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Growth, lipids, proteins, and carotenoid contents of some freshwater green microalgae under simulated day/night temperature fluctuation

C. N. Eze[1,3], I. O. Ogbonna[2], *J. C. Ogbonna[1]

[1]Department of Microbiology, University of Nigeria Nsukka, Nigeria
[2]Department of Microbiology, Federal University of Agriculture, Makurdi, Nigeria
[3]Department of Science Laboratory Technology, University of Nigeria Nsukka, Nigeria

Abstract
Day/night or seasonal temperature fluctuation is a major factor during outdoor cultivation of microalgae. In the present study, we investigated the impact of simulated day/night temperatures (20°C night and 30°C day) on the growth, lipid, protein and carotenoid contents of four local oleaginous microalgae strains under mixotrophic and heterotrophic conditions. The impact of simulated day/night temperatures on the growth and biochemical compositions varied across species and culture conditions (mixotrophic or heterotrophic). The lipid productivity by *Dictyosphaerium* sp. under heterotrophic condition was twice the value obtained at constant temperature but showed no significant ($p > 0.05$) impact under mixotrophic condition. *Desmodesmus subspicatus* elicited higher lipid (15%) and carotenoid (56%) contents under simulated day/night temperature regime than at constant temperature (30°C) ($p<0.05$). There was a negative impact on the protein content of the microalgal species under mixotrophic and heterotrophic conditions. The above results have shown that these species have high potentials for co-production of lipids, protein and carotenoid under outdoor conditions.
Keywords: Lipid productivity, day/night temperature, glycerol, carotenoid, outdoor cultivation
Corresponding author email: james.ogbonna@unn.edu.ng

Introduction

Microalgae cells are potential factories for production of various biomolecules of commercial value. Such biomolecules as lipid and carbohydrates (used for biofuel production), carotenoids (used by pharmaceutical and food industries as nutraceutics and food colourants), and proteins (used as food supplements) accumulate in microalgae cells during cultivation (Branco-Vieira et.al., 2020; Menegazzo et al., 2020). Some microalgae species have the capacity to accumulate several biomolecules simultaneously (Eze et.al., 2020) although at varying quantities across species and cultivation conditions (Cheregi et al., 2019), giving rise to biorefinery concept (Branco-Vieira et al., 2020). The biochemical compositions determine the applicability and profitability of microalgae species (Li et.al., 2020). The rationale behind biorefinery concept was to mitigate the high cost of microalgae biomass production identified as one of the prominent challenges facing the commercialization of microalgal biofuel (Eze et.al., 2020). The achievement of this concept depends very largely on large scale production of biomass and high productivity of products of interest.

Microalgae biomasses are produced in large scales either indoor or outdoor using

photobioreactors (closed system of cultivation) or in open/raceway ponds (open system of cultivation). The closed systems for cultivation are preferred because culture conditions can be controlled, leading to high productivities. The outdoor cultivation is considered cheaper (Cheregi et.al., 2019) due to free supply of sunlight in the tropics, especially in sub-Sahara Africa, which is a basic need for microalgae growth. According to Santos-Ballardo et.al. (2016), cultures grown outdoor had better performance than cultures grown indoor as it concerns lipid content and fatty acid profiles. However, the productivity is compromised by some environmental factors such as fluctuations in light intensity and temperature, depending on time and season (Sonkar et. al., 2020). Temperature is known to be a crucial factor that affects cell growth, rate of photosynthesis and composition of biomass by microalgae species (Carneiroet.al., 2020). While some microalgal species can grow well in the absence of light (heterotrophs), there is an optimal temperature for the growth of every microalgae species. Hence, temperature fluctuation as obtained in outdoor cultivation is a critical parameter to control in order to achieve high productivity (Cheregiet.al., 2019). For instance, temperature changes in the tropics especially in the sub-Sahara Africa, varies more between the day and night time than within the same season of the year (Kotir, 2011). Usually, the optimum growth temperature for many microalgae species is 30±5^{o}C (Ogbonna and Ogbonna, 2015) which is about the day time temperature in sub-Saharan African countries. For microalgae species cultivated outdoor, the drop in temperature at night below the optimum temperature obviously affects how the cells grow as well as the biochemical compositions (Carneiro et.al., 2020; Cheregiet.al., 2019). The application of temperature control system to maintain a constant temperature for optimum growth increases the cost of production(Cheregiet.al., 2019). Mattson et.al. (2019) reported that in terms of boosting algal lipids, diurnal shift in temperature exceeded nitrogen limitation in *Monoraphidium convolutum* in a large-scale outdoor cultivation. The report also suggests that the impact of diurnal temperature shift is species-specific. It is therefore important to source for local microalgae species which can adapt to diurnal shift in temperature without significantly compromising growth and productivity (Cheregi et.al., 2019). It is well known that growth and productivity by microalgae depends not only on the species but also on the culture mode. Thus, a lot of work have been done on culture conditions such as phototrophy, heterotrophy and mixotrophy to enhance accumulation of biomass as well as biochemical contents of many microalgal species (Gim et. al., 2014). Comparably, as reported by many researchers, the lipid and biomass productivities of some strains of microalgae were notably higher in mixotrophic than in photoautotrophic and heterotrophic cultivations (Gim et. al., 2014; Eze et. al., 2017). Again, the type of organic carbon source employed in mixotrophic cultivation can make the process either cost intensive (using costly substrates such as glucose) or cost effective (using cheap and available organic carbon source such as glycerol). Incidentally, there are not many microalgae species that can utilize glycerol for optimum productivity. However, such microalgae species that can utilize glycerol as organic carbon source and can perform optimally when subjected to temperature fluctuation are ideal species for outdoor cost-efficient biodiesel production.

