Optimum conditions for Cultivation of *Chlorella vulgaris* on Oil Palm Residue Extracts

Abstract

In this study Oil palm cake and Empty fruit bunch residue were obtained from Aluu, Rivers State Nigeria. The proximate, physicochemical of the extract were ascertained. The proximate result revealed that the range of Ash (8.40±0.30 - 6.60±0.40), Moisture (10.90±0.30 - 6.75±0.65), Lipid (22.05±0.35 - 14.90±0.30), Protein (1.30±0.18 -0.80±0.08), Fiber (25.50±0.50 - 39.09±0.57) and Carbohydrate (29.83±0.29 - 29.29±0.58). The physicochemical composition pH (5.15±0.02 - 5.36±0.06), Conductivity (224.15±0.45 - 481.00±2.00 µS/cm), DO (2.87±0.06 - 4.86±0.06 ppm), Nitrate (31.05±0.48 - 40.33±0.98 ppm), Sulphate (36.35±0.46 - 50.68±0.49 ppm) and phosphate (27.71±0.50 - 37.35±0.55ppm) were obtained for Oil palm cake and Empty fruit bunch respectively. Growth media ratio selected was 180:20 for empty fruit bunch:oil palm cake. The optimization revealed pH 6.0, temperature 30^{o}C, salinity 10ppm and photoperiod 12:12 day:night as optimal condition for the growth of *Chlorella vulgaris* biomass.

Keywords: oil palm cake, oil palm empty fruit bunch, *Chlorella vulgaris*

1.0 Introduction

During the last century, a great deal of study and advance, as well as applications, has been devoted to waste. These include waste minimization and treatment, environmental assessment of waste, minimization of environmental impact, life cycle assessment, phycology and biotechnology, and others (Kansal, 2002). The major reason for such huge efforts is that waste cohort constitutes one of the foremost environmental problems where production industries are concerned. Until now, an accumulative pressure has been put on discovery methods of recycling waste, for instance through cleaner production, thus mirroring rapid changes in environmental policies (Vandenberghe *et al*. 2009). Production of huge amounts of crude palm oil results in larger amounts of palm oil mill waste. It is worth considering the potential value of oil palm waste prior to its treatment through introduction of a cleaner production. Microalgae can be described as a simple unicellular photosynthetic organism that uses energy from sunlight and carbon dioxide from the atmosphere. Microalgae have been identified as an operative tool to sequester solar radiation and yield huge biomass with little or no harm on the ecosystem (Agwa *et al.*, 2012). They are cultivated in medium with reduced nitrogen and phosphorous elements. The metabolism of microalgae allows lipid and carbohydrate to be synthesized using biomass because of their vital properties (Rigano *et al.*, 1998). Utilization of the microalga *Chlorella* sp has proved effective due to its high-valued potential substances such as chlorophyll, carotene and protein amongst others, (Wijanarko *et al*., 2016b), and due to their tolerance levels for the high concentration of CO_2 and high proficiency in utilizing CO_2 during photosynthesis (de Morais and Costa 2007). *Chlorella* sp. is among the fastest growing photosynthetic organisms having carbon fixation rates in order of magnitude higher than those of land plants. It utilizes CO_2 as one of its main building blocks and is a practical option for anthropogenic CO_2 capture and sequestration (Olaizola, 2003a). The green microalgae *Chlorella vulgaris* used for this study was cultivated in low-cost carbon substrate; oil palm cake and oil palm empty fruit bunches.

2.0 Materials/Method

2.1 Collection of the oil palm waste

A sterile bag was used to collect the oil palm empty fruit bunch and oil palm cake from a palm oil mill in Aluu village in Port Harcourt, Rivers State. The oil palm empty fruit bunch and palm oil cake were properly dried to remove moisture and carefully ground thoroughly into a finely powdered form and then transported to the laboratory.

2.2 Lighting

The lighting source was natural light from sunlight. The cultures were arranged on laboratory benches from where they received sunlight through the window.

2.3 Chemicals/Reagents

Chemicals/reagents were obtained from the microbiology and chemistry stores of the University of Port Harcourt. All chemicals and reagents were of analytical grade.

2.4 Washing and sterilization

All glassware was washed and sterilized in hot air oven at 160 ^{0}C for 60 minutes and media used were also sterilized by autoclaving at 15 psi and 121^{0}C for 15 minutes.

2.5 Biosafety evaluation of the oil palm waste extract

The microbial eminence of the oil palm extract was determined, to discover the conceivable deteriogens and pathogen of health significance. The plate count assay was used to study the viability of the cells after sterilization; the incubation was done to encourage sporulation of the heat-resistant bacterial strains. Biochemical test and Analytical profile index were used to identify the pathogens that survive the second stage sterilization.

