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Enhanced lipid content in *Chlorella* sp. FC2 IITG via high energy irradiation mutagenesis

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Abstract−High energy ultra-violet radiation was used to induce mutations in the wild type strain *Chlorella* sp. FC2 IITG and the mutants obtained were screened for enhanced lipid content targeted towards biodiesel production. Screening of mutants under nitrogen starved photoautotrophic condition showed five mutant strains with higher lipid content as compared to the wild type strain. Maximum neutral lipid content of 22.26% (w/w, DCW) was found for FC2-25UV mutant strain, which was 48.4% higher than that of wild type culture. Further characterization under photoautotrophic two stage high cell density cultivation of lipid rich FC2-25UV resulted in total lipid content of 68% (w/w, DCW), which was 21.43% higher than the wild type strain with a marginal improvement of 11% in the total lipid productivity. Comparison of enzyme activity assays under nitrogen starved conditions in both the strains revealed an 18.2% and 31.25% increase in acetyl CoA carboxylase and di-acyl-glycerol transferase activity of mutant. The FAME composition analysis showed 41.5% and 24% increase in the fractions of C18:2 and C18:1 in the mutant strain when compared to the wild type strain with no significant difference in the other FAME fractions. Thus, the mutant strain could be a potential candidate for biodiesel production.

Keywords: Microalgae, Mutation, Ultraviolet Rays, High Cell Density Cultivation, Lipid Content

INTRODUCTION

Increasing anthropogenic greenhouse gases and depleting fossil fuels have prompted the search for alternate renewable and sustainable biofuels. Among several alternative fuels, microalgae are considered as one of the potential, renewable and sustainable sources of biodiesel that can meet the current energy requirements [1]. The major inherent properties of microalgae which are superior to plants are their growth rate, tri-acyl glycerol content, photosynthetic efficiency and CO_2 sequestration ability [2]. However, commercialization of microalgae based biodiesel production is still at dormancy attributed to high cost of production influenced by several factors involved in both upstream and downstream processes, which includes strain selection, low net lipid productivity, cultivation technology, energy consuming algae harvesting process, extraction and transesterification for biodiesel production [3,4].

Improving neutral lipid accumulation capability of microalgae is one of the potential ways to achieve feasibility in microalgal biodiesel production process, which can be attained via metabolic engineering and/or conventional random mutagenesis [5]. Huge species-to-species variations, unavailability of species specific genomic sequence data and lack of comprehensive knowledge on *omic* data has complicated metabolic engineering in these polyphyletic organisms. Moreover, use of genetically modified organisms in open ponds with appropriate containment and associated judicial approvals remains a major issue to be addressed [6]. Conventional mutagenesis on the other hand has been reported to be cost effective, highly reliable for a short-term strain improvement program and does not require any sequence data [7-10]. However, the critical step involved in random mutagenesis is screening and selection of the appropriate mutants, as the frequency of obtaining mutant is much less with an incidence of 10^{-3} [5].

Variety of mutagens such as ultraviolet radiation, heavy ion radiation, ethyl methane sulfonate and N-methyl-N′-nitro-nitrosoguanidine have been tested in various microalgal strains and proven to be effective [6,11]. Heavy ion radiation induced mutation of *Nannochloropsis oceanica* resulted in a strain with enhanced lipid productivity from 0.211 to 0.271 g L^{-1} day^{-1} [7]. Enhancement of carotenoid via ultraviolet radiation (UV) mutagenesis was reported for *Dunaliella bardawil* [12], while 37% increment in eicosopentaenoic acid content of *Phaeodactylum tricornutum* via UV mutagenesis was reported by Alonso et al. [13]. Random mutagenesis of *Chlorella* sp. with ethyl methane sulfonate yielded a trait with enhanced capability to grow in presence of H_2S and sequester CO_2 efficiently from biogas [14] and with enhanced lipid content [11].

Chlorella sp. is one among the well characterized microalgae for biodiesel production with maximum lipid content reported up to 47.5% (w/w, DCW) under two-stage continuous heterotrophic condition and 56% (w/w, DCW) under two-stage fed-batch photoautotrophic cultivation [15,16]. In the present study, total lipid content of *Chlorella* sp. FC2 IITG was further enhanced by mutating the

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culture using UV radiations for improved biodiesel production. The mutants obtained were screened for enhanced lipid content in comparison to the wild type strain, and the best mutant was selected for further characterization. A two-stage fed-batch photoautotrophic process targeting high cell density at the first stage followed by lipid enrichment in second stage as designed in [16] was utilized to maximize the production potential of the mutant strain. The study also captured the changes in enzyme activity under nitrogen replete and nitrogen starved conditions in both the wild type and mutant strains to understand the regulation of lipid biosynthesis.

MATERIALS AND METHODS

1. Microalgae and Cultivation Conditions

Chlorella sp. FC2 IITG is an indigenous freshwater microalga isolated from Northeast part of India [17] and grown in optimized BG11 medium containing (in g L^{-1}) urea 1.8, K_2HPO_4 0.076, $MgSO_4{\cdot}7H_2O$ 0.090, $CaCl_2{\cdot}2H_2O$ 0.067, Na_2CO_3 0.038, citric acid 0.002, ferric ammonium citrate 0.01, EDTA 0.001 and A5+Co solution (1.5 mL L^{-1}) that consists of H_3BO_3 2.86, $MnCl_2{\cdot}H_2O$ 1.81, $ZnSO_4{\cdot}7H_2O$ 0.222, $CuSO_4{\cdot}5H_2O$ 0.079, $Na_2MoO_4{\cdot}2H_2O$ 0.390 and $Co(NO_3)_2{\cdot}6H_2O$ 0.049 [16]. Two full loops of the slant culture were revived in a 250 mL Erlenmeyer flask containing 100 mL optimized BG11 media and incubated in an orbital shaker (Multitron-Pro, Infors HT, Switzerland) at 150 rpm, 28 °C under 20 μE m^{-2} s^{-1} light intensity with a light : dark cycle of 16 : 8 h with no additional CO_2 supplied. After reaching absorbance (A_{690}) 1.0, 1% (v/v) of the revived culture was used as inoculum in all the experiments.

