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[ijirset@gmail.com](mailto:ijirset@gmail.com)

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# Enhancement of Carotenoids Production from algae *Pseudanabaena limnetica* (Lemm.) *Komárek* and Accessing its Stability When Exposed to UV Radiation

Sagar Jayant Rambhiya<sup>1</sup>, Manjushri Arun Deodhar<sup>2</sup>

Ph.D. Scholar, Department of Biotechnology, KET's V. G. Vaze College of Arts, Science, and Commerce, Mithagar Road, Mulund East, Mumbai, Maharashtra, India<sup>1</sup>

Head of Department, Department of Botany, KET's V.G. Vaze College of Arts, Science, and Commerce, Mithagar Road, Mulund East, Mumbai, Maharashtra, India<sup>2</sup>

**ABSTRACT:** Nowadays natural compounds are demanded by cosmetic industries to protect skin damage caused by UV radiations and to replace chemical UV blockers in cosmetic products. Many natural compounds are safe and provide broad spectrum UV protection including carotenoids. Aim of the present study was to evaluate extraction and enhancement of carotenoid production in indigenous halophilic algal strain *Pseudanabaena limnetica* and further study its UV absorbing ability so that carotenoids can be used in cosmetic formulation. Different extraction methods viz. overnight freezing and maceration using various solvents like Hexane, acetone, ethyl acetate, ethanol and methanol were used for extraction. Out of all these, Overnight freezing technique in pure methanol proved to be the best solvent for extraction of carotenoids. The crude carotenoid extract obtained was analyzed by HPLC which showed the presence of carotenoids like Lutein, Astaxanthin and Canthaxanthin. Also enhancement in carotenoid production was studied by varying carbonate concentrations and 0.1gm/L of Na<sub>2</sub>CO<sub>3</sub> was found to produce maximum carotenoids. Further stability of carotenoids when exposed to UV radiations, with and without antioxidant Vitamin E was studied. Various combinations of Vitamin E and Carotenoid were exposed to UV radiation for 3 hours and stability was analyzed. It was found that combining Vit. E with Carotenoids increased UV absorbing ability of pigments even after 3 hours of UV exposure which was shown by its high SPF of above 20 and 5 Boot star rating. Thus carotenoids extracted from *Pseudanabaena limnetica* can be used as natural sunscreen compounds.

**KEYWORDS:** Carotenoids, Antioxidant, UV protection, *Pseudanabaena limnetica*, Sun protective factor, Boot star rating.

## I. INTRODUCTION

Culturing of the microalgae as an alternative feedstock for the biofuel production has received a lot of attention in the recent years. This superior feedstock offers several benefits such as effective land utilization, CO<sub>2</sub> sequestration, when coupled with the waste water treatment, and it does not trigger the food v/s fuel feud. But the practical difficulty in implementing technology is making the process economically affordable. Algae are the excellent source of pigments like phycobiliproteins, carotenoids, polysaccharides, lipids, long-chain fatty acids and rich proteins. These products can be sold at higher prices compared to the biofuels, which could subsidise the price of biofuels and offset the higher cost of algal cultivation. This concept is known as the "Bio-refinery concept" (Bhowmick et al. 2018). This bio-refinery concept is a novel thing and is a boon to the algal production.

Earlier the efforts have been taken in our organisation for cost effective mass cultivation of halophilic microalgal strain *Pseudanabaena limnetica* (Lemmermann) *Komárek* in specially designed 1000L photobioreactor system. The hardy halophilic strain has the ability to sustain light intensities up to 70,000 lux and temperature up to 45 °C and the reactor was operated in natural environmental conditions of Mumbai suburban region (Magar and deodhar 2018). Further the reactor system was successfully used for scrubbing unwanted CO<sub>2</sub> released from Bio-CNG production plant (Rambhiya et al. 2021).



Algae play several strategies to protect themselves from adverse environmental conditions. To protect the photosynthetic apparatus from severe light intensity the accessory pigments 'carotenoids' play a vital role. Some Xanthophylls are capable of cyclic conversions through de-epoxidation and epoxidation known as the xanthophyll cycle (Kuczynska et al. 2017). During light stress, violaxanthin is converted to zeaxanthin via the intermediate antheraxanthin, which plays a direct photoprotective role acting as an anti-oxidant and by stimulating non-photochemical quenching within light-harvesting pigments.

## II. REVIEW OF LITERATURE

Skin protection by nutritional interventions is garnering more attention now-a-days. Carotenoids have properties that may interrupt the course of assaults involved with UV radiation that eventually lead to skin damage. UV-A (320-400 nm) exposure leads to formation of Reactive Oxygen Species (ROS), especially singlet oxygen, causing oxidative stress that can damage cellular components. Carotenoids are potent singlet oxygen quenchers (Engelmann et al. 2011).

Carotenoids, their derivatives and their precursors are versatile isoprenoid compounds useful in skin care and cosmetics. All the carotenoids are derived from Phytoene and Phytofluene precursors. Carotenoid, phytoene and phytofluene are known to accumulate in human skin as well and absorb the UV light. Thus it would be reasonable to expect their role in photoprotection of skin (Martínez et al. 2015). The brown pigment fucoxanthin down regulates UV induced hyper-melanin production in skin (Shimoda et al. 2010). Zeaxanthin extracted from *Synechocystes pevalekii* is known to protect fibroblasts from UV rays and exhibits sun protective ability in UV-A region (Tendulkar and Deodhar 2018).

Present study deals with optimisation of production of UV protective carotenoids from *Pseudanabaena limnetica*, its extraction and quantification. It further deals with enhancing the stability of carotenoids with Vitamin E (tocopherol).

## III. MATERIAL AND METHODS

### 1. ALGAL CULTURE

Salt tolerant algal strain *Pseudanabaena limnetica* (Lemm.) Komárek was isolated by Mhaskar and Deodhar (2016) from salt pans of the eastern suburban region of Mumbai, India.

### 2. CULTIVATION OF PSEUDANABAENA LIMNETICA

Culture was successfully maintained in Modified Seawater BG11 Medium (MSWBG11) in standard laboratory conditions at 25°C and 16:8 hours of Day: night cycles in 250mL capacity Erlenmeyer flasks. Mass cultivation of algae was done as described by Magar and Deodhar (2018) in 60L air lift flat panel photo bioreactors. Photobioreactors were made of glass having dimensions of 50cm X 20cm X 70cm (L x B x H). Ambient air was sparged using an aquarium pump at the rate of 4LPM.

### 3. EXTRACTION OF CAROTENOIDS

Freshly harvested culture was washed and centrifuged to obtain biomass pellet, 1gm of this WET biomass was used for extraction. In another set 1gm of above WET biomass was dried in oven at 60° for 24 hours to obtain 0.1gm of DRY biomass. Both WET biomass and DRY biomass were used for extraction in 10mL and 20mL of solvents respectively. Different solvents used for extraction were Methanol, Ethanol, Acetone, Hexane and Ethyl Acetate. Two different extraction methods viz. MACERATION and OVERNIGHT FREEZING were tested using each solvent and biomass combination. In maceration method, biomass was macerated using mortar and pestle for 15mins, filtered and spectrophotometrically analysed. In Overnight freezing method each combination was incubated at 4°C for 24 hours. After incubation solvent was separated from biomass by centrifugation at 5000rpm for 15mins.

### 4. SPECTROPHOTOMETRIC DETERMINATION OF TOTAL CAROTENOIDS.

The Carotenoids extracted from above step were analysed spectrophotometrically in the range of 200nm to 540nm (ThermoFisher Scientific, Genesys 10S UV-VIS Spectrophotometer). Further Chl. A, Chl. B and total carotenoids were calculated using formula (Lichtenthaler and Wellburn 1983, Tendulkar and Deodhar 2017).

The equations specific for Methanol solvent are as follows:

$$\text{Chlorophyll A: } 15.65 (A_{666}) - 7.34 (A_{653})$$



Chlorophyll B: 27.05 ( $A_{653}$ ) – 11.21 ( $A_{666}$ )

Total carotenoids: [1000 ( $A_{470}$ ) – 3.27 (chlorophyll A) – 4.04 (chlorophyll B)]/ 229

## 5. HPLC ANALYSIS OF CAROTENOIDS

The carotenoids were analysed by injecting 10  $\mu$ L of the carotenoid extract into an HPLC system (Shimadzu, Japan) equipped with a photodiode array detector and Reverse-phase C18 column. The mobile phase consisted of solvent A (acetonitrile: methanol, 7:1 v/v) and solvent B (acetonitrile: methanol: water: ethyl acetate, 7:0.96:0.04:2 v/v). The flow rate was set at 1.0 mL/min, and pigment content was detected by measuring the absorbance at 450 nm. The chromatographic peaks were analysed by comparing the retention time against the standard.

## 6. EFFECT OF VARIOUS PHYSICO-CHEMICAL PARAMETERS ON THE GROWTH AND CAROTENOID PRODUCTION IN *P.LIMNETICA*

### 6.1. Effect of indoor and outdoor conditions on growth and carotenoid production in *P.limnetica*.

Experiment was conducted in INDOOR (in laboratory) and OUTDOOR (outside the laboratory on terrace premises) conditions to study effect of environmental conditions on the Growth and Carotenoid production of algae *P. limnetica*. Experiment was conducted in 60 Liter capacity air lift flat panel photo bioreactors that were kept in Indoor and Outdoor conditions. For INDOOR conditions the 60L photobioreactor was provided with 6 tubes x 2 feet white florescent tubes (24watts each) that gave 12000 lux intensity of light for 16:8 hour day: night cycle. For OUTDOOR operation, photobioreactor was exposed to natural sunlight of 55,000 to 70,000 lux light intensity for about 8hours and temperature was between 40°C and 45°C. Each tank was provided with MSWBG11 medium and initial inoculum size of 0.35 gm/L. Aeration to both the tanks was given at the rate of 4LPM (Hailea 7200Lph Hi-blow Hap-120 Air Pump). Total dry biomass yield of algae (gm/L) and total carotenoid content ( $\mu$ g/mL) was calculated using formula mentioned above at every 3 days interval for total of 18 days.

