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Influence of Various Nitrogen Sources on Biomass and Lipid Production by *Chlorella vulgaris*

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Authors' contributions

This work was carried out in collaboration between both authors. Author OKA designed the study, carried out all the analysis and wrote the manuscript. Author GOA critically supervised the research but both authors approved the final manuscript.

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ABSTRACT

Chlorella vulgaris is a unicellular, photosynthetic fresh water green alga with high concentration of chlorophyll. This microalga synthesizes biomass by trapping energy from the sun. It has valuable components particularly pigments and protein thus, can be utilized in the nutraceutical, pharmaceutical and agricultural industry. The organism was obtained by blooming in a 10:90 mixture of cow dung extract and pond water from fresh water pond at the African Regional Aquaculture Centre [ARAC] at Aluu, Rivers State, Nigeria. Blooming was enhanced by intermittent manual aeration under natural illumination with a bank of fluorescent tubes emiting ca15 µE/m²/s each. The isolate was cultured using a synthetic medium and identified as *Chlorella vulgaris* on the basis of its molecular characteristics by Polymerase Chain Reaction [PCR] technique. The potential of producing biomass and lipid from *Chlorella vulgaris* using three different nitrogen sources namely potassium nitrate, urea and sodium nitrate in a synthetic medium were investigated. The best growth of about 279 mgL-1 cell dry matter and 5.27% lipid content was obtained with urea as compared to the other nitrogen sources. Potassium nitrate gave 68 mgL-1 cell dry matter with about 1.53% lipid content, while sodium nitrate resulted in 236 mgL-1 cell dry matter and 0.73% lipid content. The maximum specific growth rate (µ=0.198) with a doubling time of 5.05 was recorded with urea at a concentration of 0.055 mgL-1, NaNO3 at the same concentration with urea had (µ=0.182) and a doubling time of 5.5, but KNO3 showed the least specific growth rate

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(μ= 0.169) with doubling time of 6.33. Overall, urea gave higher yields of biomass and lipid, caused small fluctuations with the medium during the algal growth.

Keywords: Biomass; Chlorella vulgaris; lipid content; nitrogen sources; urea.

1. INTRODUCTION

Due to high growth rates and photosynthetic efficiency, microalgae have recently received much attention as a potential renewable energy resource. They have vast industrial and economic potential [1] as valuable sources for pharmaceuticals, health foods, carotenoids [2-3], dyes, fine chemicals, biofuels, and may be able to solve emerging environmental problems [4]. Microalgae also have the ability to take up various kinds of nitrogen [5], and to absorb heavy metals [6-7] and phosphorus [4]. They can utilize various organic compounds particularly eutrophic compounds containing nitrogen and phosphorus for their carbon and energy sources. Major sources of eutrophication, including nitrogen; at high concentrations can cause algal blooms and other hazardous environmental problems. Microalgae growth media provide sufficient nutrients for growth of algal cells, these include important elements such as carbon, nitrogen, phosphorous and sulphur. Other essential trace elements include iron, magnesium which is important to develop balanced media for optimal microalgal cultivation [8]. The most important nutrients contributing to the biomass produced are carbon and nitrogen, they are easy to manipulate and cost effective compared with other elements. Nitrogen in the form of nitrite, nitrate, ammonia and many dissolved organic nitrogen [urea, free amino-acids and peptides] are regarded as the main nitrogen sources for microalgae [9-11]. Nitrogen is mostly supplied as nitrate and an increase in pH occurs when nitrate is supplied as the only nitrogen source. Urea is the most common nitrogen form utilized by most algae and requires the least energy to metabolize. Ammonia is the chemical form of nitrogen, readily taken up and assimilated by microalgae [8]. The nitrogen content of the biomass can range from 1% to more than 10% (w/w) and it varies between different groups. Nitrogen is known to have strong influence on metabolism of lipids and fatty acids in various microalgae. In addition, nitrogen is easy to manipulate and less expensive when compared to other factors but it is critical to enhance lipid productivity for bio-fuel production [12].

Up to 50-70% lipids of dry cell matter can be accumulated by different lipid rich species of microalgal, but the growth is generally slow. *Botryococcus braunii*, is the most well-known example of a lipid-rich species which can accumulate lipids with more than 70% of biomass at a slow doubling time of 5–7 days [13]. Some microalgae commonly double their biomass within 24 h during exponential growth [14] and this can be as short as 3.5 h [15-16].

Production of lipid can be increased under different cultural conditions [17-19], nitrogen depletion [20-21], phosphorus depletion [22], high salt concentrations [12], and high iron concentrations [23] in some microalgae. Supplementation of a culture with more nutrients increases total biomass and lipid, but the increase in cost is unacceptable in order to maintain competition between biofuels and fossil fuels. However, depletion of macroelements is a simple and an effective way to increase the relative lipid content per unit cell dry weight and reduce biomass yield. *Chlorella* needs light energy that is converted to chemical energy in the form of ATP which is used in photosynthesis, metabolism, growth and cell division, along with substrates such as nitrogen sources that are utilized in protein synthesis and cellular growth [24].

Because of their faster growth rate and easier cultivation, *Chlorella* and *Scenedesmus* strains are often used in biodiesel production, but *Chlorella* strains show only 14–30% lipid content under autotrophic growth conditions with rich nutrition [13].

