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Preliminary Study of Production of Biomass of Chlorella vulgaris Utilizing Recycled Nutrients in Sediments from Catfish Ponds

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Abstract
Feasibility of growing Chlorella vulgaris on recycled nutrients in sediments from Catfish ponds was evaluated, where a sediment filtrate-based organic media was prepared in single and double strength (100% and 50%) and tested against a conventional synthetic media (F/2) for algae culture. Findings from the study showed that sediment filtrate media (SFM) was not only able to support the growth of Chlorella vulgaris, but also yielded a significant quantity of biomass. Results for biomass density estimation showed that there was a correlation between nutrient concentration and biomass concentration because more algae concentration was recorded in the highest media concentration (100%) than the lower concentration (50%). This trend was observed for all treatments. Similarly, findings from the study also revealed that the 6th day of culture was the optimum culture period for harvesting because the highest biomass density was obtained within this period for all mediums. Furthermore, biomass yields and productivities of Chlorella vulgaris showed varied productivities and yields for both SFM and F/2 mediums. For the 3rd day, the highest productivity was obtained in f/2 medium with a productivity of 0.44g/L/day (44.3%). Similarly, in the 6th day, the highest biomass productivity was recorded in F/2 medium with a productivity of 0.17 g/L/day (17%). Even though the synthetic medium (F/2) outperformed SFM, results from the biomass productivities are promising if the growth conditions are optimized. The outcome of this study can serve as a baseline for developing technologies that will encourage the utilization and management of sediments from catfish ponds in the Kainji Lake Basin.

Keywords: Sediments, Nutrients, Chlorella, Biomass, Media

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INTRODUCTION

There has been an increase in aquaculture production over the years in Nigeria, owing to the efforts by the Federal Government of Nigeria to intensify local production of fish so as to bridge the gap between demand and supply of fish in the country. Indeed, this has yielded results because in the year 2020, Nigeria was the second highest aquaculture producing country in Africa after Egypt, with a production capacity of 261711 tonnes and this was against the production output of only 25718 tonnes in the year 2000, representing an annual growth rate of 12.30% (FAO, 2022). However, intensification of aquaculture means production of greater volumes of effluents, sediments and nitrogenous wastes from fish production systems, which when discharged causes environmental problems such as water pollution and eutrophication (Boyd *et al*., 2020; Chatla *et al.*, 2020; Sampantamit *et al.,* 2020; Ahmad *et al*., 2022).

Interestingly, some of these wastes that originate from aquaculture production facilities could serve as viable raw materials for recovery of valuable resources from them. For instance, sediments from fish ponds have been reported as reservoirs of usable nutrients which can be recovered (Drozdz *et al.,* 2020). This is because sediments from fish ponds contains macro nutrients as well as organic matter which can be used to grow crops and also to improve soil health (Ihejirika *et al*., 2012). However, till date, there are few studies that aim to explore these nutrients to grow a biological resource such as microalgae, which can be channeled back into the aquaculture value chain by producing microalgae biomass using the nutrients in the sediments.

Microalgae biomass is rich in crude protein, carbohydrate, lipids, vitamins and enzymes (Harun *et al*., 2010). Another interesting thing about microalgae biomass is the desirable array of its lipid profile because it contains polyunsaturated fatty acids (PUFA) and triglycerides (TAG) which can be used for various algal based products including health, food supplements, cosmetics and livestock feed (Wan *et al*., 2015; Ho *et al.*, 2017). Specifically, *Chlorella vulgaris* have been reported to have excellent nutritional composition with a high crude protein value ranging from 51% to 58%, on a dry weight basis, as reported by Safi *et al*. (2014). Furthermore, the protein in *Chlorella vulgaris* is rich in essential amino acids, including lysine (Li *et al*., 2013; Ballesteros-Torres *et al*., 2019). Similarly, its carbohydrate content ranges from 12% to 17% on a dry weight basis (Safi *et al.,* 2014). However, its lipid content has been reported to be variable because it depends on the growth condition with which the algae was grown with (Yeh, and Chang, 2012; Rosenberg *et al.,* 2014).

