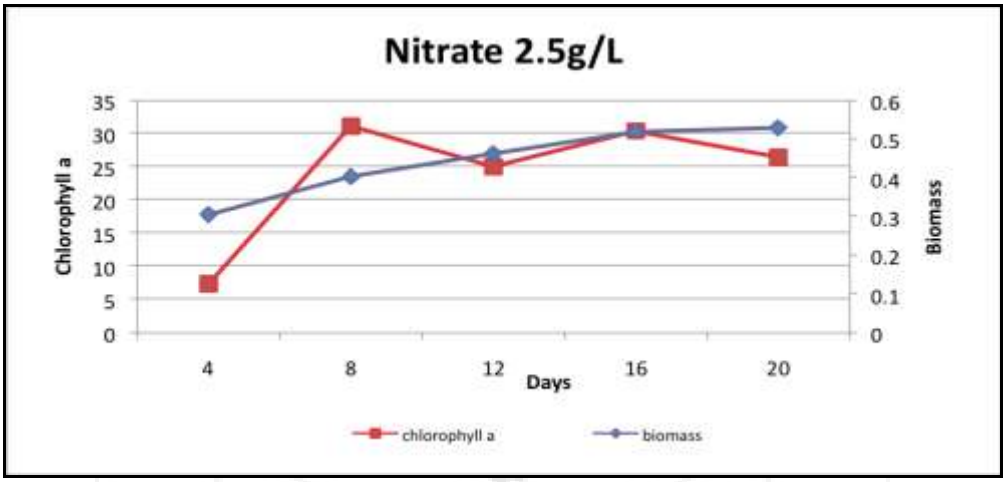

Figure 5: Correlation between chlorophyll and biomass content in 2.5g/L of sodium nitrate

When the algal culture was supplemented with high sodium nitrate content of 5.0g/L, the highest chlorophyll a content was observed on the 12th day which was recorded to be 33.99µg/ml. On the 16th day it was 32.05µg/ml and on the 20th day it declined to 27.64µg/ml **(Table 5)**

Days	Biomass (g/L)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Carotenoids (µg/ml)	Mean Absorbance
4	0.32	23.46	2.61	7.84	1.18
8	0.36	21.14	5.65	6.95	1.49
12	0.42	33.99	15.01	7.11	2.06
16	0.46	22.06	29.25	7.77	2.56
20	0.47	27.64	52.20	11.17	3.19
24	0.49	29.83	42.18	9.52	3.00

Table 5 gives the values for biomass, Chlorophyll a, b, carotenoids and mean UV absorbance in 5.0g/L of sodium nitrate.

Thus at lowest concentration of sodium nitrate highest chlorophyll a content along with highest value of biomass was observed. Thus the lowest concentration of sodium nitrate i.e. 0.625g/L was found to be beneficial for growth.

Relation between growth of Synechocystis Pevalekii and production of accessory pigments

The chlorophyll a synthesis shows a correlation with growth. Maximum chlorophyll a is produced during log phase of the organism and in stationary phase it remains constant or slightly decreases. In photosynthetic organisms as long as nutrients are available the rapid chlorophyll accumulation and cell division is observed. However when nitrogen was consumed cell division stopped, biomass yield remained the same. The consumption of nitrogen resulted in fast decrease in accumulation of chlorophyll and increase in ratio of carotenoid to chlorophyll. Nitrogen deprivation represents a feasible nutritional stress to increase carotenoids [13].

In the present experiments it is prominently observed in cultures supplemented with 1.25g/L or 2.5g/L of Nitrate. As seen in **table 3** the log phase ends on day 16 which is exhibited by highest chlorophyll a content of 31.04µg/ml. Thereafter it slightly declines to 29.16µg/ml on the day20 and 29.47µg/ml on the 24th day. Stationary phase which results due to limitations of nutrients abruptly brings about a sharp rise in values of accessory pigments chlorophyll b and total carotenoids. As seen in **table 3**, the culture supplemented with 1.25g/L of sodium nitrate, the amount of chlorophyll b produced on 12th day was 8.07µg/ml. With the onset of stationary phase it abruptly increased to 36.10µg/ml on the 16th day and on the 20th day it rises to 41.96µg/ml and on the 24th day it is as high as 51.54µg/ml.

Similarly, amount of carotenoid produced on 12th day was 5.84µg/ml. on 16th day it increases to 9.09µg/ml and on 24th day it is 11.04µg/ml.

Jalal et al (2013) while observing the growth trend in *Isochrysis* species, made similar observation. Under laboratory conditions *Isochrysis* species showed a short lag phase that lasted for about 24 hours. Subsequently the cells grow actively from day 3 to day 9. From day 10 until day 13th the cells entered into stationary phase. At this stage the microalgae begin to exhaust resources that are available to them. At this stage the chlorophyll a synthesis is markedly affected where as the carotenoid synthesis is enhanced. They also observed that maximum amount of chlorophyll a (12µg/ml) was observed on day 4 which coincides with highest growth rate. On day 10 it decreases to 4µg/ml where as the highest amount of carotenoid 7µg/ml was observed on day 10 [14].

A strong correlation between production of accessory pigments and UV A absorbance.