Previous studies have confirmed that lipid content in some micro algae strains could be increased by various cultivation conditions [25]. Becker [26] reported that the nitrogen requirement for green algae varies amongst different species. Lipid concentrations in *Chlorella vulgaris* grown under differing nitrogen concentrations vary from 14.1% to 62.9%. Green algae grown at low levels of nitrogen will have between 45% to 70% cellular lipid content which would contain lipid chains of 16:0 and 18:1 fatty acids. At higher levels of nitrogen, the lipid

content shifts to 20% and are polyunsaturated fatty acids [27-28].

This study explores the potential for the application of various nitrogen sources for the growth of *Chlorella vulgaris* for biomass and lipid production, under photoautotrophic conditions, utilizing molecular CO_2 from air to form various products of industrial importance.

2. METHODS

2.1 Microalgae Strain Isolation

The native strain microalga - *Chlorella vulgaris* used in this study was obtained from fresh water ponds at the African Regional Aquaculture Center situated at Aluu, Rivers State, Nigeria. Isolation of the alga was carried out by blooming fresh water ponds with cow dung waste extract in a 90:10 ratio with a deep green colour after five days. The bloomed culture was streaked onto an agar plate containing a synthetic medium [29] solidified with 1% agar [w/v] according to Agwa et al. [30]. Petri plates were allowed to stand for 4-7 days under controlled laboratory conditions. Individual algal cells were obtained by means of repeated re-inoculation of 0.1ml on a sterilized nutrient agar medium. The plates were incubated at room temperature under two double fluorescent lamps emitting ca15 $\mu Em^{-2}s^{-1}$. Purity of the culture was periodically checked under the microscope using X10 objective magnification and by streaking onto agar plates. Subculture was done after every four weeks of inoculation. The organism was identified on the basis of its phenotypic characteristics and molecular characterization by the Polymerase Chain Reaction [PCR] techniques using slight modification of a protocol described by Burja et al. [31].

2.2 Growth Conditions and Growth Media

Isolated mono cultures of the tested microalgae were raised and maintained aseptically in the synthetic medium consisting of 0.132 g/l Potassium nitrate, 0.066 g/l Sodium silicate, 0.066 g/l Monosodium phosphate and 0.066 g/l EDTA, autoclaved at 121℃ for 15 mins, 15psi under controlled laboratory conditions. The pH of the medium was adjusted to 8.5 with either 1N HCl or 1N NaOH solution prior to autoclaving. The growth conditions were maintained at a temperature of 28±2℃ and 12 h light cycles in a 500ml conical flask under natural illumination, aerated intermittently by manual shaking at 2 h interval for 12 h. Control flasks were set up using synthetic medium.

Influence of various nitrogen sources on the growth responses of the strain were studied in the batch culture in triplicate for 21 days. To the synthetic medium was added different concentrations of sodium nitrate [$NaNO_3$], potassium nitrate [KNO_3] and urea [$(NH_2)_2CO$. The other medium constituents and pH were kept unchanged as those of the normal synthetic medium. For inoculation, 2.0 ml inoculum of the growing culture was used for each of the treatments.

2.3 Growth Evaluation

Growth characteristics were determined from the respective growth curves developed by plotting the observed number of cells [cells / ml^{-1}] against the time of observations [days]. Cell density was determined by measuring optical density of the culture at 600 nm using a Spectrophotometer [Spectronic 20, Genesys]. Biomass was determined as cell dry matter by centrifuging about 5ml of the growing culture at 3000 rpm for 10 mins, washed with deionized water three times and dried at 50℃ in a hot air oven to a constant weight [32]. Cell numbers were determined by the direct cell count using Neubauer haemocytometer. Lipid production was determined on wet cells of *Chlorella* sp. after harvesting by the (AOAC) Official methods of Analysis [33]. Algae growth was monitored by plotting the OD600 against time. The specific growth rate and the doubling time were determined using the following equations [34]:

$$\text{Specific growth rate } (\mu): \mu = \log\left(\frac{ct2/ct1}{t2-t1}\right) \quad (1)$$

$$\text{Doubling time } (Td): Td = ln2/\mu \quad (2)$$

Where *Ct1* and Ct2 are cell density (cells ml-1) at different time points time1 (t1) and time2 (t2), respectively.

For each parameter, the average values were calculated from the data generated from three replicates of each study. Statistics analysis was done using the variance analysis [ANOVA], at a confidence level of 95% [$p < 0.05$], in order to show the differences between the means of each treatment.

3. RESULTS

The agarose gel reveals no DNA from samples in lanes 1 and 4 which served as control, but DNA

was found in lanes 2 and 3. The electrophoresis of PCR products revealed band sizes of 550bp, and corresponded to nuclear small subunits [SSU]. Lanes 5-8 was the replicate of lanes 1-4 (Fig. 1). The effect of different nitrogen source on biomass production of *Chlorella vulgaris* is presented in Figs. 2-11. Three different nitrogen sources were studied and urea salt [0.045 mgL^{-1}] gave the best result yielding a biomass of 279 mgL^{-1}, KNO_3 [0.045 mg L^{-1}] resulted in 68 mgL^{-1} biomass and $NaNO_3$ [0.075 mg L^{-1}] gave 24 mgL^{-1} biomass. Figs. 2 and 3 showed a 12 days lag period before an increase in cell mass by the 15th day at a concentration of 0.045 mgL^{-1}. Only Fig 4 gave a 3 days lag period with an increase in cell number on the 4th day at a concentration of 0.055 mgL^{-1}. No lag phase was observed from Figs 5-7 [urea treatment] with a significant increase in cells from day 0 till the end of the study showing a complete utilization of this nitrogen source by

