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GROWTH, LIPID CONTENT, AND LIPID PROFILE OF THE GREEN ALGA, *CHLORELLA VULGARIS* BEIJ., UNDER DIFFERENT CONCENTRATIONS OF Fe AND CO_2

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Abstract

The effects of Fe^{+3} (2.4E-05 and 4.8E-05 mol/L) in the growth medium, and CO_2 in air (0.036, 1, and 2%) on the growth, total lipid content (TLC), total lipid production (TLP) and fatty acid (FA) profile of the green alga, *Chlorella vulgaris* Beij., were investigated, in vitro. Hi-Fe (4.8E-05 mol Fe^{+3}/L) with 2% CO_2 provided the best growth for the alga with 460.0 ± 10 mg/L dry biomass, while the culture with lo-Fe (2.4E-05 mol Fe^{+3}/L) and 2% CO_2 provided the maximum TLC of 27.0 ± 0.8%. The maximum TLP both obtained from hi-Fe with 1% CO_2 and lo-Fe with 2% CO_2 at 116.4 ± 5.4 mg/L. Both of these lipids displayed FA profile suitable for biodiesel production, but the later condition displayed superior fuel property in terms of iodine value, cetane number, and viscosity, satisfying the specifications by both the European (EN 14214) and US (ASTM B100) standards. The study also showed the possibility of manipulating the FA composition in freshwater green alga, *Chlorella vulgaris* Beij. by varying the concentrations of CO_2 in aeration and Fe^{+3} in the growth medium.

Keywords: Microalgae, *Chlorella vulgaris*, Iron, Carbon dioxide, Fatty acid.

1. Introduction

With the world's fossil fuel resources in peril of exhaustion and its prices reaching historical highs, the quest for alternative, renewable fuel is gaining attention in

Nomenclatures

C_i	Number of carbons in fatty acid *i*
C_{ti}	Cell density at time *i*
D_i	Number of double bond in fatty cid *i*
FA_i	Fatty acid *i*
Hi-Fe	4.8E-05 mol Fe^{+3}/L
Lo-Fe	2.4E-05 mol Fe^{+3}/L
t_i	Time state *i*

Abbreviations

ACCase	Acetyl-CoA carboxylase
ASTM	American Society for Testing and Materials
CCL	Carbon chain length
CFL	Compact fluorescent light
CN	Cetane number
DU	Degree of unsaturation
EDTA	Ethylenediaminetetraacetic acid
FAME	Fatty acid methyl ester
G3P	Glyceraldehyde 3-phosphate
GC-MS	Gas chromatography - mass spectrometry
IV	Iodine value
ME	Malic enzyme
MUFA	Monounsaturated fatty acid
OD_{688}	Optical density reading at 688 nm
PBR	Photobioreactor
PDH	Pyruvate dehydrogenase
PET	Polyethylene terephthalate
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
TLC	Total lipid content
TLP	Total lipid production
UV-vis	Ultraviolet-visible

global scale. Biodiesel as an alternative to petroleum-based diesel is currently being produced from vegetable oils and animal fat, common feedstocks are coconut, soybean, canola, corn, palm and jatropha. However, the wide-scale production of these crops for biodiesel production is endangering food supply and existing local economic activities, as well as cause increases in worldwide food and commodity prices. Because of this, the search for new generation biodiesel feedstock from non-food and non-terrestrial sources, like microalgae, are being considered [1].

Microalgae have clear potential as renewable and sustainable energy source. However, the necessary technology for the production of profitable microalgae-based biodiesel is still in various states of development. The economic feasibility of algae mass culture for biodiesel production greatly depends on high biomass and valuable lipid yield. Currently, about 60-75% of the cost of algae oil is due to the algal growth [2]. Therefore, understanding the biochemical pathways for the production and optimizing the system of algal productivity of fuel precursor will enhance the prospective of producing low-cost and price competitive microalgae-derived biodiesel.