Therefore, there is a need for bioprospecting for local microalgae species that can perform optimally at day/night temperature fluctuation in terms of growth and productivity. The present study evaluated the response of four local oleaginous microalgae species to simulated night/day temperature fluctuations in comparison to optimum steady temperature of cultivation under mixotrophic and heterotrophic conditions using glycerol as the organic carbon source. The lipid, protein, and carotenoid contents of each species were analysed to assess their potentials for outdoor cost-efficient co-production of useful metabolites and biodiesel oil.

Materials and methods

Microalgae species used in this study

The microalgae species were obtained from the Department of Microbiology, University of Nigeria, Nsukka. The isolation, purification and identification have been reported elsewhere (Eze et.al., 2017; Ogbonna and Ogbonna, 2015).

Inoculum preparation

The inoculum for each microalga species was prepared by transferring 10% stock culture into 200 mL BG-11medium contained in 500 mL Erlenmeyer flasks and agitated in a rotary shaker at 100 rpm and white light illumination supplied continually at intensity of 50 μmol.m^{-2}.s^{-1}. The incubation temperature was 30°C for 10 days.

Medium composition

The BG-11medium was composed of (in gL^{-1}): $NaNO_3$, 0.25; K_2HPO_4, 0.04; $MgSO_4 \cdot 7H_2O$, 0.075; $CaCl_2 \cdot 2H_2O$, 0.027; $C_6H_8O_7$, 0.006; $C_6H_8O_7$.nFe.nNH_3, 0.006; EDTA, 0.001; $NaCO_3$,0.02; and 1.0 mL A5 + Co stock solution. The composition of the A5 + Co stock solution was distilled water, 1.0 L; H_3BO_3, 2.860 g; $ZnSO_4 \cdot 7H_2O$, 0.222 g; $MnCl_2.4H_20$, 1.81g; $CuSO_4 \cdot 5H_2O$, 0.079 g; $Na_2MoO_4 \cdot 2H_2O$, 0.390 g and $Co(NO_3)_2.6H_20$, 0.0494 g. The medium's pH was adjusted to 7.2 and 200 mL each dispensed into 500 mL Erlenmeyer flasks.

Cultivation of microalgae in batch flasks

Cultivation under constant temperature

The BG-11medium (200 mL) containing 5.0 g/L glycerol was dispensed into 500 mL Erlenmeyer flasks andthe flasks were covered with foams stuck with rubber corks for ventilation. They were sterilized at 121°C for 15 min. The growth medium, in triplicates, were seeded with 10%inoculum and the algal cultures incubated at 30^0C in a rotary shaker at the speed of 100 rpm. A set of the flasks were continuously illuminated at 50 μmol.m^{-2}.s^{-1} (mixotrophy) whereas flasks in the other set were wrapped with aluminium foil to shield the light (heterotrophy). In both cases, the cultivation was done for 8 days. Sampling (5.0 mL each) was done at 48 h intervals to measure the cell concentrations. At the end of the eight days, the culture was centrifuged at 5000 rpm for 5 min, and the cell pellet was dried in an oven at 70 °C for 24 h. The total oil contents, biomass concentrations, carotenoids and protein contents were determined.

