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High carbon (CO_2) supply leads to elevated intracellular acetyl CoA levels and increased lipid accumulation in *Chlorella vulgaris*

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ABSTRACT

Carbon dioxide, the sole carbon source in phototrophic cultivation of microalgae, is the limiting factor for photosynthesis due to its low concentration in the atmosphere (0.04% v/v). We postulate that exposure to increased CO_2 concentrations can increase the steady-state specific intracellular levels of carbon metabolic intermediates, including, the specific intracellular (si) levels of the precursor for fatty acid synthesis (acetyl CoA (AcCoA)), which in turn, appears to improve lipid accumulation. The effects of higher CO_2 concentrations on *Chlorella vulgaris* were studied. At 2.6% v/v CO_2, a 6-fold increase in volumetric lipid production was achieved. A 5.4-fold increase in the specific intracellular neutral lipid level (si-NL) from 9.6 to 52.3 mg triolein (TO)/g biomass was also obtained. Si-AcCoA was significantly elevated at 2.6% CO_2, and showed a 41, 25 and 27% increase over the air-sparged controls in the lag, log and stationary phases, respectively. Further, we show a quantitative empirical relationship between si-AcCoA and si-NL, which provides a new outlook for the design of strategies to improve lipid accumulation. In addition, favorable biodiesel characteristics were also obtained at higher CO_2. Increased si-AcCoA with increase in CO_2 concentrations, a related increase in lipid levels, and a quantitative empirical relationship between them have not been reported in the literature thus far.

1. Introduction

Increased lipid accumulation by application of environmental stresses is the most common strategy for increased lipid production by microalgae. The use of elevated temperature, light intensity, pH, metals, salinity and nutrient depletion to induce high lipid accumulation have been widely studied [1,2]. However, relatively few studies exist on increasing the carbon source, viz., carbon dioxide for lipid accumulation [3].

The primary advantage of using microalgae is its phototrophic mode of cultivation — its ability to use freely available sunlight to fix CO_2 from the atmosphere for photosynthesis. However CO_2 is known to be the limiting factor for photosynthesis due to its low atmospheric concentrations of 0.03–0.04% [3]. In addition, the affinity of the primary carbon fixing enzyme, Rubisco, for CO_2 is low [4]. Rubisco is one of the slower enzymes in nature [5] and has poor catalytic carboxylase activity [6]. Therefore, the low CO_2 concentration does not saturate Rubisco [7]. Further, unlike terrestrial plants that absorb the gas directly from the atmosphere, microalgae that normally grow in aqueous media, rely on CO_2 dissolved in the media. The availability of CO_2 is thus further reduced due to the low solubility of the gas in water and a 10,000 fold slower diffusion rate than in the atmosphere [7]. Thus, increasing the partial pressure of CO_2 during micro-algal cultivation would lead to its increased solubility and greater availability for photosynthesis, which in turn, could result in higher carbon fixation.

More importantly, carbon supply could be limiting for lipid synthesis. Lipid accumulation increases under stress when the cell is unable to utilize carbon for its otherwise regular activities such as growth [8]. The excess carbon available when stress inhibits growth is converted to lipids and is stored as energy deposits (lipid droplets) to enable cell survival [8]. Thus, an increase in carbon supply can be expected to increase the excess carbon available for TAG synthesis. This probably explains the lack of success of the TAG biosynthesis genes upregulation strategies to improve lipid accumulation [9–12] while the strategy of supplementation of acetate, which can be directly converted to acetyl CoA (AcCoA) — the precursor for fatty acid synthesis, resulted in increased oil production [13].

We propose that an increase in the C-source supply would increase the carbon metabolic intermediate (e.g. AcCoA) levels, and consequently improve the synthesis of oil (neutral lipid) precursors and the accumulation of oil. We aim to increase the C-source supply through increased CO_2 concentrations.

This study focuses on the effects of increased CO_2 concentrations on the growth and oil accumulation in the microalgae, *Chlorella vulgaris*. The composition of the lipid accumulated was analyzed and the potential biodiesel quality determined by empirical correlations. To ensure portability of data across laboratories the relevant values are presented as specific intracellular (si) levels, e.g. si-AcCoA, si-neutral lipid (si-NL), etc. Measurement of AcCoA, a central metabolite in carbon metabolism and a precursor for fatty acid synthesis, was carried out in the culture with the highest neutral lipid accumulation to understand improvement in carbon availability and its correlation with oil accumulation.

Abbreviations: si, specific intracellular; NL, neutral lipid; AcCoA, acetyl CoA

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2. Materials and methods

2.1. Microalgae and its cultivation

Chlorella vulgaris NIOT5, a marine microalga, obtained from the National Institute of Ocean Technology, Chennai, India was used as the model organism. The microalga was cultivated in 250 ml Guillard and Ryther's f/2 medium under conditions of 25 °C, 100 rpm, 1200 lx and an aeration rate of 1 vvm. An illumination regime of 12 h light and 12 h dark was used. Media were inoculated with mid log phase cells to obtain an initial cell concentration of 10^6 cells/ml.

2.2. Cultivation set up for provision of increased CO_2 concentrations

The cultures sparged with air (0.04% CO_2) at 1 vvm were treated as the controls. The influence of increasing concentrations of carbon dioxide was studied by subjecting the cultures to air enriched with carbon dioxide such that the final CO_2 concentrations were 1.3%, 2.6%, 3.9%, 5.2% and 7.8%. The CO_2 concentrations were chosen to be around 5%, which is known to yield maximum growth [14]. High pure CO_2 from a commercial cylinder was used to enrich the air with the gas. Air and CO_2 flowing at predetermined rates were mixed in a small chamber and fed through a humidifier to the culture. Humidification was necessary to prevent loss of media over the cultivation period. The higher CO_2 was supplied only during the light phase. Culture period was limited to 15 days due to time constraints. Measurement of medium pH was carried out daily in the middle of the light phase, i.e., following 6 h of excess carbon supply. Measurements were made using a bench-top pH meter from Mettler Toledo (FEP20).