2.6 Assay of physicochemical properties of the extract

The physicochemical properties of the samples were measured using the standard analytical procedure of (AOAC, 1984). The samples were analyzed for the following physiochemical parameters on getting to the laboratory: pH, chloride, nitrate, phosphate, sulphate, ammonia, total dissolved solids (TDS), conductivity, salinity, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Dissolved Oxygen (DO)

2.7 Microbiological Analyses

Isolation of microalgae

A novel synthetic medium (according to Agwa *et al.*, 2012) containing antibiotics (Tetracyclin and Nystatin to eliminate bacteria and fungi growth respectively), was used to isolate *Chlorella* species. Agar plate technique was used for isolation of the culture plates and incubated for three to five days in a shade under natural illumination (sunlight). Colonies which appeared after four days were sub-cultured for monoculture development. The colonies were identified using cultural and morphological characteristics.

Microalgae Identification and Harvesting

An aliquot of the isolate was taken and placed under the x40 objective of the microscope to identify algae based on the morphological characteristics using an algae atlas as a guide for similarity check, colour, size, shape and cell wall. some other putative signatures were used in the identification (Huss *et al.*, 1999, Ponnuswamy *et al.*, 2013).

About two to five (2-5ml) of sterile water was poured to a mono-growth of algae cells in the culture plates and a sterile wire loop was used to dislodge from the medium. They were aseptically pipetted into a sterile conical flask and cotton plugged. This was stored in a refrigerator at 4ºC until further used.

2.8 Determination of optimal wavelength

The optimal wavelength of the medium was obtained by scanning the oil palm extract from a low to high wavelength of the spectrophotometer. The optimal wavelength was determined from the point of least absorbance (Mogany, 2014 and Wang *et al.*, 2008)

2.9 Optical Density

Five milliliters of each sample were taken using a 5ml syringe after which 3ml of the collected sample was dispensed into spectrophotometer cuvette and the optical density determined using a spectrophotometer set at the wavelength of 620 nm. After 24 hours this exercise was repeated and subsequently for 7 days during blooming.

2.10 Process optimization (Light and Dark phases) for selection of biomass production.

The substrate ratio of the empty fruit bunch and oil palm cake was used to ascertain the best ratio of the two. While the novel synthetic media was used as positive controls and the un-inoculated ratio of the extract was used as negative controls.

2.11 Interaction of growth factors for the growth of *Chlorella vulgaris*

The method of Ogbonda *et al.*, (2007) was used to detect optimal points by the sigmoid graphs to determine the interaction between the microalga, oil palm extracts and operational factors such as pH, temperature, salinity and photoperiod (12:12 and 6:18).

2.12 Biomass dry weight measurement

10mls of growing culture was centrifuged at 3000 rpm for 15 mins in other to have the dry weight. The cells were washed 3 times with physiological saline and dried at 50 ºC in a hot air oven until a constant weight was obtained with a RAGWAG AS/22 weighing balance. Then brought to room temperature in a desiccator, then the net dry cell weight was determined by measuring the arithmetic difference of final weight of the filter paper and the initial weight

3.0 RESULTS

3.1 Proximate composition of oil palm cake, empty fruit bunch and algae biomass

Table 1 depicts the findings for proximate composition of Oil Palm Cake (OPC), Empty Fruit Bunch (EFB) and algae biomass used for this study.

Table 1: Proximate composition of oil palm cake, empty fruit bunch and algae biomass

Parameter	Oil palm cake	Empty fruit bunch	Algae biomass
Ash	8.40± 0.30	6.60±0.40	6.95±0.70
Moisture	10.90±0.30	6.75±0.65	5.0±0.70
Lipid	22.05±0.35	14.90±0.30	11.30±1.20
Protein	1.30±0.18	0.80±0.08	1.15±0.06
Fiber	25.50±0.50	39.09±0.57	14.52±0.49
Carbohydrate	29.83±0.29	29.29±0.58	20.52±0.49

3.2: Physicochemical composition of oil palm cake and empty fruit bunch

The data obtained for physicochemical characteristics of oil palm extract (oil palm cake, empty fruit bunch) is reported on Table 2.

Table 2: Physiochemical composition of oil palm cake, empty fruit bunch and palm oil mill effluent

Parameter	Oil palm cake	Empty fruit bunch
pH	5.15± 0.02	5.36±0.06
EC (µS/cm)	224.15±0.45	481.00±2.00
Nitrate (ppm)	31.05±0.48	40.33±0.98
Sulphate (ppm)	36.35±0.46	50.68±0.49
Phosphate (ppm)	27.71±0.50	37.35±0.55
BOD (ppm)	0.70±0.02	0.33±0.01
COD (ppm)	77.00±1.00	128.00±2.00
TDS (ppm)	149.22±0.52	326.50±4.50
DO (ppm)	2.87±0.06	4.86±0.06
Calcium (ppm)	68.80±0.59	69.13±1.01
Magnesium (ppm)	92.51±1.04	133.94±1.53
Ammonia (ppm)	23.96±0.52	29.93±0.42
Salinity (ppm)	36.75±0.59	31.29±0.94

3.3: Biostability of Oil Palm Cake and Empty Fruit Bunch

Figure 1 presents the result of tyndalization of microflora in the oil palm waste extract to certain levels of heat and heating. The initial microbial count was 90 and 100 cfu/ml and after the second day of incubation and heating the microbial count reduced to 30 and 40cfu/ml. The entire counts on the third day reduced to 0 cfu/ml. The result revealed that the exposure of the pathogens to heating and regeneration of spores were significantly affected by the heating regimes.

