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Effect of Carbon Dioxide Concentration on Cell Growth and Oil Yield of Freshwater and Marine Microalgae

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ABSTRACT

The *Chlorella saccharophila* (freshwater) and *Tetraselmis suecica* (marine) microalgae were investigated for their potential as feedstock for the production of biodiesel. Assessment was based on their ability to produce biomass and lipid accumulation. Varying concentrations of CO_2 (3, 6 and 9%) were used at 24 h light exposure. The results indicated that *T. suecica* produced higher cell yields compared to the *C. saccharophila* under all parameters tested. Statistical analysis indicated that the biomass yields achieved using CO_2 at varying concentrations were significantly different from one another. However, varying CO_2 concentrations over the range of 3 to 9% did not significantly affect the oil yields for both species over the elapsed time. Thus, from an economic stand point it is much more suitable to use CO_2 at a concentration of 3% as opposed to higher concentrations. The use of $NaHCO_3$ as a carbon source on the biomass and oil yields were also evaluated using the same experimental parameters. Results indicated that the CO_2 carbon source resulted in higher biomass and oil yields for the marine microalgae species when compared to $NaHCO_3$ carbon source. However, the *Chlorella saccharophila* species resulted in higher biomass and lipid yields using the sodium bicarbonate carbon source. This suggests that different species have a preferred carbon source. Some are better at the uptake of one source over the other. Lower oil yields were achieved using the 3% CO_2 as the carbon source compared to $NaHCO_3$ for the *Chlorella saccharophila* species. The *Tetraselmis suecica* species resulted in a slight increase in oil yield using 3% CO_2 as opposed to $NaHCO_3$. The optimal growth conditions for *Chlorella saccharophila* are the combination of nutrients, with 24 h light exposure and $NaHCO_3$ as a carbon source and those for *Tetraselmis suecica* are the ammonium nitrate, the 24 h light exposure and 3% CO_2 as a carbon source.

Keywords: Freshwater, Marine Water, Microalgae, Cell yield, Oil Yield, $NaHCO_3$, CO_2, Carbon Source.

INTRODUCTION

The demonising fossil fuel reserves and the environmental concerns associated with burning fossil fuels have accelerated the need for a renewable energy source that is environmentally friendly. Increased carbon dioxide emissions have been correlated with the amount of fossil fuel being burnt [1]. Biofuels such as biodiesel and bioethanol are promising substitution for petroleum fuel source [2]. Numerous feedstocks can be used as biomass for biofuel generation which include food waste, agricultural waste, municipal waste and both edible and nonedible oilseeds [3]. Currently, the best crops for biofuel production are oilseeds, but they are considered a food source for many people around the world [2,3]. However, microalgae have been noted to store oil that is 10 folds higher than the leading plant crop [4].

Microalgae are abundant microorganisms in nature, able to convert carbon dioxide into biomass which can then be used for biodiesel production via a transesterification reaction process [5]. Microalgae as an alternate fuel source is ideal because of their high growth rates and their ability to store higher lipids than the leading crop plants [6]. In addition, the waste that is generated after oil extraction can be used for other value added products, such as animal feed, organic fertilizers, and other biofuel products such as methane and ethanol via fermentation [7]. The amount of lipids stored in

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the microalgal cells can be manipulated by changing the environmental parameters such as temperature, pH, nutrient source, carbon source and light duration [8-10].

Biodiesel is a liquid fuel that is biodegradable and nontoxic [11]. It generates the same amount of energy (calorific value) as that produced using petroleum diesel without the resale of harsh compounds such as NOx, SOx and hydrocarbons into the atmosphere [3,12]. Biodiesel can be used in existing diesel engines without the need for much modification [2]. It is for these reasons that biodiesel is regarded as the best renewable energy source that is environmentally friendly and a viable source for replacement of the currently used petroleum source.

