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Commercial Microalgae Culture in Inorganic Fertilizer Media

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Authors' contributions

This work was carried out in collaboration between both authors. Author SEE designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author GOA supervised and approved the research. Both authors read and approved the final manuscript.

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ABSTRACT

Algal biomass production using relatively and locally available NPK formulated media has been identified as a key factor in commercial algal biomass production. The suitability of agricultural fertilizer as a growth medium for commercial algae cultivation was assessed using NPK 15:15:15, NPK 20:20:20 and composite medium of NPK 15:15:15+ BG11, while BG11 was used as a control medium. Microalgae *Chlorella* species was cultivated in these NPK formulated media at ambient temperature under solar irradiation for a period of 15 days. The cell biomass was determined by the optical density at 660nm, cell dry weight and total chlorophyll content were also determined. The maximum value for cell biomass of 0.356 mg/L, total chlorophyll content of 0.0.493 mg/ml and cell dry weight of 0.0185 mg/L achieved in the composite medium was closer to the values of 0.389 mg/L, 0.531 mg/ml and 0.2121 mg/L for cell biomass concentration, total chlorophyll content and dry cell weight respectively for BG11 medium. Although NPK 15:15:15 and NPK 20:20:20 media achieved lower values for cell biomass, total chlorophyll, and cell dry weight, there is no significant statistical difference between the media. This study suggests that inorganic fertilizer can be a relatively cost-effective and locally available substitute for commercial algae biomass production.

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1. INTRODUCTION

The growing world population has resulted in a demand for alternative and renewable energy that can replace fossil fuel with minimal environmental consequences. The potential of microalgae to yield more oil per hectare per year has drawn the attention of researchers and investors as a viable source of renewable energy. Microalgae are a group of eukaryotic unicellular photosynthetic organism that thrives in diverse aquatic habitat including soil environment. They can be unicellular flagellate, non-flagellate, colonial flagellate or colonial non-flagellate [1] with size 1 µm-50 mm depending on the species [2,3]. There are over 200,000 species of microalgae with less than 30,000 species being presently researched and not yet fully utilized [4]. The research in algae culture started in early 1950 by Oswald et al. who studied the cultivation of algae in sewage oxidation pond for wastewater treatment and biomass production [5]. Algae culture has passed through successive developmental phases in the past decades, attracting the attention of researchers and investors in exploring the untapped resources in algae for biologically active compounds and biofuel production. Microalgae and its bioactive compounds have found usefulness in industries such as aquaculture [6,7], energy [8,4], biochemical [9], wastewater [10,11] health and pharmaceutical [12,13].

Microalgae are fast-growing photosynthetic organisms capable of producing carbon hydrates, proteins, and lipids. They thrive in various aquatic habitats and on the surface of most soils [14], utilize carbon dioxide and light energy to produce biomass and oxygen [15]. Various techniques of algal culture include; photo-autotrophic, heterotrophic and mixotrophic. Algae can utilize solar energy or artificial light as an energy source and can be cultivated in open-pond or photobioreactor system, in batch or continuous culture system. Algal growth can be affected by the availability of nutrient, temperature, pH, light intensity and inoculums size. Progressively microalgae pass through various phases of growth depending on the concentration of nutrients in the medium. The major components of algae nutrient medium are nitrogen (mainly in form of nitrate, nitrate, urea or ammonia) requires as an essential component of protein, phosphorous (an essential component of nucleic acid), carbon (required for energy), vitamins and trace elements. The utilization of these nutrients for biomass production has initiated the use of algae for wastewater remediation and nutrients recovery. A study by Feng Y. [16] also reported 97% NH_4 and 96% TP removal efficiency by *Chlorella vulgaries* cultivated in artificial wastewater in column aerated Photobioreactors under batch and semi-continuous cultivation. Similarly, [17] reported 99.7% ammonia nitrogen and 75% total phosphorus removal in piggery wastewater growing algae *Chodatella* species. Microalgae can be cultured in both synthetic and locally formulated nitrate-rich medium. Some locally available media for large scale algal culture includes animal waste [18] agricultural fertilizer [19,20,21] sewerage and industrial wastewater [22,23,24,25] Formulation of culture medium with locally available materials can reduce cost, the complexity of the of synthetic medium preparation, and ultimately the cost of biomass. The cost of algae biomass production has been a challenge in industrial utilization and production of algae value-added products and commodities.

