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Simultaneous accumulation of lipid and carotenoid in freshwater green microalgae *Desmodesmus subspicatus* LC172266 by nutrient replete strategy under mixotrophic condition

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Abstract−Production of biofuel by microalgae can become economically viable if other high-value commercial products such as carotenoid are simultaneously produced. This study aimed at enhancement of lipid and carotenoid productivity by an oleaginous microalga sp. *Desmodesmus subspicatus* LC172266 by supplementing Nitrogen sources to BG 11 medium under batch or fed batch mixotrophic condition. Optimum urea supplement (0.2 gL^{-1}) to BG 11 medium enhanced carotenoid content of the *D. subspicatus* by about 3.7 fold (3.02±0.002 mgg^{-1} cell) and 3.0 fold (3.86±0.001 mgg^{-1} cell) using glucose or glycerol as organic carbon sources, respectively. Also, lipid productivity was enhanced by about 2.0 (0.07817±0.0028 $gL^{-1}day^{-1}$) (0.265±0.006 gg^{-1} cell) and 2.5 (0.08426±0.0008 $gL^{-1}day^{-1}$) (0.285± 0.02 gg^{-1} cell) fold. Fed-batch cultivation with glucose at a feed rate of 0.5 gL^{-1} per day enhanced both carotenoid and lipid productivities of *D. subspicatus* by 34% and 45%, respectively, after eight days of mixotrophic cultivation.

Keywords: Lipid Productivity, *Desmodesmus subspicatus*, Fed-batch Culture, Mixotrophic Culture, Carotenoid

INTRODUCTION

Microalgae green energy is increasingly gaining global attention due to its potential as an alternative bio-fuel resource that can replace or complement fossil fuels. Rapid and sustainable-promising advancements have been recorded in the literature in pursuance of commercial production of the desired green energy by microalgae [7,8,32,33]. However, cost intensiveness has remained an outstanding limitation to a sustainable production of biofuel by microalgae [2,35,41]. Efforts have been made to cut down on the cost of producing biofuel by microalgae through several approaches. 1. Cultivating microalgae using wastewater [21,41]. This approach reduces cost by avoiding the use of potable water and sharing the cost of production with the cost of treatment of the wastewater. 2. Enhancing the biomass/product productivity of microalgae by different techniques [8,33]. This approach reduces the cost of biodiesel production by reducing the cost of harvesting the algae biomass/product of interest [3]. 3. Co-production of biofuels and value-added metabolites of great commercial value (bio refinery concept) [2,42]. The third approach is probably the most promising because microalgae are known to accumulate other value-added products such as protein, starch, and carotenoids which have wide applications in food, feed, textile, health and pharmaceutical industries [2,42]. The increased global demand and market value for carotenoids due to rapid advancement in the knowledge of the biological roles of carotenoids in humans and animals [11,25,34] confers higher commercial value on carotenoids than other value-added products by microalgae. Consequently, co-production of bio lipid and carotenoids holds a high prospect for sustainable commercial production of biofuel. However, this prospect has not been actualized, probably because research so far on bio refinery concept by microalgae has focused on the use of cultivation strategies such as stress-dependent single phase or biphasic cultivation for accumulation of lipid and/carotenoids [19,24,31]. Unfortunately, the stress factors such as nutrient starvation, high light intensity/temperature/salinity have the disadvantage of low cell growth rate/biomass [31,36], which increases the cost of production by increased cost of downstream processing. Furthermore, the cost of energy for high light intensity or high temperature induction of lipid and/carotenoids [22,24] adds to the cost of production. The biphasic cultivation strategy which involves nutrient-replete and nutrient-limited stages apparently was reported to solve the problem of low biomass associated with stress-dependent single phase strategy [36]. However, the high cost of energy required for the transfer of culture from one phase to another and maintain culture sterility remains the bottle-neck against its cost effectiveness. Thus, the need for cost-effective cultivation strategy for simultaneous accumulation of lipid and carotenoids in microalgae towards sustainable biofuel production. Interestingly, Mixotrophy (a single stage cultivation strategy) has been reported to possess the metabolic advantages of both autotrophy and heterotrophy [9], which characterize the biphasic strategy. Mixotrophy also requires relatively low light intensity, which results in lower energy cost for the production of photosynthetic accessory pigments such as primary carotenoids [23]. The primary carotenoids (e.g., Lutein) are known to accumulate with biomass growth without stress [23] as against secondary carotenoids (e.g., Astaxan-

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thin) which have stress-stimulated accumulation in microalgae [22]. It is therefore necessary to explore the potential of single stage nutrient-replete mixotrophy as a better alternative to the double-stage hetero/mixo-phototrophic cultivation. In literature, there are scant reports on nutrient replete technique as a strategy for cost effective simultaneous production of lipid and carotenoids in microalgae under mixotrophic condition. In the present study, we investigated the effect of different nitrogen sources on simultaneous accumulation of lipid and carotenoid in *Desmodesmus subspicatus* using glucose or glycerol as carbon sources in BG11 medium under mixotrophic condition. The effects of urea nitrogen supplementation at optimized concentration to sodium nitrate on enhanced simultaneous lipid and carotenoid productivity by *D. subspicatus* were also investigated. The effect of fed batch cultivation using different glucose feed rates on the simultaneous enhancement of lipid and carotenoid accumulation under mixotrophic condition was also investigated.