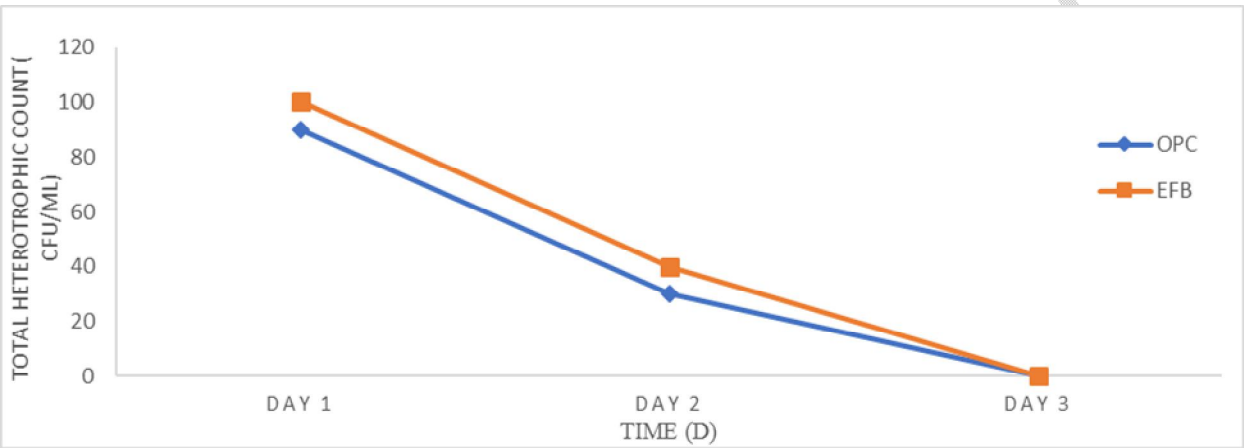

Fig 1: Biostability study on the Oil Palm Cake and Empty Fruit Bunch

3.4: Wavelength for monitoring growth of Oil Palm Cake, Empty Fruit Bunch and control.

Figure 2 denotes the response of varying wavelength and optical densities. The results determine a corresponding fall in the absorbance of the formulations as the wavelength increased. The wavelength was increased manually from 500-800nm, the corresponding absorbance level fell from 0.9nm to 0.1nm. The selected wavelength exhibited better accuracy for the biomass monitoring and optimization investigations. The difference in the growth pattern for the positive control and the optimal growth point is obvious in the results presented

A correlation exists between the wavelength tested and the optimal or commonly used points.

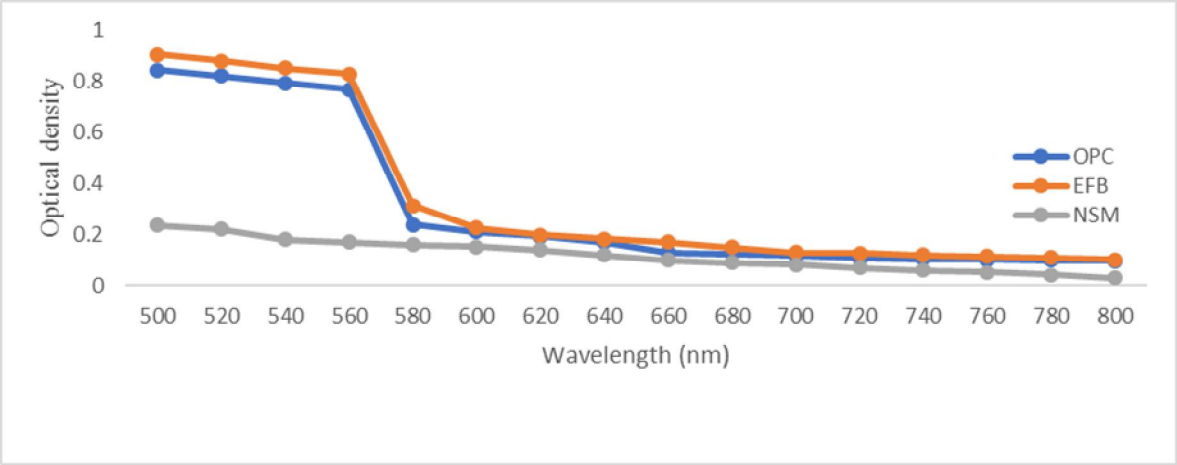

Fig 2: Wavelength selection for the Oil Palm Cake, Empty Fruit Bunch and control.