Simulated day/night temperatures

The 200 mL culture medium containing 5.0 g/L glycerol in 500 mL Erlenmeyer flasks as in section 2.4.1 above were seeded with 10% inoculum in triplicates. The cultures were incubated in a rotary shaker at varied temperatures (20^0C at night (6.0 pm - 6.0 am) and 30^0C at day (6.0 am - 6.0 pm) under continuous illumination at 50 μmole/m^2/s intensity and agitation speed of 100 rpm for eight days. The cell concentrations and biochemical compositions were determined as in section 2.4.1 above.

Analytical Methods

Cell growth rate

UV/VIS spectrophotometer (GENESYS 10S UV–Vis, Thermo Fisher Scientific, MA, USA) was used to measure the optical density (OD_{680}). The biomass concentrations were determined from the OD vs dry cell weight calibration curve. The specific growth rate, μ (day^{-1}) was calculated from Eq. 1

$$1/t \times \ln(X_m/X_0) \quad \text{..... Eq. 1.}$$

Here, X_0 (g L^{-1}) and X_m(g L^{-1}) stand for the cell concentration on day 4, and day 8respectively while t (day) is 4 days.

Measurement of Lipid Concentrations

A known dry weight of each of the microalgae biomass (0.2 g) was pulverized mechanically with the aid of a mortar and pestle. The method of Bligh and Dyer (1959) was used to determine the total lipid content.

Carotenoid and protein contents of the cells

Cell pellets were recovered from the 5.0 mL broth sample by centrifuging at 5000×g for 5 min and washing with distilled water (twice). The carotenoid was extracted by suspending the cell in 5.0 mL 90% (v/v) methanol and centrifuging at 5000×g for 5 min. The absorbance of the extract was measured at 470nm and 660 nm using UV-VIS spectrometer. Total carotenoid (mg L^{-1}) was calculated from Eq. 2 as reported by Lichtenthaler (1987)

$$(1000A_{470} - 44.76A_{666}/221) \quad \text{..... Eq. 2}$$

The pulverized sample (0.1g) was dissolved in 3.0 mL 1 M NaOH, and boiled for 20 min to extract the protein content (Rausch, 1981). The protein was recovered by centrifuging at 5000xg for 5 min and quantified with Coomassie Brilliant Blue (Bradford, 1976).

Statistical analysis

All the experiments were carried out in three replicates (n = 3). Significance differences were tested and the means separated using Analysis of Variance (ANOVA) and Least Significance Difference (LSD) respectively. The results were expressed or plotted as means ± S.E.

Results and Discussion

Effect of low-high temperature (20:30°C) regime on cell growth and biomass production

The cell concentrations of each microalgal species increased but varied across species during the period of cultivation at constant or simulated diurnal temperature shift both under mixotrophic and heterotrophic conditions (Fig. 1). This suggested that these microalgae species have the potentials for growth under varied temperatures (Yang et. al., 2016). It may also have indicated that the various species were able to utilize glycerol for their growth under the different culture conditions tested. One of the advantages of this outcome was the cost-effective production of the algal biomasses by the use of glycerol, a by-product of biodiesel production. Impressively, the price of glycerol has been reported to decrease as the biodiesel production expands (Yang et. al., 2012) most probably due to high quantity of glycerol by-product and consequent reduced market demand. For instance, in the United States of America, the price of refined glycerol was about US$0.3 per pound in 2007 which was much lower than US$0.7 per pound obtained before biodiesel production was expanded (Yang et. al., 2012). The variation in cell concentrations across the species and culture conditions (Fig. 1) suggested that the responses of the cells were both species-specific and culture condition- dependent. The growth curves (Fig. 1 A and C) revealed that the stationary growth phase was reached on day 8 of cultivation at constant temperature for both mixotrophic and heterotrophic conditions. However, the cells cultivated at simulated diurnal temperature shift both under mixotrophic and heterotrophic conditions showed evidence of continued growth even on the 8th day (Fig. 1 B and D). The reason for this characteristic growth was not clear. However, the varied temperatures were a simulation of outdoor day/night temperatures in the tropics from where the organisms were isolated. There were more pronounced lag phases in the growth curves of cells at low-high temperature regimes than at constant temperatures under heterotrophic condition (Fig. 1 C and D). However, no lag phase was observed for mixotrophic condition both at constant and varied temperatures. On the whole, cell growth was significantly higher under mixotrophic than heterotrophic conditions ($p<0.05$). This suggests that the cells more readily assimilated glycerol in the presence of light than in darkness. Xu *et al*. (2019) also, after cultivation of *C. vulgaris* in municipal wastewater for 15 days under high and high-low temperature conditions did not observe any significant lag phase, which according to the authors was an indication that *C. vulgaris* adapted very well in both culture conditions. On the contrary, *N. oculata* was reported with distinct lag phase in a phototrophic culture under constant and sinusoidal temperature regimes (Tamburic et. al., 2014).