MATERIALS AND METHOD

Source of *Chlorella vulgaris* Pure Culture

Chlorella vulgaris was isolated from earthen Catfish ponds at Monai Cluster fish farm located at the Southern Basin of Kainji Lake, Nigeria. Pure culture of *Chlorella vulgaris* was obtained by repeated sub-culturing on f/2 medium until a mono-algal culture of *Chlorella vulgaris* was obtained, as described in the procedure by Ovie and Ovie (2010). *Chlorella vulgaris* was identified using microscopic and macroscopic techniques using algal identification guide for freshwater algae by Van-Vuuren *et al*. (2006). To obtain pure culture with 99% purity, substantial level of quality control was achieved. This was conducted by repeated sub-culturing to and fro from nutrient agar enriched with f/2 medium to liquid f/2 medium followed by frequent microscopic observations to ensure that the cultures meet at least 98-99% purity.

Bioreactors

Simple improvised batch feed reactors were set up using 750ml plastic bottles with a working volume of 500ml. The plastic bottles were fitted with flow valves using aquarium aeration rubber pipes for exchange of gas to and fro the bioreactors so as to diffuse atmospheric carbon dioxide into the reactors and also to keep the algal cells in continuous motion. Prior to use, the reactors were sterilized by rinsing the bottles with 60% ethyl alcohol, with rigorous shaking and then a final rinse with sterile distilled water. All cultures were ran in batch cultures in mixotrophic condition (Simultaneous photoautotrophic and heterotrophic growth modes, 12hours:12 hours) over a nine (9) day period. Photo bioreactors were fitted with an aerator which provided agitation in the reactors to keep the algae cells in suspension so as to prevent aggregation.

Figure 1: Set-up of improvised bioreactors

Growth conditions of Bioreactors

a. Method of Cultivation: Mixotrophic (12:12) hours of phototrophic and heterotrophic modes respectively.
b. Nutrient supply: Batch operated
c. Temperature: 26-29◦C (optimum: 28.90 ◦C)
d. pH: 6.2 – 8.7 (optimum: 7.5)
e. Aeration: 5L/min output for 180min/day (900L/day), approx. 43m^3/L/day per reactor.
f. Duration of culture with maximum yield: Day 6

Preparation of Growth Mediums

Sediment Filtrate Media

Growth mediums for the study were prepared using sediments from fish ponds as the primary resource. After collection, the sediments were weighed homogenized in sterile distilled water and thereafter conditioned in a reservoir in the laboratory for 3 days. During the conditioning period, the sediments were manually agitated for a duration of 10 minutes (3-4 times daily). The resultant liquid obtained was carefully siphoned, filtered and sterilized at 121◦C for 15 minutes using an autoclave. This medium was prepared in two concentrations (100% and 50%).

F/2 Media

A synthetic medium (f/2) medium was also prepared and it served as the control. F/2 medium was prepared using the standard modified protocol of Guillard and Ryther (1962). The procedure involved preparation of stock solutions of nitrate, phosphate and trace metals, which were then combined and adjusted for pH. The final medium was filtered, sterilized and stored in a sterile container at 4°C. In preparation of stock solution of nitrate, 7.5g of $NaNO_3$ (sodium nitrate) was measured and dissolved in 100ml of sterile distilled water. For phosphate stock solution, 0.5g of NaH_2 PO_4 H_2O (sodium dihydrogen phosphate) was measured and dissolved in 100ml of sterile distilled water also. For trace metal solution, vitamin B12, biotin and thiamine vitamin solutions, 1ml of each was measured and mixed with 1 liter of sterile distilled water respectively. Prior to use, this media was also prepared in single strength (50%) concentration by diluting with equal volume of sterile distilled water and double strength (100%) without any dilution.

