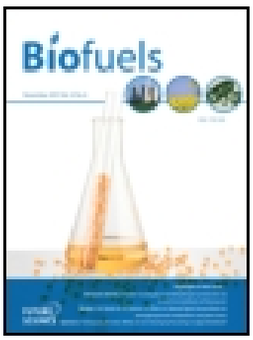

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Potential production of biodiesel from green microalgae

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ABSTRACT

This work involves the study of the potential production of biodiesel from green microalgae. The green microalgae were cultured and treated with different nutrient concentrations of 0, 0.175, 0.35, 2 and 4 g in the laboratory where they were later centrifuged. The 4 g treatment had the highest growth of 0.186 cell/h, followed by the 2 g treatment of 0.151 cell/h within 24 h. The 0, 0.175 and 0.35 g had similar growth rates which were lower than the 4 and 2 g. Oil was extracted from the centrifuged cultured algae sample and the raw algae sample using hexane via Soxhlet extractor. The total oil extracted from 15 g of the centrifuged sample dry biomass and 30 g of the raw sample dry biomass were 2.038 and 3.826 g, giving a yield of 13.6 and 12.8%, respectively. The amounts of hexane recovered from the centrifuged sample and the raw sample were 46.67 and 53.33%, respectively. The results suggest hexane can be used to extract algal oil which can be transesterified to biodiesel which is more environmentally friendly than fossil diesels.

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Green microalgae; biodiesel; Soxhlet extractor; hexane; algal oil

Introduction

The big picture of energy demand in the world is based on the need for electricity, heat and transport. However, the majority of energy worldwide is provided by fossil fuel sources. There are major challenges facing the use of fossil fuel. For instance, rapid depletion of the stocks of fuel, price increments, coupled with carbon dioxide emissions to the atmosphere resulting in global warming. This has necessitated the need to switch from conventional fossil fuel energy sources to alternative energy sources which release less carbon dioxide [1,2]. Biofuels and biodegradable fuel sources as renewable energy have gained substantial attention [3–5]. The need to reduce dependence on petroleum-based fuels as well as to tackle the problem of global warming has led to an increased interest in algae-based biofuels [6,7]. One source that has gained most attention is green microalgae for biodiesel production. This is due to several advantages of green microalgae: high growth rate [6], short time to maturity stage [8], large biomass production and small land area requirement [1–3] and biodegradability of the biodiesel [4]. Other key advantages are high composition of fatty acid containing compounds which are the best component for the production of biodiesel and can be controlled by varying the growth conditions [9–13], the ability to produce several different types of lipids based on their species [7], the potential to fix 40–50% of global organic carbon such as CO_2 (although they constitute only 0.2% global biomass) via photosynthesis [14]. Green microalgae also release significant amounts of oxygen to the atmosphere, thus supporting life on our planet as shown in Equation (1):

$$6\,CO_2 + 6\,H_2O + \text{light energy} \rightarrow C_6H_{12}O_6(\text{sugars}) + 6\,O_2 \quad (1)$$

Production of oil from microalgae has been extensively studied. Chisti reported the oil content in 14 different microalgae species to be 15–75% dry weight [11]. Oil produced from microalgae was 15–300 times greater than other conventional crops on basis of area [15]. Different solvents and different combinations of solvents for algae oil extraction have been employed: Folch method (2:1 v/v chloroform-methanol) [16], Bligh and Dyer method (1:2 v/v chloroform-methanol) [17] and Matyash method, a modification of the Folch and Bligh and Dyer method with methyl-tert-butyl ether [18]. However, these combinations or methods are environmentally unfriendly when used on a large scale [19]. Thus, superior solvents such as ethanol, butanol, hexane, esters, ethers and their respective combinations have been tried [20]. For example, 7.3 and 9.2% of alga oils were produced from two microalgae species, *Spirogyra* sp. and *Oedogonium* sp., respectively, using hexane-ether solution [21]. Also, 62.04 ± 2.42% and 40.71% ± 4.26% algae oils were extracted from the microalga *Scenedesmus obliquus* using ethanol and hexane, respectively [22]. Though the ethanol had higher yield, it also extracted green pigments which

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required further purification, thereby making the use of hexane more suitable. Using hexane alone had been reported to be feasible and effective [23,24]. In addition, hexane is less expensive hence can be used in a large scale to reduce production costs.

