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Cultivation of microalgae using Cassava wastes as a growth media.

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Abstract.
One of the renewable sources of energy, microalgae is a good source of feedstock for the production of various biochemicals because of their unique properties. In this study, the proximate and the physicochemical analysis of the cassava wastes (effluent and peels) revealed the presence of vital nutrients for microalgae growth. The growth media was formulated using combinations of the cassava effluent and cassava peels with pond water from the African Regional Aquaculture Centre (ARAC), Aluu, Rivers State, Nigeria in varying ratios. The growth conditions were monitored as cell density (OD 600nm), lipid productivity (mg/l/day) and dry matter (g/l) for 14 days at an ambient temperature, under natural light. Algal blooming occurred in the mixtures on day 4 with a deep green colouration and there was a slight decrease in optical density recorded on day 6 for the cassava effluent. The microalgae were able to convert the wastes into organic macromolecules (lipids); stored in their cells as biomass which can be modified to serve as value added products. The Cassava peels were found to give a higher lipid productivity of 40.7mg/l/day than the effluent with a lipid productivity of 26.1 mg/l/day. The physicochemical parameters recorded showed a very high variation in the BOD; 9200mg/l, 291mg/l and TDS; 18,180mg/l, 9,200mg/l for the cassava effluent and peels respectively compared to the standard values of WHO; 24mg/l and 500mg/l for the BOD and TDS respectively. Cassava wastes which can constitute a great nuisance to the environment if discharged on both soil and water bodies without proper treatment, have proven to be of a great value and resource if used for the cultivation of microalgae. The use of Cassava wastes for the cultivation of microalgae is therefore an attractive economic venture and also a demonstration of Sustainable Resource Management (SRM).

Key words*: Biomass, Cassava effluent, Cassava peels, lipids, Microalgae, Pollution.*

Introduction

Cassava (*Manihot esculanta*) is a root tuber crop that is widely cultivated in the tropical regions of the world as a shrubby perennial that can grow to a height of 6-8 feet and is usually propagated by plant in short section of the stem (Oboh, 2005). Cassava is the third-largest source of food carbohydrates in the world (FAO, 2012) and is also a major staple food in the developing world, providing a basic diet for over half a billion people. It is one of the most drought-tolerant crops, capable of growing on marginal soils. Nigeria is the world's largest producer of cassava. It is consumed as fermented products either as foo foo, starch, lafun or processed into garri. The technology of processing cassava roots involves essentially; peeling, grating, fermenting, de-watering and sieving. These processes increase the qualities of cyanogenic glycosides and cassava waste products lost into the wash water used (Eze, 2010). Though there are different types of wastes generated from processing of cassava tubers as shown in table 1, Oboh (2005) identified two important wastes to include cassava peels and cassava effluent.

In processing of cassava, the peel which is the outer covering of the cassava root is removed and contains the outer thin, brown and thick leathering parachymatous inner covering (Atulegwu and Nnamdi, 2011). Cassava peels constitute about 20-35% of the weight of the tuber, especially in the case of hand peeling (Obadina *et al*., 2006). Consequently, a large amount of cassava peel waste is generated annually. In Nigeria, cassava peels produced were about 450,000 tons annually with an increasing trend (FAO, 2006).They are discarded as waste and allowed to rot in the open resulting in health and environmental hazards (Atulegwu and Nnamdi, 2011). The vegetation and soil around the heaps of the peels are rendered unproductive and devastated due to biological and chemical reactions that take place between the continuously fermenting peels, soil and the surrounding vegetation (Olanbiwoninu and Odunfa, 2012). The waste could also constitute breeding grounds for flies and insects which are carriers of diseases (Omotosho and Sangodoyin, 2013).

Table 1: Types of wastes generated from different unit operations of cassava processing and their environmental impact.

