



Full Length Article

Effect of High Temperature on Activities and Lipid Production in Mutants of *Chlamydomonas reinhardtii*

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Abstract

Mostly organisms do not have the ability to cope with the high temperatures and this is one of the contributing environmental factors which can significantly affect the growth and lipid production capacity of that particular organism. In this study, we have tried to increase the growth and total lipid production of green microalgae, *Chlamydomonas reinhardtii* at high temperature because we know that they can be potentially utilized as novel raw materials for the production of biodiesel. We examined closely the effects of two different cultivation temperatures i.e., 25°C (control) and 33°C (high temperature). We have determined the biomass and lipid productivity of these strains. We also have done the biochemical analysis using carbohydrate, protein, chlorophyll and carotenoids content. Detailed fatty acid methyl ester (FAME) analysis of these strains has also been done using the GC-MS. Our results revealed that there is a strong negative correlation between biomass accumulation and lipid contents of the three selected mutant strains. Interestingly we found a differential preference of temperature among these algal strains, like CC-4033 grew faster at 25°C but CC-1171 showed high growth at 33°C. Our results showed that CC-1173 strain performed equally well at both selected temperature points. Similarly lipid contents were also changed at given temperatures in the order CC-4033 >CC-1171 >CC-1173 at 33°C. In all three mutant strains FAMES were identified which are considered as the suitable biodiesel components. Antioxidant enzymes like catalase and ascorbate peroxidases were found to be low at 33°C in all the studied strains. Here we suggest that CC-4033 strain can be considered as a potential mutant among all three mutants strains studied for the exploration of new renewable energy. © 2018 Friends Science Publishers

Keywords: Yellow in dark mutants; *Chlamydomonas reinhardtii*; Fatty acids; Stress biomarker; ROS

Introduction

Recently microalgae have gained remarkable attention for biomass, feed stock production and tendency to produce polyunsaturated fatty acids and pigments like carotenoids or antioxidants (Chisti, 2008; Singh and Gu, 2010). Therefore, one developed bioreactors mainly offer optimal conditions for algae growth but along with that the yield of a special product should also be increased (Rangel-Yagui *et al.*, 2004; Williams and Laurens, 2010; Lam and Lee, 2012). Microalgae use light and carbon dioxide and their photosynthetic efficiency for the production of biomass is higher than plants (Benemann, 1997; Miao and Wu, 2006). When microalgae are exposed to the environmental factors like changing temperatures, light-dark cycles fluctuations and also nutrient availability (e.g., N₂, CO₂, Phosphates or Silicates), the responses are usually the changes in the metabolic cycles and their carbon allocation patterns. There should be quantitative methods that can be helpful in measuring the carbon flow from photosynthetic primary products into the component of interest. There are many

microalgal species that have been given preference for their capacity of biodiesel production based on their lipid accumulation potential.

Lipids are considered to be very important for biodiesel production so there is a wide range of cultivation approaches that can be tested for the enhancement of lipid content in microalgae with the induction of biotic and abiotic stresses (Griffiths and Harrison, 2009; Roleda *et al.* 2013). Recent reports have shown the increased (20-40%) lipid contents of microalgae even though the biomass productivities have been compromised in stress conditions (Shekh *et al.*, 2013). However, lipid content with stress can change the fatty acid methyl ester (FAME) signature of the microalgae. It is very important as it defines the biodiesel quality from microalgae lipid (Ramos *et al.*, 2009; Cha *et al.*, 2011). Although several studies have reported the lipid production increase but detailed interrogation of changes in FAME composition under various cultivation conditions of selected microalgal strains including the stress is very limited, therefore stress induced lipid production needs special attention specifically for different strains. In this

regard, the strains that can tolerate different ranges of temperature are of key importance for the cultivation of microalgae at large scale. There are many microalgal strains that can develop tolerance and ability to grow over a wide range of temperatures but the responses and adaptations are highly dependent basically on the origin of a certain strain (De Boer *et al.*, 2005; Li and Qin, 2005). Critical evaluation of the effect of environmental temperature on the growth of microalgae at large scale cultivation is significantly important.