OBJECTIVES

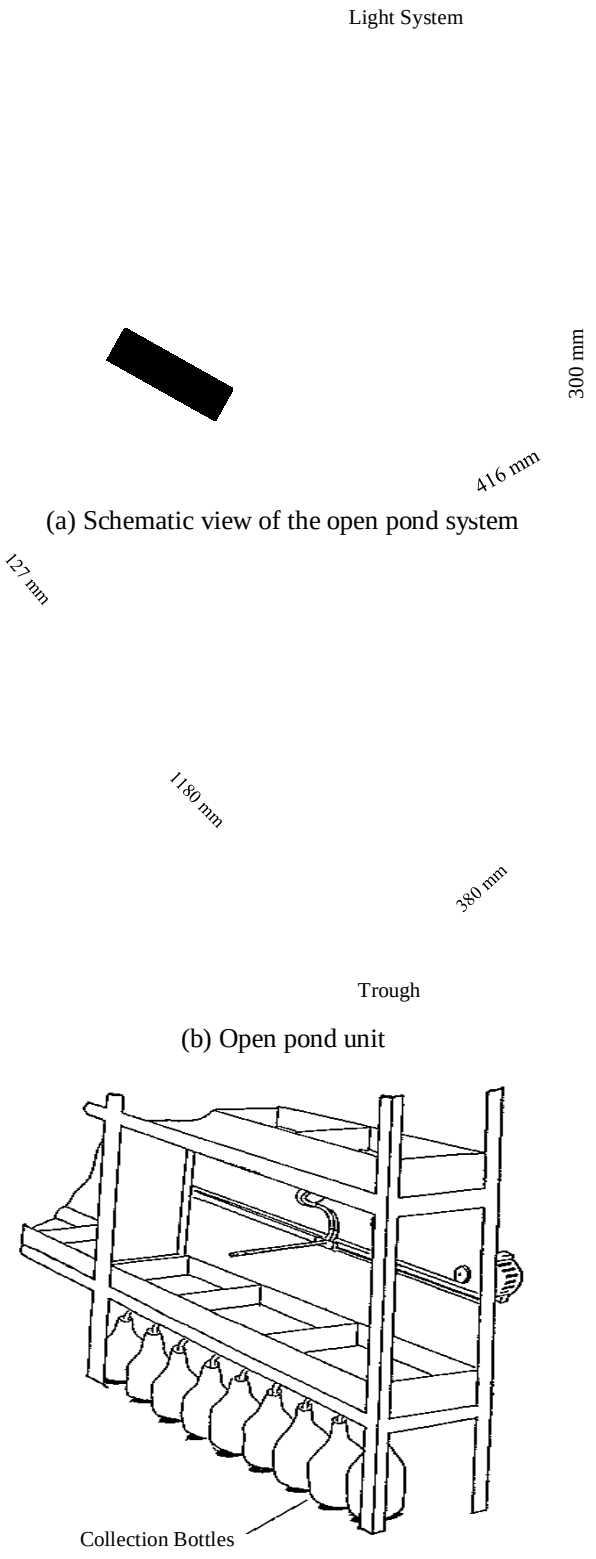
The aim of this study was to investigate the possibility of increasing the microalgae cell growth rate and oil yield by exposing the microalgae to various carbon sources ($NaHCO_3$ and CO_2) in a specially designed pilot scale open pond system. The specific objectives were: (a) to study the effect of CO_2 concentration in the air on biomass yield and oil content at three levels (3:97, 6:94 and 9:91 v/v CO_2 to air) and (b) to evaluate the effect of carbon source ($NaHCO_3$ and CO_2) on the microalga biomass yield and oil content.

MATERIALS AND METHODS

Experimental Apparatus

A fully automated multiple open pond system (Figure 1) consisted of a frame, 18 open pond units, a cooling unit, a lighting unit, a supernatant collection unit and control unit was used in this study.

