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Biochemical Composition of *Chlorella vulgaris* Grown on Sugarcane Molasses

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The quest for green and sustainable biofuel to serve as alternative to the conventional fossil fuel have remained a grey area in biotechnology. The *Chlorella vulgaris* was isolated from the African Regional Aquacultural Centre Aluu, Port Harcourt, Nigeria. Sugarcane Molasses modified Bold Basal medium was used to cultivate the Microalgae mixotrophically. The algal culture was incubated at room temperature for 15 days with continuous aeration and 12:12 hour photoperiod under artificial illumination of 2000 lux. The proximate composition of the biomass showed 6.28%wt, 67.37% wt and 11.35%wt of moisture, volatile organic matter and Fixed carbon content respectively. The ultimate composition of *Chlorella* biomass revealed that Carbon was 42.46% while Oxygen content was 27.93%. Nitrogen content was 6.62% while Sulphur content was 0.82% while hydrogen content was 6.74%. The study further identified that algal biomass from *C. vulgaris* has the potential of serving as both nutraceuticals and bioenergy feedstock. There is need for further studies around the algae oil oriented optimization as a veritable tool for biotechnological advancements.

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1. INTRODUCTION

The Global energy consumption is has evolved over the years and this has remained complex for quite some time now. These changes have mainly attributed to population, economic activity, commercial and technological advancements increases, so also the energy-use and its fluctuations. Hence, the global energy-use varies across the world depending the available resource and technological advancements [1]. Although fossil fuels are still being produced, under various geochemical processes, they are consumed faster than they are formed. The sources of these fuels are therefore finite and exhaustible (Gollakota et. al. 2018). In addition, fossil fuels are found to be major contributors to greenhouse gas (GHGs) emissions to the biosphere, and in 2006 energy associated CO_2 emissions were estimated at 29 Gtonnes [2]. The treaty signed in 1997 in Kyoto, known as Kyoto protocol, advocated for a 5.2% reduction in worldwide greenhouse emissions from the 1990 levels [3].

Microalgae are photosynthetic microorganisms that utilize sunlight, CO_2, minerals and wastewater. They do not require large area of arable land for their cultivation, compared to terrestrial plants [4]. They have been used in the production of animal feed, cosmetics, polymers and cosmetics [4], but the current interest in the use of Microalgae is for the production of biofuels, was motivated because they can accumulate as much as 70% of the dry weight large lipid fractions and other compounds. However, Microalgae was found to remediate effluents [5], hence suitable for growth in wastewater feedstock. Microalgae require carbon dioxide, light, pH, temperature and nutrients. Carbon dioxide supplies carbon for the production of Biomass, the sources of CO_2 for Biomass production can come from industrial exhaust (15% CO2 above). Light supplies energy, though if it is much can affect growth due to photoinhibition. pH provide suitable medium for growth, a pH of 6-8 though there exist acidophilic algae that can grow in pH as low as 2-3. Temperature (mainly 20-30 degrees) for ideal growth, though biomass production increases with increase in temperature. However different species have different adaptability with respect to pH and temperature [6]. Generally, extraction and production of biofuels from microalgal biomass is more expensive and technologically more challenging, than growing crops. Its production requires light, inorganic nutrients, water, CO_2 and temperature regime that has to be controlled and monitored closely [7,8].

Biofuels are referred to liquid, gas and solid fuels principally produced from biomass. A variety of fuels can be produced from biomass such as ethanol, methanol, biodiesel, Fischer-Tropsch diesel, hydrogen and methane' (Demirbaş, 2006). Therefore, biofuels are renewable fuels created from animal fats or plant oils. They are cleaner than petroleum based diesel, non-toxic, and biodegradable [9]. Biofuels are classified into Primary; which include firewood, wood chips, pellets, animal waste, forest and crop residues and secondary fuels which are categorized into 1st, 2nd and 3rd Generation fuels. Compared to other source of biofuels, microalgae is the most important renewable fuel crop because of the following advantages [10-13]: Higher yield of Biomass and fuels, Higher growth rates and photon conversion efficiency, Higher CO_2 sequestration, Does not necessarily require fresh water to grow, because it can grow on brackish/saline water, seawater and waste water. Microalgae can utilize phosphorus and Nitrogen