2. High Energy Radiation Mutagenesis Using Ultraviolet Rays

Fresh exponential growing revived cell suspension (5 days grown culture) of FC2 at a concentration of 1.4×10^7 cells mL^{-1} was used for UV mutagenesis. Cell culture of 7 mL was taken as thin film in a 90 mm petri dish for mutation with UV irradiation of wavelength ~254 nm from a UV-C lamp (T8-TUV/G30, 30W, Philips, Holland). The plate was kept 20 cm from the light source and samples of 0.5 mL were taken at different exposure time over 60 minutes. This preliminary experiment was performed to determine the time of exposure required to achieve 98±2% mortality, which may induce substantial mutagenesis. The 0.5 mL samples collected over 60 minutes time period were added to 0.5 mL fresh optimized BG11 medium in microcentrifuge tubes and incubated overnight under dark at 28 °C to avoid photo-repair and to stabilize the mutants [18]. After overnight incubation, the cells were plated in petri plates containing optimized BG11 medium for isolation of the colonies and incubated under the photoautotrophic condition, as mentioned in the above section. The number of colonies formed was counted to obtain the mortality rate, and the best time of exposure for inducing mutagens was obtained.

3. Screening of the UV Mutants

The UV mutants obtained were screened for neutral lipid accumulation under nitrogen-starved photoautotrophic growth condition in 250 mL Erlenmeyer flasks containing 100 mL optimized BG11 medium devoid of nitrogen source. The cultures were incubated at 28 °C in an orbital shaker at 150 rpm, under 20 μE m^{-2} s^{-1} light intensity with a light : dark cycle of 16 : 8 h, and a neutral lipid accumulation was obtained through Nile-red based neutral lipid analysis. The best strain with maximum neutral lipid content was further selected for detailed characterization and further evaluation to understand its production potentials. The screening experiments were conducted in triplicate and the data were represented as mean±standard error.

4. Two Stage High Cell Density Cultivation for Production of Lipid Rich Biomass of the Mutant

A two-stage cultivation process was developed to obtain high cell density in the first stage followed by enrichment of neutral lipid content in the second stage [16]. The experiment was conducted in a 3 L automated photobioreactor (Bioconsole ADI 1010, Applikon, Holland) containing optimized BG11 medium. The bioreactor was operated at 28 °C, agitator speed of 400 rpm, and aeration at 1 vvm with 1% (v/v) CO_2 with initial light intensity of 250 μE m^{-2} s^{-1} was provided from cool fluorescent lamps for a light : dark cycle of 16 : 8 h. The pH of the medium was maintained at 7±0.4 using CO_2. High cell density cultivation of the mutant was achieved in the first stage by intermittent feeding of the nutrients urea and phosphate coupled with stepwise increase in light intensity from 250 to 450 μE m^{-2} s^{-1}. The concentrations of the limiting nutrients urea and phosphate were maintained at levels not less than 90% of their optimal concentration 1.8 g L^{-1} and 0.076 g L^{-1}, respectively, by intermittent feeding after end of every light cycle. The light intensity was maintained at 250 μE m^{-2} s^{-1} for the initial cultivation period up to 112 h, followed by 350 μE m^{-2} s^{-1} till 10th day (232 h) of cultivation, and then a constant light intensity of 450 μE m^{-2} s^{-1} was maintained throughout the fed-batch experiment. Once the stationary phase was reached, the cells were harvested and re-suspended in the nitrogen free (devoid of urea) optimized BG11 medium for lipid enrichment. The mutant strain was evaluated under this condition in terms of biomass, total lipid content and lipid productivity and compared with the wild type strain. Sampling was performed at regular intervals to obtain dynamic profiles of growth, intracellular lipid accumulation and substrates utilization [16].

5. Analysis of Growth, Substrate Utilization and Intracellular Biomass Compositions

A known volume of the broth was centrifuged at 8,000 × *g* for 10 minutes at 4 °C, and the supernatant was collected for analysis of extracellular substrate utilization, while the pellet was utilized for the measurements of growth and intracellular biomass compositions. Cell density was monitored by measuring the absorbance at 690 nm (A_{690}) using a UV-Visible spectrophotometer (Cary 50, Varian, Australia) correlated with the dry cell weight (DCW) using separate correlation equations for mutant and wild type strains. For wild type strain under photoautotrophic nutrient sufficient condition, one cell density corresponded to 0.232 g L^{-1} dry cells (R^2=0.99), and for nutrient starved condition, one cell density corresponded to 0.24 g L^{-1} dry cells (R^2=0.98) [16]. For the mutant strain under photoautotrophic nutrient sufficient condition, one cell density corresponded to 0.241 g L^{-1} dry cells (R^2=0.99), and for nutrient starved condition, one cell density corresponded 0.253 g L^{-1} dry cells (R^2=0.99). Estimation of urea and phosphate in the supernatant involved using DAB (Di-acetyl monoxime, thiosemicarbazide method) as prescribed by [19] and ascorbic acid method [20], respectively. Intracellular chlorophyll contents were estimated

using the method provided by Muthuraj et al. [17]. The total chlorophyll content of the cells was expressed as the sum of chlorophyll *a* and *b*. Dynamic profile of neutral lipid accumulation in the biomass was obtained by Nile-red based assay method [17], and the total FAME along with fatty acid composition was obtained through *in situ* transesterification method prescribed by Kumar et al. [3] and analyzed using gas chromatograph equipped with an flame ionization detector (All the protocols are detailed in supplementary material S1).

6. Enzyme Activity Assays

For analysis of the intracellular enzyme activities, 20 mg (equivalent dry cell weight) of fresh algal cells was collected at each time point and washed twice with Tris-HCl (100 mM) buffer supplemented with ascorbic acid (20 mM) of pH 6.9. The cells were resuspended in the buffer and quickly frozen at −196 °C in liquid nitrogen and stored at −80 °C for enzyme extraction. The cells were thawed and about 15 μL of protease inhibitor cocktail complex was added to the cells before sonication to inhibit the protease activity. For enzyme extraction, the cells were subjected to sonication for 20 minutes at 30% amplitude for cycles of 10 s ON and 10 s OFF at 4 °C. After complete disruption, the cell lysate were collected through centrifugation at 13,000 × *g*, 4 °C for 30 minutes, and the contents were stored for not more than 24 hours. The enzymes AGPase (ADP-gluco-pyrophosphorylase) and ACCase (Acteyl CoA Carboxylase) involved in the first committing step of starch and fatty acid biosynthesis steps, respectively, were chosen for the study along with G3PDH (Glycerol 3-phopshate dehydrogenase) and DGAT (Di-acyl glycerol acyl transferase) involved in the triacyl glycerol (TAG) biosynthesis pathway. The activities of G3PDH, AGPase and ACCase were conducted in the supernatant obtained, while the assay for DGAT was conducted in the microsomes isolated from the pellet debris by ultracentrifugation (Optima TLX Ultracentrifuge, Beckman Coulter, USA) at 80,000 × *g*, 4 °C for 1 hour. All the enzyme activity was measured and represented in terms of specific activity (U mg^{-1} soluble protein); the detailed protocol is in supplementary material S1. The protein content of the crude was obtained through Lowry's method (details in supplementary material S1).

