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Full Length Research Paper

Heterotrophic cultivation of *Chlorella* sp. using different waste extracts

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Heterotrophic growth of *Chlorella* sp. using glucose as carbon source was investigated using different animal waste extracts. The *Chlorella* cells were obtained from a fresh water pond by blooming and cultured under different growth conditions. Artificial illumination with forced aeration resulted in 2.30 mg/ml (pig); 2.39 mg/ml (goat); 1.43 mg/ml (cow dung); 2.12 mg/ml (grass cutter) and 2.52 mg/ml (poultry) and lipid content of 4.25%; 4.49%; 10.16%; 8.15% and 8.66% respectively. Artificial illumination with unaerated growth condition gave 1.74 mg/ml; 1.71 mg/ml; 1.16 mg/ml; 1.64 mg/ml and 2.06 mg/ml of dry matter and lipid content of 2.40%; 2.13%; 9.92%; 4.97% and 8.39% respectively. The natural condition showed 1.99 mg/ml; 1.64 mg/ml; 2.30 mg/ml; 2.58 mg/ml and 2.77 mg/ml of dry matter with 7.78%; 4.03% 12.99%; 19.56% and 22.71% lipid content respectively. There were significant difference (P<0.05) between the different cultural conditions and the waste extracts. The proximate composition of the *Chlorella* cells under the heterotrophic growth conditions revealed about 38.30% of crude fat content compared to 13.20% from an autotrophic *Chlorella* cell. This result shows that heterotrophic cultivation of *Chlorella* cells using poultry waste extracts under natural condition is feasible as a potential waste utilization, management and pollution control alternative.

Key words: Animal waste extracts, *Chlorella* cells, dry matter, heterotrophic growth, lipid content.

INTRODUCTION

Microalgae have long been recognized as an efficient biological system to harvest solar energy for producing biomass and a great variety of metabolites. Attention has been drawn to the accumulation of high-value nutrients in the cells of microalgae (Shi et al., 2000). The ability of microalgae to adapt their metabolism to varying cultural conditions provides opportunities to modify control and thereby maximize the formation of targeted compounds. Certain criteria must be met by microalgae for heterotrophic production which include (1) the ability to divide and metabolize completely in the dark; (2) the ability to grow on an inexpensive and easily sterilized media; (3) the ability to adapt rapidly to the new environment and withstand hydrodynamic stresses (Wen and Chen, 2003).Various systems are used for production of microalgae which can be autotrophic, mixotrophic and heterotrophic. Variations in climatic conditions, characteristics of the microalgae as well as the method used, determine yield and productivity of the products (Ogbonna et al., 2000). Autotrophic microalgae (*Chlorella* sp.) can grow heterotrophically (light-independent), if supplemented with a preferred carbon source (Shi et al., 2000; Lee, 2004; Chen and Chen, 2006; Qiao et al., 2009). Heterotrophic cultivation has been known for decades, as it is regarded as the most practical and promising way to promote productivity of biomass, high levels of lipids and less protein than photosynthetic algae (Grima et al., 2003; Olaizola, 2003; Miao and Wu, 2006).The system is based on the addition of glucose, acetate or glycerol as the main carbon source (Octavia Perez-Garcia., 2010). Light-independent heterotrophic growth eliminates light as a growth factor and significantly reduces the cost of cultivation. Production of algal biomass and high cell density cultures are desirable in order to reduce the cost of processing.

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These systems provide a high degree of growth control and also lower harvesting costs due to the higher cell densities achieved as there is less biomass loss during the dark phase (Brennan and Owende, 2008). The set-up costs are minimal, although the system uses more energy than the production of photosynthetic microalgae because the process cycle includes the initial production of organic carbon sources via the photosynthesis process. This reduces the impact of biomass loss during dark respiration and decreases the amount of organic substances utilized during growth. These features infer that heterotrophic production can be an important part of the microalgae-to-bioresource process. Azma *et al*. (2010) cultivated the green microalgae, *Tetraselmis suecica* in heterotrophic conditions in total darkness in natural sea water. The cell concentration obtained by this method of cultivation was 2-3 times higher than that obtained from the photoautotrophic culture. Miao and Wu (2006) also studied *C. protothecoides* and found that the lipid content in heterotrophic cells could be as high as 55%, which was 4 times higher than in autotrophic cells under similar conditions. Hence, they concluded that heterotrophic cultivation could result in higher production of biomass and accumulation of high lipid content in cells. The heterotrophic cultivation of *Chlorella* sp. using different animal waste extracts under different cultural conditions was investigated. The goal is to establish an inexpensive and sustainable protocol for the cultivation of *Chlorella* sp. for the purpose of biodiesel, biochemical and biopharmaceutical products.

MATERIALS AND METHODS

Cultural conditions

Blooming was first carried out using a 10:90 mixture of cow dung extract and pond water from fresh water ponds within the Niger Delta Region of Nigeria. Then 1ml of the blooms was aseptically inoculated into flasks containing 300 ml of the different animal waste media as broth media, 10g L^{-1} glucose was added to the basal medium and these were maintained at 28±2^{o}C (Miao and Wu, 2006; Xu *et al*., 2006). The glucose stock solution was sterilized by filtration through a whatman filter paper. The following set ups were prepared (i) Unaerated condition (ii) Aerated condition using an aquarium pump supplying about 150 bubbles per minutes (Abu and Epegu, 2006). (Options (i) and (ii) were mounted in a dark chamber at a height of about 30 cm from the bench top) but the chambers were sealed (iii) dark condition in a total dark room aerated by manual shaking at 2h interval for 12h. Triplicate samples were taken at the end of each period to monitor algal concentration by measuring the optical density, biomass as cell dry matter, population of cells as cell number and lipid content from the wet algal cells. Wet and dry cells of *Chlorella* sp. were subjected to proximate analysis.