As seen in **table 3** when *S. pevalekii* was supplemented with 1.25g/L of nitrate the stationary phase was achieved on day 16 which resulted in sharp increase in production of accessory pigments. On day 12 the amount of chlorophyll b was 8.07µg/ml and carotenoid produced was 5.84µg/ml. When stationary phase was achieved it drastically increased chlorophyll b production. On the 16th day it was 36.10µg/ml and carotenoid was 9.09µg/ml. At this stage a sharp increase in absorbance was observed. On the 12th day the mean UV absorbance in the A region was 1.55 which increased to 2.90 on the 16th day and on the 20th day it was 3.02.

Similar observations were made when the culture of *S. pevalekii* was supplemented with 2.5g/L of sodium nitrate. As seen in **table 4**, the stationary phase was achieved on day 20. This was depicted by a drastic increase in the accessory pigments. The chlorophyll b content on day 16 was 33.24µg/ml, which increased to 47.93µg/ml on day 20. Same was the case for carotenoid content. The carotenoid content an interval prior to stationary phase i.e. day 16 was 8.7µg/ml that escalated to 12.03µg/ml on day 20 when the stationary phase was achieved. The UV A absorbance was also found to increase on day 20. On day 16 the mean UV A absorbance was 2.71 and on day 20 the absorbance recorded was the highest i.e. 3.10.

Effect of various sodium nitrate concentration on chlorophyll B and its relationship with the mean UV-A absorbance and carotenoid content

Lower concentrations of nitrate are more conducive for production accessory pigments and UV absorbance. In the present work with *S. pevalekii* growing in Zarrouk's medium, standard concentration of nitrate is 2.5g/L. As seen in **table 4** for this concentration the stationary phase is achieved on the 16th day which is depicted by highest chlorophyll content i.e. 30.42µg/ml. The highest chlorophyll b content 47.93µg/ml was produced on the 20th day. And on this day the absorbance in the A region was highest i.e. 3.10 **(Fig 6)**.

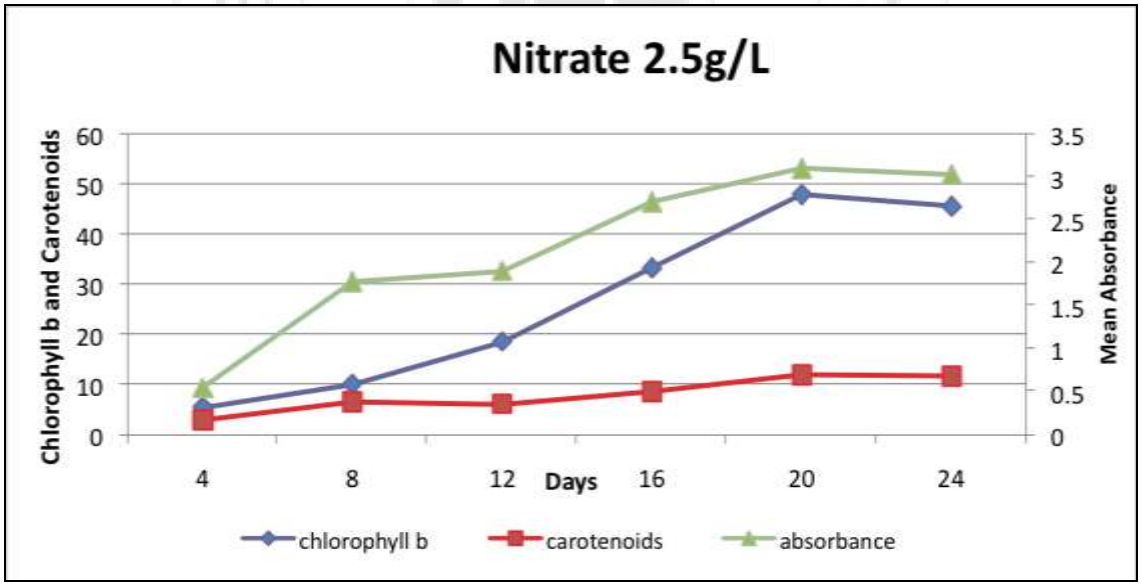

Figure 6: Relation between chlorophyll b, carotenoids and mean UV-A absorbance in 2.5g/L of sodium nitrate

When nitrate concentration was reduced to 1.25g/L though the stationary phase was achieved on 16th day the highest chlorophyll b and carotenoids were formed on the 24th day. The highest chlorophyll b produced was 51.54µg/ml, which was higher than highest chlorophyll b content (47.93µg/ml) when the culture was supplemented with 2.5g/L of nitrate. The relative absorbance was also higher i.e. 3.12. Total carotenoid content was slightly lesser **(table 3 and 4)**

When nitrate supplied was still lesser that is 0.625g/L the stationary phase was not achieved till 24th day. On the 24th day biomass was 0.60g/L. The chlorophyll b was 53.86µg/ml and mean absorbance was 3.19 **(fig 7)**