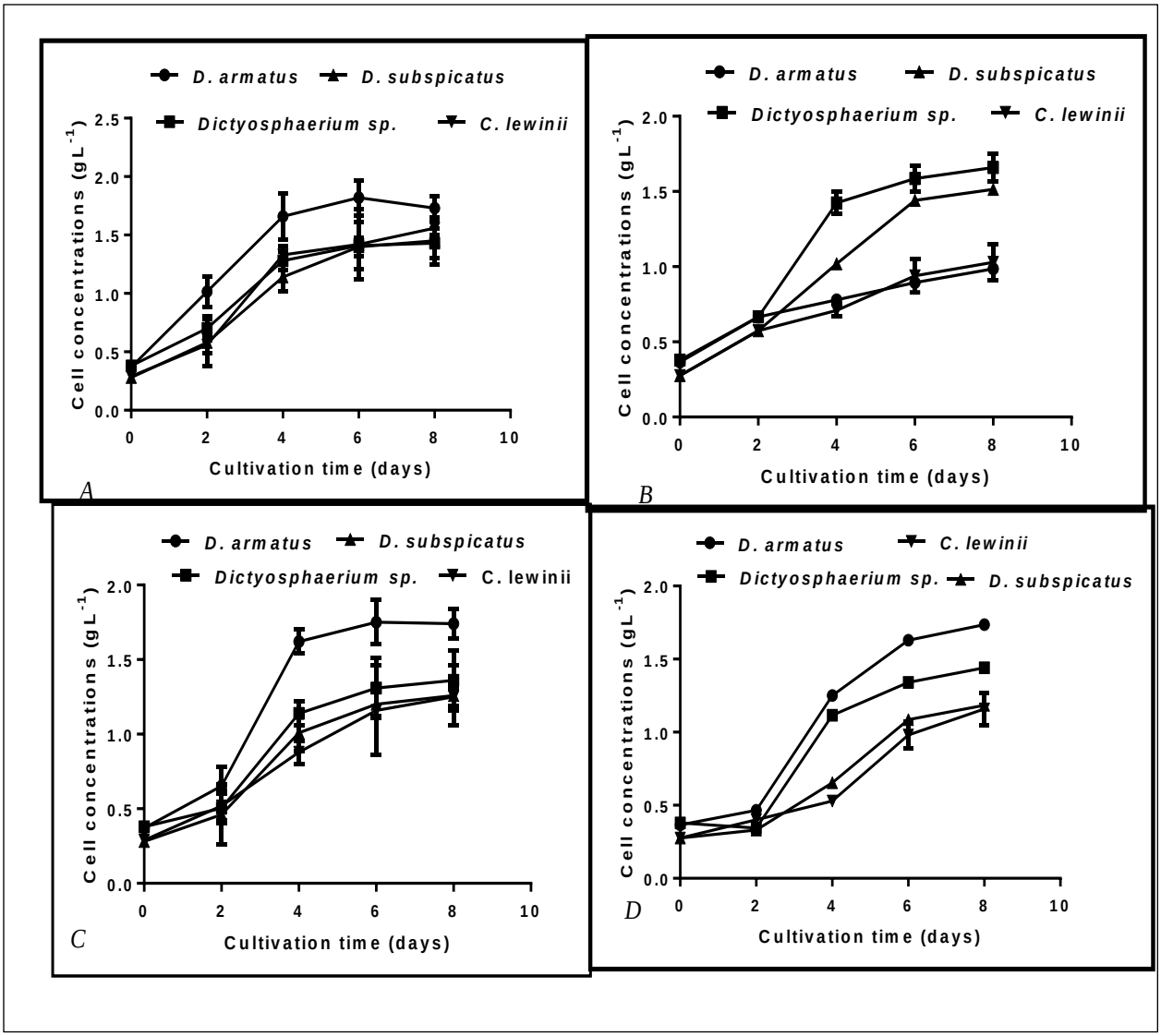

Figure 1: The growth curves of microalgae species cultivated under mixotrophic condition at constant temperature (A), varied temperature (B), and heterotrophic condition at constant temperature (C), varied temperature (D) for eight days.

The different algal species had comparable cell biomass and growth rates at constant temperature under mixotrophic cultivationcondition. However, when compared to simulated diurnal temperature shift under the same culture condition, the cell biomass and growth rates of *D. armatus* and *C. lewinii*were significantly lower ($P<0.05$). This report did not agree with Ogbonna and Tanaka (1996) who reported that addition of glucose to *Chlorella pyrenoidosa* culture at night promoted continuous cell growth both at day and night. The variation in the reports may be due to either differences in the microalgae species cultivated or culture conditions such as type of organic carbon sources and operational temperatures. The sub-optimal temperature of 20°C employed at night may have given rise to nighttime biomass loss due to suppressed metabolic activities of these isolates to levels that could not be compensated for as was seen at optimal temperature (30°C) (Carneiro et. al., 2020). *Dictyosphaerium* sp. and *D. subspicatus* recorded higher cell biomasses at high-low temperature regimes than other species ($p<0.05$) and about the same cell growth rates compared to values obtained at constant