All organisms possess their own reactive oxygen species (ROS)-scavenging systems i.e. they can control the mechanisms of ROS accumulation. Similarly microalgae like plants have their own specific mechanism that involves schematic enzymatic and non-enzymatic reactions because they have chloroplasts which act as the ROS factories (Foyer and Noctor, 2009). Potent antioxidants like glutathione and ascorbate among membrane-localized carotenoids in plants and algae are part of the non-enzymatic ROS-scavenging systems. These antioxidant molecules act as a major line of defence against H₂O₂ that is produced in the chloroplast as a result of many reactions. Enzymes like catalase and heme peroxidase including ascorbate peroxidase are present for H₂O₂ removal (Foyer and Noctor, 2009). Plants and algae have the capacity to activate several defence systems at a time for the efficient scavenging of different ROS but certain conditions still can lead excess in ROS production and cause significant damage to the cell. For this there should be a regular feedback that should activate more aggressive mechanisms in the cell to get rid of damaged components and maintain ROS levels under control.

In the present study, yellow in dark mutants of *C. reinhardtii* was grown in temperature stress conditions. We used various biochemical parameters to analyze the immediate response of *C. reinhardtii* to the temperature stress. This study experimentally evaluates the effects of high temperature on microalgae biomass and lipid composition for their potential use as a biodiesel feed stock. We tried to investigate whether the temperature has any effect on the forbearance to oxidative stress in the green alga, *C. reinhardtii*, in order to clarify whether temperature acts as a signal transducer in the response to environmental stress. This study will be important not only for understanding of temperature tolerance to regulate lipid biosynthesis but will also help to develop strategies for microalgal based integrated biodiesel and bio-ethanol production.

Materials and Methods

Organism and Growth Condition

Chlamydomonas reinhardtii is a single celled green alga that belongs to Phylum Chlorophyta. In this study we selected three yellow in dark mutant strains of *C.*

reinhardtii (i) CC-1171 (y6 mt+), (ii) CC-4033 (y5 NIT+ mt +) and (iii) CC-1173 (y7 mt+) (Chlamydomonas Resource Center, <http://www.chlamycollection.org/>) were selected in this study. All the algal strains were grown on Murashige and Skoog medium including vitamins instead of TAP media for two weeks.

Experimental Conditions

Three strains of yellow in dark mutants of *C. reinhardtii* were grown at 25°C under the cool white light fluorescent bulbs with constant illumination from the algal stock. Well grown cells were then grown at 25°C followed by heat stress at 33°C for 8 days. All the experiments were carried out in bio-triplicates in 1 L flasks containing 500 mL of MS. Physiological changes in the algal culture under all the experimental conditions were observed and recorded with the help of camera (D5200 Nikon Tokyo, Japan).

Estimation of Growth and Biomass Productivity

50 mL of algal culture grown for 8 days was centrifuged at 5000 rpm for 10 min and the harvested biomass was dried at 60°C to get the constant dry cell weight (Rai *et al.*, 1991). Following equation was used to calculate the biomass productivity

$$P = \frac{X_2 - X_1}{T_2 - T_1}$$

In this equation the dry cell mass (mg/L) is denoted by X1 and X2 and the time by T1 and T2, respectively.

Protein, Chlorophyll, Carotenoid, Carbohydrates and Enzymatic Assays

Lowry's method was used to determine the total protein content, this method uses bovine serum albumin as a standard (Lowry *et al.*, 1951). Photosynthetic Pigments and carotenoids content were determined by the method described by Pancha *et al.* (2014). Total carbohydrate was determined by its content using the phenol sulfuric acid method.

Selected algal strains were pelletized by centrifugation and homogenized in 0.1% w/v TCA solution. After centrifugation at 10,000 rpm for 10 min supernatant was taken, 10 mM phosphate buffer (pH 7.0) and 1 M KI was added to the supernatant. Absorbance reading was taken at 390 nm by spectrophotometer. A calibration curve was prepared using the known concentrations of H₂O₂ and the concentration in μmol from the fresh weight in the sample was determined.

Algal samples were homogenised in 80% ethanol solution and were centrifuged at 3000 g for 5 min. 20% trichloroacetic acid (w/v) solution (containing 0.01% butylated hydroxytoluene (w/v) plus and minus 0.65% TBA (w/v)) was added in supernatant. MDA compounds were recorded by taking absorbance at 532 nm while

absorbance at 440 and 600 were used for correction of anthocyanin and sugar content. The MDA equivalents were determined by using an extinction coefficient of $157 \text{ mM}^{-1} \text{ cm}^{-1}$ (Hodges *et al.*, 1999).