The aim of this study is to assess the suitability of agricultural fertilizer as a growth medium for commercial algae cultivation.

2. MATERIALS AND METHODS

2.1 Algal Strain Isolation and Culturing

Pond water sample containing microalgae was collected from (ARAC) African Regional Aquaculture Centre, Aluu, Rivers State and University of Port Harcourt Fish pond. Agar plating techniques were used to isolate algal strains from the pond water. The algal strains were isolated by modifying the method of [26]. The isolated strains were sub-cultured in BG11 medium in natural illumination and subsequently subculturing every seven days to maintain fresh algal culture. The isolates were purified by repeated streaking on solid media and identified to the genus level using standard laboratory procedures and reference materials.

2.2 Culturing Parameters

2.2.1 Temperature and light

The temperature of the medium was measured daily with (Mercury thermometer) to ensure the

optimum temperature for algae growth is maintained. The culture was placed natural illumination in the outdoor sunlight, the daily illuminance was measured with the Lux meter (Lx 9621) and the value recorded. The culture was placed below a shade to prevent photo-inhibition and overheat by direct sunlight.

2.2.2 Carbon supply and pH monitoring

The pH of the culturing medium was adjusted within the range of 8.2-8.5; which is the optimal pH for algal growth. This is measured with a pH meter (PHEP, pocket-size pH meter, Hanna Instruments). The pH of the media was measured every 3 days. Sodium bicarbonate was used as a carbon source for algae growth at the dose of 4g per litre in NPK 20:20:20 and NPK 15:15:15 medium.

2.2.3 Aeration

Aeration of the culture was done to prevent sedimentation and to ensure all algae cells are exposed to adequate nutrient and sunlight. Aeration was achieved by constantly bubbling of air from the aquarium pump, (Resun air pump, AC99041) at the rate of 9L/min and pressure of 0.014mpa.

2.2.4 Nutrient

The media used in the experiment are factory grade agricultural fertilizer NPK 15:15:15; (Table 2) and NPK 20:20:20 (Table 1) purchased from the local market and the BG-II medium (Table 3) prepared with of analytical grade chemicals. The BG11, NPK 15:15:15 and NPK 20:20:20 were all prepared by accurately weighing 0.5 g/L of each of the salts, and dissolved in distilled water. The reactors were all autoclaved at 121°C, 15 psi for 15 min, while media were sterilized by membrane filtration with Millipore membrane filter size of 0.45 µm and 25 mm diameter.

2.3 Analytical Procedures

2.3.1 Algae biomass concentration

Optical density was used to measure biomass concentration in the culture. The amount of light absorbed by the suspended algal cell is assumed to be directly related to biomass concentration [27]. Optical density was determined using a spectrophotometer at 600 nm wavelength with 5 ml of the growing culture. A blank absorbance was prepared by filling the cuvette with 5 ml of distilled water to calibrate the spectrophotometer (Spectronic 20, Genesys Thermos, USA) at 600nm wavelength. The cuvette containing 5 ml of algae was placed in the spectrophotometer and the corresponding absorbance value was recorded for each sample.