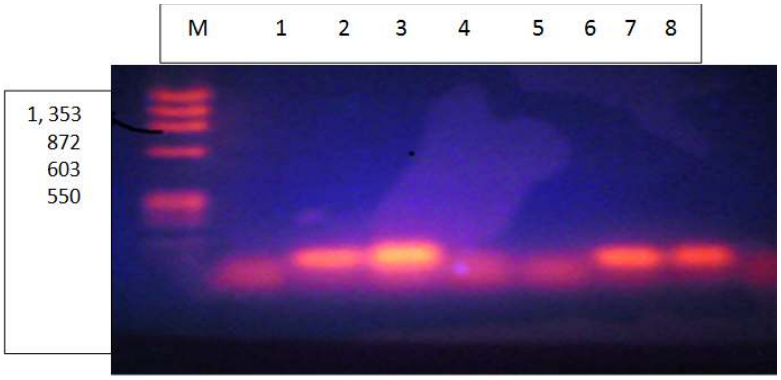

Fig. 1. Gel electrophoresis of the amplified PCR products for the detection of *Chlorella vulgaris* genes

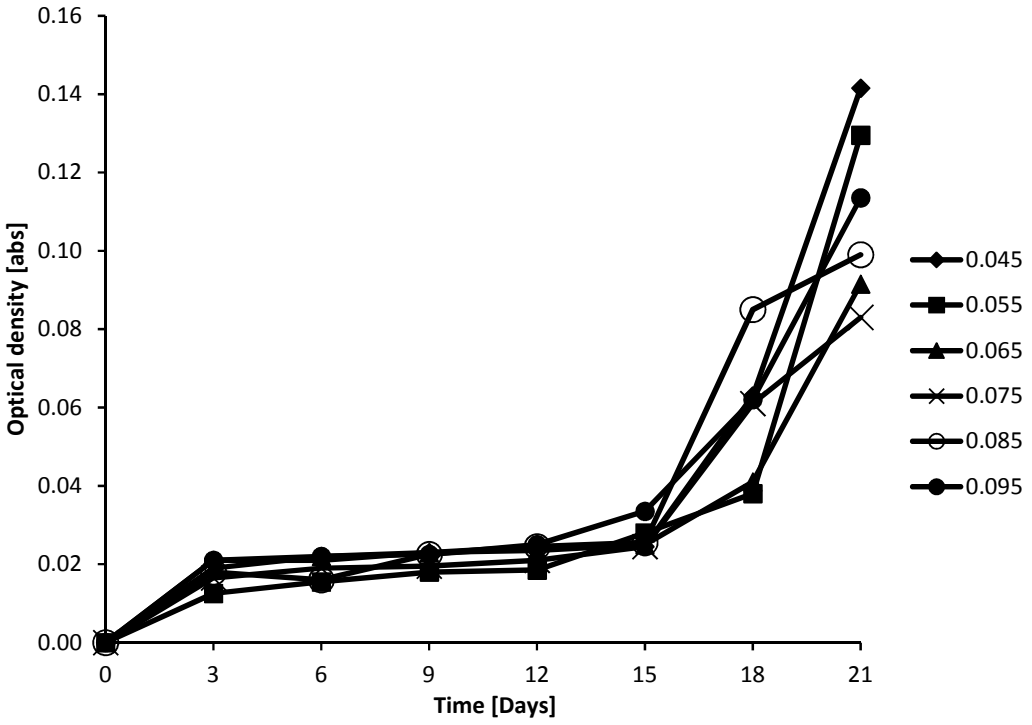

Fig. 2. Effect of nitrate [KNO_3] on *Chlorella vulgaris* measured as optical density

the microalgae. The same growth phase was observed in Figs. 8-10 but utilization of the nitrogen source occurred at higher concentrations. No significant difference was found between treatments in KNO_3 and $NaNO_3$ [P>0.05] but a significant difference was seen in urea between treatments [P<0.05]. Fig. 11 illustrates the lipid content of the microalga with different nitrogen sources. The lipid production was highly significant between all the treatments [P<0.05]. The maximum growth rate (μ=0.198) with a doubling time of 5.05 was recorded with urea at a concentration of 0.055mgL^{-1} $NaNO_3$ at the same concentration with urea had (μ=0.182) at a doubling time of 5.5, but KNO_3 showed the least growth rate (μ= 0.169) with doubling time of 6.33.

4. DISCUSSION

Chlorella vulgaris is a non-motile, unicellular freshwater green microalga, which is known to accumulate large amounts of protein and lipid [35]. The phenotypic characteristics of *Chlorella* sp. have been described; this microalga is a simple unicellular green alga with small spherical cells, cup-shaped, (Fig. 1) and gregarious [36] Huss et al. [37] and Krientz et al. [38] stated that *Chlorella* is a coccal green alga with small spherical cells. According to Sharma, et al. [39] *Chlorella vulgaris* is a green, spherical, single celled fresh water microalga belonging to the phylum Chlorophyta. The chloroplast DNA of *Chlorella* sp. revealed a band size of 550bp corresponding to nuclear SSU (Fig. 2). The primer sequence for *Chlorella vulgaris* showed that Nuclear SSU primers typical of *Chlorella vulgaris* are amplified in lanes 2 and 3.This agrees with the findings of Burja et al. [31] who showed a genetic sequence of about 578bp fragment of the 16S rRNA gene present within the chloroplast genome. For this reason, we identified the isolate in this study as *Chlorella vulgaris*. Nitrogen is an important constituent of cell protein and protoplasm needed for algal growth and it affects the productivity of microalgae. Microalgae are capable of utilizing various dissolved forms of inorganic and organic nitrogenous sources. An essential criterion for mass production of microalgae varies from species to species; it is based on the selection and utilization of a suitable nitrogen source [40]. A wide variety of nitrogen sources, such as ammonia, nitrate, nitrite and urea, can be used for growing microalgae [26]. Urea, KNO_3 and $NaNO_3$ were used to investigate the effect of nitrogen sources on Chlorella vulgaris. The lowest concentration of urea [0.045 mgL-1] gave a biomass yield of

