Full Length Article

Lipid production under a nutrient-sufficient condition outperforms starvation conditions due to a natural polarization of lipid content in algal biofilm

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ABSTRACT

Attached algae cultivation for biofuel production has received proliferated attention given its advantage to reduce harvesting/dewatering costs and contamination risk. In this study, the effects of various nutrient starvation strategies on lipid production through the attached cultivation of *Ettlia* sp. YC001 was investigated. A detailed study on compositional variation over the depth of biofilms was followed to understand the phenomena within the biofilms under various nutrient conditions. As a result, the nutrient starvation strategy enhanced the lipid content of biofilms by 3–7 %, however, it limited biomass growth significantly. Ultimately, lipid production with sufficient nutrient supply outperformed those with nutrient starvations, reaching up to the maximum lipid productivity of 6.5 ± 0.1 g/m^2/day. Compositional analyses over the depth of biofilms identified that high lipid accumulation was induced in the top layer of the biofilm regardless of medium condition as the top layers were naturally under stress from nutrient deficiency and high light exposure.

1. Introduction

Lipids in microalgae are promising resources that can be converted into biofuels, bioplastics, and other valuable biochemicals to sustainably replace petroleum-based products [1]. Despite their potential, the current microalgal lipids production system has several mechanical and biological hurdles to meet its economic feasibility [2,3]. One of the biological hurdles is that high lipid accumulation in the cell body negatively affects cell growth, thus often requiring a two-step process to achieve high lipid productivity: cell growth and lipid accumulation [2]. Stressful condition of key environmental factors for algae such as salinity, pH, temperature, light intensity, and nutrients is known to induce triacylglycerol (TAG) synthesis and accumulate TAG in dense lipid bodies located in the cytoplasm [4]. Among stressful conditions, nutrient starvation, typically of nitrogen and phosphorous, is known to be the most effective strategy used in the two-step process to induce lipids accumulation in microalgal cell bodies after achieving high cell

growth [5–7]. Lipid productivity, however, is not effectively improved as the nutrient starvation process sacrifices biomass productivity [2]. Furthermore, as nutrient-depleted condition diminishes the photosynthetic activity and viability of algal cells, the culture could face a higher risk of contamination.

The attached cultivation has been receiving increasing attention, especially for biofuel production, as it has several advantages over the conventional suspended cultivation including reduced harvesting/dewatering process cost and risk of contamination. Those advantages come from its unique cultivation mechanism, which accommodates algal cells to grow as a biofilm attached to a porous hydrophilic substrate. The substrate separates the liquid layer from biofilm while allowing water and nutrient to diffuse into biofilm, maintaining it hydrated. Whilst the attached cultivation system certainly has great potential for economic lipids production, the effect of the two-step nutrient starvation strategy on lipid productivity for the attached cultivation are narrowly studied [8]. Furthermore, a review study on lipid production

through the attached algae cultivation reported that studies on nutrient starvation in the attached cultivation system had different results primarily due to a lack of understanding of the exact phenomena within the biofilm in response to the nutrient starvation [7].

Since diffusion is the main mechanism of medium supply to the biofilm, the nutrient concentration gradient within the biofilm is crucial as it is one of the driving forces of diffusivity. The nutrient concentration gradient within the biofilm provides an uneven environment through the layers of algal cells, thus the characteristics of algal cells with respect to the depth of biofilm must be thoroughly studied, especially during the nutrient alteration processes. In the previous studies on astaxanthin production through the attached cultivation of *Haematococcus pluvialis*, a higher concentration of astaxanthin in the top layer of the biofilms was observed presumably due to its longer exposure to the light and carbon dioxide [9,10]. Although the change in composition over the depth of algal biofilm has been continuously reported, no study specifically focused on change in lipid contents and other pigments over the depth of algal biofilm has yet been reported. Along with the nutrient starvation experiment, a thorough investigation of the compositional gradient within the algal biofilm produced for lipids production is, therefore, necessary.

In the present study, the effects of various nutrient starvation strategies on lipid productivity of attached cultivation of *Ettlia* sp. YC001 was comprehensively investigated. To elucidate the effect of nutrient starvation on algal biofilm, compositional variations over the depth of algal biofilms were analyzed. Through this study, potentials to improve lipid productivity of the attached cultivation of *Ettlia* sp. for economically feasible biofuel production were identified.

2. Materials and methods

2.1. Microalgal strain and inoculum preparation

The freshwater green microalgal species, *Ettlia* sp. YC001 (KCTC 12109BP), was obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). *Ettlia* sp. YC001 was maintained on a TAP agar plate for long storage, and suspended in BG-11 media and photoautotrophically cultivated when inoculums were needed for the attached cultivation. The seed culture was prepared in an autoclaved 500 ml bottle with an air inlet (working volume was 400 ml). The continuous light intensity of 200 μE/m^2/s with 2 % CO_2 was used for all seed preparation purposes.