temperature under mixotrophic condition. This suggests that *Dictyosphaerium* sp. and *D. subspicatus* adapted to the simulated outdoor temperatures without compromising biomass production. Such is an indication of potential for outdoor mass cultivation under mixotrophic condition. The cell biomass accumulated by *D. armatus* under heterotrophic and mixotrophic conditions both at constant temperature were the same with the values obtained at simulated outdoor temperatures under heterotrophic condition ($P>0.05$), but significantly higher than the values obtained by other species either at constant or simulated diurnal temperature shift ($p<0.05$) (Fig 2 A and B). This report demonstrated the potential of *D. armatus* to accumulate high biomass in natural outdoor-cultivation condition with light during the day (mixotrophy) and without light (heterotrophy) at night. The advantage of this outcome is that external light supply is not needed at night to maintain continuous optimum biomass accumulation. The implication is reduced cost of microalgae biomass production at commercial scale which mitigates the high cost of bio products by microalgae. However, the biomass concentration was lower than the value produced by *C. vulgaris* (1.62 gL^{-1}) under low-high temperature conditions (4-35^{o}C) using a waste medium (Xu et. al., 2019). The reason for the lower biomass in the current study may be due to lower concentrations of nutrient in theBG-11 medium than waste medium and, the smaller temperature difference (10^{o}C) in the current study than the (31^{o}C) reported by Xu et al. (2019).

