Original Research Article

COMMERCIAL MICROALGAE CULTURE USING AGRICULTURAL FERTILIZER AS GROWTH MEDIA.

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* sp was cultivated in these NPK formulated media at ambient temperature under solar irradiation for a period of 15days. The cell biomass was determined by the optical density at 660nm, cell dry weight and total chlorophyll content was also determined. The maximum value for cell biomass of 0.493 mg/l, total chlorophyll content of 0.356mg/L and cell dry weight of 0.0185mg/L achieved in the composite medium was closer to the values of 0.531 mg/L, 0.389mg/L and 0.2121mg/L for cell biomass concentration, total chlorophyll content and dry cell weight 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 agricultural fertilizer can be a relatively cost-effective and locally available substitute for commercial algae biomass production.

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 potentials 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 or non- flagellate, colonial flagellate or colonial non-flagellate ([1]; with size ranges of 1µm- 50mm 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],

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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 lightning as an energy source and can be cultivated in open-pond or photo bioreactor system, in batch or continuous culture system. Algal growth can be affected by the availability of nutrient, temperature, pH, light intensity and inoculum size. Progressively microalgae pass 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); required as an essential component of protein, phosphorus; an essential component of nucleic acid, carbon; requires for energy, vitamins, 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 [16] also reported 97% NH4 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* sp. 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]), sewage and industrial wastewater [22], [23] [24], [25] Formulation of culture medium with locally available materials can reduce cost, preparation and the complexity of synthetic medium, and subsequently the cost of biomass produced. 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.

MATERIAL and METHODS

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 raw 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 sub culturing 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.

Culturing Parameters

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 allowed the experience natural illumination from the outdoor sunlight, the daily illuminance was measured with the Lux

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meter (Lx 9621) and the value recorded. The culture was placed below a shade to prevent photo-inhibition and overheat by direct sunlight.

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 the portable 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.

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.

Nutrient

The media used in the experiment are factory grade agricultural fertilizer NPK 15:15:15; (Table 1.3) and NPK 20:20:20 Table 1.2) purchased from the local market and the BG-II medium (Table 1.1) prepared with of analytical grade chemicals, and The BG11, NPK 15:15:15 and NPK 20:20:20 were all prepared by accurately weighing 0.5g/L of each of the salts, and dissolved in distilled water. The reactors were all autoclaved at 121^{o}C, 15psi for 15min, while media were sterilized by membrane filtration with Millipore membrane filter size of 0.45µm and 25mm diameter.

Table 1.1 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_2O5)	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

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Table 1.2. Chemical Composition of NPK 15:15:15

Components	Concentration
Nitrogen (N)	15%
Phosphorus (P_2O5	15%
Potassium (K_2O	15%
Trace elements Sulphur, Boron, Zinc, Copper, Manganese, Iron, Molybdenum	

Table 1.3 Composition of BG-II medium

REAGENT	QUANTITY PER LITER
$NaNO_2$	1.5g
$K_2HPO_4 \cdot 3H_2O$	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 ($C_6H_8O_7 \cdot xFe \cdot yNH_3$)	0.006 g
Na_2Mg-EDTA	0.001 g
Na_2CO_3	0.02 g
Microelement stock solution	1 mL
MICROELEMENT STOCK SOLUTION	**PER LITER**
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

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Analytical Procedures

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 600nm wavelength with 5 ml of the growing culture. A blank absorbance was prepared by filling the cuvette with 5ml of distilled water to calibrate the spectrophotometer (Spectronic 20, Genesys Thermos, USA) at 600nm wavelength. The cuvette containing 5ml of algae was placed in the spectrophotometer and the corresponding absorbance value was recorded for each sample.

Cell Dry Weight

Culture growth was estimated by measuring the dry cell weight of the culture broth by modified [26] method. About 5ml of growing culture was sampled every 3days, centrifuged at 3000rpm for 10minutes. The harvested cells were washed 3times with distilled water and dried with pre-weighed Whatman filter paper (pore size 0.45µm) the filter papers containing algae cells were dried at 105^0C for 4 hours and weighed to determine the dry cell weight in mg/l.

Specific Growth Rate. µ (mg/L /d)

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

$$\mu\ (mg/L/d) = \frac{\text{Ln } Bc2/Bc1}{\Delta t} \quad \text{Eqn. (1)}$$

Bc2 & Bc1= final and initial biomass concentration (mg/L) during the exponential phase.
Δt = cultivation time (days).