This work investigated the possibility of producing algae oil from microalgae using hexane as an extracting solvent via Soxhlet extractor. Inorganic fertilizer was used as nutrient treatment during culturing of the freshwater green microalgae [25] which were characterized using a digital microscope. The growth rates and algae oil yields are presented and discussed.

Materials and method

Materials and equipment

Liquid hexane, ethanol (70%), iodine tincture and diethyl ether were obtained from the Chemistry Department of the University of Cape Coast-Ghana chemical store room. Yara Vita fertilizer was purchased from a licensed fertilizer retailer in Cape Coast Kotokruba market. Whatman filter paper number 4, pipette, hot plate, beakers, microscope (1000x), test tubes, two Soxhlet extractor set ups, Gilson pipette, 500 ml flat bottom flasks, centrifuge (brand 5702R), zooplankton and phytoplankton net, rotary evaporator, Eppendorf tubes and hemocytometer were obtained from the Chemistry and Microbiology Departments of the University of Cape Coast-Ghana.

Sampling and sample preparation

A sample was taken from the science botanical garden pond behind the science building of the University of Cape Coast-Ghana. The pond was easily accessible. The nature and location of the pond indicated the presence of phytoplankton (freshwater green microalgae). The sampling was conducted twice. The first sample was collected for centrifugation and serial dilution for the growth process. In that case, 1 L of the pond water which contained the microalgae was taken to the laboratory. The algae were concentrated and prepared as stock for the serial dilution by centrifuging at 1000 rpm for 4 min [26]. The second sample (the raw sample) was collected for sun drying without centrifugation. Thus 5 L of the pond water was collected and sun dried.

Isolation of algal species using serial dilution method

Serial dilution methodology was utilized by a chemist in the chemical laboratories to achieve minute and fine molar concentrations of chemicals and compositions. These studies applied the same methodology to isolate (separate) and categorize individual colonies present in the sample collected. It was realized that it would be cost effective to isolate and collect a particular colony from the freshwater microalgae which was sampled rather than buying those algal samples.

To start the serial dilution method, all apparatuses which were used for the process were cleaned to avoid contamination. Ten test tubes were arranged in a test tube rack and filled with 9 ml of distilled water. Then 1 mL of the centrifuged stock sample which had higher concentrations of the microalgae was transferred into the first test tube: 1 ml was taken from the first test tube and poured into the second test tube; 1 ml from the second test tube was taken and poured into the third test tube. The process was repeated for the 10 test tubes as depicted in Figure 1. Once the process was completed, three of the test tubes were randomly selected to serve as inoculum for the culturing process. Three test tubes were replicated of one another. This procedure was repeated five times for four different nutrient concentration treatments and a control setup.

Microalgae were grown over seven successive days in 500 mL flat bottom flasks illuminated from above with a 5200 lux daylight and/or fluorescent light.

Reproducibility experiments were conducted using three replicates for each nutrient treatment and kept under constant aeration. The culture growth was monitored closely to determine the variation in the number of cells throughout the period of growth.

A hemocytometer was used to count the algae cells. A growth curve was derived for the respective treatment and regulated to a model to differentiate between the different stages of growth. The growth rate was evaluated by applying the exponential phase of the growth curve, which characterizes the number of cellular splitting per day and the subsequent replication time. The stipulated division time or generation time was determined from the growth rate as reported by Stein [27].