One promising approach to reduce the input costs of microalgae cultivation is the direct utilization of CO_2 from flue gases or by means of the integrated microalgae fuel and carbon capture process. However, the growth response and tolerance of various microalgal species to the concentration of CO_2 is variable. For example, *Scenedesmus obliquus* reportedly grow best at 9% CO_2 and lipid production at 12% CO_2 [3], *C. vulgaris* at 1% CO_2 [4], *Isochrysis galbana,* at 5% CO_2 [5] and *Chlorococcum sp.* at 6% CO_2 [6]. This study highlights the use of indigenous freshwater green algal strain, *Chlorella vulgaris*. The goal of the study were to improve the growth and lipid content of the algae, and investigate the effects of different concentration of Fe^{+3} in the growth medium and CO_2 in aeration on growth, lipid accumulation and fatty acid profile of the lipid extracted.

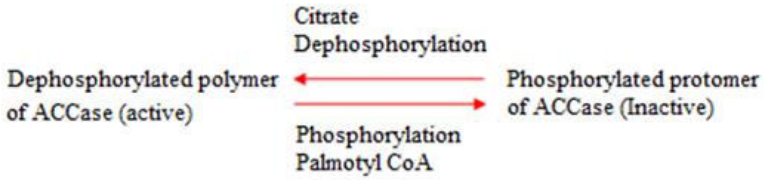
2. Microalgae Biomass and Lipid

2.1. Biomass pathway

For eukaryotic algae, photosynthesis takes place in specialized organelles known as chloroplast; the product is a three (3) carbon sugars glyceraldehyde 3-phosphate (G3P). This molecule is then converted to either glucose or pyruvate are used for structural purposes and, under certain circumstances, as storage material like lipid.

2.2. Lipid pathway

The fatty acid biosynthesis in algae also happens in the chloroplast. The first committed step in this process is the carboxylation of acetyl-CoA to form malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACCase). This process is often the rate-limiting step in the fatty acid biosynthesis [7, 8]. Acetyl-CoA is generated in the chloroplast from pyruvate via reaction catalyzed by the pyruvate dehydrogenase (PDH). In the mitochondria, acetyl-CoA is also generated primarily from two sources: fatty acid oxidation, as well as via PDH reaction, but in order for these acetyl units to be utilized for fatty acid synthesis they must be present in the cytoplasm. The mitochondrial acetyl-CoA is converted to cytoplasmic acetyl-CoA by a series of malic enzyme (ME) reactions [8, 9]. The rate of fatty acid synthesis is controlled by the equilibrium between the protomeric and polymeric ACCase. The control of the ACCase involves phosphorylation-dephosphorylation reactions. Metabolically, this conformational change is enhanced by citrate and is inhibited by palmitoyl-CoA [10]. The reaction is summarized as follows:



The accumulation of citrate in the cytoplasm shifts equilibrium to the polymeric ACCase, thus activating fatty acid biosynthesis. However, the overexpression of ACCase could inhibit fatty acid synthesis [8]. Palmitoyl-CoA promotes polymer disaggregation and is a primary feedback inhibitor of fatty acid synthesis.

3. Experimental

The freshwater algal strain, *Chlorella vulgaris*, was obtained from the Institute of Biological Sciences, University of the Philippines Los Baños, Laguna. The strain was originally isolated from Laguna Lake and was maintained in standard BG-11. *C. vulgaris* cells were cultivated in 6L capacity clear PET bottles with 5L working volume at 28 ± 2 $^{\circ}$C, illuminated under 12/12 hr light/dark cycles using cool white CFL at 200 μmol/photons m^2/s. The growth medium, BG-11, was prepared with different levels of Fe^{+3} of 2.4×10^{-5} (lo-Fe) and 4.8×10^{-5} mol/L (hi-Fe), as Ferric-citrate ($C_6H_5FeO_7$) complexed with EDTA. Algal culture was aerated continuously at 3L/min, with different levels of CO_2 of 0.036, 1 and 2%. The pH of the culture was allowed to change naturally. Algal sampling was every 2 - 3 days, wherein algal growth and the pH were measured. The algal cultures were terminated, and the biomass harvested at day 32. The initial algal concentration was the same for all growth conditions with 3×10^6 cells/ml or 0.1 optical density reading at 688 nm (OD_{688}).