2.2. Media preparation

BG-11 was used as a basis of media used in this study, which consists of 1.5 g/L of $NaNO_3$, 0.23 mM of K_2HPO_4, 0.3 mM of $MgSO_4 \cdot 7H_2O$, 0.24 mM of $CaCl_2 \cdot 2H_2O$, 0.031 mM of Citric Acid$\cdot H_2O$, 0.021 mM of $5NH_4 \cdot Fe2(C_6H_4O_7)$, 0.0027 mM of $Na_2EDTA \cdot 2H_2O$, 0.19 mM of Na_2CO_3, and 1 ml/L of BG-11 trace metals solution of 46 mM of H_3BO_3, 9 mM of $MnCl_2 \cdot 4H_2O$, 0.77 mM of $ZnSO_4 \cdot 7H_2O$, 1.6 mM of $Na_2MoO_4 \cdot 2H_2O$, 0.3 mM of $CuSO_4 \cdot 5H_2O$, and 0.17 mM of $Co(NO_3)_2 \cdot 6H_2O$. $NaNO_3$ and K_2HPO_4 were adjusted for different nitrogen and phosphorus concentration, respectively. The pH of the medium was adjusted in a range of 7–7.5 using 1 N of HCl and NaOH prior to all experiments including inoculum preparation.

2.3. Attached cultivation system and biofilm growth analysis

A culture of *Ettlia* sp. was inoculated on the porous membrane substrate (nitrocellulose membrane, 47 mm diameter, 0.45 um pore size, Milipore) with an inoculum size of 30 mm and a surface density of 2.5 g/m^2. The inoculated membrane substrates were then placed on the source layer, another porous membrane that provides medium through diffusion. The medium was not recirculated in order to keep the nutrient concentration constant throughout the cultivation period and was

Table 1
List of the name of five different medium and their nutrient conditions. The plus symbol (+) indicates a presence of respective chemical at the concentration of normal BG-11 and the minus symbol (−) indicates an absence of respective chemical.

	ANR (All nutrient-replete)	ND (Nitrogen depleted)	PD (Phosphorous depleted)	NPD (N and P depleted)	AND (All nutrient depleted)
NaNO3	+	−	+	−	−
K2HPO4	+	+	−	−	−
	Normal BG-11		N/P modified BG-11		Distilled water

supplied to the source layer periodically at a rate of 1.5 ml every 2.5 min. Experiments were conducted under the optimum condition for lipid production of *Ettlia* sp. YC001 with a light intensity of 500 μE/m^2/s with a 7 % CO_2 concentration, which was found in the previous study [11]. The growth of biofilm was measured daily using the wet weight measurement method developed in the previous study [11]. Three replicates of sample were used to measure biofilm growth and biomass composition analyses. [11].

For the nutrient starvation steps, five different medium conditions: all nutrient-replete (ANR), nitrogen depleted (ND), phosphorus depleted (PD), N and P depleted (NPD), and all nutrient-depleted (AND) medium, were prepared (Table 1). Depleted condition for each N and P indicates no addition of $NaNO_3$ and K_2HPO_4, respectively. The AND media refers to sterilized distilled water without any chemicals added. For the experiment to determine the effect of nitrogen and phosphorous concentrations on biomass growth, nitrate concentrations at 1.8 mM, 5.3 mM, 10.6 mM, 17.7 mM, 53.0 mM and 105.9 mM, and phosphate concentrations at 0.023 mM, 0.069 mM, 0.138 mM, 0.230 mM, 0.689 mM, and 1.378 mM, were used respectively, while concentrations of other chemicals in the medium remained unchanged. The starvation step was scheduled to be introduced on the 4th day of the cultivation where the surface biomass productivity was found to be at the highest [11]. Nutrient-replete BG-11 medium (ANR) determined in this study was supplied for all conditions for the first 4 days, and then the media were changed to four different mediums (ND, PD, NPD, and AND) on day 4 for each respective starvation condition and the starvations were continued until day 12.

2.4. Biofilm sectioning

Biofilm was sectioned into three layers (top, middle, and bottom layer) for the analyses of compositional variation over the depth of biofilm. Harvested biofilms were first frozen at −70 °C and placed on the ice to keep the biofilm frozen while sectioning. Using a micro-blade, biofilms were sectioned into layers by gently scraping from the top of the biofilm. A total of three layers, the top, middle, and the bottom layers were successfully scraped (Fig. 1). Each collected biomass was then rinsed with DW three times and then centrifuged to collect EPS-free biomass before lyophilization. The dry biomass of each layer was measured for their dry weights and then used for further analysis.

