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Research Article

Photon up-conversion increases biomass yield in *Chlorella vulgaris*

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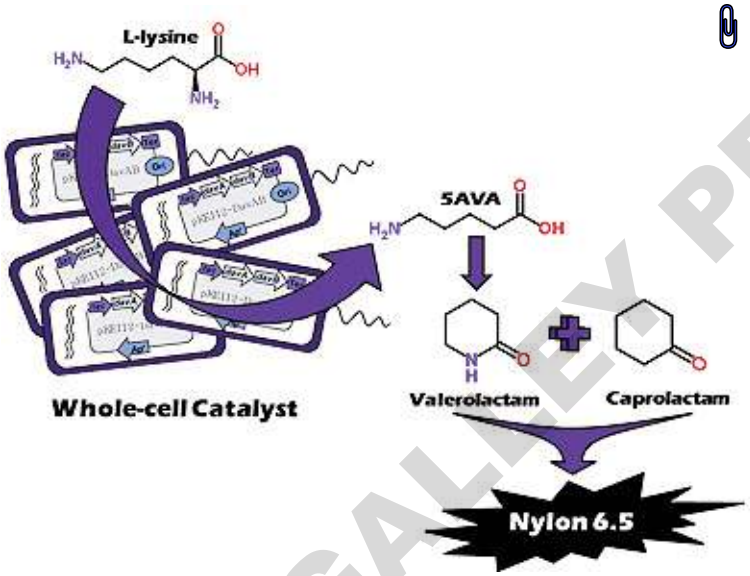

Photon up-conversion is employed as a novel strategy for improving microalgal growth and lipid productivity. In this study, authors found that photon up-conversion increases the biomass yield, specific growth rate, intracellular neutral lipid and reactive oxygen species of the microalgae *Chlorella vulgaris*. They conclude up-conversion can be used to increase the utilization range of the electromagnetic spectrum for improved cultivations of photosynthetic systems such as plants, algae and microalgae. ■**Please check the changes are ok**■

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Research Article

Photon up-conversion increases biomass yield in *Chlorella vulgaris*

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Photon up-conversion, a process whereby lower energy radiations are converted to higher energy levels via the use of appropriate phosphor systems, was employed as a novel strategy for improving microalgal growth and lipid productivity. Photon up-conversion enables the utilization of regions of the solar spectrum, beyond the typical photosynthetically active radiation, that are usually wasted or are damaging to the algae. The effects of up-conversion of red light by two distinct sets of up-conversion phosphors were studied in the model microalgae *Chlorella vulgaris*. Up-conversion by set 1 phosphors led to a 2.85 fold increase in biomass concentration and a 3.2 fold increase in specific growth rate of the microalgae. While up-conversion by set 2 phosphors resulted in a 30% increase in biomass and 12% increase in specific intracellular neutral lipid, while the specific growth rates were comparable to that of the control. Furthermore, up-conversion resulted in higher levels of specific intracellular reactive oxygen species in *C. vulgaris*. Up-conversion of red light (654 nm) was shown to improve biomass yields in *C. vulgaris*. In principle, up-conversion can be used to increase the utilization range of the electromagnetic spectrum for improved cultivation of photosynthetic systems such as plants, algae, and microalgae.

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Keywords: *Chlorella vulgaris* · Lipid productivity · Photosynthetically active radiation · Photon up conversion · Reactive species

1 Introduction

For the development of bio-oil as an alternative energy source, microalgae are considered promising due to their ease of cultivation in all seasons, lower requirements for arable land and freshwater, higher growth rates, productivity, and efficient photosynthesis [1–4]. Despite these advantages, many challenges hinder the commercialization of algal bio-oil. Incomplete utilization of the light source and damage to algal metabolism caused by heating effects of solar radiation [5] are two significant hurdles, among many others, to the commercialization of algal production systems. Microalgae absorb solar radiation in the range of 400 to 700 nm, known as photosynthetically active radiation (PAR), for photosynthesis. A large fraction of the solar spectrum, i.e. 51.3% of the incident solar energy [6], is thus left unutilized by the photosynthetic apparatus. Algae that are grown with sunlight in photobioreactors (PBR) or open ponds face challenges due to overheating [7]. The increase in temperature is primarily caused by infra red and near infra red radiation in sunlight, which are not constructively utilized by microalgae. Utilization of light beyond PAR and elimination of the heating effects of sunlight could result in improved photosynthesis that may translate into higher biomass yields and increased lipid productivity.