Sampling and analysis

Optical density

Optical density (OD) at 600 nm was obtained using the method of Agwa *et al*. (2012). About 5 ml of the growing culture was obtained and the optical density determined using the spectrophotometer (Spectronic 20, Genesys, Thermos, USA)

Cell dry matter

Cell dry matter was determined using the method of Agwa *et al*. (2012). About 5 ml of the growing culture was harvested by centrifugation at 3000 rpm for 10mins. The cells were washed (3x) with physiological saline dried at 50^{0}C in a hot air oven to a constant weight.

Cell number

The number of cells was obtained using the improved Neubauer cytometer counting chamber. About 1 ml of the culture was diluted tenfold and at least 5 squares were counted and the average value recorded as cells/ml (Agwa *et al*., 2012).

Lipid extraction

Wet extraction procedure according to Agwa *et al*. (2012) was adopted. Cells were harvested by Centrifuging 100 ml of the culture at 3000rpm for 15mins; the supernatant was decanted into an empty centrifuge tube leaving the wet paste at the bottom. To about 40 mg of the wet cells was added 1ml of water, 2.5ml methanol and 1.25 ml 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, 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^{o}C) leaving the lipid which was then reweighed using an analytical weighing balance (Setra BL-410S, USA).

Proximate composition

Moisture and ash were determined by the air oven

Table 1: proximate analysis of *chlorella* cells

	HETEROTROPHIC		AUTOTROPHIC	
Parameters (%)	**Wet**	**Dry**	**Wet**	**Dry**
Crude Protein	28.26	24.43	56.0	40.8
Crude Lipid	37.03	38.30	10.3	13.2
Carbohydrate	18.93	20.46	13.7	24.3
Ash	0.96	2.65	1.5	4.4
Moisture content	6.32	3.37	6.6	4.8
Others	8.50	10.79	11.9	10.5

Table 2: Autotrophic proximate analysis of *Chlorella* cells

Parameters (%)	**Wet**	**Dry**
Crude Protein	56.0	40.8
Crude Fat	10.3	13.2
Carbohydrate	13.7	24.3
Ash	1.5	4.4
Moisture content	6.6	4.8
Others	11.9	10.5

method (AOAC, 1990). Crude protein was determined by the micro- Kjeldahl method (AOAC, 1990) and the conversion factor from nitrogen to protein was 6.25. Crude lipids were determined by the soxhlet extraction method of Egan *et al*., (1981). Total carbohydrate content was determined by using the Anthrone method (Osborne and Voogt, 1978). The crude fibre content was calculated by difference using the formula: Crude fibre = 100 – (% protein + % TAC + % moisture + % fat + % ash).

Statistical analysis

Analysis of variance (ANOVA) method was used to ascertain the significant difference that existed between the optical density, cell dry matter, cell number and lipid produced at the various cultural conditions and the different waste extracts.

RESULTS

The results of the heterotrophic growth of *Chlorella* sp. under different conditions was measured as optical density at 600nm, cell dry matter, cell number and lipid content from wet cells. Proximate analysis of the wet and dry cells of *Chlorella* sp. can be seen in Table 1. High levels of crude fibre are evident, while the crude protein and moisture content of the wet cells were slightly higher than that of the dry cells. But the crude fat, carbohydrate content and ash content of the dry cells were higher than that of the wet cells. The values obtained higher except with the crude protein which was significantly higher from the other values obtained. The growth of *Chlorella* sp. under heterotrophic conditions is typified in Figure 1-3 measured as optical density with the animal waste extracts as blank. Poultry waste had the highest absorbance at 600nm (1.92abs) among all the animal waste extracts, while cow dung waste extracts gave the least absorbance (1.47abs) under the artificial and natural illumination. The same was observed with the results obtained from the cell dry weight (Fig 4-6) and cell number (Fig 7-9) under artificial illumination. But under natural illumination (dark) poultry waste was the highest ($P<0.05$), followed by pig waste and goat waste extracts. Figure 10-12 represents the lipid production of *Chlorella* sp: under artificial illumination, here cow dung gave the highest lipid content of (9.92%) and goat waste had the lowest lipid content (2.13%). Artificial illumination (aerated) cow dung gave the highest lipid content of about 10.16%, while pig waste ((4.25%) gave the lowest result. Natural illumination (dark) resulted in the poultry waste (22.71%) having the highest lipid content ($P<0.05$) and goat waste (4.03%) the lowest.

DISCUSSION

Microalgae are the most efficient primary producers of biomass and important alternative energy source (Demirbas, 2006; Nakamura, 2006). Most applications of microalgae use light because microalgae are very efficient solar energy converters, and they can produce a great variety of useful metabolites autotrophically (Lebeau and Robert, 2006). Light-independent heterotrophic growth, wherever possible, has significant economic advantage over light-dependent growth in mass-producing microalgae (Chaumont, 1993; Borowitzka, 1999). These systems provide a high degree of growth control and also lower harvesting costs due to the higher cell densities achieved (Chen and Chen, 2006). Heterotrophic growth of microalgae presents significant economic advantage over the more common autotrophic cultivation. Under heterotrophic growth, *Chlorella* sp. grown alone had slightly larger populations and had higher growth rates than under autotrophic growth, because more ATP and NAD(P)H for metabolic processes is available, which is unrelated to autotrophic carbon assimilation (Yang *et al*., 2000). The efficiency of growth and glucose uptake from different animal wastes was compared with the autotrophic regimes of *Chlorella* cells. Heterotrophic growth of *Chlorella* cells was superior to autotrophic growth. The highly technical viability of heterotrophic production compared to autotrophic is shown in Table 1. Heterotrophic growth of *Chlorella* sp. resulted in complete disappearance of chlorophyll in cells