Type of operation	Type of waste generated	Expected environmental impact
1. Peeling	Peels with high fibre and high cyanide content; the cyanide can diffuse into rivers or ponds.	Can contaminate ground water supply during rain. Foul odor. High HCN concentration in the waste water can be a problem if used directly on land. Dissipation is rapid if passed to waterways. Organic matter is a problem, causing high BOD and COD, and eutrophication of waterways and foul odors.
2. Washing	Organic matter, soil.	Little impact.
3. Squeezing	Effluent with high content of cyanide and organic matter (mainly starch).	High HCN may kill plants if effluent is allowed to run out on land. Dissipation should be rapid if released into waterways. Organic content may contaminate ground water supply and cause eutrophication of surface water and foul odor.
4. Sieving	Fibrous waste.	If exposed to rain, the seepage of organic material from stored waste could contaminate the ground water
5. Sedimenting	Starch residue, waste water.	Large amounts of solid waste can degrade the aesthetics of a processing environment, high organic matter can cause high BOD and COD and eutrophication of water ways.

The other type of waste is the cassava effluent or waste water. The cassava effluent is the waste water generated from the processing of cassava or the liquid squeezed out of its mash. By its nature, cassava processing for extraction processes produces large amounts of effluent high in organic content, if untreated may be displayed in the form of stagnant effluent ponds from which strong odors emanate, its effect on the environment is significant as the air we breathe becomes contaminated with the odor emanating from it, resulting in adverse respiratory health problems (Eze, 2010). In many areas where traditional processing is practiced, the effluent is normally discharged beyond the "factory" wall into roadside ditches or fields and allowed to flow freely, settling in shallow depressions (Ehiagbonare *et al*., 2009). Eventually this will percolate into the subsoil through infiltration or flow into streams and other surface water sources thereby causing pollution of the underground water reservoir, agricultural surface water and the subsoil (Ehiagbonare *et al.*, 2009; Morenikeji, 2010). Domestic animals and man that consume this water might be infected with diseases and other health problems such as stomach pains, diarrhoea; it may also have effect on plants as vegetation is hardly noticed on such areas (Ogundola and Laiasu, 2007) causing environmental degradation. The waste water can also contaminate other soil properties because of its Total Solid, Total Organic Carbon, Nitrogen and Phosphorus (Osunbitan *et al*., 2000). Additionally, runoff after heavy rainfalls or storms can cause eutrophication and pollution when these nutrients are carried to the ground water before proper treatment (Eze, 2010).

Both cassava peels and effluent contain a number of contaminating substances amongst which is cyanide. Cyanogenic glucosides in these cassava wastes are in various concentrations depending on the variety and growing conditions (Ehiagbonare *et al.*, 2009). Cyanide, being an acidic component will naturally have an inhibiting action on the biological degradation of cassava wastes and in sewage may inhibit the usual degradation processes as well (Olayiwola, 2013). Additionally, the presence of simple and complex cyanide and their break down products – cyanohydrins and hydrogen cyanide has been a cause of concern because of their possible effects on health and environment (Okunade and Adekalu, 2013). Atulegwu and Nnamdi (2011) revealed that cyanide forms complexes with zinc and hydrogen to form an acidic complex called hydrogen cyanide acid which is a major threat to the environment. The unbroken down cyanogenic glycoside - Linamarin and Lotaustrlin which constitute potent toxicant to the soil, soil organisms, water and plants when degraded and leached into the soil has been reported by Madamombe (2006) to also liberate poisonous cyanide in the body. The deleterious effects of cyanide even at low concentrations have been widely reported (Onabolu *et al*., 2001; Oluwole *et al*., 2003). The lethal dose of hydrocyanic acid (HCN) taken by mouth is 0.5-3.3mg/kg body weight and it is rapidly absorbed from the gastrointestinal tract. Consumption of water and food products containing large amounts of cyanide can cause acute intoxication, with symptoms of dizziness, headache, nausea, vomiting, sometimes death (Omonoma and Akinpelu, 2010), and many instances of cyanide poisoning in humans and animals ((Uhegbu *et al.*, 2012). Cyanide has also been implicated as a causative agent in certain diseases such as Leber' Optical Atrophy, Tobacco

Amblyopic, Tropical Ataxic Neuropathy (TAN), Endemic Goiter and Cretinism Syndrome (Oluwole and Onabulu, 2004). A number of enzymes like catalase, superoxide dismutase, nitrate reductase have been reported to be inhibited by cyanide (Oluwole *et al*., 2002) but the most important effect of cyanide is the inhibition of tissue respiration (Lehininger, 2005).