### 6.2. Effect of various Carbonate concentrations on growth and carotenoid production in *P.limnetica*.

MSWBG11 contains 0.02 gm/L of  $\text{Na}_2\text{CO}_3$  as the standard concentration for the growth of *P.limnetica*. Different concentration of  $\text{Na}_2\text{CO}_3$  viz. 0.01 gm/L, 0.02 gm/L, 0.1 gm/L, 0.2 gm/L were used to study its effect on growth and biomass yield of algae as well as total carotenoid production. Starting inoculum size of 0.35 gm/L was used to inoculate different concentration of  $\text{Na}_2\text{CO}_3$  containing medium. The experiment was carried out in 60L capacity flat panel Photobioreactors in outdoor environmental conditions as mentioned in above section 6.1. The amount of total dry biomass yield (gm/L) and total chlorophyll a, b and carotenoid content evaluated using formula mentioned in section 4.

## 7. STABILITY STUDIES OF CAROTENOIDS

### 7.1. The effect of UV-exposure on stability of Carotenoids

Crude Carotenoid was extracted from dried biomass of *P.limnetica* as described in section 3. Analytical grade Vitamin E was procured from HiMedia Labs (Mumbai). Phytosqualan is a ready to use cosmetic base which was used to dissolve carotenoids and Vit. E (Yasham speciality ingredients Pvt. Ltd., Mumbai) for the experiment. Each sample was exposed to UV-A and UV-B radiations using UV-A Philips tube (wavelength maxima 340nm to 400nm) and UV-B bulb (Philips, wavelength maxima 280nm- 300nm) respectively. Both tubes were places at the distance of 5cm from the sample plates having radiation intensity of 3750  $\mu$ W/cm<sup>2</sup> which is equal to 3.75 milli J/sec cm<sup>2</sup>, measured using UV-meter (Lutron UV-340A model). Each samples were exposed to UV-A and B radiation for total of 3 hours, giving total irradiance of 27 J/cm<sup>2</sup>.

Here Carotenoid Control (1000 $\mu$ g/ml) and Vit. E control (1000 $\mu$ g/ml) were exposed to UV radiations. The effect of UV exposure on stability of carotenoid and Vit.E was studied spectrophoto-metrically in the range of 200 to 400nm at every 60mins interval for 3 hours duration. At each interval Sun Protective Factor (SPF), UV-A/UV-B Ratio, Boot Star Rating and Critical wavelength were calculated as per formula (Donglikar and Deore2016).

### 7.2. Synergistic effect of Vit. E with Carotenoid on stability of carotenoids when exposed to UV radiation

Different combination of Carotenoids and Vit.E concentrations were taken viz. TEST 1 (carotenoid 1000 $\mu$ g/mL + Vit. E 1000 $\mu$ g/mL), TEST 2 (carotenoid 1000 $\mu$ g/mL + Vit. E 2000 $\mu$ g/mL), TEST 3 (carotenoid 2000 $\mu$ g/mL + Vit. E 1000 $\mu$ g/mL). Exposure condition and irradiance was kept same as described in section 7.1. The effect of UV exposure on stability of three test samples viz. TEST 1, TEST 2 and TEST 3 was studied spectrophoto-metrically in the range of



200 to 400nm at every 60 mins interval for 3 hours duration. At each interval Sun Protective Factor (SPF), UV-A/UV-B Ratio, Boot Star Rating and Critical wavelength were calculated as per formula given by (Donglikar and Deore 2016).

#### IV. RESULTS AND DISCUSSION

##### 1. CULTIVATION AND SCALE UP OF *PSEUDANABAENA LIMNETICA*

The indigenous algal strain isolated from salt pan areas of Mumbai suburbs is capable of withstanding harsh tropical environmental conditions (Mhaskar and Deodhar 2016). Its capability to survive in these high light intensities of 70,000 lux and temperatures of 45°C has been thoroughly evaluated. Culture was successfully maintained in standard laboratory conditions at 25 °C and 16:8 hours of day: night cycles in 250mL capacity Erlenmeyer flasks. The scaling up of 250mL culture to 60 litres was done using air lift flat panel photo bioreactors in outdoor environmental conditions. Ambient air was sparged using aquarium pump at the rate of 4LPM in order to keep the culture sufficiently floating in the medium. Media used was Modified Sea water BG11 media as per the composition provided by Magar and Deodhar (2018). This culture growing in 60Litre capacity photo bioreactor was used for further experiment and studies.

##### 2. EXTRACTION OF CAROTENOID:-

###### 2.1. Extraction of Carotenoids using different methods:-

Extraction of carotenoids from algal biomass is one of the crucial steps involved in obtaining carotenoids. There are various methods that are employed for extraction of carotenoids viz. complete cell disruption (milling, ultrasound, microwave, freezing, thawing or chemical attack), organic solvent extraction, from biomass green solvent mediated extraction, supercritical fluid mediated extraction and *in-situ* extraction. Organic solvent mediated extraction of carotenoids from biomass is one of the most commonly used methods (Guedes et al. 2011). Carotenoids have varied polarity some of which are polar are extracted using polar solvents like Ethanol and those which are non-polar are extracted in non-polar solvents like Hexane (Mezzomoand Ferreira 2016).

Optimizing the solvent for extraction was an important part of this study. The parameters to be studied were minimum biomass utilization, best extraction solvent, and number of extraction steps involved. To fulfil this aim, algal biomass used for the extraction of carotenoids was harvested from 60Litre photobioreactors operated under standard conditions. Two different extraction methods viz. MACERATION and OVERNIGHT FREEZING were tested using various solvents as explained in section 3 of materials and method. Further concentration of total carotenoids was calculated for each solvent treatment using formula for each solvent as given in section 4 of material and methods.

Carotenoids are known to have inherent UV absorbing ability and thus has photo-protective role in photosynthesis. These pigments absorb UV radiations and protect skin from photo-damage. Earth is continuously exposed to high UV radiations, specifically UV-A and UV-B. UV-C is rather absorbed and reflected back by ozone layer. Thus for cosmetic point of view compounds that possess UV absorbing ability in UV-A and UV-B regions are of prime importance. Here the carotenoids extracted from *P.limnetica* were analysed for providing maximum UV absorbing ability.

###### 2.2. Effect of various extraction methods on UV absorptive ability of carotenoids extracted from *P.limnetica*.

###### 2.2.1. Effect of various solvents on Extraction of carotenoids:

Irrespective of any extraction method maceration or overnight freezing or for both wet and dry biomass, solvent plays a major role in amount of carotenoid extracted as well as UV absorptive ability of extract. Polar solvents like methanol and ethanol had highest capacity to extract carotenoids from algal biomass. E.g. when the maceration of wet biomass was performed using different solvents as shown in Table 1, the highest amount of carotenoids obtained was 136.178 µg/mL. In acetone the amount of carotenoids produced was 101.602 µg/mL. Contrary to this when the extraction was done in nonpolar solvents like hexane or ethyl acetate very less amount of carotenoids were extracted. In hexane the amount of carotenoids extracted was only 0.810 µg/mL whereas in ethyl acetate the amount was 45.428 µg/mL.

###### 2.2.2. UV absorptive ability of carotenoids extracted by different methods:

The UV absorptive ability of carotenoids extracted with different procedures was also studied using spectrophotometric analysis. The absorption spectra were studied at different wavelengths from 200nm to 540nm. The wavelengths between 200nm to 400nm represent UV visible region which can be divided in to UV-C (200nm to 280nm) UV-B region (Between 280nm to 320nm) and UV-A (320nm to 400nm). The region between 400nm to 540 nm represents the part of visible region at which different carotenoids show absorption maxima.

Polar solvents like methanol and ethanol had highest absorption in both UV and visible regions. For example in maceration method when dry biomass was used, methanol extract had UV absorbance of 3.218 in UV-B region while in UV-A region it was 3.750. It also showed a peak between 420nm to 500nm which suggest the extract is rich in carotenoids.

Contrary to this when the extraction was done in nonpolar solvents like hexane or ethyl acetate it had negligible absorption in UV region (**Figure 1 and Table 1**). For example maceration of dry biomass in hexane showed negligible absorbance of 1.131 in UV-B region and in UV-A region the absorbance was 0.657.

### 2.2.3. Effect of extraction process:

When maceration method was used and solvent of extraction was methanol in wet biomass the amount of carotenoids produced was 136.178  $\mu\text{g/mL}$ . Whereas when overnight freezing method was used for wet biomass and methanol was used as solvent there was a small increase in total amount of carotenoid, it was 139.888  $\mu\text{g/mL}$ .

### 2.2.4. Choice of biomass Dry/wet plays a substantial role in extraction process.

When overnight freezing method was used, methanol was used as solvent for wet biomass amount of carotenoid produced was 139.888  $\mu\text{g/mL}$  whereas when dry biomass was used for extraction the amount of carotenoid extracted was substantially better it was 143.934  $\mu\text{g/mL}$ . This was also reflected in UV absorptive ability of extract. The overnight freezing of wet biomass had UV absorbance in B region that was 3.233 and in A region the absorbance was 2.967. But when the dry biomass was subjected to freeze drying in methanol the absorbance in B region was substantially increased to 4.003 and in A region it was increased to 3.866 (**Figure 2 and Table 1**). Thus overnight freezing method using dry biomass and methanol as solvent were shortlisted for further extraction.