2.5. Total lipids and fatty acids composition analyses

Lipids analyses of the harvested biomass were conducted based on the modified Folch method [12,13]. The collected biomass was dewatered by centrifugation and lyophilized at −70 °C for 2 days. Obtained dry biomass was ground using a mortar, weighed, and placed in a 15 ml glass tube for the treatment of chloroform/methanol (2:1, v/v) solution for lipids extraction. Phase separation was achieved by adding water, followed by centrifugation for 10 min. The lower phase was handled with a syringe and transferred into a pre-weighted aluminum dish to evaporate solvents in a fume hood. After the evaporation overnight, the
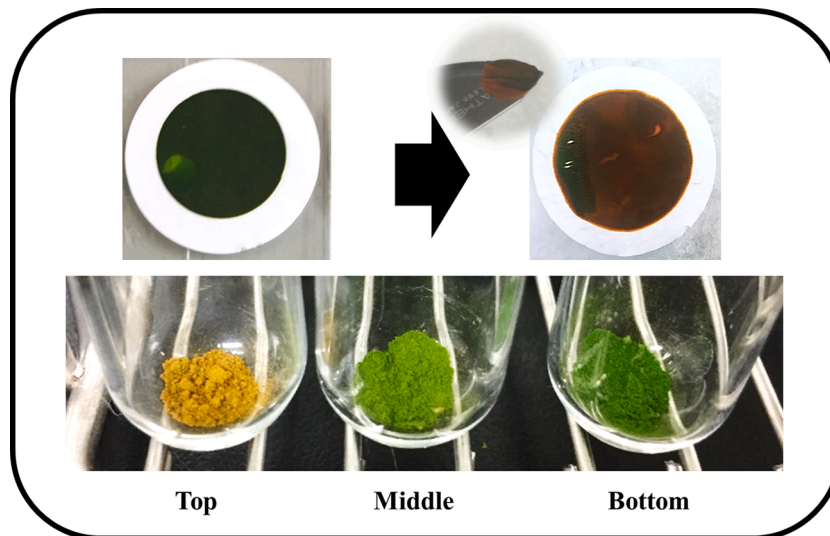
Fig. 1. Pictures showing biofilm sectioned by micro-blade into three different layers: top, middle, and bottom layers. The picture at the bottom shows the successfully isolated biomass of each layer (from left to right, top layer, middle layer, and bottom layer).
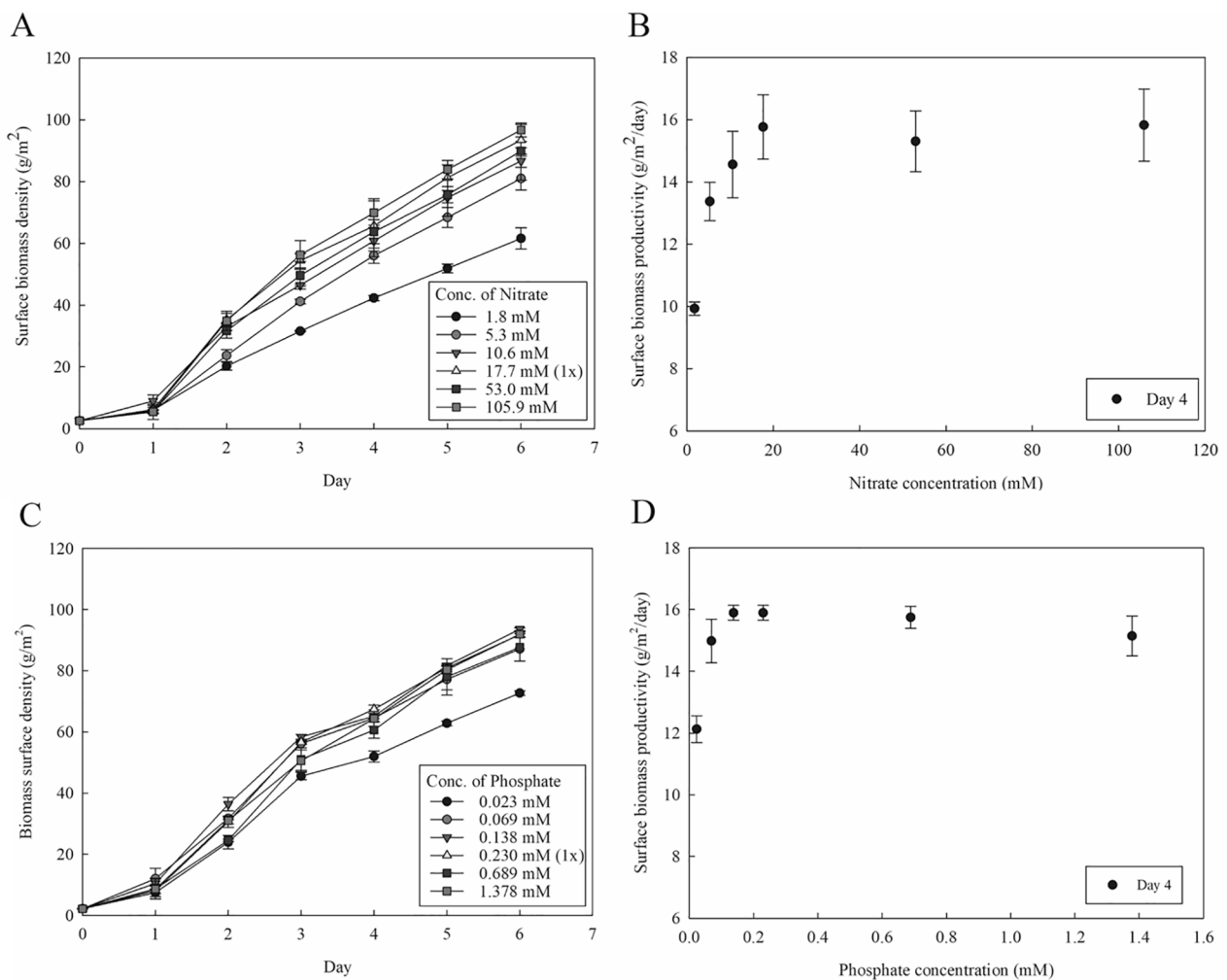