MATERIALS AND METHODS

1. Microalgae Identification

The microalgae *Desmodesmus subspicatus*, which was isolated from Maiduguri, Nigeria (LC172266) was obtained from the Department of Microbiology, University of Nigeria, Nsukka. The isolate was identified as reported by [8].

2. Inoculum Preparation

The microalgae strain was subcultured in the same laboratory using BG 11 growth medium. The inoculum was prepared by transferring 10% stock culture into a 500 mL Erlenmeyer flask containing 200 mL BG 11 medium. It was incubated under continuous light illumination (50 $\mu mol \cdot m^{-2}s^{-1}$) in a rotary shaker (100 rpm and 30 °C) for ten days.

3. Preparation of Culture Medium

The growth medium used was BG 11 which was composed of (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; $C_6H_8O_7 \cdot nFe \cdot nNH_3$, 0.006; EDTA, 0.001; $NaCO_3$, 0.02; urea, 0.2; and 1.0 mL A5+Co stock solution. The A5+Co stock solution was prepared by dissolving H_3BO_3, 2.860 g; $ZnSO_4 \cdot 7H_2O$, 0.222 g; $MnCl_2 \cdot 4H_2O$, 1.81 g; $CuSO_4 \cdot 5H_2O$, 0.079 g; $Na_2MoO_4 \cdot 2H_2O$, 0.390 g and $Co(NO_3)_2 \cdot 6H_2O$, 0.0494 g; in one liter of distilled water. The pH of the culture medium was adjusted to 7.2.

4. Cultivation of Microalgae in Batch Flasks.

BG 11 medium (200 mL) containing either 5.0 gL^{-1} glucose or glycerol as the carbon source in 500 mL Erlenmeyer flasks covered with foams stuck with rubber corks for ventilation were sterilized at 121 °C for 15 min. The seed culture (10%) was inoculated in the sterile growth medium in triplicate and the algal cultures were incubated at 30 °C in a rotary shaker at the speed of 100 rpm under 50 $\mu mol \cdot m^{-2}s^{-1}$ continuous light illumination for eight days. The microalgae culture (5.0 mL) was sampled at two-day intervals to measure the cell concentrations and carotenoid content. At the end of the eight days, 5.0 mL of the culture was centrifuged at 5,000 rpm for 5 min, and the cell pellet was dried in an oven at 70 °C for 24 h. The total oil content and biomass concentration were determined.

4-1. Effect of Nitrogen Sources on Cell Growth and Lipid Production

BG 11 medium (200 mL), containing 0.25 gL^{-1} of either urea, yeast extract, ammonium nitrate or sodium nitrate and 5.0 gL^{-1} glucose in 500 mL Erlenmeyer flasks were seeded with 10% inoculum. The algal cultures were replicated three times and incubated as described before. The microalgae cultivation lasted for eight days during which the cultures were sampled at two-day intervals to measure the cell growth. The cells were harvested after cultivation and dried as described before. The total oil content and biomass concentrations were analyzed.

4-2. Optimization of Urea Nitrogen Source Supplement

Urea was optimized in 200 mL of BG 11 medium (pH=7.2) containing 5.0 gL^{-1} of glucose or glycerol and either 0.1, 0.2, 0.5 or 1.0 gL^{-1} of Urea in 500 mL Erlenmeyer flasks. The algal cultures were replicated three times and seeded with the 10% inoculum. The microalgae cultures were incubated and sampled as described before to measure the cell growth and carotenoid contents. The cells were harvested after cultivation and dried at 70 °C for 24 h. The total oil content and biomass concentration were analyzed.

5. Cultivation of Microalgae in Fed Batch for Improved Lipid and Carotenoid Productivities

Erlenmeyer flasks (500 mL) containing 200 mL of BG11 and 0.2 gL^{-1} of Urea were used for the cultures in three replicates. To each of the replicates, 0.2 gL^{-1}, 0.5 gL^{-1} or 1.0 gL^{-1} of glucose was added on daily basis. The experiment was set up in a rotary shaker as described in section 2.4 above under mixotrophic condition. The cultures were sampled at two-day intervals for the measurement of cell growth and carotenoid contents.

