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Responses of algae to high light exposure: prerequisite for species selection for outdoor cultivation

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Abstract
The outdoor cultivation of algae relies on the exposure of algae to unusual conditions than that encountered in laboratory or natural habitat. The experimental outcome of algae grown under artificial light (AL) differs by all mean when they are exposed to sunlight (NL), where they are exposed to uncontrolled distribution of light. Further, NL is often accompanied by factors like change of dark light cycle, seasonal or daily variation which contributes to the lowered productivity of algae. The photosynthetic apparatus must adjust to these environmental factors for its proper functioning, and sustainable growth. The current study deals with observations of initial responses of algal growth under NL followed by changes of chlorophyll *a* fluorescence during transition of algae from laboratory scale to NL. The photosynthetic performance is routinely probed using changes in chlorophyll *a* performance which is useful indicator of integrity of photosynthetic apparatus and its functionality. The eight algal species were monitored for chlorophyll *a* fluorescence changes after exposure to NL (≥1500 µmol photons $m^{-2}s^{-1}$) and correlated to their biomass productivity. The algal species- not all showed the same growth response, the alga *Chlorella saccharophila* proved to be the best case of NL acclimation.

Key words: Acclimation. Algae. Biomass productivity. High light. Non photochemical quenching. Photobleaching.

Introduction

In the last decades, the use of microalgae as source of value added compounds and energy alternative to fossil fuels has attracted great attention followed by their mass cultivation (Ugwu *et al.*, 2008). Various efforts have been made to improve the mass culture yield using sunlight as the energy source (Dismukes *et al.*, 2008). One of the most critical factors for biomass production is the efficiency of light utilization of algae under given light conditions. These organisms photosynthetically convert CO_2 and minerals to biomass (Duong *et al.*, 2012). The process of photosynthesis has been optimized in algae over three billion years of evolution (Price *et al.*, 2012); but it still remains inefficient at converting solar energy to chemical energy and biomass, due to inevitable loss of light energy via heat dissipation (Stephenson *et al.*, 2011). The theoretical efficiency of solar conversion efficiency (13%) drops up to 8% due to reductions in the efficiency of photon utilization and biomass accumulation. The further drop in efficiency can be evident in growth limiting or unfavourable conditions like high or low temperature, day length (Farquhar *et al.*, 1982). Algae can experience and must adjust to wide daily and seasonal fluctuations in temperature and light. The photosynthetic process of algae can respond to these changes over a wide range of time scales; seconds to minutes (regulation of photosynthesis) (Raven and Geider, 2003); hours to days (acclimation) and up to thousands of years (adaptation). It is generally known that low light intensity promotes an increase in the chlorophyll pigment protein complexes (large antenna size) of both PSI and PSII for the increased light harvesting. Similarly, high light intensities promote a reduction in light harvesting pigment protein complexes (smaller antenna size). This response appears to be well conserved in all photosynthetic systems. In spite of the ability to adapt to changes in light intensity, photosynthetic organisms are damaged upon exposure to excess visible light due to photoinhibition characterized by lowered growth rates. Regulation is the early response to sudden change in the environment which is characterized by transient, physiological change and molecular perturbations in a cell. Prolonged exposure to stress factor often induce a phenotypic alteration with no change in the genotypic expression of alga; leading to stable, long term adjustments that reflect a developmental response to the new environmental conditions. In contrast adaptation usually reflects the genetic changes in a cellular genome which distinguish different taxa from the strain (ecotype). The responses however are dependent upon the light prehistory of alga and can impart the advantage in acclimation of changing light conditions.