Continuous discharge of these wastes has accentuated the adverse effect of cassava waste to the environment and biodiversities (Atulegwu and Nnamdi, 2011). These wastes also contain varying concentration of heavy metals either as simple metals or complexes (Igbozuruike *et al*., 2009). From Table 1 a summary of the various wastes that can be generated from cassava processing and their environmental impact can be seen. The cassava waste generally contains large amounts of inert materials- BOD and COD, about 180kg of COD per tonne of roots. Arimoro *et al*., (2008) showed a depletion of dissolved oxygen, depression in ph values, elevation of BOD and Nitrate values in the tropical stream of southern Nigeria as a result of the discharge of these wastes. The wastes pose a serious threat to the environment and the quality of life of the people in the rural areas where the processing units are mainly located.

It is important to note that about 60% of the Nigerian populace still source for domestic and sometimes drinking water from ponds, streams, shallow wells and other ground water sources. This justifies the concern for increases in the level of pollutants in surface and ground water thus making ineffective and inefficient waste disposal a pressing environmental problem (Coker *et al.*, 2009; Morenikeji, 2010). With these major threats to the environment by the cassava processing industry and increasing future demand for cassava especially in Nigeria, improper cassava waste handling may become a problem. Therefore algal growth with cassava wastes is thus an added advantage because these wastes have proven very useful for the growth of microalgae because they contain the following essential compositions; Moisture 0.82%, Ash 2.71%, crude fibre 4.40%, crude protein 2.69%, crude lipid 3.92% and total carbohydrate 85.46% (Sarkiyayi and Agar, 2010), in addition to essential ions like nitrate, sulphate and phosphorus which are required for their growth.

Microalgae are generally microscopic algae, found in fresh water or marine systems which have an extraordinary potential for cultivation as energy crops (Tredici and Materassi, 2004; Neboh *et al.*, 2014). Their common feature is their oxygenic photosynthesis similar to that in higher plants and they make large contributions to the equilibrium of the earth's atmosphere by producing oxygen and removing carbondioxide (Agwa *et al.*, 2011). Microalgae apart from been used as single cell proteins, are projected as living cell factories for the production of biofuels and various beneficiary biochemicals used in food, aquaculture, poultry and pharmaceutical industries (Anand, 2010). Microalgae have been discovered to have the highest oil or lipid yield among various plant oils, and the lipid content of some microalgae has up to 80% triglyceride that can be converted into biodiesel through transesterification (Chisti, 2007). The need for renewable energy supplies that do not cause environmental harm nor compete with food supply have heightened interest and driven economic sustainability to the development of biological sources that cannot affect food crops but can be used for energy applications (Chisti, 2007; Feng *et al*., 2011). Renewable energy products from microalgae can be more environmentally sustainable, cost effective and more profitable if combined with processes such as waste management and its utilization (Agwa *et al.*, 2012b). Microalgae are therefore an unexploited resource representing one-third (1/3) of the world's plant biomass.

Besides the energy value that can be obtained from these wastes if properly converted, the pollution potential of the cassava wastes are also minimized within the industrial environment if used to cultivate microalgae (an eco-friendly technology); thus this research was therefore, undertaken to scientifically explore the possible application of these wastes for the cultivation of microalgae, thereby reducing environmental pollution and providing excellent use for bioenergy production.

Materials and Methods

Sample collection

Water samples containing the microalgae were aseptically collected from the African Regional Aquaculture Centre (ARAC), Aluu, Rivers State. The Cassava peels and the effluent were also collected from a cassava mill industry in Alakahia town, Rivers State and stored in sterile containers placed on ice jackets and transported to the laboratory for analysis.