Table 1. Chemical composition of some selected Algal species [6]

Microalgae species	Lipid content (%)	Protein (%)	Carbohydrates (%)
Chlorella vulgaris	14–22	51–58	12–17
Chlorella pyrenoidosa	2	57	26
Scenedesmus obliguus	12-14	50-56	10-17
Scenedesmus dimorphus	16-40	8-18	21-52
Prymnesium paryum	22-38	28-45	21-52
Spirulina maxima	6-7	60-71	13-16

from waste water sources (e.g. industrial and municipal wastewaters, agricultural run-off and concentrated animal feed operations); thereby achieving waste water bioremediation. Microalgae growth does not compete with arable land for agricultural production because it can use marginal areas like seashores land and deserts which are unsuitable for agricultural purpose. Microalgae growth does not require the use of fertilizers and pesticides, thereby reducing environmental pollution. Microalgae production is not seasonal and can grant multiple harvests. It allows the inducement of cultures thereby achieving high concentration of feedstock like biomass, oil and starch. Microalgae have negligible environmental impact like deforestation. After extraction of Oil, microalgae residue produces by-products like biopolymers, proteins, animal feeds, pigments, polysaccharides and fertilizers. Microalgal biomass is also a renewable source for nutraceuticals, aquaculture feed, fine chemicals, and cosmetics.

Chlorella vulgaris is a unicellular *e*ukaryotic organism having an average diameter of about 3 µm [14]. Though *Chlorella* is an inhabitant of fresh water it can still grow in wastewater and some of its species can grow in marine environment. However, it is resistant to different cultivation conditions and temperature ranges (15 and 40˚C) [15,16]. Chlorella's cell rigidity is connected to its hemicellulotic cell wall. Moreover, the main body of cells in flagellate species and non-flagellate species is protected by glycoprotein and firm polysaccharide walls respectively. Open pond systems utilize Autotrophic cultivation for *Chlorella* strains while mixotrophic and heterotrophic cultivations are mainly achieved by addition of limiting nutrients which could be used in agro-allied services [14,15,17]. Because of its rich and diverse uses; *Chlorella* is the most cultivated microalgae and has been used in the production of many industrial products. *Chlorella* is rich in lipid, protein, vitamins, antioxidants, minerals, carotenoids and polysaccharides [17].

2. MATERIALS AND METHODS

2.1 Water Sample Collection

Water sample was collected from New Calabar River, Port Harcourt, Rivers State. Water Sample was collected in a sterile container and was transferred to laboratory for the study. The microalgal culture (*Chlorella vulgaris*) was obtained from a Fish pond in African Regional Aquacultural Centre Aluu, Port Harcourt, Rivers state.

2.2 Growth conditions and Monitoring

Chlorella spp. was isolated from African Regional Aquacultural Centre, Aluu, Rivers State Nigeria. The pure strain was isolated using a solidified Brilliant Green media, supplemented with 100µg/ml Nystatin and 62.5 µg/ml Chloramphenicol and 0.02mg/L Cyanocobalamin was added as a source of trace elements. Additional *Chlorella* sp. biomass was obtained from the Department of Microbiology, University of Port Harcourt. The algal culture was incubated at room temperature for 15 days with continuous aeration and 12:12 hour photoperiod under artificial illumination of 2000 lux. The microalgal culture was separated by centrifuging at 4000 xg. The biomass pellet was dried at 80^0C for about 1 hour until stable weight has obtained. The biomass productivity was calculated using the standard formula. The biomass accumulated was measured using UV visible spectrophotometer at wavelength of 680 nm while the cell dry weight was determined from the cell pellets after centrifugation and dewatering. The pellets were dried in a muffle furnace at over 600^oC. The dried pellets were placed in a desiccator over night after which the biomass was weighed in triplicates.

$$Biomass\ productivity\ \left(\frac{g}{L}.d\right) = \frac{1}{t}\frac{(w_2 - w_1)(g)}{volume(L)} \quad (2.1)$$

Where w_1 = Initial weight (g), w_2 – Final weight (g), t-time/duration of the experimental run (day).