6. Analytical methods

6-1. Cell Growth Rate

The cell concentration was determined by measuring the optical density (OD680) using a UV/VIS spectrophotometer (GENESYS 10S UV-Vis, Thermo Fisher Scientific, MA, USA). The specific growth rates μ (day^{-1}) were calculated as $1/t \times \ln(X_m/X_0)$, where X_m and X_0 (g L^{-1}) were the cell concentrations on day 8 and day 4, respectively, and t (day) was the time between the two measurements (four days).

6-2. Measurement of Lipid Concentrations

A known dry weight of *D. subspicatus* was weighed out in a mortar and pulverized mechanically using a pestle. The method of Bligh and Dyer [12] was used to determine the total lipid content.

6-3. Determination of Carotenoid Content

An aliquot (5.0 mL) of culture broth was sampled and centrifuged at 5,000 ×g for 5 min and rinsed twice with distilled water. The pellet was extracted with 5.0 mL 90% (v/v) methanol and centrifuged at 5,000 ×g for 5 min. The carotenoid content of the supernatant was determined by UV-VIS spectrometer using the following equation [16]:

$$\text{Total carotenoid (mg } L^{-1}) = (1000A470 - 44.76A666/221)$$

6-4. Statistical Analysis

All the experiments were performed in three replicates (n=3). Analysis of Variance (single classification) was used to test for significance differences. Least significance difference (LSD) was used to separate the means. The results were expressed or plotted as means±S.E.

RESULTS

1. Cultivation of *D. subspicatus* with Different Nitrogen Sources

Urea gave the highest cell concentration of 2.65 gL^{-1} and the least lipid content of 0.14 gg^{-1} cell, while sodium nitrate gave the highest lipid content of 0.26 gg^{-1} cell and a comparable cell concentration (1.503 gL^{-1}) with ammonium nitrate and yeast extract (Fig. 1). However, the lipid productivity obtained with urea (0.041 $gL^{-1}day^{-1}$) compared favorably with the value obtained by sodium nitrate (0.039 $gL^{-1}day^{-1}$). The lipid productivity of these nitrogen sources can be ranked as (Urea=sodium nitrate)>(yeast extract)>(Ammonium nitrate). Hence, urea was selected for optimization as a supplement to Sodium nitrate (0.25 gL^{-1}) in BG 11 medium using glucose or glycerol as the carbon source. The glucose concentration was exhausted within four days in the culture medium containing urea and within eight days in the medium containing sodium nitrate and ammonium nitrate (Fig. 2). However, the culture containing yeast extract had about 1.7±0.1 gL^{-1} residual glucose after eight days of cultivation. The different nitrogen sources impacted differently on the pH of culture medium. The pH of culture medium containing ammonium nitrate decreased to 6.9, while the culture medium containing urea decreased to 6.68 on the 8th day of cultivation (Figure not shown). The pH of culture medium containing yeast extract decreased to 5.04, while the one of sodium nitrate culture medium maintained about the same pH at the end of cultivation (figure not shown). The maximum total carotenoid content obtained with urea was 3.15 mgg^{-1} cell, which is about 3.8-fold higher than the value obtained with sodium nitrate (0.82 mgg^{-1} cell). Yeast extract elicited the least carotenoid content of 0.46 mgg^{-1} cell as shown in Fig. 1.

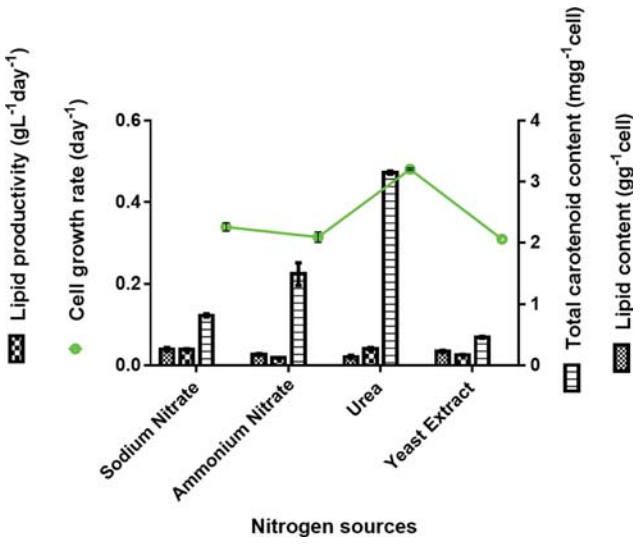

Fig. 1. Effect of nitrogen sources (0.25 gL^{-1}) on cell growth rate, lipid productivity, lipids and total carotenoid contents of *D. subspicatus* under mixotrophic condition. The initial glucose concentration was 5.0 gL^{-1}.