The frame (244 cm in width x 41 cm in depth x 283 cm in height) consisted of three shelves (76 cm apart) and housed the open pond, light, cooling, water collection and control units. Each shelf was divided vertically into two sides by a 1.2 cm thick plywood sheet to provide a better control of light and feed. The open pond unit consisted of six ponds, each was made of galvanized steel and was divided into three compartments (each was 38 cm in length x 38 cm in width x 12.5 cm in height and can hold up to 18 L). The lighting unit provided 430 hectolux of illumination per shelf (480 µmol m^{-2} s^{-1}) using a mixture of fluorescent and incandescent lamps (six 40 W cool white fluorescent lamps 122 cm in length and four 100 W incandescent bulbs) mounted on each shelf, that sit 100 cm away from the ponds. A cooling unit was designed to continuously remove the heat produced by the lamps to avoid heating of the algae on the upper and middle shelves. A 5 cm diameter PVC pipe (having 6 mm diameter holes spaced 6 cm apart and facing out) was placed under the backside of the ponds. Two metal blocks placed under each pond provided a 5 cm space between the pond and the lighting system of the shelf below it. A 5 cm diameter PVC pipe was attached vertically to the left side of the frame and acted as a manifold through which air was blown by means of a motor



(a) Schematic view of the open pond system

(b) Open pond unit

(c) Collection unit

Figure 1. Experimental Apparatus.

driven fan (Model AK4L143A Type 821, Franklin Electric, Bluffton, Indiana, United States of America). The supernatant from each tray was collected in a separate container (2.7 L each) located at the bottom of the system. The outlets were connected to plastic tubes of 1 cm outside diameter, which were passed through a solenoid valve.

A computer was used to operate and control the various components of the open pond system and record the various measurements. The light intensity was measured using a Quantum Sensor, SQ-316 Series (Apogee, Logan, Utah, United States of America). The pH was measured using pH electrodes (EW-59001-65, Cole Parmer, Montreal, Quebec, Canada). The temperature was measured using thermocouples (WD-08541-12, Nova-Tech International, Houston, Texas, United States of America). A basic computer program (BASIC Stamp Editor v2.5) allowed the configuration of the operating frequency and duration of the light, aeration unit and collection system. The computer was connected to a data coordinator (cDAQ-9178, National Instruments) which had 24 digital output ports and 24 digital input ports. The digital output ports were connected to electronic circuits which were responsible for the lighting, cooling and collection systems.

Microalgae

One freshwater microalgae (*Chlorella saccharophila*) and one marine (*Tetraselmis suecica*) were selected based on their ability to yield high biomass and store lipids [13].

The freshwater strain *Chlorella saccharophila* was selected for study because of its high lipid content (45%). This strain is capable of achieving a biomass yield of 3.88 g/L, which is not the highest among the freshwater species, but can however be offset by the fact that it achieves the highest lipid content. This results in a lipid yield of 1.75 g/L. The highest biomass yielding algae *Scenedesmus obliqus* of 4.34 g/L only achieves a lipid content of 38%, which intern results in a lipid yield of 1.69 g/L. *Chlorella saccharophila* is a green unicellular microalga belonging to the Chlorella genus [14]. The cells have an average size of 7.3 µm [15]. The cells contain a single chloroplast enclosed in a spherical or subspherical form. These cells reproduce asexually through production of non-motile autospores [16]. This species is able to use glucose [17], bicarbonate and carbon dioxide as the carbon source for growth [18]. The optimal temperature and pH for growth are 20-24°C and 7.5-8, respectively.