3.1. Algae growth monitoring

The algal cell population was measured as OD_{688} using Perkin-Elmer UV-Vis Spectrometer Lambda 8000. OD_{688} was positively correlated with cell density, with $R^2 = 0.9931$. The relation was found by the equation:

$$y = [\,7.9607x - 0.4323] \quad (1)$$

where, y and x is equal to the cell density and optical density reading at 688 nm, respectively. The cell density or cell count was determined following the procedure given by Martinez et al. [11] using the Improved Neubauer haemocytometer, 1/10 mm deep. Algae growth was monitored by plotting the OD_{688} against time. The specific growth rate and the doubling time were determined using the following equations [12]:

Specific growth rate (μ): $$\mu = \frac{log(C_{t_2}/C_{t_1})}{(t_2 - t_1)} \quad (2)$$

Doubling time (T_d): $$T_d = ln\,2/\mu \quad (3)$$

where C_{t_1} and C_{t_2} are cell density (cells ml^{-1}) at different time points time1 (t_1) and time2 (t_2), respectively.

3.2. Dry biomass concentration

The dry biomass concentration of the algae culture at day 32 was determined by filtering 10 ml of the algal culture onto 4.7 cm Whatman GF/C glass fiber filter, pre-conditioned and pre-weighted, using a vacuum filter. The filtered samples were washed with 200 ml distilled water to removed excess salts, then placed in pre-weighted aluminium dish and dried in the oven at 105 °C for 16 hours; the dried samples were transferred in desiccator and allowed to cool for 24 hours. The dry biomass of the sample used was determined gravimetrically. The dry biomass concentration, calculated as the ratio of the dry biomass to the volume of the sample used. Reported values were averages of three measurements.

3.3. Total lipid extraction

The total lipid was extracted from dry algae powder using chloroform: methanol mixture (2:1, v/v), in three (3) steps. At each step, 100 mg algae sample was soaked in 5 ml solvent for 8 hours; after which, the mixture was centrifuge at 4000 rpm for 5 minutes. The solvent-lipid phase was carefully transferred to a pre-weighted aluminium dish, the solvent evaporated to dryness under vacuum for 5 hours. The total lipid was determined gravimetrically, the % total lipid calculated as % ratio of dry weight of extracted lipid to dry algae biomass used. Reported values were averages of three measurements.

3.4. FAME fatty acid analysis

Samples of crude algae oil were submitted to the Philippine Institute of Pure and Applied Chemistry in Ateneo de Manila University. In which, the FAME was extracted and the fatty acid analyzed using GC-MS. Reported values were averages of two repeated readings.

3.5. FAME fuel property

The average carbon chain length and degree of unsaturation of the algal lipid calculated based on the GC-MS FAME fatty acid analysis, using the following equations [13]:

$$\text{Ave. carbon chain length} = \sum\left(\frac{\%FA_i}{100} * C_i\right) \quad (4)$$

$$\text{Ave. degree of unsaturation} = \sum\left(\frac{\%FA_i}{100} * D_i\right) \quad (5)$$

The values of iodine value, cetane number, and viscosity of the algal lipid were obtained based on the correlation study by Hoekman et al. [13].

4. Results and Discussion

The effect of the concentrations of Fe^{+3} in the growth medium and/or CO_2 in aeration on the growth (expressed either as OD_{688} or dry biomass yield), lipid accumulation (lipid content), and FAME fatty acid distribution of *C. vulgaris* were analyzed in this paper. One-factor and interaction effect were statistically analyzed with design of experiment using Design Expert 7 with three (3) replicates and $p \leq 0.1000$ as level of significance. The fuel quality in terms of the iodine value, cetane number, and viscosity are presented in this section.