The biomass productivity was calculated using the following formula

$$Lipid\ productivity\ \left(\frac{g}{L}.d\right) = \frac{Lipid\ content\ of\ cells(g/g)\ x\ Dry\ cell\ weight\ (g/L)}{Cultivated\ period(d)} \quad (2.2)$$

2.3 Proximate and Ultimate Analysis

Waste paper was used as a binder to convert the algal biomass into pellets. Proximate analysis followed the ASTM standard (D5373-02) and the ultimate analysis was conducted using and (ASTM 2003; Jenkins et al. 2008)

2.4 Proximate Analysis

The moisture content was determined by weighing 3g of the biomass into a ceramic crucible and it was heated to about 105°C for 2 hours. Subsequently, the dried biomass was weighed and the content was expressed in percentage (2.3). However, the Ash content was determined by weighing 3g of the biomass into a ceramic crucible and it was heated to about 550°C in a muffle furnace for 3 hours and it was calculated using equation 2.4. And the volatile matter was determined by subjecting the biomass to a temperature of 950˚C for about 7mins then calculated using equation 2.5. However, the fixed carbon was determined by difference using equation 2.6

a. Moisture Content

$$Moisture\ Content\ (\%) = \frac{(g-x}{g}\ x\ 100 \quad (2.3)$$

Where g is the weight of sample, x is the Weight of after drying (g –x) is the Loss in weight

b. Ash Content

$$Ash\ (\%) = \frac{x}{g}\ x\ 100 \quad (2.4)$$

g is the weight of Sample and x is the weight of ash

c. Volatile Matter

$$Volatile\ Matter\ (\%) = \frac{x-y}{g}\ x\ 100 \quad (2.5)$$

g is the weight of sample, x is the of dry matter, y is weight of residue

Fixed Carbon (%) = *100 – (Volatile matter + Ash + Moisture Content* (2.6)

2.5 Ultimate Analysis

Ultimate analysis determines the chemical properties there in the biomass and it comprises of the Carbon, Hydrogen, Oxygen, Nitrogen and Sulphur contents. They are determined via the following formula:

a. Carbon Content

$$Carbon\ (\%) = \frac{(B-T)X\ MX0.003X\ 100X\ 1.33}{g} \quad (2.7)$$

g is the weight of sample, B is the blank titre, T is the titre value (of the sample) and M is the molarity of the acid used

b. Nitrogen Content

$$Nitrogen\ (\%) = \frac{(T\ X\ M\ X\ 0.14\ X\ DF}{g}\ x\ 100 \quad (2.8)$$

g is the weight of sample, T is the titre value, M is the molarity of the acid used and DF is the Dilution factor

c. Hydrogen content

$$Percentage\ Hydrogen\ (\%) = \frac{wt\ of\ H2O\ X\ 0.11119\ X\ 100}{wt\ of\ pellet} \quad (2.9)$$

d. Sulphur content

$$Sulphur\ (\%) = \frac{x-0.1373}{g}\ x\ 100 \quad (2.10)$$

g is the weight of sample and x is the weight of BaSO4

e. Oxygen Content

Percentage Oxygen (%) = 100 – (*C* + *H* + *N* + *S* + *%Ash*) (2.11)

3. RESULTS AND DISCUSSION

3.1 Growth Studies of *C. vulgaris* on Formulate Media

The results presented in Figs. 1 and 2 shows the patterns of growth of *Chlorella vulgaris* using the formulate-media under laboratory conditions. Fig. 1 shows comparative performance of the *C. vulgaris* using the Bold Basal Medium and the Modified BBM media. The study revealed that there was an acclimatization stage of the growth which represents the lag phase between Day 1 to Day 3 of the monitoring from 0.14 OD units to 0.15 OD units. There was a steepy increase in the growth representing the exponential phase

between the day 3 to day 10 as presented in the increase from 0.15 OD units to 0.45 OD units. The growth went into a stationary growth phase between the 10th day to the 15th day of the monitoring using the modified BBM. The study further recorded a significant yield. Fig. 2 shows the chlorophyll accumulation pattern of the *Chlorella vulgaris* using Bold Basal Medium, Sugarcane Molasses and a 1:1 mixture of BBM: SM-M. There was a short lag phase for the first two days of the study. There was an exponential phase between days 4.0 at day 11, with an increase in the chlorophyll absorption from 0.1 to 0.35 OD units. There was a decline in the accumulation between day11 and day 15.