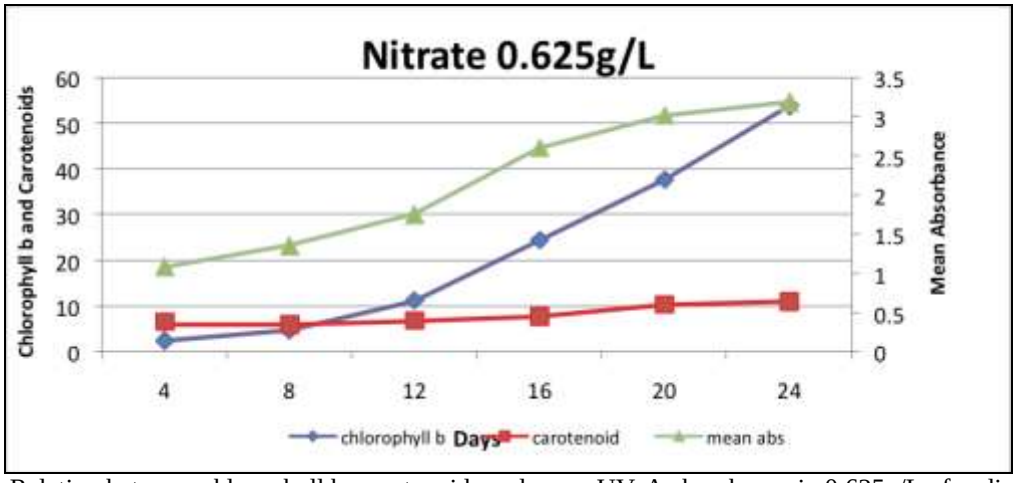

Figure 7: Relation between chlorophyll b, carotenoids and mean UV-A absorbance in 0.625g/L of sodium nitrate.

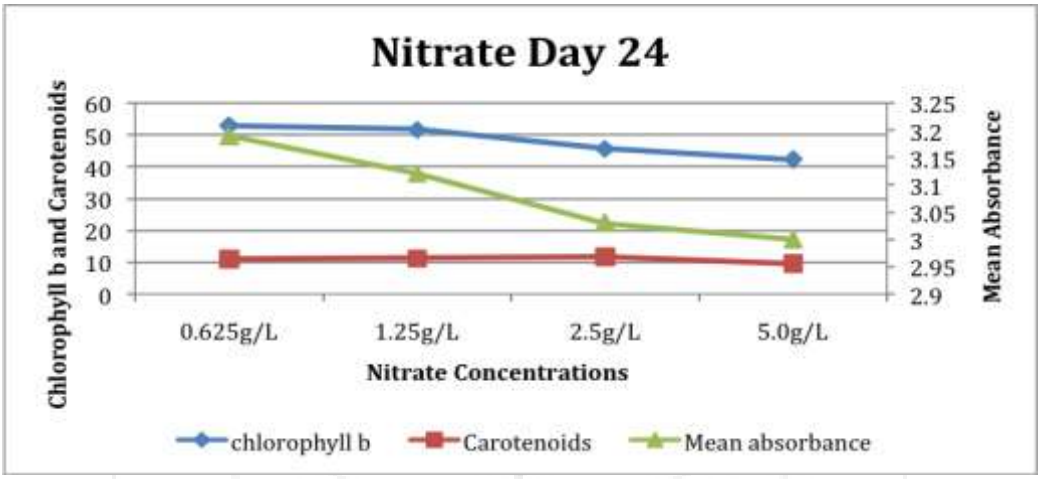

Figure 8: Relation between chlorophyll b, carotenoids and mean UV-A absorbance in various concentrations of sodium nitrate on the 24th day.

Thus the favourable nitrate concentration is 0.625g/L for biomass as well as the UV absorbance.

Nitrogen is considered to be a key nutrient that significantly affects the algal growth and metabolism. Limitation of nitrogen greatly reduces chlorophyll synthesis and substantially improves carotenogenesis. Nitrogen deprivation for synthesis of β- Carotenes in *Dunaliella* and Astaxanthin in *Hematococcus* is elaborately studied [5], [15], [16].

The influence of nitrogen concentrations on growth, carotenoid production and production of phenolic compounds and α- Tocopherols was studied by **El Baky in 2008** [17]**.** The experiments were conducted at same set of sodium nitrate concentrations on *Spirullina maxima.* The highest growth and high chlorophyll content was achieved at 2.5g/L. But carotenoid concentration was increased with decrease in concentration of nitrate. At 2.5g/L of nitrate the carotenoid content was 7.32mg/g. When the nitrate concentration was reduced to 1.25g/L it was increased to 12.35mg/g. At 0.625g/L of nitrate carotenoid production was as high as 16.53mg/g.

The antioxidant tocopherols were also found to be increasing with nitrogen depriviation in a study conducted on *Nannochloropsis oculata* [18].

Effect of various Sulphate Concentration on biomass production

As the organism was isolated from hot sulphur springs it was essential to find out the sulphate requirement for the organism. For setting up the experiment to study the effect of various sulphate concentrations on the growth, chlorophyll a and b content, Carotenoid content and the mean UV-A absorbance, the inoculum added was 0.3g/L as standardized in the Inoculum size experiment. The nitrate concentration of 0.625g/L, optimized in the previous experiment was used. Zarrouk's medium contains Potassium sulphate (K_2SO_4) as the sulphate source. The different concentrations of potassium sulphate considered in this experiment were 1g/L, 2g/L, 3g/L and 4g/L

The effect of various sulphate concentrations on the growth of *S. pevalekii* is depicted in **Fig 9 and 10**. When 1g/L concentration of potassium sulphate was used the biomass recorded on the 4th day was 0.35g/L. It increased to 0.56g/L on the 12th day of the experiment. The highest biomass recorded was 0.64g/L on the 16th day after which it declined to 0.53g/L on the 20th day and 0.52g/L on the 24th day. As seen in the graphical representation in **Fig 10** the lag phase was observed up to 8 days after which started the log phase. The log phase was observed from 8 to 16 days.