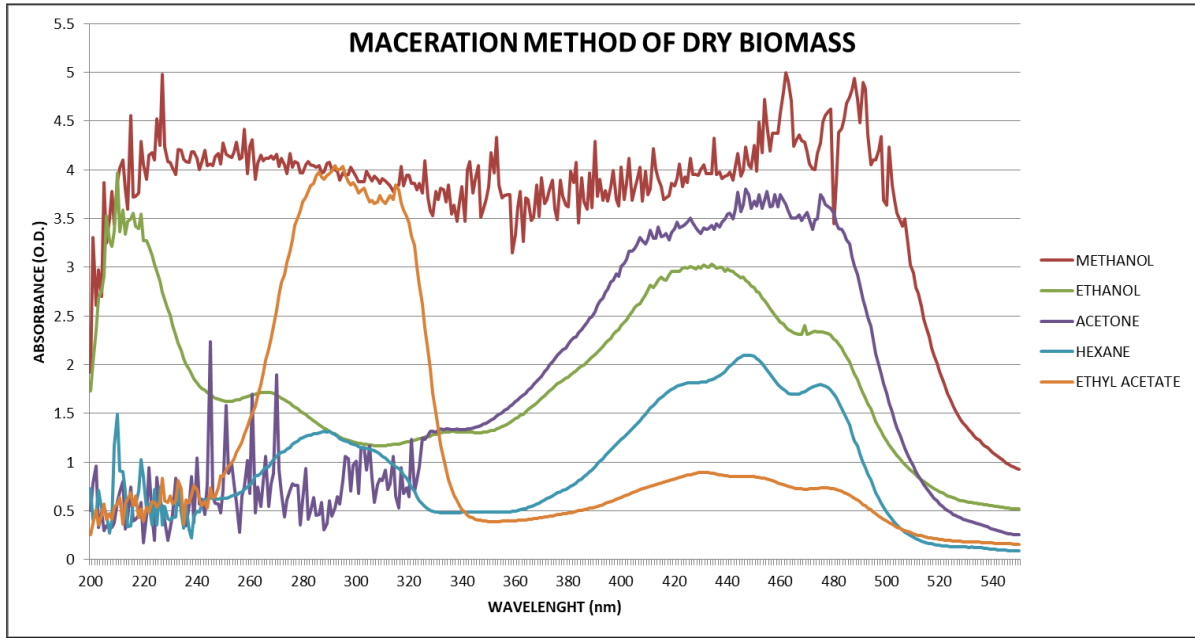
### 2.2.5. Carotenoid productivity of *P.limnetica*.

The highest carotenoid was yielded when dry biomass was extracted by freeze drying using 20mL methanol the amount of carotenoid extracted was 139.888  $\mu\text{g/mL}$ . But in above experiment 0.1 gm of dry biomass was extracted in 20 mL of methanol. Hence when the experiment was conducted in batch mode, productivity of *P.limnetica* was found to be 28.787  $\mu\text{g/mg}$  of dried biomass.

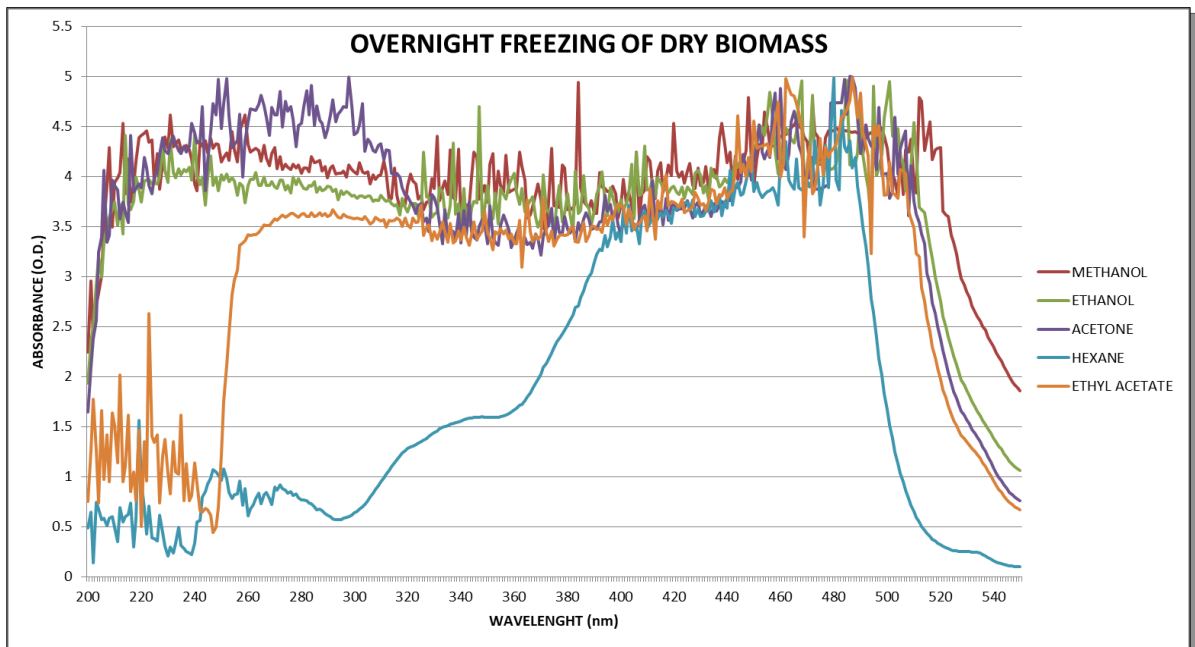
Recently **Singh et al. (2019)** discussed high production of carotenoids in green algae *Asterarcysquadricellulare*. When algae was cultured in BBM medium the biomass produced in 24 days was 0.85 mg/mL and carotenoid produced was 35  $\mu\text{g/mg}$  under continuous light conditions and when all culture conditions were standardised the biomass was increased to 1.9 mg/mL and the carotenoid content was increased to 118  $\mu\text{g/mg}$ .

**Table 1. Average UV absorbance values for UV-A, UV-B and UV-C**

Methods	Solvents	Methanol	Ethanol	Acetone	Hexane	Ethyl acetate
Maceration of Wet Biomass	Total carotenoid extracted $\mu\text{g/mL}$	136.178	91.031	101.602	0.810	45.428
	UV-C (200-280nm)	3.113	3.189	0.537	0.123	0.456
	UV-B (280-320nm)	3.218	3.329	0.822	0.137	0.923
	UV-A (320-400nm)	2.917	2.938	1.814	0.048	0.653
Maceration of Dry Biomass	Total carotenoid extracted $\mu\text{g/mL}$	119.190	68.827	70.097	14.713	13.448
	UV-C (200-280nm)	4.012	2.246	0.660	0.715	1.192
	UV-B (280-320nm)	3.943	1.245	0.747	1.131	3.792
	UV-A (320-400nm)	3.750	1.619	1.819	0.657	0.727
Overnight freezing of wet biomass	Total carotenoid extracted $\mu\text{g/mL}$	139.888	139.576	134.300	0.636	79.358
	UV-C (200-280nm)	3.077	3.002	4.163	-0.051	1.174
	UV-B (280-320nm)	3.233	3.269	4.364	-0.079	1.639
	UV-A (320-400nm)	2.967	2.958	2.715	0.046	1.322
Overnight freezing of dry biomass	Total carotenoids extracted $\mu\text{g/mL}$	143.934	127.618	129.233	53.054	125.421
	UV-C (200-280nm)	4.143	3.889	4.282	0.678	2.010
	UV-B (280-320nm)	4.003	3.795	4.348	0.847	3.579
	UV-A (320-400nm)	3.866	3.749	3.502	2.137	3.461



**Figure 1: Absorbance of carotenoids from DRY biomass in different solvent using MACERATION METHOD**



**Figure 2: Absorbance of carotenoids from DRY biomass in different solvent using OVERNIGHT FREEZING METHOD**

### 3. HPLC ANALYSIS OF CAROTENOID EXTRACT

The Crude Carotenoid extract obtained from above extraction procedure was further analysed by HPLC. The extract was evaporated at room temperature till dry pigment was obtained and the same was dissolved in 1mL of 100% HPLC grade methanol. The samples were then subjected to HPLC analysis.

The peaks obtained at various time intervals were compared with standard samples of standard pigments like Lutein, Chlorophyll B, Canthaxanthin, and Astaxanthin. **Table 2** gives the retention time of standard samples. Their concentration is represented as Area %.

The Crude Carotenoid extract showed many different peaks at different retention times (**Figure 3**). Peak 1 at retention time 3.898mins had peak area of 10.497%. It did not match with any available standards and was described as Unknown 1. Also Peak 2 at retention time 5.829mins having 4.599% Area did not match with standards and was mentioned as Unknown 2. Peak 3 obtained at retention time of 6.554mins matched with Astaxanthin standard peak given in **Table 2**. Area % of this compound was calculated to be 1.600. Peak 4 eluted at retention time 8.716mins was coinciding with the standard of Lutein (retention time 8.519 mins). Its area% was comparatively larger i.e. 12.805. Other peaks viz. Peak 5 and 6 at retention time 10.632mins having area% 1.783 and at retention time 18.548mins having area% 2.053 matched with retention times of Canthaxanthin and Chlorophyll B of standard peaks respectively. Compared to 4 known carotenoids eluted, Lutein was in highest concentration in the sample having % area of 12.805 (**Table 3**). Apart from above peaks, Highest peak (Peak 7) of all was obtained at retention time of 22.265mins having Area % of 28.51. This peak did not match with any standards available and was labelled as Unknown 3 (**Figure 3**). Thus HPLC analysis suggests the presence of Lutein, Astaxanthin and Canthaxanthin carotenoid pigments in the sample.