4.1. Effect of Fe and CO_2 on the growth of *C. vulgaris*

Increases in Fe^{+3} and/or CO_2 significantly improved the growth of the algae relative to the control (lo-Fe with 0.036% CO_2) ($p < 0.1000$) (Fig. 1). The growth rate increased from 0.0798 day^{-1} to 0.1024 day^{-1} and the doubling days shortened from 8.68 to as short as 7 days. Overall, the growth of *C. vulgaris* in the control was the slowest. In addition, the control appeared to have entered the stationary phase at day 28, while the rest of the culture continued to grow at exponential rate, up until the final culture day (day 32). The growth of the algae was the fastest in hi-Fe with 2% CO_2. Analysis of interaction between Fe and CO_2 showed no significant effect on the growth of the algae ($p > 0.1000$).

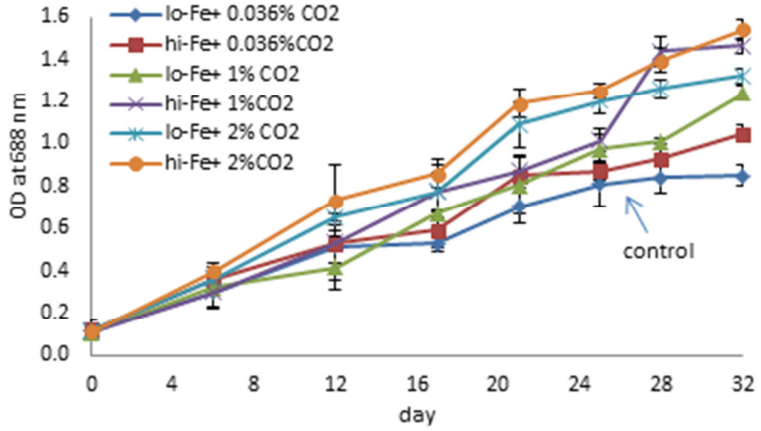


Fig. 1. Growth response of *C. vulgaris* grown under different concentrations of Fe^{+3} and CO_2, n = 3.

4.2. Effect of Fe on growth

In Fig. 2, algal growth as dry biomass yield in lo-Fe with CO_2-enriched aeration were significantly increased compared with under ambient aeration (0.036% CO_2) ($p < 0.1000$), however, between 1 and 2% CO_2 dry biomass yield has no significant difference ($p > 0.1000$). At hi-Fe, dry biomass yield showed increasing trend with increasing CO_2 up to 2%. Thus, it appeared hi-Fe condition enhanced the capacity of *C. vulgaris* for CO_2 fixation at high levels of CO_2. This result was similarly

observed in *S. obliquus*, *C. meneghiniana*, and *C. vulgaris* [3, 14, 15]. The result is consistent with the role of iron as an important co-factor in photosystem I and photosystem II, iron generally affect the light harvesting, photochemical energy conversion, electron transfer, and carbon fixation efficiency. The increase in iron must have resulted to the increase in photosynthesis that led to the increase in microalgae biomass [16].

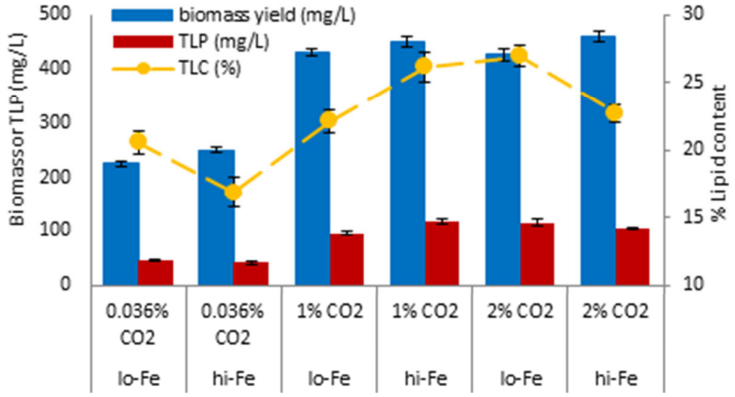


Fig. 2. Dry biomass, total lipid content (TLC), and total lipid production (TLP) of *C. vulgaris* grown under different concentration of Fe^{+3} and CO_2.