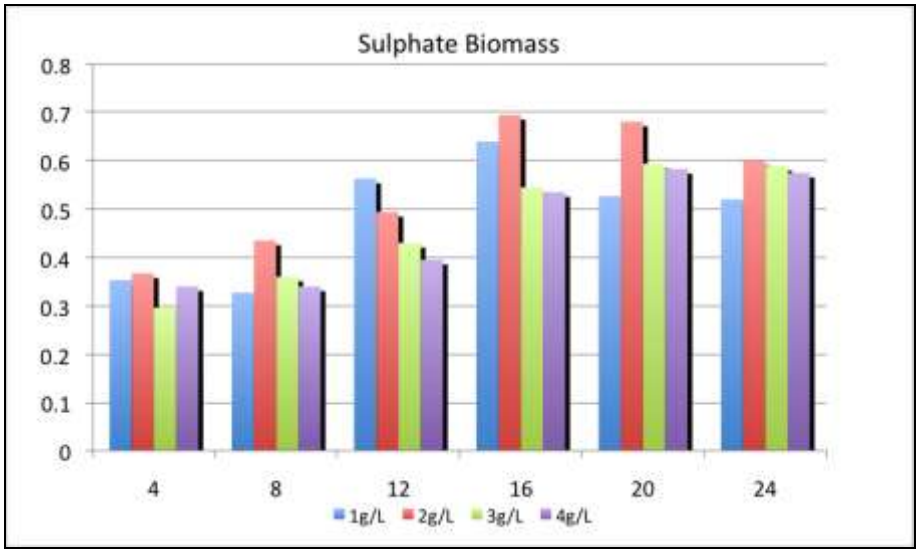

Figure 9: consolidated growth pattern in different concentrations of sulphate in a bar graph representation.

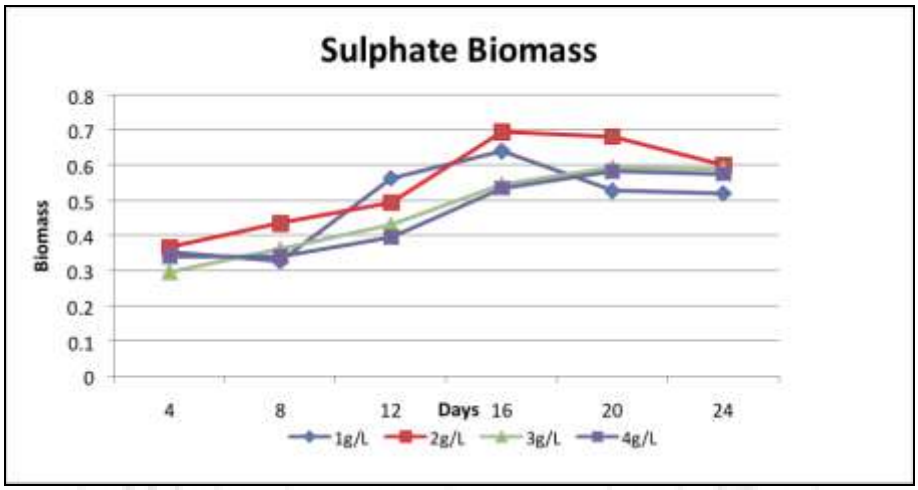

Figure 10: Consolidated growth pattern in different concentrations of sulphate in a line graph representation

The little more concentration of potassium sulphate appeared to be conducive for growth in *S. pevalekii* **(Fig 9 and 10)**. When 2g/L of potassium sulphate was added to the medium, the biomass on the 4th day was 0.37g/L, which gradually increased to 0.44g/L on the 8th day. The 12th day recorded a biomass reading of 0.49g/L. The highest biomass recorded for this concentration was on the 16th day and that was 0.69g/L which was much higher than the biomass produced by *S. pevalekii* supplemented with other concentration of sulphate **(Fig 9 and 10).** As depicted in **(Fig 10)** the lag phase was almost absent and the log phase was seen up to 16 days. Then there was stationary phase on the 20th day which showed a slight decline in the biomass to 0.68g/L and then to 0.60 on the 24th day, thus indicating that the stationary phase was achieved on the 16th day itself.

The higher concentrations 3g/L and 4g/L were not supporting the growth of *S. pevalekii* **(Fig 9 and 10).** When 3g/L of potassium sulphate was used, on the 4th day the biomass was 0.30g/L, which increased to 0.36g/L on the 8th day. The 12th day showed an increase in the biomass to 0.43g/L. On the 16th day the biomass further increased to 0.55g/L. The highest biomass recorded was on the 20th day, which was 0.59g/L whereas at 1g/L sulphate concentration the highest biomass achieved was as high as 0.69g/L. On the next interval the biomass did not show much decrease in the biomass (0.59g/L). For this concentration of sulphate the stationary phase was achieved on the 20th day. This indicates that the algal culture gradually adapts to the increasing concentration of potassium sulphate.

Silimilarly, for 4g/L sulphate the highest biomass produced was on 20th day. It was 0.58g/L.

Hence 2g/L of potassium sulphate was suitable for biomass production

Effect of different sulphate concentrations on chlorophyll a content.

As discussed earlier, the chlorophyll a synthesis shows a correlation with growth. Maximum chlorophyll a is produced during log phase of the organism and in the stationary phase it remains constant or slightly decreases.