Algal cells were sonicated in cold extraction buffer (50 mM Tris-HCl (pH 7.8), 1 mM MgCl_2 , 1 mM EDTA, along with 1% w/w polyvinylpyrrolidone (PVP), 1 mM ascorbate was added to this buffer in case of APX assay. Following the centrifugation at $15,000 \times g$ for 20 min, supernatant was taken and used as crude extract enzyme activities assay. Catalase (CAT) activity was determined spectrophotometrically. Decrease in absorbance at 240 nm was recorded up to 150 sec. Changes in absorbance observed at 290 nm were recorded to evaluate Ascorbate peroxidase (APX) activity (Nakano and Asada, 1981).

Lipid Extraction

Total lipid was extracted according to method described by Bligh and Dyer (1959). Algal cells were centrifuged at 10,000 rpm for 10 min and washed with distilled water. Wet weight was recorded and cells were kept at 60°C to dry. Methanol and Chloroform (2:1) was added in dried algal cells and kept for 18 h at 25°C . For layer separation chloroform and water was added followed by vigorous shaking. Following the centrifugation upper oil layer was collected in clean pre-weighed glass vial and procedure was repeated thrice. Glass vials were kept in hot oven to get dried weight. Total lipid content was calculated by following equation

$$\begin{aligned} \text{Total Lipid content} \\ &= \text{weigh of glass vial with Lipid} \\ &- \text{weigh of empty glass vial} \end{aligned}$$

Lipid content was expressed as % dry cell weight.

Fatty Acid Profiling

Part of the lipid fraction was trans-esterified through the method described by Parsaeimehr *et al.* (2015) and qualitatively characterized by a gas chromatograph, model 7890 GC (Agilent, Santa Clara, USA) equipped with HP-5 column and FID detector.

Statistical Analysis

All the experiments were done in bio-triplicate and data represent the mean value. For significance, t-tests and standard errors with significance criteria of $P < 0.05$ were used for the statistical analysis.

Results

High Temperature Effects on Microalgal Growth

In the present study, we have found a significant difference in the dry mass and productivity when three yellow in dark mutants of *C. reinhardtii* were grown at 25°C and 33°C . The DCW of CC-1171 grown at 33°C ($1.509 \pm 0.9 \text{ g/L}$) was

found to be higher when compared to the cultures grown at 25°C (0.982 g/L) while CC-4033 showed a decrease in DCW when grown at 33°C (0.98 g/L) with respect to that grown at 25°C (1.609 g/L). No significant difference was seen in DCW of CC-1173 grown at 25°C ($0.726 \pm 0.012 \text{ g/L}$) and 33°C ($0.749 \pm 0.05 \text{ g/L}$) as shown in Fig. 1A.

Biomass productivity of CC-1171 was also increased with elevating the temperature. As compared to the culture grown at 25°C (65.5 mg/L/day), 1.53 fold higher biomass productivity (100 mg/L/day) was found in the culture grown at 33°C . The Biomass productivity of CC-4033 was decreased at 33°C whereas again there is non-significant change in the biomass productivity of CC-1173 as shown in Fig. 1B.

As far as the growth rate among the three strains is concerned, no significant differences were seen among CC-4033 and CC-1173 but CC-1171 have shown a reduced growth rate as the temperature increases as shown in Fig. 1C. The maximum specific growth rate (μ_{max}) of CC-1173 at 33°C was 0.194 day^{-1} higher than that at 25°C (0.179 day^{-1}). With the elevation in temperature, the differences in the μ_{max} were reduced from 0.23 day^{-1} and 0.2 day^{-1} to 0.21 day^{-1} and 0.14 day^{-1} in CC-4033 and CC-1171, respectively. Increase in temperature affected microalgal growth because the colour changes of the cells from green to yellow/brown were highly visible in the culture. The intensity of green colour is the indication of growth. The lighter green to colourless is indicating the low number of cells and kinetics at the increased temperatures as shown in Fig. 1D.