Media preparation

Inorganic nutrients were applied in this study. Yara Vita fertilizer of the following composition was used to prepared nutrients of known concentrations for the growth process: 5% Boron (B), 5% Zinc (Zn), 0.1% Copper (Cu), 0.1% Manganese (Mn), 0.1% Molybdenum (Mo), 5% Nitrogen (N), 7.5% Phosphate (P_2O_5), 5% Potash (K_2O), 5% Magnesium (Mg), 5% Sulphur (S). This served as a media for the cells of the freshwater microalgae to use as their source of food for growth/multiplication, and any essential alterations (addition of fresh water/evaporation) completed should also effect nutrient concentrations changes in the system.

A total of 0.175, 0.35, 2 and 4 g of nutrient were weighed using a weighing balance and then dissolved in a small amount of distilled water in a 200 ml beaker. Further distilled water was added to top up the beaker to the 200 ml mark. The volume of the mixture was then suction filtered to remove the particles which

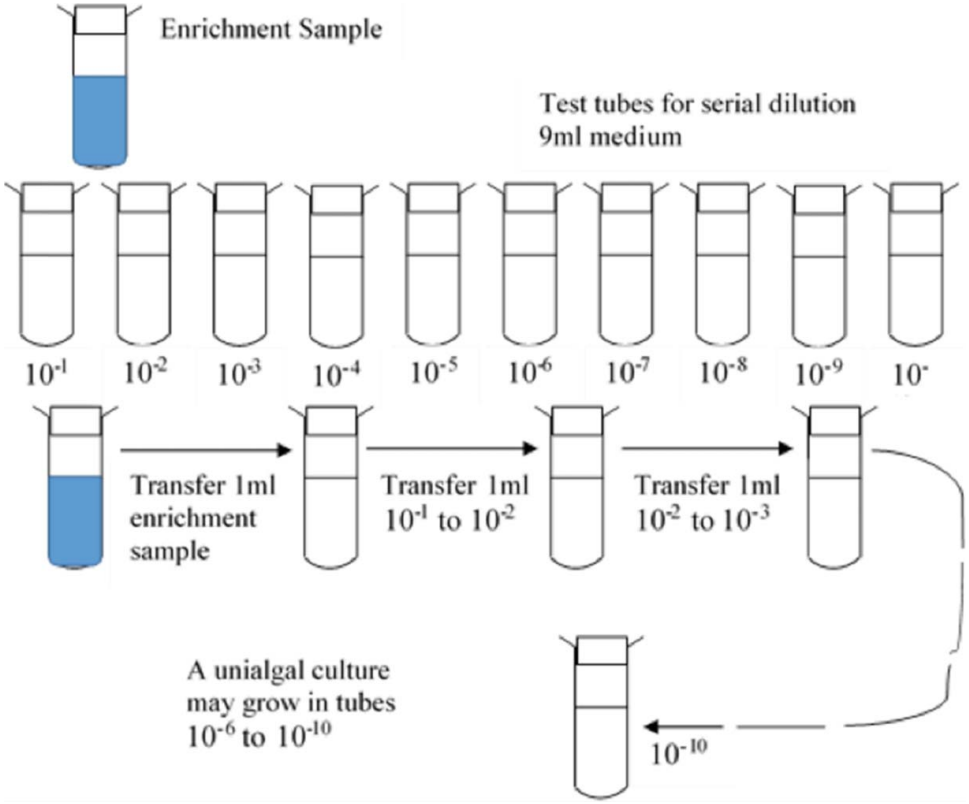

Figure 1. Schematic representation of the serial dilution method.

remained in the water after the dissolution. The 200 ml nutrient solution was poured into a 500 ml flat bottom flask, 300 ml of distilled water was added to make up to 500 ml. This process was repeated twice more. The three test tubes which were randomly selected as the inocula were then added separately to three flat bottom flasks for culturing. All measurements were triplicated for effective reproducibilty.