Table 1. Chemical composition of NPK 20:20:20

Components	Concentration
Total nitrogen	20%
Nitrate-nitrogen (N-$N0_3$)	6%
Ammonical-Nitrogen (N-NH_4)	4%
Urea-nitrogen (N-NH_2)	10%
Phosphorus (P_2O_5)	20%
Potassium (K_2O)	20%
Micro-nutrients	
EDTA	Chelated
Iron (Fe)	1000ppm
Manganese (Mn)	500ppm
Boron (B)	200ppm
Zinc (Zn)	150ppm
Copper (Cu)	110ppm
Molybdenum (Mo)	70ppm

Table 2. Chemical composition of NPK 15:15:15

Components	Concentration
Nitrogen (N)	15%
Phosphorus (P_2O_5)	15%
Potassium (K_2O)	15%
Trace elements	
Sulphur, Boron, Zinc, Copper, Manganese, Iron, Molybdenum	

2.3.2 Cell dry weight

Culture growth was estimated by measuring the dry cell weight of the culture broth by modified [26] method. About 5 ml of growing culture was sampled every 3 days, centrifuged at 3000rpm for 10 minutes. The harvested cells were washed 3 times with distilled water and dried with pre-weighed Whatman filter paper (pore size 0.45 µm). The filter papers containing algae cells were dried at 105°C for 4 hours and weighed to determine the dry cell weight in mg/L.

2.3.3 Specific growth rate µ (mg/L /d)

The biomass concentrations (Bc, mg/ calibration curve was used to create the growth curve of biomass density over time to determine the specific growth rate (µ, /d).

$$\mu\ (mg/L/d) = \frac{\ln Bc2 \div Bc1}{\Delta t} \quad (1)$$

(mg/L) during the exponential phase.
Δt = cultivation time (days).

Table 3. Composition of BG-II medium

Reagent	Quantity per litre
$NaNO_2$	1.5 g
$K_2HPO_4 \cdot 3H_2O$	0.004 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.027 g
Citric acid ($C_6H_8O_7$)	0.006 g
Ammonium ferric citrate ($C6H8O7 \cdot xFe \cdot yNH_3$)	0.006 g
Na_2Mg-EDTA	0.001 g
Na_2CO_3	0.02 g
Microelement stock solution	1 mL
Microelement stock solution	**Per litre**
H_3BO_3	2.860 g
$MnCl_2 \cdot 4H_2O$	1.810 g
$ZnSO4 \cdot 7H_2O$	0.222 g
$Na_2MoO4 \cdot 2H_2O$	0.390 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$CO(NO_3)_2 \cdot 6H_2O$	0.0494 g

2.3.4 Generation time (d)

The algae generation time (G) is the time required for the algal cell to divide and multiply

$$G = Ln2/\mu \quad (2)$$

G= generation time in days, μ= specific growth rate

2.3.5 Chlorophyll determination

The procedure for the chlorophyll analysis was adopted from [28]. A 5 ml of algal suspension was collected at 3 days intervals from each of the reactors. The samples were centrifuged at 3500 rpm for 15 minutes, the supernatant was discarded and the residual pellet suspended with 95% Dimethyl sulphate (DMSO). 5 ml of DMSO was added to the sampled broth and homogenized, the sample was kept in a water bath at 70°c for 5 minutes, and left to cool to room temperature. The extract was centrifuged at 3500 rpm for 5 mins and pigment was read at 660 nm wavelength after blanking with distilled water.

2.4 Statistical Analysis

Statistical analysis was carried out using SPSS software. The cell biomass concentration, total chlorophyll and cell dry weight were analysed using one-way ANOVA and Post Hoc Turkey HSD.

3. RESULTS AND DISCUSSION

3.1 Cell Biomass Concentration

The algae growth rate is determined by the cell biomass concentration in the media. The varied nutrient composition in each of the media has a different effect on algal growth. In this study, the media exhibit varied algae growth phases at different incubation days. Fig. 1 illustrates the growth curve of *Chlorella* in the NPK media studied. The maximum biomass concentration of 0.356 mg/L was obtained in the composite medium, with the highest cell biomass achieved on culture day 11 as shown in Fig. 1. The NPK 20:20:20 medium has the rapid exponential phase; the stationary phase was attained on culture day 7 with the cell biomass concentration of 0.239 mg/L. The maximum cell biomass produced in the medium containing NPK15:15:15 + BG11 could be attributed to having advantages of both NPK and synthetic elements of BG11 in the medium. On the other hand, rapid growth observed in the early culture days of NPK20:20:20 may be as a result to the varied nitrogen source which includes 10% of urea, 4% of ammonia and 6% of nitrate. [29] observed that algae prefer ammonium to nitrogen when both are present as a source of nitrogen in the growth medium. The less energy required for ammonium uptake relative to other forms of nitrogen in algal culture leads to rapid growth in the induction phase of the algae culture.