High Temperature Effects on Pigments Composition

In this study, we also have measured the changes in photosynthetic pigments to see the effects of elevated temperature on photosynthesis of *C. reinhardtii*. Chlorophyll a content of CC-1171, CC-4033 and CC-1173 enhanced as temperature elevated. As compared to culture grown at 25°C (1.73 ± 0.19), 2.61% higher chlorophyll a content was observed in CC-1171 at 33°C . Increase in CC-1173 and CC-4033 was found 1.1 and 2.04%, respectively in the culture grown at 33°C . Chlorophyll b increased 0.56% in CC-1171 when grown at 33°C , while CC-4033 showed decreased chlorophyll b content at 33°C . Increase in Chlorophyll b content of CC-1173 was 0.35% as shown in Table 1. Carotenoids, an important antioxidant, increased significantly in CC-1171, CC-4033 and CC-1173. Carotenoids accumulation was observed to 6.6% higher in CC-1171 at 33°C . Carotenoids content was recorded to be enhanced by 1.6 and 4.7% in CC-1173 and CC-4033, respectively. Chlorophyll a/b ($4.67 \mu\text{g/mL}$) and carotenoids/chlorophyll a + b ratio was highest ($0.413 \mu\text{g/mL}$) in 33°C grown culture which are indicative of the active stress due to decrease in light harvesting complex and PS II activity as shown in Table 1. Increase in carotenoid content of algal cells was previously reported as part of a defence mechanism against photo-damage.

Table 1: Effect of temperature on pigments composition of *C. reinhardtii*. All data is means \pm SE of bio-triplicates. * ($P < 0.05$) denotes significant difference between control and stressed strains

Algal Strains	Treatment	Chlorophyll a ($\mu\text{g/mL}$)	Chlorophyll b ($\mu\text{g/mL}$)	Chlorophyll a + b ($\mu\text{g/mL}$)	Carotenoids ($\mu\text{g/mL}$)	Chlorophyll a/b ($\mu\text{g/mL}$)	Carotenoids/Chlorophyll a + b ($\mu\text{g/mL}$)
cc-1173	Control	3.73 \pm 0.35*	3.73 \pm 0.35*	9.91 \pm 0.89*	1.16 \pm 0.5*	0.604 \pm 0.01*	0.115 \pm 0.05*
	Stress	7.87 \pm 1.12	5.05 \pm 0.26	12.92 \pm 1.37	3.02 \pm 0.36	1.55 \pm 0.15	0.233 \pm 0.005
cc-4033	Control	2.05 \pm 0.6*	3.31 \pm 1.25	5.36 \pm 1.7	1.34 \pm 0.18*	0.645 \pm 0.1*	0.266 \pm 0.07*
	Stress	6.25 \pm 1.55	1.39 \pm 0.3	7.66 \pm 1.4	7.66 \pm 1.4	4.67 \pm 1.55	0.413 \pm 0.02
cc-1171	Control	1.73 \pm 0.19*	3.15 \pm 0.35*	4.88 \pm 0.55*	0.45 \pm 0.06*	0.55 \pm 0.001*	0.093 \pm 0.003
	Stress	6.26 \pm 2.56	4.92 \pm 0.76	11.18 \pm 3.08	3.46 \pm 1.13	1.26 \pm 0.4	0.307 \pm 0.01

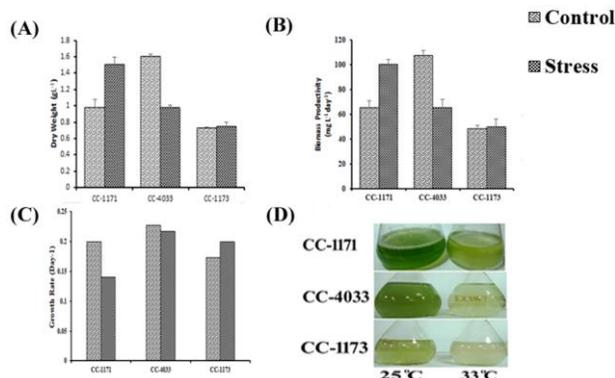


Fig. 1: Effect of temperature on DCW and biomass productivity of *C. reinhardtii*

(A) The average dry weight (g/L) of *C. reinhardtii* (CC-1171, CC-4033, CC-1173) at 25°C and 33°C (B) Biomass productivity (mg/L/day) (C) Growth Rate (day^{-1}) of *C. reinhardtii* (CC-1171, CC-4033, CC-1173) at 25°C and 33°C (D) Growth phenotype. Data is represented as means of bio-triplicates and error bars represent standard deviations

High Temperature Effects on Biochemical Composition

As the lipids and carbohydrates are hydrophobic in nature and are present in the reduced states, they are considered as the preferred storage products in the microalgae under various stress conditions. Cells use these carbohydrates and lipids to cope with the adverse stress conditions for their survival and proliferation.