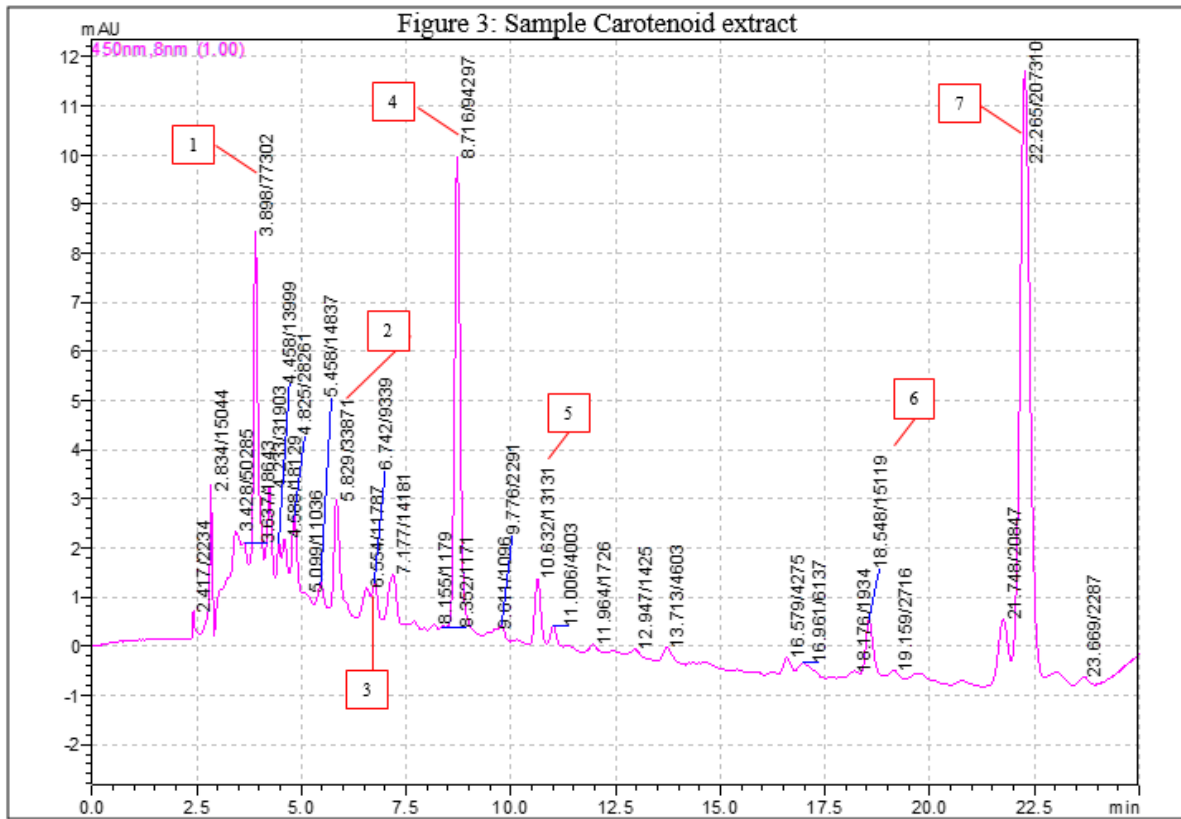
**Table 2: Retention time and Area of Standard Carotenoids**

Sr. No.	Carotenoid	Time (mins)	Area	Height	A/H	Area%
1	Lutein	8.519	1030310	109493	9.4100	90.125
2	Chlorophyll B	18.463	232013	19224	12.0690	68.150
3	Canthaxanthin	10.849	225404	22042	10.2260	73.788
4	Astaxanthin	6.379	3671934	410996	8.9340	86.186

**Table 3: Retention time and Area of Sample Carotenoid Extract**

Peak No.	Carotenoid	Time (mins)	Area	Height	A/H	Area%
1	Unknown 1	3.898	77302	8220	9.4040	10.497
2	Unknown 2	5.829	33871	2638	12.8370	4.599
3	Astaxanthin	6.554	11787	808	14.5840	1.600
4	Lutein	8.716	94297	9689	9.7320	12.805
5	Canthaxanthin	10.632	13131	1363	9.6320	1.783
6	Chlorophyll B	18.548	15119	1179	12.8270	2.053
7	Unknown 3	22.265	207310	12419	16.6930	28.51





#### 4. EFFECT OF INDOOR AND OUTDOOR ENVIRONMENTAL CONDITIONS ON GROWTH OF ALGAE AND CAROTENOID PRODUCTION.

In this experiment effect of different environmental conditions viz. INDOOR (in laboratory) and OUTDOOR (outside the laboratory on terrace premises) on the Growth and Carotenoid production of algae *P. limnetica* was evaluated. Experiment was conducted in two 60 Liter capacity air lift flat panel photo bioreactors that were kept in Indoor and Outdoor conditions. For indoor conditions the 60L photobioreactor was provided with 12000 lux intensity of light for 16:8 hour day: night cycle. For outdoor operation, photobioreactor was exposed to natural sunlight of 55,000 to 70,000 lux light intensity for about 8hours and temperature was between 40°C and 45°C. Aeration of 4LPM was provided in each tank. Total biomass yield of algae (gm/L) and total carotenoid content (µg/mL) was calculated and estimated at every 3 days interval for total of 18 days.

##### 4.1. Effect of indoor and outdoor conditions on growth and biomass production:

When algae were cultivated in indoor conditions, initial inoculum was 0.38 gm/L on day 0. It experienced a negligible lag phase and entered into a log phase by day 3 when the biomass increased to 0.47 gm/L. The log phase continued till day 15 and biomass increased from 0.47 gm/L to 0.73 gm/L. After day 15, there was negligible increase in biomass and on day 18<sup>th</sup>, indicating stationary phase. The biomass yield on 18<sup>th</sup> day was 0.76 gm/L. On the other hand when algae was cultivated in outdoor environmental conditions, initial biomass was 0.37gm/L on day 0. Cultures immediately entered log phase and the biomass sharply increased 0.74 gm/L on day 3. At indoor conditions it took 18 days to achieve this much biomass. In outdoor conditions, the log phase ended comparatively early on 6<sup>th</sup> day and thereafter there was negligible increase in biomass. On day 6<sup>th</sup> it was 1.09gm/L, on day 9<sup>th</sup> it became 1.16gm/L Biomass yield on day 12 was 1.27 gm/L and then it increased slightly on day 15 to obtain maximum biomass of 1.33 gm/L indicating the stationary phase because of depletion of nutrients. On day 18, biomass yield dropped to 1.21 gm/L indicating death phase (Figure 4, Table 4).

This indicated that the cultures thrived very well in outdoor conditions and biomass production was almost doubled outdoor compared to indoor.

##### 4.2. Effect of indoor conditions on Chlorophyll A content:

Earlier, chlorophyll was regarded as a tool to measure growth of algae (Boyer et al., 2009). In indoor conditions when the nutrients were amply available and the cultures were in log phase there was continuous increase in amount of Chlorophyll A in algal culture. It was 0.45 µg/mL on day 3 and 13.21 µg/mL on day 9 (Table 4). The highest chlorophyll content 32.74



µg/mL was observed on 12<sup>th</sup> day. After 12<sup>th</sup> day the amount of chlorophyll appears to decrease indicating the stationary phase. It became 28.75 µg/mL on 15<sup>th</sup> day and the lowest on 18<sup>th</sup> day 24.20 µg/mL.

**4.3. Effect of indoor conditions on Chlorophyll B and carotenoids production:**

With the onset of stationary phase and as nutrient availability decreased, the sharp decline in Chl. A containment was associated with abrupt increase in amount of chlorophyll b and carotenoids in culture.

On 12<sup>th</sup> day when the cultures were at the end log phase, the amount of Chl. A was 38.18 µg/mL, Chl. B was 35.85 µg/mL and carotenoids was only 11.049 µg/mL. On 15<sup>th</sup> day as log phase ends the amount of Chl. A sharply decrease to 28.75 µg/mL and that is associated with a prominent increase in Chl. B which is 47.40 µg/mL and amount of carotenoids was increased to 16.29 µg/mL. The same trend was observed on 18<sup>th</sup> day. Amount of Chl. A still decreased to 24.20 µg/mL and Chl. B became very high 50.23 µg/mL and carotenoids concentration was increased to 16.410 µg/mL. Similar observation was made by **Tendulkar and Deodhar (2017)** in their studies on carotenoids production in *Synechosystespevalekii*. Many scientific papers in the literature have dealt with the stimulation of carotenoid biosynthesis by cell, grown in culture media with low concentrations of inorganic nitrogen, phosphorus and sulphur (**Forjan et al. 2007**).