High light is one such factor which induces rapid changes in the algal cellular machinery and its acclimation. On a smaller time scale, algae cope up with the inefficiencies of light conversion by reorganising or altering its photosynthetic machinery. Investigations of acclimation have partly been made with cultures of algae in

laboratory, partly by studying natural population; and it is already clear that excess light could decrease photosynthesis by bleaching chlorophyll and/or damaging photosynthetic enzymes, in a process called photodamage. The extent of photodamage however can be species specific. Presumably the constraints in the extent of acclimation involve the greater fitness of alga when growing in a habitat close to its optimum than when acclimated to a very different habitat. In other words, a particular species might have greater fitness in high light due to its genetic make up than the other (Fujita *et a.l*, 1989). Primary regulation involves the lowered activity of RUBISCO (Zhang *et al.*, 2002), state transitions (Fujimori et al., 2005) and activation of xanthophylls cycle (Demmig and Adams, 1996); the extent of which depends upon the photon flux density to which algae are exposed and they are more restrictive under low PFD (photon flux density) than under high PFD. These balances are required to protect the organism from the detrimental effects of excess light while maintaining sufficient pools of ATP and NADPH for cellular metabolism (Maxwell *et al.*, 1994).

To date, there is enormous mounting interest in the production of third generation biofuels and value added products from algae which is limited by cost of algae biomass production (Chisti Y, 2007). The selection of an energy and cost effective production strategy is major player in achieving competitive biomass production for number of outcomes. This includes the selection high biomass producing strain, suitable locations for algal cultivation, efficient methods of cultivation and harvesting algal biomass and further efficient product extraction. Here we focus on the first step, the selection of high biomass producing strain and possible key players affecting the domestication of algae to outdoor cultivation. While shifting algae from artificial controlled laboratory conditions to uncontrolled outdoor systems, the efficiency of photosynthesis is usually lowered at each scale up level. Despite the numerous reports of photoinhibition occurring within natural environment, there is paucity of information as to the nature of damage to photosynthesis under environmental conditions. Such information, if investigated may find use during the cultivation algae for biomass production or other value added compounds under outdoor systems which are often limited by knowledge about algal acclimation to changing environment. Under current study, eight species of algae were grown in specially designed glass chamber with controlled temperature and solar energy as a light source. The study aimed at the screening of algal species with high light tolerance and high biomass productivities. The current work finds application in strain selection procedures for outdoor cultivation.

Material and methods

Algal strain and culture medium

The green algae under study were procured from various culture collection centers as mentioned in table 1. The maintenance of cultures in different collection center is reported at 25±2 °C temperature and light intensity of 55±5 µmol photons m^{-2} s^{-1}. The cultures were maintained on respective growth media (table 1).

Table 1. Details of species procured from different culture collection centers and their respective media.

Species	Collection center	Serial number	Growth medium
Chlorella saccharophila	UTEX, Austin, Texas, USA	UTEX 247	MBG-11
Chlorella minutissima		UTEX 2219	MBG-11 MN
Chlorella protothecoides		UTEX 25	
Chlorella regularis		UTEX1807	MBG-11
Haematococcus pluvialis		UTEX	MES-volvox
Nannochloropsis occulata		UTEX 2164	ES
Chlamydomonas reinhardtii	Chlamydomonas resource center	CC-503 cw92 mt+	HS
Parachlorella keslerii	IIT Chennai, India	-	Walne's

Growth and maintenance conditions

The receipt of algal cultures from respective culture collection centers was followed by their maintenance under controlled conditions of light and temperature in incubator shaker (figure 1); at 28±2 °C and continuous shaking at 100 rpm. The cultures were continuously illuminated with light intensity of 50±5 µmol photons m^{-2} s^{-1} using an array of cool white fluorescent tube lights placed above the flasks. The cultures were grown in 100 ml Erlenmeyer flask, in respective liquid media (table 1). The algal species were maintained on respective agar (1%) media under same controlled growth conditions.

The algae were grown under outdoor conditions in specially designed environmental chamber (EC) which mimics the natural environment (Rathod *et al.*, 2016). The alga was subjected to seasonal and diurnal light variation (0-1500 µmol photons m^{-2} s^{-1}) (figure 2), with a controlled temperature of 28 °C in environmental chamber. Alga was cultivated in polythene bag reactors (2000 ml) with provision of aeration through tubings so as to keep the cells in suspension and to allow homogenous mixing (figure 1).