7. Statistical Analysis

All the experiments were conducted in triplicate and the data are expressed as mean±standard error. The significant difference in specific enzyme activities of both the wild type and mutants was analyzed through one-way analysis of variance using Tukey's method.

RESULTS AND DISCUSSION

1. UV Mutagenesis and Screening of the UV Mutants

UV mutagenesis has been widely used as a conventional technique to generate improved strains of plants and microorganisms. The efficiency of UV radiation and its dosage required to induce mutations in the microalga *Chlorella* sp. FC2 IITG was determined in the present study. Exposure of *Chlorella* sp. FC2 IITG cells to UV-C lamp depicted exponential decrease in the survival rate of the cells with linear increase in exposure time as shown in Fig. 1. The number of viable colonies decreased exponentially from

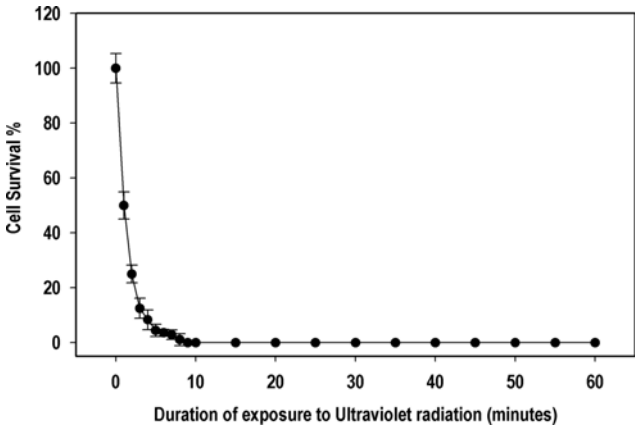

Fig. 1. *Chlorella* sp. FC2 IITG cell survival percentage as dependent on exposure to ultra-violet radiations over a time period up to 60 minutes.

1 to 10 minutes of exposure with a significant increase in the mortality rate from 0 to 100%, respectively, which confirms the lethality of UV radiations on the microalga FC2. Exposure to UV induced mutations in cyanobacteria *Synechocystis* PCC6803 [21] and *Chlorella pyrenoidosa* [22], which supports the present study. High frequency of mutation is usually expected at high mortality rate, and therefore an exposure to 8 minutes with 98% kill was chosen as the minimal exposure time to achieve high number of potential mutants [23]. A total of 36 colonies were obtained from the mutation experiments after three weeks of incubation at 28 °C. About 30 individual colonies were isolated, and all these strains were taken for further screening based on intracellular neutral lipid accumulation. The screening experiments were conducted under shake flask nitrogen starved photoautotrophic conditions to obtain the strains with maximum lipid content and were compared with the wild type strain. Among the 30 strains, five strains exhibited higher neutral lipid content than the control culture with the maximum obtained for FC2-25UV containing 22.26±0.67% (w/w, DCW) of neutral lipid. A significant variation in the neutral lipid content was observed from 0.7 to 22.26% (w/w, DCW), which shows the efficiency of UV radiation to induce mutation in the microalgal strains (Fig. 2). Even though about 30 mutant colonies were obtained, only 16.6% of the total colonies showed increased lipid content as compared to the wild type strain, whereas the remaining strains were undesirable with low lipid content, and many of the strains were unable to survive with repeated sub cultivations. Conventional mutagenic strategies are random and the probability of getting desirable mutants is very minimal. For instance, exposure of *Chlorella minutissima* to 2 M concentration of ethyl methane sulfonate resulted in several colonies; however, only seven colonies were desirable based on their ability to grow faster as compared to the wild type strain. Among them, only three strains could survive the sub culturing protocols [11], which remains the major disadvantage with the conventional mutagenesis. Ultraviolet radiation induces the formation of thymine dimers in the DNA molecules, thereby enabling transition of guanine-cytosine to adenine-thymine or by causing deletion of adenine-thymine base pairs in the DNA [21]. The mutant strain 25UV showed 48.4% increase in

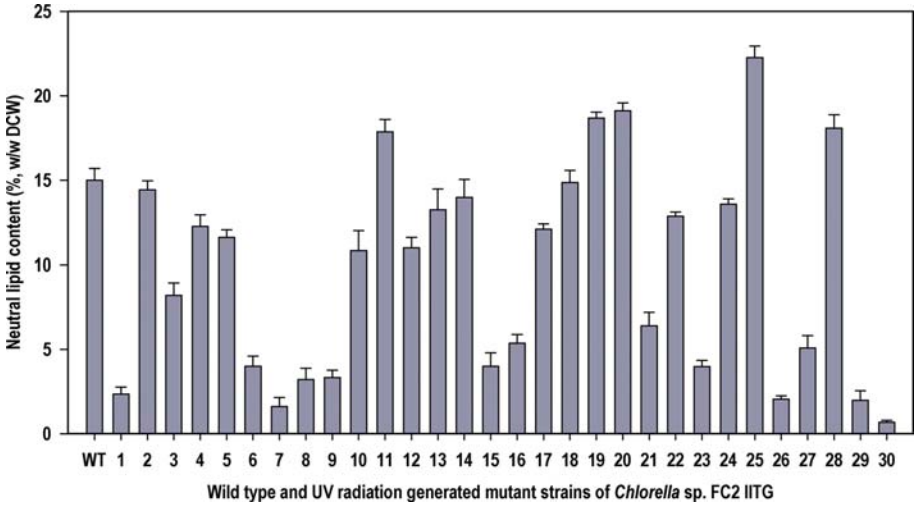

Fig. 2. Screening of 30 UV mutant FC2 strains for enhanced lipid content in comparison to the control wild type culture under nitrogen starved photoautotrophic condition.

neutral lipid content when compared with the wild type culture FC2-WT, and therefore was chosen as the best mutant strain for further characterization and evaluation (Fig. 2). A similar increase in lipid content up to 28% was observed in the mutant strain of *Nannochloropsis* HP-1 obtained through heavy-ion radiation [7].