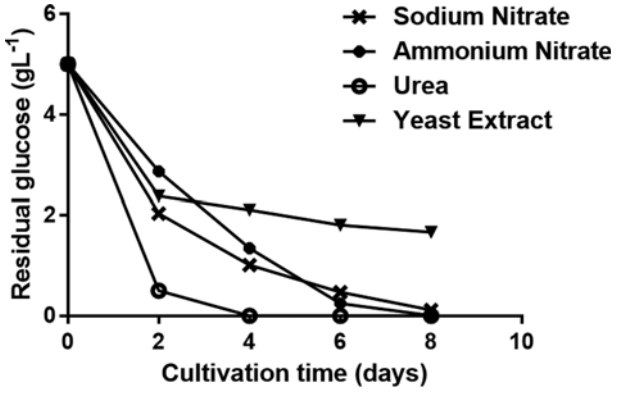

Fig. 2. Effect of nitrogen sources (0.25 gL^{-1}) on glucose utilization by *D. subspicatus* under mixotrophic condition. The initial glucose concentration was 5.0 gL^{-1}.

2. Optimization of Urea Concentration

Supplement of Urea (0.2 gL^{-1}) was the optimum for cell growth

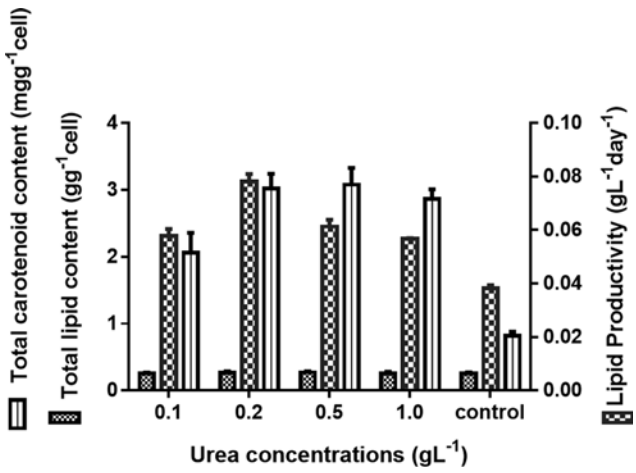

Fig. 3. Effect of urea concentration in BG 11 medium on lipid productivity and carotenoid content of *D. subspicatus* using glucose (5.0 gL^{-1}) as the carbon source under mixotrophic condition.

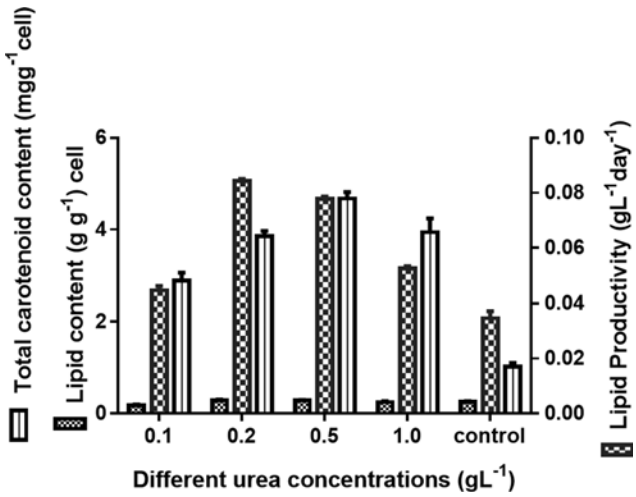

Fig. 4. Effect of urea concentration in BG 11 medium on lipid productivity and carotenoid content of *D. subspicatus* using glycerol (5.0 gL^{-1}) as the carbon source under mixotrophic condition.

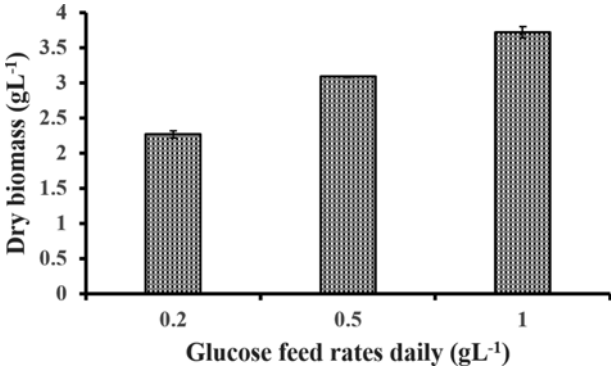

Fig. 5. Effect of glucose feed rates on *D. subspicatus* cell concentrations after eight days of cultivation in a medium containing 0.2 gL^{-1} of urea under mixotrophic condition.