4.3. Effect of CO_2 on growth

The concentration of CO_2 has significant effect on the growth of the alga ($p < 0.1000$). During growth, the pH of the algal cultures significantly and exclusively influenced by the concentration of CO_2 ($p < 0.1000$). At ambient aeration (0.036% CO_2), the pH of the algal cultures were observed to rise in the range of 7.9-8.8. On the other hand, CO_2-enriched aeration caused acidification of the growth medium causing a rapid downward shift of pH. With 1% CO_2, the pH of the cultures up to day 12 ranged 7.8-8.0, after which remained in 6.9-7.5. At 2% CO_2 aeration, the pH of the culture after day 6 remained between 7.1- 7.4.

From Fig. 3, the downward shift in the average pH of the culture towards neutrality generally showed increased in algal biomass yield. This observation is consistent with the common trend found in the literature in many algal species, including *C. vulgaris, Thalassiosira pseudonana, Thalassiosira oceanic and S. obliquus* [17-20]. The pH of the medium close to nuetrality allowed the easy uptake of CO_2 into the pH nuetral intracelluar fluid in algae cells via simple diffusion [5], and increased the bioavailability of some essential nutrients, like Fe^{+3} which is most stable in aqueous solution at pH < 8.1 [21].

4.4. Effect of Fe and CO_2 on the lipid accumulation of *C. vulgaris*

The interaction of Fe^{+3} and CO_2 has significant effect on the lipid content in *C. vulgaris* ($p < 0.1000$). In Fig. 2, lipid content under lo-Fe showed increasing trend with increasing CO_2. Under hi-Fe, the lipid content significantly increased with 1% CO_2 and decreased both with 0.036% and 2% CO_2, all compared to the corresponding result obtained under lo-Fe. This response was surprisingly

different with the common trend found in the literature, wherein Fe augmentation with ambient or moderately high-CO_2 aeration is associated with an increase in lipid content; as were reported for *S. obliquus* [3] and *C. vulgaris* [4, 15]. The differences with the present result may be due to several reasons. First, all published data by Liu et al. [15] pertains to a marine strain of *C. vulgaris*, while the present study refers to a freshwater strain. Secondly, different growth media and culture condition (i.e. illumination) were used in the present study. Third, Abd El Baky et al. and Liu et al. [3, 15] both employed $FeCl_3$ as source of iron while the present study used Fe-citrate as source. Lastly, the interaction of Fe added as ferric citrate and CO_2 must have affected the activity of the enzyme acetyl-CoA carboxylase (ACCase) that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, in the biosynthesis of lipid. In hi-Fe with 0.036% CO_2, condition must have caused modest increased in the bio available Fe^{+3} in the growth medium due to its low solubility in pH 7.9 – 8.8, resulting to improved carbon uptake from 0.036% CO_2 and possibly some from citrate. On the other hand, in hi-Fe with 2% CO_2, it is possible that the 2% CO_2 already provided enough carbon for uptake. Hence, in both conditions, high amount of citrate must have remained unutilized in the medium. This citrate must somehow affect the enzyme ACCase, the same way as the citrate found inside the algae cells. The presence of citrate in the medium in both hi-Fe with 1% CO_2, and hi-Fe with 2% CO_2 in addition to the natural production of citrate during the citric acid cycle resulted in the overexpression of the enzyme ACCase, which is known to inhibit lipid synthesis [8]. On the other hand, at hi-Fe with 1% CO_2 condition, the bio-available concentration of Fe^{+3} in the medium was improved, also due to its increased solubility in pH 6.9 – 7.5. Hence, it is possible that there were enough carbon acceptor from both 1% CO_2 and citrate in the medium, leaving no or small amount of citrate in the medium that could significantly influence the activity of the enzyme ACCase. Nonetheless, the citrate in the cytoplasm must be enough to shift the equilibrium to the polymeric ACCase that activated the fatty acid biosynthesis in the microalgae (Sec 2.2.).