Figure 1. Algal cultivation in (A) incubator under controlled light (50 µmol photons m^{-2} s^{-1}) and temperature (28° C), with continuous shaking at 100 rpm; and (B) environmental chamber under sunlight (0 -1500 µmol photons m^{-2} s^{-1}) and controlled temperature (28±2 °C).

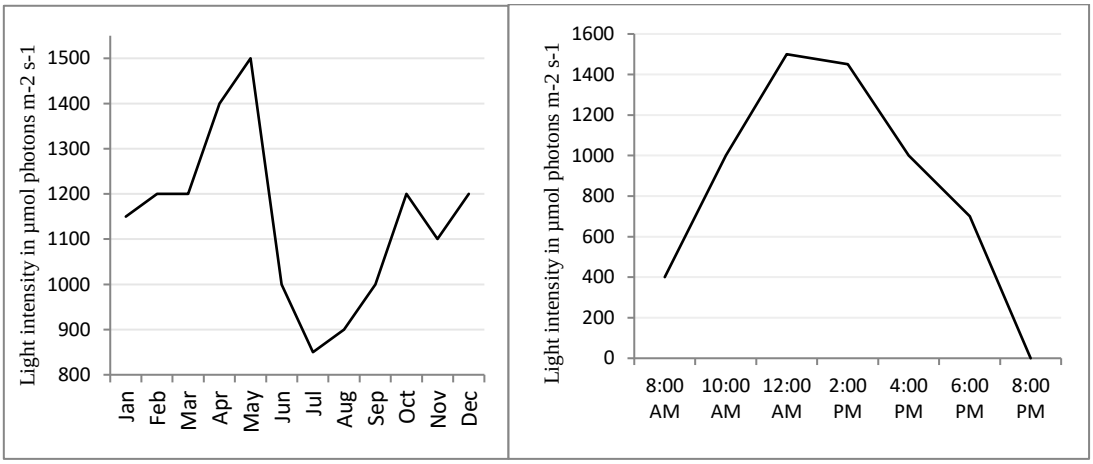

Figure 2. Seasonal and diurnal variation of light intensity in environmental chamber experienced by algae. (A) Monthly PAR received by algae in polythene bag reactors under NL. The recorded PAR indicates the averaged 12'O clock light intensity values measured at the side surface of reactors facing the sun. The values indicate the averaged light intensity for each year from 2012-2015. (B) The average PAR values in the month of may, indicating day night variation of light intensity to which alga is exposed during peak summer.

Experimental procedures

The algal species were grown and maintained initially in artificial low light (AL) conditions in incubator shaker. The algal cultures were allowed to grow for minimum of 10 generations before exposure to sunlight (EC). The culture was supplemented with fresh media at the end of exponential phase in each passage. The algae were transferred from AL (50 µmol photons $m^{-2}s^{-1}$) to sunlight (NL) (1500 µmol photons $m^{-2}s^{-1}$) during the peak

summer season. In both the cases cells were grown in their respective growth media, with initial constant cell number (10^7cells/ml) for experimental monitoring. The experimental runs were monitored for their photosynthetic performance and biomass productivity in EC. The experiment was performed in three replicates. The light intensities and temperature readings were recorded the in environmental chamber on hourly basis during entire experimental run.