When 1g/L of sulphate concentration was used the chlorophyll a content measured on the 4th day was 7.22µg/ml. This increased to 29.99µg/ml on the 8th day. The highest biomass measured was on the 16th day and also the highest chlorophyll a content observed was on the same day (33.73µg/ml). This marks the end of the log phase and the beginning of the stationary phase of the culture. After this the culture now shows a decline the biomass values as well as the chlorophyll a content. On the 20th and the 24th day there was seen a decrease in the chlorophyll a content as

compared to the concentration measured on the 16th day. This was also supported by the decrease in the biomass seen. **Fig11** shows the correlation between the biomass and chlorophyll a content when 1g/L of Potassium sulphate was used. **Table 6** gives the values for Biomass, Chlorophyll a, b, Carotenoids and mean UV absorbance in the A region for 1g/L of potassium sulphate

Table 6: Values for Biomass, Chlorophyll a, b, Carotenoids and mean UV absorbance in the A region for 1g/L of potassium sulphate

Days	Biomass (g/L)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Carotenoids (µg/ml)	Mean Absorbance
4	0.35	7.22	4.13	6.45	1.22
8	0.33	29.99	5.63	6.39	1.68
12	0.56	27.86	13.84	5.71	1.67
16	0.64	33.73	12.75	6.47	2.03
20	0.53	29.59	37.75	10.09	2.98
24	0.52	31.47	41.85	12.51	3.11

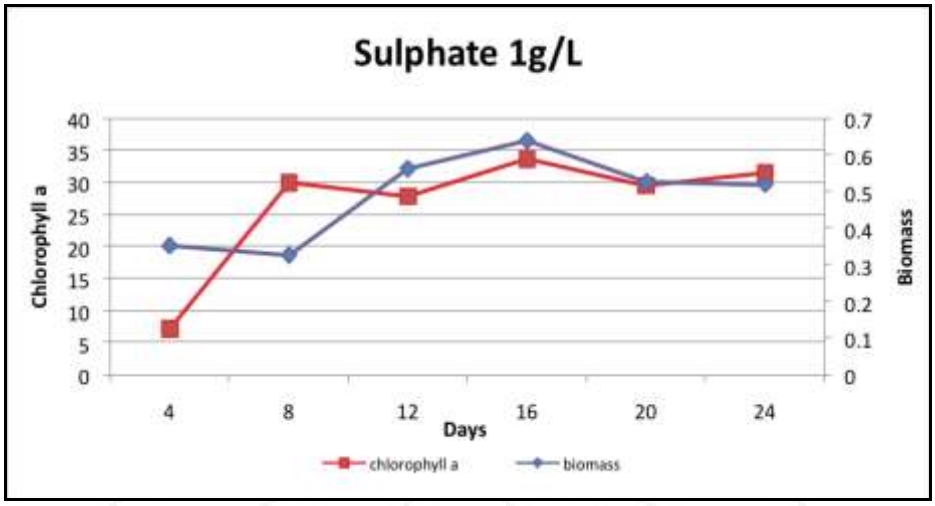

Figure 11: Correlation between the biomass and chlorophyll a content when 1g/L of Potassium sulphate was used

When 2g/L of sulphate was supplemented to the algal culture the chlorophyll a content on the 4th day was 22.98µg/ml. On the 8th day it was 32.93µg/ml. There was not much decrease seen in the chlorophyll a content on the 12th day. However the chlorophyll a content showed a decrease in its value on the 16th day that was found to be 27.47µg/ml. This was not in accordance with the biomass on the same day. The stationary phase for the culture was achieved on the 20th day after which the chlorophyll a content also shows a decline on the next harvest interval. The chlorophyll a content on the 20th day was 33.48µg/ml and on 24th day it was 22.23µg/ml. These values were found to decrease with the decreasing amount of biomass. **Fig 12** shows the correlation between the biomass and chlorophyll a content in 2g/L of potassium sulphate. **Table 7** gives the values for Biomass, Chlorophyll a, b, Carotenoids and mean UV absorbance in the A region for 2g/L of potassium sulphate.

Table 7 gives the values for Biomass, Chlorophyll a, b, Carotenoids and mean UV absorbance in the A region for2g/L of potassium sulphate.

Days	Biomass (g/L)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Carotenoids (µg/ml)	Mean Absorbance
4	0.37	22.98	4.85	5.81	1.30
8	0.44	32.93	12.46	8.29	2.15
12	0.49	32.35	12.98	7.07	1.88
16	0.69	27.47	30.94	12.51	2.65
20	0.68	33.48	17.77	6.50	2.26
24	0.60	22.23	17.18	4.99	1.63

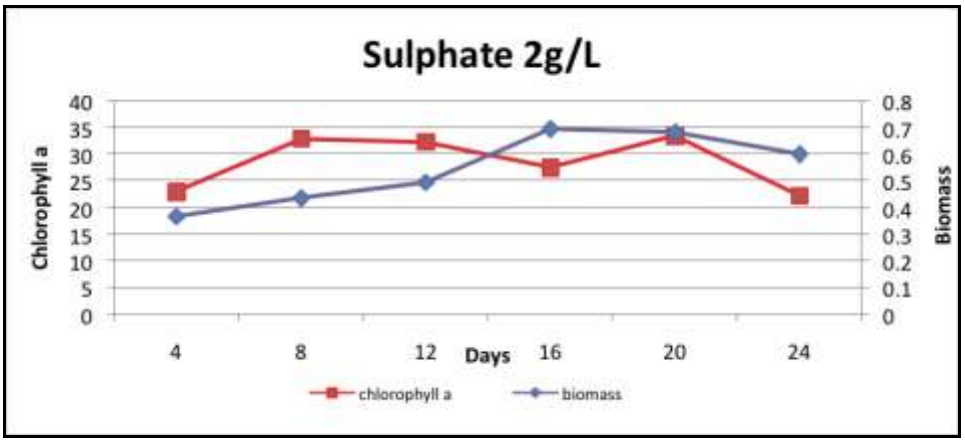

Figure 12: Correlation between the biomass and chlorophyll a content in 2g/L of potassium sulphate