Effects of high temperature on lipid content and lipid productivity, carbohydrates and total protein content of three yellow in dark mutants of *C. reinhardtii* is shown in Fig. 2. As cultivation temperature is increased from 25°C to 33°C, CC-4033 produced total lipid content of 528.2 ± 6.2 mg/g of dry weight which was 55.77% of total dry weight. However, at 33°C total lipid content in CC-1171 and CC-1173 decreased to 15.38% and 33.12% respectively which was lower than lipid produced at 25°C 55.5% and 49.5% respectively. Lipid productivity was also increased in CC-4033 while decrease in lipid production was observed in CC-1171 and CC-1173 (Fig. 2B).

In the present study, a slight increase in carbohydrate accumulation was observed in CC-1171 and CC-4033 grown at 33°C which was 32.7 ± 5.1 $\mu\text{g/mL}$ and 35.3 ± 5.1 $\mu\text{g/mL}$ respectively when compared to carbohydrate accumulation at 25°C, which was 27.7 ± 9.2 $\mu\text{g/mL}$ and

31.2 ± 5.1 $\mu\text{g/mL}$, respectively. However, in case of CC-1173, at 33°C accumulation decreased to 32.3 ± 4.6 from 43.5 ± 1.5 $\mu\text{g/mL}$ at 25°C as shown in Fig. 2C.

Total protein content of CC-1171 mutant of *C. reinhardtii* was significantly decreased at 33°C. Whereas, CC-4033 and CC-1173 have shown a very low protein content even at 25°C which showed a slight decrease at 33°C (2D).

Fatty Acid Methyl Ester (FAME) Signature

The comparative analysis of total lipids accumulation in three mutant strains grown at high temperature have shown that lipid accumulation generally decreased with increasing temperature in CC-1171 and CC-1173 but in CC-4033 lipid accumulation generally increased with increasing temperature as shown in Fig. 2A. The fatty acid composition revealed that all strains showed the presence of same fatty acids but the amount of these fatty acids varied in all the strains at different temperatures. The predominant acids present in cultures grown under standard conditions (non-treated control) were palmitic, linoleic, oleic, α -linolenic acids at 8 days of incubation. The fatty acid methyl ester (FAME) composition that changed under high temperature at 08 days of incubation were palmitic (6.98-7.2% in CC-1171, 7.46-6.89% in CC-4033 and 1.46-6.22% in CC-1173), oleic (5.53-5.21% in CC-1171, 5.42-5.79% in CC-4033 and 36.1-5.07% in CC-1173), linolenic (6.3-4.8% in CC-1171, 5.21-5.63% in CC-4033 and 2.6-4.8% in CC-1173), α linoleic (56.86-58.84% in CC-1171, 57.5-56.43% in CC-4033 and 48.61-59.29% in CC-1173) acids were most prevalent (Table 2).

Cellular Oxidative Stress Levels at High Temperature

Reactive oxygen species like Superoxide (O_2^-), Hydrogen peroxide (H_2O_2) and Hydroxyl (OH^\cdot) radicals are the reactive oxygen species that can cause severe oxidative damage whereas oxygen itself when present has no harm to the cells. To further examine the responses of *C. reinhardtii* to high temperature, ROS level was quantified. At 33°C, the intracellular production of ROS level increased considerably in all three mutants as compared to the control as shown in Fig. 3A. MDA content (0.9 ± 0.05 nmol g^{-1} FW) was also lowest in 25°C grown culture as shown in Fig. 3B. By increasing the cultivation temperature to 33°C it resulted in 0.58% increase (1.43 ± 0.152 nmol g^{-1} FW).