2. High Cell Density-lipid Rich Cultivation of the Mutant Strain FC2-25UV

The present study targeted enhancing the neutral lipid productivity further via a process engineering strategy which involves high cell density cultivation in the first step followed by lipid enrich-

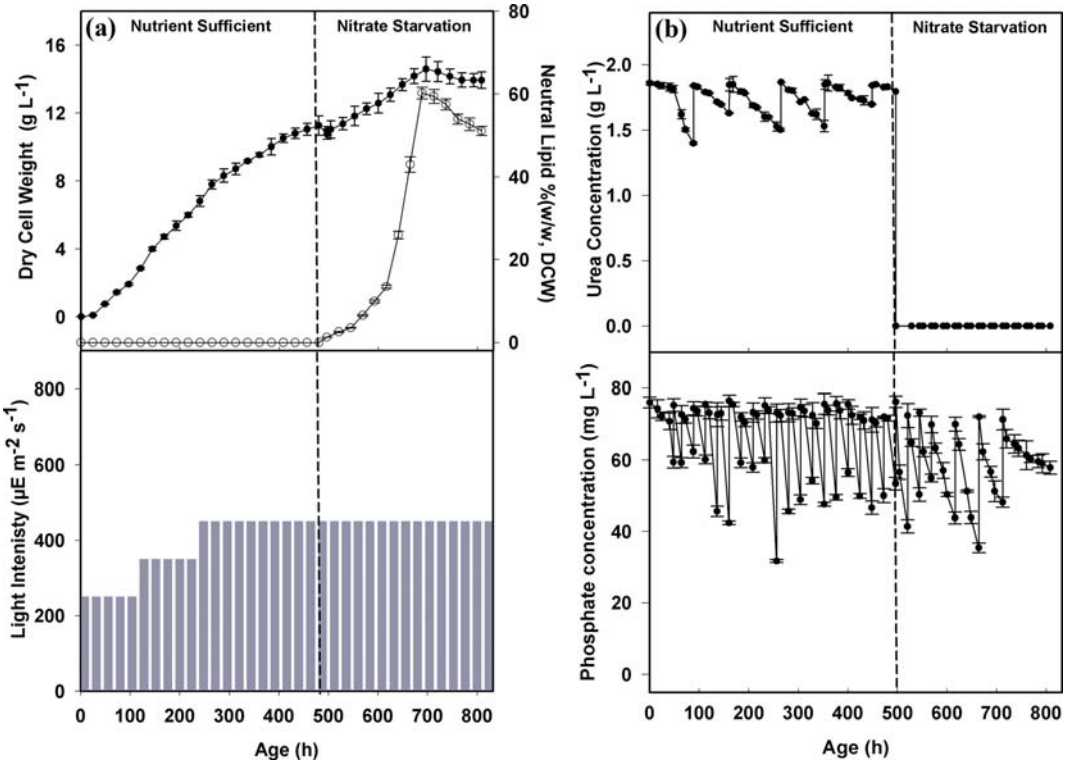

Fig. 3. Dynamic profiles for growth and lipid production of FC2-25UV grown under two stage cultivation mode, which comprises nutrient sufficient growth stage I for high cell density growth of 25UV and nitrogen starved stage II supporting the lipid production. (a) Biomass formation (●), neutral lipid production (○); (b) step-wise increase in light intensity from 250 to 450 µE m^{-2} s^{-1} for a light : dark cycle of 16 : 8 h; (c) intermittent feeding and utilization of urea (●) and (d) intermittent feeding and utilization of phosphate (○). The experiments were conducted in an automated bioreactor of 3.0 L volume at 28 °C, 400 rpm aerated with 1% (v/v) CO_2 and dynamic increase in light intensity from 250 to 450 µE m^{-2} s^{-1} for a light : dark cycle of 16 : 8 h.

ment through nitrogen starvation in the second step. The high cell density cultivation was achieved via dynamic increase in light intensity along with intermittent feeding of the limiting nutrients, while lipid enrichment was achieved in the second stage by re-suspension of the high dense culture in the nitrogen starved media [16]. Similar strategic enhancement of neutral lipid was reported by Karemore et al. [24] with nitrogen limited supply, and the studies in wild type FC2 strain showed substantial increase in neutral lipid content as a response to nutrient starvation [16,17,24]. The substrate utilization profiles along with the intermittent feeding and the dynamic increase in light intensity are depicted in Fig. 3 for the mutant strain FC2-25UV, whereas the profiles for the WT strain are depicted only in Muthuraj et al. [16]. The optimum light intensity for the growth of algal cells varies with the cell density and growth conditions. With a constant light intensity supplied to the reactor, the available light to the cells will decrease with increasing cell density attributed to cell shading effect. Thus, a single optimum light intensity may not be suitable for growth of microalgae under photoautotrophic conditions. Therefore, the light intensity supplied to the reactor needs to be increased simultaneously with increase in the cell density to achieve a sufficient light availability per cell. In the high cell density cultivation, optimum nutritional conditions were maintained via substrate feeding, which in turn resulted in rapid growth of the organism, leading to early crowding of the cells in bioreactor and associated light attenuation. Thus, dynamically increasing the light intensity up to 450 $\mu E\ m^{-2}\ s^{-1}$ did not result in decrement of growth rate, instead supported the growth to the maximum attributed to the increased light availability per cell. The strategy worked well with the FC2-WT as demonstrated in our previous study [16]. Maximum total lipid content of 68% (w/w, DCW) was observed in case of mutant strain FC2-25UV (Fig. 3), which was 21.4% higher than the total lipid content of wild type strain (56% w/w, DCW), a study conducted separately in our laboratory [16]. Maximum neutral lipid content of 60% (w/w, DCW)

Table 1. Comparison of the biomass and lipid productivity of various mutants and wild type strain available in the literatures with the FC2-25UV mutant strain

Strains	Biomass productivity ($mg\ L^{-1}\ day^{-1}$)	Lipid content[a] % (w/w, DCW)	Lipid productivity[a] ($mg\ L^{-1}\ day^{-1}$)	% Increase in lipid content[d]	% Increase in lipid productivity[d]	Reference
Chlorella sp. FC2-25UV mutant[b]	503	68	346	21.43%	11%	Present study
Chlorella sp. FC2-Wild Type	675	56	313			
Nannochloropsis sp. LARB-202-3 mutant[c]	578	46.6	294	No significant difference	22%	[5]
Nannochloropsis sp. LARB-202-2 mutant[c]	517	48.3	271		12.45%	
Nannochloropsis sp. wild type	456	47.4	241			
Nannochloropsis oceanica IMET1 HP-1 mutant[e]	550	NA	271	NA	28.44%	[7]
N. oceanica wild type	520	NA	211			
Nannochloropsis sp. mutant[c]	12.22	50.8	6.20	49.42%	65%	[26]
Nannochloropsis sp. wild type	11.12	34	3.77			
Chlorella pyrenoidosa mutant[c]	492	39.89	196.26	83.15%	86.44%	[27]
C. pyrenoidosa wild type	580	21.78	105.27			
Desmodesmus sp. mutant S5[c]	40.95	48.41	19.82	20%	20%	[28]
Desmodesmus sp. wild type S81	28.15	40.41	16.56			
Desmodesmus sp. mutant G3[c]	38.92	46.01	17.92	29.024%	29.01%	[28]
Desmodesmus sp. wild type G41	32.26	35.66	13.89			
Synechocystis sp. PCC6803 mutant[c]	99.78	32.8	32.72	153%	198%	[29]
Synechocystis sp. PCC6803 wild type	84.43	12.97	10.95			
Chlorella minutissima CM2 mutant[c]	167	32	53.44	19%	32%	[11]
C. minutissima CM5 mutant[c]	220	36	79.2	33.33%	96%	
C. minutissima CM7 mutant[c]	240	42	100.8	56%	149%	
C. minutissima wild type	150	27	40.5			