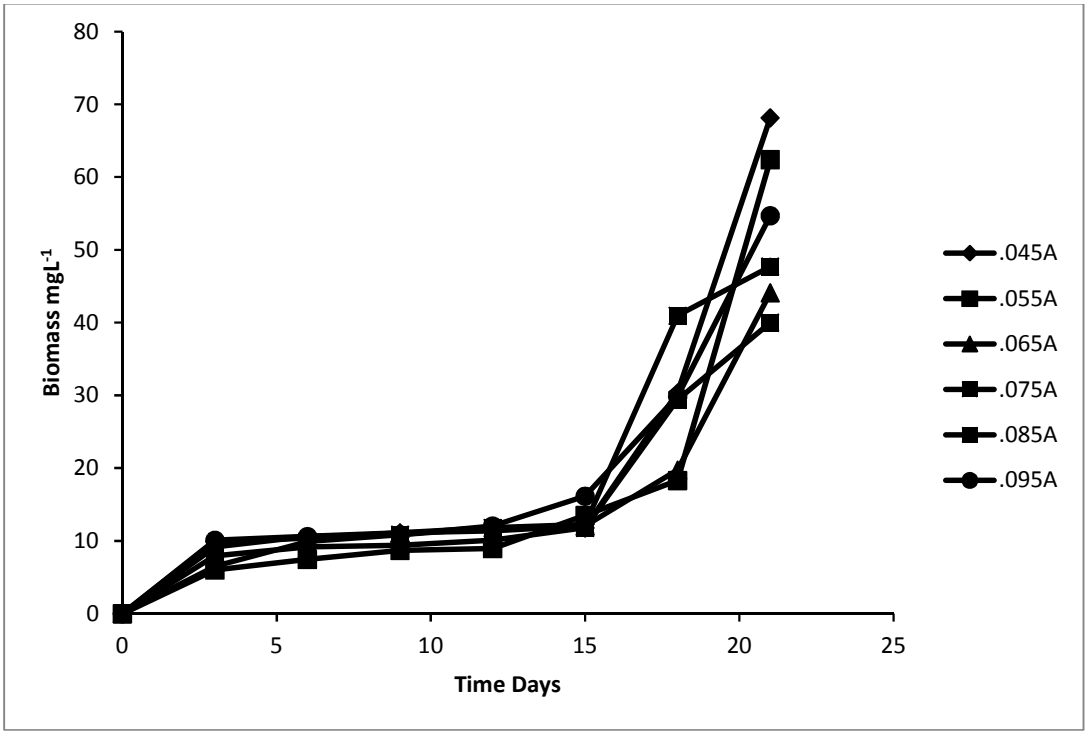

Fig. 3. Effect of nitrate [KNO_3] on *Chlorella vulgaris* measured as dry matter

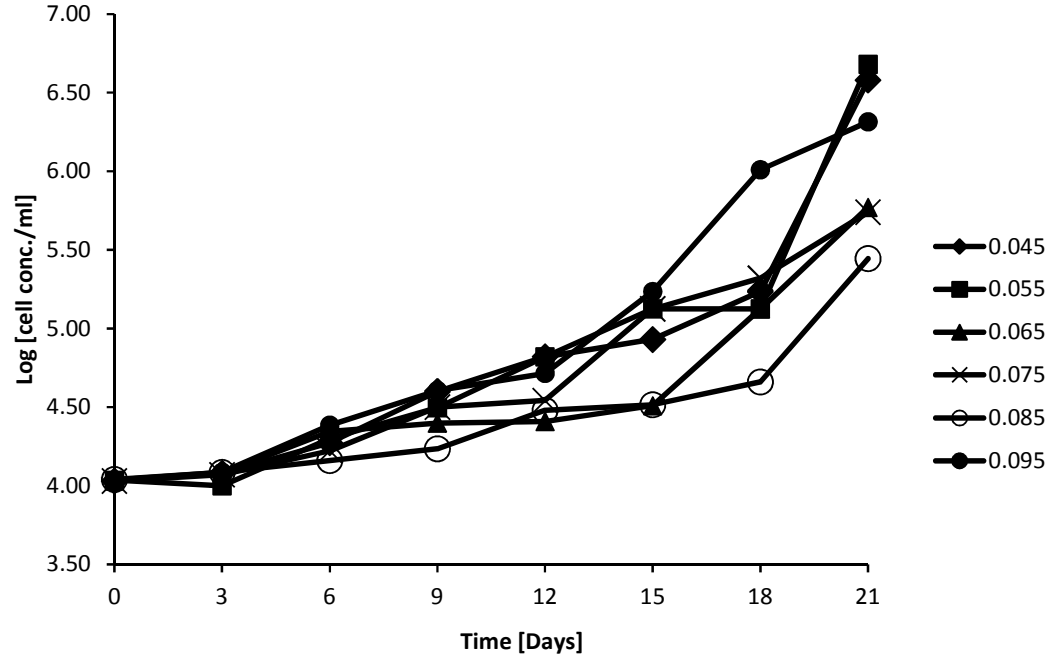

Fig. 4. Effect of nitrate [KNO_3] on *Chlorella vulgaris* measured as cell number