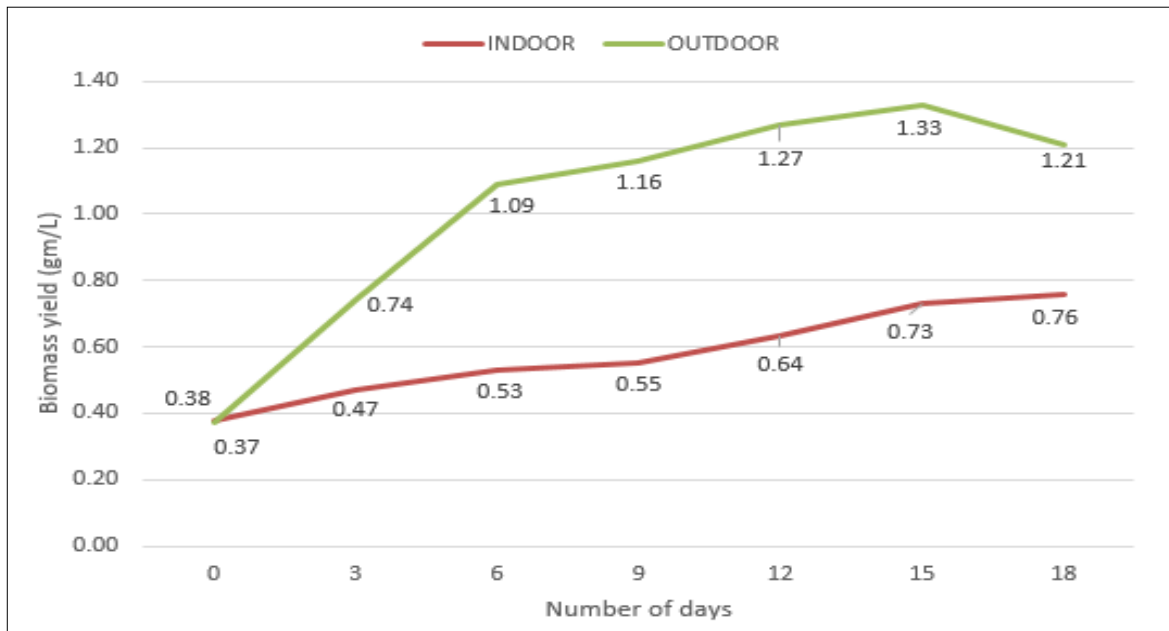


Figure 4: Effect of Indoor and Outdoor Conditions on Dry Biomass yield (gm/L)

Days	Chlorophyll a (µg/mL)	Chlorophyll b (µg/mL)	Total carotenoid (µg/mL)	Biomass yield (gm/L)
0	0.19	0.69	0.164	0.38
3	0.45	0.66	0.205	0.47
6	2.02	8.36	1.090	0.53
9	13.21	24.64	6.730	0.55
12	38.18	35.85	11.049	0.63
15	28.75	47.40	16.290	0.73
18	24.20	50.23	16.410	0.76



#### 4.4. Effect of outdoor conditions on Chlorophyll A content of algae:

As discussed earlier in outdoor condition log phase ended very early hence the highest Chl. A content was found on 9<sup>th</sup> day it was 25.44 µg/mL. There after there was decline in amount of Chl. A on 15<sup>th</sup> day it was 23.21 µg/mL and on 18<sup>th</sup> day it still reduced to 23.12 µg/mL (Table 5).

Contrary to this in indoor conditions the highest Chl. A obtained was as high as 38.18 µg/mL. High irradiance is known to cause photo inhibition of chlorophyll A.

#### 4.5. Effect of outdoor conditions on PSII pigments Chl. B and carotenoids:

Inhibition of Chl. A in outdoor condition was associated with sharp increase in pigments of Photosystem II. On 6<sup>th</sup> day amount of Chl. B was 10.81 µg/mL. On 9<sup>th</sup> day when log phase was at end, it sharply increased to 52.60 µg/mL. On 12<sup>th</sup> day it was 65.98 µg/mL. Maximum Chl. B was observed on 18<sup>th</sup> day, it was 69.52 µg/mL (Table 5).

Days	Chlorophyll a (µg/mL)	Chlorophyll b (µg/mL)	Total carotenoid (µg/mL)	Biomass yield (gm/L)
0	0.10	1.02	0.195	0.37
3	0.42	0.81	0.198	0.74
6	5.72	10.81	2.994	1.09
9	25.44	52.60	12.683	1.16
12	25.29	65.98	16.505	1.27
15	23.21	68.71	17.849	1.33
18	23.12	69.52	19.055	1.21

Similarly carotenoids content on 6<sup>th</sup> day was only 2.994 µg/mL which increased to 16.505 µg/mL on 12<sup>th</sup> day and at the end of experiment on 18<sup>th</sup> day it was as high as 19.055 µg/mL. Whereas in indoor experiment it was 16.410 mg/mL at the end of experiment.

The best part of the experiment is this carotenoids increase is without harming biomass productivity. In outdoor condition the biomass productivity is almost doubled and it was associated with carotenoids production which is not observed in other carotenoids producing algae.

The light source plays a crucial part in photosynthesis. Theoretically, the growth rate of vegetative algae cells increases with increasing light intensity and the maximum growth rate is achieved at specific light intensity which is called constant saturation of irradiance. If irradiance is increased beyond this light intensity photo-damage starts. High irradiance ceases growth and leads to elevated level of ROS. The cell activates non-photochemical quenching of chlorophyll molecule as photo-protective mechanism. This defence strategy involves synthesis of secondary carotenoids such as Astaxanthin, Lutein and Xanthophylls (Faraloni and Torzillo 2017, Wu et al. 2020).

Celekli and Donmez (2005) studied the role of two different light intensities on growth and β-carotene production of *Dunaliellasalina* isolated from Hypersaline Lake. At 32 kerg/cm/s, NaCl 15% and pH 9 the biomass cell count was 42 x 10<sup>6</sup> cells per mL and β-carotene produced was 0.08 ng/cell. When light intensity was increased the β-carotene was increased to 0.136 ng/cell but cell count was decreased to 1.3x10<sup>6</sup> cells per mL.

Recently Xu et al. in 2019 screened five different halotolerant strains of *Dunaliella sp.* for their capacity for photo-protection against high light stress and production of carotenoids. The different light intensities used were 200, 500, 1000, and 1500 µmol m<sup>-2</sup> s<sup>-1</sup>. For The two strains DF17 and UTEX 2538 the growth rate increased with increase in light intensity and was highest at 1500 µmol m<sup>-2</sup> s<sup>-1</sup>. The total carotenoids content was also higher.



*Haematococcus lacustris* is a promising source of Astaxanthin production. But that is light sensitive microalgae and shows a sluggish growth when subjected to high light irradiance. At relatively low light intensities of around  $20\text{--}30 \mu\text{mol m}^{-2} \text{s}^{-1}$  the organism shows the growth rate of  $0.15\text{--}0.23 \text{d}^{-1}$ . **Kaewpintong et al. (2007)** observed morphological changes in vegetative cells when light intensity was greater than  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  which was associated with accumulation of Astaxanthin. This is because the cell activates Non Photochemical Quenching (NPQ) at high irradiance. However when the cells are bubbled with elevated level of  $\text{CO}_2$  NPQ decreases. Recently **Wu et al. (2020)** investigated combined effect of increased light intensity and carbon enrichment on *H. lacustris*. Three different light intensities used were low intensity  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ , medium intensity  $55 \mu\text{mol m}^{-2} \text{s}^{-1}$  and high intensity  $110 \mu\text{mol m}^{-2} \text{s}^{-1}$  with or without supplementation of 1%  $\text{CO}_2$  v/v. The highest growth rate of  $4 \times 10^5$  cells per mL was observed in high intensity with  $\text{CO}_2$  supplementation which was 4-6 folds than non- $\text{CO}_2$  groups. The low light intensity group without  $\text{CO}_2$  supplementation gave cell count was  $1.2 \times 10^4$  cells per mL. But all  $\text{CO}_2$  groups showed low level of Astaxanthin with average of  $1.5 \times 10^{-11}$  g/cell, whereas in Non  $\text{CO}_2$  groups the highest Astaxanthin content of high light group was found to be  $4.9 \times 10^{-11}$  g/cell.

In present communication it was concluded that high carbonate in the form of  $\text{Na}_2\text{CO}_3$  enhances dissolution and availability of  $\text{CO}_2$  to algal cells and thus leads to enhanced biomass production. In next experiment effect of high carbonate on the production carotenoid pigment was analysed.

## 5. EFFECT OF VARIOUS CARBONATE CONCENTRATION OF GROWTH AND CAROTENOID PRODUCTION OF ALGAE.

Among various stresses applied for enhanced carotenoid production in algae, nutrient-deficiency by reducing concentration of few chemical components improves carotenoid accumulation in algae but hinders active cell growth and biomass production. To overcome this problem, several studies have been carried out on the supplementation of inorganic carbon in the form of gas or solids which not only enhances pigment production but also supports cell growth. **Srinivasan et al. (2018)** studied the effect of supplementation of Sodium bicarbonate on biomass production, photosynthetic activity, biochemical constituents, nutrient uptake and antioxidant response of *Dunaliella salina* during macronutrient deficit conditions.

In present research the main objective was using algal cultures for mitigating industrial  $\text{CO}_2$  and for scrubbing higher amount of  $\text{CO}_2$  the concentrated  $\text{Na}_2\text{CO}_3$  enriched medium was used where the concentration of  $\text{Na}_2\text{CO}_3$  used was 0.2 gm/L instead of 0.02 gm/L in BG11 medium. The objective of present research was to study how the elevated level of  $\text{Na}_2\text{CO}_3$  particularly effect carotenoid production in *P. limnetica*.