Fig. 6. Effect of glucose feed rates on lipid and carotenoid contents of *D. subspicatus* after eight days of cultivation in a medium containing with 0.2 gL^{-1} of urea under mixotrophic condition.

rates and lipid productivity of *D. subspicatus* using glucose or glycerol as the carbon source (Figs. 3 and 4). The lipid productivity obtained with 0.2 gL^{-1} urea supplement was 100% (0.0782 $gL^{-1}day^{-1}$) and 125% (0.0843 $gL^{-1}day^{-1}$) more than the control (0.0383 $gL^{-1}day^{-1}$) (0.0346 $gL^{-1}day^{-1}$) (without urea supplement) using glucose or glycerol as the carbon source, respectively. The lipid productivity in media containing different urea concentrations can be ranked as 0.2 gL^{-1}>0.5 gL^{-1}>1.0 gL^{-1}>0.1 gL^{-1}>control. However, the urea concentration of 0.5 gL^{-1} was more productive in terms of carotenoid content than the control or other urea concentrations tested. Glucose supported higher cell growth rates, while glycerol supported higher carotenoid contents with all the concentrations of urea tested (Figs. 3 and 4). However, the biomass and lipid content obtained with either glycerol or glucose varied depending on the concentrations of urea administered (Figs. 3 and 4).

3. Fed-batch Cultivation

It was observed that a daily addition of 1.0 gL^{-1} and 0.5 gL^{-1} glucose yielded comparable lipid productivity of 0.133±0.004 $gL^{-1}day^{-1}$ and 0.143±0.0003 $gL^{-1}day^{-1}$, respectively (Fig. 6). However, there were significant ($p<0.05$) differences in the algal biomass

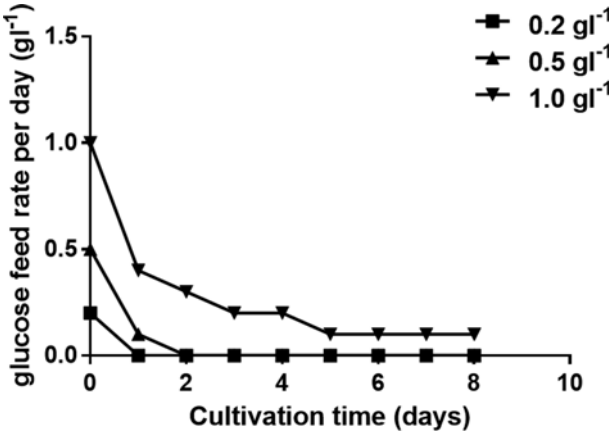

Fig. 7. Effect of glucose feed rates on glucose utilization by *D. subspicatus* under mixotrophic condition.

(Fig. 5) and lipid content (Fig. 6) obtained with the two glucose feed rates after eight days of cultivation. The daily addition of 0.5 gL^{-1} glucose was selected with a total of 3.50 gL^{-1} glucose consumed at the end of the eight-day fed batch cultivation. The total carotenoid content obtained by daily addition of 0.2 gL^{-1} (3.65 mgg^{-1} cell) or 0.5 gL^{-1} (3.5 mgg^{-1} cell) glucose concentrations was significantly higher ($p<0.05$) than the value obtained by daily addition of 1.0 gL^{-1} glucose (2.3 mgg^{-1} cell) (Fig. 6).

There was no residual glucose in the algal cultures receiving 0.2 gL^{-1} and 0.5 gL^{-1} glucose feed rates per day from the second day until the eighth day of cultivation (Fig. 7). The algal culture with 1.0 gL^{-1} glucose feed rate per day contained residual glucose until the eighth day of cultivation.

DISCUSSION

1. Effect of Different Nitrogen Sources on *Desmodesmus subspicatus*

Nitrogen in various forms such as nitrate, ammonium, peptone, yeast extract or urea has been reported as one of the most important macronutrients in culture medium that affect growth and intracellular metabolite accumulation in various microalgae species [6, 20]. In the present report, different nitrogen sources elicited different metabolic responses in *D. subspicatus* which may be due to differences in assimilating different nitrogen sources by the same microalgae species [20,43]. The high biomass/low lipid content by urea-N and high lipid content by Sodium nitrate-N as reported in the present work is consistent with some reports on *Chlorella vulgaris* [10] and *Scenedesmus dimorphus* [27]. Although Kumar et al. [10] reported that nitrate was the best nitrogen source for both high biomass and lipid productivity in *Chlorella vulgaris* after 14-day cultivation period, Minyuk et al. [14] reported urea-fed cultures of *Chromochloris zofigiensis* with lipid content significantly higher than that of nitrate-fed cultures for 16-day cultivation. The high carotenoid induction by urea in the present report is consistent with the report on *Chromochloris zofigiensis* [14]. However, in *Haematococcus pluvialis*, and *Chlorella vulgaris*, nitrate was reportedly the best nitrogen source for carotenoid accumulation com-