Photon up-conversion is the process of converting light of lower energy to that of higher energy via excitation of multiple photons [8] using crystal systems called up-conversion phosphors (UCPs). Up-conversion has been applied in assays and medical imaging: (i) to make luminescent optical labels [9]; (ii) in photodynamic therapy to treat cancer [10,11]; (iii) in solar cells to improve its efficiency [12]; (iv) in molecular solar energy storage [13];

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Abbreviations: PAR, photosynthetically active radiation; **PBR**, photobioreactor; **ROS**, reactive oxygen species; **si**, specific intracellular; **si-ROS**, specific intracellular reactive oxygen species; **TTA**, triplet triplet annihilation; **UCP**, up-conversion phosphor

and (v) in IR photon detection [14]. However, most of these applications require further research in order to be commercialized.

Our idea was that up-conversion could be used to overcome the light-related challenges facing microalgae cultivation. Of particular interest to this work is an up-conversion system that is excited by diffuse light unlike most other phosphor systems, which require coherent light sources such as a LASER. Such systems have used sensitizer-emitter systems to achieve up-conversion [12]. The up-conversion involved the triplet triplet annihilation (TTA) mechanism and was brought about in a stepwise manner via a system of two crystals: a sensitizer and an emitter/annihilator, which transformed the infra red region of the solar spectrum into blue wavelength. TTA essentially occurs via the transfer of energy from the triplet state sensitizer molecule to the triplet state emitter molecule finally leading to singlet delayed fluorescence. The sensitizer, excited by a long wavelength radiation, undergoes inter-system crossing to its triplet excited state. This energy is then transferred to the triplet state annihilator molecule causing the sensitizer to go back to the ground state. The sensitization cycle is repeated to generate yet another triplet annihilator molecule thus enabling TTA and consequently a singlet delayed fluorescence [15].

It would be desirable to use up-conversion of infra-red radiation to enhance algal cultivation, as it would have the additional benefit of mitigating the heating effects attributed to infra-red radiation. Despite our best efforts, we could not procure such up-conversion phosphors. Earlier studies in our laboratory [16] have shown that cultivations with blue light yielded higher biomass and neutral lipid levels compared to red light. Based on these results, two sets of UCPs capable of up-converting red light were used in this study. Our previous work also indicated that an increase in the specific levels of intracellular reactive oxygen species (ROS) such as hydroxyl and superoxide radicals coincided with the increase in the light energy (frequency). Reactive species, of which ROS is one kind, are known to play multiple important roles in biological systems [16]. Hence, monitoring ROS levels was relevant to the current study since red light is up-converted to higher energy levels. The principle of up-conversion was thus demonstrated with two sets of UCPs and the feasibility of its use to enhance the cultivation of *C. vulgaris* was explored for the first time. The effect of the UCPs on microalgal growth, lipid productivity, and ROS generation were studied.

2 Materials and methods

2.1 *Chlorella vulgaris* cultivation

The microalgae, *C. vulgaris* NIOT5 was obtained as a gift from the National Institute of Ocean Technology, Chennai, India. The cells were grown in shake flasks containing 12× Guillard and Ryther's f/2 medium (pH 7.2) as earlier studies in our laboratory found these media to be optimal [16]. Atmospheric carbon dioxide was the carbon source. The flasks were incubated in an illuminated orbital shaker (Orbitek model, Scigenics Biotech Pvt. Ltd., Chennai, India) at 25°C ambient temperature and 100 rpm with a photoperiod of 16 h light and 8 h dark. The culture flasks were inoculated to obtain an initial concentration of 1×10^6 cells/ml; the relevant calculations are shown in the Supporting information. These were mid log phase cells (in the 7^{th} and 8^{th} day of growth) cultivated in a typical f/2 medium. All data points were measured in triplicates and also each 20 day long experiment was repeated at a different time.