(NSM---Novel Synthetic Medium)

3.5: Growth of *Chlorella vulgaris* in different ratios of Empty Fruit Bunch and Oil Palm Cake

Figure 3 designates the growth performance of the diverse ratios of the Empty Fruit Bunch and Oil Palm Cake. The results depict 180:20 which is also equivalent to the 80:20 for the Empty Fruit Bunch (EFB) and Oil Palm Cake (OPC) as the best growth respectively for the different samples tested. The entire growth formulation of the substrate terms the substrate capacity to support the growth of the algae. The Positive control made with the two novel synthetic media had a much lower growth performance to the other ratios.

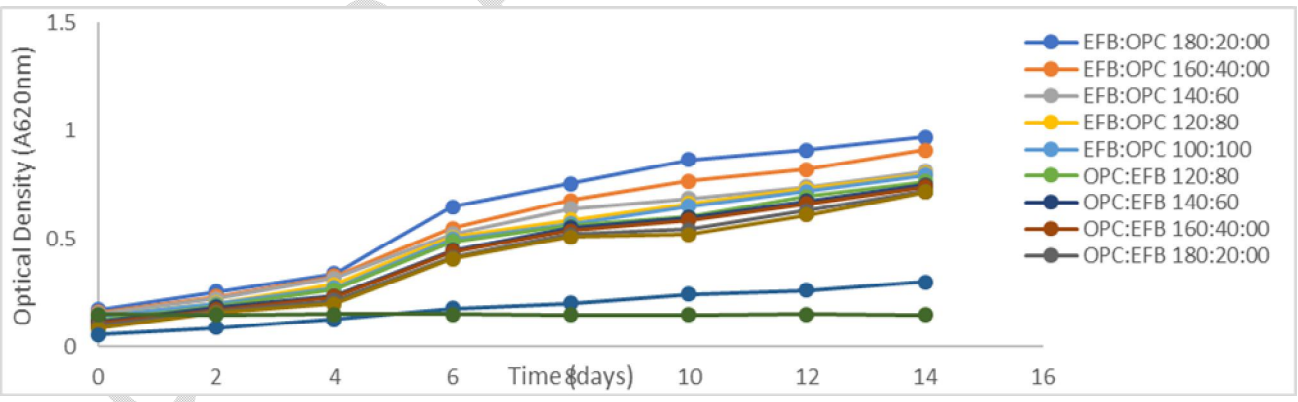

Fig 3: Growth selection of *Chlorella vulgaris* in different ratio of Empty Fruit Bunch and Oil Palm Cake

3.6: Optimization processes for *Chlorella vulgaris* growth

The following parameters were optimized for the growth of *Chlorella vulgaris* with temperature, salinity, pH, photoperiod, photoinhibition.

Figure 4 describes the effect of different temperature variations on the optimal conditions for the biomass production. The results present the growth pattern of the 30ºC which had a lag phase between the first days. The negative control which was uninoculated extract. The positive control was novel synthetic medium inoculated with the 3-day old culture of the Chlorella had no significant increase. 20 ºC and 35ºC had noticeable growth on the medium, after the exponential increase of the cell, suggesting the presence of soluble nutrients to the algal cells at the temperature levels tested.

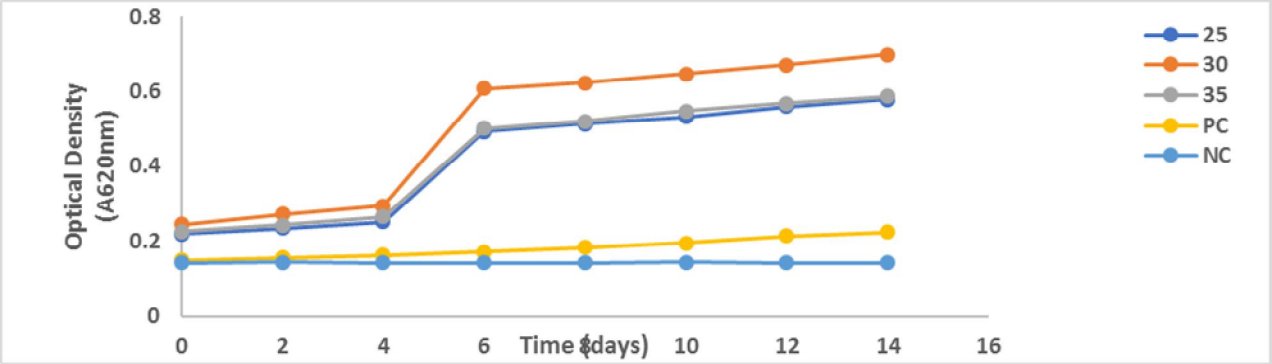

Fig 4.: Effect of incubation temperature on *Chlorella* growth
Key: PC= Positive Control
NC= Negative Control