Monitoring of Algae Growth Performance and Yield

Chlorella vulgaris growth was monitored using spectrophotometric method as described in the procedure by Nasir *et al*. (2015). The growth was monitored by determining the biomass density using spectrophotometric method at 650nm (Nasir *et al*., 2015). After spectrophotometric measurements, values obtained were transposed into algae cell counts by comparing with cell counts obtained from standards of cell counts obtained from known spectrophotometric readings of respective algae growth mediums for each culture duration (3, 6 and 9 days). This was achieved using the values from standard curve to estimate the microalgae cell number in each case.

Measurement of Biomass Concentration

Biomass concentration of *Chlorella vulgaris* was achieved using gravimetric approach (ISO, 2000) (ISO-14242/2). This was achieved by concentrating and estimating the density of the biomass of suspended Chlorella cells in each reactor. The dry method of microalgae density estimation was done by quantifying the density of suspended algae. This was achieved by the same method as the method of measurement of total suspended solids (TSS) in the sample using the vacuum pump filtration method as described by (APHA, 1999). In this method, total mass of suspended solids was filtered using standard 1.2µm grade GF/C 7cm disc (glass fiber filter paper). Prior to filtration, the filter paper was weighed, dried in the oven at 105◦C for 1 hour (Lepcha, 2016) and kept in the desiccator for temperature stability. The initial weight of the filter paper (W_1) was then measured before placing it on the funnel to start the sample filtration. 10ml of the medium from each reactor was filtered through the filter paper, where the vacuum pump was connected to hasten the filtration process. When the filtration process was complete, the filter paper was placed in a glass dish and oven dried overnight. The final weight of the filter paper after drying (W_2) was measured and the biomass weight was computed using the equation:

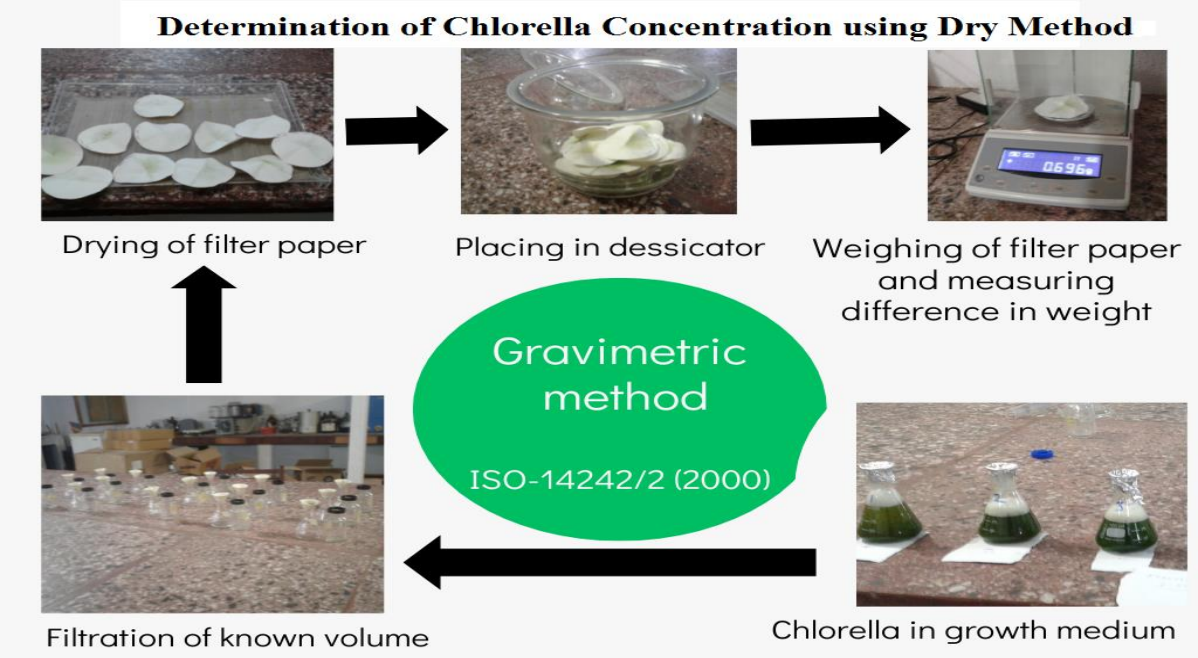
$$\textbf{Biomass weight (mg/l)} = \frac{Wf-Wi}{Vs\ (ml)} x\ 1000$$