2.2 Characterization of the up-conversion systems

The two sets of sensitizer (0.1 mM) – emitter (1 mM) systems used in our studies were designated set 1 and set 2, and were as follows:

Set 1: (la), 2, 3, 7, 8, l2, l3, l7, l8-octaethylporphyrin palladium (PdOEP) and (lb), 9, 10-diphenylanthracene (DPA)

Set 2: (2a), meso-tetraphenyl-tetrabenzoporphyrin palladium ($PdPh_4TBP$) and (2b), 9,10-bis(phenylethynyl)anthracene (BPEA)

The UCPs were purchased from Sigma Aldrich. Transformation of light by these UCPs was confirmed using fluorescence spectroscopy. The set l and 2 phosphors transformed the red light at 654 nm to 470 and 510 nm, respectively.

2.3 Design of the experimental set up

The experiments were carried out by placing the culture flasks over specially designed polymethylmethacrylate holders containing the UCP. The disc shaped holders were fabricated to the following specifications: radius 5 cm; thickness 2 mm; and capacity 15.71 ml. High-quality acrylic sheets of high transparency were used for fabricating the holders. The material was also ascertained to be inert to toluene.

Up-conversion phosphors set 1 and 2 were housed within the holder and kept between the light source and the culture flask. Light was provided by means of LEDs emitting at a wavelength of 654 nm and 4000 lux intensity, and a PPF of approximately 363.9 $\mu E/m^2s$ or 66.6 W/m^2 was found to be available to the algal cells. The LEDs were characterized [17] using a spectrometer (Ocean Optics USB 2000). Intensity of the electroluminescence spectra of the LEDs were recorded over a range of wavelengths.

Cells cultivated in red light without the inclusion of up-conversion phosphors (this cultivation condition was

designated as R), served as a control for the up-conversion experiments carried out with both phosphor systems. Cultivations with set 1 and 2 UCPs are designated RU1 and RU2, respectively.

2.4 Quantification of biomass/growth

Biomass concentrations were recorded by counting cells in an improved Neubauer's chamber under 40× magnification using a binocular microscope (Labomed Vision 2000). Protocols for cell viability were as previously described [18].

2.5 Measurement of ROS

2.5.1 Hydroxyl free radicals

Intracellular hydroxyl radicals were measured using the dye 2-[6-(4-amino) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF) (Invitrogen) according to Setsukinai et al. [19] and were calibrated against hydroxyl radicals generated by a Fenton type reaction [20] between ferrous sulphateheptahydrate (Merck) and hydrogen peroxide (Sigma–Aldrich, Bangalore, India).

2.5.2 Superoxide free radicals

The intracellular superoxide radicals were measured using the dye DihydroEthidium (DHE) (Sigma) and were calibrated against superoxide radicals generated by the reaction between potassium superoxide (KO_2) (Sigma) and dimethyl sulfoxide (DMSO) (Merck) in the presence of Crown ether (Sigma) according to Valentine et al. [21]. All measurements of radicals were made in a fluorescence spectrophotometer (LS 55, Perkin Elmer, Llantrisant, UK). Values were expressed as amount per cell and thus termed specific intracellular ROS.

2.6 Measurement of lipids

Neutral lipids were estimated according to Chen et al. [22], using the dye Nile Red and were calibrated against the triglyceride triolein (TO), both from Sigma–Aldrich. These were measured in a fluorescence spectrophotometer, (LS 55, Perkin Elmer, Llantrisant, UK) and values were expressed as amount per cell thus termed specific intracellular lipids.