A separate study was also conducted where the pH of f/2 medium was brought down to 6.81 ± 0.14 using HCl. The cultures at pH 8.1 ± 0.18 (the inherent pH of f/2 medium) were treated as the controls. Both sets of cultures were sparged with air (0.04% CO_2) at 1 vvm.

2.3. Biomass growth

Daily cell counts were recorded using an improved Neubauer's chamber to monitor the growth of the microalgae, and the corresponding cell dry mass measured.

2.4. Measurement of neutral lipid accumulation

Neutral lipid accumulation in the cells was quantified using the fluorescent dye Nile red as previously described, with minor modifications [15]. Pretreatment of micro-algal cells with DMSO was carried out prior to the assay in order to permeabilize the cell. Several concentrations of DMSO viz. 25, 50, 75 and 100% were tested. The optimal cell concentration for the assay was found to be 10^6 cells/ml. Therefore, the cell concentrations were normalized to 10^6 cells/ml for the assay, and the measured values were expressed as specific intracellular neutral lipid (si-NL). Fluorescent intensity measured in a fluorescence spectrophotometer, (LS 55, Perkin Elmer) was calibrated against a standard triglyceride, tri-olein (TO), from Sigma-Aldrich.

2.5. Biomass composition

For all cultures, carbohydrate and protein estimations were carried out on the day of harvest (day 15). Cell pellets obtained after harvesting were washed with phosphate buffer, pH 7, followed by distilled water to remove any salts and traces of media. Pellets were then lyophilized for further quantifications. The total lyophilized biomass obtained was measured. 1 mg of each of the lyophilized pellets was then suspended in phosphate buffer for the analyses. The carbohydrate concentration in the suspension was estimated through the phenol sulfuric acid method [16]. For protein estimation, the suspension was sonicated to disrupt the cells and facilitate protein release. Sonication was carried out on ice using QSonica (Q700) for a total on time of 4 min with pulses of 10 s on and 10 s off. The lysates were centrifuged and the supernatant used for protein estimation according to Bradford [17]. Absorbance was measured using a UV–Vis spectrophotometer (V 630, JASCO, Tokyo, Japan) and calibrated against glucose and BSA standards to deduce the carbohydrate and protein contents, respectively.

2.6. Extraction and analysis of acetyl CoA

Si-AcCoA levels of the controls and the highest si-NL yielding CO_2 treated cultures (2.6% CO_2) were measured and compared. Si-AcCoA levels on days 2, 9 and 15 that correspond to the lag, log and stationary phase of growth respectively, were measured. Si-AcCoA measurements were carried out with the 'Acetyl-Coenzyme A Assay Kit' (Catalog Number MAK039) from Sigma Aldrich. Extraction of the metabolite was carried out as per the instruction on the kit with minor modifications based on optimization studies for the microalgae. Culture volume corresponding to 10^9 cells were harvested. The pellet was suspended in 500 μl of ice-cold 1 N perchloric acid and sonicated as described in Section 2.5. The homogenate was then centrifuged at 10,000*g* for 5 min. The supernatant was neutralized with 3 M $KHCO_3$, incubated on ice for 5 min and centrifuged at 10,000*g* to obtain the supernatant, which was then used for AcCoA analysis. The value so obtained was designated as specific intracellular acetyl CoA (si-AcCoA).

2.7. Trans-esterification and fatty acid profiling using GC–MS

Fatty acid trans-esterification and analysis of the fatty acid methyl esters (FAMEs) were carried out according to Balan and Suraishkumar [18]. FAME analysis was conducted using a GC–MS (Clarus 600/600S) instrument from Perkin Elmer.

2.8. Replication and statistical analysis

All cultivations and measurements were carried out in triplicates and values have been expressed/plotted as average ± standard deviation. The experiments were repeated later to confirm reproducibility of results. One way ANOVA and Tukey's multiple comparisons test were performed using Minitab 16. Level of significance used was 0.05 for all tests.

3. Results and discussion

3.1. Effect of increased CO_2 on growth rates

Cell concentrations were plotted against time to obtain the growth curve shown in Fig. 1. The figure shows that the growth profiles of all cultures are comparable. To better quantify the effect of increasing concentrations of CO_2 on the growth of microalgae, the logarithmic specific growth rates for the cultures were calculated.

In the exponential growth phase where cells actively multiply, growth follows a first order rate equation [19] that can be represented by

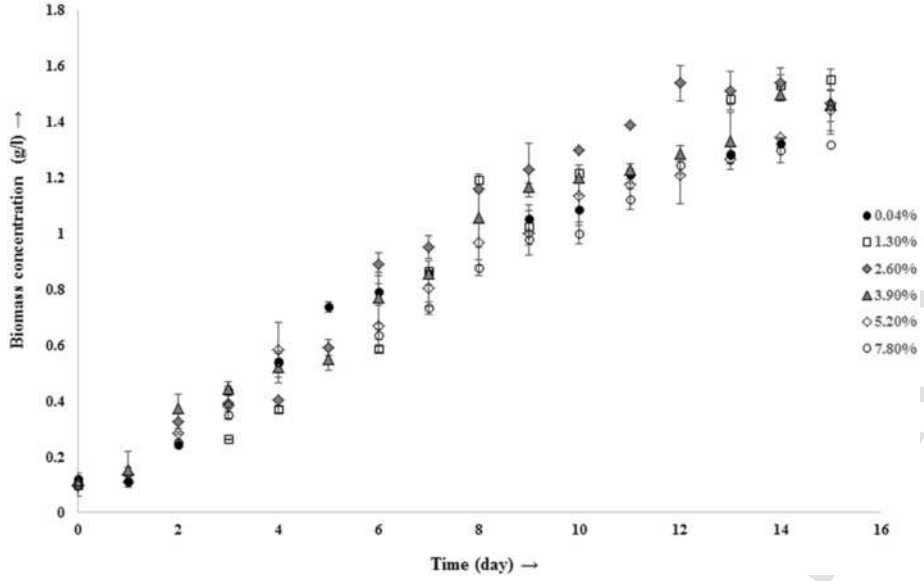

Fig. 1. Growth curves for *Chlorella vulgaris* cultivated under different CO_2 concentrations for 15 days. The cultures were sparged with 0.04% (control), 1.3%, 2.6%, 3.9%, 5.2% and 7.8% CO_2. The maximum specific growth rates of the different cultivations, calculated along the logarithmic phase, were comparable to control. Data points are the mean values obtained from triplicate experiments. The error bars represent ± 1 standard deviation (SD) of the corresponding data point.

$$\frac{dx}{dt} = \mu x \tag{1}$$

where x is the cell concentration (g/l) and μ the specific growth rate (per day). Upon integration, we obtain the equation

$$x = x_o e^{\mu t} \tag{2}$$

where x and x_o correspond to the cell concentrations at time t and zero respectively.