The concentrations of the measured masses of the Yara Vita fertilizer were calculated to be 350, 700, 4000, 8000 and 0 mg/L for 0.175, 035, 2, 4 g and control, respectively. In the process of preparing a stock solution which contained a mixture of compounds as in the case of Yara Vita fertilizer, it was prudent to dissolve each individually in a minimal volume of water before mixing, then combine and dilute to volume. After the nutrient and the samples were combined in the flat bottom flasks, the treatments were then monitored every 24 h for cell count using a microscope and a hemocytometer.

Preparing the sample for hemocytometer count

A total of 1 ml of the medium in the 500 mL flat bottom flask was drawn with a pipette containing the cells of the freshwater microalgae and was appropriately prepared before applying onto the hemocytometer. The hemocytometer was cleaned using 70% ethanol. The shoulders of the hemocytometer were moisturized and the coverslip was affixed using mild pressure and tiny circular motions. The phenomenon of Newton's rings was seen when the coverslip was correctly affixed, hence the depth of the chamber was confirmed.

Preparing the cell suspension

The cell suspension to be counted was properly mixed by gently swirling the flask holding the cells. A total of 1 ml of cell suspension was sampled in an Eppendorf tube before the cells could settle. Pipetted cells were gently mixed to avoid lysing of the cells. Approximately 100 μL was drawn and placed into a new Eppendorf and two drops of iodine tincture was added to the Eppendorf tube. This was mixed gently to aid the cells in the counting procedure.

A Gilson pipette was used to sample cell suspension containing iodine tincture. The hemocytometer was carefully filled by gently placing the end of the Gilson tip at the brink of the chamber. Maximum care was taken in the process not to exceed the capacity of the chamber.

The sample was taken from the pipette by the application of capillary action and care was taken for the sample to flow to the brink of the grooves. The pipette was reloaded and filled for the second chamber. The hemocytometer was placed on the microscope stage. The microscope was focused on the grid lines of the hemocytometer employing the 10x objective lens. The set of 16-corner square of the hemocytometer was focused on as shown by the circle in Figure 2.

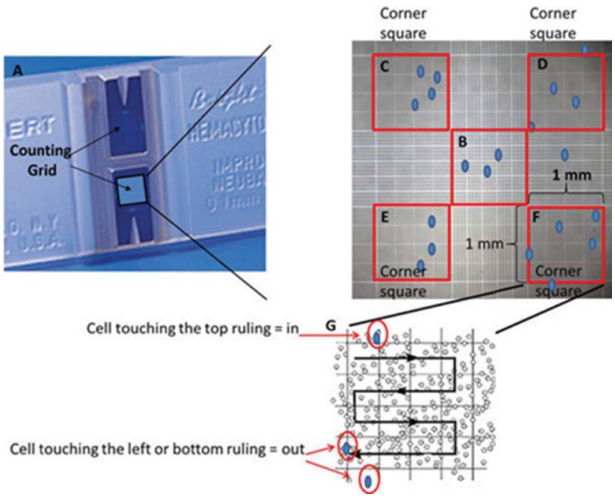

Figure 2. Depiction of the hemocytometer used to count algal cells.

Calculating cell density

Some basic calculations are needed to evaluate cell density. Measurements required: number of cells in a square, area of the square, height of the sample, and dilution factor. The objective was to find the number of cells in 1 ml of original solution.

The algal cells were seen as blackish spots and sometimes in a colony. Cell concentrations were calculated as expressed in Equation (2):

$$\frac{cell\ counts\ x\ dilution\ factor}{area\ (haemocytometer)\ x\ depth\ (haemocytometer)}\left(\frac{cells}{ml}\right) \quad (2)$$

The cell count was done every 24 h for seven days and recorded. The data from the cell counts was used to obtained a growth curve for the freshwater microalgae.

Identification of algae

The cultured freshwater green microalgae were visually observed and characterized using a microscope. The identification was performed with the aid of a phytoplankton identification manual [28]. When viewed under the microscope, most of the cells were green which categorized them as chlorophyte (green microalgae) as seen in Figure 3.