Slower growth was observed in the NPK 15:15:15 during the exponential phase but achieved maximum growth of 0.268 mg/L on culture day 9, a value higher than NPK20:20:20. Although BG11 is considered a superior nutrient medium for algae growth, the agricultural fertilizer media provided required support needed for algal biomass production. Higher cell biomass and longer stationary phase were observed in the composite and the BG11 media, but there was no statistically significant difference among the media. This result agrees with [30] who observed that NPK fertilizer and macrophyte can supply adequate nutrients and may replace synthetic medium for large scale algae cultivation. Similarly, the study by [31] suggested that agricultural fertilizer can provide required nutritional support for algae growth though the Bristol medium has relatively higher cell biomass

concentration when compared with fertilizer medium.

3.2 Effect of the NPK Medium on the Total Chlorophyll

The result of the total chlorophyll analyses Fig. 2. showed the BG11 and NPK15:15:15+BG 11 achieved the highest total chlorophyll content 0.531mg/ml and 0. 494 mg/ml respectively at the stationary phase. Both NPK 20:20:20 and NPK 15:15:15 medium had a short stationary phase with total chlorophyll content of 0.245 mg/ml and 0.284 mg/ml respectively. Since high chlorophyll content is related to the high nitrogen content of the culture medium, the high chlorophyll observed during the lag phase of NPK 20:20:20 which occurred between day 1-day 5, maybe as a result of ammonium presence in the medium, reduced form of nitrogen which is absent in others. Ammonium is known to be generally preferred by algae in place of other forms of nitrogen as it can be directly converted to an amino acid in the cells without further reduction [32]. However, photosynthesis inhibition occurred in NPK 20:20:20 when excess ammonium was transported to the cell leading to the impediment of ATP formation in the chloroplast, thus reducing the total chlorophyll content of the algae. This agrees with [33], who observed a 50% reduction in photosynthesis activity of *Scenedemus obliques* cultured in high rate algal pond at 0.76 nm free ammonia. On the other hand, all the media exhibit a significant reduction in chlorophyll content with nutrient depletion from culture day 9 for both NPK 15:15:15 and NPK 20:20:20, while the chlorophyll decline for BG11 and NPK+BG11 occurred on culture day 11. In nitrogen depleting conditions, the chlorophyll serves as a nitrogen source that supports algal cell division and reproduction, while total depletion of nitrogen leads to the non-photosynthetic activity due to inability of the chlorophyll to facilitate the metabolic change as a result of failure to capture light and CO_2 required for the photosynthesis [34]. Thus, there is a linear relationship between biomass production and total chlorophyll content in algae.

3.3 Effect of NPK Medium on Cell Dry Weight

Fig. 3 illustrated the result of the *Chlorell*a Sp growth measured as the cell dry weight. The highest cell dry weight of 0.0185 mg/L was observed in the composite media of NPK and BG11 medium with the stationary phase lasting from culture day 9 to 13. The lowest cell dry weight of 0.0122 mg/L was observed in NPK 20:20:20 medium at the stationary phase lasting from culture day 9 through day 13.