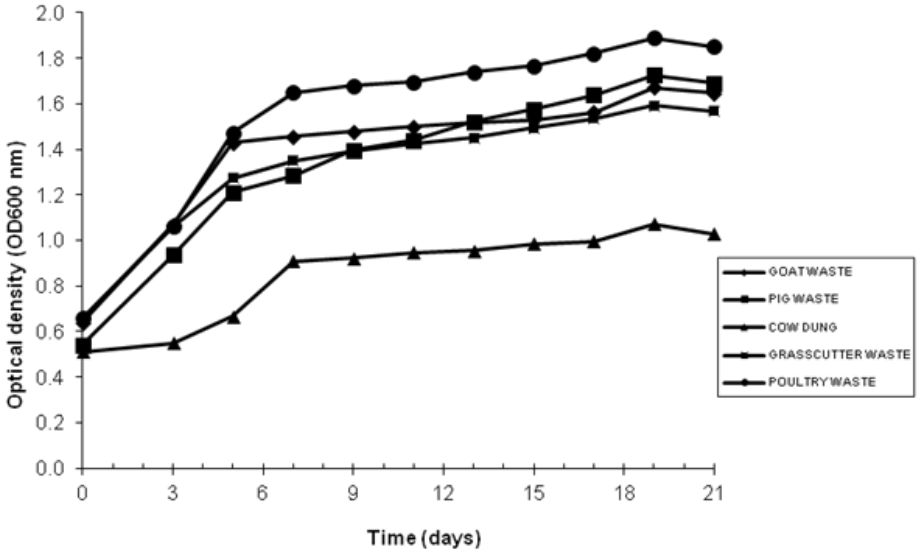

Figure 1. Heterotrophic growth of *Chlorella* sp. in broth media (aerated) measured as optical density.

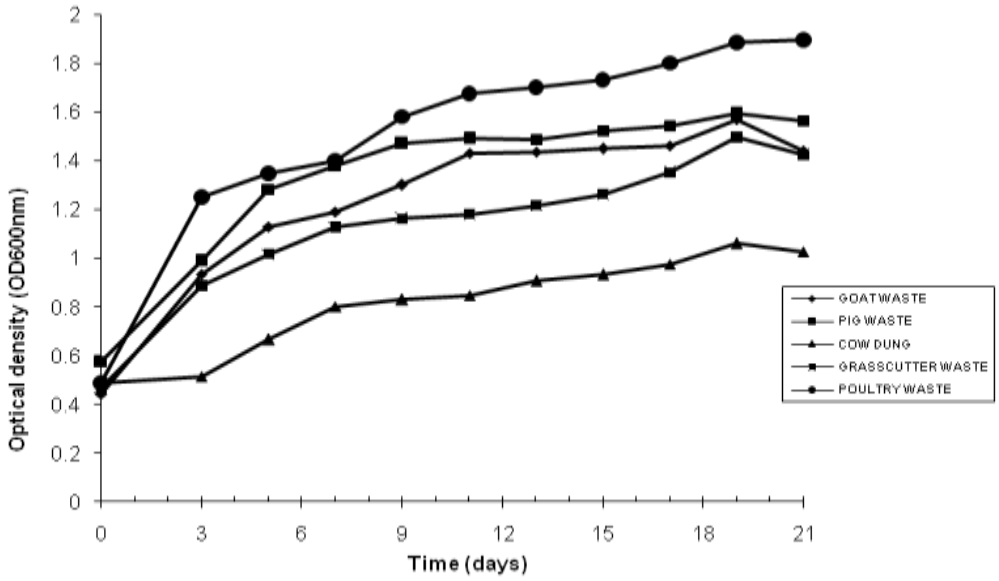

Figure 2. Heterotrophic growth of *Chlorella* sp. in broth media (unaerated) measured as optical density

with the media turning yellow. This resulted in high accumulation of lipid content in cells. But autotrophic growth revealed about 13.2% lipid content with autotrophic production. Lipid content in heterotrophic cells reached up to 38.30% which is about three times that in autotrophic cells (Miao *et al*., 2006). According to Xu *et al.* (2006) heterotrophic cells resulted in 55.2% which is about four times that in autotrophic cells with the substrate glucose with the cells appearing light yellow and greasy. Li et al. (2007) carried out heterotrophic cultivation of *Chlorella protothecoides* and the lipid content reached up to 48%. Consequently, Feng *et al.* (2011) culturing of *Chlorella vulgaris* with waste water treatment achieved a total lipid content of 42%. These researchers concluded that higher production of biomass and lipid content in cells could result from heterotrophic cultivation of microalgae.