The marine microalgae strain *Tetraselmis suecica* was selected for this study because of its high biomass yield of 4.48 g/L and comparatively high lipid content. This species achieves a lipid content of 23% which is not the highest among the other species but can, however, be offset by the fact that it achieves the highest biomass yield. This results in a lipid yield of 1.03 g/L, while the *Chaetoceros muelleri*, with the highest lipid content of 34%, only achieves a biomass yield of 0.98 g/L, which results in a lipid yield of 0.33 g/L. *Tetraselmis suecica* grows as single cells. They are motile and can be compressed or curved, but they are never twisted [19]. The cells are spherical or elliptic with a length of 35 µm and a width of 14 µm. This species is able to use both sodium bicarbonate [20] and carbon dioxide [21] as the carbon source for growth. The optimal temperature and pH for growth are 18-24°C and 7-9, respectively [22].

Experimental Design

Two set of experiments were carried out. In the first set of experiments, the selected freshwater (*Chlorella saccharophila*) and marine (*Tetraselmis suecica*) microalgae species were grown in an open pond system using $NaHCO_3$ as a carbon source. The sodium bicarbonate ($NaHCO_3$) was administered at a concentration of 1300 mg/L. Ammonium nitrate was used for the marine microalgae and a combination of nutrients (ammonium nitrate, ammonium sulfate and ammonium phosphate) was used for the freshwater algae as sources of nitrogen at the optimum light exposure (24 h) as recommended by [13]. The light intensity was kept at 480 $\mu mol/m^2\ s^1$ and the nitrogen content, pH and temperature were kept constant at 70 mg/L, 8.3-8.9 and 22°C, respectively. In the second set of experiments the effects of carbon dioxide concentration on the algae biomass and oil content were evaluated. Carbon dioxide was administered at concentrations of 3, 6 and 9% (v/v in air). The algae were exposed to full light exposure (24 h and the light intensity was kept at 480 $\mu mol/m^2\ s^1$). A combination of nutrients (ammonium nitrate, ammonium phosphate and ammonium sulfate) was used as nutrient for the freshwater microalgae and ammonium nitrate was used for the marine microalgae. The nitrogen content, pH and temperature were the same as in the first set of experiment. The best results obtained with CO_2 were compared with those obtained with $NaHCO_3$.

Preparation of Liquid Medium for Inoculum Growth

The freshwater microalgae medium was prepared on algal proteose medium (ATCC Catalog Medium No. 847, American Type Culture Collection, Manassas, Virginia, United States of America) and was made up by adding 1 g of proteose peptone (Difco 0120) to 1 L of Bristols solution (Table 1). Bristols solution was prepared by adding the following amounts from the prepared stock solutions: 10 mL of $NaNO_3$, 10 mL of $CaCl_2$, 10 mL of $MgSO_4\ 7H_2O$, 10 mL of K_2HPO_4, 10 mL of KH_2PO_4, 10 mL of NaCl, 0.05 mL of $FeCl_3$ and 940 mL of distilled water. The stock solutions were prepared as follows: 10 g of $NaNO_3$ in 400 mL of distilled water, 1g of $CaCl_2$ in 400 mL of distilled water, 3 g of $MgSO_4\ 7H_2O$ in 400 mL of distilled water, 3 g of K_2HPO_4 in 400 mL of distilled water, 7 g of KH_2PO_4 in 400 mL of distilled water and 1 g of NaCl in 400 mL of distilled water.

The marine microalgae medium was prepared in F/2 medium [23]. The trace element liquid medium stock solution (Table 2) was prepared by the addition of 4.16 g of Na_2 EDTA, 3.15 g of $FeCl_3 \cdot 6H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.022 g of $ZnSO_4 \cdot 7H_2O$, 0.01 g of $CoCl_2 \cdot 6H_2O$, 0.18 g of $MnCl_2 \cdot 4H_2O$ and 0.006 g of $Na_2MoO_4 \cdot 2H_2O$ into 1 L of

to 125 mL Erlenmeyer flask containing 25 mL of F/2 liquid media and then left to grow at room temperature for 2 weeks at a photocycle of 14 h light and 10 h dark. The mixture was then transferred to a 500 mL Erlenmeyer flask containing 250 mL of F/2 liquid media and was left to grow for 2 weeks at a photocycle of 14 h light and 10 h dark. Finally, the media was transferred from the 500 mL flask into a 30 L bioreactor containing 25 L of F/2 liquid media and left to grow for 2 additional weeks at a cycle of 14 h light and 10 hour dark.