Dewatering

Dewatering removes/separates the water from the freshwater microalgae which has been harvested from a freshwater pond. This was conducted in two ways. First, the dewatering process, where the microalgae were extracted from the medium using a centrifuge (brand: 5702 R) to concentrate the biomass and then sun dried to remove the remaining moisture from the microalgae. Second, after the freshwater microalgae was sampled from the freshwater pond with the aid of the zooplankton and phytoplankton net, 5 liters of the microalgae was sun dried for five days at ambient temperature 25–28°C without centrifugation.

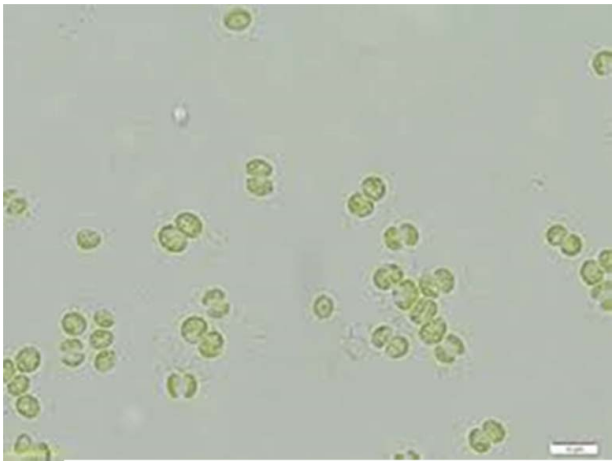

Figure 3. Green microalgae capture using a microscope.

Lipid extraction using hexane as solvent

The sun-dried algae were crushed into a fine powder. The algae powder was then dispensed into a paper container and enclosed to withstand any solid algae discharge. The container was arranged within an extraction chamber and successfully prepared for hexane extraction as shown in Figure 4. Two Soxhlet extraction set-ups were prepared for the extraction process: 150 ml of hexane was added to the distillation flask of the first set-up which contained the 15 g of the centrifuged cultured dry sample; 300 ml of hexane was added to the second set-up which contained 30 g of the raw dry sample. The extraction process was carried out at 65°C for 60 cycles in 3 h. The crude oil was

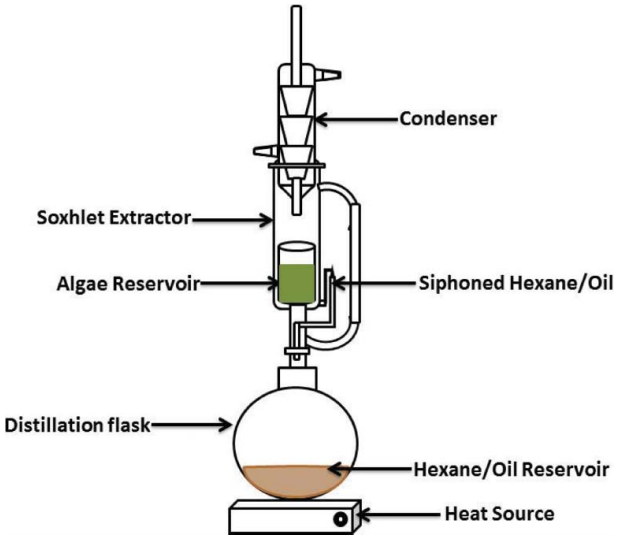

Figure 4. Soxhlet extraction set up for the algae oil extraction process.

collected by evaporating the hexane from the hexane/oil reservoir using a rotary evaporator [29].

Tests for the presence of lipids

This is a qualitative experiment which depicts the presence of oil in a substance. This qualitative test was performed to determine the presence of lipids in the algae oil extracted.

Translucency test procedure

This involved filter paper, a hot plate and ether solvent. Filter paper was taken and a drop of the algae oil was placed on it. The filter paper was placed on the hot plate and heated to 60°C for 5 min. The filter paper was removed and immersed in ether, after which it was air dried and the spot examined for translucency.