Proximate Analysis

Typical analysis of the micronutrients of the cassava peels such as Moisture content, crude protein, crude ash, crude fibre, total carbohydrate and lipid were carried out according to the procedures of Agwa *et al*. (2012b).

Assay of Physicochemical Properties

Nitrate, Sulphate, Calcium and Magnesium were among the parameters determined according to the standard methods of APHA (1998). Hydrogen ion concentrations were carried out using an automatic digital pH meter (model Mettle Delta- 340) made in England. Total Dissolved Solids (TDS) were estimated by gravimetric method as described by Trivedi and Raj (1997) while the

Biochemical Oxygen Demand (BOD) and Dissolved Oxygen (DO) were determined according to the procedures of Agwa *et al.* (2012a).

Sample preparation

The Cassava peels were sun dried, ground into fine powder using a Panasonic electric blender, model (MX-J110P) to obtain a cassava peel with particle size of 80/100 mesh. Extracts were prepared by dissolving 1g of ground cassava peels in 100ml of distilled water and sterilized to destroy the pathogens and filtered using whatman's filter paper (No 1). 100ml mixture of the pond water to the cassava peels water were made to achieve the following ratios; 90:10, 80:20, 70:30, 60:40 and 50:50 and labeled A to E respectively to induce blooming of the algae in 500ml cotton plugged conical flasks. Appropriate controls were also set up, containing only cassava extracts (Control F) and the other containing only Pond water (Control G). Likewise for the cassava effluent, 100ml of the mixture of the effluent and pond water were also made to achieve the same ratio as the cassava peels. Appropriate control was also set up, containing only the cassava effluent (Control H). The flasks were aerated manually and was intermittently shaken every 2hrs for 16hrs and finally incubated for 14 days under natural light source.

Isolation and Identification

The technique of Agwa *et al.*, (2012a) was used for the isolation of the microalgae. Using 1ml Pasteur pipette, an aliquot of the sample was placed in a watch glass and viewed under X40 objective of the Hm-lux leitz light microscope. A micro pore Pasteur pipette fitted with a rubber bulb was used to suck out cells of the microalgae, transferred into another watch glass containing 1ml of normal saline to obtain a unialgal culture of the microalgae and the process was continued until the microalgae were identified according to the scheme of Richmond (2004) based on their cultural and morphological characteristics.

Analysis

Optical Density

The Optical Density (OD) was determined using a spectrophotometer (Spectronic 721 model) set at 600nm. About 5ml of the growing culture were removed aseptically, placed in the cuvette after blanking and the absorbance was measured at 600nm.

Cell dry weight

Cell dry weight was determined (Anaga and Abu, 2006). About 5ml of the growing culture was harvested by centrifugation at 3000rpm for 10mins. The cells were washed (3x) with physiological saline dried at 50^{o}C in a hot air oven to a constant weight.

Lipid Extraction

The wet extraction procedure was adopted (Agwa *et al*, 2012b). Cells were harvested by centrifuging 100ml of the culture at 3000rpm for 15mins; the supernatant was decanted into a centrifuge tube leaving the wet paste at the bottom. To about 40mg of the wet cells was added 1ml distilled water, 2.5ml methanol and 1.25ml chloroform. The mixture was mixed for 10mins, thereafter centrifuged at 1000rpm for 5mins and the supernatant transferred into the centrifuge tube containing the initial supernatant. To the residue at the bottom of the centrifuge tube was added another 2.5ml methanol, 1.25ml chloroform and 1.0ml water, mixed and the extraction procedure repeated. The lower chloroform phase containing the extracted lipids was transferred into a pre-weighed 50ml Erlenmeyer flask, diluted with chloroform to 10ml and brought to dryness in a rotary evaporator (30-35^{0}C) leaving the lipid which was then reweighed using an analytical weighing balance (Setra BL-410S, USA).