Preparation of Algae Production Media

The freshwater production medium is a modification of the Fitzgerlad medium [24]. The preparation of the stock solutions for this media is shown in Table 3. The medium was made up by the addition of 1 mL of each of the stock solutions A, B, C and D to 1 L distilled water (Table 4).

A modification of the F/2 media [23] was used as the production medium for the marine microalga. The medium was modified by eliminating the addition of sodium nitrate. The medium consists primarily of autoclaved ocean water (Halifax Waterfront, Halifax, Nova Scotia, Canada). Table 5 shows the elemental analysis of the components present in the marine water which was performed at the Mineral Engineering Center of Dalhousie University.

Experimental Protocol

To each compartment in the open pond system a total of 4.75 L of freshwater production media was added. The amount of nutrient was added to the production medium. This solution was enriched with the desired carbon source (1.3 g/L of sodium bicarbonate, 3% CO_2, 6% CO_2 or 9% CO_2). To this, 250 mL of *Chlorella saccharophila* inoculum was added to each compartment. The cells were exposed to 24 h light and left to grow for 10 days. Every other day, 100 mL sample was taken for experimental analyses. The samples were analyzed for pH and biomass yield. At the end of the run the biomass was harvested from the liquid media using a Sorvall T1 Centrifuge (Thermo Scientific, Marietta, Ohio, United States of America). The supernatant from the centrifuge tubes was decanted and the cells were collected for biomass yield and oil content analyses. The marine medium was used with marine algae and the same procedure was followed using the ammonium nitrate nutrient system.

Microalgae Biomass Determination

The freshwater mciroagla yield was determined by measuring the optical density at 484 nm from a standard curve between the cell count and optical density. The number of Colony Forming Units (CFU) for *Chlorella saccharophila* was determined using a series of dilutions. A test tube containing 9 mL of autoclaved distilled water and a 1 mL aliquot sample was added to the tube. The contents of the tube were vortexed (Thermolyne Maxi Mix, Thermolyne Corporation, Hampton, New Hampshire, United States of America) to distribute the cells. A 1 mL aliquot of this solution was added to another tube that had been autoclaved with 9 mL of distilled water. This tube was again vortexed to distribute the cells. This was repeated 7 times to obtain dilutions of 1:1, 1:10, 1:100, 1:1000, 1:10 000, 1:100 000, 1: 1 000 000. For each of the dilutions made, 0.1 mL of the solution was added to a petri dish containing solid freshwater medium. The plates were sealed with parafilm, inverted and incubated at room temperature (~24°C) at a photocycle of 14 hours light and 10 hours dark for 3 days. The plates were then

Table 3. Formulation of stock solutions for *Chlorella Saccharophila* production medium.

Stock Solutions (per 200 mL)	Composition	
A	24.648 g	$MgSO_4•7H_2O$
B	1.360 g	KH_2PO_4
	8.700 g	K_2HPO_4
C	1.392 g	$FeSO_4•7H_2O$
	1.864 g	EDTA tri Na
D	0.620 g	H_3BO_3
	0.340 g	$MnSO_4•H_2O$
	0.057 g	$ZnSO_4•7H_2O$
	0.018 g	$(NH_4)_6Mo_7O_{24}•4H_2O$
	0.027 g	$CoCl_2•6H_2O$
	0.024 g	KBr
	0.017 g	KI
	0.023 g	$CdCl_2•5/2H_2O$
	0.091 g	$Al_2(SO_4)_3(NH_4)_2SO_4•24H_2O$
	0.040 mg	$CuSO_4•5H_2O$
	0.560 mL	H_2SO_4 (97%)

Table 4. Components of freshwater production medium.