3.7: Effect of salinity on *Chlorella* growth

Figure 5 shows the response of *Chlorella sp.* to saline environments. The superlative growth was observed for the 10 ppm of sodium chloride, the log phase started from the day 0 (0.228 abs) to day 2 (0.246 abs). Also, the 15ppm had a high biomass growth. The positive control had a lag phase on the first day and an exponenstial phase followed. The number of cells created was limited by growth factor and as a result the rate of cell growth matches the cell death. The negative control remained unchanged for the entire growth period. Congruently other salinity intensities had different growth but not significant.

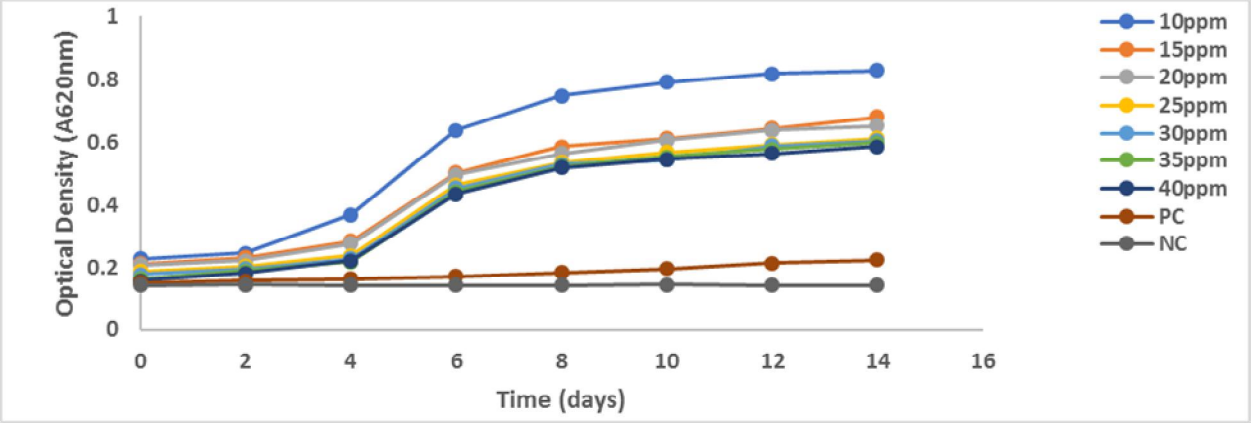

Fig 5: Effect of salinity on *Chlorella* growth
Key: PC= Positive Control
NC= Negative Control

3.8: Effect of different pH values on *Chlorella* growth using mixture of Empty Fruit Bunch and Oil Palm Cake Extract

Figure 6 is the response of the biomass feedstock to fluctuating level of the pH ranges. The result recommends that the pH 6.0 had the best biomass growth and build up with the lag phase of between 0-3days. Biomass build up pH at 7-9.0 had no striking significant difference.

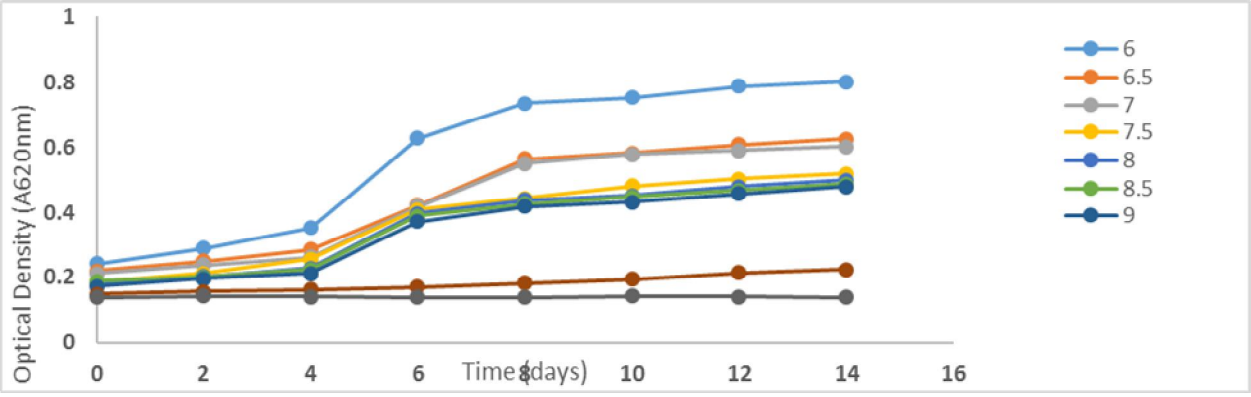

FIG 6: Effect of different pH values on *Chlorella* growth using mixture of Empty Fruit Bunch and Oil Palm Cake extract
KEY: PC= Positive Control
NC= Negative Control

3.9: Effect of photoperiod on *Chlorella* growth using Oil Palm Cake and Empty Fruit Bunch

Figure 7 presents the growth pattern of the isolates after their previous exposure to the 12:12 and 6:18 photoperiod for the blend of the oil palm extract, the study suggests the later photoperiods had a significant response compared to the negative and the positive control. The study observed an increase in the population of the cells with a log/exponential phase between 0 - day 3. The positive control was observed to have corresponding rise in biomass but performed least.