Under the different cultural conditions with the substrate glucose, the cell growth reached maximum value with the cow dung waste under artificial illumination

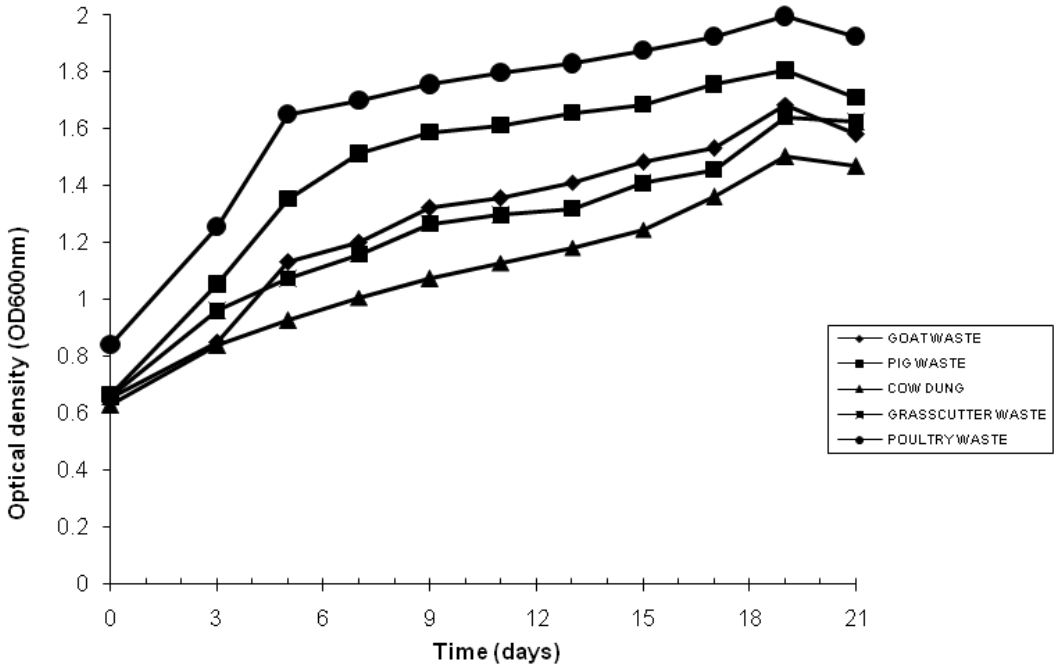

Figure 3: Heterotrophic growth of *Chlorella* sp. in broth media (dark) measured as optical density

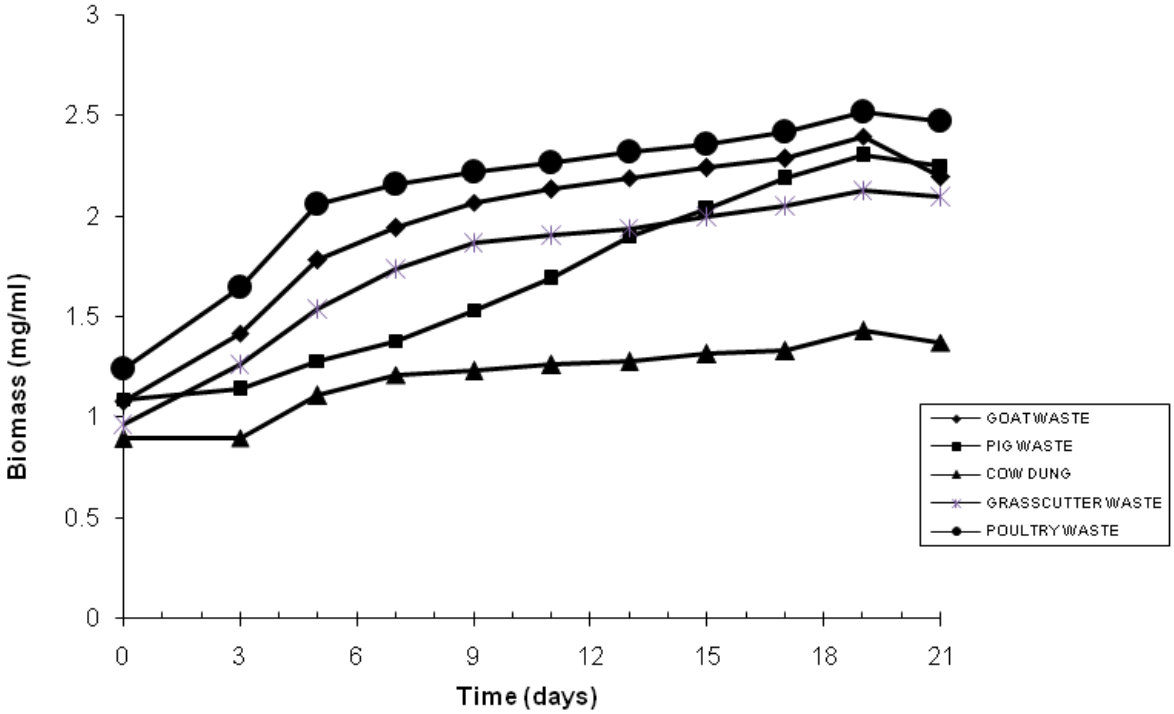

Figure 4. Heterotrophic growth of *Chlorella* sp. in broth media (aerated) measured as dry weight

(unaerated) yielding 9.92% of lipid content at a biomass of 2.06mg/ml, while aerated resulted in 10.16% lipid content at a biomass of 2.52mg/ml with the same waste extract. But in the dark, the poultry waste gave 22.71% lipid content at a biomass of 2.77mg/ml. Heterotrophic cultivation resulted in higher production of biomass and accumulation of high lipid content in cells using poultry waste as a suitable substrate under natural conditions. Iyovo *et al.* (2010) produced a nutrient rich digestate feasible for the production of biodiesel with the