Fig. 2. (A) Effects of nitrate concentration on surface biomass density and (B) surface biomass productivity on day 4. (C) Effects of phosphate concentration on surface biomass density and (D) surface biomass productivity on day 4.

dish was weighed and the weight of total lipids was calculated. An aliquot of the solvent containing lipids was used with a known amount of heptadecanoic acid (C17:0) as an internal standard, and sulfuric acid and additional methanol for the reaction at 100 °C for 20 min to carry out transesterification. The produced fatty acid methyl esters (FAMEs) in the organic phase were filtered into a vial and analyzed using a gas chromatograph (HP6890, Agilent, USA) with a flame ionized detector and an HP-INNOWAX column (30 m × 0.32 mm × 0.5 μm, Agilent, USA). The fatty acid composition was identified and quantified by a comparison of the retention times and peak areas of the internal FAME standards.

2.6. Pigment extraction and analysis by HPLC-UV

Content and composition of photosynthetic pigments were measured using High-Performance Liquid Chromatography (HPLC, Dionex Ultimate 3000, Thermo Fisher Scientific, USA) with 1 Dionex Acclaim™ 120 5um C18 column (Thermo Fisher Scientific, USA). UV–visible detector (Ultimate 3000 VWD Variable Wavelength Detector, Thermo Fisher, Scientific, USA) was used at 440 nm. The column oven temperature of the HPLC was maintained at 25 °C. Two eluents (methanol: 0.5 M ammonium acetate = 8:2 (v/v), methanol:acetone = 7:3 (v/v)) were supplied at the flow rate of 0.8 ml/min for analysis. The ratio of two eluents flowing into the column gradually changed as a pre-set time interval. Each peak of the chromatograph was compared with those of chlorophyll *a*, beta-carotene, violaxanthin, and zeaxanthin (Sigma-Aldrich, USA), and each standard was prepared with a concentration gradient of 0.2/1/5/10/50 ug/ml. Concentrations of each pigment were acquired using calibration curves with individual standards.

3. Results and discussion

3.1. Effects of nitrate and phosphate concentrations on biomass growth

Nutrient uptake from the source water through the membrane into the biofilm is mainly driven by diffusion supported by capillary force and evaporative force [14]. Concentrations of nutrients in the source water, therefore, are key parameters in the attached cultivation system. In this study, surface biomass density and productivity at various concentrations of sodium nitrate (source of nitrogen) and potassium phosphate (source of phosphorous) were compared with a continuous medium supply at a constant nutrient concentration to determine the effect of nutrient concentration on biofilm growth.