[a]Represents the total lipid content and total lipid productivity

[b]Represents the mutation by ultra-violet radiations

[c]Represents the mutation by ethyl methane sulfonate

[d]Represents the percentage increase in lipid content & productivity in the mutants with respect to the wild type strain

[e]Represents the mutation by heavy ion radiation; NA-not available

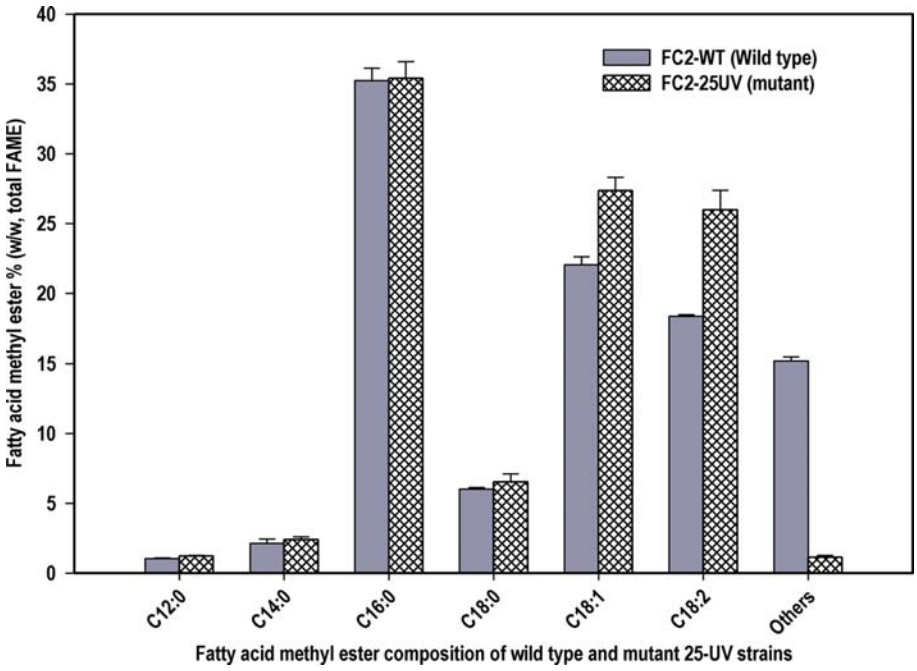

Fig. 4. Percentage composition of fatty acids among the total fatty acid composition for the wild type strain FC2 and the mutant strain FC2-25UV.

was obtained for the mutant strain (Fig. 3(a)), which was 17% higher as compared to the wild type strain with 50% (w/w, DCW). However, the mutant strain FC2-25UV showed maximum biomass titer of 14.59 g L^{-1}, which was 21% less than the wild type FC2 strain (17.73 g L^{-1}). If the total lipid content of the broth is considered (i.e., in terms of g L^{-1}), then the wild type strain showed total lipid titer of 8.86 g L^{-1}, whereas the mutant strain showed 8.75 g L^{-1} of total lipids. However, the maximum total lipid productivity for the mutant strain was found to be ~11% higher (346 mg L^{-1} day^{-1}) when compared with the total lipid productivity of control wild type strain 313 mg L^{-1} day^{-1}. The neutral lipid productivity of the mutant strain was found to be 305.37 mg L^{-1} day^{-1}, whereas the wild type strain showed 286 mg L^{-1} day^{-1}. This signifies that the neutral lipid or the total lipid accumulated in the biomass of the mutant strain was achieved earlier as compared to the wild type strain, thereby reducing the process time. This marginal increase in lipid productivity and marginal variation in the lipid titer in terms of g L^{-1} may be due to the un-optimized growth conditions provided for the mutant strain FC2-25UV, and it is expected to have significant improvement in biomass titer (g L^{-1}) and lipid productivity by optimizing the process parameters for the mutant strain. For instance, the frequency of intermittent phosphate required for the growth of FC2-25UV (Fig. 3(d)) was found to be less when compared with the frequency required for the wild type culture [16]. Optimizing the growth conditions such as light intensity for the mutants of *Nannochloropsis* sp. LARB-202-2 and LARB-202-3 resulted in increased lipid productivity compared to the wild type strain [5]. This supports the requirement of process parameter optimization for the mutant strain to achieve higher biomass productivity, which in turn will improve the lipid titer in the broth and productivity. However, the lipid productivity of the strain FC2-wild type and mutant FC2-25UV was found significantly higher than many other algal wild type strains and mutants characterized for lipid production in the literature (Table 1). Thus, under optimized cultivation conditions and fed-batch operation mode with dynamic increase in light intensity, the strain FC2-25UV could outlast the wild type strain FC2 and many of other algal strains characterized in the literature for biodiesel production. The fatty acid composition of the mutant strain was analyzed using a GC in terms of fatty acid methyl esters. The analysis showed major fractions of C16:0 (palmitate), C18:0 (stearate), C18:1 (oleic acid), C18:2 (linoleic acid), which comprised more than 75% (w/w) of the total fatty acids in the mutant strain with very less significant changes in the composition when compared with the wild type strain (Fig. 4). However, 41.5% and 24% increase in the fractions of C18:2 and C18:1 was found in the mutant strain when compared to the wild type strain, which are considered to be the key elements for suitable biodiesel [16]. Thus, the mutant strain could be a potential candidate for biodiesel production.