2.7 Measurement of pigments

Pigments were measured using a protocol by Lichtenthaler [23], which involved extraction of the pigments from 1.0 mL of algal sample in 100% methanol and measurement at 665 nm and 450 nm in a UV-Vis spectrophotometer (V 630, JASCO, Tokyo, Japan) for chlorophyll and carotenoids respectively. This assay was calibrated with commercial standards for chlorophyll and β-carotene from Sigma. Chlorophyll was calculated using $y = 0.046x$, where y is the optical density and x, the chlorophyll concentration (μg/ml).

2.8 Statistical analysis

A one way ANOVA was performed on all results ~~in order~~ to ensure that the differences in the results across the various cultivations for a particular experiment were indeed relevant. The significance of these differences was assured from the p values that were found to be < 0.05. All data are expressed as ± SD. Error bars shown in the figures are the standard deviation values of the particular parameter.

3 Results

3.1 Design of a suitable system for *C. vulgaris* cultivation with up-conversion

The initial design concept was to have the UCPs present in the medium for effective utilization of the altered wavelengths. In such a case, it would be necessary to dissolve the UCP uniformly in an appropriate medium that is compatible with the cultivation medium, since UCPs are not soluble in aqueous solutions. In the literature, toluene has been used to dissolve UCPs [12], but it was found to be immiscible with the aqueous growth medium used in this study, and hence was unsuitable.

Two other solvents were tested, namely DMSO and dioxane. The UCPs did not exhibit up-conversion when they were dissolved in DMSO, and thus it was not suitable solvent. The UCPs dissolved in dioxane retained up-conversion activity, and hence, further studies were done with dioxane. The growth of *C. vulgaris* was first characterized in media containing 0.1, 0.2, 0.5, 1 or 2% v/v dioxane. At all concentrations of dioxane used, no growth of *C. vulgaris* was observed and all cells died within a day of inoculation. Hence it was found that dioxane is toxic to *C. vulgaris* and dioxane is therefore an unsuitable solvent for the purpose of dissolving UCPs in medium for *C vulgaris* cultivation.

The inability to find a suitable solvent to allow the UCPs to be present in the medium meant that, the UCPs needed to be physically separated from the cells, and the light needed to pass through the UCPs before reaching the cells. Specially designed holders were required for containing the UCPs dissolved in toluene. Polymethylmethacrylate was chosen over glass for making the holders because polymethylmethacrylate is known to be inert to solvents such as toluene, DMF, chloroform, dioxane, ethanol, and phenol. Disc shaped holders, with their diameter equal to the base of the culture flasks, were constructed to contain the UPCs and were placed above the LEDs. The culture flasks were then placed over the holders so that light from the LEDs first passed through the

UCPs in the holders and the light up-converted prior to illuminating the culture flasks. The use of such holders, while eliminating the toxic effects of the UCPs on the microalgae, also made handling of the cultures much easier.

The up-conversion behavior of set 1 and 2 UCPs were verified using fluorescent spectroscopy. The UCPs dissolved in toluene, were excited with red light of 654 nm and the emission peak was scanned for in the wavelength range 300 to 700 nm. An emission peaks of 470 and 510 nm were obtained with UCP set 1 and 2, respectively. Although up-conversion phosphors had not been employed previously for cultivation of any organism, they are known through other studies to retain their up-conversion characteristics over many days without undergoing photo bleaching [11].

3.2 Increased growth rate of *C. vulgaris* with up-conversion

To understand and compare the effects of the up-conversion of red light on microalgal growth, the biomass concentrations have been plotted with respect to time in Fig. 1. The figure clearly shows a significant increase in the biomass concentrations in RU1 as compared to the control. The lag phase appears to extend till the third to fifth day followed by the logarithmic phase that lasts until the 16th day.

RU1 with a biomass concentration of 4.11×10^7 cells/mL (Table 1) was 2.85 times that of control (1.44×10^7 cells/mL) on the 20th day of cultivation. RU2 on the other hand showed only a 30% increase in biomass concentration. RU1 showed a significantly higher specific growth rate of 0.425 day^{-1} compared to that of R (0.129 day^{-1}) and RU2 (0.135 day^{-1}); the growth rates of R and RU2 were comparable.