In current experiment effect of various Carbonate concentrations on total biomass yield and carotenoid production was studied. Algae *P. limnetica* was provided with  $\text{Na}_2\text{CO}_3$  as the carbonate source and different concentrations viz. 0.01 gm/L, 0.1 gm/L and 0.2 gm/L were tested against control (0.02 gm/L). Experiment was conducted in 60 Litre flat panel photobioreactors with 4 LPM of ambient air and kept in outdoor environmental conditions which experienced natural sunlight of 55,000 to 70,000 lux intensity for about 8 hours and temperature was between  $40^\circ\text{C}$  and  $45^\circ\text{C}$ . Total biomass yield of algae (gm/L) and total carotenoid content ( $\mu\text{g/mL}$ ) was calculated and estimated at every 3 days interval for total of 18 days.

### 5.1 Effect of different carbonate concentration of growth of algae:

*P. limnetica* was grown under high irradiance in outdoor condition and supplemented with different concentration of  $\text{Na}_2\text{CO}_3$ . The biomass produced at 3 day interval for 15 days was interpreted as shown in **Table 6**. As concentration of carbonate increases it supports the growth of biomass. At the end of 15 days the biomass produced at 0.01 gm/L of  $\text{Na}_2\text{CO}_3$  was 0.70 gm/L. At 0.02 gm/L of  $\text{Na}_2\text{CO}_3$  the biomass produced on 15<sup>th</sup> day was increased to 0.83 gm/L. The highest Biomass was produced was 1.15 gm/L on 15<sup>th</sup> day when 0.2 gm/L of  $\text{Na}_2\text{CO}_3$  was supplied.

### 5.2. Effect of various carbonate concentration on Chlorophyll A:

As discussed earlier, chlorophyll A is the indicator of growth. When biomass increase is observed there is simultaneous increase in chlorophyll A content also.

Hence as seen in **Table 6** increase in  $\text{Na}_2\text{CO}_3$  concentration also contributed to increase in Chl. A level. The cultures supplemented with 0.01 gm/L of  $\text{Na}_2\text{CO}_3$  produced  $12.67 \mu\text{g/mL}$  of chlorophyll at the end of 15<sup>th</sup> day. And cultures supplemented with 0.2 gm/L of  $\text{Na}_2\text{CO}_3$  created the drastic increase in Chl. A. It was as high as  $37.94 \mu\text{g/mL}$  at the end of 15 days.

### 5.3. Effect of various carbonate concentrations on Chlorophyll B and carotenoids production:

As explained in previous section accessory pigments like Chl. B and carotenoids start increasing when culture enters stationary phase.

As seen in **Table 6** the highest carotenoid production was achieved when cultures were supplemented with 0.1gm/L of Na<sub>2</sub>CO<sub>3</sub>. At this concentration the amount of carotenoids produced was 17.094 µg/mL. Further increase in Na<sub>2</sub>CO<sub>3</sub> Concentration caused little decline in carotenoids. It was 15.037 µg/mL at the end of 15 days. At this concentration Chlorophyll B was at highest level it was as high as 60.00 µg/mL.

This tells that addition of 0.1 gm/L of Na<sub>2</sub>CO<sub>3</sub> supported good growth of algae and highest carotenoids but double the amount of Na<sub>2</sub>CO<sub>3</sub> i.e. 0.2 gm/L support maximum biomass yield but not carotenoid production (**Table 6**).

**Srinivasan et al.(2015)** studied the effect of various concentrations of Sodium Bicarbonate on growth and β-carotene production in algal species *Dunaliellasalina*. Various concentrations of sodium bicarbonate considered were 0, 50, 100, 150, 200 mM and experiment was conducted for 28 days. As concentration of sodium bicarbonate increased from 0 to 200 mM, biomass increased from 0 to 100mM where maximum biomass yield in *D. salina* was obtained at 100mM sodium bicarbonate, after which increase in the concentration did not support growth and total biomass yield reduced. Total carotenoids were found to be maximum on day 21<sup>st</sup> and sodium bicarbonate concentration of 150mM, where culture has reached stationary phase of its growth. Total carotenoid was 7.10 ±0.08µg/mL and maximum β-carotene was 180.06 ± 0.07 µg/100mg of sample. He proved that supplementing sodium bicarbonate upto 150mM was beneficial for maximum growth as well as high β-carotene content beyond which it proved detrimental to algae *D. Salina*.

Thus for overall high biomass yield and total carotenoid production 0.2gm/L of Na<sub>2</sub>CO<sub>3</sub> considered for further experiments.

Table 6: Effect of different Carbonate Concentration on carotenoid production							
Concentrations	DAYS	0	3	6	9	12	15
0.01gm/L	Biomass yield (gm/L)	0.37	0.4	0.59	0.74	0.84	0.7
	Chl. A	6.58	5	8.35	12.82	15.26	12.67
	Chl. B	2.24	1.08	7.14	16.88	17.18	22.09
	Carotenoids	5.094	4.263	4.624	8.196	10.352	11.656
0.02gm/L	Biomass yield (gm/L)	0.4	0.43	0.46	0.62	0.83	0.83
	Chl. A	6.53	11.53	15.64	21.14	23.61	13.8
	Chl. B	2.34	17.63	22.31	24.3	30.16	47.65
	Carotenoids	4.852	5.939	8.545	13.042	14.825	16.648
0.1gm/L	Biomass yield (gm/L)	0.37	0.38	0.57	0.77	0.88	1
	Chl. A	5.48	10.89	17.59	18.63	30.15	30.3
	Chl. B	1.37	16.65	23.84	43.31	48.73	49.62
	Carotenoids	4.553	5.861	7.455	13.594	16.278	17.094
0.2gm/L	Biomass yield (gm/L)	0.32	0.57	0.76	0.81	0.94	1.15
	Chl. A	5.54	11.79	25.6	28.03	31.49	37.94
	Chl. B	1.74	37.38	42.27	47.96	49.36	60
	Carotenoids	4.1	0.735	7.443	10.824	11.605	15.037

### 6. EFFECT OF VITAMIN E SUPPLEMENTATION ON IMPROVING STABILITY OF CAROTENOIDS TO UV EXPOSURE

Carotenoids are pigments associated with chlorophyll to protect it from photo-oxidation. Being able to quench free radicals carotenoids themselves are unstable molecules. They are prone to immediate oxidation as soon as removed from the tissues. Oxidation of carotenoids leads to loss of their antioxidant and UV- absorbing ability.



Stability of lycopene in oil phase and its conversion from trans to cis isomer have been studied under different storage conditions of light and temperature (Urbonaviciene et al. 2017). After 63 days in dark condition about 26% lycopene was converted to cis- form and under light 29% was converted to cis- form.

Stability of 2 Carotenoids like  $\beta$ -carotene, Lycopene and 2 Xanthophylls like Lutein and Neoxanthin to UV irradiation was studied in hexane solvent, the lycopene was more prone to bleaching due to absence of rings at terminal end of hydrocarbon chain (Cvetkovic and Markovic 2008).

Various commercial; anti-oxidants are available that are used in food and cosmetics to protect compounds from being oxidized. In order to use carotenoids for its application in Cosmetics or as natural organic colour, it is important to access its stability and combine it with suitable antioxidant to enhance its UV protective nature. Stability of beta carotene in oil-in-water (O/W) emulsion stabilised with Gum Arabic has been studied by Liu et al.(2015). The photo-oxidation rate was studied at 45°C at 450 W/m<sup>2</sup>. The impact of antioxidants on thermal stability of  $\beta$ -carotene in O/W emulsion was also studied. Out of 3 different antioxidants viz.  $\alpha$ -Tocopherol, Tertiary Butyl Hydroquinone (TBHQ) and Ascorbylpalmitate, the most effective antioxidant was  $\alpha$ -Tocopherol. Hence In present work Vit.E was chosen as anti-oxidant to protect photo-degradation of carotenoids.

In present experiment, Carotenoids extracted from dried biomass of *P.limnetica* and Vit. E in different proportions were initially dissolved in Phytosqualan (PS) and then exposed to UV radiation. Phytosqualan is a ready to use base derived from Squalene molecule found in plants. It is very stable compound and is used to formulate various skin care cosmetic products. UV absorbing ability as well as stability of Carotenoids in various concentrations individually and along with Vit.E was studied. Different combination of Carotenoids and Vit.E concentrations were taken as given in Table 7.

UV-A and UV-B radiations were provided for the period of 3 hours by UV-A Philips tube (wavelength maxima 340nm to 400nm) and UV-B bulb (Philips, wavelength maxima 280nm- 300nm) respectively. UV-A and UV-B tubes were placed at the distance of 5cm from the sample plates. Irradiance was measured using UV-meter (Lutron UV-340A model). Radiation intensity of UV-A and UV-B together at the distance of 5 cm was found to be 3750  $\mu$ W/cm<sup>2</sup> which is equal to 3.75 milli J/sec cm<sup>2</sup>. Carotenoids and Vit.E were exposed to UV-A and UV-B radiation for 3 hours thus giving total radiation of 27 J/cm<sup>2</sup>.The effect of UV exposure on stability of carotenoid and vitamin E was studied spectrophotometrically in the range of 200 to 400nm at the interval of one hour. At each interval Sun Protective Factor (SPF), UV-A/UV-B Ratio, Boot Star Rating and Critical wavelength were calculated as per formula (Donglikar and Deore 2016).