Results and discussion

Treatment of cultured algae with varying amount of Yara Vita nutrients

The serial dilution method performed resulted in the growth of unialgal species of freshwater microalgae in the flasks. The cells were grown over a period of 24–264 h at increments of 24 h. The isolation process using the serial dilution method was done to achieve one or two species of freshwater microalgae growing in at least one of the flasks. Table 1 shows the growth rates of cultured algae treated with variable amounts of Yara Vita nutrients (0, 0.175, 0.35, 2 and 4 g) labelled as A, B, C, D and E. It was observed that as the nutrient concentration increased, the growth rates generally increased from left to right of Table 1. Algae growth rates for the control, 0.175 and 0.35 g were similar with differences of ±0.000 to ±0.009 (0–22.5%). For example, the algae growth rates for the control, 0.175 and 0.35 g at 24 h were 0.040, 0.031 and 0.031 cell/h, respectively, whereas at 240 h were 0.003, 0.003 and 0.004 cell/h, respectively. Nutrient concentrations of 2 and 4 g resulted in exponential algae growths as compared to control, 0.175 and 0.35 g over the entire duration. This effect is clearly depicted in Figure 5 which indicates the growth phases for the cultured microalgae species (lag, exponential, phase of declining and death phase).

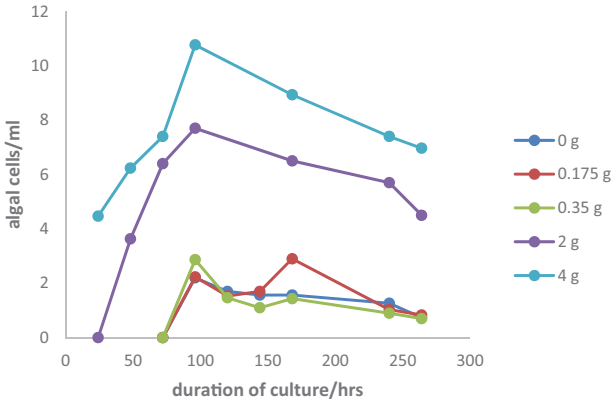

Figure 5. Effects of different nutrient concentrations of Yara Vita fertilizer on the growth of the freshwater microalgae.

From Table 1 it can be observed that cell count was not monitored 24/7. Cell count was not recorded at 24 and 48 h for control, 0.175 and 0.35 g treatments. The maximum growth for 2 and 4 g treatments occurred at 24 h after which the algal started reducing in growth, hence the growth at 120 h and 144 h were ignored and continued with 168 h.

The 4 g showed the highest growth followed by the 2 g as shown in Figure 5. This growth may be a result of the higher nutrient contents of 2 and 4 g concentrations. This suggests that a sufficient amount of nutrients is a key condition required for algae growth when cultured, although it must be regulated. Growth rates in the first 72 h for the 0, 0.175 and 0.35 g sample treatments were higher than the rest of the durations.

This may be attributed to decreasing nutrient concentrations, hence the decreasing growth rates of the algae in subsequent hours.

Table 2 shows the result for analyses of variance analysis (ANOVA) for the various treatments. Cultures of nominally the same species often showed wide variations in cell growth rates.

Comparing the control medium with the 4 g nutrient treatment, which was the best result obtained,

Table 2. ANOVA results for the data obtained during the cell count period.

Source of Variation	SS	*df*	MS	$F_{observed}$	*p*-value	$F_{critical}$
Between groups	227.31	4	56.83	39.45	1.53E-11	2.69
Within groups	43.21	30	1.44			
Total	270.52	34				

Table 1. The growth rates of cultured algae treated with variable amounts of Yara Vita nutrients.