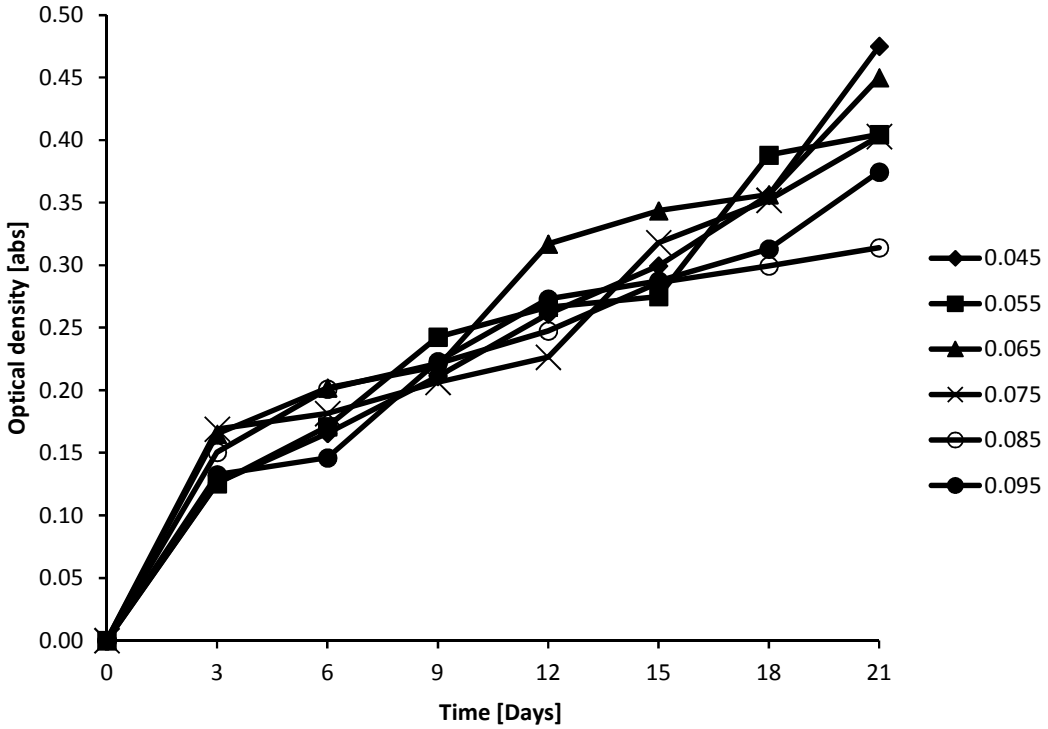

Fig. 5. Effect of nitrate [UREA] on *Chlorella vulgaris* measured as optical density

about 279 mgL-1. In large scale, urea can be used for microalgae cultivation as a very efficient nitrogen source, because it is very cheap compared to other nitrogenous nutrients available for microalgae. This makes it economically feasible and accessible for industrial production of microalgal fuel [41]. Urea plays a role in growth and metabolism regulation;

its concentrations have a strong influence on cell division; low concentrations of urea enhance vegetative growth of algal cells. At higher concentrations, urea is converted to ammonia leading to ammonia toxicity; this will disrupt the cell constituent resulting in chlorophyll decomposition [42] Urea increases growth due to urea utilization, and therefore enhances vegetative growth of the algae [43]. Cleavage of urea molecule allows its fast utilization by the alga and their degradation involves two specific enzymatic systems [urease and urea amydolyase]. Growth rate was higher with urea nitrogen sources as compared with other nitrate sources due to the extra nitrogen quantities from urea metabolism [44]. It is cheaper than nitrate and has no effect on the final chlorophyll content [45,10]. Piorreck et al. [27] observed that diminishing nitrogen concentration in a green algae medium, including *Chlorella vulgaris*, lead to a lipid increase, reaching approximately 45%. Becker [26], reported urea to be the best nitrogen source of culturing *Chlorella* among other organic sources of nitrogen. Shi et al. [46] recorded the highest specific growth rate when cultivated in a medium with urea as the nitrogen source with a biomass productivity of approximately 18.7 g/l. Nitrate has been recognized as the preferred nitrogen source for many algal species [45,10] . Many investigators such as Shi et al. [46], Kangama and Rumei [47], Ganuza et al. [48] (2009) and Shen et al. [49] indicated that nitrogen sources have no significant effect when added at concentrations between 0.85 and 1.7 g/l. Our investigation shows that lower concentrations of nitrogen sources had significant effect on the biomass and lipid content of the microalga. *Spirulina platensis* could utilize urea in fed-batch and batch culture [10,50]. The use of urea by the marine microalga *Isochrysis galbana* resulted in higher fatty acid contents than the use of nitrate and nitrite [50] similarly the growth rate and total lipid content of *Chlorella* sp. also varied with the level of urea concentration used during cultivation [21]. Consequently, an optimized supply of urea or deficiency of $NaNO_3$ is considered to be a cultivation strategy for microalgae lipid production. Studies have revealed that urea could replace nitrate as a nitrogen source and this could support growth. Urea seems to be the most effective nitrogen source for providing the alga with sufficient carbon and at the same time nitrogen comparable to a nitrate source [51]. From our studies, the results revealed urea to be the most suitable nitrogen source for growing the *Chlorella vulgaris* strain considering the lipid and biomass productivity. Thus urea serves as a complementary carbon source [52] and enhanced the vegetative growth of the microalga.