3.3 Reactive oxygen species generated with up-conversion

The specific intracellular reactive oxygen species (si-ROS) measured at a specific time every day have been plotted against time in Fig. 2. Both plots reveal a decreasing trend in si-ROS levels over time with the maximum values being recorded in the lag phase. At the end of the cultivation period, using the up-conversion phosphors, RU2 displays the highest levels of both si-ROS (9.63×10^{-16} moles $OH^{\bullet}$/cell and 8.3×10^{-12} moles O_2^-/cell). RU1 on the other hand was found to contain levels comparable to the control.

3.4 Specific lipid levels with up-conversion

The time profile of specific intracellular (si) neutral lipids in the cultivations is compared in Fig. 3. The si-neutral lipid content was observed to decrease over time in all cases. However, up-conversion due to set 2 phosphors

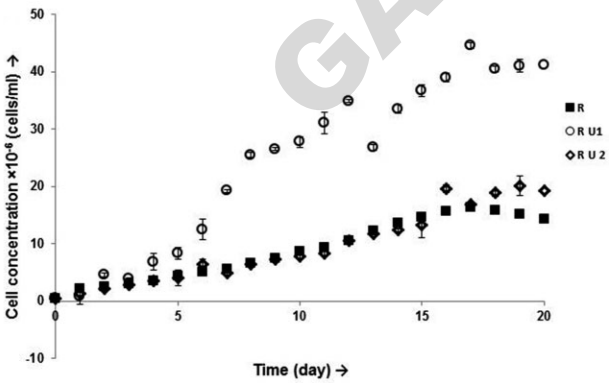

Figure 1. Cell growth profiles in various cultivation setups. Growth profiles of the cultivations of RU1 and RU2, subjected to red light up-converted by UCP1 and UCP2 respectively, have been compared with the control cultivation R subjected to red light without up-conversion. All data are expressed as mean ± SD, n = 3, in two independent sets of experiments. Data as analyzed by one way ANOVA was statistically significant with $p = 0.005$. Dunnet's comparisons with control revealed RU1 to be significantly different from R.

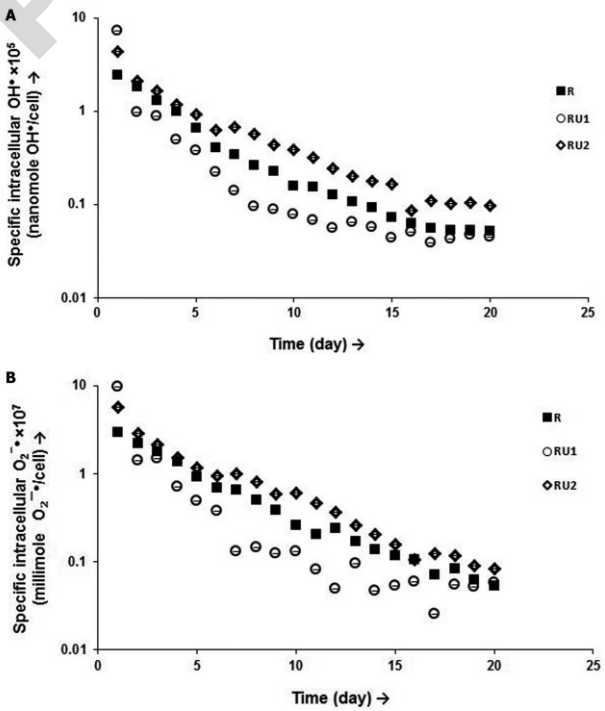

Figure 2. (**A**) Si-hydroxyl free radical time profile of the cultivations. (**B**) Si-superoxide free radical time profile of the cultivations. The si-ROS levels of UCP1 and UCP2 treated cultivations, RU1 and RU2 respectively, are compared with that of the control cultivation R. All data are expressed as mean ± SD, n = 3, in two independent sets of experiments. Data analyzed by one way ANOVA in was statistically significant with $p < 0.001$. Dunnet's comparisons with control revealed both RU1 and RU2 to be significantly different from R.