Table 2: Identification and quantification of fatty acids from CC-1171, CC-4033 and CC-1173 strains after temperature shift to 25 or 33°C after 08 days of incubation

Strains	Temperature (°C)	Fatty acid concentration (% of Total Fat)							
		C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
CC-1171	25	6.369048	6.982143	7.041667	7.041667	6.041667	5.535714	6.303571	56.86905
	33	6.309091	7.2	6.787879	5.569697	5.569697	5.212121	4.836364	58.84848
CC-4033	25	6.456376	7.469799	6.651007	5.986577	5.463087	5.42953	5.212121	57.50336
	33	6.154799	6.896108	7.062008	5.998651	6.037793	5.796351	5.636068	56.43521
CC-1173	25	4.788889	1.466667	1.988889	1.988889	2.477778	36.10556	2.605556	48.61111
	33	5.99636	6.228389	7.343039	5.288899	5.928116	5.077343	4.854413	59.29254

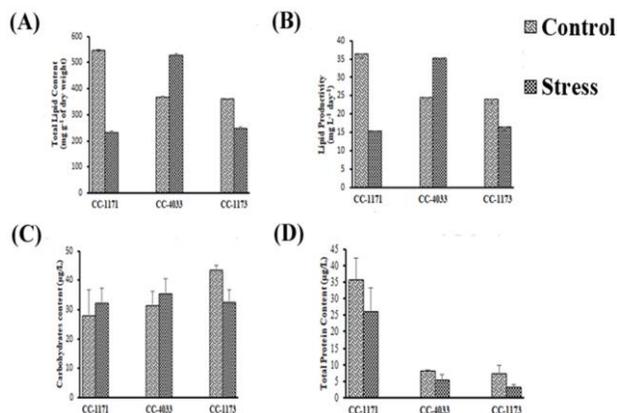
Effect of High Temperature on the Antioxidant Enzyme Activity

Antioxidant defence mechanisms of the photosynthetic organisms have evolved strongly for the balanced ROS levels. This is so because these organisms utilize the enzymatic antioxidant molecules as SOD, CAT, and APX. We also determined the general enzymatic antioxidant response of *C. reinhardtii* grown under high temperature. Activities of antioxidant enzymes in *C. reinhardtii* substantially changed when the cells were exposed to high temperature. The CAT activity in algae progressively decreased to the level below the control with increase in temperature from 25°C to 33°C. The pattern of APX activities was similar to the CAT in algae exposed to high temperature as shown in Fig. 3C and D.

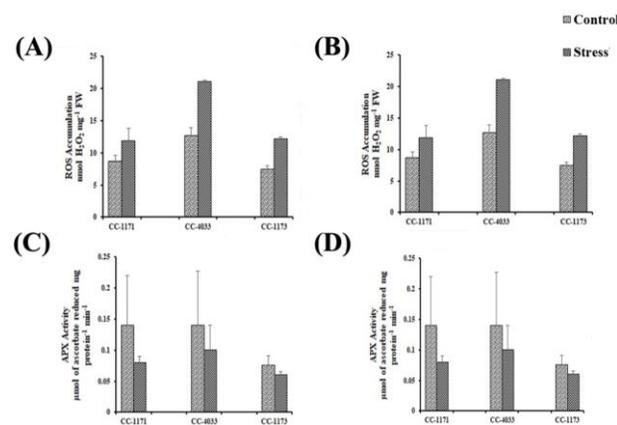
Discussion

Processes like gamete formation, carbohydrates or lipids accumulation, autophagy induction or degradation of various cellular elements affects the growth efficiency of micro-algae which ultimately depends on level and duration of the stress (Li *et al.*, 2012). Microalgae should be adapted to these changes by producing biochemical products which will ultimately ensure their survival under stress (Lim *et al.*, 2012a). Photoautotrophic microalgae are considered to be more important for sustainable biodiesel production but they have a limiting factor of biomass and lipid slow yields (Parsaeimehr *et al.*, 2015). *C. reinhardtii* is a unicellular green alga, which is used as a model organism in many fields of molecular biology. For more understanding about the optimum conditions necessary for the growth of *C. reinhardtii* in a lab setting, we focused on how temperature can affect mutant forms.

Optimization of high biomass yield of microalgae through growth conditions is really getting importance. Cultivation temperature plays an important role for the efficient growth of microalgae. Cultivation temperature effects differently on various microalgae and is variable among the species. Different species have different optimum temperatures e.g., *Chlorella vulgaris* grows at 30 Parsaeimehr C, *Nannochloropsis oculata* at 20°C (Converti *et al.*, 2009), while the optimum temperature for *Acutodesmus dimorphus* as shown to be 35°C (Chokshi *et al.*, 2015). Wang *et al.* (2016) have shown that the optimal temperature for the growth of *Chlorella sorokiniana* was 26°C. We observed that the mutant *C. reinhardtii* CC-1171