Fig. 2 shows the effects of concentrations of nitrate (A and B) and phosphate (C and D) of the media on surface biomass density and surface biomass productivity. As a result, the optimum nitrate and phosphate concentrations were found where biomass productivity no longer increased with a higher nutrient concentration. Concentrations of nitrate were 1.8 mM, 5.3 mM, 10.6 mM, 17.7 mM, 53.0 mM and 105.9 mM, which are 0.1x, 0.3x, 0.6x, 1x, 3x, and 6x of the typical nitrate concentration of BG-11 medium. In Fig. 2A, biomass growth under the nitrate concentration of 1.8 mM showed the least growth with surface biomass density at around 60 g/m² on day 6, whereas biomass growth under higher nitrate concentrations showed substantially better biomass growth with an incremental trend from 80 to 95 g/m² of surface biomass density as nitrate concentration increased from 5.3 mM to 105.9 mM, respectively. In Fig. 2C, the results from biomass growth under different phosphate concentrations showed similar tendencies. Concentrations of phosphate were 0.023 mM, 0.069, 0.138, 0.230, 0.689, and 1.378 mM, which are 0.1×, 0.3×, 0.6×, 1×, 3×, and 6× of the typical phosphate concentration of BG-11 medium. Biomass growth seemed limited under phosphate concentrations of 0.023 mM, which resulted in growth up to 73 g/m² on day 6. Biomass growth under higher phosphate concentrations showed improved biomass growth with surface biomass densities at around 85 to 90 g/m².

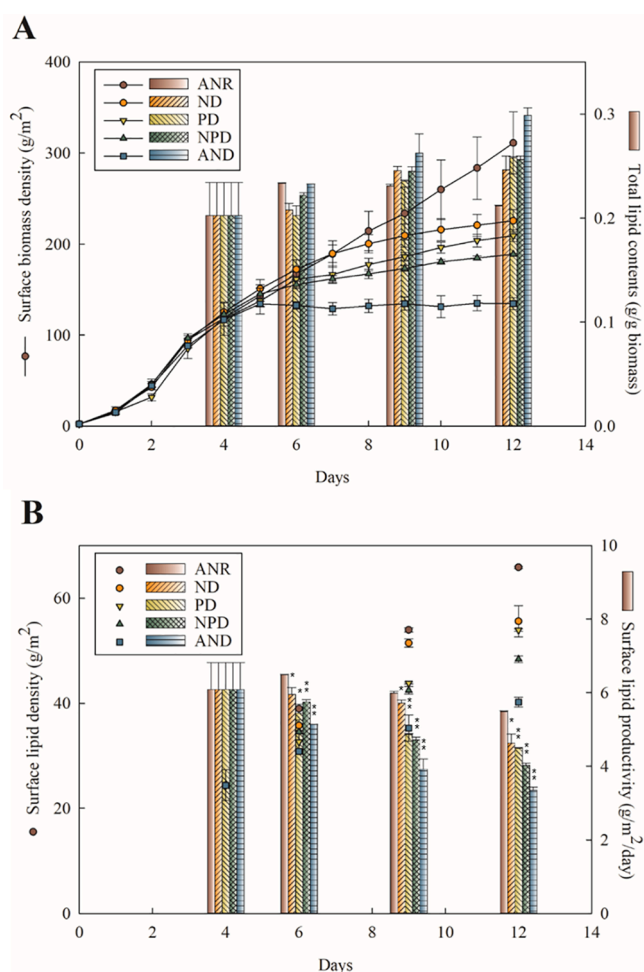Effects of nitrate and phosphate concentrations on the surface

Fig. 3. Effects of various nutrient starvation steps including the ANR (red), ND (orange), PD (yellow), NPD (green), and the AND (blue) on (A) surface biomass density (lines), lipid contents (bars), (B) surface lipid density (dots), and productivity (bars).

biomass productivity on day 4 are shown in Fig. 2B and D, respectively. Surface biomass productivities on day 4 were chosen to be compared as the surface biomass productivity reached its maximum on day 4 [11]. Surface biomass productivity continuously improved as nitrate concentration increased from 1.8 mM to 17.7 mM and phosphate concentration from 0.023 mM to 0.138 mM and reached the maximum surface biomass productivity of around 16 g/m²/day.

Importantly, a further increase in either nitrate or phosphate concentration did not result in a noticeable improvement in surface biomass productivity. These results indicate that 17.7 mM or higher nitrate concentration and 0.138 mM or higher phosphate concentration is presumably high enough to transfer sufficient amounts of nitrate and phosphate into the biofilm. Considering the optimum nutrient concentrations can be species-dependent, similar results with different microalgal species are previously reported as well [10,15,16]. For the following starvation study, 17.7 mM of nitrate concentration and 0.138 mM of phosphate concentration were used for the sufficient condition of either nutrient.