Where $\mathbf{W_f}$ and $\mathbf{W_1}$ are final and initial weights of filter paper respectively and **Vs** is the volume of sample used)

The biomass yield per time interval was computed using the relation:

Biomass Yield (mg/l) = B_{t1}-B_{t0} (where Bt_1= Biomass measurement at time 1 and Bt_0 = Biomass measurement at time 0). Similarly, biomass productivity will be computed using the equation:

$$\% \textbf{ Biomass Productivity (mg/l/day)} = \frac{Biomass\ yield\ (mg/l)}{Number\ of\ days}\ x\ 100$$



Physicochemical Analysis

Physicochemical analysis involving nutrient analysis and other physicochemical variables were assessed. Parameters were pH, temperature, conductivity, total alkalinity, nitrate (NO_3-), nitrite (NO_2^-), ammonia (NH_3) and phosphate as orthophosphate (PO_4^{3-}). In the procedure, temperature was measured with the aid of a mercury-in glass thermometer while pH was determined with the aid of a hand-held digital pH meter (pHep®, HANNA, USA). Other chemical parameters were measured using standard procedures as described in the manual of official methods (AOAC, 2019).

Statistical Analysis

Statistical analysis was conducted using SPSS (IBM, version 20). Means of growth densities of Chlorella vulgaris in all treatments were subjected to one way analysis of variance to determine if there was significant difference between the cell counts. Post-Hoc test involving least significant difference was used to separate means that were significant using pair-wise comparison.

RESULTS

Optical Density Measurement (650nm).

Result for growth of *Chlorella vulgaris* cultured under mixotrophic condition in batch cultures using sediment filtrate medium (SFM) and f/2 medium recorded notable growth, particularly in the 6th day of culture. In the duration of the 9 day culture cycle, cell densities of *Chlorella vulgaris* recorded varied densities where growth at day 3 was found to increase in all treatments with F/2 medium (100%) showing the highest cell density of 4.90 x 10^6 cells/mL. This clearly indicated that the synthetic medium (F/2) supported better growth of *Chlorella vulgaris* at the early growth cycle. Similarly at the 6th day of the culture cycle, the highest cell density of 6.0 x 10^7 cells/mL was also observed in the F/2 (100%) treatment, further validating its superiority over the organic medium (SFM medium) at a higher concentration. However, the organic medium (SFM) also recorded a cell density of 6.0 x 10^6

cells/mL at 100% concentration, which was a good performance as well. However, on day 9, the cell densities declined in all treatments, with only F/2 (50%) showing the highest density (3.90×10^6 cells/mL).

The decline in growth from the 9th day of culture in all treatments was basically why the culture cycle was terminated from the 9th day of culture in all treatments (Table 1). Generally, results showed a correlation between the growth of *Chlorella vulgaris* and nutrient concentration because 100% medium concentration recorded higher growth than the lower concentration (50%) from the 3rd day of culture and this trend cut across for both mediums.

Table 1: Mean Cell Density of *Chlorella vulgaris* cultured mixotrophically over a 9 day cycle by Optical density Measurement

	SFM 100%	**SFM 50%**	**F/2 100%**	**F/2 50%**
Day 0	$3.20x10^{5a}$	$3.20x10^{5a}$	$3.20x10^{5a}$	$3.20x10^{5a}$
Day 3	$4.60x10^{6b}$	$3.80x10^{6b}$	$4.90x10^{6b}$	$4.80x10^{6b}$
Day 6	6.0×10^{6b}	$4.50x10^{6b}$	$6.20x10^{7a}$	$2.50x10^{7a}$
Day 9	$3.70x10^{6c}$	$3.50x10^{6b}$	$2.10x10^{6a}$	$3.90x10^{6a}$

*Means with different superscript letters on same column are significantly different ($p<0.05$)
KEY: **SFM** – Sediment filtrate Media **F/2** – F/2 Media.