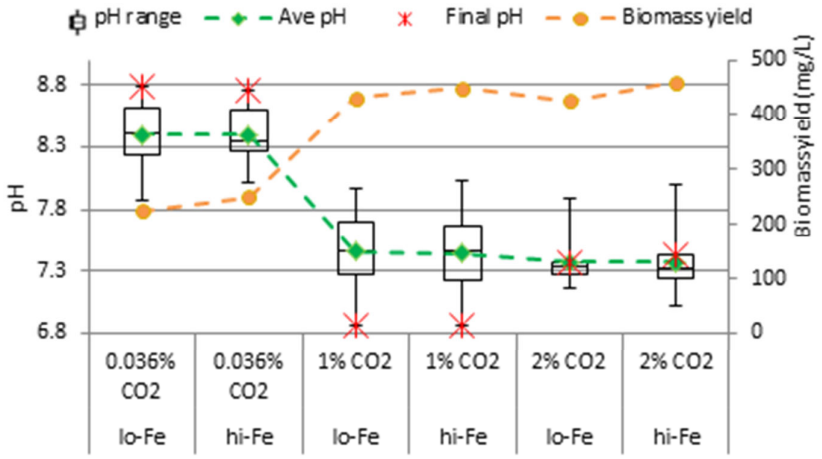


Fig. 3. Effect of pH on biomass yield of *C. vulgaris*.

4.5. Total lipid production of the *C. vulgaris*

There were two (2) culture conditions that yielded the highest total lipid, at hi-Fe with 1% CO_2, and at lo-Fe with 2% CO_2 of 116.4±5.4 mg/L. This yield accounted for 2.6-fold increase compared to the control (lo-Fe with 0.036% CO_2). These results were due to simultaneous increased in the biomass and lipid content in *C. vulgaris.*

4.6. Effect of Fe and CO_2 on the fatty acid in *C. vulgaris*

Table 1 shows the fatty acid in *C. vulgaris* were mainly distributed between the C12 – C18 with palmitic (C16:0), oleic (C18:1), linoleic (C18:2) and linolenic acid (C18:3) as dominant constituents. Except in lo-Fe with 2% CO_2, containing palmitic and linoleic acids as its only constituents. These fatty acids are common in several algae species including in *C. vulgaris* [13,22], and in some commercially known biodiesel feedstock such as camelina, canola, corn, jatropha, rapeseed, safflower, soy and sunflower [15].

Additionally, Table 1 shows the fatty acid saturation and carbon chain length remarkably changed in lo-Fe with 2% CO_2. Two other growth conditions, hi-Fe with 0.036% CO_2, and hi-Fe with 2% CO_2, showed slight alteration on the saturation and carbon chain length of the fatty acid in *C. vulgaris*, all compared to the control (lo-Fe with 0.036% CO_2). Generally, 2% CO_2 seem to be a proper condition for accumulation of high amounts of SFAs such as C12:0 and C16:0. This observation was in agreement with the result reported by Tsuzuki et al. on *C. vulgaris* [23]. However, with hi-Fe, this effect seem to be moderated in favor of the synthesis of longer carbon chain length. The possible reason was the high CO_2 fixation and CO_2 consumption at 2% CO_2 that might affect the enzymatic desaturation and elongation reactions in lipid synthesis, as was observed by Abd El Baky et al. in *S. obliquus* [3].

Table 1. GC-MS FAME Fatty acid distribution, degree of unsaturation and carbon chain length of the lipid extracted from *C. vulgaris* grown under different concentrations of Fe^{+3} and CO_2.