Component	Amount (mL)
A	1
B	1
C	1
D	1
Distilled Water	996

Table 5. Elemental analysis of autoclaved ocean water used for marine production media.

Element	Amount (mg/L)
Na	10 254.00
Mg	1 078.00
S	1 010.00
K	395.00
Ca	386.00
Sr	6.79
Si	2.80
P	0.10
Ba	0.05
Al	0.05
Ni	0.04
Zn	0.02
Mo	0.01
Cd	0.01
Co	0.01
Cu	0.01

Microalgae Oil Content Using CO_2 as a Carbon Source

The oil yield results are depicted in Table 6. Analysis of the variance (ANOVA) was performed on the oil yield data as shown in Table 9. The effects of microalgae type on oil yield were significant at the 0.003 level. However, the effect of CO_2 concentration and the interactions between microalgae type and CO_2 concentration were not significant. Tukey's grouping was used to test the differences among the levels of each parameter as shown in Table 10. The two microalgae *Chlorella saccharophila* and *Tetraselmis suecica* were significantly different from one another at the 0.05 level. The highest mean oil yield (4.18%) was obtained from the freshwater microalgae species. The CO_2 concentrations were not significantly different from one another at the 0.05 level. The highest mean oil yield (3.16%) was obtained with the 3% CO_2 concentration.

Effect of Microalgae Type

The effect of the microalgae type on the oil content is illustrated in Figure 7. *Chlorella saccharophila* achieved the highest oil yields at all CO_2 concentration. It produced average oil yields of 4.71, 3.90 and 3.59% while *Tetraselmis suecica* produced average oil yields of 2.09, 1.01 and 2.43% at the 3%, 6% and 9% CO_2 concentrations, respectively. These results are similar to those of Pittman et al. [47] which indicated that the marine microalgae produce much lower oil yields compered to freshwater microalgae. The oil yields obtained from *Chlorella saccharophila* were 4 times higher than those obtained from *Tetraselmis suecica*, despite the higher biomass yields obtained from the *Tetraselmis suecica* species.

Sharma et al. [48] stated that the occurrence and extent to which lipids are produced by microalgae is species/strain specific. Pittman et al. [47] stated that different species use their energy for different metabolic pathways. In this study a trade-off between cell generation and lipid accumulation was seen among the species. The marine species used most of its energy for cell generation as opposed to lipid while the freshwater spices used most of its energy for oil accumulation as opposed to cell generation.

Demirbas [7], Moheimani, [33], Sobczuk et al., [49], Sukenik et al., [50], Wagenan et al., [51] and Pagnanelli et al., [52] reported a lipid content in the range of 36-47% and 15-23% for *Chlorella saccharophila* and *Tetraselmis suecica* species, respectively. The differences in the lipid content are attributed to the different nutrient systems used and the culture age before harvest.

Effect of Carbon Dioxide Concentration

The effect of carbon dioxide concentration on the oil yield is illustrated by Figure 8. As the carbon dioxide concentration was increased from 3% to 9%, the oil yield decreased from 4.7% to 3.6% for the freshwater microalgae (*Chlorella saccharophila*). On the other hand as the carbon dioxide concentration was increased from 3% to 6% the oil yield decreased from 2.1% to 1.0% for the marine microalgae (*Tetraselmis suecica*). However, a further increase in CO_2 concentration to 9% increased the oil yield to 2.43%.

It should be noted that the trends in Figure 9 are the opposite of the trends of cell yield shown in Figure 5. The higher oil yield obtained for the freshwater microalgae at the 3% CO_2 concentration can be attributed to the trade-off of lower cell generation, and the lower oil yields obtained when the CO_2 concentration was increased to 9% is a result of increased cell division. Similarly, the variation in lipid content for the marine (*Tetraselmis suecica)* microalgae species can also be attributed to the variation in biomass yield caused by variation in CO_2 concentration and the tolerance of the species to the acidity caused by higher CO_2 concentrations. The results showed that from an economic stand point, the 3% CO_2 concentration is the most optimal condition for lipid accumulation in both species.