Kinetic and Yield Parameters

The lipid content, C_L (mg/l) was calculated using the equation.

$$C_L = \frac{W_{2\,(g)} - W_{1\,(g)}}{W_{DW\,(g)}} \quad \ldots\ldots\ldots\ldots (1)$$

Where W_1 and W_2 are the weight of the extraction flask before and after extracting the oil. W_{Dw} is the weight of the dry algae biomass.

The lipid productivity P_L (mg/ L/ day) was calculated by using the equation:

$$P_L = \frac{C_L}{t} \quad \ldots\ldots\ldots\ldots (2)$$

Where C_L (mg/ L) is the lipid content and t is the duration of the cultivation.

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) was used to calculate the mean and Standard Deviation (SD). The Post hoc test (Scheffe and Duncan) was used to test for the significant difference at p-values < 0.05 within the groups measured at 95% confidence level.

Results and Discussion

The results of the proximate analysis and physicochemical parameters are shown in tables 2 and 3. The samples were found to support the growth of the microalgae because they contained essential nutrients required for their growth. These nutrients include sulphate, nitrate, and phosphate amongst others. Anaga and Abu (1996) reported that these ions are necessary for the stimulation of microalgal growth causing a good blooming of the organisms. There was effective blooming under natural light within 4-8days with a deep green colouration indicating the presence of the microalgae. The results showed that the cassava effluent and the cassava peels with the pond water complemented each other, however, the controls did not show any sign of blooming. Algal blooming occurred first in the 20:80 mixture of both effluent to pond water and peels to pond water respectively on day 4 of exposure to sunlight, this is in accordance with the study carried out by Budiyono and Kusworo (2011); Neboh *et al*., 2014.

Table 2: Proximate composition of cassava peels

PARAMETER	**V/W (%)**
Carbohydrate	27.7
Protein	2.438
Lipid	2.8
Moisture	13.6
Ash	15.9
Fibre	49.562

Table 3: Comparison of the physicochemical parameters of the cassava effluent, peel extract and pond water to that of standards for drinking water.

Parameter	Cassava effluent	Cassava peel extract	WHO Water standard	FEPA	Pond Water
NO_3(mg/l)	0.88	18.09	0.2	< 0.01	0.55
SO_4(mg/l)	130.72	0.69	100	1000	5.72
PO_4(mg/l)	168	584	-	-	-
BOD(mg/l)	9200	291	24	10-20	2.79
COD(mg/l)	-	1755	-	-	-
TDS(mg/l)	18,180	9200	500	-	63.75
DO(mg/l)	-	-	6.8	>1.0	5.8
Ca(ppm)	3.67	32.0	22.1	-	26.50
Mg(ppm)	34.927	32.0	0.2	-	12.0
pH	5.54	7.09	6.5-8.5	5-9	7.8

Favourable growth conditions also encouraged the proliferation and growth of the microalgae. Apart from nutrients, aeration and light also enhanced the process of photosynthesis. Microalgae cultures as reported by Rocha (2003) become denser gradually and block light from reaching deep into the flasks. Janssen (2005) noted that, light is distributed by mixing as each algal cell moves through dark and light zones of the flask. Mixing is the next factor that can expose the cultures throughout the flasks to moderate light intensity and improve the light absorption; it will also expose the cells to low and high light cycle and

save energy because the low light phase, channels the energy in photosynthesis into downstream metabolic processes (Perez, 2008). According to Anand (2010), the aeration will not only prevent settling of cells but will also ensure that the nutrients are well distributed to the cultures.

The physicochemical parameters of the cassava effluent in comparison to standards for drinking water showed a very high variation in the effluent from that of the standards. Neboh *et al*. (2013) stated that any water contaminated to this level is neither good for domestic use nor is supposed to be discharged directly into the environment without proper treatment.