3.2. Effects of nutrient starvation strategies on lipid production

Nutrient starvation is one of the well-known effective strategies used to induce lipid accumulation for enhanced lipid productivity. However, there is only a limited number of studies on the effect of nutrient starvation strategy on lipid production through the attached algae
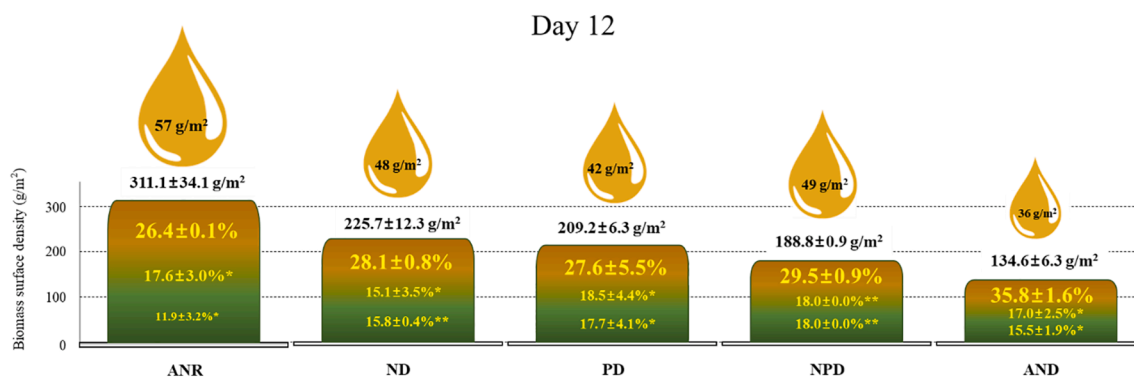
Fig. 4. The polarization of lipid contents within the algal biofilm grown under various medium conditions. Surface lipid densities are shown in the yellow droplet image above each biofilm image. The height of each biofilm corresponds to its surface biomass density and is shown on the top of each biofilm image. The lipid content of biomass at each layer is shown in the respective layers of the biofilm.

cultivation, and most importantly, there is a lack of understanding of the phenomena within the biofilm during the starvation process.

Nitrogen and/or phosphorus starvations during the attached cultivation of *Ettlia* sp. YC001 were conducted and the results of biomass growth along with their lipids contents and productivity are shown in Fig. 3. Surface biomass densities over the entire cultivation period of 12 days under all five medium conditions are shown in Fig. 3A as a line graph. Under the ANR condition, a positive control in this study, surface biomass density continued to grow up to 298 g/m^2, while all nutrient-depleted conditions (ND, PD, NPD, and AND) showed limited growth after the starvation and reached up to 225, 209, 189, and 134 g/m^2 on day 12, respectively. Under the ND condition, biomass continued to grow for 3 additional days, then biomass growth rate started to decrease on day 7, while under both PD and NPD conditions, the biomass growth rate decreased after 2 days the starvation started. Under the AND condition, biomass growth stopped only a day after starvation started and remained unchanged throughout the rest of the cultivation period. Biomass continued to grow for a certain period after the media were changed to the ones with depleted nutrients because biofilm consists of extracellular polymeric substance (EPS) that stores nutrients by adsorption as a reservoir, which can be utilized by microalgae either directly or after being broken down into smaller sizes by co-existing microbes [7]. On day 12, the final surface biomass density under the ND, PD, and NPD conditions decreased by about 25–30 % of the final surface biomass density under the ANR condition. Surface biomass density under the AND condition was 134 g/m^2 on day 5 and remained constant for the rest of the cultivation period, which is about 40 % of the final surface biomass density under the ANR condition.

The bar graph in Fig. 3A shows changes in the total lipid contents of biomass grown under each media condition. Throughout the cultivation period, the lipid content of the biofilm under the ANR condition was around 20–23 %. As compared to the ANR, total lipid contents under the ND, PD, NPD, and AND conditions started to increase on day 9 to 24.6 ± 0.4 %, 23.6 ± 0.1 %, 24.5 ± 0.4 %, and 26.3 ± 1.9 %, respectively. Eventually, total lipid content under the ND, PD, NPD, and AND conditions on day 12 increased to 24.6 ± 1.3 %, 25.8 ± 0.1 %, 25.6 ± 0.3 %, and 29.9 ± 0.7 %, respectively. The results clearly show that all nutrient-depleted conditions increased lipids contents of the biofilms, where the ND, PD, and NPD conditions increased lipid contents by 2–3 % points while the AND condition increased lipid content almost by 7 % points on day 12.