3. Mapping Key Specific Enzyme Activity to Understand the Carbon Partitioning Mechanism in the Mutant and Wild Type Strain

To capture the metabolic changes in the mutant strain leading to increased neutral lipid content, the key enzymes involved in the biosynthetic pathways of carbohydrate and lipid metabolism were analyzed for their activity and compared with the wild type strain. The lipid biosynthesis pathway involves three sequential steps categorized as fatty acid synthesis, acyl chain prolongation and triacyl glycerol formation, while carbohydrate biosynthesis involves the polysaccharide biosynthesis from Glucose-6-phopshtae (G6P) node. AGPase and ACCase are the primary enzymes involved in the first committing step towards carbohydrate and fatty acid biosyn-

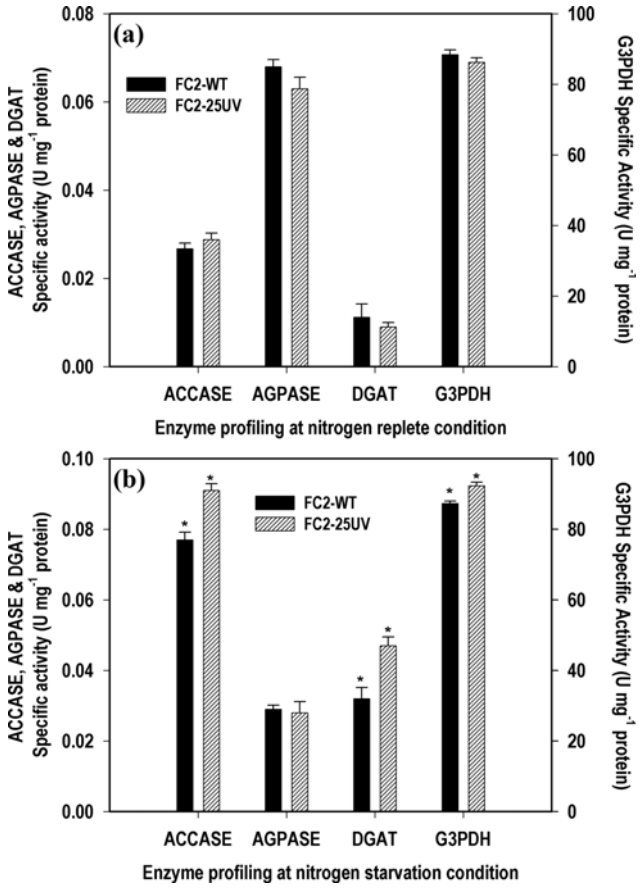

Fig. 5. Profile of specific enzyme activity of the rate limiting enzymes Acetyl CoA carboxylase, ADP-Glucopyrophosphorylase, Diacyl glycerol transferase and Glycerol-3-phosphate dehydrogenase (ACCase, AGPase, DGAT and G3PDH, respectively) involved in carbohydrate and tri-acyl glycerol biosynthetic pathways in both the wild type FC2-WT and FC2-25UV mutant strain. Statistical analysis was used to assess the significant difference in specific enzyme activity of respective enzymes from wild type strain and mutant strain using Tukey's model based one way analysis of variance. The symbol * shows that the specific enzyme activity is significantly different between the wild type and mutant strain with p-value<0.05.

thesis, respectively, whereas the enzymes G3PDH and DGAT are involved in the first and last step of tri-acyl glycerol biosynthetic pathway, respectively. Thus, the carbon partitioning between the fatty acid biosynthesis and carbohydrate biosynthesis was well captured by analyzing the activities of ACCase and AGPase, while the carbon flux towards tri-acyl glycerol biosynthesis was captured via measuring the activity levels of G3PDH and DGAT.

Samples were collected for the enzyme assays from both the wild type strain and FC2-25UV during the nutrient sufficient phase (88 h of cultivation) supporting high cell density cultivation and the nitrogen starved phase (688 h of cultivation) supporting the lipid enrichment of the two stage fed batch experiment. No significant variation in the enzyme activity was observed between the wild type strain and FC2-25UV mutant strain during the growth supporting nutrient sufficient phase (Fig. 5). However, a significant increment in the ACCase activity up to 18% was observed in the mutant strain when compared to the wild type strain during the nutrient starvation phase (Fig. 5). Similarly, a 31.25% increase in DGAT activity of the mutant strain was observed when compared to the wild type strain, which may be the reason for increased neutral lipid accumulation and total lipid productivity in the mutant strain. DGAT is the terminal enzyme that supports the formation of tri-acylglycerol, the precursor for biodiesel production [2]. Thus, increased activity of DGAT during the starvation phase will directly support the generation of TAG, thereby augmenting biodiesel production, which is a significant achievement in the present study. Several metabolic engineering reports have also identified overexpression of ACCase, DGAT and G3PDH as an approach to enhance neutral lipid accumulation in microalgal strains [29,30]. The other enzymes AGPase and G3PDH did not show any significant variation in their activity among the wild type and mutant strain (Fig. 5).

Comparison of the specific enzyme activities revealed decreased activity of AGPase with concomitant increase in ACCase, DGAT and G3PDH under nitrogen starved condition as compared to nutrient sufficient condition in both the strains. Under nitrogen starved conditions, the organism experiences stress and therefore switches its metabolism towards accumulation of high energy compounds such as neutral lipids instead of carbohydrates. This redirection of carbon flux from carbohydrate biosynthesis to neutral lipids is observed with concomitant reduction in AGPase activity under nitrogen starved condition along with increased activity of ACCase, DGAT and G3PDH involved in the neutral lipid biosynthesis. For instance, about three-fold increment in the activity of ACCase was evidenced in both the WT and FC2-25 UV strains under nitrogen starved condition as compared to nitrogen sufficient condition. *Nannochloropsis oculata* cultivated under nitrogen starvation with high light density showed 3 folds increment in the ACCase activity as compared to control nutrient sufficient conditions [31]. Similar increment in the ACCase activity and expression was evidenced in *Monoraphidium* sp. under fluvic acid treatment [32]. Expression studies conducted in *Chlorella pyrenoidosa* also revealed thirty-fold increased expression of *accD* gene which forms the major subunit of ACCase [33]. In addition to that, about 3- to 5.22-fold increment in the DGAT activity was evidenced in both the strains under nitrogen starved condition as compared to the nutrient sufficient condition. Increased expression of DGAT genes under nitrogen starved conditions and their role in augmenting neutral lipid biosynthesis have been reported in numerous studies [31,32] and up to seven-fold increment in DGAT activity was witnessed in *Nannochloropsis oculata* under nitrogen deprived conditions [31]. AGPase activity showed ~2.3-fold decrement under nitrogen starved condition depicting the redirection of carbon fluxes from carbohydrate biosynthesis towards neutral lipid biosynthesis, which is also supported by Radakovits et al. [30]. Glycerol-3-phosphate dehydrogenase involved in the formation of glycerol from dihydroxy acetone phosphate showed increased activity under nitrogen starved condition only in the FC2-25UV mutant strain. Recent studies on the expression of G3PDH in *Chlamydomonas reinhardtii* showed increased accumulation of G3PDH under nitrogen deprivation [34]. In contrast, the wild type strain did not show significant increment in the G3PDH activity, which may be a reason for reduced and delayed neutral lipid accumulation in the

wild type strain as compared to the mutant strain. Thus, the mutant strain was able to contribute maximum carbon flux towards the lipid biosynthesis rather than the biomass formation during the starvation phase, as observed from the increased activity of enzymes involved in lipid and TAG biosynthesis. Therefore, these strains, both the wild type and the mutants, can be the potential candidates for large scale biodiesel production.