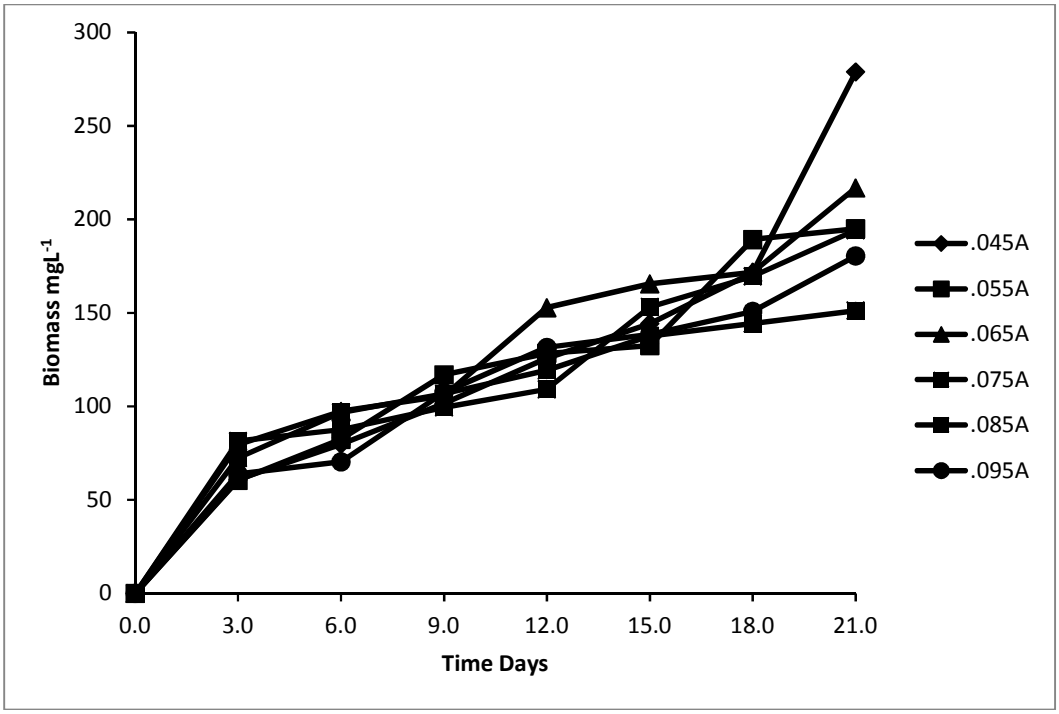

Fig. 6. Effect of nitrate [UREA] on *Chlorella vulgaris* measured as dry matter

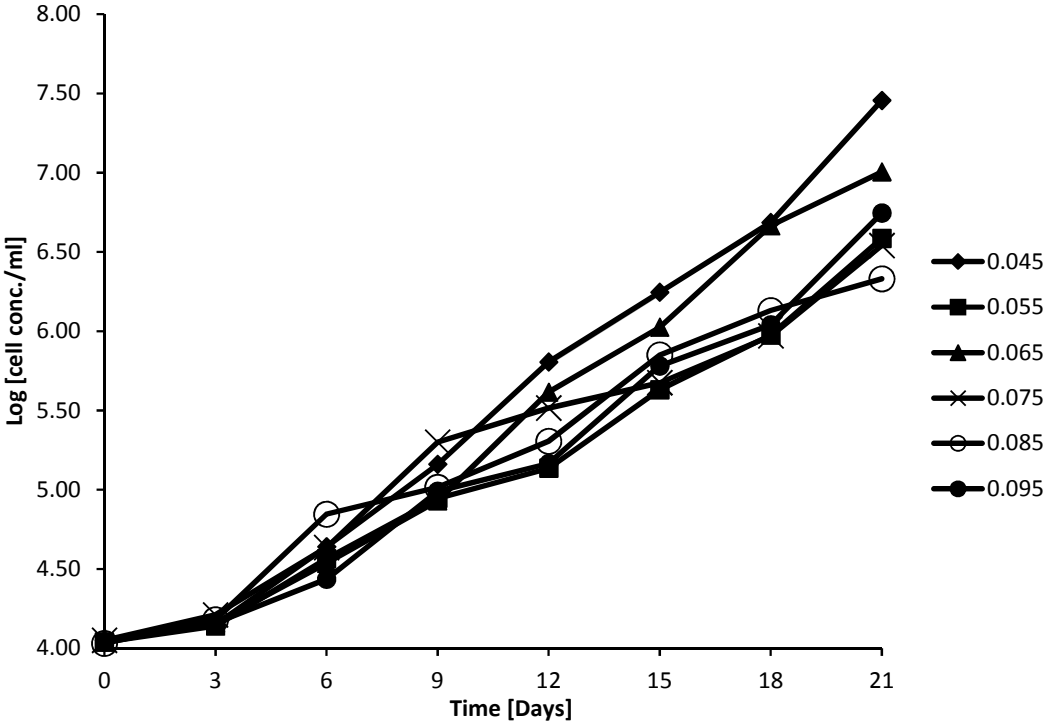

Fig. 7. Effect of nitrate [UREA] on *Chlorella vulgaris* measured as cell number