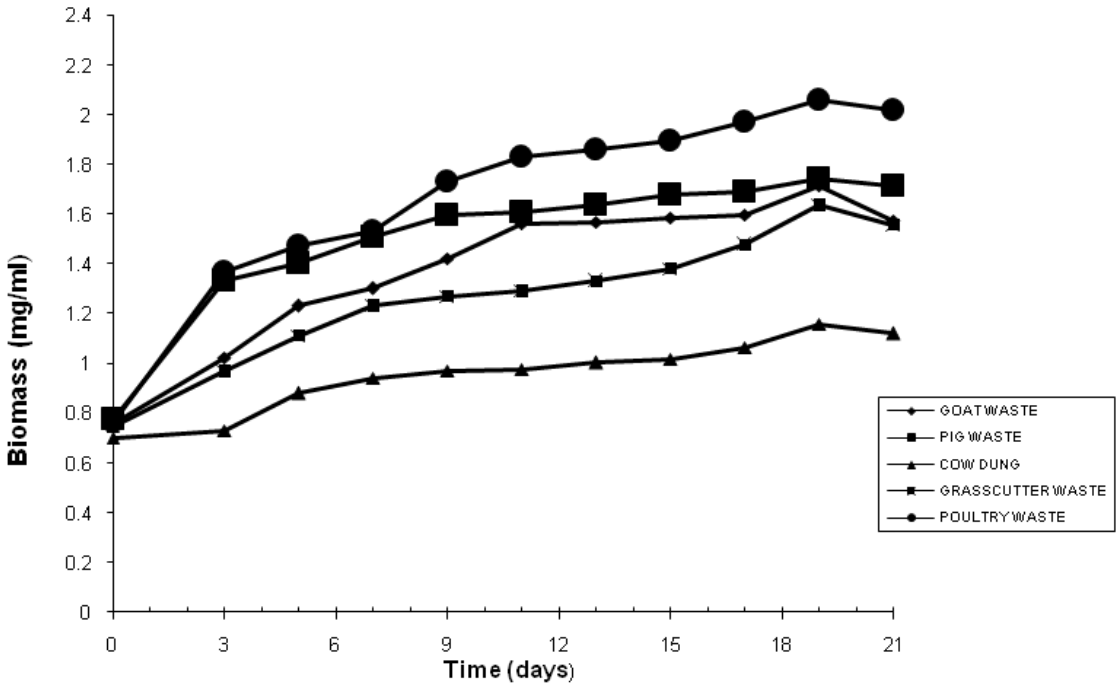

Figure 5: Heterotrophic growth of *Chlorella* sp. in broth media (unaerated) measured as dry weight

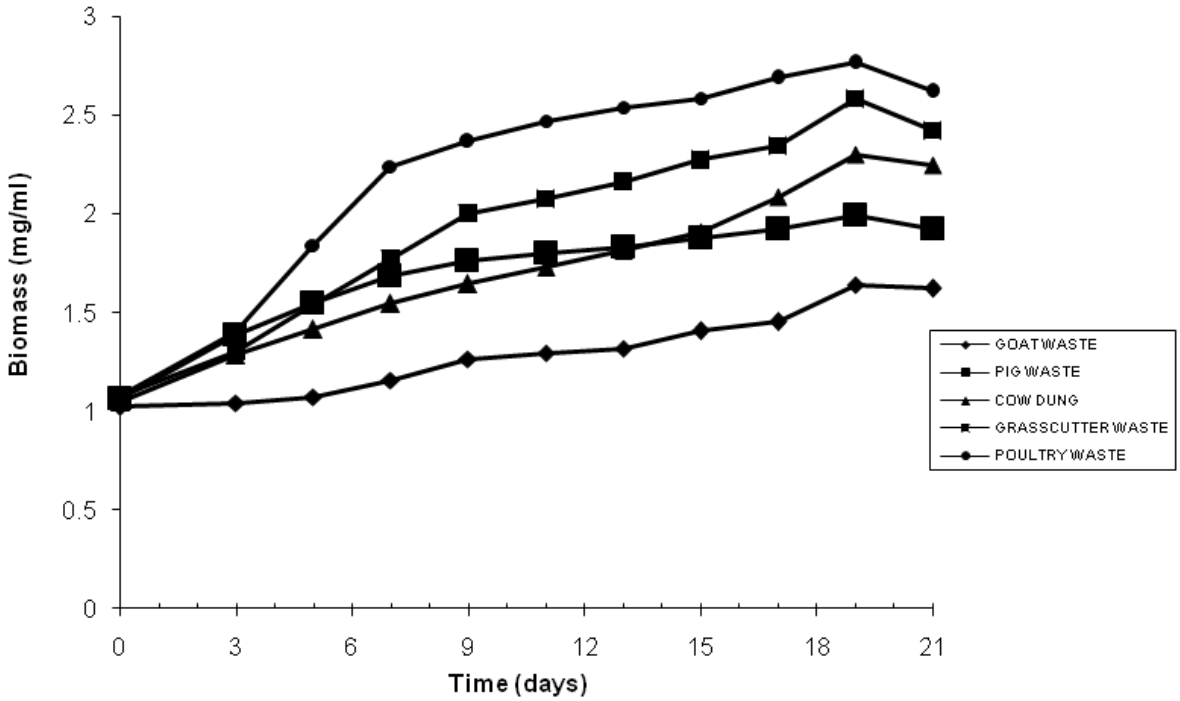

Figure 6. Heterotrophic growth of *Chlorella* sp. in broth media (dark) measured as dry weight

microalgae *Chlorella vulgaris* using poultry waste. Agwa et al., (2012) recorded similar findings with *Chlorella* sp. when using different animal wastes extracts under natural illumination. The total crude fat reported in our work points to the high potential for lipid production by this specie of *Chlorella*. The chain sequential processing of