FAME fatty acid	0.036%CO_2 lo-Fe	0.036%CO_2 hi-Fe	1% CO_2 lo-Fe	1% CO_2 hi-Fe	2% CO_2 lo-Fe	2% CO_2 hi-Fe
	% Composition					
C12:0	2.9	7.3	3.6	4.3	n.d.	5.7
C16:0	13.3	17.1	10.6	10.9	69.8	19.9
C18:1	18.4	16.4	18.9	17.2	n.d.	16.7
C18:2	35.6	35.4	37.3	40.2	30.2	37.1
C18:3	29.9	23.8	29.6	27.3	n.d.	20.6
SFA	16.2	24.4	14.2	15.2	69.8	25.6
MUFA	18.4	16.3	18.9	17.2	0.0	16.7
PUFA	65.5	59.3	66.9	67.5	30.2	57.6
Ave DU	1.8	1.6	1.8	1.8	0.6	1.5
Ave CCL	17.6	17.2	17.6	17.5	16.6	17.3

C12:0, lauric acid; C16:0, palmitic acid; C18:1, oleic acid; C18:2, linoleic acid; and C18:3, linolenic acid; SFA: saturated fatty acid; MUFA: mono-unsaturated fatty acid; PUFA: poly-unsaturated fatty acid; DU: degree of unsaturation; CCL: carbon chain length; n.d., not detected.

Analysis of the lipid from hi-Fe with 1% CO_2, and lo-Fe with 2% CO_2, from which the highest total lipid were obtained, Table 1 shows remarkably different lipid profile, while Table 2 shows acceptable fuel quality for both of these lipids. However, the lipid from lo-Fe with 2% CO_2 displayed superior fuel quality, expected to comply both the European (EN 14214) and US (ASTM B100) standards for the iodine value, cetane number and the kinematic viscosity.

Table 2. Comparison of the FAME property of the *C. vulgaris* to biodiesel standards.

	1% CO_2, hi-Fe	2% CO_2, lo-Fe	EN 14214	ASTM B100
Ave. DU	1.8	0.6		
Iodine number	147	59	Max. 120	None
Cetane number	51	59	Min. 51	Min. 47
Ave . CCL	17.5	16.6		
Kinematic viscosity mm/s^2	2.75 – 4.61*	2.75 – 4.61*	1.9 - 6.0	3.5 – 5.0

DU: degree of unsaturation; CCL: carbon chain length
* value range for average carbon chain length of 13.4 – 19.1 [13].

5. Conclusions

The effects of different concentration of Fe^{+3} in the growth medium, 2.4E-05 (lo-Fe) and 4.8E-05 mol/L (hi-Fe), and concentration of CO_2 in aeration, 0.036, 1, and 2% by volume, on the growth, total lipid content (TLC), total lipid production (TLP), and fatty acid (FA) profile of the green alga, *Chlorella vulgaris* Beij., were investigated, in vitro. Based on this study, several conclusions can be drawn.

- The augmentation of Fe^{+3} improved the growth of *C. vulgaris* grown at 0.036%, 1%, and 2% CO_2 aeration. Hence, the addition of Fe could be a viable strategy in enhancing the growth of *C. vulgaris* under moderately high CO_2 aeration.
- Increasing the concentration of CO_2 in aeration up to 2% caused downward shift of the pH close to 7.0, this appeared favourable for algal growth. The condition of hi-Fe and 2% CO_2 provided the best condition for the growth of *C. vulgaris*. The concentration of Fe^{+3}, supplied as Ferric-citrate complexed with EDTA, and its interaction with CO_2 influenced the lipid accumulation in *C. vulgaris*. Lo-Fe with 2% CO_2 aeration provided the best condition for lipid accumulation in *C. vulgaris.*
- The degree of unsaturation and the chain length of fatty acid were dependent on the concentration of CO_2 and Fe^{+3}. The lo-Fe with 2% CO_2 provided proper condition for accumulation of high amount of SFA and synthesis of short carbon chain FA. This result suggested the possibility of manipulating the lipid composition in freshwater green alga, *C. vulgaris* Beij. by varying the concentrations of CO_2 in aeration and Fe^{+3} in the growth medium.

Acknowledgements

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