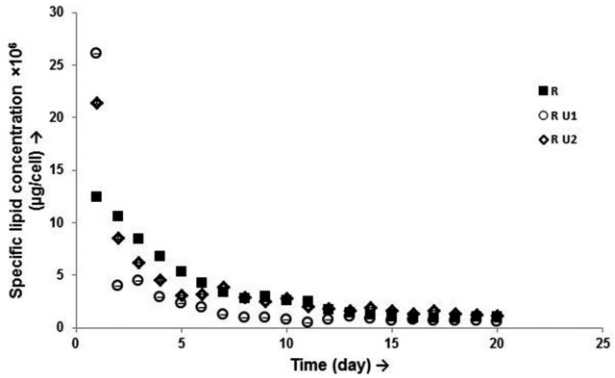

Figure 3. si-neutral lipid time profiles of the cultivations. Specific neutral lipid values of the cultivations RU1 and RU2 treated with light up-converted by UCP1 and UCP2, respectively, are compared to the control cultivation R. All data are expressed as mean ± SD, n = 3, in two independent sets of experiments. Data analyzed by one way ANOVA was statistically significant with $p < 0.001$. Dunnet's comparison with control revealed both RU1 and RU2 to be significantly different from R.

resulted in a 12% increase in si-neutral lipid as compared to the control value of 1.01×10^{-6}µg TO/cell (Table 1). Conversely, set 1 phosphor treated cultivations with 0.574×10^{-6}µg TO/cell were only half the value of the control.

3.5 Contrasting effects of up-conversion on specific chlorophyll and carotenoid contents

Specific chlorophyll content from the various cultivations are compared using a plot of specific intracellular chlorophyll against time as shown in Fig. 4A. The specific chlorophyll content increases during the lag phase until day 4 and is maintained at an almost constant level during the logarithmic phase. With the onset of stationary phase, specific chlorophyll content increases further in the up-conversion cultivations but remains constant in the controls. Up-conversion using both phosphors significantly increases chlorophyll content by several folds. The highest specific chlorophyll content is seen in RU2 (7×10^{-7}µg/cell) which is 14.2 fold greater than control (Table 1). RU1 (4.17×10^{-7}µg/cell) also shows an 8.4 fold increase in specific chlorophyll.

The time profiles of specific carotenoids in all cultivations (Fig. 4B) show an increase over time. However, in both cultivations with up-conversion, the values were reduced to 2.5×10^{-8} and 4.58×10^{-8}mg/cell in RU1 and RU2 respectively.

The ratio of specific chlorophyll content to specific carotenoid content is often used as a stress indicator [24] and is plotted against time in Fig. 4C. In all cases, the ratios follow a trend similar to that of specific chlorophyll. The various characteristics of the cultivations have been compared in Table 1.

4 Discussion

A 2.85 fold increase in biomass concentration and a 3.2 fold increase in the specific growth rate of RU1 compared to the control was observed. This shows that growth is accelerated due to up-conversion with set 1 phosphors. Up-conversion by set 2 phosphors also increased biomass concentration by 30%, although the specific growth rates were comparable to the control. Growth of the microalgae was thus seen to be highest when treated with UCP set 1, followed by that treated with UCP set 2, and then the control red light.

In general, reactive oxygen species levels are indicators of oxidative stress in cells [25] and their pseudo-steady state levels have been proposed as markers of stress [16]. Thus the levels of specific intracellular reactive oxygen species were measured ~~in order~~ to evaluate oxidative stress in the microalgae due to up-conversion. Both si hydroxyl and si superoxide were found to be highest in RU2 and the lowest in RU1. This suggests that up-conversion by set 1 phosphors results in less oxidative stress in *C. vulgaris* cells compared to the control condition. This was contrary to our expectations that an increase in energy of radiation, as a result of up-conversion, would bring about an increase in si ROS. Treatment with set 2 UCPs