Measurement of Biomass Density of *Chlorella vulgaris*

Results showed that the biomass concentration of *Chlorella vulgaris* in both growth mediums were statistically similar across all treatments on day 3 and the biomass concentrations recorded in all treatments ranged from 0.60 ± 0.6 g/L/day for SFM (50%) to 1.33 ± 0.6 g/L/day for F/2 (100%.)

However, on day 6 significant Results for biomass density estimation also showed that there was a correlation between nutrient concentration and biomass concentration because more algae concentration was recorded in the highest media concentration (100%) than the lower concentration (50%). This trend was observed for all treatments. Similarly, observations also showed that the 6th day of culture was the optimum culture period for harvesting because the highest biomass density was obtained within this period in all treatments. However, SFM treatments (100% and 50%) all showed lower biomass concentrations compared to biomass concentrations recorded in the F/2 mediums (50% and 100%).

Likewise, by day 9, the biomass concentrations were statistically similar across all treatments again, ranging from 0.33 ± 0.5 g/L/day for SFM 50% to 0.67 ± 1.2 g/L/day for SFM 100% and F/2 100%. These results generally indicated that more biomass concentrations were obtained in day 6 and this trend cut across all the treatments (Table 2).

Table 2: Biomass Concentration of *Chlorella* sp. Cultured over a 9 day period

	Day 3 (g/l/day)	**Day 6 (g/l/day)**	**Day 9 (g/l/day)**
SFM 100%	1.00 ± 1.0^a	0.67 ± 1.2^a	0.67 ± 1.2^b
SFM 50%	0.60 ± 0.6^a	0.34 ± 0.5^a	0.33 ± 0.5^b
F/2 100%	1.33 ± 0.6^a	2.33 ± 0.6^b	0.67 ± 0.9^b
F/2 50%	1.00 ± 0.6^a	1.33 ± 0.5^b	0.50 ± 0.5^b

*Values are means ± SD
* Means with different superscripts on the same column are significantly different ($p<0.05$).
KEY: **SFM** – Sediment filtrate Media **F/2** – F/2 Media

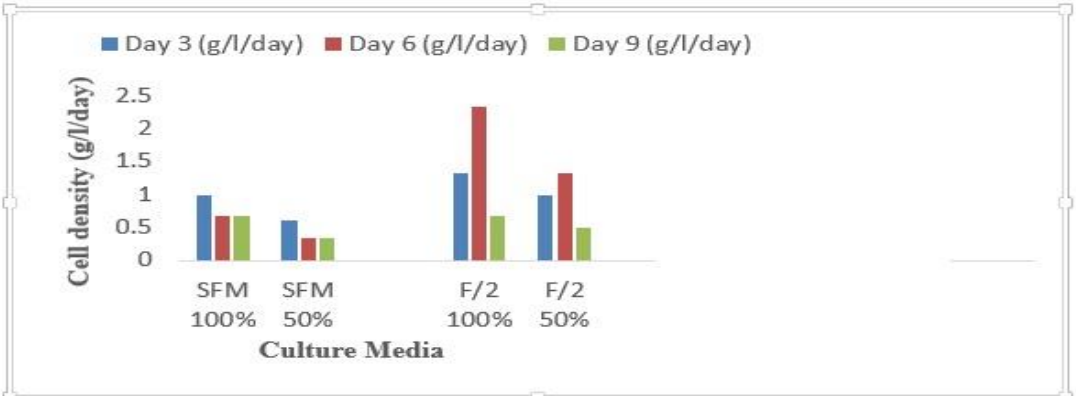


Figure 1: Cell concentration of Chlorella vulgaris in growth mediums

Biomass Yields and Productivities of *Chlorella vulgaris*

Result for biomass yields and productivities of *Chlorella vulgaris* showed varied productivities and yields for both SFM and F/2 mediums. For the 3rd day, the highest productivity was obtained in f/2 medium with a productivity of 0.44g/L/day (44.3%). Similarly, in the 6th day, the highest biomass productivity was recorded in F/2 medium with a productivity of 0.17 g/L/day (17%). Again, it was generally observed that there was a positive correlation between nutrient concentration and biomass yields and productivities because higher yields and productivities were obtained in the highest nutrient concentrations (100%) for all treatment (Figure 2).