The pH of the cassava effluent was found to be acidic; this plays a part in determining both the qualitative and quantitative abundance of micro flora (Federov, 2003) in both soil and river where these wastes are discharged. It could be inferred then that more hydrogen ion became available, lowering the pH value of contaminated soil and affecting the pattern of microbial population. The TDS was very high; 18,180mg/l and 9200mg/l for the cassava effluent and peels respectively compared to that of WHO (63.75mg/l). This implies that the TDS can absorb heat from the sun and transfer the same to water bodies. Turbidity can also be increased as a result of this and has the capacity to depress light penetration into a given water body thus affecting fish feeding habits, the growth of phytoplankton and photosynthetic activity of plants (Ehiagbonare *et al.*, 2009). The high level of turbidity in the effluent may result in unpleasant colour of the water, indicative of the presence of colloidal solids and large number of micro-organisms which may be harmful. The value of BOD of the waste water and the peels; 9200mg/l and 291mg/l were high compared to that of FEPA (10 - 20mg/l) and WHO (24mg/l). This increase resulted in the depletion of oxygen which is utilized by the micro-organisms for the breakdown of organic matter and thus actively encouraging the growth of anaerobic than aerobic organisms. When oxygen becomes less available, it prompts denitrifying bacteria to reduce available nitrate to gaseous nitrogen that enters the atmosphere with a resultant negative effect (Madigan, 2003). The proximate analysis of the cassava peels was found to correspond with that of Kadashi (2005); Julie *et al.*, (2009). But the moisture content value was lower than that reported by Frederick (2008) whose analysis was on fresh cassava weight. The difference in moisture content as compared to that of literature was due to loss of water during the drying process.

Figure 1and 2 are line graphs showing the optical densities of both the cassava peels and the effluent. The wastes were found to support the growth of the microalgae, the optical densities of both wastes showed no lag phase, indicating that they served as a good substrate for the growth of the microalgae.