Table 1. Various characteristics of the different cultivations

Parameter	R[a)]	RU1[b)]	RU2[c)]
Specific growth rate (day^{-1})	0.129 ± 0.001	0.425 ± 0.022	0.135 ± 0.021
Biomass Concentration (cells/ml)	$(1.44 \pm 0.01) \times 10^{7}$	$(4.11 \pm 0.11) \times 10^{7}$	$(1.92 \pm 0.17) \times 10^{7}$
si-neutral lipid (µg TO/cell)	$(1.01 \pm 0.008) \times 10^{-6}$	$(5.74 \pm 0.01) \times 10^{-7}$	$(1.14 \pm 0.0004) \times 10^{-6}$
si-hydroxyl radical (moles $OH^{\bullet}$/cell)	$(5.15 \pm 0.276) \times 10^{-16}$	$(4.48 \pm 0.04) \times 10^{-16}$	$(9.63 \pm 0.008) \times 10^{-16}$
si-superoxide radical (picomoles O_2^{-}/cell)	5.26 ± 0.09	5.7 ± 0.07	8.3 ± 0.141
Specific chlorophyll (µg/cell)	$(0.49 \pm 0.24) \times 10^{-7}$	$(4.17 \pm 0.4) \times 10^{-7}$	$(7 \pm 0.01) \times 10^{-7}$
Specific carotenoid (mg/cell)	$(5.85 \pm 0.001) \times 10^{-8}$	$(2.5 \pm 0.02) \times 10^{-8}$	$(4.58 \pm 0.003) \times 10^{-8}$

a) Control cultivated in red light in the absence of up-conversion phosphors
b) Cultivation in light up-converted by set 1 up-conversion phosphors
c) Cultivation in light up-converted by set 2 up-conversion phosphors
All values correspond to day 20, the day of harvest. All data are expressed as mean ± SD, n = 3, in two independent sets of experiments.