Considering both biomass growth and their lipids contents change combined, surface lipids density and productivity for all medium conditions are shown in Fig. 3B. Surface lipid densities under the ANR, ND, PD, NPD, and AND conditions on the final day (day 12) were 65.9 ± 0.2, 55.6 ± 3.0, 53.9 ± 0.2, 48.4 ± 0.6, and 40.2 ± 0.9 g/m^2, respectively. Surface lipid productivities under the ANR, ND, PD, NPD, and AND conditions on the final day (day 12) were 5.5 ± 0.0, 4.6 ± 0.2, 4.5 ± 0.0,

4.0 ± 0.1, and 3.4 ± 0.1 g/m^2/day, respectively. Surface lipid productivities were all at the highest on day 6 with 6.5 ± 0.1, 6.0 ± 0.2, 5.4 ± 0.2, 5.8 ± 0.1, and 5.1 ± 0.0 g/m^2/day under the ANR, ND, PD, NPD, and AND conditions, respectively. Despite enhanced lipids accumulation under nutrient-depleted conditions, higher biomass productivity under the ANR condition resulted in a higher surface lipid density and productivity because nutrient-depleted conditions sacrificed biomass productivity significantly. As a result, the ANR condition allowed the highest lipid productivity, and the starvation strategy did not increase lipid productivity as normally expected in a typical suspended cultivation system. A similar result was reported by Cheng et al that although nitrogen shortage increased the content of hydrocarbon, overall lipid productivity decreased since cellular growth was inhibited [17]. The authors also mentioned that the crude-hydrocarbon productivity was higher with non-circulating treatment than those with circulating treatment presumably due to nutrient shortage in the circulating medium, which is in line with the result from the present study considering non-circulating treatment as nutrient sufficient condition and circulating treatment as nutrient-limited condition [17]. Ji et al also reported that total lipid and TAG productivity through the attached cultivation of *Aucutodesmus obliquus* decreased with low nitrogen in the source water with the minimum compromise of the biomass productivity [15]. Because the biofilm offers a heterogeneity of composition of algal cells, particularly with respect to its depth, investigation of compositional variation over the depth of the biofilm under various nutrient conditions is critical to understand the aforementioned phenomena more accurately.

3.3. Compositional variation over the depth of biofilm

Immobilized microalgal cells in a biofilm have a compositional variation over depth due to gradients in key parameters such as water, nutrients, CO_2, and light [14,18]. In an effort to understand such phenomena explicitly, particularly during the starvation process, biofilms grown under various nutrient conditions were sliced into three different layers (top, middle, and bottom layer), and analyzed.

Fig. 4 shows the variations in lipid contents over the depth of the biofilms grown under various nutrient conditions (ANR, ND, PD, NPD, and AND) harvested on day 12. Variations in lipid contents over the depth were observed in all biofilms, where the lipid content was generally higher in the top and lower in the bottom regardless of medium condition. Lipid contents in the top, middle, and the bottom of the algal biofilm were respectively 26.4 ± 0.1 %, 17.6 ± 3.0 %, and 11.9 ± 3.2 % under the ANR condition, 28.1 ± 0.8 %, 15.1 ± 3.5 %, and 15.8 ± 0.4 % under the ND condition, 27.6 ± 5.5 %, 18.5 ± 4.4 %, and 17.7 ± 4.1 % under the PD condition, 29.5 ± 0.9 %, 18.0 ± 0.0 %, and 18.0 ± 0.0 % under the NPD condition, and 35.8 ± 1.6 %, 17.0 ± 2.5 %, and 15.5 ± 1.9 % under the AND condition. This phenomenon of

Table 2

Results of elemental analysis (carbon, nitrogen, and phosphorous), total lipids, fatty acid compositions, and pigment analysis (chlorophyll *a*, chlorophyll *b*, and lutein) of biomass at each layer of biofilm (top, middle, and bottom) grown under different medium conditions (ANR, ND, PD, NPD, and AND).