Measurement of photosynthetic parameters

The chlorophyll *a* fluorescence parameters were monitored for algal suspensions using a pulse amplitude modulated fluorimeter (Dual-Pam-100, Heinz Walz, Effeltrich, Germany). For all the measurements the cells were kept in darkness for 10 minutes immediately after sampling and then used for chlorophyll *a* fluorescence analysis. The sample was illuminated with a modulated light (< 0.3 µmol photons $m^{-2}s^{-1}$) from a light emitting diode (with peak wavelength at 660 nm); and minimal fluorescence (F_o) was determined, following exposure to far red illumination (at 710nm) for 10s to ensure maximal PSII reoxidation., a 300 ms saturation pulse (SP) (6,000 µmol photons $m^{-2}s^{-1}$) was used to determine the maximal fluorescence yield (F_m). Dark adapted values for F_m and F_o were measured to obtain maximal quantum yield of photosystem II, F_v/F_m; $F_v=F_m-F_o$, (Krause & Weis, 1991). The light adapted readings of Minimal (F_o') and maximal (F_m') fluorescence were recorded in presence of actinic light, 1200 µmol photons $m^{-2}s^{-1}$ (growth light intensity). The value of NPQ was monitored under steady state photosynthesis) and calculated as (Fm- Fm')/Fm' (Genty *et al.*, 1989). The value indicate the ratio of Y(NPQ) to Y(NO), that is regulated heat dissipation to non regulated heat dissipation. The parameter Y (II) was calculated as described by (Klughammer and Schreiber, 2008): Y (II) = (Fm'- F)/Fm'.

Determination of biomass productivity

10 ml of culture was used for determination of the final biomass productivity of algae at the end of the log phase. The culture was centrifuged in a pre-weighed centrifuge tube and the algal cell pellet was washed thrice with distilled water to remove and trace of the medium salts. The pellet was allowed to dry at 60 °C overnight or till a constant weight was obtained. The biomass productivity was expressed as weight of dry biomass produced per unit volume of culture medium per unit time and calculated as follows:

$$\text{Biomass productivity } \left(\frac{\text{g}}{\text{l. d}}\right) = \frac{\text{Weight of tube with algae(g)} - \text{Weight of empty tube (g)}}{\text{Volume of culture processed (ml)} \times 1000 \times \text{number of days of growth}}$$

Statistical analysis

The experiment was performed in three replicates and all the values are presented as ±SD.

Result and discussion

Algal growth in incubator and first exposure to sunlight

One of the important criterions suggested by Chisti Y (2007) for algal growth under outdoor conditions is selection of local strains which are assumed to have competitive advantage over local geographical, ecological and climatic conditions. Microalgae are found not only in aquatic ecosystems but also exist in extreme environment like volcanic waters, saline waters (Duong *et al.*, 2012). Such conditions support the robust and opportunistic algae with superior survival skills. The responses of each algal species to slight changes in environment can be drastically different. And hence as opposed to only manipulating strains for enhanced biomass production, a more efficient strategy can be used. That includes in depth and systematic identification of a strain with inherent advantage of being robust under given geographical and climatic conditions.

In the current study, eight different algal species procured from different culture collections and were screened for their photosynthetic performance and biomass production after acclimation to artificial low (AL) and high light (NL) conditions. As shown in table 2, all algal species could grow in AL conditions with maximum biomass productivity (0.12 g $l^{-}d^{-}$) obtained for *C. saccharophila* and least biomass productivity was for *C. protothecoides* (0.03 g $l^{-}d^{-}$). The algal growth at AL (50±5 µmol photons m^{-2} s^{-1}), can be attributed to its previous AL exposures in culture collections owing to light prehistory. On contrary, not all the cultures subjected to NL conditions grow. The responses of algal growth to NL conditions are shown in table 2. The variation of growth responses to NL are majorly contributed by the changes of light intensities from AL to NL transition. Under such transfer alga might experience damage to its photosynthetic assembly and thus exhibit reduced growth.

Table 2. The responses of different algae to incubator (50 µmol photons m^{-2} s^{-1}) and sunlight after their 1st exposure.