3.2 Proximate and Pigment Composition of *Chlorella* Biomass

The moisture content of the *Chlorella* biomass was 6.28%wt. The volatile organic matter was 67.37% wt. The fixed carbon was 11.35%wt. The Ash content was 15.43%w/w. The HHV content was 19.04 %w/w. The ultimate composition of *Chlorella* biomass revealed that Carbon was 42.46% while Oxygen content was 27.93%. Nitrogen content was 6.62% while Sulphur content was 0.82% while hydrogen content was 6.74%. The result presented in Table 3 showed the biomass had 7-12000 µg/g beta carotene, Astaxantin, Cantaxantin, Chlorophyll-a and Chlorophyll-b was 550,000 µg/g, 362,000 µg/g, 250-9630 µg/g and 72-5770 µg/g. The vitamins B7, B12, B9, B3 and C was 191.6 mg/100g, 125.9 mg/100g, 23.8 mg/100g and 26.9 mg/100g.

3.3 Discussion

The present study evaluated the potential of modified Bold Basal medium fortified with sugar cane molasses and Bold Basal Medium. The specific growth rate was 0.041 mg/Lday^{-1} using 1:1 fortified sugar molasses while sugarcane molasses had a lower SGR of 0.054 mg/Lday^{-1}.

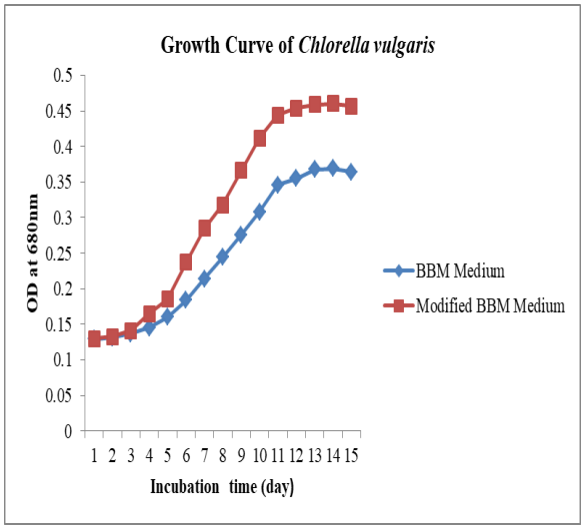

Fig. 1. Growth pattern studies of *Chlorella vulgaris*

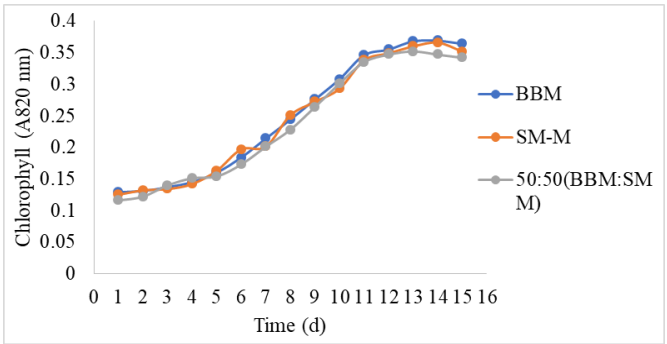

Fig. 2. Chlorophyll accumulation studies of of *Chlorella vulgaris*
Key: BBM-Bold Basal Medium

Table 2. Proximate composition of *Chlorella vulgaris* biomass

Parameter	Concentration (wt %)[a]
Moisture	6.28
Volatile Matter	67.37
Fixed Carbon[b]	11.35
Ash	15.43
HHV (MJ/Kg	19.04

a-As received basis, b- by difference

Table 3. Ultimate composition of *Chlorella vulgaris*

Parameter	Concentration (wt %)[c]
Carbon	42.46
Hydrogen	6.74
Nitrogen	6.62
Oxygen	27.93
Sulphur	0.82