Thus, taking natural logarithm on both sides,

$$\ln x = \ln x_0 + \mu t \tag{3}$$

To obtain the logarithmic specific growth rate, natural logarithm of the cell concentration was plotted against time. The slope across the logarithmic phase (linear portion of the curve) corresponds to μ. Since the specific growth rate is highest in the logarithmic phase, the value obtained was termed the maximum specific growth rate for the particular culture (μ_{max}). The specific growth rate estimation is included in the supplementary file (Fig. S1(a)–(f)).

The maximum specific growth rate was 0.22 ± 0.01, 0.26 ± 0.01, 0.23 ± 0.01, 0.21 ± 0.01 and 0.2 ± 0.01 day^{-1} for the cultures treated with 0.04, 1.3, 2.6, 3.9, 5.2 and 7.8% CO_2, respectively. Contrary to previous studies in the literature [20], these μ_{max} values indicate negligible effects of increased CO_2 supply on the microalgal growth. Statistical analysis revealed no significant differences between the growth rates. A comparison of the dry cell mass obtained on day 15 at different CO_2 concentrations is shown in Table S1 of the supplementary file. The values indicate that the cells are slightly heavier or larger when sparged with 1.3%–5.2% CO_2. This observation was in agreement with the results obtained by Gardner et al.; the size of cells sparged with 5% CO_2 was larger than those sparged with atmospheric CO_2 concentration [21]. Cell mass at 7.8% CO_2 however, was much lower than the controls indicating a comparatively smaller cell size.

The pH of inoculated medium was around 7.5. CO_2 was sparged only during the light phase and it caused a decrease in medium pH. The pH reached a stable value within 45 min after the start of CO_2 supply. Cessation of the CO_2 supply allowed the medium pH to increase back to values comparable to the control culture media. Stable pH values were observed 45 min into the dark phase. Fig. S2 in the supplementary file traces the medium pH across the culture period. These values refer to the measurements taken in the middle of the light phase, i.e. after six hours of CO_2 supply. The value on day zero, however, corresponds to the pH measured immediately following inoculation and prior to CO_2 supply. pH increased from 7.5 to 8 in the controls as the culture growth advanced. This has been commonly observed during micro algal culture and has been attributed to the loss of CO_2 from the medium [22]; CO_2 uptake by the cells occurred at a rate faster than it could be supplied into medium, which resulted in an increase in medium pH [22]. However, the cultures treated with CO_2 seemed to maintain a stable pH in the light phase throughout the duration of the culture. On day 15, the medium pH was 8.07 ± 0.16, 6.89 ± 0.005, 6.81 ± 0.04, 6.52 ± 0.07, 6.37 ± 0.01, 6.25 ± 0.02 in cultures sparged with 0.04. 1.3, 2.6, 3.9, 5.2 and 7.8% CO_2 respectively. *Chlorella vulgaris* is known to withstand a wide range of pH ranging from 3 to 9 [23,24]. However, optimal growth has been reported between values 6.5 to 8 [25]. Both acidic and alkaline pH are known to retard growth [24]. A change in external pH leads to a change in the pH gradient between the cell and the surrounding medium. Cell survival would be dependent on its ability to cope with this new pH gradient [25]. *Chlorella vulgaris* is said to have a good carbon concentrating mechanism that allows it to withstand such a wide pH range [26]. This mechanism probably explains the lack of a significant change in μ_{max} despite a decrease in the medium pH.

3.2. Si-neutral lipid increased up to 540% of the controls

The si-NL content was measured across the cultures using the optimized Nile Red assay. Pretreatment of cells with 100% DMSO yielded the best results for lipid extraction among the tested DMSO

concentrations, and hence it was used for all further measurements. Si-NL was expressed in equivalents of mg tri-olein (TO) since the fluorescent values were calibrated against the triglyceride tri-olein. The values correspond to 1 g of biomass and are thus 'specific' intracellular values. Si-NL for all cultures were plotted against time in Fig. 2. Elevated CO_2 supply significantly improved lipid accumulation at all the CO_2 concentrations studied, as well as led to an earlier onset of lipid accumulation than the controls. Neutral lipid accumulation was observed from the mid log phase (days 4–5) in CO_2 treated cultures but only in the late log phase/early stationary phase (day 10) in control. Neutral lipid accumulation in the 7.8% CO_2 treatments was not appreciable until day 8. The highest amount of accumulation was observed with the use of 2.6% CO_2. At all concentrations of CO_2, lipid accumulation was found to continuously increase during the duration of the cultivation. To clearly highlight the dramatic improvement and early accumulation, the si-NL content in all cultures, as measured on day 10, is represented in Fig. 3. Comparison of values on day 10 was carried out since appreciable accumulation in the controls was noticed only from day 10 onwards. Further, it indicated the early accumulation in the CO_2 treated cultures. Lipid accumulation in the culture sparged with 2.6% CO_2 reached 52.3 mg TO/g biomass compared to 9.6 mg TO/g biomass in the controls, a 5.4-fold increase in lipid accumulation. Si-NL levels increased to > 4.5 times that of the controls in the cultures sparged with 1.3% and 3.9% CO_2, where the levels achieved were 43.7 and 45 mg TO/g biomass, respectively. Treatment with 5.2% CO_2 also led to over a three-fold increase. Fig. 3 clearly shows that the lipid accumulation achieved with the use of high CO_2 supply was several-fold higher than the control. Although the 'relative benefit' of increasing the CO_2 content to 7.8% was reduced as compared to 2.6%, a 140% increase in si-NL compared to control was still achieved. Lower si-NL in the cultures sparged with higher CO_2 concentrations as compared to 2.6% CO_2 may be a result of the decreased medium pH. As discussed in Section 3.1, the desirable pH for optimal growth of *Chlorella* is 6.5 to 8. Use of the higher CO_2 concentrations (5.2 and 7.8%) led to decreases in pH beyond this desirable range. Although *Chlorella* spp. are relatively acid tolerant, this may have interfered with the microalgae's optimal metabolic state. Changing media pH has been stated to limit algal growth via metabolic inhibition [22].