In case of 3g/L of sulphate concentration, the chlorophyll a content on the 4th day was found to be 12.46µg/ml. On the 12th day it was found to be 30.62µg/ml and on the 8th day the amount recorded was 34.72µg/ml. The 12th day showed a small decline in the chlorophyll a content to 33.62µg/ml. The highest chlorophyll a content recorded was on the 16th day 33.79µg/ml and on the 24th day it was found to be 33.67µg/ml. **Table 8** gives the values for Biomass, Chlorophyll a, b, Carotenoids and mean UV absorbance in the A region for 3g/L of potassium sulphate.

Table 8: Values for Biomass, Chlorophyll a, b, Carotenoids and mean UV absorbance in the A region for 3g/L of potassium sulphate

Days	Biomass (g/L)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Carotenoids (µg/ml)	Mean Absorbance
4	0.29	12.46	2.17	3.67	0.75
8	0.36	34.72	9.63	7.85	1.96
12	0.43	30.62	8.49	6.41	1.66
16	0.55	30.57	11.36	5.9	1.71
20	0.59	32.79	17.98	7.97	1.11
24	0.59	33.67	22.34	8.38	2.43

When 4g/L of sulphate was supplied for algal growth, the chlorophyll a content measured on the 4th day was 7.08µg/ml. This concentration increased to 32.60µg/ml on the 8th day of the experiment. The value of chlorophyll a measured on the 16th day was the highest i.e. 35.05µg/ml and it gradually showed decrease in the subsequent intervals indicating that the culture has reached its stationary phase. The stationary phase was achieved on the 20th day when the chlorophyll a content was found to be 33.63µg/ml. The chlorophyll a content measured on the 24th day was 31.68µg/ml which showed a further decrease. **Table 9** gives the values for Biomass, Chlorophyll a, b, Carotenoids and mean UV absorbance in the A region for 4g/L of potassium sulphate

Table 9: Values for Biomass, Chlorophyll a, b, Carotenoids and mean UV absorbance in the A region for 4g/L of potassium sulphate

Days	Biomass (g/L)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Carotenoids (µg/ml)	Mean Absorbance
4	0.34	7.08	1.84	2.43	0.48
8	0.34	32.60	8.18	7.43	1.95
12	0.39	29.81	8.72	6.41	1.65
16	0.54	35.05	19.57	7.39	2.49
20	0.58	33.63	22.09	6.69	2.38
24	0.58	31.68	23.25	7.36	2.33

The increase in the sulphate concentrations did not have a direct effect on the chlorophyll a content overall. This is because the chlorophyll a concentration was always related to the biomass content.

Relation between Growth of S. Pevalekii with Production of Accessory Pigments and UV A Absorbance.

As discussed earlier, the chlorophyll a synthesis shows a correlation with growth. Maximum chlorophyll a is produced during log phase of the organism and in stationary phase it remains constant or slightly decreases.
Also the production of accessory pigments is enhanced on nutrient deprivation. A profound effect is seen on accessory pigments production when the concentration of nutrients is lower and stationary phase is achieved. Similar effect was observed when lower concentration of potassium sulphate was used for the growth of *S. pevalekii* for the production of accessory pigments.

When the culture was supplemented with maximum concentration of sulphate i.e. 4g/L. The stationary phase was achieved on the 20th day. On this day the chlorophyll a content was 33.63µg/ml. The highest chlorophyll b content was 23.25µg/ml that was obtained on the 24th day. Similarly the highest carotenoid obtained was also on the 24th day (7.36µg/ml). The mean UV absorbance was found to be almost similar on the 24th as well as the 20th day **(Table 9).**

When the sulphate concentration was reduced to 3g/L, the stationary phase achieved was on 24th day that was depicted by highest chlorophyll a content of 33.67µg/ml. On this day itself the chlorophyll b content was also found to be the highest i.e. 22.34µg/ml. the total carotenoid content was 8.38µg/ml and the mean absorbance was 2.43 **(Table 8).**

When the sulphate supplied was still reduced to 2g/L the maximum biomass was obtained on 16th day and the stationary phase was achieved on the 20th day which was depicted by highest chlorophyll a content 33.48µg/ml. On this day the chlorophyll b content was also the highest i.e. 17.77µg/ml. However the chlorophyll b content, carotenoid content and the mean UV absorbance slightly decreased on day 24. The values for chlorophyll b, carotenoid content and UV absorbance on day 24 were 17.18µg/ml, 6.50µg/ml and 2.26 respectively **(Fig 13)**.