	Biomass productivity (g l^{-} d^{-})		First exposure to sunlight	
	AL	NL	Growth	Pigment Bleaching
Chlorella sachharophila	0.12	0.22	+	+
Chlorella regularis	0.1	0.16	+	+
Chlamydomonas reinhardtii	0.09	0.12	+	+
Parachlorella kessleri	0.05	0.1	+	++
Chlorella minutissima	0.09	0.06	+	++
Chlorella protothecoides	0.03	Poor growth during subsequent passages	-	++
Haematococcus pluvialis	0.06		-	+++
Nannochloropsis occulata	0.09		-	+++

- No growth, + growth and + , ++, +++ indicates increasing level of pigment bleaching with cell death

Sunlight as a stress factor

In the initial exposure to NL conditions; the algal species, *C. protothecoides*, *H. pluvialis and N. occulata* could not survive more than 24 hours. The obvious reason for poor growth of algae under NL is increased light intensity than required for the process of photosynthesis. It is known that algae modify their photosynthetic pigment content through a regulatory process in changing environment (Raven and Geider, 2003). Algae growing under low light levels achieve the maximal absorption of light by increasing the light harvesting antenna pigments (large antenna) which provides them the competition advantage over others for capturing maximum light for survival. The sudden transfer of the culture to high light exposes this machinery to light intensities more than it was adapted to. In high light conditions, such a design of large antenna might lead to excess absorption of light than that can be handled by algal photosynthetic apparatus. In natural habitat, algae usually show movement away from bright light under high light (phototaxis) unlike in outdoor systems where they do not have opportunity to avoid high light levels and are restricted to their growth site. For a very reason the three species were possibly photoinhibited by overabsorption of light. The photobleaching events in these species indicate the rapid accumulation of triplet chlorophylls by excessive excitation of photosynthetic apparatus and production of damaging reactive oxygen species (ROS) molecules. Although the process is reversible, we did not see reversal of photobleaching in these algae possibly due to prolonged exposure to NL. To reduce the light received by each cell in the reactor system we adopted maintaining high density inoculums for supporting algal growth during initial exposures.

Role of initial inoculum density and light penetration

Initial inoculums density of the culture plays a very important role in cell growth and final biomass productivity of algae as it influences the light availability to individual cells in the culture. The initial cell density determines the time period for which algal cells are exposed to light as well as the time of dark-light cycle due to mutual shading of the cells (Wang *et al.*, 2013). Lower inoculum densities expose the algal cells to high light irradiances which could possibly lead to photoinhibition whereas high cell densities limit the light penetration into the culture vessel, thus creating a photo-limited environment. To avoid the photoinhibition during algal growth under NL, various approaches were undertaken (figure 3), which were found to support the survival of species under high light for considerable time. The AL, high density cultures (≥10^{10} cells/ml) (Chen *et al*., 2012), were transferred to the NL when the light exposure was least damaging i.e. pre dawn or post dusk so that culture adapts to slowly increasing light rather than its sudden exposure to high light at full sunlight. With such high density cultures, the adaptation process required assistance in regular replenishing of fresh nutrients, as the high density cultures were observed to utilize nutrients much faster and reach stationary phase within two to three days. The maintenance of cultures in the same volume of media each time during its subculture allowed the survival due to

high density of culture. Further to lower the potential damage due to NL, in the initial phase of growth the cultures were kept in the shady places (light intensity still more than AL, 700-800 µmol photons $m^{-2}s^{-1}$) of NL and later transferred to direct sunlight. Although the same strategy was applied to all eight species, the three species namely *C. protothecoides*, *H. pluvialis and N. occulata* could not survive. It can possibly be due to the insufficient photoprotective mechanisms or higher time scales required for the acclimation. The growth of algae under NL can be supported by one or the more mechanisms of protection (figure 4); decreasing the light absorption by reducing the cross sectional area of PSII (small antenna size), loss of excess energy as heat by non photochemical quenching (NPQ), and efficient quenching of ROS. Such photoprotective mechanism in turn cause downregulation of PSII, this can limit the photosynthetic electron transport to CO_2 fixation, as cell engages itself in handling high light stress rather than CO_2 fixation. In case of the three species which could not survive under NL the probable reason can be insufficient photoprotective mechanisms than photodamage leading to cell death.