Duration culture/h	Control (A)	Growth rate (cell/h)	0.175g (B)	Growth rate (cell/h)	0.35g (C)	Growth rate (cell/h)	2g (D)	Growth rate (cell/h)	4g (E)	Growth rate (cell/h)
24	–	–	–	–	–	–	3.6 ± 1.3	0.151	4.5 ± 1.8	0.186
48	–	–	–	–	–	–	6.4 ± 1.2	0.133	6.2 ± 2.2	0.130
72	2.9 ± 1.2	0.040	2.2 ± 0.8	0.031	2.2 ± 1.0	0.031	7.7 ± 1.8	0.107	7.4 ± 2.4	0.103
96	1.5 ± 0.9	0.015	1.5 ± 0.4	0.016	1.7 ± 0.8	0.018	6.5 ± 2.1	0.068	10.8 ± 2.7	0.112
120	1.1 ± 0.3	0.009	1.7 ± 0.4	0.014	1.6 ± 0.1	0.013	–	–	–	–
144	1.4 ± 0.2	0.010	2.9 ±	0.020	1.6 ± 1.2	0.011	–	–	–	–
168	0.9 ± 0.4	0.005	1.0 ± 0.2	0.006	1.3 ± 0.6	0.008	5.7 ± 0.8	0.034	8.9 ± 1.6	0.053
240	0.7 ± 0.1	0.003	0.8 ± 0.1	0.003	0.7 ± 0.2	0.004	4.5 ± 0.9	0.019	7.4 ± 1.8	0.031
264	0.7 ± 0.1	0.003	1.6 ± 1.4	0.006	1.0 ± 0.1	0.004	4.8 ± 1.1	0.018	7.0 ± 1.3	0.026

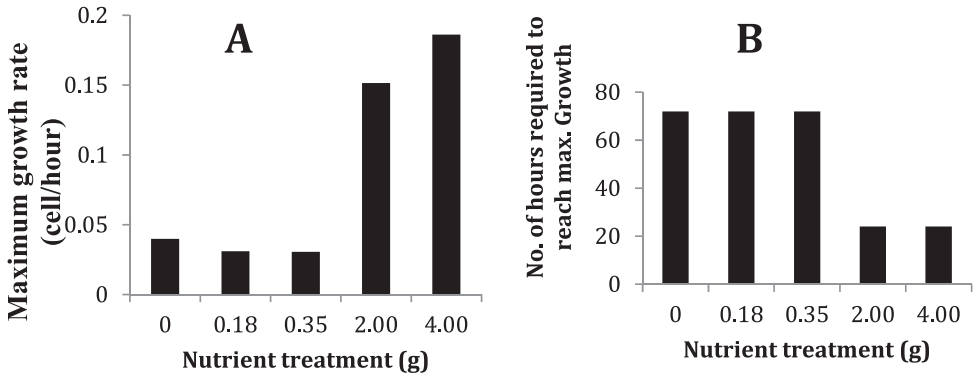

Figure 6. (A) Maximum growth rate and (B) time taken for nutrient treatments.

there were significant differences with respect to the cell density. The *p*-value was less than 5% ($p < .05$) which is indicated by $F_{critical} < F_{observed}$ (2.6896 < 39.4548).

In Figure 6A, the growth rate of the cells generally increased proportionally with increased nutrient in the medium of the culture. The experimental nutrient treatment of the 4 g showed significant changes as compared to the other treatments. The 2 g experimental nutrient treatment also showed significant differences when compared with the control, 0.35 and 0.175 g experimental nutrient treatment. This indicates that the nutrients applied in the culturing process had positive impacts on the cell growth. The confidence interval for the 4 g treatment was 7.46 ± 2.04. Figure 6B shows the time taken for each of the treatments to reach their maximum growth rate. The growth rate of each of the treatments was found by dividing the mean cell counts by the growth period. The maximum growth rate for each of the nutrient treatment was selected. The time taken for each treatment to reach their maximum growth rate was determined from the duration of culture.

It was observed that the first three treatments reached their maximum growth after 72 h of the cultured period while the last two treatments reached their maximum growth within 24 h of the growth period. This indicates that the higher the nutrient concentration, the faster the growth of the freshwater microalgae.