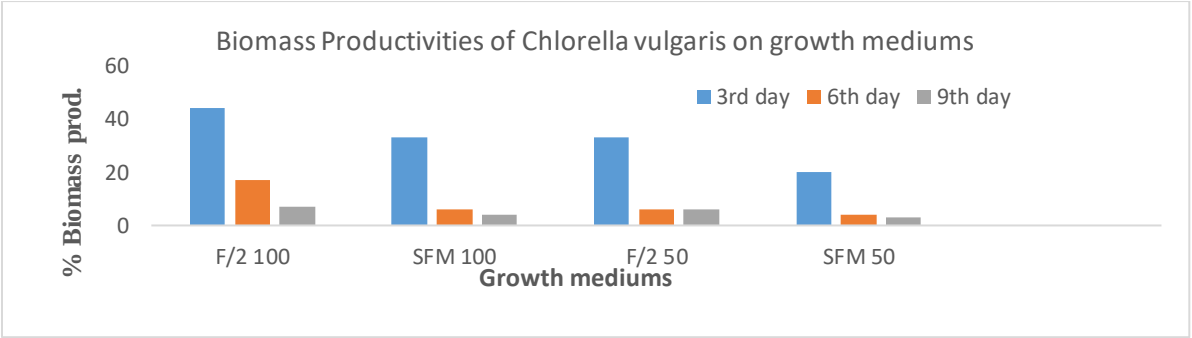


Figure 2: Biomass productivities of Chlorella vulgaris on SFM and F/2 mediums.

Physicochemical Variables in Reactors

Results for physicochemical analysis showed minor variations in pH, conductivity, total alkalinity and temperature values in all reactors of respective nutrient mediums, throughout the culture duration (Days 0, 3. 6 and 9) respectively. This trend applied to both the single (50%) and double (100%) nutrient media concentration respectively

a) pH

The pH in bioreactors ranged from 7.5 to 8.9, with the optimum pH of 8.5 sampled in reactors on day 6. It was generally observed that at day 9, there was a drop in pH from alkalinity to around neutrality, with pH range of 7.5-7.7 in reactors of both nutrient mediums.

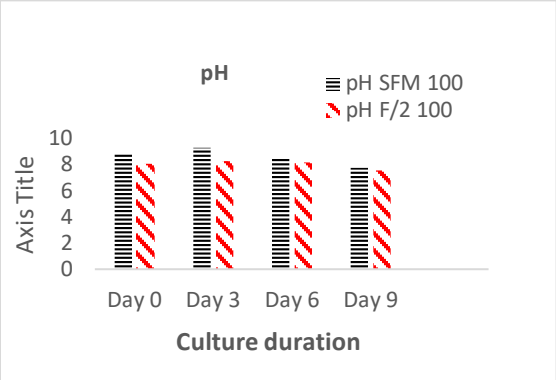


Figure 3: pH in bioreactors

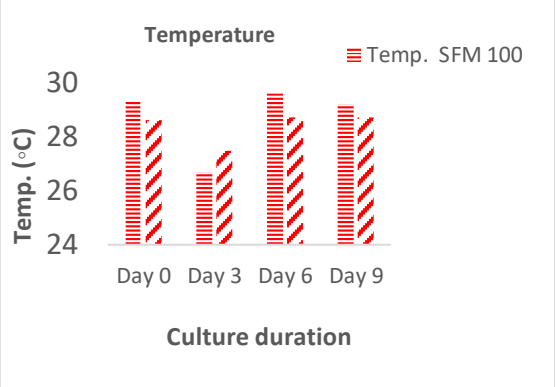


Figure 4: Temperature of bioreactors

b) Temperature

Temperature in all bioreactors ranged from 26◦C to 29◦C and the optimum temperature that was observed to yield the highest algae biomass was 28.9◦C which almost coincided with the temperature value recorded in all reactors at the 6th day of culture.

c) Ammonia

Ammonia concentration was observed to increase from day 0 to day 3 across all reactors. Subsequently, from day 3 onwards, ammonia utilization by Chlorella vulgaris was eminent by gradual decrease.