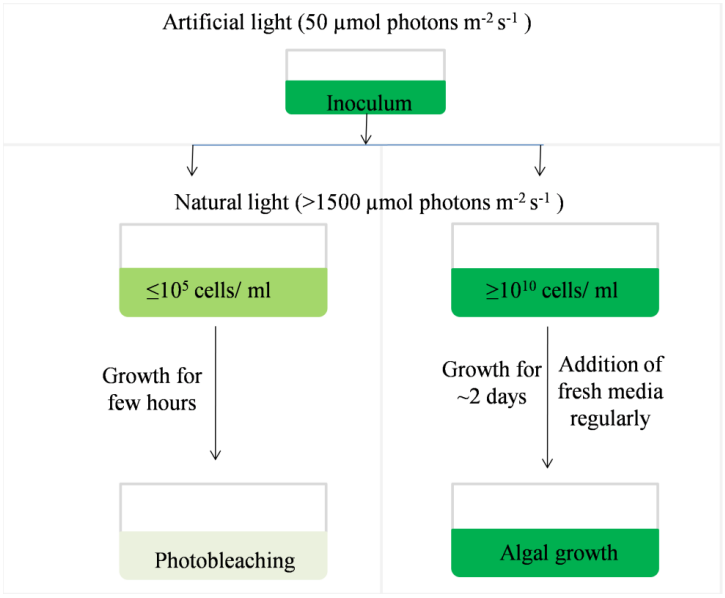

Figure 3. Effect of initial cell density on growth of algae and approach undertaken to support algal growth under high light

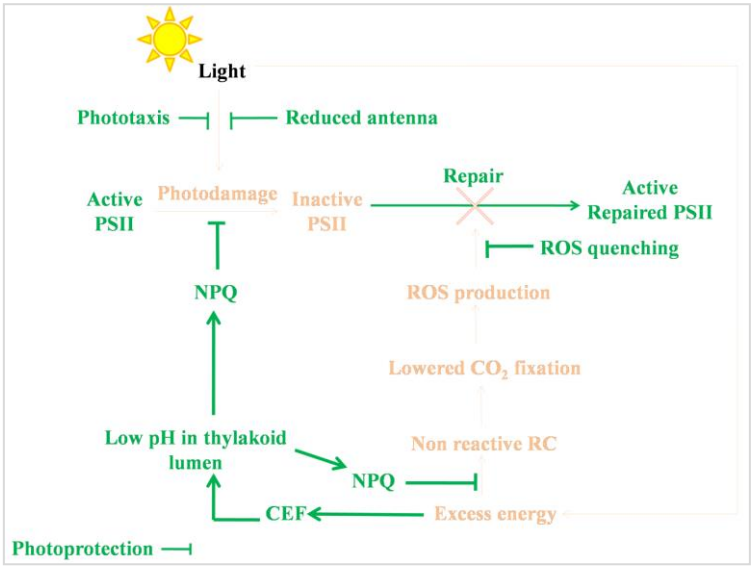

Figure 4. A theoretical model of algal responses to excess energy. The green arrows indicate the photoprotective mechanisms whereas pink arrows indicate the possible photodamage to algal photosynthetic machinery in absence of photoprotective mechanisms.