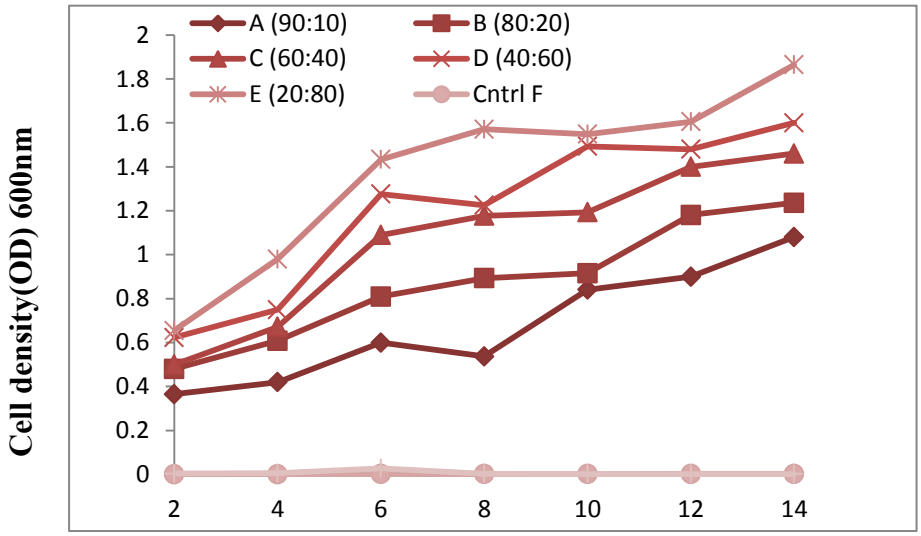

Figure 1: Optical density of Cassava waste peel to Pond water

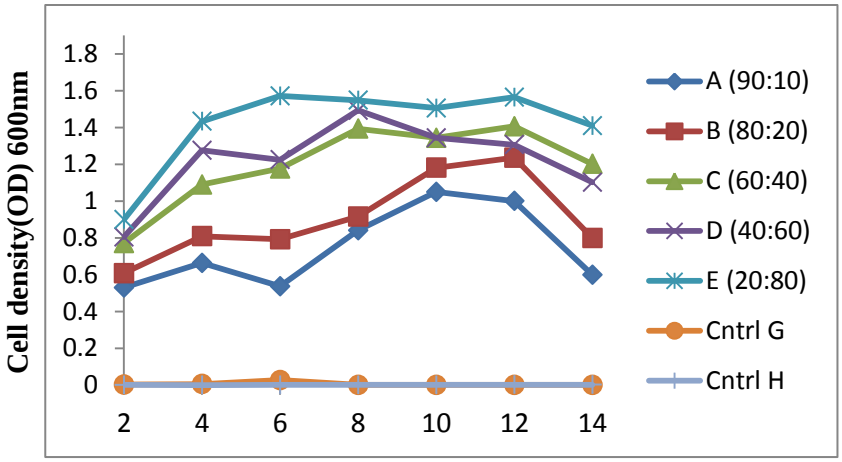

Figure 2: Optical density of Cassava effluent to Pond water

The results also showed a steady increase in optical density of all the mixtures from day 1 to 5, but on the 12th day there was a decline in the OD reported for the cassava effluent, showing that probably the organisms have reached a decline owning to a reduction in the available nutrients. On the other hand, the OD for the Cassava peels continued to increase as a result of the availability of some nutrients present in the waste and its unique characteristics. This trend of growth also affected both the dry weight and the lipid productivity of the wastes. The cassava peels were found to produce more lipid than the effluent. The highest lipid productivity obtained on the 14th day were 40.7mg/l/day and 26.1mg/l/day with respective dry weight of 5.71(g/l) and 3.66(g/l) for the 20:80 ratio of both the cassava peels and effluent respectively (Figure 3 and 4).