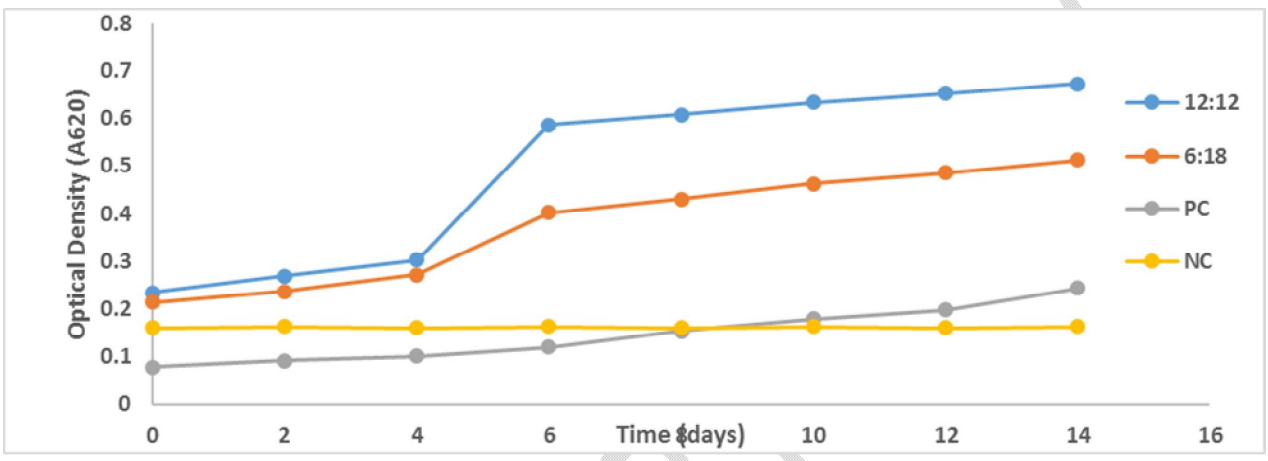

Fig 7: Response of *Chlorella* sp. to photoperiod
Key: PC= Positive Control
NC= Negative Control

3.10 Effect of photoinhibition to *Chlorella* growth

Figure 8 shows the growth curve of the cultured chlorella cells under 12:12 and 6:18 periods. The result suggests there is no significant difference in the growth between the periods of exposure to light. The positive control was observed to have increased from day 2 to day 5.

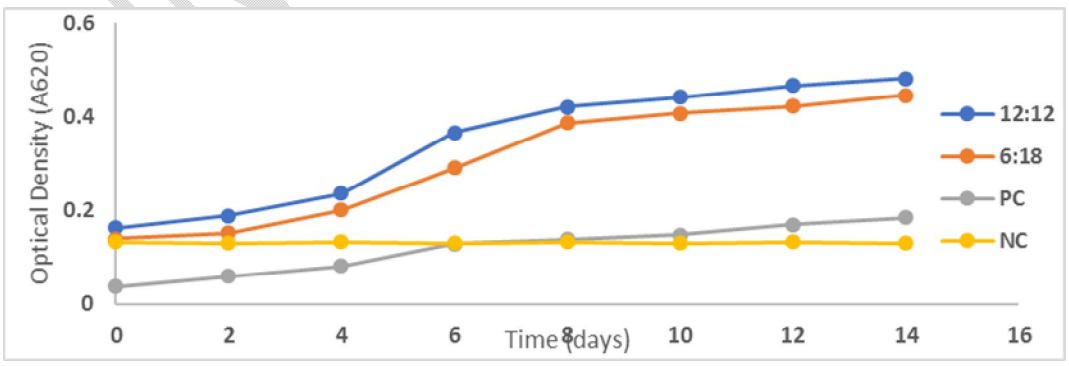

Fig 8: Effect of photoinhibition on the *Chlorella* sp.

KEY: PC= Positive Control

NC= Negative Control

4.2 Discussion

Agricultural industries produce an enormous quantity of agro industrial residue and there is a necessity to exploit these wastes to yield valuable products. Oil palm residues are one of the wastes spawned in large amount by oil palm mill processing industries and during the manufacturing of palm oil (Ehirim, 2004).