SPF or Sun protective factor is a measure of any compound protecting the skin from UV-B radiations. Other parameters like Critical wavelength indicates the degree of broadband protection given by sunscreen. It gives one shortest wavelength at which 90% of sun’s energy is absorbed. If the critical wavelength is 370 nm or greater, the formulation can be presumed to have broad spectrum protection (UV-A and UV-B). Another system called Star Rating was developed in UK that gave rating of 1 to 5 stars to a compound or formulation depending on their UV-A to UV-B absorbance ratio. If UVA/B ratio is higher than 0.86 it is regarded as 5 star rating. Thus higher boot star rating indicates ability of a compound to absorb in the UV-A region (Donglikar and Deore 2016)

As seen in Figure 5 the control sample of carotenoid (1000 $\mu$ g/ml) when analysed on spectrophotometer prominently shows absorbance in UV-A region as it absorbs in the wavelengths ranging from 320 to 400nm. It also shows a peak which partially covers B region (wavelength from 242nm to 272nm). In previous report by Tendulkar and Deodhar(2018) carotenoids extracted from *Synechocystispevalekii* was also UV-A absorber.

**Table 7: Different combinations of Carotenoid and Vit. E in Phytosqualan**

Combination	Carotenoid ( $\mu$ g/ml)	Vit. E ( $\mu$ g/ml)	Ratio Carotenoid : Vit. E
CONTROL CAROTENOID	1000	0	-
CONTROL VITAMIN E	0	1000	-
TEST 1	1000	1000	1:1
TEST 2	1000	2000	1:2
TEST 3	2000	1000	2:1



**6.1.Effect of UV radiations on Carotenoids:**

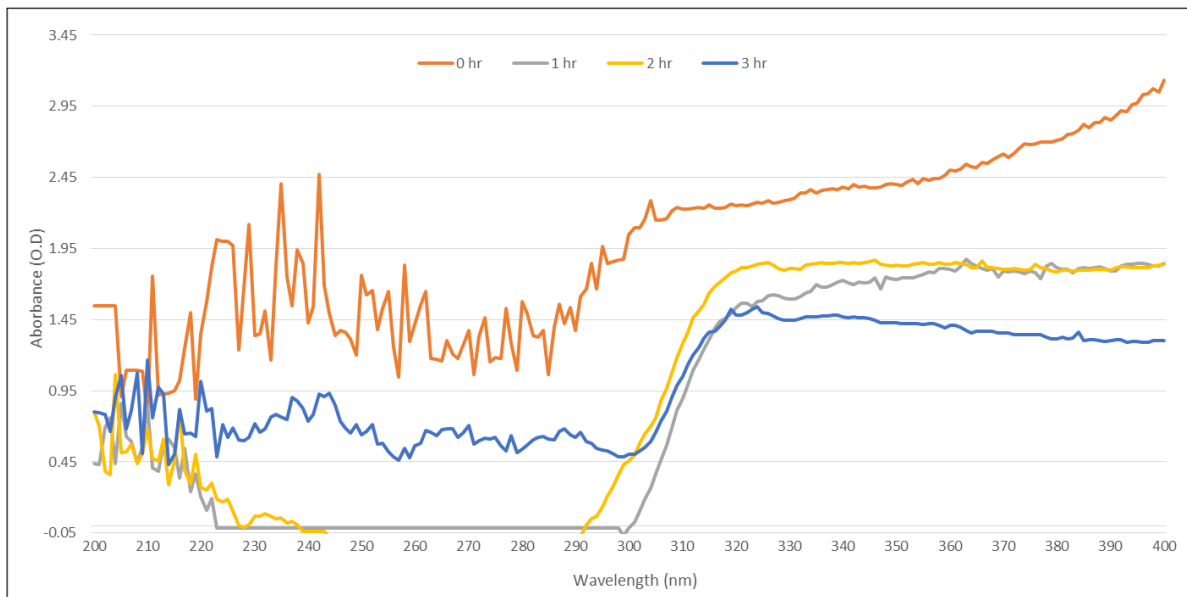
When Control sample containing only carotenoid (1000µg/ml) was exposed to UV radiation, at 0 hours the average UV-A of the Control was 2.559 (Table 8). After exposure to UV radiation, the mean absorbance in UV-A region decreased to 1.749 after 1 hour of exposure. It was further decreased to 1.393 after 3 hours of UV exposure.

The mean absorbance at UV-B region at 0 hour was 1.901 which decreased to 0.806 after 3 hour of exposure. As seen in Table 8 exposure to UV radiations also resulted in decrease of SPF from 19.008 at 0 hour to 8.059 after 3 hour of exposure. At the end of 3 hours the UV-A to UV-B ratio increased from 1.347 to 1.729. And hence boot star rating was maintained at 5 star.

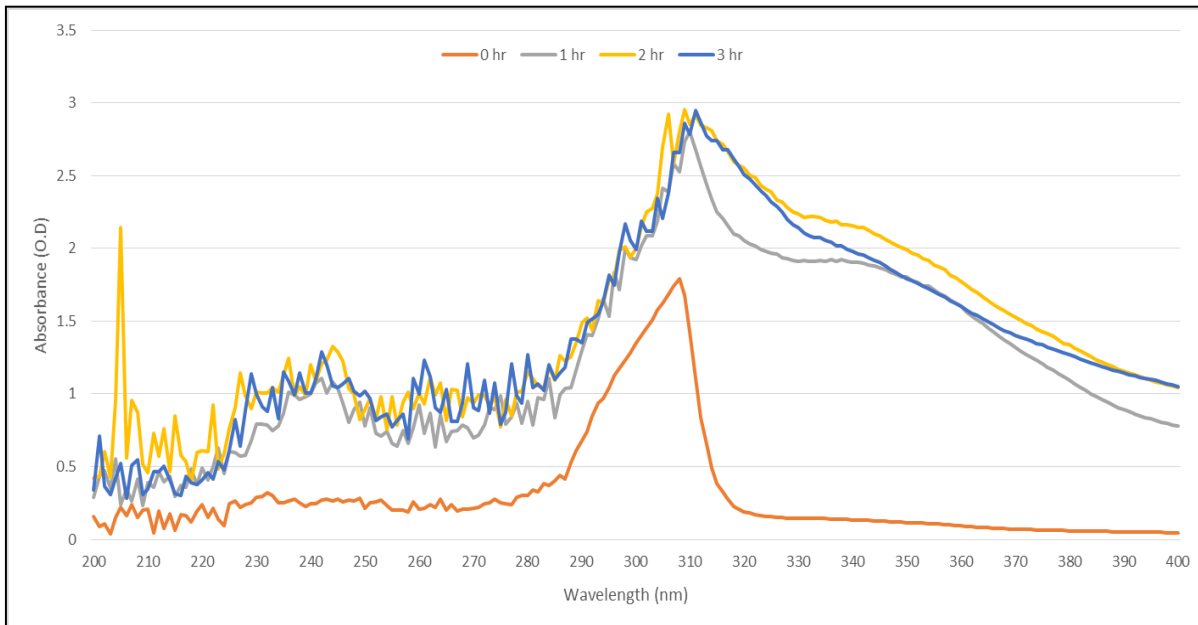
**6.2.Effect of UV exposure on Vit. E:**

Figure 6 shows the spectrophotometric analysis of Vit. E (1000 µg/ml). It shows a peak (290 to 315nm) which covers UV-B region. Comparatively it shows lesser absorbance in UV-A region. At 0 time its absorbance is UV-A and UV-B region was 0.097 and 0.881 respectively. When Vit.E was exposed to UV radiations the average absorbance in A region was sharply increased from 0.097 at 0 hour to 1.483 after 1 hour and 1.629 at 3 hours of exposure. Similarly UV-B absorption increased from 0.881 to 1.824 after 1 hour exposure. Further after 3 hours of exposure average UV-B value increased to 2.012.

This increase in UV-A absorption also resulted in increased UV-A/UV-B ratio from 0.110 at 0 hour to 0.810 at the end of 3 hour and increased boot star rating from 1-star at 0 hour to 4-star after 3 hour of exposure (Table 8). Also increase in UV-B absorption resulted in increased SPF value from 8.807 at 0 hour to 20.120 after 3 hour of UV exposure.



**Figure 5: Graph of Carotenoid (1000µg/ml)**



**Figure 6: Graph of Vit. E (1000µg/ml)**

Table 8: UV Protective ability of Carotenoids and Vit. E						
	Sample	UV- A	UV- B	SPF	UV-A/UV-B	Boot Star Rating
0 Hour	Carotenoid	2.559	1.901	19.008	1.347	*****
	Vit. E	0.097	0.881	8.807	0.11	*
1 Hour	Carotenoid	1.749	0.416	4.163	4.203	*****
	Vit. E	1.483	1.824	18.236	0.813	****
2 Hour	Carotenoid	1.825	0.633	6.328	2.884	*****
	Vit. E	1.735	2.051	20.51	0.846	****
3 Hour	Carotenoid	1.393	0.806	8.059	1.729	*****
	Vit. E	1.629	2.012	20.12	0.81	****

**6.3.Synergistic effect of carotenoids and Vit. E:**

To study synergistic effect of carotenoids and Vit.E when exposed to UV radiations, three different combinations were used Viz. 1000 µg/ml of carotenoids and 1000 µg/ml of Vit.E (TEST 1), 1000 µg/ml of carotenoids and 2000 µg/ml of Vit. E (TEST 2), 2000 µg/ml of carotenoids and 1000 µg/ml of Vit. E (TEST 3). The absorption spectrum of combination of carotenoids with Vit.E shows one absorption maxima between 280nm to 400nm which covers both UV-A and UV-B region respectively.

Though carotenoid individually is prone to degradation on exposure to UV radiation, when it is added to Vit.E the combination synergistically shows different action. When the combination is exposed to UV radiation for 3 hours, their absorptive ability appears to be increasing in both UV-A and UV-B regions and this increase in activity is seen in all 3 combinations.