pared with urea and other nitrogen sources [10]. By and large, nitrogen sources in the nutrient medium obviously have specie-specific effect on the biomass and intracellular metabolites of microalgae species [15,20]. The high cell growth rate and low lipid accumulation of *D. subspicatus* with urea (Fig. 1) can be attributed to high organic carbon (glucose) assimilation (Fig. 2) at the expense of lipid synthesis [41]. According to some researchers, urea dissociates to form CO_2 and ammonium in solution via the urea amido hydrolase pathway (urease enzyme), thus providing nitrogen and carbon nutrients at the same time [14,37] to support the growth of microalgae species. Li et al. [40] believed the higher support to cell growth by urea when compared to NH_4Cl and NH_4NO_3 during heterotrophic cultivation of *Chlorella sorokiniana* was related to decrease in pH due to ammonium assimilation. The poor support to microalgal growth by NH_4Cl and NH_4NO_3 as reported by Li et al. [40] also correlates with the present report since NH_4NO_3 proved to support the least cell growth (Fig. 1) though under mixotrophic culture condition. According to some reports, urea is the preferred nitrogen source for large scale microalgal cultivation because it is less expensive as compared to other N sources [14]. Although the type of carotenoid was not determined in the present work, the increased content at increased biomass by *D. subspicatus* under continuous low light intensity in the present work may suggest that they are growth-dependent/primary carotenoids [23,47]. The positive commercial implication is that of increased carotenoid productivity due to increased biomass content as against low biomass content associated with carotenoid production under stress [31,36]. A two-stage (biphasic) cultivation strategy has been reported as a break-through from low cell biomass under nutrient starvation cultivation [13,14,41], for production of lipids and/carotenoids by microalgae. Caprio et al. [13] reported a biomass concentration of about 1.6 gL^{-1} by *Scenedesmus* sp. after twenty-two days of cultivation and lipid content of over 50% after twenty-nine days of cultivation in a two-stage phototrophic/heterotrophic (Olive Mill waste water) cultivation under constant illumination (24 h) with 80± 10 $\mu E\ m^{-2}\ s^{-1}$. The results in terms of biomass production are lower than the current report on single strategy (3.09 gL^{-1}), although the differences in the organic carbon sources, microalgae species as well as light intensity may be contributory factors. Minyuk et al. [14] reported dry weight biomass of 1.97 gL^{-1} and 1.57 gL^{-1} by *Chromochloris zofigiensis* using sodium nitrate and urea nitrogen sources, respectively, after 32 days of two-stage cultivation. This report, though very recent, was also far below the present report probably due to differences in the microalgae species and some culture conditions. Yen and Chang [17] in a two-stage phototrophic/mixotrophic fed-batch (glucose or glycerol) cultivation strategy recorded a biomass of 7.4 gL^{-1} by *C. vulgaris*, which is higher than the current report under single stage strategy. However, the cost implications of lengthy cultivation period (1st stage plus 2nd stage), the cost of changing from one stage of cultivation to another as well as the cost of supply of high light intensity stress still remain cost intensive hurdles which so far have not been justified by the increased or sustained biomass concentration achieved by the biphasic strategy.