When the overall pattern of production of chlorophyll b was studied it was found that higher concentrations of sulphates did not encourage chlorophyll b/accessory pigment concentration. Thus it can be concluded that 2g/L of sulphate is conducive for biomass production whereas 1g/L of potassium sulphate helps in enhancing the concentration of accessory pigments

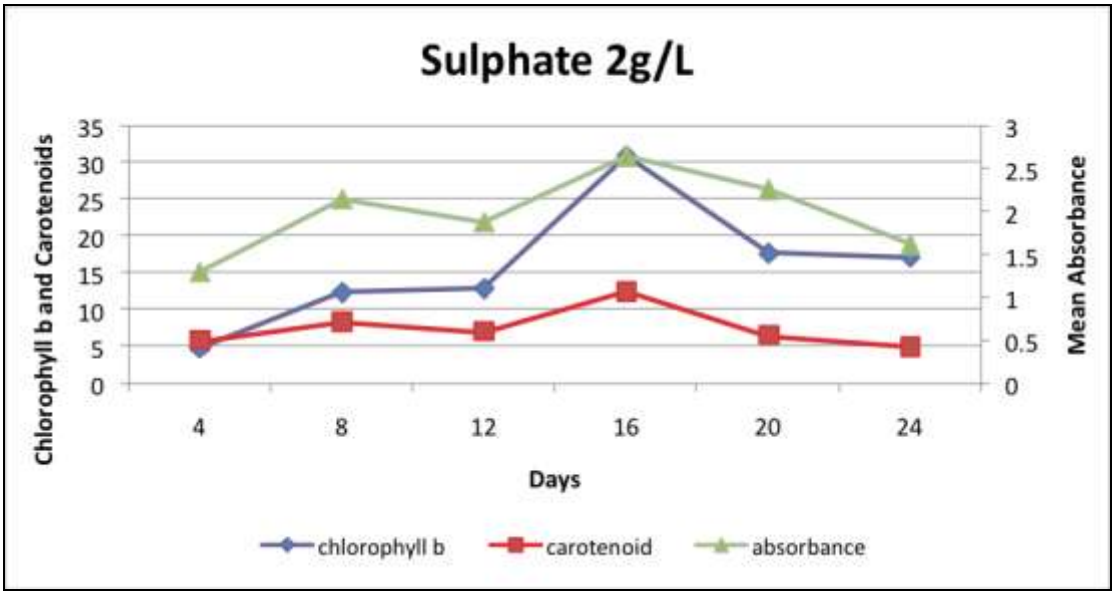

Figure 13: Relation between chlorophyll b, carotenoids and mean UV-A absorbance in 2g/L of potassium sulphate

When 1g/L of potassium sulphate was used in Zarrouk's medium for the growth of *S. pevalekii* the stationary phase was obtained on the 20th day. The log phase ended on day 16 which showed a chlorophyll a content of 33.73µg/ml. As the stationary phase was set in on the 20th day, the chlorophyll a content decreased to 29.59µg/ml. this decrease in chlorophyll a corresponded to an increase in chlorophyll b and carotenoid production. The chlorophyll b content on day 16 was 12.75µg/ml which showed an abrupt increase in the content to 37.75µg/ml on the 20th day when the stationary phase began. Also the carotenoid content increased from 6.47µg/ml on day 16 to 10.09µg/ml on the 24th day (Fig 3.14).

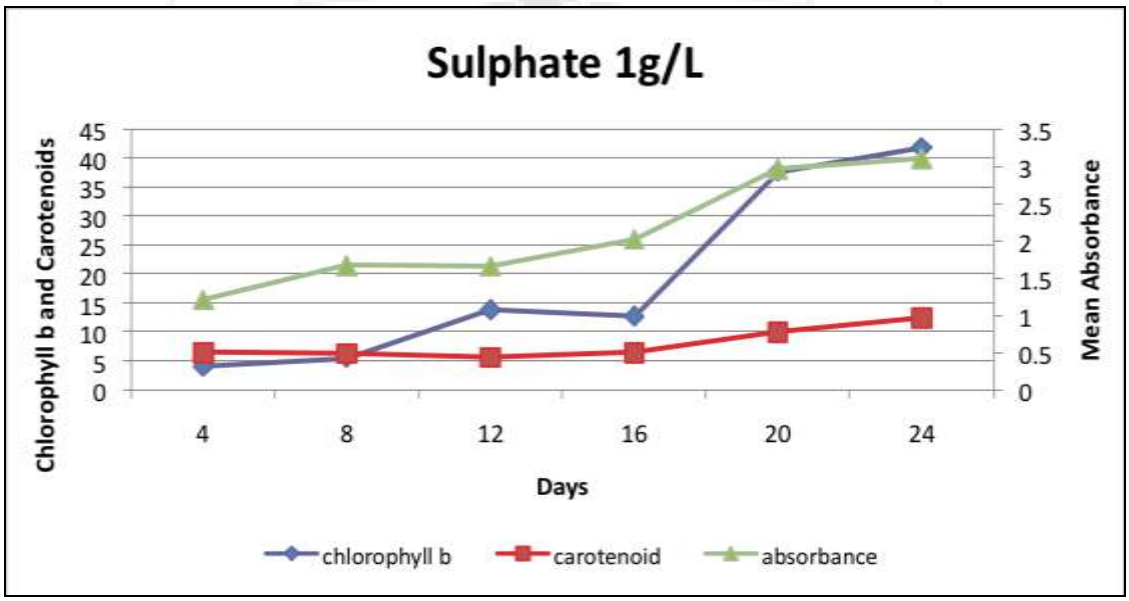

Figure 14: Relation between chlorophyll b, carotenoids and mean UV-A absorbance in 1g/L of potassium sulphate

The effect of different sulphate concentrations on the total carotenoid production, chlorophyll b content and UV absorbance on day 24 is well depicted in **fig 15**.