**Fig. 2:** Effect of temperature on biochemical composition of *C. reinhardtii*

(A) Total Lipid content (mg/g of DCW) (B) Lipid productivity (mg/L/day) (C) carbohydrate content ($\mu\text{g/ml}$) (D) Total Crude protein content ($\mu\text{g/ml}$). Data is mean of bio-triplicates and error bars show standard deviations

**Fig. 3:** Effect of High temperature stress ROS accumulation (A), MDA content (B) and on the activities of ascorbate peroxidase (APX) (C) and catalase (CAT) (D) in yellow in dark mutants of *C. reinhardtii*. The cells were incubated at 25°C and 33°C. The ROS accumulation, MDA content and enzyme activities were determined. Values are the means \pm SD ($n = 3$)

et al., 2015). Wang *et al.* (2016) have shown that the optimal temperature for the growth of *Chlorella sorokiniana* was 26°C. We observed that the mutant *C. reinhardtii* CC-1171

was superior to CC-1173 and C-4033 in order of biomass accumulation and biomass productivity at 33°C as shown in Fig. 1. This suggests that temperature is considered a main contributing factor in algal growth; a possible explanation is the increase in temperature increases the kinetic energy of a system. Higher kinetic energy increases the number of collisions between the enzymes and substrates of the light reactions, ultimately resulting in a higher photosynthetic rate (Lambers *et al.*, 2008).

Microalgae fulfil their energy demands by the process of photosynthesis and hence it is very important for sustaining the metabolism. Photosynthetic efficiency depends on the chlorophyll and carotenoid levels of microalgae and they need to maintain a proper balance between chlorophyll and carotenoid levels in order to efficiently utilize all the carbon (Zhang *et al.*, 2002). Ledford and Niyogi (2005) observed that the carotenoid accumulation under stress protects the algal cells from damage. In the present study, significant difference ($p < 0.05$) in the photosynthetic pigments (chlorophyll a and b) and carotenoids was found when yellow in dark mutant of *C. reinhardtii* was allowed to grow at 33°C. Our results are fully in concomitance with the previous findings of Chokshi *et al.* (2015).

Microalgae have a tendency to produce higher amounts of organic compounds like lipids or carbohydrates which actually contributes for their use in biofuel production. Temperature has been found to have a major effect on the fatty acid composition of microalgae. Microalgae have various mechanisms to cope with high temperature stress, as the temperature gets high these mechanisms are turned on. Consequently, there is an increase in lipid production which ultimately results in the increased biodiesel production. In present study, for lipid accumulation and lipid productivity CC-4033 was found superior to CC-1171 and CC-1173 at 33°C. In *C. reinhardtii* it has been shown previously that biomass productivity is reduced with an increase in temperature. Similar results have been obtained for *H. pluvialis* which showed that under various stresses lipid content is increased and the biomass productivity is decreased (Saha *et al.*, 2013). Our results also corroborate with the finding that lipid content is enhanced and biomass production is decreased when the temperature is increased.

The main fatty acids (FAs) among these strains were C16:0, C16:3, C18:3. Culture temperature has also influenced FA composition of CC-4033, CC-1171 and CC-1173. As the temperature is increased, the proportions of C16:0, C18:1, saturated FAs (SFAs), and monounsaturated FAs (MUFAs) remained almost unaffected in CC-1171 and CC-4033 but in CC-1173, proportions of C16:0, 16:1 and C18:0 increased and C18:1 decreased drastically from 36.10% to 5.07%, while the proportions of C16:3 and C18:3 increased in CC-1171 and CC-1173 but decreased in CC-4033. Processes like photosynthesis, ion permeability and respiration influence the physical properties of membrane