NPQ as a stress response

Exposure of AL cultures to NL for first time, only a part of harvested light is used for photosynthetic electron transport and protection of photosynthetic apparatus, while the major part of harvested energy cause damage to the apparatus, either by inactivation of PSII or photooxidation of its components. In case of *C. Protothecoides, H. Pluvialis, N. occulata* could not survive even with subsequent acclimation approaches, which can be attributed to inability of algae to respond via regulatory processes (figure 4). A possible reason for lack of photoprotection can be lack of NPQ induction in these algae where thermal dissipation does not occur. It is reported by Alberosi *et al.*, (2010) that the fastest response to high light stress is provided by NPQ which consists of the thermal dissipation of the excited chlorophylls either in singlet or triplet forms by activation of xanthophylls cycle. Probably in the species which could not survive with repeated exposures to high light levels the formation of triplet chlorophylls may exceed than the rate of it's quenching by xanthophylls with associated oxidative damage (Hideg *et al.*, 1998). Further the ROS generation can result in massive photodestruction in these species than others due to lack of NPQ indicating role of carotenoid (Baroli & Niyogi, 2000). The role of specific protein PsBs has been recently reported in possible induction of NPQ, which is highly susceptible to ROS degradation. Other than the differences in NPQ machinery, all these responses are species as well as time dependent and may take several minutes to years for their activation. Although media components and reactor designing can contribute to such reduced growth we focus further on the five species with promising growth potential. Five different algal species which survived in NL (*C. saccharophila*, *C. reinhardtii*, *C. regularis*, *P. kessleri*, and *C. minutissima*) were monitored for their photosynthetic performance and biomass productivity.

Measurement of photosynthetic parameters and biomass productivity

Photoinhibitory damage is thought to occur when PSII cannot utilize all excitation energy via photochemistry and further fails to dissipate excess energy in regulatory manner. To study these responses in the five species of algae which could acclimate to NL were studied for the photosynthetic parameters and biomass productivity. We monitored the chlorophyll *a* fluorescence as measured by dual-pam-100 in these cultures to understand the steady state photosynthetic responses and related it to their possible photoprotective mechanisms. The quantum yields of PSII and biomass productivity are shown in figure 5. The Y(II) value for *C. saccharophila* was found to be 0.62±0.05 and the remaining energy was dissipated at heat. For the algae acclimated to NL conditions even though the maximum photochemistry is expected, the heat dissipation cannot be excluded as the maximum value of Y(II) cannot exceed beyond 0.6-0.8 in algae which is the theoretical maxima for solar energy conversion efficiency. Further this efficiency is reported to decrease under stress conditions like high light, high or low temperature, and nutrient availability. If the value of Y(II) is affected, it is expected that the excess energy then should participate in the safe dissipation without causing any damage to algal cell or photosynthetic apparatus specifically; however such responses are species dependent. The responses are not only dependent on the light history but the nutritional history of the species. The distribution of heat dissipation in *C. saccharophila* indicates the higher activity of Y(NPQ) (0.26±0.05) than Y(NO) (0.12±0.02) indicating regulated loss of excess energy though xanthophylls cycle. As reported by (Mathur et al, 2009), xanthophylls are act as a quencher of triplet chlorophylls thus relieving stress on reaction center chlorophylls. Further, under NL conditions the xanthophyll pigments (specifically Zeaxanthin) brings about the physical separation of LHC antennae from PSII reaction centers which lower the light harvesting capacity of PSII complex thus imparting photoprotection. Although a minor fraction (0.12±0.2) of energy was lost through formation of closed reaction centres Y(NO) in this alga. On contrary, *C. minutissima* exhibited least Y(II) (0.12±0.05) amongst all other species, with maximum heat loss through non regulatory pathway Y(NO) (0.49±0.02). The loss of excitation energy through Y(NO) in case of *Chlorella minutissima* can be attributed to rapid accumulation of closed PSII reaction centers, which cannot utilise the incoming energy and thus leading to overreduction of electron carriers. The alga indeed showed high Y(NPQ) value (0.39±0.05) indicating the photoprotective responses were active but not sufficient to overcome the photodamage. In case of *C. regularis* the value of Y(II) was found to be 0.58±0.02 which only slightly decreased as compared to *C. saccharophila*. In case of *C. reinhardtii* a decreased Y(II) was noted than that compared to *C. saccharophila* and *C. regularis* indicating greater loss in heat dissipation than in photochemistry. The higher YNPQ) in this case denoted the photoprotective mechanisms. The decreased photochemistry Y(II) with elevated photoprotection Y(NPQ) were evident in case of *P. kessleri* similar to *C. reinhardtii* however the decrease in Y(II) by 62% and 81% was noted as compared to *C. saccharophila* and *C. reinhardtii.* The Y(NPQ) in this case was increased by 54% than that in *C. saccharophila* indicating increased photoprotection in this alga and downregulation of PSII at greater extent. The least value of Y(II) was noted for .*C minutissima* with maximum Y(NO) value indicating formation of non reactive RC and loss of excess energy in non regulated manner indicating susceptibility of this alga to high light than that of *C. saccharophila.* The alga, *C. saccharophila* exhibited highest biomass productivity (0.22 g $l^{-}d^{-}$) amongst all five species, followed by *C. regularis* (0.18 g $l^{-}d^{-}$). In case of *C. reinhardtii* and *P. kessleri* the biomass productivity noted was 0.12 g $l^{-}d^{-}$ while *C. minutissima*