Generation Time (d)

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

$$G = Ln2/\mu \quad \text{Eqn. (2)}$$

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

Chlorophyll Determination

The procedure for the chlorophyll analysis was adopted from [28]5ml of algal suspension was collected every 3 days interval from each of the reactors. The samples were centrifuged at 3500 rpm for 15minutes, the supernatant was discarded and the residual pellet suspended with 95% Dimethyl sulphate (DMSO). 5ml of DMSO was added to 5ml and homogenized, the sample was kept in a water bath at 70^oC for 5minutes, removed and left to cool to room temperature. The extract was centrifuged at 3500 rpm for 5mins and the pigment read at a 660nm wavelength at photo spectrometer using distilled water as blank and reading recorded.

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Statistical Analysis

The experimental results were analyzed with one-way analysis of variance (ANOVA). The significance between the media was analyzed using least significant difference (LSD) test at 5% level of significance.

RESULTS AND DISCUSSION

Cell Biomass Concentration

In this study, NPK 15:15:15 and NPK 20:20:20 agricultural fertilizer was used as a nutrient media for algae cultivation. The composite medium (NPK 15:15: 15 + BG 11) was formulated with BG11 serving as a control medium. The *Chlorella* sp was cultured photo-autotrophically for 15 days in ambient temperature under solar irradiation. The Algal growth phases of induction, exponential, stationary and decline phase were achieved in all the media at different incubation time. A maximum biomass concentration of 0.494mg/L was obtained in the composite medium, with the highest cell biomass achieved on culture day 9 as shown in figure 1.0. 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 in the medium. The NPK 20:20:20 medium has the rapid growth phase with the stationary phase achieved on culture day 7 with the cell biomass concentration of 0.245mg/L. This rapid growth observed in the early culture day may be attributed to the varied nitrogen composition of NPK 20:20:20 which includes 10% of Urea, 4% of ammonia and 6% of nitrate. Algae preference for ammonia nitrogen compares to the nitrate form of nitrogen ([29]) due to less energy and time required for the nitrate uptake may be responsible for the rapid early growth in NPK 20:20:20 relative to other experimented media.

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Comment [P50]: Engage command of clear parameter; e.g. Cell biomass concentration or algal growth
Note: engage consistency on the results and discussion.
e.g.
Cell Biomass Concentration;
- Presentation for synopsis of results,
- Explanation of results
- References to previous research
- Present Figure for cell biomass concentration

Algal growth;
- Presentation for synopsis of results,
- Explanation of results
- References to previous research
- Present Figure for algal growth

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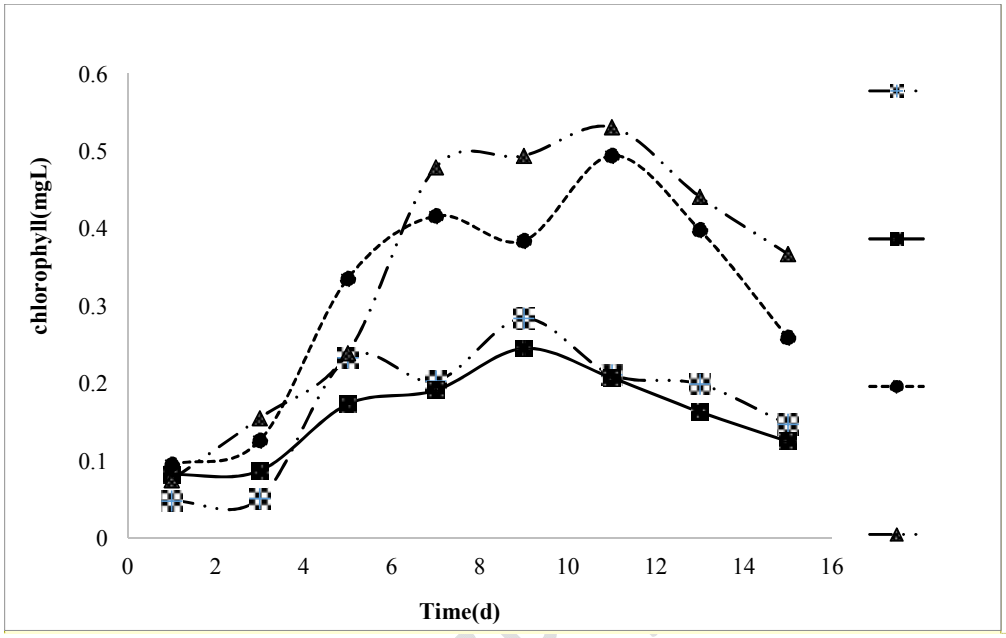

Figure 1.0. The growth curve of *Chlorella* sp in various NPK media.