3.3. Volumetric lipid production increased up to 600% of the controls

Neutral lipid accumulation is generally observed in algal cells only in the stationary phase when the cells are under stress such as nutrient deprivation, and therefore are unable to multiply [12,27]. Process strategies thus trigger accumulation only after sufficient biomass has been achieved since conditions that are employed for TAG accumulation prevent growth. A compromise between cell growth and lipid accumulation is thus necessary and considered a severe disadvantage of the process since it increases operation time [9]. In the current study, lipid accumulation began in the log phase in the CO_2 treated biomass while the control exhibited appreciable accumulation only in the stationary phase. Thus, increased carbon supply achieved simultaneous growth and lipid accumulation. Since the specific growth rates in all cultures were comparable to the controls, and the si-neutral lipid values were higher with excess CO_2, the overall volumetric lipid production increased. Volumetric lipid production here is defined as the micrograms of lipid obtained per unit volume of media. Increased si-lipid in 2.6% CO_2 led to a volumetric lipid production of 68 mg TO/l media on day 10, compared to 11.5 mg TO/l in the controls. This indicated a six-fold increase in lipid production. Significant increases were observed in all the other treatments as well. The volumetric lipid production values were 4.6, 4.7, 3.6 and 2.7 mg TO/l in the cultures sparged with 1.3%, 3.9%, 5.2% and 7.8% CO_2, respectively. A given value of total volumetric lipid production would be achieved in lesser operation time, which in turn, would improve process economy.

Aeration with excess CO_2 however, led to a decrease in the medium pH with time. To investigate the contribution of this change in pH towards lipid accumulation, a separate experiment was con-

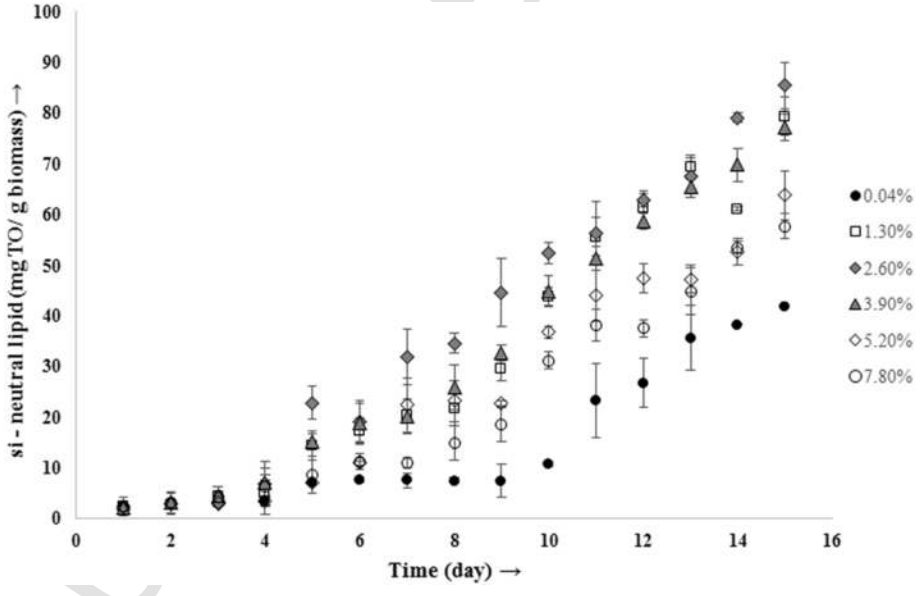

Fig. 2. Time profile of the specific intracellular (si) neutral lipid accumulation in the cultures sparged with 0.04% (control), 1.3%, 2.6%, 3.9%, 5.2% and 7.8% CO_2. Elevated CO_2 values triggered an early onset of neutral lipid accumulation, from day 5. Neutral lipid levels with elevated CO_2 were significantly higher compared to control. Si-neutral lipid is expressed in equivalents of mg tri-olein (TO) per gram of biomass. Neutral lipid was analyzed by fluorimetry with the dye Nile red. The fluorescent intensity was calibrated against the triglyceride tri-olein and thus the values are expressed in equivalents of mg TO. The values correspond to 1 g of biomass and thus are 'specific' intracellular (si) values. Data points are the mean values obtained from triplicate experiments. The error bars represent ± 1 standard deviation (SD) of the corresponding data point.

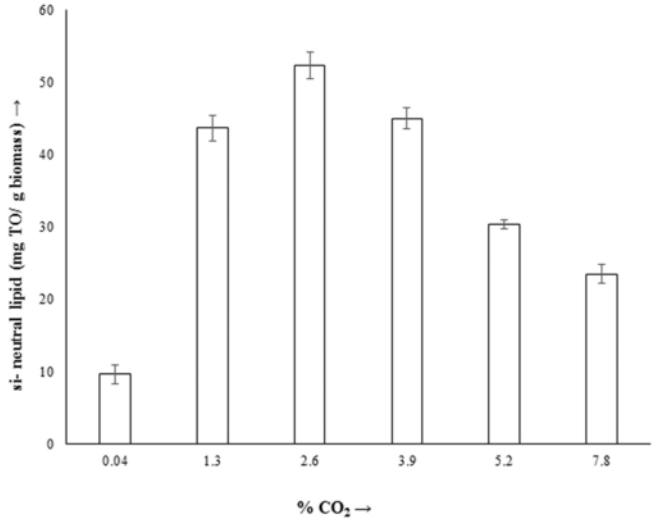

Fig. 3. Specific intracellular (si) neutral lipid levels in the cultures sparged with 0.04% (control), 1.3%, 2.6%, 3.9%, 5.2% and 7.8% CO_2, on day 10. Si-neutral lipid, expressed in equivalents of mg tri-olein (TO) per gram of biomass, was significantly higher in the cultures treated with elevated carbon supply; a five-fold increase was obtained with 2.6% CO_2. Neutral lipid was analyzed by fluorimetry with the dye Nile red. The fluorescent intensity was calibrated against the triglyceride tri-olein and thus the values are expressed in equivalents of mg TO. The values correspond to 1 g of biomass and thus are 'specific' intracellular (si) values. Data points are the mean values obtained from triplicate experiments. The error bars represent ± 1 standard deviation (SD) of the corresponding data point. The differences in the data are significant across all CO_2 concentrations, $p < 0.001$.