2. Effect of Urea Supplementation on *Desmodesmus subspicatus*

Nitrogen supplementation has the capacity to change cell metabolism significantly and contributes to lipid synthesis [27]. The supplementation of urea to some medium for microalgae cultivation has been reported with varying optimal supplements of 1.5 gL^{-1} urea to BG II medium [26] and 0.2 gL^{-1} urea to Beneck's medium [4]. The difference in the reports could be attributed to the difference in the strains of the microalgae or basal nitrogen sources in the media used. However, the present results correlate with the report by Wijanarko [4] (0.2 gL^{-1} urea optimal supplement) though the basal nitrogen source in Beneck's medium is sodium nitrite against sodium nitrate in BG 11 medium used in the present report. The decreased biomass content by higher urea supplement (0.5 gL^{-1} and 1.0 gL^{-1}) (figure not shown) which correlates with the report by Wijanarko [4] was probably due to excess of intracellular ammonium, which inhibits the formation of Adenosine triphosphate (ATP) in the chloroplast following substrate activation leading to growth inhibition [4]. However, this may be specie or basal medium-specific [26]. The use of urea and another nitrogen source as a cultivation strategy has been reported to increase chlorophyll, protein and lipid content in *Chlamydomonas reinhardtii* [1] and achieve maximal lutein production in *Auxenochlorella protothecoides* though under heterotrophic condition [44]. The reason for the improved productivity by two nitrogen sources in the present work is not clear, although there are reports of nitrogen nutrient replete conditions enhancing lipid and carotenoid productivities on the basis of increased cell biomass [24]. However, it does appear that the rapid urea assimilation, which is preferred to sodium nitrate by some microalgae species [27] channeled the metabolic flux of the microalgae to biomass and carotenoid accumulation while sodium nitrate which was assimilated later because of its oxidized nature [38], resulted in lipid accumulation [20]. Furthermore, Zhu et al. [27] reported an increased biomass up to 3.0-3.5 gL^{-1} and algal lipid accumulation reaching 25.6% of dry weight by co-cultivation of *Chlorella vulgaris* and *Scenedesmus dimorphus* in media with three nitrogen sources for ten days. The high biomass increase was attributed to the capacity of microalgae to assimilate not only inorganic nitrogen (nitrate and ammonium) but also organic urea [27]. However, lipid productivity achieved was lower than what was achieved by monoculture, double nitrogen source in the present report. The variation may be due to differences in microalgae species, concentrations of nitrogen sources in the media or type of media used. Some authors even reported that urea alone has the potential to increase both the lipid and carotenoid contents because of its dual resource for carbon and nitrogen [39].

3. Improvement of Lipid and Carotenoid Productivity by Glucose Fed Batch Cultivation of *D. subspicatus*

There was enhancement in lipid productivity (0.11235 $gL^{-1}day^{-1}$ to 0.1429 $gL^{-1}day^{-1}$) amidst slight decrease in biomass concentration (3.2 gL^{-1} to 3.09 gL^{-1}) of *D. subspicatus* by daily addition of 0.5 gL^{-1} glucose (fed-batch mode) when compared with batch culture containing 10.0 gL^{-1} glucose (optimum) (batch mode) (data not shown) for the same period of cultivation. This agrees with Chirslip and Torpee [5] who also reported that glucose concentration at low level in fed-batch cultivation gave a slightly lower cell dry weight for marine *Chlorella* sp. and *Nannochloropsis* sp., and enhanced lipid production when compared to batch cultivation. Xie et al. [47] also reported lower biomass productivity by *Chlo-*

rella sorokiniana FZU60 during eight days Na-acetate fed-batch compared to batch culture, and which was attributed to decreased light penetration with increasing biomass concentration, which decreased the inorganic carbon metabolism even when the organic carbon metabolism was active [46]. However, Chen et al. [23] reported that a five day feeding interval of nutrient medium increased microalgal biomass concentration of *Chlorella sorokiniana* MB-1-M12 from 3.07 gL^{-1} (batch cultivation) to 6.84 gL^{-1} (fed-batch). The variation in the effect of fed-batch process on the microalgae may be due to differences in the strains as well as the type or composition of feed. Fed batch process undoubtedly leads to a better control of nutrient concentration (glucose addition) [47], thereby making the process cost effective and more economically viable. For instance, in the present study, lower glucose concentration (3.5 gL^{-1} in fed-batch against 10 gL^{-1} in batch) was consumed with resultant increased productivity. The carbon-to-nitrogen ratio of the culture medium may have influenced the simultaneous accumulation of lipid and carotenoid in the fed-batch cultivation as observed by Braunwald et al. [39] in batch cultivation of *Rhodotorula glutinis.* The researchers asserted that there is no "head or tail" relationship between the synthesis of the two pathways. However, the combination of urea and sodium nitrate may have increased the nitrogen concentration in the medium, which has been reported to enhance carotenoid (Lutein) content in *Desmodesmus* sp. [48], *Chlorella sorokiniana* [6], carotenoid and biomass yield in *Trentepholia arborum* [8] and, in addition to ammonium, improved lipid productivity in mixed culture of *C. vulgaris* and *S. dimorphus* [27]. Also, the two nitrogen sources may have played individual roles in terms of carotenoid and lipid productivity. For instance, urea as demonstrated in this study favored biomass and carotenoid accumulation while sodium nitrate favored lipid content accumulation (Fig. 1). The carotenoid content, as the light-harvesting complex [39], could have increased in size/concentration to enhance light absorption for photosynthesis since organic carbon source (glucose) was exhausted in the fed-batch culture medium (Fig. 7). Furthermore, fed-batch cultivation under a nitrogen nutrient-replete condition may have created a cyclic mixotrophic-phototrophic condition (Mixotrophic when glucose was added and photoautotrophic when glucose was exhausted prior to addition of glucose to the culture medium the next day) that triggered the simultaneous accumulation of lipid and carotenoid content of the microalgae. Fed-batch strategy has been reported to enhance solely lutein content in *Chlorella sorokiniana* MB-1-M12 [23] and *Chlorella sorokiniana* FZU60 [47] up to 6.30 mgg^{-1} and 9.25 mgg^{-1} respectively. These were outstanding carotenoid accumulations, but the experiment lacked bio refinery concept since there was no record of simultaneous accumulation of either lipid or any other intracellular content in both studies. And this makes it difficult for comparison with the present result. In addition, *Chlorella sorokiniana* MB-1-M12 was an improved strain obtained by random mutagenesis of the native strain *Chlorella sorokiniana* MB-1. More importantly, both high carotenoid con-