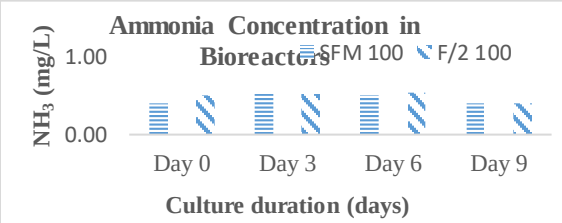


Figure 5. Ammonia concentration

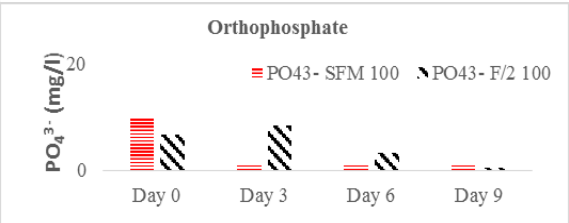


Figure 6. Orthophosphate concentration

d) Phosphate (Orthophosphate)

There was a drastic reduction in orthophosphate concentrations in all growth mediums. There was almost a 95% orthophosphate utilization rate by *Chlorella vulgaris* in all bioreactors of both growth mediums, signaling aggressive orthophosphate assimilation by *Chlorella vulgaris* to sustain its growth.

DISCUSSION OF RESULTS

In the study, growth of *Chlorella vulgaris* was supported in both growth mediums (SFM and F/2). Also, another major finding from the study was that growth was observed to be higher in the treatments with the highest media concentration (100%) than the lower concentration (50%). According to Ovie *et al.* (1986), the two most important components of any algal growth nutrient media are phosphate (source of phosphorus) and nitrate, which serves a source of nitrogen. This statement strongly agrees with a major finding from this work where it was observed that *Chlorella vulgaris* aggressively assimilated orthophosphate from all of the growth mediums much faster than other nutrients. Other researchers also reported similar trend and one of those was in the work of Miao *et al.* (2022), where they reported aggressive orthophosphate assimilation efficiency by *Chlorella vulgaris* to sustain its growth (Miao *et al.*, 2022). The possible explanation to this activity by *Chlorella vulgaris* could be

attributed to the fact that it prefers phosphorus as a nutrient resource than nitrogen (Aravinthan *et al*., 2014; Wu *et al*., 2014). Sequel to this, Alketife *et al.* (2017) specifically reported that *Chlorella vulgaris* prefers phosphorus as a nutrient source than nitrogen, as shown by the optimal P, N, and C concentrations in bioreactors from their study. Similarly, Wu *et al.* (2014) reported that *Chlorella vulgaris* prefers phosphorus as a nutrient source, with an optimum ratio of 16:1 nitrogen to phosphorus for growth in their medium. In addition, cell division is disrupted in *Chlorella vulgaris* when the algae suffer from the condition of Phosphate deficiency (Brembu *et al.* 2017). The efficient capacity of *Chlorella vulgaris* to convert excess orthophosphate in their medium into metabolic products such as Phospholipids, nucleic acids and energy can be the primary reason for the high phosphate requirement of *Chlorella vulgaris* in its growth medium (De-Melo, *et al.*, 2018;Taufikurahman *et al*., 2020).