#### 6.4. Effect of UV exposure on combination of Carotenoid and Vit. E:

Test 1 included carotenoid 1000 µg/ml and Vit. E 1000µg/ml. Average absorbance of combination in UV-A region at 0 hour was 2.626. It was observed that on exposure to UV radiations for 1 hour and 2 hour, activity of the Test 1 in UV-A region increased to 2.724 and 2.764 respectively as given in **Table 9**. Further after 3 hour exposure, absorbance in UV-A region reduced to 2.382.

Absorbance of Test 1 in UV-B region at 0 hour was 2.411 which was initially reduced to 1.424 after 1 hour of exposure. But when it was further exposed to 2 or 3 hours to UV radiation its absorbance was increased. After 2 hours the mean absorbance in UV-B region was 2.035 and it increased to 2.059 after 3 hours of exposure. This was also reflected in SPF. Initially SPF of Test 1 combination was 24.111 which was slightly decreased to 20.588 after 3 hour of UV exposure.

As average absorbance in UV-A region increased, it resulted in increased UV-A/UV-B ratio from 1.089 at 0 hour to 1.913 after 1 hour of exposure. More exposure to UV led to little decrease of UV-A/UV-B ratio from 1.913 to 1.157 after 3 hours but this value was still higher than A/B ratio at zero hour. At zero time A/B ratio was 1.089 and after 3 hours of exposure it was 1.157 indicating that its 5-star Boot Star rating was maintained even after 3 hour of UV radiation.

In TEST 2(**Figure 7**), concentration of Vit.E was increased to 2000µg/ml and carotenoid was same as in test 1 i.e. 1000µg/ml. Initial average UV-A absorbance was 1.930. When sample was exposed to UV radiations, tremendous increase in UV-A absorbance was observed. Absorbance increased from 1.930 at 0 hour to 2.848 after 1 hour of exposure and further increased to 3.533 and 3.660 after 2 hours and 3 hour of UV exposure respectively.

Similarly absorbance in UV-B region showed high increase from 0.889 at 0 hour to 1.653 after 1 hour of exposure and further it increased to 2.403 and 2.415 after 2 hour and 3 hour of exposure respectively. This increase in UV-B region resulted in increase of SPF value of Test 2 combination. At 0 hour SPF value was 8.886 which got doubled after 1 hour exposure to give value of 16.529. It further increased to 24.154 after 3 hour of UV exposure. On the other hand, though there was increase in average UV-A absorbance from 1.930 to 3.660 after 3 hour, UV-A/UV-B ratio declined from 2.172 to 1.515 after 3 hours of UV exposure. Though there was decrease in UV-A/UV-B ratio over the period of 3 hours Boot star rating remained constant which is 5-star as seen in **Table 9**.

In Test 3 carotenoid was increased to 2000 µg/ml and Vit.E was 1000µg/ml. When the combination was exposed to UV radiation the initial average UV-A absorbance at zero time was 2.531 which was increased to 3.972 after 2 hours and after 3 hours it was as high as 4.009.

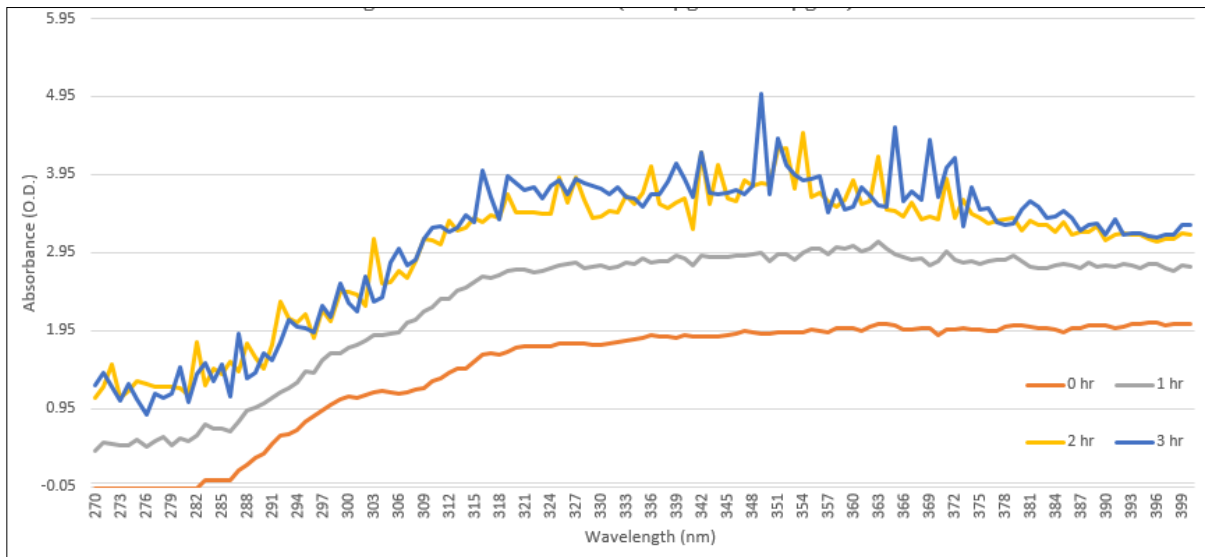
The average UV-B absorbance for test 3 was 1.424 which was increased to 2.579 at 2nd hour and at 3rd hour it was increased to 2.712. The SPF was 11.535 at zero time for test 3 which was increased to 14.240 at 1 hour, and 25.790 after 2 hours. After 3 hours of exposure SPF was 27.171. The initial UV-A/UV-B ratio was 2.194 which decreased to 1.475 at 3 hour but Boot star rating was maintained 5 star.

**Table 9: Effect of UV exposure on combination of Carotenoid and Vit. E**

	Sample	UV-A	UV-B	SPF	UV-A/ UV-B	Boot Star Rating
0 Hour	Test 1	2.626	2.411	24.111	1.089	*****
	Test 2	1.930	0.889	8.886	2.172	*****
	Test 3	2.531	1.154	11.535	2.194	*****
1 Hour	Test 1	2.724	1.424	14.240	1.913	*****
	Test 2	2.848	1.653	16.529	1.723	*****
	Test 3	2.485	1.077	10.772	2.307	*****
2 Hour	Test 1	2.764	2.035	20.350	1.358	*****
	Test 2	3.533	2.403	24.029	1.470	*****



	<b>Test 3</b>	3.972	2.579	25.790	1.540	*****
<b>3 Hour</b>	<b>Test 1</b>	2.382	2.059	20.588	1.157	*****
	<b>Test 2</b>	3.660	2.415	24.154	1.515	*****
	<b>Test 3</b>	4.009	2.717	27.171	1.475	*****



**Figure 7: UV-absorbance of Combination of Vit. E and Carotenoids after 3 hours of UV radiation**

When test 1 test 2 and test 3 are compared, test 1 contains carotenoids and Vit. E in 1:1 Ratio. This combination though initially shows good absorption in both UV-A and UV-B region after UV exposure it shows little decline. Absorbance in UV-A region which is initially 2.626 declines to 2.382 and SPF declines from 24.111 to 20.588. It means amount of Vit.E becomes insufficient to protect carotenoid degradation.

In test 2 the ratio of carotenoid to Vit. E is 1:2. The amount of carotenoid is comparatively less. It is reflected in the ability to absorb UV-A region. The mean UV-A absorption is 1.930 and UV-B absorption is only 0.889. It is reflected in SPF it is only 8.886. But after exposure to UV radiation for 3 hours it shows substantial enhancement in SPF. It increases from 8.886 to 24.154. Absorbance in A region also is improved from 1.930 to 3.660. Hence carotenoid and Vit. E in ratio 1:2 is very effective after UV radiation.

In test 3 the ratio of Carotenoid to Vit. E is 2:1. This combination also works well after UV exposure. Initially the average UV-A absorption is 2.531 is substantially increased to 4.009 and the initial SPF of 11.535 increases to 27.171 after 3 hours of exposure. Thus any combination of carotenoid and Vit. E is very effective against UV exposure (Table 9).

Thus combination of carotenoids with Vit. E not only increases the stability of carotenoid but the combination also add to the UV protective ability. We further intend to incorporate the carotenoid in cream formulation and use it for protecting skin from sun exposure.

**V. CONCLUSION**

The crude extract of carotenoid was efficiently extracted in 100% methanol solvent using Overnight freezing method over maceration. HPLC analysis of the crude extract showed presence of Lutein, Astaxanthin and Cantaxanthin carotenoids. Further carotenoid production was enhanced using 0.1gm/L of carbonate source Na<sub>2</sub>CO<sub>3</sub>. Further stability of extracted carotenoids was studied by exposing it with UV radiation and addition of Vit. E in



carotenoids enhanced the UV absorbing ability of carotenoids even after 3 hours of exposure. Addition of Vit. E in carotenoids enhanced SPF and always showed 5 boot star rating. This proved that similar combinations of carotenoids and Vit. E can be used in the cosmetic formulation as a potential sunscreensing agent.

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#### VII. DECLARATIONS

##### Funding

No funds were provided for this research work conducted.

##### Conflict of interest

The author(s) declare that they have no competing interests.

##### Ethics approval

N. A.

##### Consent to participate

All authors have contributed equally and voluntarily in the conduct of study and preparation of the manuscript.

##### Consent for publication

The article is sent for publication upon consent from all authors.

##### Availability of data and material:

Data generated during the conduct of this study has been included in the manuscript.

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