Table 1. Comparison of carotenoids produced by microalgae reported by various workers

Microalgae species	Carotenoid content (mgg^{-1})	Cell biomass (gL^{-1})	Culture conditions	Reference
Desmodesmus subspicatus LC172266	3.65	3.09	BG II medium, 0.2 gL^{-1} Urea-N supplement, 0.5 gL^{-1} glucose fed-batch daily, 30 °C; light: 50 μmol·$m^{-2}s^{-1}$, 8 d.	Present study
Ostreococcus lucimarinus	431.1 μgL^{-1}	ND	Artificial seawater medium, trace elements, vitamins; 12-h light/12-h dark cycle; light: 150 μmol $m^{-2}s^{-1}$, salinity: 15 gL^{-1} NaCl. 7 d.	[22]
C. zofingiensis	0.113 mgL^{-1} day^{-1}	1.57±0.01	BBM medium, Biphasic: photo-mixotrophy: (1st stage; nutrient replete, 120 μmol m^{-2} s^{-1}, 20±1 °C, 17 d. 2nd stage; 50 mmol L^{-1} sodium acetate, light: 240 μmol PAR quanta m^{-2} s^{-1}, 24-25 °C, 15 d.	[14]
Isochrysis galbana	1.21 mgL^{-1}	1.55×10^{7} cells mL^{-1}	Walne's medium, 72 mgL^{-1} $NaNO_3$-N, 25 °C, 25 gL^{-1} salinity, 12 : 12 h light : dark cycle, light: intensity of 80 mmol photonsm^{-2}·s^{-1} 13 d.	[28]
Nannochloropsis oceanica CCALA 978	$1{,}100\times10^{-3}$ μg mL^{-1}	980 mgL^{-1}	Seawater, f/2 nutrients, 25 °C 16 : 8-h light-dark photoperiod, light: 100 μmol photons m^{-2} s^{-1}, two stage (Nitrogen, Phosphorus stress) 21 d.	[31]
Desmodesmus subspicatus KX235325	4.6 mgg^{-1}	2.72	BBM, NPK fertilizer supplemented, light: 39 μmol m^{-2} s^{-1}, 12 : 12 h light and dark cycle, 45 d.	[18]
Chlorella sorokiniana MB-1-M12	6.30 mgg^{-1}	2.6	BG-11 medium, 6 gL^{-1} sodium acetate, fresh medium fed-batch, light: 150 μmol·m^{-2}·s^{-1}, 28 °C 15-21 d.	[23]
Scenedesmus quadricauda (FACHB-1297)	1.75-2.0 mgL^{-1}	1.03	BG11 medium, 25±1 °C light: 60 μmol m^{-2} s^{-1}, 4.0 gL^{-1} xylose, 14 d.	[30]

tent reported were products of modified sodium acetate fed-batch cultivations. For instance, Chen et al. [23] fed a 100-fold concentrated fresh BG11 medium containing 6.0 gL^{-1} sodium acetate into the microalgal culture at designated time intervals. Xie et al. [47] pulse-fed sodium acetate into the culture at different time intervals and supplemented concentrated BG11 medium to the microalgal culture to provide sufficient nutrients when 90% of the initially available nitrate had been consumed. It is noteworthy that acetate hydrolysis forms acetic acid, which affects cell membrane integrity and needs to be used with membrane protectant [45]. Minyuk et al. [14] also suggested that acetate should be used with caution. The carotenoid production by *D. subspicatus* in the present work is higher than the values reported by some researchers and lower or compares favorably with other reports by microalgae species (Table 1).

In conclusion, *Desmodesmus subspicatus* LC172266 has the potential to utilize both inorganic (sodium nitrate) and organic (urea) nitrogen sources in a single stage culture for enhanced productivity. Nitrogen-nutrient replete mixotrophic fed-batch condition at low light intensity was effective in inducing simultaneous accumulation of carotenoid and biodiesel oil by the microalga strain. Under the culture conditions of the experiment, *D. subspicatus* is presented with the potential for cost-effective and sustainable commercial production of biofuel.

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