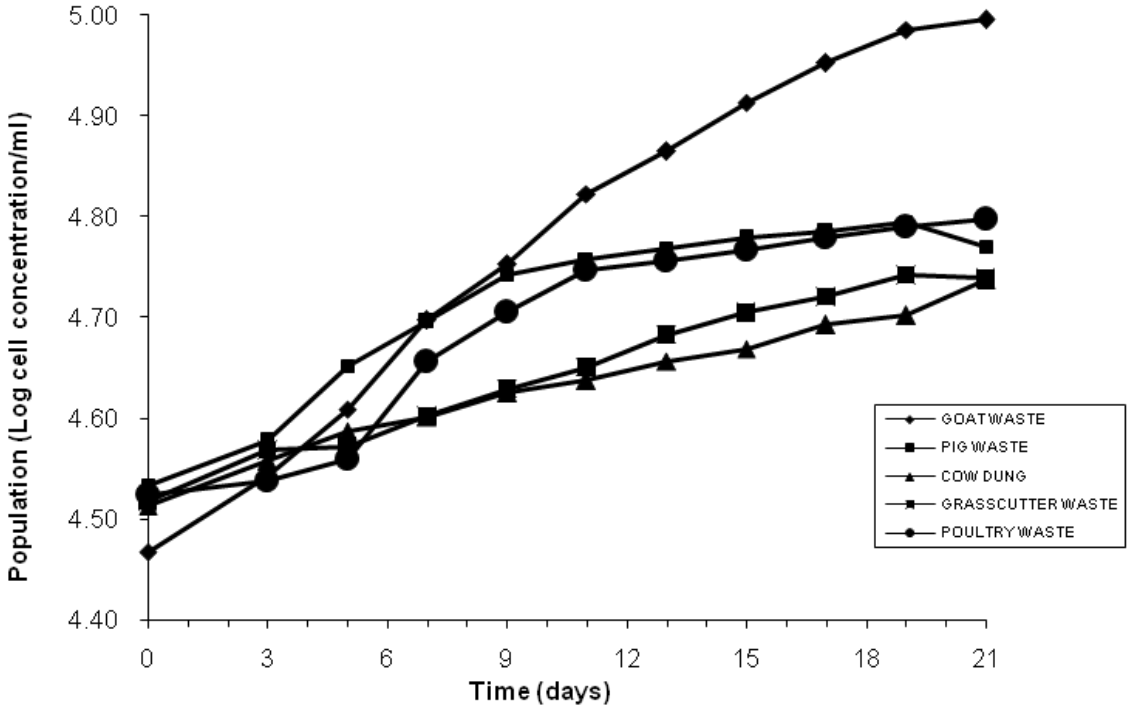

Figure 7. Changes in population of *Chlorella* sp. in broth media (aerated) during heterotrophic growth

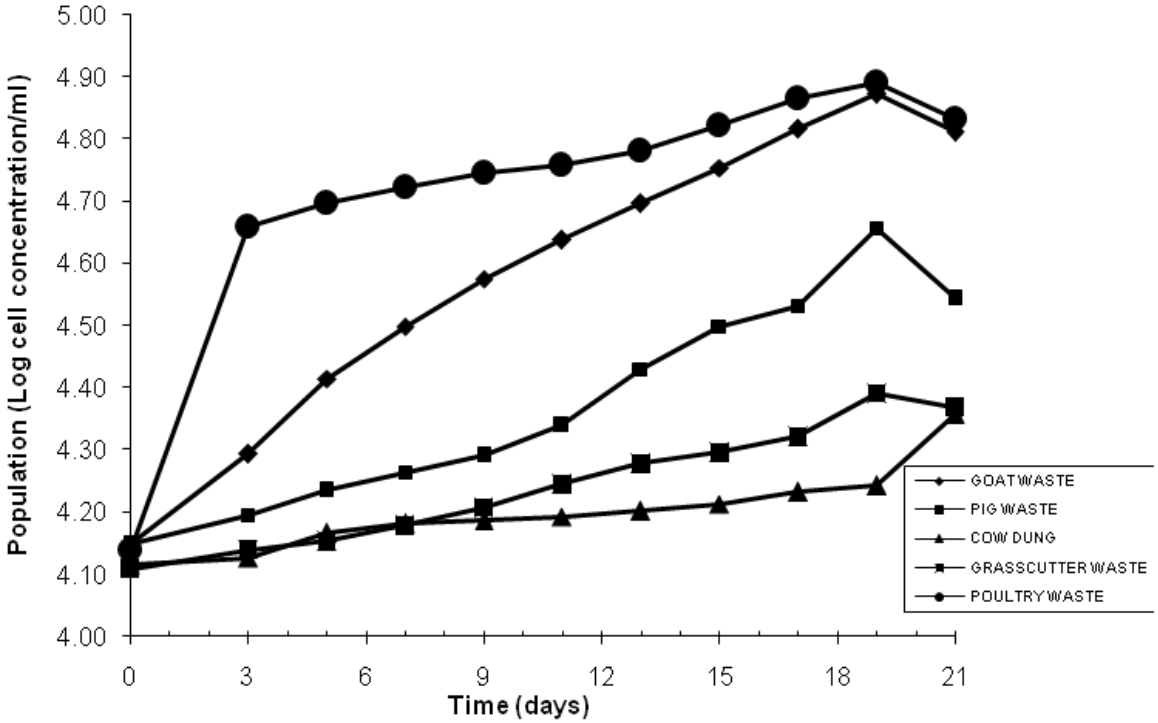

Figure 8. Changes in population of *Chlorella* sp. in broth media (unaerated) during heterotrophic growth

nutrients involving poultry and *Chlorella* sp. is a value addition to waste-destined raw materials. In this way heterotrophic cultivation of *Chlorella* is a good balance of raw material demand and energy costs.