CONCLUSIONS

This study once again confirms conventional UV mutagenesis as an effective means to get desired traits of microalgae. The FC2-25UV mutant strain exhibited ~18% higher neutral lipid content in terms of % (w/w, DCW) as compared to the wild type in the strategic process designed for high cell density lipid rich cultivation under photoautotrophic condition. Maximum lipid productivity of 346 mg L^{-1} day^{-1} was obtained for the mutant strain, which was higher than other microalgal mutants characterized so far. Enzymatic assays showed increased specific activity of ACCase, G3PDH and DGAT in mutant as a reason for increased lipid content, thus confirming the potential of the mutant developed.

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ETHICAL STANDARDS

The experiments comply with the current laws of the country in which they are performed.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

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Supporting Information

Enhanced lipid content in *Chlorella* sp. FC2 IITG via high energy irradiation mutagenesis

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MATERIALS AND METHODS

1. Analysis of Growth

Cell density was monitored by measuring the absorbance at 690 nm (A_{690}) using a UV-Visible spectrophotometer (Cary 50, Varian, Australia). A known volume of algal biomass from the broth was collected and centrifuged at 8,000 × *g*, 4 °C for 10 minutes followed by washing with normal saline (0.8% w/v sodium chloride) to make the biomass free from media components. The obtained cell pellet was further subjected to drying at a temperature of 60 °C till a constant weight was reached and the dry cell weight was obtained gravimetrically. The absorbance values were converted in to dry cell weight (DCW) through appropriate calibration equations.

The biomass productivity (P_B, mg L^{-1} day^{-1}) under different cultivation conditions were calculated based on the following equation

$$P_B = \frac{X_f - X_0}{t_f - t_0} \quad \text{(A1)}$$

where, X_0 and X_f were the dry cell weight (g L^{-1}) obtained at initial (t_0) and final (t_f) time points (in days) respectively. Specific growth rate of the cells was calculated based on the following equation

$$\mu = \frac{\mathrm{Ln}(X_2/X_1)}{(t_2 - t_1)} \quad \text{(A2)}$$

where, X_1 and X_2 were the dry cell weight (g L^{-1}) obtained at initial (t_1) and final (t_2) time points (in days) respectively.

2. Analysis of Urea Utilization

The urea concentration in the medium was estimated using diacetyl monoxime method as prescribed by Wybenga et al. [1]. The method uses 1 : 1 : 1 mixture of mixed acid reagent (containing sulfuric acid, ferric chloride and orthophosphoric acid), mixed colour reagent (containing 2% w/v diacetyl monoxime and 0.5% w/v thiosemicarbazide) and distilled water as coloring reagent which on reaction with urea at 100 °C forms a pink color product. The reagents are prepared freshly before the assay and a maximum of 0.1 mL sample was used in the analysis. The absorbance of the pink colored product was measured at 540 nm. One absorbance at 540 nm corresponds to 1.876 g L^{-1} of urea.

3. Analysis of Phosphate Utilization

Phosphate estimation was carried out using ascorbic acid method with potassium hydrogen phosphate (dibasic) as standard [2]. Combined reagent (0.32 mL) comprising (5 N) sulfuric acid, (0.018 M) antimony potassium tartrate, (0.102 M) ammonium molybdate and (0.1 M) ascorbic acid was used for estimating the phosphate content in the supernatant of 2.0 mL. The absorbance was read at 880 nm after incubation for 10 minutes at room temperature and the correlation between phosphate concentration vs corresponding absorbance (One A_{880} corresponds to 8 mg L^{-1} of phosphate).

4. Analysis of Intracellular Chlorophyll Formation

The chlorophyll estimation was carried out using the method provided by Pruvost et al. [3] which uses 100% methanol for extraction at 45 °C. An absorbance scan of wavelength from 400 to 800 nm was performed and the following equations given by Ritchie [4] for organisms containing chlorophyll *a* and chlorophyll *b* were used for quantification. Total chlorophyll content of the cells was expressed as the sum of chlorophyll *a* and *b*.

$$\text{Chlorophyll } a \text{ (mg L}^{-1}) = (16.52 \times [A_{665} - A_{750}]) - (8.09 \times [A_{652} - A_{750}]) \quad \text{(A3)}$$

$$\text{Chlorophyll } b \text{ (mg L}^{-1}) = (27.44 \times [A_{652} - A_{750}]) - (12.17 \times [A_{665} - A_{750}]) \quad \text{(A4)}$$

5. Analysis of Intracellular Neutral Lipid Accumulation

For Nile-red based neutral lipid analysis, cell pellet with absorbance 0.7 re-suspended in 1.0 mL of 25% (v/v) dimethyl sulfoxide was used. Nile red was added to the re-suspended pellets at the concentration of 4 μg mL^{-1} and incubated at 50 °C in a water bath for one minute. The fluorescence spectra was obtained in a spectrophotometer (Fluoromax 3, Horiba, USA) with excitation at 480 nm and emission in the region 550 to 650 nm. The auto-fluorescence of algal cells and the intrinsic fluorescence of Nile red were subtracted from the fluorescence of Nile red neutral lipid complex obtained at 580 nm. Triolein (Supelco, USA) was used as standard for Nile-red based neutral lipid estimation.