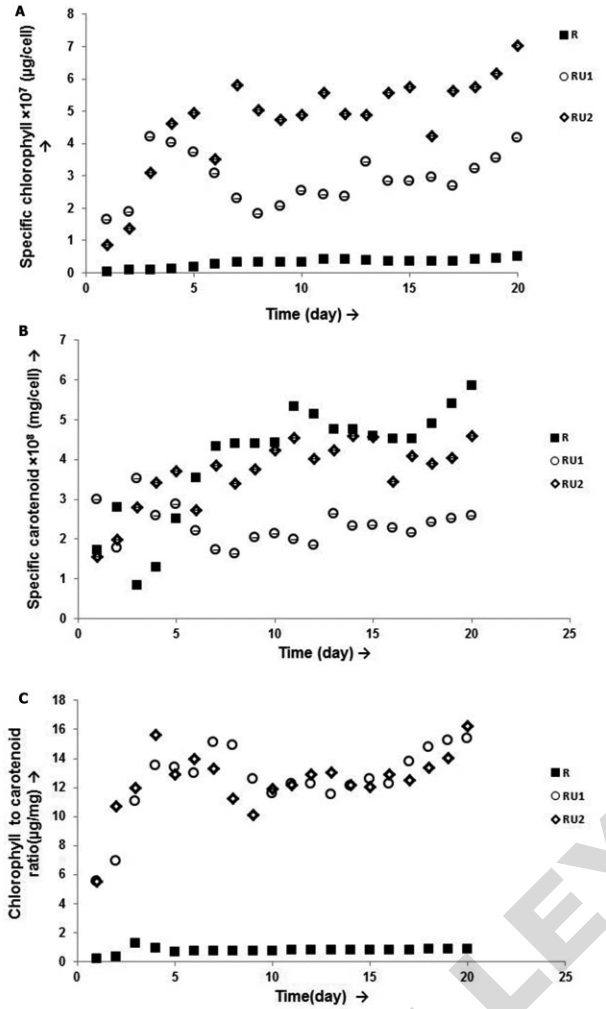

Figure 4. (**A**) Specific chlorophyll time profiles of the *C. vulgaris* cultivations. Chlorophyll values were recorded in both UCP1 and UCP2 treated cultivations (RU1 and RU2 respectively) and compared to the control (R). (**B**) Specific carotenoid time profiles of the *C. vulgaris* cultivations. Specific carotenoid contents of the various cultivations are compared. All data are expressed as mean ± SD, n = 3, in two independent sets of experiments. (**C**) Ratio of specific chlorophyll to specific carotenoid contents in various *C. vulgaris* cultivations. Data of specific chlorophyll and specific carotenoid values analyzed by one way ANOVA was statistically significant with $p = 0.001$ and $p < 0.001$ respectively. Dunnet's comparison with control revealed both RU1 and RU2 to be significantly different from R.

on the other hand did increase the oxidative stress as inferred from the si ROS measurements. Higher levels of si ROS in the lag phase, as noted in all cultivations, is consistent with our earlier observations [16] but the reasons remain speculative at this stage. The specific levels decreasing over time is probably a result of the cells adapting to the culture conditions.

Specific neutral lipid measurements indicated that up-conversion using set 2 phosphors improved lipid accumulation, while set 1 phosphors exhibited poor lipid accumulation and levels were found to be lower than in the control. Further, it was noted that an increase in biomass occurred at the expense of specific neutral lipid production. RU1, which exhibits the highest specific growth rate and biomass concentration, had the lowest specific neutral lipid content. On a comparative basis, increased neutral lipid content in RU2 is seen along with decreased biomass production (although biomass production was still greater than the control). This observation is in agreement with literature where lipid accumulation often occurs under conditions that compromise growth, thereby leading to an overall decrease in biomass production [26]. Since, lipids are storage compounds in microalgae they are accumulated in response to nutrient deficiencies [27], particularly during nitrogen limitation. However, prolonged periods of elevated temperature, light intensity, minerals, pH, heavy metals, radiation, and salinity have also been shown to result in lipid accumulation [28]. These conditions, however, are also associated with increased oxidative stress [25]. This link between oxidative stress and lipid accumulation was also found in the pattern of si-ROS and si neutral lipids observed in this study. A linear correlation between si ROS and si neutral lipids as reported in our previous study [16], was also observed here in all cultivations. The lowest levels of si-neutral lipid values were seen in RU1, which also exhibited the lowest si-ROS values. In the same way, RU2 displayed the highest levels of both si-neutral lipids and si-ROS. Furthermore, the effects of ROS on chloroplasts, more specifically on photosynthetic efficiency in terms of PSI/PSII ratios and antenna size, were reported in our earlier study [29]. Our results indicate that cultivations exposed to up-conversion by set 2 phosphors experienced greater oxidative stress and displayed higher lipid accumulation but lower biomass concentrations. The opposite was observed in cultivations with set 1 phosphor mediated up-conversion.

Thus, it could be concluded that while both the phosphors brought about up-conversion and a significant increase in biomass yields, set 1 is better suited for applications that target biomass. However, owing to the periodic replacement of phosphors, our study estimates a cost increment of $39.09 and $271.46 for set 1 and 2 UCPs respectively, for each 10 L cultivation of 20 days in a PBR. The cost estimate was based on a PBR designed with outer acrylic tubes concentric to the glass tubes carrying the culture. The UCP system was contained within the annular space formed so that light from the source is up-converted upon passing through the tubes and then illuminates the culture. It should be noted that an increase in specific growth rate and the consequent increase in biomass productivity as seen in up-conversion with set 1 phosphors, could decrease the operation period required in applications where biomass is of interest, thereby decreasing the associated cost. Since up-conversion of light of 654 nm has already been shown to increase bio-

mass yields, up-conversion of infra red (IR) radiation to PAR could perhaps improve the yields further due to greater utilization of the solar spectrum and a reduction of heating effects, thus leading to a decrease in the associated costs.

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The authors declare no financial or commercial conflict of interest.

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