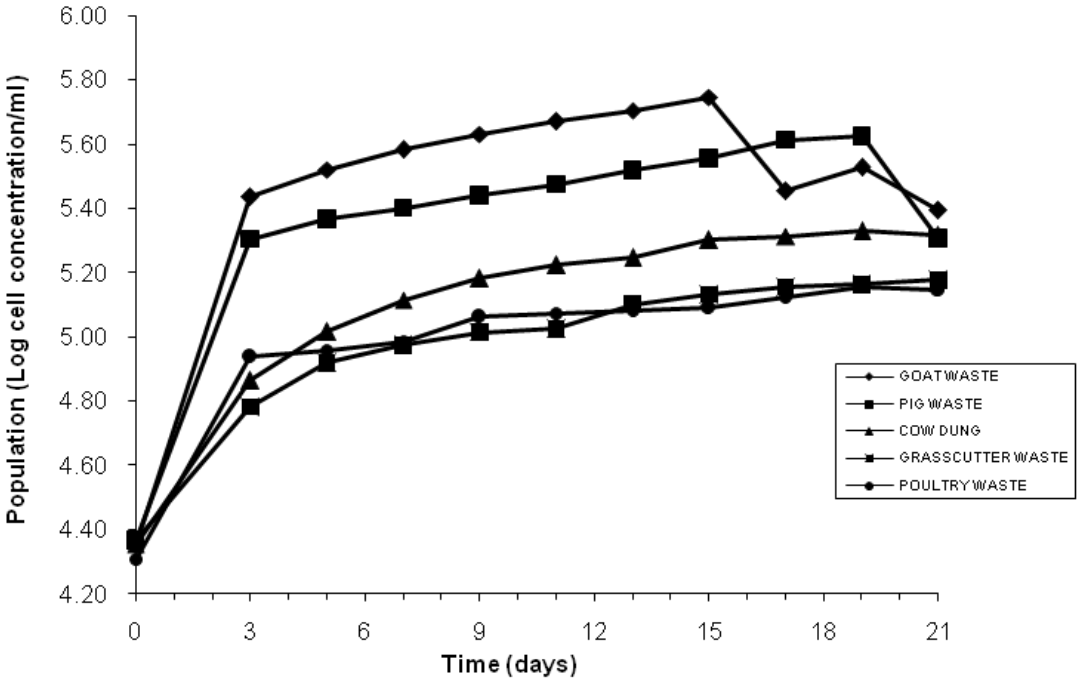

Figure 9. Changes in population of *Chlorella* sp. in broth media during heterotrophic growth

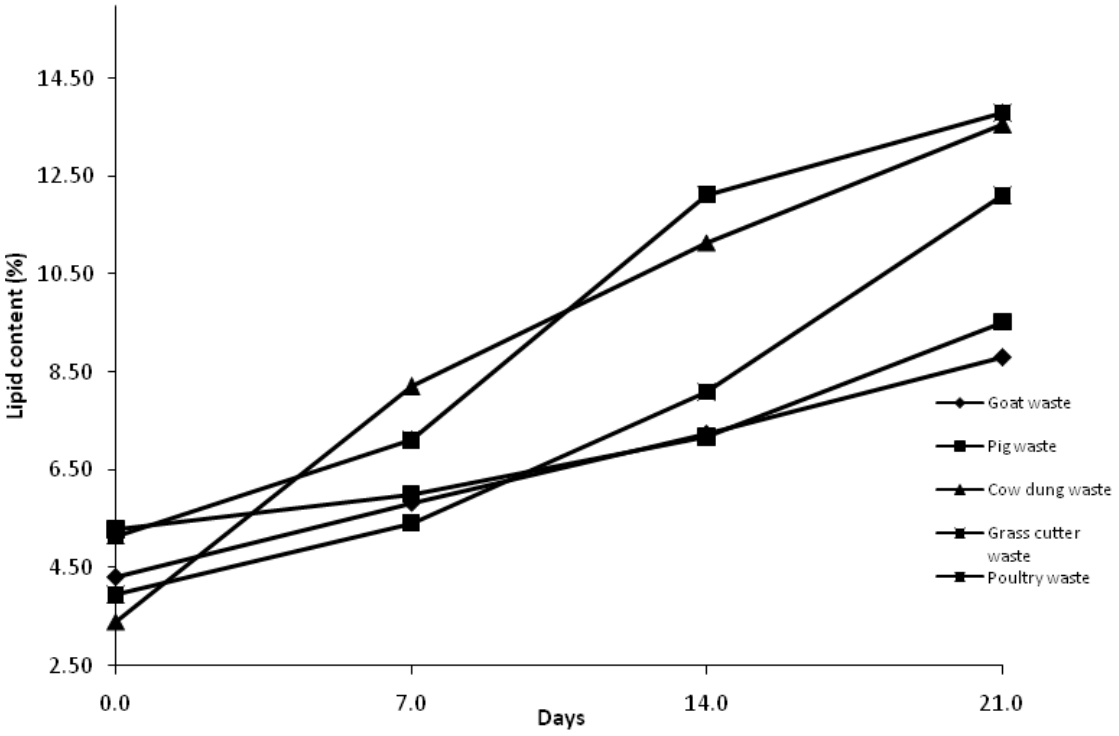

Figure10. Lipid Production by *Chlorella* sp. under heterotrophic growth Condition (Aerated)

CONCLUSION

In order to improve the economic benefits of *Chlorella* sp. culture and efficient utilization of different animal wastes, the effect of different animal wastes extracts on the growth, the biomass and lipid production of *Chlorella* sp.