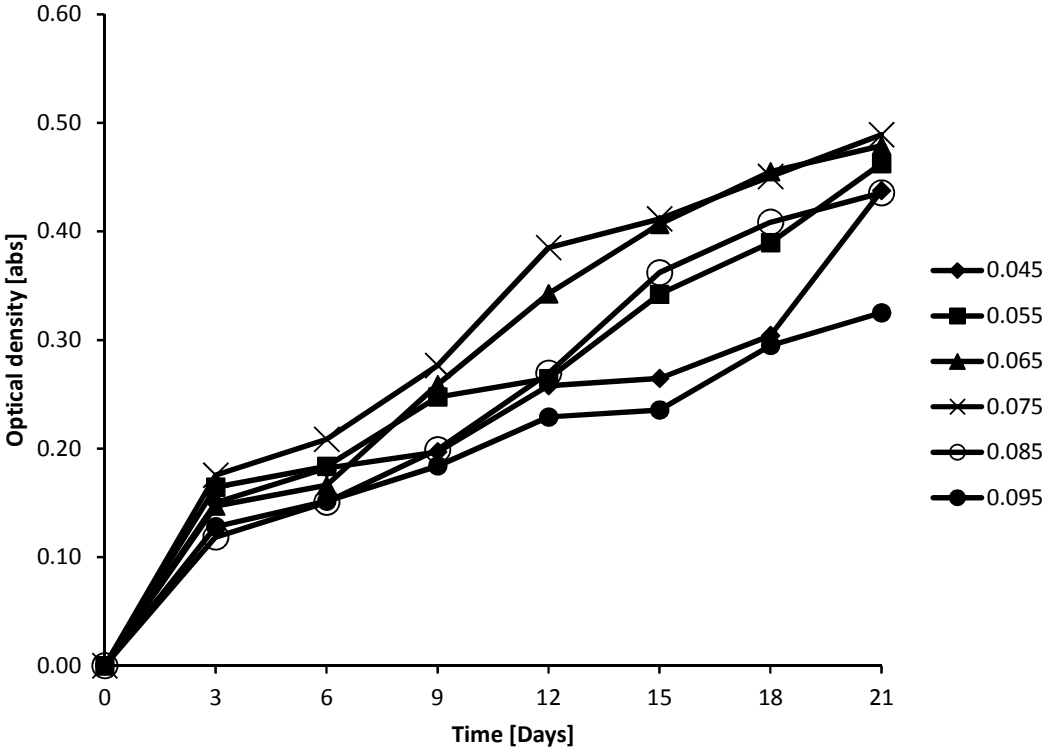

Fig. 8. Effect of nitrate [$NaNO_3$] on *Chlorella vulgaris* measured as optical density

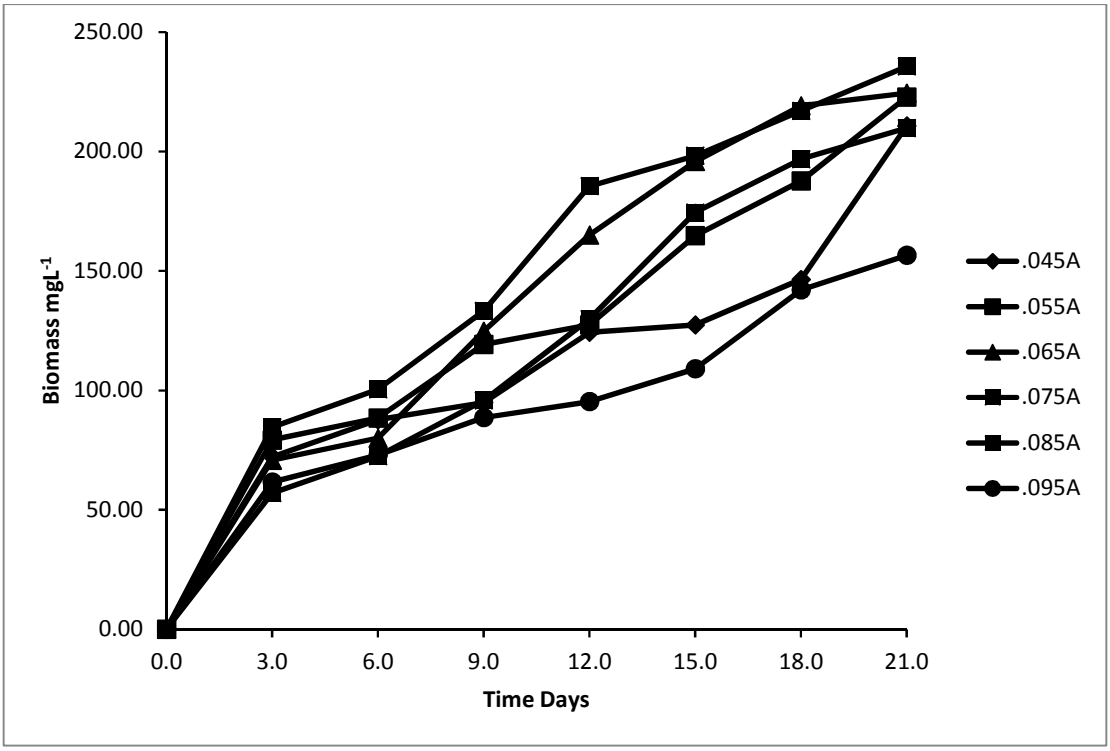

Fig. 9. Effect of Nitrate [$NaNO_3$] on *Chlorella vulgaris* measured as dry matter