ducted at medium pH 8.1 ± 0.18 (control) and 6.81 ± 0.14 (corresponding to treatment with 2.6% CO_2 that achieved the highest lipid accumulation) and no difference in si-NL levels were observed between the two as seen in Fig. S3. Therefore, based on this observation, it was expected that the decrease in medium pH did not lead to any increase in lipid accumulation. pH stress has been used to increase TAG accumulation; a rise in pH of media was observed to yield TAG accumulation in *Chlorella* sp., *Scenedesmus* sp. and *Coelastrella* sp. [28].

In general, the increase in lipid accumulation may be attributed to the greater availability of carbon under high CO_2 supply. The presence of low CO_2 has been previously associated with minimal TAG accumulation due to carbon limitation [21] A number of studies in the literature [9,10,29,30] indicate that TAG synthesis increases due to the redirection of carbon towards its synthesis. For example, nitrogen limitation in the presence of ample carbon is the most common strategy employed to boost TAG accumulation. Carbon fixed, which is otherwise utilized to synthesize products of nitrogen metabolism, is diverted to starch and TAG synthesis due to the absence of nitrogen [9,29,30]. There are studies where starch-less mutants of *Chlamydomonas* accumulate very high levels of lipid deposits in comparison to the wild type. Restructuring of the carbon skeleton to fatty acid synthesis was suggested as the possible reason [11]. All these studies indirectly indicate that more carbon was available for TAG synthesis.

It is known from the literature [10,11,31], that an increase in lipid accumulation may not always result through overexpression of key enzymes involved in the synthesis of fatty acid and TAG. Studies on overexpression of TAG biosynthesis genes in diatoms, plant seeds and *Chlamydomonas* showed no significant increase in lipid accumulation [11]. Up-regulation of Acetyl CoA carboxylase (ACCase), a key enzyme in fatty acid synthesis, in these organisms also resulted in very low increases in lipid productivity [10,31]. It was found that although the transformants had very high ACCase activity, no significant changes were observed in the lipid content [10,31]. The possibility of carbon limitation for TAG synthesis has however been overlooked in all these reports involving ACCase.

3.4. Biomass composition

The carbohydrate and protein measurements were carried out to study any effect of increased carbon supply on the biomass composition. These contents are expressed as percentage of biomass in Fig. 4. The differences in the biomass composition across the cultures treated with varying CO_2 concentrations were not statistically significant compared to the controls. The results suggest that, the increases in si-NL accumulation observed in all cultures treated with excess CO_2, did not occur at the expense of the synthesis of proteins and carbohydrates. This is probably because only carbon in excess of that used for their synthesis was directed towards si-NL synthesis. It is already known from the literature that under favorable conditions, most of the carbon fixed is used for protein synthesis [9]. Only carbon in excess of that required for daily cell activities is converted to storage products such as starch and TAGs [5]. Since TAG synthesis is more energy expensive than starch/glycogen formation [5], the latter may be favored over TAG accumulation. As a result, TAG synthesis occurs at high rates only when the cell has exhausted on its capacity to synthesize starch [13]. Therefore, it is probable that any excess carbon would overflow to TAG biosynthesis. In addition, nitrate and phosphate depletion in the media may have favored lipid accumulation over growth. The levels of these nutrients could consequently influence biomass composition. A nutrient re-supplementation study observed that exhaustion of nitrate from the media influenced cell growth and biomass [32].

3.5. Elevated acetyl CoA levels at 2.6% CO_2

Increased carbon supply, as postulated above, should lead to increased carbon availability for uptake, and consequently, higher intracellular metabolite levels. Since AcCoA is a central metabolite in carbon and energy metabolism, changes in its concentration will significantly influence the metabolic state of the cell. Studies on bacteria and fungi observed that AcCoA levels change with the type and concentration of carbon sources used [33–35]. Thus, we expected the

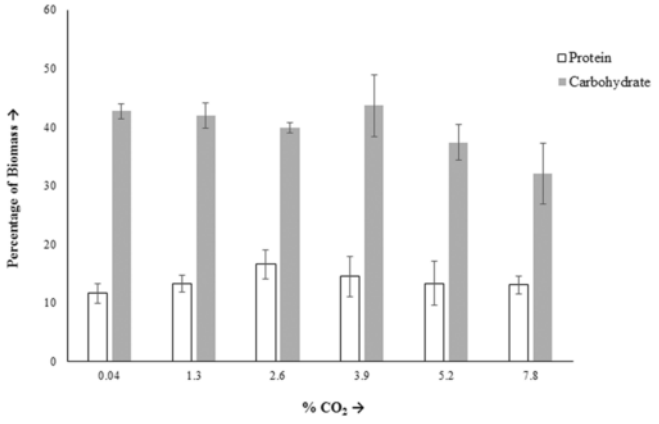

Fig. 4. Percentage contribution of carbohydrate and protein in the biomass of the cultures sparged with 0.04% (control), 1.3%, 2.6%, 3.9%, 5.2% and 7.8% CO_2, measured on day 15. Biomass compositions across various CO_2 treatments were comparable with no statistical differences, which indicate that the elevated CO_2 supplied in this study did not affect the proportion of these biomass components. Data points are the mean values obtained from triplicate experiments. The error bars represent ± 1 standard deviation (SD) of the corresponding data point.

steady state intracellular levels of AcCoA to reflect the increased carbon source. It is also the first key intermediate for fatty acid synthesis and thus can indirectly influence TAG accumulation in microalgae. Since the culture sparged with 2.6% CO_2 gave the highest si-NL accumulation, si-AcCoA levels from this culture were compared to that of control. Si-AcCoA extracted from the biomass in the different phases of growth were compared in Fig. 5 to see the effect of the CO_2 treatment on the intracellular concentration in the different phases. Measurements were carried out at three different time points, i.e. days 2, 9 and 15 that correspond to the lag, log and stationary phases, respectively.