bilayers resulting in lipid compositional changes that help maintain normal metabolic functions under different temperature regimes. A number of evidences have shown that algae generally increase their relative amount of unsaturated FAs environmental temperatures are low (Jiang, 2002; Sheng *et al.*, 2011). It has been noticed that microalgae upon exposure to lower growth temperature tends to increase its PUFA contents (Fulke *et al.*, 2010) and *C. reinhardtii* has shown a sharp increase in its PUFA content when cultured at extreme positive (38.5°C) or negative shift (0–15°C) in optimum temperature (Morowvat *et al.*, 2010; Shekh *et al.*, 2016). An increase in the PUFA content may be helpful for microalgae to adapt to unfavourable environmental conditions (An *et al.*, 2013). A type of *C. reinhardtii* mutant have shown a shift in neutral lipids (NL) content instead of polar membrane lipids after a temperature increase from 22 to 34°C (Yao *et al.*, 2012). Previously it was mentioned that the percentage of unsaturated fatty acid and lipid content were both down-regulated when the culture temperature increased from 25°C to 35°C in *Chlorella vulgaris* (Converti *et al.*, 2009). This is consistent with our data indicating that temperature can have impacts on lipid content and lipid composition simultaneously. The increased lipid content at 25°C can be interpreted as more lipid accumulation with a high percentage of unsaturated fatty acids that can help the yellow in dark mutants of *C. reinhardtii* to maintain membrane fluidity under temperature stress.

Carbohydrates are the end products of photosynthesis and they can be accumulated either as starch in the plastids or they can play their role as the basic component of the cell walls (Rangel-Yagui *et al.*, 2004). Different species produces carbohydrates differently i.e., the carbohydrate production is variable among the species. Appropriate and felicitous cultivation conditions can significantly improve the carbohydrates production ability of microalgae. It has been observed that microalgae strains showing high carbohydrate productivity and suitable sugar composition are highly advisable for biofuels or chemical production. It has been reported that *C. reinhardtii* accumulates more sugars as the temperature decreases (Valledor *et al.*, 2013). Our present work showed that CC-4033 has the highest carbohydrates productivity which is approximately 1.8% higher at 33°C when compared to at 25°C.

Reactive oxygen species (ROS) levels increases with abiotic stress, as a result the oxidation of different biomolecules such as proteins, nucleic acids and lipids is increased that can be considered as very harmful for the cellular redox homeostasis (Torres and Dangl, 2005). Reactive oxygen species such as H₂O₂, O²⁻ or OH have seen to be generated constantly as by-products of various oxygen metabolisms particularly taking place in mitochondria, chloroplasts and peroxisomes (Apel and Hirt, 2004). It is the responsibility of the antioxidant enzymes which are present in several organelles to take control of the

ROS production under normal conditions (del Río *et al.*, 2006). During stress, the ROS overproduction can lead to serious cellular damage which subsequently affects many cellular functions through the modification of oxidizing proteins, nucleic acids. This all ends up in the induction of lipid peroxidation (Mittler *et al.*, 2004). The present study showed the increased level of ROS in the cells exposed to high temperature. In lipid peroxidation the ROS attack polyunsaturated fatty acids of the cell membrane which subsequently induces a chain reaction, lipid hydroperoxides act as the intermediate products of this chain reaction which results in the destabilization and disintegration of the cell membrane (Melegari *et al.*, 2012). Several pieces of evidence indicate that the increase in temperature is associated with lipid peroxidation (Ali *et al.*, 2005; Hasanuzzaman *et al.*, 2013). Lipid peroxidation produces MDA which acts as a natural biomarker that can be helpful in indicating the extent of cell damage produced by these ROS. In agreement with this evidence, we showed that the exposure of *C. reinhardtii* to high temperature results in enhanced MDA content. Effect of temperature stress on H₂O₂ concentration, antioxidant defence system and MDA contents in yellow in dark mutants of *C. Reinhardtii* are shown in Fig. 3.

Presently, microalgae are considered as a source of potential raw material for biofuel production so it is of higher importance that their lipid production mechanisms and the factor affecting them should be known. By utilizing this knowledge, we can optimize the cultivation of commercially important microalgae and can find ways to grow them in a cost-effective or even profitable manner. This study explained the thermo-tolerance response of *C. reinhardtii* (CC-1171, CC-4033, CC-1173) from the sides of ROS level, MDA content, protective enzyme activity, fatty acid content, morphological analysis and soluble protein, which contributes the understanding of biological high temperature-tolerant mechanisms.

Conclusion

Our findings suggest that temperature is a critical environmental factor that can modulate the amount and composition of algal fatty acids. Three *C. reinhardtii* strains were analyzed with different stress conditions and biochemical analysis showed that the CC-4033 strains can be considered as a potential mutant strain for exploration of new renewable energy source. Fatty acid profiling of this mutant strain has also supported that high temperature is one of the major factors which affect productivity of microalgae, we believe that it has a promising future in algal oil and biodiesel production.

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