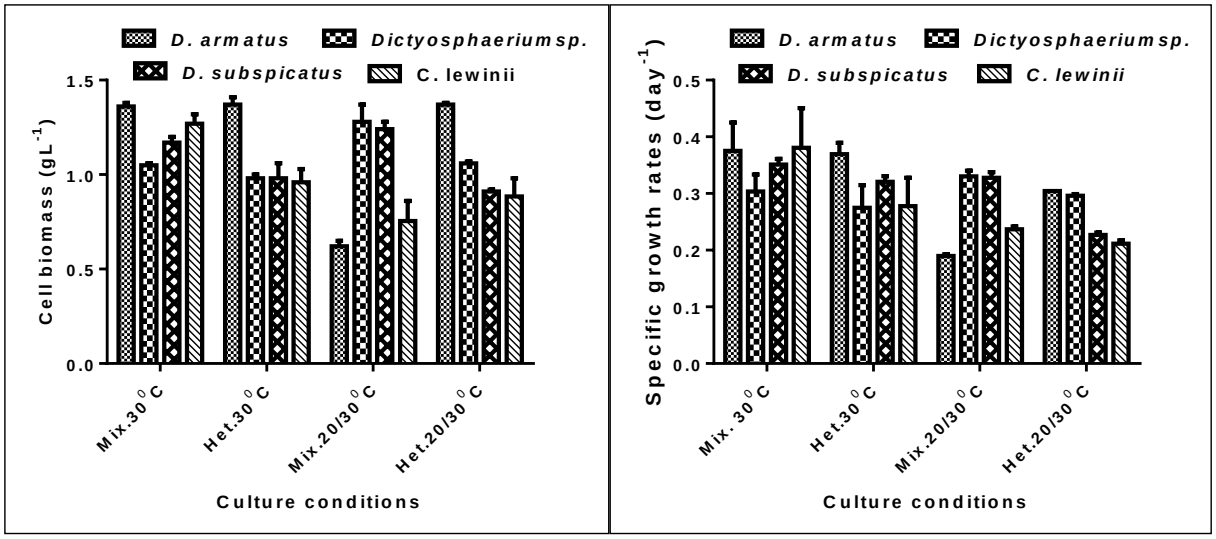

Figure 2: The Cell biomass (A) and Specific growth rates (B) of microalgae species cultivated under mixotrophic or heterotrophic conditions at constant or varied temperatures for eight days.

Effect of low-high temperature (20:30^{o}C) regimes on biochemical composition of the microalgae

The biochemical compositions of the microalgal species varied under different culture conditions (Fig. 4). For *D. armatus, Dictyosphaerium* sp. and *C. lewinii*, at simulated diurnal temperatures, the lipid content decreased under mixotrophic condition. A similar report was given on *Nanochloropsis oceanic* cultivated outdoor at 28:18^{o}C temperature regime (Carneiroet.al., 2020). This means that the decrease in lipid production at the low temperature (20^{o}C) could not be fully compensated for by the increase in the lipid production at the optimal temperature (30^{o}C). However, this concept may not hold true in heterotrophic cultivation as most of the isolates under the said culture condition had increased lipid content at low-high temperature than at constant temperature (Fig. 3). It is however, important to note that reports exist on increased lipid content by *C. vulgaris* (Xu et.al., 2016) and some *Chlorella* strains (Yang et. al., 2016) at

high-low temperature regimes. As discussed earlier, the advantage of heterotrophic accumulation of lipids at simulated diurnal temperature changes can lead to cost reduction in biodiesel production by such microalgae strains. Furthermore, the protein content decreased significantly at simulated diurnal temperature shifts when compared to the values at constant temperature both under mixotrophic or heterotrophic condition ($p<0.05$). This observation agreed with the findings of Eze et. al. (2020) and Xu et. al. (2019) that protein accumulation was probably inhibited by lipid synthesis, although, lipid synthesis was only favoured under heterotrophic condition in the current study. Some other reports (Cuhel et. al., 1984; Kakinum et. al., 2006; Rhee and Gotham, 1981) also affirmed the impact of temperature on the protein content in algal cell. However, in this study, the intrinsic properties of the various isolates in conjunction with the culture condition probably contributed to the variations in the isolates' protein contents under the two temperature regimes. For *D. subspicatus*, it accumulated higher lipid content at simulated diurnal temperature shifts than at constant temperature both under mixotrophic and heterotrophic conditions ($p<0.05$), but comparable protein contents under heterotrophic condition ($p>0.05$). This flexible quality of *D. subspicatus* in the current study may be as a result of intrinsic ability possessed by the isolate (Skurupsi et. al., 2012). Also, it could be an indication that such isolate would be a good feedstock for biodiesel production in tropical countries where diurnal temperature shifts are obtainable. It has been previously noted that microalgae strains that thrive under adverse environmental conditions have great potential for commercialization (Cheregi et al., 2019). The lipid content achieved by *D. subspicatus* at low-high temperature regime under mixotrophic condition was significantly higher than the value reported by Xu et al. (2019*)* and lower than the value reported by Yang et al. (2016). However, the response by *C. lewinii* to diurnal temperature shift was the opposite (Fig. 4) of that of the *Chlorella* strains reported by Yang et al. (2016) in terms of lipid and protein contents. The differences in culture media/conditions and species strain may be accountable for the variations in the results. There was low accumulation of carotenoids at constant or low-high temperature regimes by the microalgal species under heterotrophic conditions (Table 1) obviously due to absence of light supply to the culture media. The carotenoid content of the cells under mixotrophic conditions were higher than the values recorded under heterotrophic conditions at the two temperature regimes tested ($p<0.05$). *D. subspicatus* elicited significant increase in carotenoid contents while others recorded lower values at simulated diurnal temperature shift compared to constant temperature under mixotrophy ($p<0.05$) (Table 1). The ability of *D. subspicatus* to accumulate carotenoids alongside lipids at constant temperature has been reported elsewhere (Eze et. al., 2020). Light is one of the major factors that trigger carotenoids accumulation by microalgae probably because of its photo-damage protective function (Liu and Lee, 2000; Tjahjono et. al., 1994). The carotenoid contents of *D. subspicatus* at low-high temperature regimes may be due to an intrinsic property of the species rather than stress-related (Eze et. al., 2020).