The Empty Fruit Bunch and Oil Palm Cake were both utilized as good source of nutrients for the growth of *Chlorella vulgaris*. Protein composition is vital for biosynthesis of active enzymes and co-enzymes, comparatively protein content is needed for functional system and supplementation (Nnamani *et al*., 2009). The protein content for OPC, EFB and algae biomass is (1.30±0.18, 0.80±0.08 and 1.15±0.06). Carbohydrate composition is a significant nutrition for algal growth (Harun et al., 2010). OPC and EFB had carbohydrate level of 29.83± 0.29 and 29.29±0.58 which favored the growth of the microalgae. It has been pragmatic that about 80% of the entire cost of production in microalgae cultivation is carbon substrate (Christi, 2007). These carbohydrates synthesized are amalgamated into the cell wall as a prospective feedstock for citric acid production. The grinded oil palm waste underwent aqueous extraction and was analyzed for their physicochemical properties. Agwa *et al*. (2012a) recounted a huge amount of organic and inorganic nutrients tied to agro residue, these residues serve as reservoir of nutrients. pH of a medium affects bioavailability of nutrients. The pH of the oil palm waste was (OPC=5.15±0.02, EFB=5.36±0.06). Clair et al., 2003 reported that extreme pH conditions may impede the growth of organisms.OPC and EFB were found to have high sulphate and nitrate content. Sulphate (ppm) (OPC=36.35±0.46, EFB=50.68±0.49) and Nitrate (ppm) (OPC=31.05±0.48, EFB=40.33±0.98). According to Qiang and Milton (2010), these ions are essential to enhance the growth of *Chlorella* sp. Anaga and Abu (1996), also reported that the ions have been noted to be compulsory for simulation of microalgal growth triggering blooming of the organisms.

Optimal conditions are fundamental for cultivation of microalgae. (Cho *et al*., 2007). In this study, the optimal temperature recorded with the oil palm waste was 30^{o}C. The results of this study agree with the findings of Cho *et al*. (2007) who reported the same temperature. Converti et al. (2009) reported that temperatures above 30^{o}C do not favour maximum growth activity in *Chlorella vulgaris*. Salinity is a quantity of ions and salts present in a medium for proper growth of microalga (Ruangsomboon, 2012). Alkhamis and Qin (2013) reported that a negative trend in the growth rate of the microalga was observed when the NaCl concentration was elevated above 15ppm, which affected the growth of cells. In this study, salinity concentrations of 10ppm had highest biomass quality as seen in figure 5. The work of Cho *et al*. (2007) suggested that an optimum salinity of 10ppm was recorded. The optimal pH was observed to be 6.0, this agrees with the reports of Pandey and Tiwari (2010). They reported that pH lenience of 7.0 is critical for the growth microalgae and a high throughput of biomass was observed. The determination of the

photoperiod is a crucial procedure in the selection of media activity, for the growth of microalgae which states that more biomass was accumulated in the 12:12 period of growth cultivation than in 6:18 period. The result of this study agrees with the report of Mata *et al*. (2010) which states that 12h day and light encouraged the production of biomass. But Jacob-Lopes *et al*. (2009) investigations revealed that 24h dark cultivation conditions lower biomass production. After inoculation of the microalgae to oil palm extract in a ratio of 180:20 of EFB and OPC, at day 0 the cell density was almost zero because the cells have to adapt to the physiological conditions of the new environment. During the day three, the (lag phase) the microalgal showed slow growth as the cells allocate most resources to the physiological adaptation induced by the new environment (Becker 2004). The lag phase was proceeded by a rapid exponential phase which is characterized by cell doubling, doubling will continue at a constant rate so both the number of cells and the rate of population doubles each consecutive time period. As the nutrient becomes exhausted, the growth phase is decelerated and the cell biomass augmented linearly. Then the growth phase remained stationed till lapse of incubation period because the nutrient has depleted and the biomass concentration decreased.

4.0 **CONCLUSION**

Elaeis guineensis extract is a low-cost carbon substrate effective for the growth of Microalgae with high biomass production. Microalgae cultivation with oil palm extract generated high cell growth than cultivation in a synthetic medium.

5.0 REFERENCES

A.O.A.C. Official methods of analysis, (1984): Robert C. Rund (ed). Associate Chapter Editor; office of Indiana State Chemists and Commissioner.Pp.20-120.

Agwa, O. K.; Ibe, S. N. and Abu, G. 0. (2012). Biomass sand lipid production of a fresh water algae *Chiorella* sp. Using locally formulated media. Inter. Resear. *J. MicrobioL* 3: 288-295.

Agwa, O.K., Ibe, S.N. and Abu, G.O. (2012a). Assessment of cow dung waste for the laboratory cultivation of *Chlorella* for lipid production. *Astan Journal of Microbiology and Biotechnology of Environmental Science.* 14:1-6.