Sediment Filtrate Medium (SFM) displayed substantial growth on Day 3 and 6, indicating an initial positive response to the medium, but not as much as F/2 medium. However, the subsequent decline in cell count by Day 9 in all two mediums suggests that there may be the possibility of nutrient exhaustion and other possible factors that could possibly induce stress on the *Chlorella vulgaris* population over time. The decline in cell count observed in SFM could be mainly attributed to the impact of nutrient exhaustion, since the growth was basically ran in batch cultures. In a batch culture system, the initial nutrient supply is determinate and does not get replenished throughout the experiment. This can lead to a decline in nutrient availability with time as chlorella population and consumption rate increases (Liu *et al.*, 2017). At the beginning of the batch culture, the medium contains an initial abundance of nutrients from the sediment extracts. This abundance likely supported the substantial growth observed on day 3 to day 6. However, as the Chlorella population grew, it utilizes available nutrients for photosynthesis, cellular division and metabolic processes (Panahi *et al.*, 2019; Piasecka and Baier, 2022). Consequently, in a batch culture, these nutrients are not replenished, leading to a gradual depletion of essential elements. With fewer nutrients available, Chlorella cells may experience a decrease in growth rate and overall metabolic activity, leading to stress (Chen *et al.*, 2017). According to Koza *et al*. (2022), nutrient scarcity can induce stress on microorganisms, affecting their physiological state and potentially leading to a decline in cell viability. This stress might contribute to the observed decrease in cell counts over time. With respect to *Chlorella vulgaris*, both nitrogen and phosphorus are essential nutrients required for its growth and proliferation. Nitrogen is required for protein synthesis and insufficient nitrogen levels can lead to decreased growth rates and lower productivity (Chen *et al*. 2017). Similarly, Phosphorus is also essential for energy transfer and cell division and insufficient phosphorus levels can also lead to decreased growth rates and lower productivity as well (Chen *et al*. 2017). However, as earlier stated, *Chlorella vulgaris* has more appetite for phosphate than nitrogen in its growth medium (Aravinthan *et al.*, 2014; Wu *et al.*, 2014; Alketife *et al.*, 2017). Furthermore, pollutants such as heavy metals present in sediment could impart a negative effect on the growth of *Chlorella vulgaris* because heavy metals can inhibit the growth of Chlorella by interfering with various cellular processes, such as photosynthesis, respiration and even nutrient uptake (León-Vaz *et al.,* 2021; Pang *et al*., 2021). This is because heavy metals like copper, cadmium, lead and mercury have all been reported to inhibit the growth of Chlorella at high concentrations (Expósito *et al*., 2021). The results indicated that the synthetic medium (F/2) supported better growth and higher biomass concentrations, particularly during the initial growth phase, compared to the organic medium (SFM). However, by the end of the study period, the differences in biomass concentrations across treatments were not statistically significant.

With regards to the relationship between medium type and medium strength as well as Chlorella yield`, results showed positive correlation between media strength and media type for the organic mediums. From the results of Pearson's correlation coefficients between media strength (100% and 50%) as well as media type (SFM and F/2) and Chlorella yields, a positive relationship between growth mediums of similar type and weak positive correlation for mediums of dissimilar nature.

CONCLUSION

Major findings from this research study was that recycled nutrients in sediments from catfish ponds can be used to produce biomass of *Chlorella vulgaris* in batch mixotrophic culture condition. Even though the cell density and biomass productivities of the synthetic medium (F/2) was higher than the organic medium (SFM), it can be said that biomass production from waste nutrients in sediments from earthen fish ponds can significantly lower the cost of algae production compared to using synthetic mediums. Furthermore, this can serve as a baseline study towards developing technologies that will encourage the management of sediments from catfish ponds through utilization. Ones sediments from catfish ponds are projected as raw materials for nutrient recovery, it presents a viable opportunity towards recycling back the nutrients present in the sediments into the aquaculture value chain.

Based on the findings from this study, it is recommended that further studies focus on up-scaling the bioreactors and also optimization of Chlorella growth conditions, so as to obtain a good quantity of Chlorella biomass for potential applications. By ensuring bioreactor scale-up and growth condition optimization, an innovative, cost-effective and environmentally friendly technology to algae cultivation, that simultaneously addresses waste management in aquaculture systems will be produced.

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