The si-AcCoA values varied from 32.7 to 84.3 nmol/g biomass. In both the controls and 2.6% CO_2 treated cultures, si-AcCoA in the log phase increased steeply from low levels in lag phase. In the controls, the level almost doubled from 32.7 nmol/g biomass in the lag phase to 61.7 nmol/g biomass in the logarithmic phase. A similar increase was observed even in the cultures sparged with 2.6% CO_2 where si-AcCoA levels increased from 46.3 to 77.4 nmol/g biomass. The si-AcCoA levels in the stationary phase were also higher than in the log phase.

A comparison between the si-AcCoA of the two cultures reveals higher levels in the cultures sparged with 2.6% CO_2 in all phases of growth. This indicates increased availability of intracellular AcCoA due to increased carbon supply that possibly facilitated its higher uptake. In other words, excess carbon relative to available nitrogen and/or phosphorus could have resulted in increased AcCoA levels. It is reasonable to assume from this observation, that the si-AcCoA levels in the other cultivations sparged with higher CO_2 concentrations, were also higher than in the control cultivation, due to increased carbon supply. The lag phase cells of 2.6% CO_2 exhibited a significant 41% increase in si-AcCoA over the control cells whereas the log phase cells showed a 25% increase. The metabolite content in the stationary phase cells of 2.6% CO_2 also showed a 27% increase with respect to the controls. AcCoA levels have been associated with the growth of the cell [33], although an increase in the metabolite levels do not always result in improved growth rates [35].

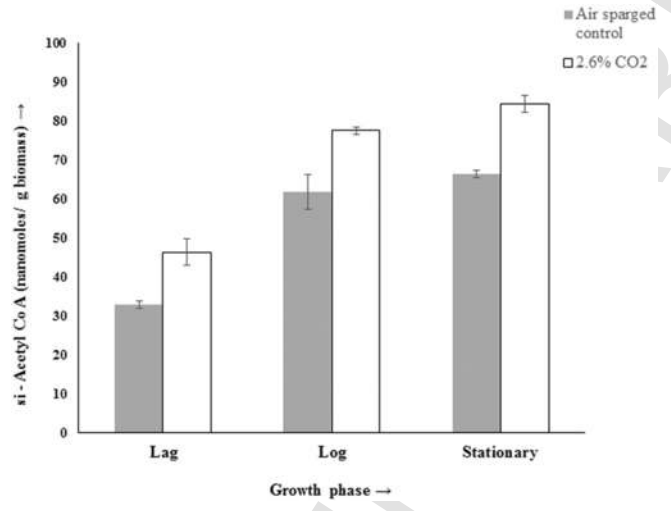

Fig. 5. Specific intracellular (si) Acetyl CoA levels in the lag (day 2), log (day 9) and stationary (day 15) phases of control culture (sparged with atmospheric CO_2 concentration) and the culture sparged with 2.6% CO_2. Si-Acetyl CoA was higher in the culture sparged with 2.6% CO_2, in all phases of growth. The values correspond to Acetyl CoA in 1 g of biomass and thus are 'specific' intracellular values. Data points are the mean values obtained from triplicate experiments. The error bars represent ± 1 standard deviation (SD) of the corresponding data point. The difference between the si-Acetyl CoA of the two cultures were significant in each growth phase, $p < 0.05$.

AcCoA has been considered as a potential regulatory branch point in carbon metabolism [9]. Although not completely understood, it is thought to play a major role in determining the fate of the carbon fixed. AcCoA is channeled towards the TCA cycle, which further gives rise to amino acids (among other products) such as glutamine and glutamate. These are in turn, used for the production of other amino acids and cofactors [9,11,36]. Oxaloacetate from the TCA cycle may be transformed into phosphoenolpyruvate, which is used for gluconeogenesis. Glucose so obtained can be stored as starch as has been studied in *Chlamydomonas* [11]. AcCoA can also be converted to malonyl CoA that is further utilized for fatty acid synthesis. Under conditions of nitrogen deprivation, the pathways leading to nitrogen containing compounds would not be active. Thus, the carbon originally channeled towards the syntheses of these compounds is channeled towards other pathways. As a result, storage compounds like starch and TAGs accumulate under these conditions. Likewise, TAG accumulation is also observed in starch mutants.

Under the conditions used in the study, higher partial pressure of CO_2 caused a higher dissolved CO_2 in the media for uptake. This implies an increase in fixed carbon to result in improved levels of intracellular metabolites. Since AcCoA is considered a key interconnected intermediate in carbon metabolism, its levels could rise as a direct result of higher carbon fixation. As discussed earlier in Section 3.4, carbon present in excess of that required for proteins and carbohydrates are stored as TAGs. Therefore, the 'increased' levels of the intracellular metabolite AcCoA could be directed towards fatty acid synthesis that could consequently improve TAG synthesis and accumulation.

3.6. Relationship between si-acetyl CoA and si-neutral lipids

The si-neutral lipid contents, as discussed above, also showed a dramatic increase (5.4-fold) over the controls in 2.6% CO_2 along with the si-AcCoA levels. AcCoA is a precursor for fatty acid synthesis, which in turn, is assembled into TAG bodies. Therefore, an increase in this precursor level could result in increased TAG synthesis. Although, fatty acid synthesis is argued to occur mostly in the chloroplast in microalgae, Avidan et al. have suggested that an increase in total AcCoA biosynthesis coupled with increased chloroplastic Co A levels could result in increased TAG accumulation [37]. It was noted above that lipid accumulation began early in excess CO_2 treated cells as compared to the control cells. A 41% increase over control in si-AcCoA levels in the lag phase cells of 2.6% CO_2 probably contributed to the earlier accumulation of neutral lipid in this culture.