Alkhamis, Y and Qin, J. G. (2013) Cultivation of *isochrysis galbana* in phototrophic, heterotrophic, and mixotrophic conditions. *BioMedical Research International* 9: 1-9

Anaga, A., and Abu, G.O., (1996). A laboratory-scale cultivation of *Chlorella* and *Sp.irulina* using effluent from a fertilizer company in Nigeria. *Bioresource Technology*. 58, 73–95.

Becker, E. W. (2004). Large scale cultivation in Microalgae-Biotechnology and Microbiology. Cambridge University Press, Cambridge. Pp. 63-171.

Chisti, Y. (2007) Biodiesel from microalgae. *Biotechnology Advances*. 25:294-306

Cho, S.H., Ji, S.C., Hur, S.B., Bae, J., Park, I.S. and Song, Y.C. (2007) Optimum temperature and salinity conditions for growth of green algae *Chlorella ellipsoidea* and *Nannochl- oris oculata*. *Fisheries Science* 73 (5) 1050–1056.

Converti, A., A. A. Casazza, E. Y. Ortiz, P. Perego, and M. Del Borghi. 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of

Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. *Chemical Engineering and Processing* 48(6):1146-1151.

De Morais, M. G. and Coasta, J. A. V. (2007). Isolation and selection of microalgae from coal fired thermoelectric power plant for biofixation of CO_2. *Energy Conversion and Management* 48: 2167-2173.

Ehirim N. C. and M. A. C. A. Odii, (2004). Economics of Palm Oil Marketing in Owerri, Imo State, Nigeria, *J. Technol*. Edu. in Nigeria, (9), 71-81.

Huss, V.A.R, Elke C. F. C., Hartmann, M. H, Loboucek, A., Seidel, B. M., Wenzeler, P and Erich K. (1999) Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato (chlorophyta) *Journal of Phycology* 35: 587–598

Jacob-Lopes, E., Scoparo, C.H.G., Lacerda, L.M.C.F and Franco, T.T. (2009) Effect of light cycles (night/day) on CO_2 fixation and biomass production by microalgae in photobioreactors. *Chemical Engineering Process* 48:306–310

Kansal, A. (2002). Solid waste management strategies for India. *Indian Journal of Environmental Protection*. 22(4): 444-448

Mata, T. M., Martins A.A and Ceatano, N.S. (2010). Microalgae for biodiesel production and other appplications: a review. *Renewable Sustainable Energy Review.* 14: 217-232

Mogany, T (2014) Optimization of Culturing Conditions and Extraction Method for Phycocyanin Production from a Hypersaline Cyanobacterium. Student thesis, Durban University of technology.

Nnamani, C.V., Oselebe, H.O and Agbatutu, A. (2009). Assessment of nutritional values of three underutilized indigenous leafy vegetable of Ebonyi state, Nigeria. *African Journal of Biotechnology*. 8: 2321-2324.

Ogbonda, J. C., Odii C. A. and Tanaka, H. (2007). Light requirement and photosynthetic cell cultivation-development process for efficient light utilization in photobioreactors. *Journal of Applied phycology.*

Olaizola, M. (2003a). Commercial development of microalgal biotechnology: from test tube to the market place. *Journal of Biomolecular Engineering*. 20: 459-506.

Pandey, J.P and Tiwari, A. (2010). Optimization of biomass production by *Sp.irulina platensis.* *Journal of Algal Biomass* 1 (2) 20-32

Ponnuswamy, I., Madhavan, S and Shabudeen, S. (2013). Isolation and Characterization of Green Microalgae for Carbon Sequestration, Waste Water Treatment and Bio-fuel Production *International Journal of Bioscience and Biotechnology*. 5, 2-17.

Qiang,H. and Milton, S. (2010). Novel Chlorella sp.. and uses. *Akron Biotechnology*. 21:5-68

Rigano,V.D.M., Vona, N., Esp.orto,S., Carillo, P., Carfagna, S. and rigano, C.(1998). The physiological significance of light and dark ammonium metabolism in *Chlorella sorokiniana. Phytochemistry*. 47: 177-181

Ruangsomboon, S., 2012. Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, Botryococcus braunii KMITL 2. *Bioresource Technol*., 109: 261-265.

Vandenberghe, L.P.S., Soccol, C.R., Pandey, A., and Lebeault, J. (2009). Microbial production of citric acid. *Braz. Arch. Biol. Technol.* 42 (3): 263 –276.

Wang B, Li Y, Wu N, Lan C. (2008) Carbon (iv) oxide bio-mitigation using microalgae. *Applied Microbiology and Biotechnology*; 79(5):707–718.

Wijanarko, A., Dianursanti, H., Soemantojo, W. and Ohtaguchi, K. (2006b). ´´Effect of light illumination alteration on chlorella vulgaris Buitenzorg's CO_2 fixation in bubble column photobioreactor." *International journal for Algae*.8: 53-60.