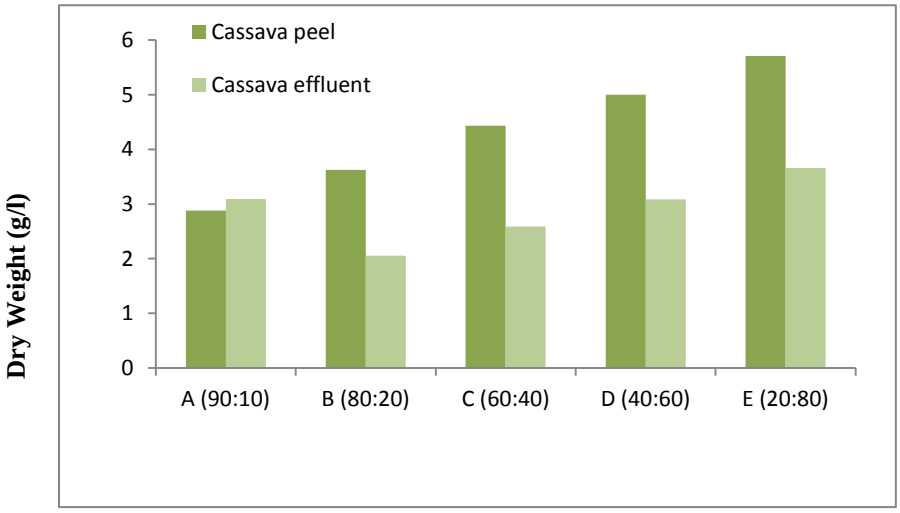

Figure 3: Growth of microalgae on Cassava wastes measured as dry weight

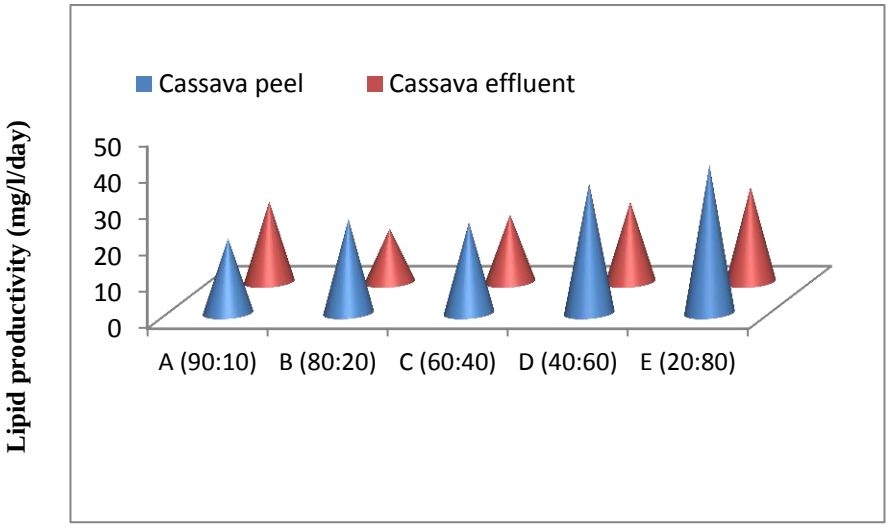

Figure 4: Lipid productivity of microalgae grown on different cassava wastes

This could be attributed mainly to the micronutrients as obtained in the proximate analysis, such as lipid, carbohydrate, nitrogenous substances from the crude protein of the cassava peels. Nutrient stress as reported by (Kalpish *et al.*, 2012) have been found to increase lipid production. Nitrogen is the most growth limiting factor for microalgae and would be the first nutrient to be depleted during algae cultivation (Kalpish *et al.*, 2012). Earlier studies by Richmond (2004) shows that nitrogen constitutes about 7% - 10% of microalgae dry cell weight and it is the most important nutrient for microalgae growth. Cassava peels have also been reported to have a high starch content and a high C:N ratio compared to the effluent(IFAD, 2010). Even though the effluent has been found to undergo nutrient stress such as nitrogen / phosphorus starvation (Hu *et al.*, 2008), more lipid production was expected but owning to the fact that the peels themselves naturally contain a high lipid coupled with that produced within the cultivation period explains the reason for the higher lipid content in the cassava peels.

In addition, environmental stress condition when nutrients are limited, invariably cause a steady decline in cell division rate. However, active biosynthesis of fatty acids is maintained in some algae species under such conditions provided there is enough light and CO_2 available for photosynthesis (Kalpish *et al.*, 2012) as was seen in the cassava peels. Miao and Wu, (2006); Hu *et al.*, (2008) identified lipids as a precursor for biodiesel production. Triacylglycerides (TAGs) generally serve as energy storage in microalgae that once extracted can easily be converted to biodiesel through transesterification reactions (Chisti, 2008). Recently the rise in petroleum prices and the need to reduce green house gas emission has seen a renewed interest in large scale biodiesel production from microalgae (Chen, 2011).

The Post hoc test (Scheffe and Duncan) showed a significant difference for optical density, dry weight and lipid by the microalgae between the cassava effluent and peels, within the groups measured at 95% confidence level (p-values < 0.05).

Conclusion

It is evident from this study that Cassava wastes are very good sources of essential nutrients required for the cultivation of microalgae. Though the cassava peels proved to be a better substrate than the effluent due to its unique features as revealed in its proximate analysis. However, the microalgae through their photosynthetic machinery, were able to convert both wastes into organic macromolecules (carbohydrate, lipids, and proteins) stored in the cell as biomass. The lipids especially can be useful for biodiesel while the protein and carbohydrate can be converted to other useful chemicals instead of discharging them on land or water in an unplanned manner causing pollution of underground water reservoir and the subsoil. This is in support with the studies carried out by Nwaogwu *et al.*, (2007); Ehiagbonare *et al.*, 2009 and Budiyoro and Kusworo (2011), that cassava wastes can be used in the cultivation of microalgae for the production of biomass such as biofertilizer, biofuels, protein etc. Thus, the wastes which are considered as polluting can be channeled towards the culture of microalgae on a large scale thereby adding more value to them and increasing their potential towards the demonstration of sustainable resource management.

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