The medium with NPK 15:15:15 has lower growth during the exponential and linear phase, but achieved maximum growth of 0.284mg/L on culture day 9 higher than NPK 20:20:20. Although BG11 is considered a superior nutrient medium for algae growth, the agricultural fertilizer media provided required support needed for algal biomass production as shown in figure 1.0. While the composite medium achieved cell biomass concentration and longer stationary phase closer to the BG11; a known growth medium, the analysis of variance of the cell biomass concentration and the incubation time of the experiment showed no significant difference among the media. This result agrees with [29] 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 [30] 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.

Effect of the NPK Medium on the Total Chlorophyll

The result of the Chlorophyll analyses showed the BG11 and NPK15:15:15+BG 11 achieved the highest total chlorophyll content 0.383mg/L and 0. 351mg/L at the stationary phase of the algae growth respectively shown in figure 2.0. Both NPK 20:20:20 and NPK 15:15:15 medium had a short stationary phase with total chlorophyll content of 0.238mg/L and 0.268mg/L respectively as shown in figure 2.0. 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

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Engage cell biomass concentration for y axis title not chlorophyll

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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 [31]. 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 [32], who observed a 50% reduction in photosynthesis activity of *Scenedemus obliques* cultured in high rate algal pond at 0.76nm 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 [33]. Thus, there is a linear relationship between biomass production and total chlorophyll content in algae.

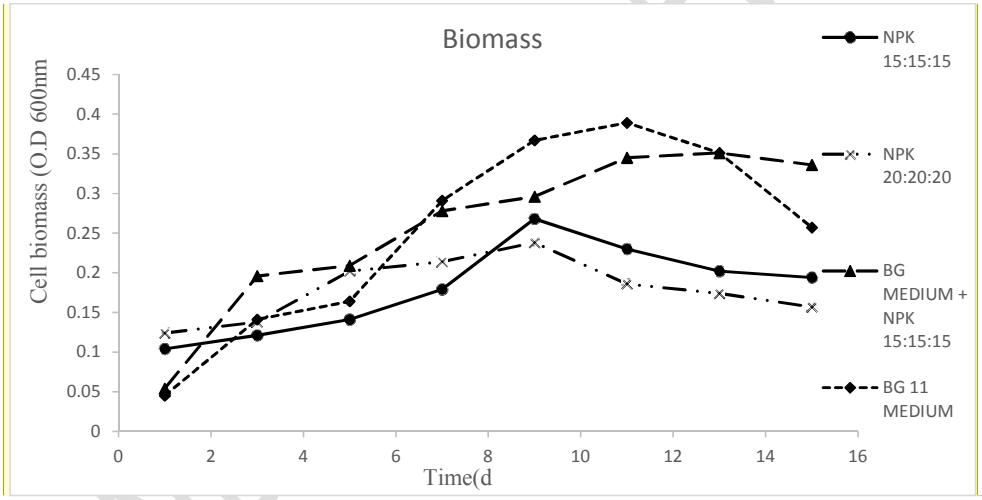

Figure 2.0. Total Chlorophyll content of *Chlorella* sp in various NPK media

Effect of NPK Medium Cell Dry Weight.

To determine the suitability of the agricultural fertilizer for commercial dry biomass algae production, microalga *Chlorella* sp was cultured NPK 15:15:15, NPK 20:20:20, and composite

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medium of NPK15:15:15 & BG11 formulated media under photoautotrophic condition. Figure 3.0 shows the biomass concentration measured as dry cell weight, the transition from the lag, exponential, linear phase, stationary and death phase for all the media. The highest cell dry weight of 0.0185mg/L was observed in the composite media of NPK and BG11 medium, figure 3.0.with the stationary phase lasting from culture day 9 to 13. The lowest cell dry weight of 0.0122mg/L was observed in NPK 20:20:20 medium at the stationary phase lasting from culture day 9 through day 13. Studies have revealed that various nitrogen sources have different effects on algae growth. 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 (Table1.1), although same quantity of NPK (0.5mg/L) was used to formulate the media. This agrees with [34] 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, [35] and [36] also reported inhibition of algal cell growth as a result of a high concentration of nitrogen in the media.