Similar results were reported in the literature. Widjaja et al. [36] noted that the microalgae species *Chlorella vulgaris* in lipid yields of 20%, 28% and 25% at the CO_2 concentration of 0, 0.33 and 0.83%, respectively. Huang and Su [53] noted that for the microalgae species *Chlorella vulgaris* grown using 0%, 15% and 50% CO_2 concentrations resulted in lipid yields of 34%, 35% and 36%, respectively. The findings are similar to those obtained in this study since they indicate that varying the CO_2 concentration does not significantly influence the lipid content. The variation in oil yield can be attributed to the varying cultivation periods, variation in nutrient systems and the effectiveness of the oil extraction methods used.

Effect of Carbon Source

Biomass

The cell yields shown in Table 6 for *Chlorella saccharophila* species which was obtained using $NaHCO_3$ as

Table 9. Analysis of the variance for oil yield using CO_2 as the carbon source.

Source	DF	SS	MS	F	P
Total	17	58.206			
Model					
Algae Species (S)	1	30.147	30.147	14.26	0.003
CO_2 Concentration (C)	2	1.464	0.732	0.35	0.714
S*C	2	1.228	0.614	0.29	0.753
Error	12	25.367	2.114		

DF: Degree of freedom; SS: Sum of square; MS: Mean of square; R^2= 56.42%

Table 10. Tukey's grouping on oil yield by algae species and CO_2 concentration.

Factors	Level	N	Mean Yield	Tukey's Grouping*
Species	Marine Water	9	1.590	A
	Freshwater (v/v)	9	4.178	B
CO_2:Air	3:97	6	3.164	A
	6:94	6	2.994	A
	9:91	6	2.493	A

*Groups with the same letter are not significantly different from each other at the 0.05 level.

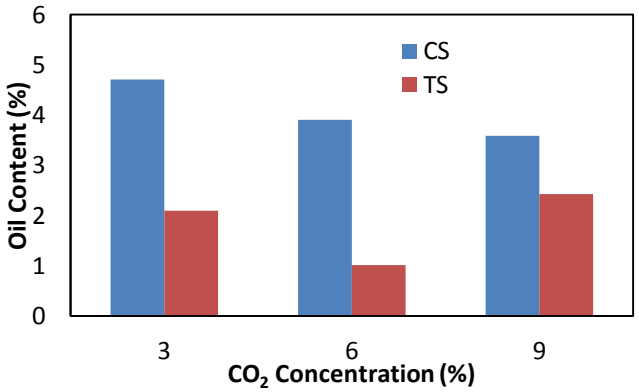


Figure 7. Effect of microalgae type on the oil yield at varying CO_2 concentrations (CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica*).