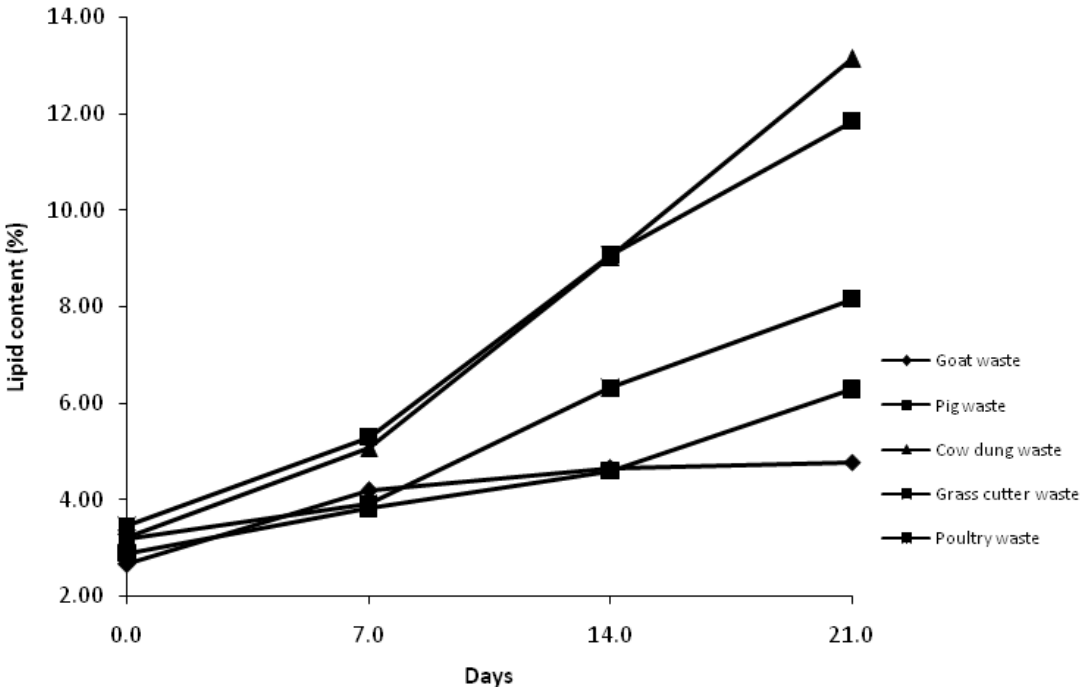

Figure11. Lipid Production by *Chlorella* sp. under heterotrophic Condition (Unaerated)

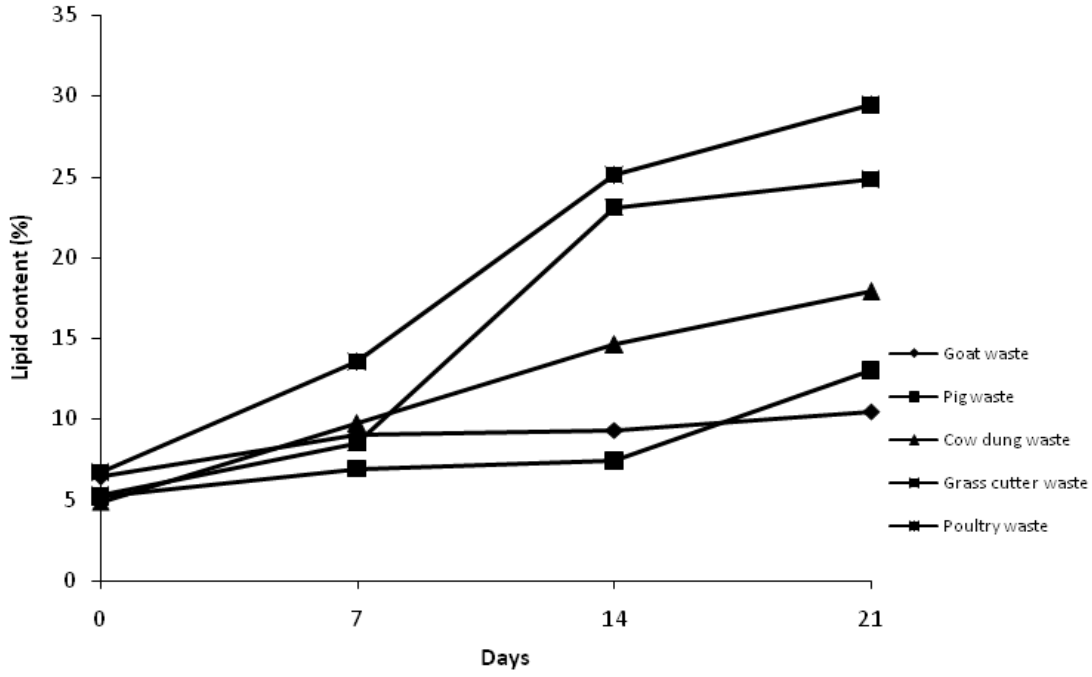

Figure12. Lipid Production by *Chlorella* sp. monitored heterotrophic Condition (dark)

were studied. The transformation of raw material by poultry into high use nutrients for *Chlorella* heterotrophic growth would enhance environmental sustainability and support renewable energy resources with various value added products.

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