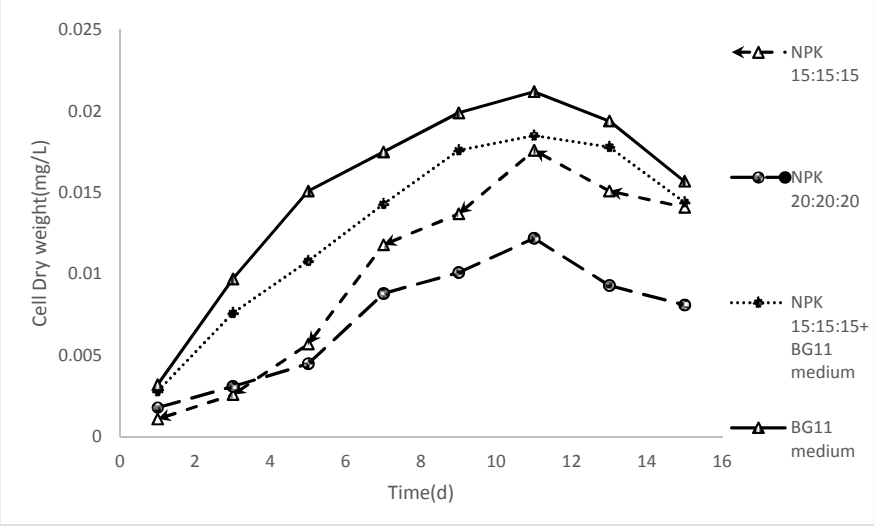

Figure 3.0. Cell Dry Weight of *Chlorella* sp in various NPK media

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

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stationary phase from both NPK of 0.0176mg/l and composite media of 0.0185mg/L were close to the value of 0.0212mg/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 formulated for optimal growth, this experiment has shown that little modification of the NPK medium will provide a relative low cost and better nutrient medium for commercial algae biomass production [29] [21]. The growth rate and the generation time were also calculated for each of the medium shown in figure 4.0 and 5.0 respectively. The maximum value of 0.111mg/L/d for growth rate and minimum generation time of 6.24d was achieved in the composite medium of NPK and BG11 compared to other media, including the BG11 medium. The minimum value of 0.082mg/L/d for growth rate and the maximum generation time of 8.45d was also observed for the NPK 20:20:20, this shows that concentration of nitrogen plays important role in the growth and algal biomass production.

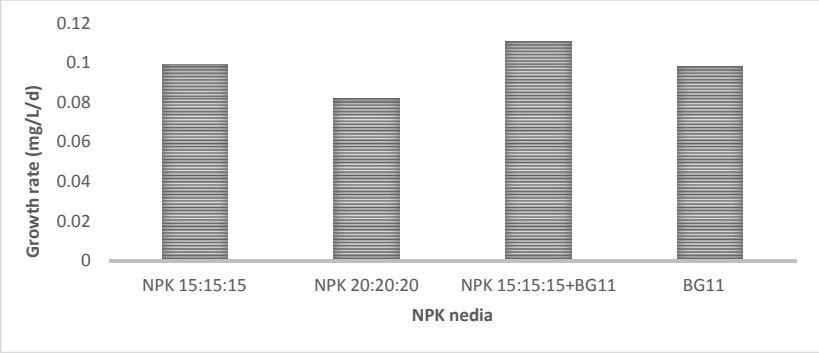

Figure 4.0. The growth rate of *Chlorella* sp in NPK media

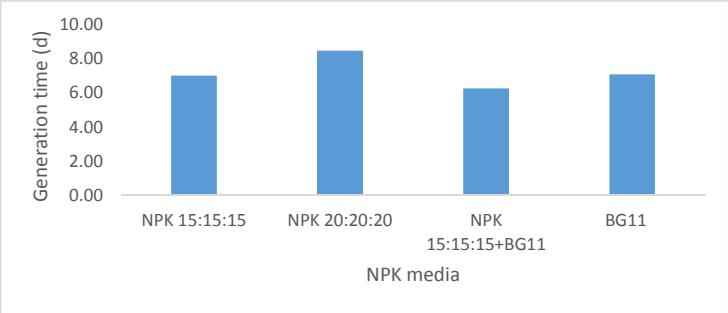

Figure 5.0. The Generation Time of *Chlorella* sp in NPK media

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Sub title: growth rate of *Chlorella* sp
- Presentation for synopsis of results,
- Explanation of results
- References to previous research

Sub title: generation time of *Chlorella* sp
- Presentation for synopsis of results,
- Explanation of results
- References to previous research

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Conclusion

The result from this study has suggested agricultural fertilizer can be a low cost and efficient substitute for the synthetic growth medium for commercial algae biomass. Although a variety of agricultural fertilizers can be used to formulate growth medium, emphasis should be on the NPK with a lower percentage of nitrogen concentration. More so, media modification with essential elements and experimenting with various concentrations of NPK will achieve optimal nutrient media formulated with locally available and cost-effective growth medium for commercial algae biomass production.

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Comment [P68]: Engage the measured parameters (growth, chlorophyll, cell biomass and cell dry weight) in relation to fertilization treatments in the conclusion

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Comment [P71]: revise complex sentence

References

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