When the sulphate concentration was 3g/L the chlorophyll b concentration was found to be 22.34µg/ml with carotenoid concentration being 8.38µg/ml. The mean absorbance was 2.43. When the concentration of sulphate was decreased to 2g/L the concentration of chlorophyll b was found to decrease to 17.18µg/ml. the carotenoid content was 4.99µg/ml and the mean absorbance was 1.63.

However when the concentration of potassium sulphate was further decreased to 1g/L the concentration of chlorophyll b was found to drastically increase to 41.83µg/ml and also the carotenoid content was 12.52µg/ml. The mean absorbance was recorded to be 3.11 which was the maximum. On comparing the concentrations of chlorophyll b, carotenoids and mean absorbance for all the concentrations on Day 24 it was clearly observed that 1g/L of potassium sulphate showed the best results.

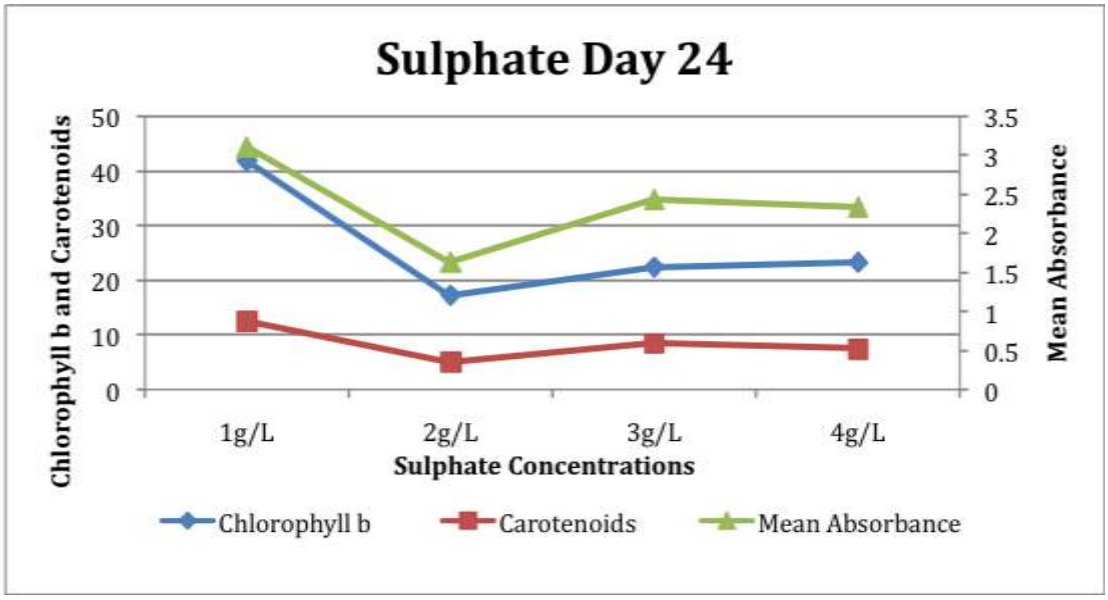

Figure 15: Relation between chlorophyll b, carotenoids and mean UV-A absorbance in various concentrations of potassium sulphate on Day 24

Similar studies on *Nannochloropsis gaditana* are also reported. On starvation or on providing limiting concentration of sulphur, nitrate or phosphorus led to increase of carotenoid/ chlorophyll ratio [19].

Also **Shaker S** [20] et al in **2017** studied the effect of sulphur. Iron and manganese depriviation/ starvation on naturally isolated strain of *Dunaliella salina*. The report suggests that sulphur, iron and manganese deprivation reduced the growth of *Dunaliella salina* cells however the β- Carotene yield was significantly increased. The β- Carotene yield was elevated from 6.75mg/L in basic nutrient rich medium to 14.616mg/L in sulphur deficient medium.

4. Conclusion

The complete study carried out is for the optimization of the nutrient media for maximum production of biomass as well as UV absorbing pigment. Deciding the optimum concentration of inoculum size is very crucial. The thermotolerant strain *Synechocystis pevalekii* showed enhanced growth when 0.3g/L of inoculum size was used for all the experiments.

The various concentrations of Sodium nitrate used were 0.625g/L, 1.25g/L, 2.5g/L and 5g/L. The lower concentration of nitrate 0.625 g/L was responsible for highest biomass production. In the organism, as long as nutrients were available rapid cell division and chlorophyll a synthesis was observed. However, when nutrients were depleted there was a sharp increase in production of accessory pigments like chlorophyll b and carotenoids. The UV absorbance also drastically increased with the onset of the stationary phase. Thus lower concentration of nitrate (0.625g/L) was conducive for both biomass production as well as production of UV absorbing carotenoids.

The different concentrations of potassium sulphate used were 1g/L, 2g/L, 3g/L and 4g/. 2g/L of sulphate was conducive for highest biomass production. But though this concentration was contributing to highest biomass production, the total carotenoid concentration and UV absorbance declined. Hence 1g/L of sulphate was decided as optimum concentration for carotenoid production and UV absorption. The current findings will help in better production of UV protective pigment by the microalgae, with the media cost being less; thus enabling its maximum extraction followed by its incorporation in sun protective formulation.

5. Acknowledgement

We appreciate the help provided by Dr. B. B. Sharma (Principal, V. G. Vaze college of Arts, Science and commerce, Mulund, Mumbai) for providing excellent lab facilities and also for his constant support.

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