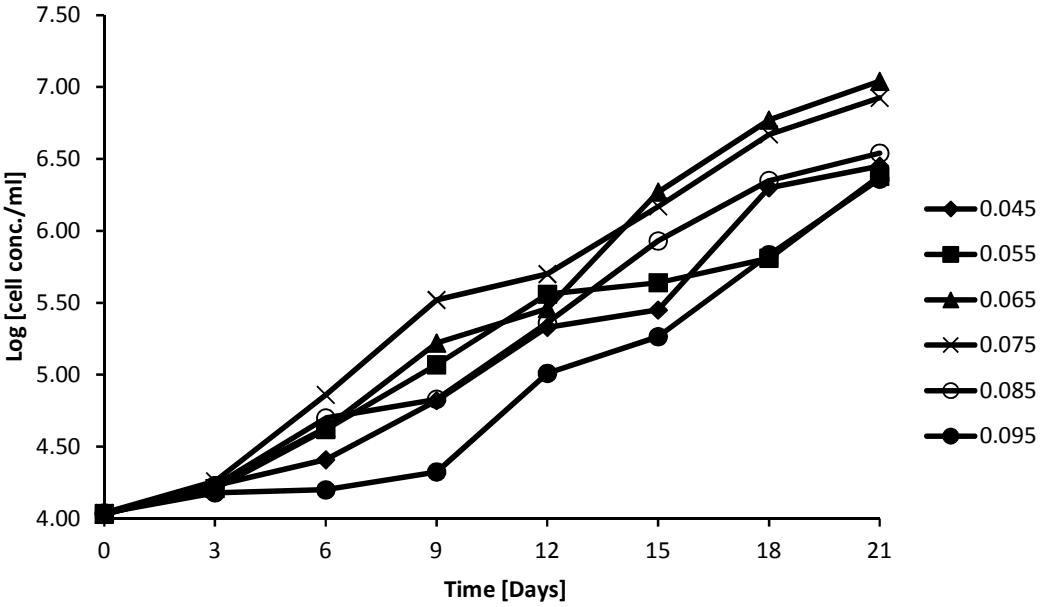

Fig. 10. Effect of nitrate [$NaNO_3$] on *Chlorella vulgaris* measured as cell number

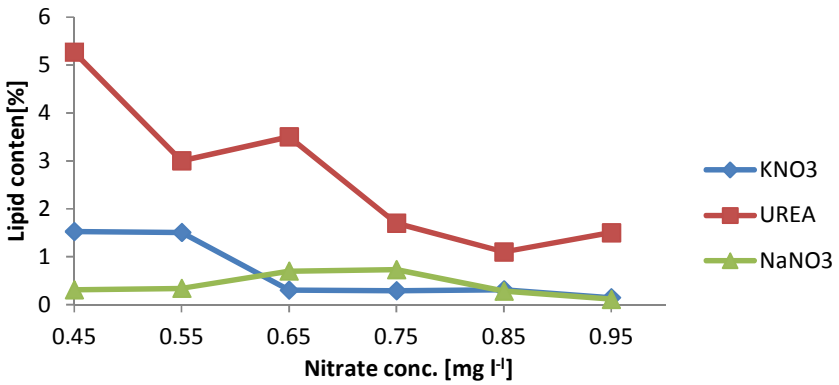

Fig. 11. Lipid content of *Chlorella vulgaris* with different nitrate sources

Table 1. Specific growth rate of *Chlorella vulgaris* with different nitrogen sources

Concentration	KNO_3		$NaNO_3$		Urea	
	SGR (μ)	DT (d)	SGR(μ)	DT (d)	SGR(μ)	DT(d)
0.045	0.0677	14.77	0.152	6.578947	0.1827	5.47
0.055	0.072	13.89	0.182	5.494505	0.1977	5.05
0.065	0.0753	13.28	0.0767	13.03781	0.1767	5.66
0.075	0.1587	6.33	0.107	9.345794	0.1067	9.37
0.085	0.0433	23.09	0.171	5.847953	0.1157	8.64

Key: SGR= Specific Growth Rate; DT= Doubling Time

Lipid production and accumulation in microalgae increases due to limiting nitrogen levels. Lipid accumulation occurs in the early stationary phase, allowing for the intervals between harvests to be shortened, this can result in higher overall biomass yield [23]. Lipid content was found to be higher with urea [5.27%] than in KNO_3 [1.53%] and $NaNO_3$ [0.73%]. This is in agreement with Nadia et al. [53] who studied the effect of some nitrogen sources on growth and lipid of *Chlorella* sp. The result indicated that urea is the most appropriate nitrogen source for biomass and lipid production by *Chlorella* sp.

5. CONCLUSION

Considering the lipid content of algal biomass produced in media containing different nitrogen sources, cultures grown in media containing urea had higher lipid content than those grown in cultures using the potassium and sodium inorganic salts. The results indicated that urea was the most appropriate nitrogen source for growth of *Chlorella* sp. These results may be attributed to the ability of *Chlorella* to metabolize urea which is usually hydrolyzed into ammonia by urea carboxylase enzyme [and sometimes urease] before its nitrogen is incorporated into cells and bicarbonate. The presence of urea in any media causes changes in intracellular fatty acid content; C_{16} fatty acids are converted to C_{18} by an additional carbonyl group.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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