6. Analysis of Fatty Acids Methyl Esters (FAME) Derived from Microalgae

FAME analysis was carried out for the freeze dried algal cell mass harvested at the end of the batch. A sequential two step direct transesterification method was employed which involves alkali catalyst in first step and acid catalyst in second step. In the first step of direct transesterification, 1.0 mL of 2% (w/v) NaOH in methanol was added to 30 mg lyophilized biomass followed by incubation at 90 °C in a shaking water bath at 150 rpm for 20 minutes. In

the second step, 1.0 mL 5% (v/v) H_2SO_4 in methanol was added to the mixture and was further incubated at the same conditions for 20 minutes [5]. The reaction mixtures after direct transesterification were cooled down to room temperature after completion of first and second incubation. Deionized water and hexane of equal volume (1.0 mL) were added to cooled transesterified mixture to obtain the FAME in hexane layer. The hexane layer was washed twice with water to remove any aqueous impurities and used directly for GC analysis after filtration through 0.2 μm filter. FAME was analyzed directly in GC equipped with flame ionization detector (GC-FID, Varian 450, Netherlands) and SLB-IL100 column (30 m×0.25 mm i.d., 0.20 μm film thickness). For GC analysis, nitrogen at a constant flow rate of 0.4 m s^{-1} was used as the carrier gas, split ratio of 1 : 20, oven temperature 140 °C (5 min) followed by ramping at a rate of 3 °C min^{-1} till 220 °C followed by 5 minutes holding. The injector and detector temperature was kept at 250 °C and the injection volume of 1 μL was used for analysis. FAME mix C14-C22 (Supelco, USA) was used as the standard for GC-FID and the lipid quantified using this method re- presents the total lipid of the biomass in % (w/w, DCW). The lipid productivity (P_L, mg L^{-1} day^{-1}) was calculated as follows:

$$P_L = L_c \times P_B \quad \text{(A5)}$$

where, L_c represents the total lipid content determined through FAME analysis or the neutral lipid content of the organism in % (w/w, DCW) determined through Nile-red based assay method.

7. Enzymatic Assays

7-1. AGPase Activity Assay

ADP-gluco-pyrophosphorylase catalyzes the first committing step in carbohydrate biosynthesis which involves the formation of ADP-glucose (ADP-glc) and pyrophosphate (PPi) from glucose-1-phopsate and ATP. AGPase activity was measured by the method proposed by Fusari et al. [6]. The PPi released in the AGPase reaction is further broken down to inorganic phosphate by the activity of pyrophosphatase enzyme which was quantified to obtain the activity of the AGPase enzyme. Thus, one unit (U) of AGPase represents the amount of AGPase required to produce 1 μmol of PPi in one minute at 28 °C. The reaction was carried out in mixture containing 50 mM Hepes (pH 8.0), 7 mM $MgCl_2$, 0.5 mM glucose-1-phosphate, 1.5 mM ATP, 0.0015 U mL^{-1} pyrophosphatase, 0.2 g L^{-1} Bovine serum albumin (BSA) and enzyme extract of 20 μL in a total volume of 200 μL for 10 minutes at 28 °C. The reaction was terminated by addition of malachite green color reagent and the absorbance of the mixture was read at 650 nm. A standard was set along with every assay containing known concentration of sodium pyrophosphate. Appropriate blanks were taken in the absence of substrates along with enzyme extract to nullify any interferences in the crude.

7-2. ACCase Activity Assay

A discontinuous spectrophotometric assay developed by Willis et al. [7] was employed to measure the activity of Acetyl-CoA carboxylase which involves two steps. In the first step, Acetyl-CoA was converted in to malonyl-CoA and the remaining acetyl-CoA in the reaction mixture was allowed to react with oxaloacetate to form citrate and CoA-SH. The formed CoA-SH was read by reaction with DTNB (di-thio-bis-nitrobenzoic acid) to form a colored product which was read at 412 nm in a UV-Visible spectrophotometer. One unit of ACCase activity is defined as the amount of enzyme required to consume 1 μmol of acetyl-CoA per minute at 28 °C. Units of specific enzyme activity (U mg^{-1}) are expressed as micromoles per minute per milligram of protein. The 20 μL crude enzyme extract was added to an assay mixture containing 1 mM acetyl-CoA, 15 mM potassium bicarbonate, 5 mM $MnCl_2$ and 5 mM ATP, 20 μL of BSA 1 g L^{-1}, 20 μL biotin (3 g L^{-1}) in 100 mM phosphate buffer at pH 8. Aliquots were removed every 2 minutes for a time period of 10 minutes and the reactions were stopped by the addition of 10% (w/v) trifluoroacetic acid. The level of acetyl-CoA remaining in each aliquot was determined via a citrate synthase assay, in which the formation of the yellow compound acidthiophenolate was followed spectrophotometrically at 412 nm. A standard curve was constructed every time by measuring the change in absorbance of citrate synthase reaction containing defined amounts of acetyl-CoA instead of supernatant and graphing the change in absorbance *vs.* concentration of acetyl-CoA.

7-3. DGAT Activity Assay

In this fluorescence-based DGAT Assay, activity was measured by monitoring the released CoA-SH from a DGAT mediated reaction [8]. 7-Diethylamino-3-(4-maleimidophenyl)- 4-methylcoumarin (CPM), a coumarin derivative reacts with the sulfhydryl (-SH) group of CoA-SH and forms a highly fluorescent product that was readily detected with excitation and emission wavelengths of 390 and 469 nm, respectively. The assay condition consist of 0.25 μg of total protein, 50 mM HEPES, pH 7.5, 1% Triton X-100, 10% DMSO, 312.5 μM oleoyl-CoA, and 625 μM 1, 2-Di Oleyl Glycerol. The reaction was initiated by the addition of enzyme extract and carried out for 30 min at room temperature (28 °C). The reaction was stopped by SDS at a final concentration of 0.1% (w/v) and incubated for an additional 30 minutes. Then one-tenth volume of CPM at 1 mM was added to the reaction, and the plate was incubated at room temperature for another 30 minutes followed by detection of fluorescent signal (excitation at 390 nm; emission at 469 nm). Appropriate blanks were taken for individual samples comprising of all components along with enzyme extract in the absence of substrates (1, 2-Di Oleyl Glycerol and oleoyl-CoA). A standard curve of CoA-SH (ranging from 0 to 100 μM CoA-SH) was generated together with assay to calculate the amount of CoA-SH produced in the DGAT reaction. One unit of DGAT activity is defined as the amount which will catalyze the transformation of 1 nano mole of the DAG (diacylglycerol) per minute. Units of specific enzyme activity (U mg^{-1}) are expressed as nano moles per minute per milligram of soluble protein.

7-4. G3PDH Activity Assay

G3PDH activity was determined by measuring the changes in absorbance at 340 nm, 28 °C due to NAD+ formation from NADH using a spectrophotometer [9]. The reaction mixture of 300 μL contained pH 6.9 buffer solution (33.3 mM Hepes, Tricine and MES), 0.2 mM NADH, 1 mM dihydroxy acetone phosphate (DHAP) and enzyme extract. Appropriate blanks were taken for individual samples comprising of all components along with enzyme extract but absence of the substrate (DHAP). A standard curve of NADH (ranging from 0 to 100 μM CoA-SH) was generated together with assay to calculate the amount of NAD+ produced in the G3PDH

reaction. G3PDH activity (U) is defined as the amount of enzyme that caused per micromoles NADH oxidation or per micromoles NAD+ reduction per minute.

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