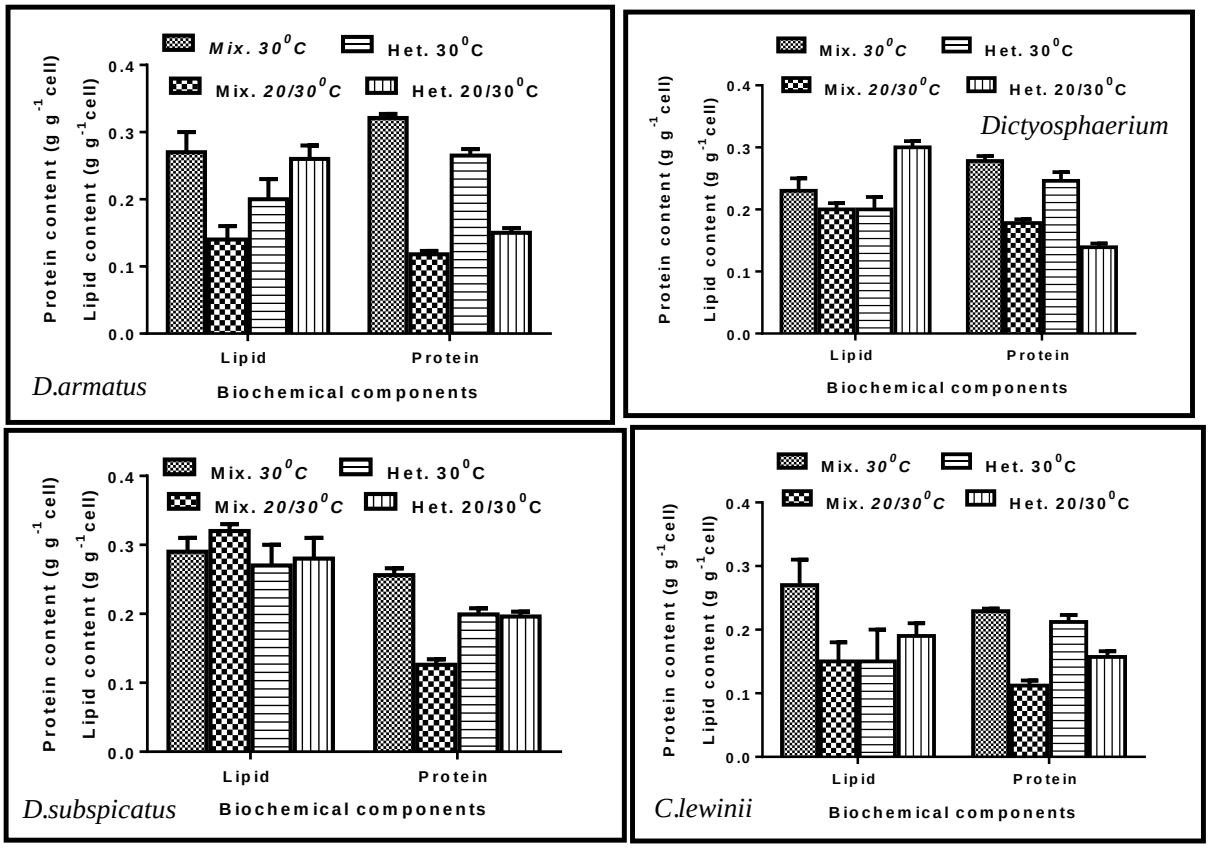

Figure 3: The lipid and protein contents of microalgae species cultivated at constant or varied temperatures under mixotrophic or heterotrophic conditions for eight days.