A plot of the si-AcCoA content versus the corresponding si-NL content is shown in Fig. 6. The graph indicates that the si-NL increased exponentially with si-AcCoA and a quantitative relationship was derived from the data. The equation $y = 0.0187e^{0.6608x}$, where y and x correspond to si-NL (mg TO/g biomass) and si-AcCoA (nmol/g biomass) respectively, is an empirical correlation that indicates a significant effect of AcCoA levels on the neutral lipid accumulation. In other words, the equation shows that an increase in si-lipid can be obtained with the accumulation of sufficient levels of AcCoA, as has been previously suggested [11]. It was discussed earlier in Section 3.4 that only carbon in excess of that required for general cell activities may be used for TAG synthesis. Thus, it can be expected that beyond a threshold level of AcCoA, which is needed to produce all essential cell products as well as the storage product starch, any additional AcCoA is employed for fatty acid production. This could consequently lead to higher TAG synthesis. Thus, a surge in TAG synthesis is expected beyond this threshold level of si-AcCoA, which explains the exponential nature of the obtained correlation. The correla-

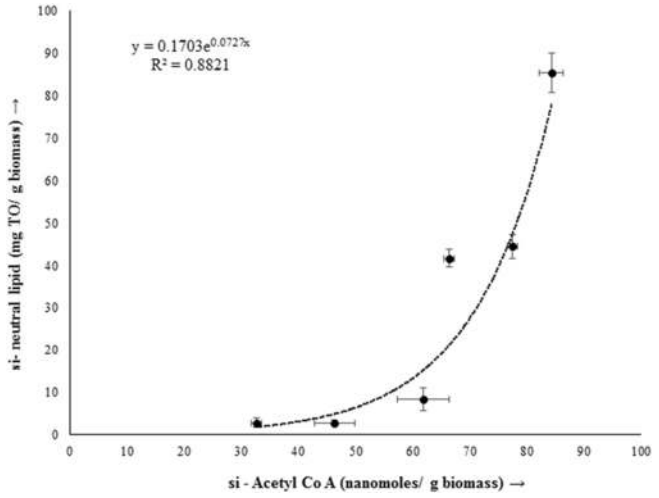

Fig. 6. Specific intracellular (si) Acetyl CoA versus the si-neutral lipid content in *Chlorella vulgaris*. Si-neutral lipid increased exponentially with increase in si-Acetyl CoA. Si-neutral lipid is expressed in equivalents of mg tri-olein (TO) per gram of biomass. Neutral lipid was analyzed by fluorimetry with the dye Nile red. The fluorescent intensity was calibrated against the triglyceride tri-olein and thus, the values are expressed in equivalents of mg TO. Both the neutral lipid and Acetyl CoA values correspond to 1 g of biomass and are thus 'specific' intracellular (si) values. Data points are the mean values obtained from triplicate experiments. The error bars represent ± 1 standard deviation (SD) of the corresponding data point.

tion further implies that the ability of an organism to bring about Ac-CoA accumulation could be used as an additional screening method for selection of microorganisms, designing of appropriate culture conditions and genetic manipulations for increased lipid accumulation.

3.7. Lipid profile with elevated CO_2

The relative percentages of the various fatty acid methyl ester (FAME) contents obtained from the GC–MS chromatograms were analyzed. The distribution of these FAME contents in the various cultures can be seen in Fig. 7, and it showed significant variations across the cultures. Table 1 records the total relative percentage of saturated (SFA) and unsaturated fatty acid (UFA) contents. The total % SFA accounts for the percentage of fatty acids contributed by the sum of all saturated fatty acids detected in the sample i.e. myristic, palmitic, stearic and arachidic methyl esters. The total % UFA accounts for the contribution of palmitoleic, oleic, linoleic and α-linoleic methyl esters. The relative % SFA increased from 41.7% in the controls to 52% in 1.3% CO_2 but fell to 27.9% in 2.6% CO_2 treated cultures. The % SFA in the remaining CO_2 treated cultures were also lower than the controls indicating an overall shift in the fatty acid contents from saturated to unsaturated in the higher CO_2 range used in the study.

An increased unsaturation, as seen in the lipid profile of most cultures in the current study, is generally associated with a decrease in oxidative stability. However, a deduction based on just the percentage saturation of the fatty acids is misrepresentative. % SFA alone is not a sufficient measure of the desirable production scenario, in terms of the diesel quality. The composition of the SFA (the relative amount of the individual SFA components such as myristic acid, stearic acid, palmitic acid, and arachidic acid) is more important than just a combined measure of saturation and unsaturation for determining the diesel quality. Similarly, unsaturated fatty acids also impart desired qualities. A number of factors such as the fatty acid chain length, branching, alcohol used for trans-esterification, etc., also influence the overall quality of the resulting biodiesel to varying extents [38]. Unsaturation improves cold flow properties and lubricity of the fuel whereas saturation improves the oxidative stability and ig-