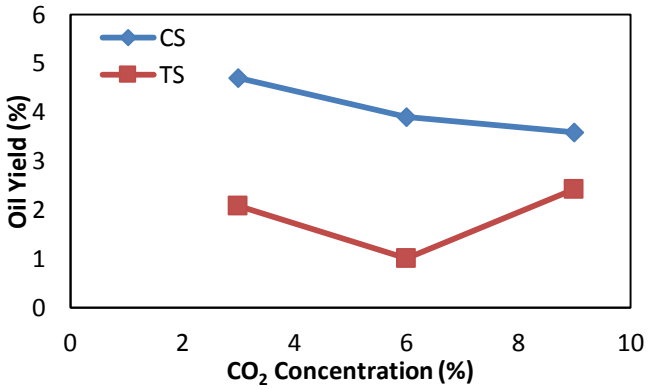
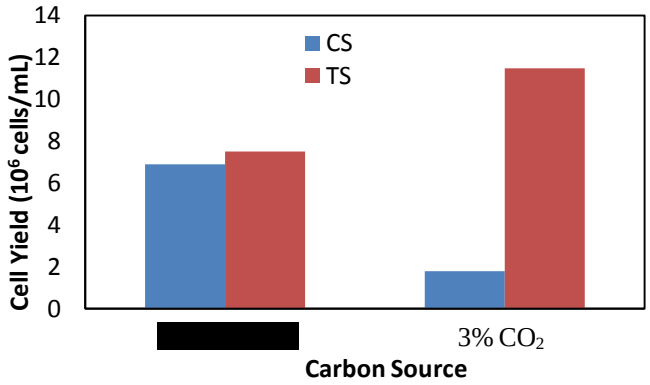
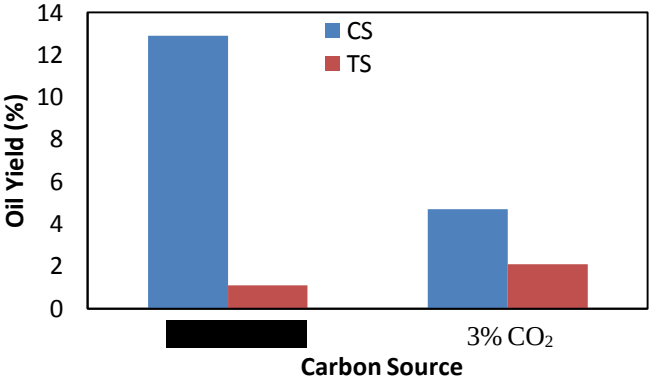


Figure 8. Effect of CO_2 concentration on the oil yield of freshwater and marine microalgae (CS-*Chlorella saccharophila*, TS-*Tetraselmis suecica*).

a carbon source ($0.689x10^6$ cells/mL) were 74% higher than those achieved using 3% CO_2 ($0.181x10^6$ cells/mL). The cell yield for *Tetraselmis suecica* species that resulted while using $NaHCO_3$ ($0.750x10^6$ cells/mL) were 53% lower than those achieved using 3% CO_2 ($1.148x10^6$ cells/mL). These results can be attributed to the cells ability to convert the carbon source into the preferred form for uptake and the abundance of the carbon source. The direct uptake of bicarbonate through an active transport system has only been noted in certain species [54]. In addition, some species have better extracellular carboanhydrase activities which allow them to convert the carbon source into different forms [54-56].



(a) Cell Yield



(b) Oil Yield

Figure 9. Effect of carbon source on the cell yield and oil yield of freshwater and marine microalgae (CS-*Chlorella saccharophila*, TS- *Tetraselmis suecica*).

Devgoswami et al. [46] studied the *Chlorella* microalgae species and noted biomass productivity of 82 and 189 mg/L/d using sodium bicarbonate and CO_2, as the carbon source, respectively. Moheimani [33] noted that the *Chlorella* sp. and *Tetraselmis suecica* grown using CO_2 as a carbon source resulted in biomass yields that were 6 and 23% higher than those obtained using $NaHCO_3$ as the carbon source. Goswami et al. [35] reported that the *Selenastrum sp*. grown using $NaHCO_3$ (20-100 mg/L) and CO_2 (4.4-8.2 g/L) resulted in biomass productivities in the range of 689-1102 mg/L/d and 667-889 mg/L/d, respectively. In this study, the biomass productivity achieved using $NaHCO_3$ and 3% CO_2 for *Chlorella saccharophila* was 106.8 mg/L/d and 28.1 mg/L/d, respectively. The biomass productivity achieved using $NaHCO_3$ and 3% CO_2 for *Tetraselmis suecica* was 116.3 mg/L/d and 177.9 mg/L/d, respectively. Variation in the values achieved in this study and those of the literature are attributed to the different species and different cultivation methods used.

Oil yield

The oil yields obtained with $NaHCO_3$ and 3% CO_2 as carbon sources are shown in Figure 9. The oil yield achieved

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