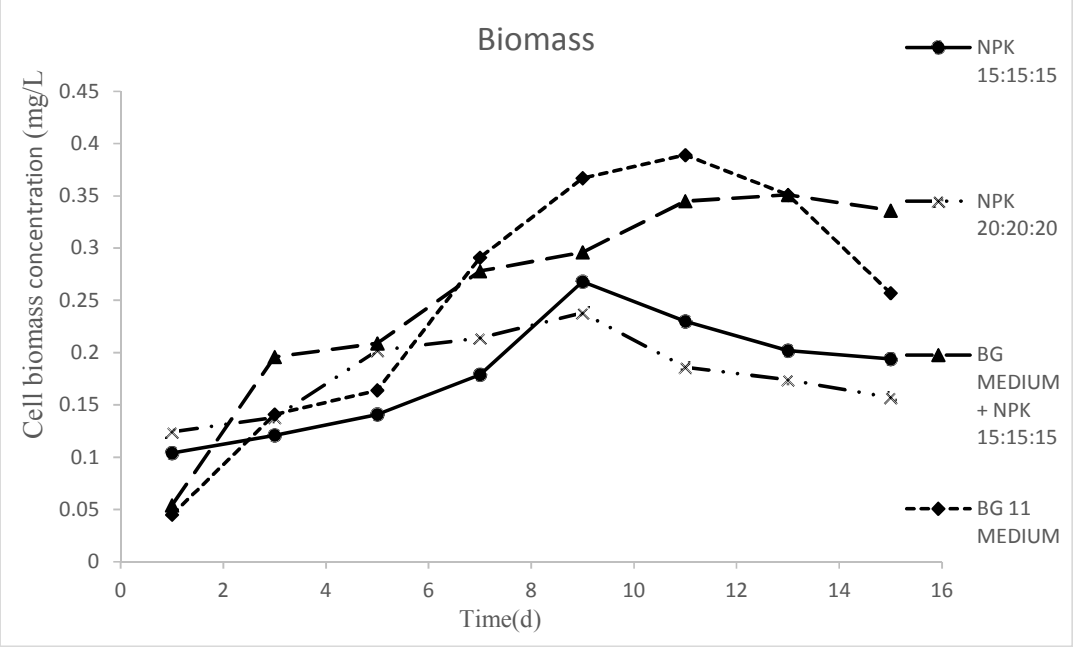

Fig. 1. The growth curve of *Chlorella* sp in NPK media

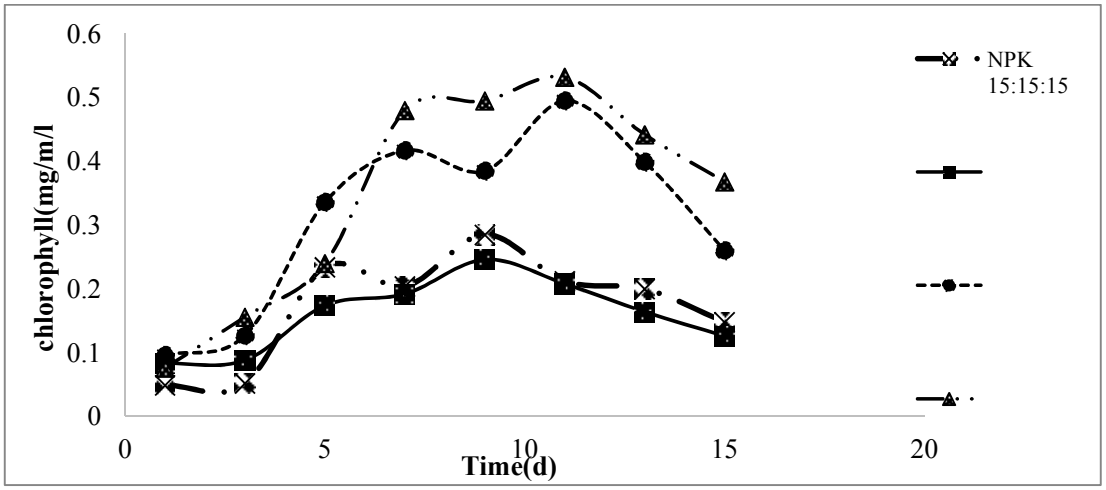

Fig. 2. Total chlorophyll content of *Chlorella* sp in NPK media

The lower cell dry weight observed in NPK 20:20:20 medium when compared with NPK 15:15:15 medium could be due to high urea and ammonium nitrogen present in NPK 20:20:20 (Table 1), although the same quantity of NPK (0.5 mg/L) was used to formulate the media. The increase in cell dry weight was observed in all the media from culture day 5 through day 11, which is considered an exponential phase of the algae growth. The dry cell biomass obtained at stationary phase from both NPK of 0.0176 mg/l and composite media of 0.0185 mg/L was close to the value of 0.0212 mg/L obtained from the BG11, which is a synthetic medium. Although the BG11 medium has been modified over the years with all the essential elements for optimal growth, this experiment has shown that little modification of the NPK medium will provide a relatively low cost and better nutrient medium for commercial algae biomass production as observed by [30,21]. When compared statistically to the significance level ($P<0.05$), there were no significant differences were found in cell dry weight between media studied. This study also observed that sources and concentrations of nitrogen in the medium have different effects on algae growth. This agrees with [35] who observed a decline in the growth of *Chlorella Pyrenoidosa* due to ammonium toxicity caused by the high concentration of urea in the media. Additionally [36,37] also reported inhibition of algal cell growth as a result of a high concentration of nitrogen in the media.