Soxhlet extraction of oil from the algae

A total of 15 and 30 g, respectively, of the centrifuged cultured sample and the raw sample biomass were used for the extraction process. The translucent test indicated the presence of lipids in the samples implying that the oil extracted can be transesterified to produce biodiesel using methanol and a catalyst. Table 3 shows the amount of solvent recovered and the mass of oil obtained from the algae. About 46.67% of the hexane was recovered from the centrifuged sample and it was lower than the hexane recovered from the raw sample (53.33% recovery). Thus, 150 ml of hexane was used for 15 g of the centrifuged sample and a recovery of 80 ml was made, whilst 300 ml of hexane was used for 30 g of the raw sample and a recovery of 140 ml was obtained.

Table 3. Solvent recovery and the mass of oil obtained.

	Centrifuged cultured sample	Raw dried sample
Initial volume of solvent	150 ml	300 ml
Solvent recovered at the end of extraction	80 ml	140 ml
Mass of algae oil obtained	2.04 g	3.83 g

The higher solvent recovery in the raw sample as compared to that of the centrifuged cultured sample may be due to the higher amount of solvent used for the raw algae though by mass it was doubled proportionally to the volume of solvent.

Figure 7shows the yield of the samples used in the extraction process. It was observed that 2.038 and 3.826 g of oils were produced from a biomass of 15 g of the centrifuged cultured sample and 30 g of the raw sample, respectively. The centrifuged culture sample had 13.6% yield which was higher than the raw sample yield of 12.8%.

This might have occurred as a result of the presence of other materials in the raw sample which hindered the extraction of the oil from the microalgae. However, since microalgae grow rapidly and abundantly

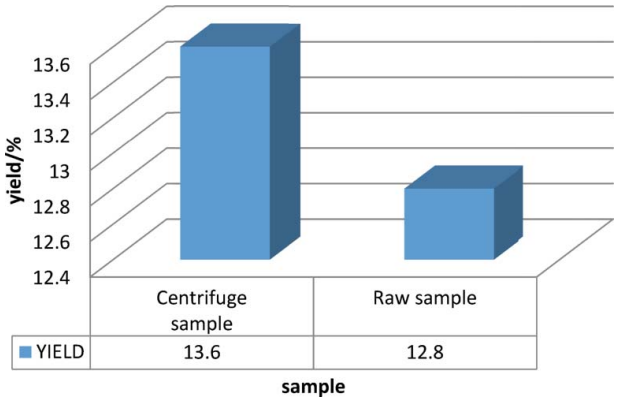

Figure 7. Oil yield of the centrifuged cultured and the raw sample via extractor.

throughout the year, and the method of oil extraction is simple, the 12.8 and 13.6% yield can be optimized to produce better yield. Similar studies reported lower yields of 4.8 [30], 7.3 and 9.3% using different algae species [21].

Conclusion

This present study investigated the potential production of biodiesel from green microalgae. The results of the study revealed that the cultured algae treated with 4 g Yara Vita nutrient had the highest growth of 1.186 cell/h, followed the 2 g treatment, and they were recorded within the first 24 h. Contrarily, the lower treatments (0, 0.175 and 0.35 g) reached their maximum growth of 0.04, 0.031 and 0.031 cell/h, respectively, within 72 h. These imply that the higher the nutrients concentration, the faster the growth of the freshwater microalgae. Also, 0.038 g of oil was generated from 15 g of the centrifuged cultured algae giving a yield of 13.6%. Conversely, 3.826 g of the oil was obtained from 30 g of the raw algae sample giving a yield of 12.8%. The lower yield in the raw sample compared to the centrifuged sample may be attributed to the presence of impurities which might have hindered the oil extraction.

The results of the study therefore suggest that biodiesel which is more environmentally friendly than fossil diesel can be produced from the abundant and sustainable green microalgae using hexane for less $CO_{2(g)}$ emissions. Moreover, since algae grow easily and abundantly throughout the year, obtaining a yield of 12.8 or 13.6% is very significant.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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