showed least biomass productivity (0.06 g $l^{-1}d^{-1}$). The results indicate that the photoprotection in each algal species depends upon the activation of different biosynthetic pathways under high light as regulatory response. The global change in the algal photosynthetic machinery like changes in the light harvesting complexes (LHC) (antenna size) or enhanced regulatory pathways (NPQ) for excess energy dissipation might allow alga to acclimate to high light over long term exposure. However, the responses of each species can be different and the extent of photoprotection is dependent upon NPQ machinery of each alga. The maximum photochemistry with least damage can efficiently sustain the algal growth under high light and can be used as effective parameter to probe potential of biomass productivity of algae.

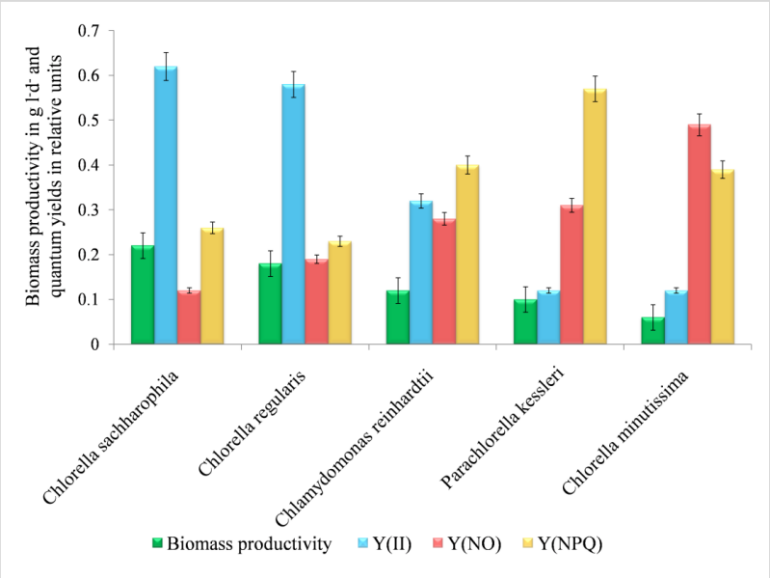

Figure 5. Comparison of biomass productivity (■) and photochemical parameters that is Y(II) (■), Y(NO) (■) an Y(NPQ) (■) for different algae under current study exposed to high light.

Conclusions

Light is the important resource that can hamper the growth of algae. Clearly under current study, different algal species have varying responses to high light, which drastically affected their biomass production. Although algae have developed efficient ways to tackle the photoinhibition events, such regulation is complex under natural environment and hence the survival of alga depends upon the inherent ability to reorganise its photosynthetic apparatus and the enhancement of photoprotective responses so as to sustain under extreme conditions. The algae with enhanced regulation can possibly prove to be suitable candidate for growth under large scale cultivation.

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