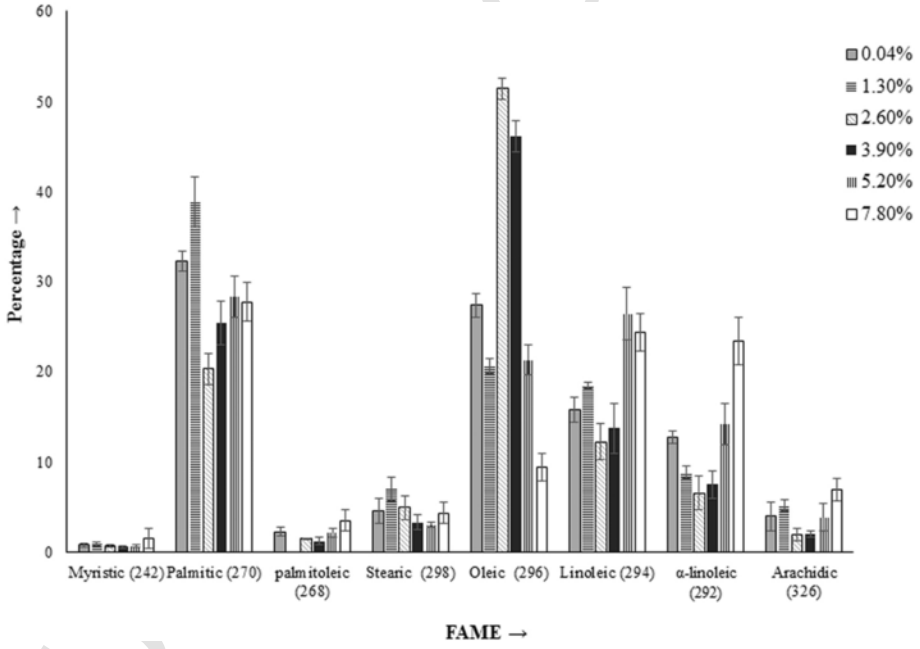

Fig. 7. The relative percentage fatty acid methyl ester (FAME) contents in the biomass of the cultures sparged with 0.04% (control), 1.3%, 2.6%, 3.9%, 5.2% and 7.8% CO_2. Fatty acid composition was determined through gas chromatography. The changes in the individual FAME contents led to variations in the total percentage saturated fatty acid content (sum of myristic, palmitic, stearic and arachidic acid methyl esters) of the cultures in the different treatments. Data points are the mean values obtained from triplicate experiments. The error bars represent ± 1 standard deviation (SD) of the corresponding data point. Variations in oleic, α-linoleic and linoleic acid methyl esters were significant with $p = 0.002$, 0.026 and 0.04 respectively.

Table 1
Total percentage saturated (SFA) and unsaturated fatty acid (UFA) contents.

% CO_2	0.04%	1.3%	2.6%	3.9%	5.2%	7.8%
% SFA	41.7	52.0	27.9	31.3	35.9	40.6
% UFA	58.3	48.0	72.1	68.7	64.1	59.4

nition quality. Increased chain lengths improve the cetane number and the heat of combustion, and increased CH_2 moieties increase melting points and thus degrade cold flow properties. Based on all these factors, biodiesel with methyl oleate as the major component has been previously recommended [39]. Even genetic manipulations have been carried out to achieve high percentage of oleic acid in soybean oil [40]. Cultivation of the microalgae in 2.6% and 3.9% CO_2 achieved this requirement. The major fatty acid in the biomass of these cultures was oleic acid as against palmitic acid in control thereby indicating improved biodiesel quality. It is known that palmitic acid is further extended and incorporated into complex lipids [41]. Possibly, this process happens at a higher rate at 2.6% CO_2 compared to the other CO_2 levels used in this study. This could have resulted in lower palmitic acid content in this culture.

However, with increasing concentrations of CO_2 beyond 1.3%, the total % SFA content showed an increasing trend and reached 40.6% in 7.8% CO_2 treated cells. Although the total % SFA in this culture was not significantly different from the controls, the individual fatty acid contents showed significant variation. The polyunsaturated fatty acids (PUFA), linoleic and α-linoleic acid, were higher at 5.2 and 7.8% CO_2. An increase in α-linoleic acid, an essential fatty acid, is of value for nutritive applications and may contribute to the economic viability of this method.

3.8. Desirable fuel characteristics with elevated CO_2 supply

Significant differences in the fatty acid compositions of the lipid at various CO_2 concentrations prompted further assessment for the fuel applications of the lipid. For a more robust quality prediction of the biodiesel, evaluation of several biodiesel parameters were carried out. Existing empirical formulae based on the fatty acid composition of the lipid accumulated, were used for the calculation of important biodiesel characteristics such as cetane number, kinematic viscosity, density and gross calorific value [42]. The calculated values, as shown in Table S2 of the supplementary file, showed that all parameters are well within the ranges prescribed by standard bodies such as the American Society for Testing of Materials (ASTM D6751-15), the European standards (EN 14214) and Indian standards (IS 15607) thereby ensuring its quality for use as biodiesel.

The use of excess CO_2 for microalgae cultivation, at the concentrations used in this study, is thus effective in improving lipid accumulation as well as the resulting biodiesel quality. CO_2 in high concentrations as required for such a strategy can be derived from economic sources such as flue gases that can have CO_2 concentrations as high as 15% [43]. Several studies have explored the plausibility of flue gas bio-mitigation using microalgae and indicate that the gas may be used for algal cultivation without toxic effects [43–45].

4. Conclusion

The use of increased carbon supply (CO_2) to improve neutral lipid accumulation was demonstrated in the current study. Elevated carbon supply achieved up to a 6-fold increase in volumetric lipid production and a 5.4-fold increase in neutral lipid accumulation over the cultures sparged with atmospheric CO_2 concentration. With increased CO_2, si-neutral lipid accumulation was triggered earlier, in the logarithmic phase of growth indicating decreased operation time and therefore a better process economy. Neutral lipid thus accumulated, when analyzed for its fatty acid composition, was found to have desirable biodiesel characteristics. The use of excess CO_2 is thus seen as an effective strategy for improved bio-oil production.

To see whether increased carbon supply may have increased lipid accumulation via the increase in precursor levels, si-AcCoA within the cell was measured at elevated CO_2 supply. Increases in this metabolite level under conditions of 2.6% CO_2, as compared to the controls, indicated improved availability of carbon in response to increased carbon supply. Likewise, it can be expected that the other cultivations sparged with higher CO_2 concentrations in this study, may also have higher si-AcCoA than control (0.04% CO_2). The si-AcCoA levels were further correlated with si-neutral lipid accumulation, and an empirical correlation was obtained between the two. To date, no studies showing increased intracellular AcCoA levels in response to increased carbon supply or a quantitative relationship with neutral lipid accumulation in microalgae have been reported in the literature. The correlation further indicates that improved neutral lipid accumulation can be effected by improving precursor availability. This information could be employed for the selection of culture conditions and the design of process strategies for enhanced lipid accumulation.

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Appendix A. Supplementary data: Maximum specific growth rate estimations, pH profiles, cell mass and biodiesel parameters

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.08.011.

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