a- By ash and free dry basis

Most heterotrophic cultivation the use of glucose-rich medium through a set of Embden Meyerhof Parnas (EMP pathway) routes [18,19,20]. The higher metabolic fluxes of the physiological metabolism rather than the passive processes had more optimal specific growth rate of microalgae when cultured in the presence of glucose [18-21]. Other scientific reports suggested there is more aerobic processes occurs via heterotrophic and mixotrophic cultivations of *Chlorella* sp. than energy generated from autotrophic cultivation, although energy from photon energy and reception by pigments by microalgae *Chlorella vulgaris* grown in mineral salt medium containing organic sources of carbon. Furthermore, microalgae grown in media containing fortified with Sugarcane molasses presented a presented a better growth profile than that of the autotrophic medium (Bold Basal Medium) as observed in the present study. Metabolic process and yield rates tend to be higher with heterotrophic sources of nutrient than with carbon-rich sources. This is because glucose has a direct metabolic route as agreed by *C. protothecoides* cultivated in shake flasks with sucrose-containing media [18,21]. Alves da Silva et al. [18] also observed the microalgae had μmax values of 0.011 and 0.013 hr^{-1} in Bold Basal medium and other mineral salt media supplemented with glycerol, while the μmax values were 0.010 and 0.015 hr^{-1} in BB (Bold Basal Medium) and NPK media supplemented with acetate, respectively [18]. Bonini and Bastos [22] reported μmax for *C. vulgaris* cultivated in aerobic+ glycerol (0.09 hr^{-1}) while in acetate (0.007 hr^{-1}) based media. However, Chen and Walker (2011) obtained a high μmax for *C. protothecoides* grown in shake flasks containing glycerol-based media (0.029 hr^{-1}) these values are close to that found in the present study. Sugarcane molasses is a cost effective substrate and feasible material for industrial scale farming of algae (Nascimento et al., 2016). However, it is necessary the microalgae have the enzyme invertase bioconversion into soluble and absorbable nutrient and assimilation [19]. Glycerol is utilized through physiological process, then fed into the electron transport chain. The pentose-phosphate pathway appears to be inhibited when glycerol which is not encountered through a number of reductions of NADH through Embden Meyerhof Parnas (EMP pathway). The utilization of pyruvic acid requires the synthesis of secondary metabolites is seen growth curves [22]. This is a probable explanation, as it was exactly what was observed here for the adaptation phases in cultivations with sucrose, glycerol and acetate as carbon source in relation to the other substrate.

The proximate composition of microalgal biomass correlates with the molecular morphology and physiological activities of the microalgae including activities such as induction and synthesis of vital compound such as lipids and vitamins. The present study recorded the following, moisture content of the *Chlorella* biomass was 6.28%wt while volatile organic matter was 67.37% wt. The fixed carbon was 11.35%wt. The Ash content was 15.43%w/w the HHV content was 19.04 %w/w. The ultimate composition of *Chlorella* biomass revealed that Carbon was 42.46% while Oxygen content was 27.93%. Nitrogen content was 6.62% while

Sulphur content was 0.82% while hydrogen content was 6.74%. These findings corroborate the findings of Jabeen et al. [23] where they reported that Fixed carbon, Volatile organic matter and Ash was 84.3%wt, 10.4%wt, and 5.3%wt respectively. Furthermore, Adamakis et al. [24] reported 34%wt carbon content of *Chlorella* biomass. It is pertinent to note that previous study of Bi and He [25] reported an ultimate composition of the *Chlorella* biomass was 58%, Nitrogen was 6.8%wt, Oxygen was 27.5%wt and Sulphur was 0.4%wt and a hydrogen composition of 7%wt. The lower concentration of Sulphur makes the oil a "sweet" oil since it is devoid of fractions that can contribute to the pollution index. The concentration of the nitrogen could be linked with the concentration of the nucleic acids in the cell after the extraction that could have contaminated the oil. The Carbon concentration gave an indication into the high and low heating values of the *Chlorella* oil. However, the Oil seems to have better quality considering the lower sulphur content which is a critical factor in Biodiesel as to avoid environmental challenges.

4. CONCLUSION

This study has established the feasibility in cultivation and extraction of oil from a wide array of Chlorella vulgaris using modified conditions could yield a number of biodiesel-quality oil as veritable tool for biofuel production. The study underscored the application of mixotrophic cultivation using agrowaste in the commercial farming of microalgae for high biomass and lipid production. This study serves underscores the need for Government agencies to fund research in the area of biorefinery design and bioprocess development for biofuel production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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