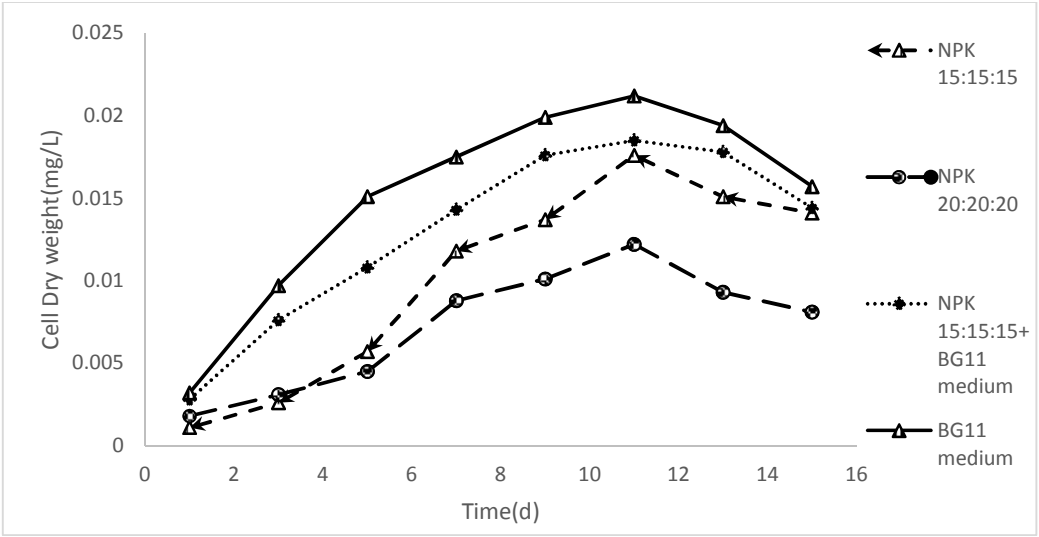

Fig. 3. Cell dry weight of *Chlorella* sp in NPK media

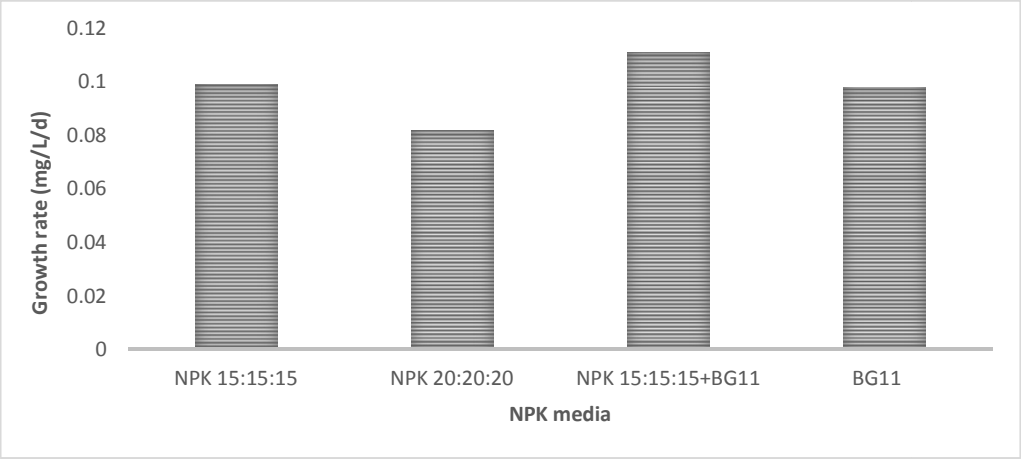

Fig. 4. The growth rate of *Chlorella* sp in NPK media

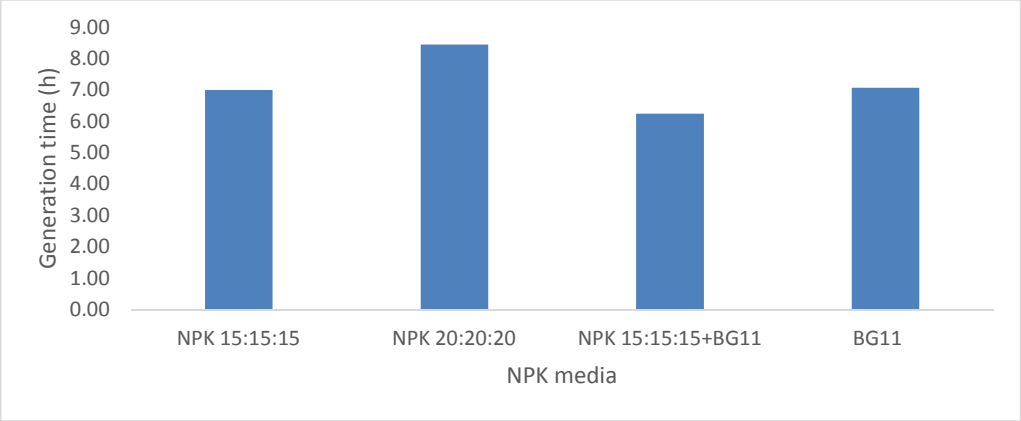

Fig. 5. The generation time of *Chlorella* sp in NPK media

3.4 Determination of Growth Rate and Generation Time of *Chlorell*a Sp

The growth rate and the generation time were calculated for each of the media as shown in Figs. 4 and 5. respectively. The maximum value of 0.111 mg/L/d for growth rate and minimum generation time of 6.24h was achieved in the composite medium of NPK and BG11 compared to other media, including the BG11 medium. The minimum value of 0.082 mg/L/d for growth rate and the maximum generation time of 8.45h were also observed for the NPK 20:20:20, this shows that concentration of nitrogen plays important role in the growth and algal biomass production. Nitrogen is a limiting nutrient in algae culture, this study observed a low growth rate in (NPK 20:20:20) medium with a higher concentration of nitrogen. This could be explained by the decline in algae growth as a result of higher nitrate reductase activity leading to higher accumulation of nitrate inside the algae cell. This agrees with [38] who reported a decrease in algae growth rate when nitrogen concentration was increased during *Chlorella* culture. Thus the rate of nitrogen uptake and assimilation by algae cells is determined by the concentration and source of nitrogen in the medium.

4. CONCLUSION

The result of this study has suggested that inorganic fertilizer can be a cost-effective and efficient substitute for the synthetic growth medium for commercial algae biomass production. Formulation of algae growth media with as low as 0.5 g/L of NPK will provide a low-cost nutrient media in large scale algae biomass production Though variety of the fertilizer with different concentration of nitrogen, phosphorus

and potassium can be used; low nitrogen concentration has shown to stimulate cell biomass concentration, chlorophyll and cell dry weight. In addition, modification of the NPK media with essential elements has shown to promote exponential algae growth in *Chlorella* species studied; further study is required for various algae species to ascertain the nutrient requirement for optimal biomass production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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