Table 1: Comparison of the effect of different culture conditions on carotenoid contents of microalgae isolates cultivated for eight days using 5 gL^{-1} glycerol as organic carbon source. Agitation speed = 100rpm. Light intensity = 50 $\mu mol.m^{-2}.s^{-1}$

	Carotenoid content (mgg^{-1}cell).			
Culture conditions	***D. armatus***	***Dictyosphaerium* sp.**	***D. subspicatus***	***C. lewinii***
Mixotrophy at30^{0}C	0.91±0.02	1.15±0.02	1.02±0.02	1.20± 0.02
Heterotrophy at30^{0}C	0.56± 0.01	0.12±0.01	0.36±0.03	0.20± 0.01
Mixotrophy at20/30^{0}C	0.70± 0.01	0.40±0.02	1.60±0.02	1.08± 0.02
Heterotrophy at20/30^{0}C	0.35± 0.01	0.15±0.01	0.28±0.01	0.24± 0.01

Effect of low-high temperature (20:30°C) regime on lipid productivities by the microalgae species

Lipid productivities varied across the microalgal species and between the different culture conditions tested at low-high or constant temperature regimes (Table 2). However, the values reported for each species was a reflection of combined output of lipid and biomass contents which were discussed earlier. Lipid productivity is a product of the biomass concentration and the lipid content of the cells. Thus, it is a useful index of the potentials of the cell strains for commercial applications (Eze et al., 2017). The low- high temperature regime had no significant effect on lipid productivity by *D. armatus* and *D. subspicatus* under heterotrophic condition, and *Dictyosphaerium*sp. under mixotrophic condition compared to constant temperatures ($p>0.05$). However, under heterotrophic condition, *Dictyosphaerium* sp. at low-high temperature yielded twice the lipid productivity obtained at constant temperature. *D. subspicatus,* under mixotrophic condition yielded significantly higher lipid productivity ($p<0.05$) at low-high temperature compared to the value obtained at constant temperature. Conversely, high-low temperature regime impacted negatively on *C. lewinii* under both mixotrophic and heterotrophic conditions compared to constant temperature. These variations could be due to unique intrinsic properties of each of the species influenced by either mixotrophic or heterotrophic conditions. Therefore, depending on the culture conditions, the microalgal species except *C. lewinii* could be good candidates for outdoor cultivation and production of biodiesel. It is interesting to know that these microalgae strains are not currently reckoned with among the strains employed for outdoor mass cultivation for biodiesel/bioproducts production. *Dictyosphaerium* sp. and *D. subspicatus* expressed more robust qualities, having the capacity to maintain high productivities at simulated day/night temperatures under both mixotrophic and heterotrophic conditions.

Table 2: Comparison of the effect of different culture conditions on lipid productivities of microalgae isolates cultivated for eight days using 5 gL^{-1} glycerol as organic carbon source. Agitation speed = 100rpm. Light intensity = 50 $\mu mol.m^{-2}.s^{-1}$

	Lipid productivity ($gL^{-}day^{-1}$).			
Culture conditions	***D. armatus***	***Dictyosphaerium* sp.**	***D. subspicatus***	***C. lewinii***
Mixotrophy at 30^0C	0.05658±0.003	0.03019±0.002	0.04241±0.002	0.04286± 0.003
Heterotrophy at 30^0C	0.05138± 0.001	0.02458±0.001	0.03308±0.002	0.018± 0.002
Mixotrophy at $20/30^0$C	0.01085± 0.001	0.03146±0.002	0.04883±0.001	0.01384± 0.002
Heterotrophy at $20/30^0$C	0.04504± 0.002	0.04015±0.002	0.03145±0.001	0.02102± 0.001

Conclusion

The response of some local isolates of microalgae species to day/night temperature fluctuations can be exploited for co-production of lipid and carotenoid, leading to reduction in the cost of biodiesel. *Dictyosphaerium* sp. and *D. subspicatus* demonstrated good adaptation to day/night temperature changes under heterotrophic and mixotrophic culture conditions respectively. The duo could be resourceful in outdoor mass cultivation of microalgae for cost-efficient production of biodiesel in the tropics.

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