Conditions	Layers	Elements, wt %			Lipids, wt %	Fatty acids compositions, %											Pigments, wt %			
		C	N	P		C10:0	C14:0	C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n3	SFA	MUFA	PUFA	Total	Chl a	Chl b	Lutein
ANR	TOP	54.8 %	3.3 %	0.6 %	26.4 %	1.3 %	0.0 %	17.4 %	5.4 %	4.4 %	46.9 %	15.6 %	10.5 %	22.7 %	51.6 %	25.7 %	2.3 %	1.8 % (79.5 %)	0.1 % (3.1 %)	0.4 % (16.7 %)
	MID	50.6 %	4.3 %	1.2 %	17.6 %	4.1 %	0.0 %	16.4 %	3.7 %	3.5 %	34.4 %	16.9 %	10.8 %	26.7 %	42.4 %	30.9 %	3.3 %	2.4 % (72.5 %)	0.2 % (5.1 %)	0.7 % (20.8 %)
	BOT	47.8 %	5.3 %	1.6 %	11.9 %	5.6 %	1.2 %	16.7 %	2.3 %	2.2 %	24.9 %	15.3 %	12.3 %	31.9 %	33.8 %	34.3 %	4.5 %	3.4 % (75.9 %)	0.3 % (5.6 %)	0.7 % (16.5 %)
ND	TOP	54.2 %	1.8 %	0.1 %	28.1 %	0.9 %	0.0 %	17.0 %	3.3 %	3.2 %	36.7 %	14.0 %	10.1 %	24.8 %	47.0 %	28.2 %	1.0 %	0.7 % (70.0 %)	0.0 % (3.9 %)	0.2 % (19.8 %)
	MID	50.9 %	2.1 %	0.2 %	15.1 %	2.1 %	0.0 %	21.1 %	3.0 %	3.1 %	35.1 %	16.6 %	12.4 %	28.2 %	40.8 %	31.0 %	1.2 %	0.8 % (70.9 %)	0.1 % (4.4 %)	0.3 % (24.0 %)
	BOT	49.7 %	2.6 %	0.2 %	15.8 %	2.4 %	0.0 %	21.0 %	2.9 %	3.2 %	34.8 %	16.7 %	13.1 %	28.2 %	40.1 %	31.7 %	1.4 %	1.0 % (71.5 %)	0.1 % (4.8 %)	0.3 % (20.6 %)
PD	TOP	56.1 %	3.2 %	0.0 %	27.6 %	1.6 %	0.0 %	14.7 %	3.6 %	3.2 %	36.0 %	13.3 %	10.7 %	23.4 %	47.7 %	28.9 %	1.8 %	1.2 % (64.9 %)	0.1 % (5.4 %)	0.5 % (24.8 %)
	MID	51.0 %	4.2 %	0.1 %	18.5 %	3.3 %	0.0 %	13.6 %	3.6 %	2.2 %	22.6 %	12.9 %	10.3 %	27.9 %	38.3 %	33.8 %	2.6 %	1.8 % (71.2 %)	0.1 % (5.3 %)	0.6 % (23.5 %)
	BOT	50.3 %	5.2 %	0.1 %	17.7 %	4.5 %	0.0 %	13.5 %	2.0 %	2.0 %	21.9 %	12.7 %	10.0 %	30.0 %	35.9 %	34.1 %	3.5 %	2.6 % (74.5 %)	0.2 % (5.2 %)	0.6 % (18.4 %)
NPD	TOP	55.6 %	2.0 %	0.3 %	29.5 %	0.6 %	0.0 %	17.6 %	4.2 %	2.8 %	36.0 %	14.7 %	7.9 %	25.1 %	47.9 %	27.0 %	0.7 %	0.4 % (60.1 %)	0.1 % (7.0 %)	0.2 % (32.9 %)
	MID	51.1 %	2.5 %	0.5 %	18.0 %	0.7 %	0.0 %	19.1 %	3.4 %	2.6 %	32.8 %	15.1 %	7.8 %	27.5 %	44.4 %	28.1 %	0.8 %	0.5 % (64.9 %)	0.1 % (7.1 %)	0.2 % (29.2 %)
	BOT	50.8 %	3.2 %	0.7 %	18.0 %	0.8 %	0.0 %	19.6 %	3.3 %	2.6 %	32.3 %	15.1 %	7.8 %	28.2 %	43.8 %	28.1 %	1.0 %	0.7 % (68.6 %)	0.1 % (7.1 %)	0.2 % (23.5 %)
AND	TOP	58.3 %	2.3 %	0.4 %	35.8 %	0.0 %	0.0 %	18.9 %	5.5 %	3.2 %	40.8 %	12.3 %	8.0 %	24.9 %	52.2 %	22.9 %	0.7 %	0.4 % (61.3 %)	0.0 % (3.5 %)	0.2 % (34.7 %)
	MID	53.2 %	2.8 %	0.5 %	17.0 %	0.0 %	0.1 %	26.9 %	5.9 %	3.4 %	49.4 %	12.4 %	8.1 %	28.6 %	52.1 %	19.3 %	1.0 %	0.7 % (71.0 %)	0.1 % (5.7 %)	0.2 % (23.5 %)
	BOT	52.4 %	3.5 %	0.8 %	15.5 %	0.4 %	0.5 %	27.5 %	6.0 %	3.5 %	50.4 %	12.4 %	8.1 %	29.3 %	51.9 %	18.9 %	1.4 %	1.1 % (75.8 %)	0.1 % (6.8 %)	0.2 % (17.4 %)

polarization in lipid contents of algal cells in biofilm implies that each layer is under a different influence on lipid production. Similar studies reported the unevenness in light and nutrients distributions over the depth of the biofilm resulted in a higher accumulation of astaxanthin in the upper part of the biofilm as astaxanthin accumulation is also induced by high light stress and nutrient limitation [9,10]. In the same context, neutral lipids synthesis in the algal cells is induced by stressful conditions, not only from nutrient deficiency but also accompanied by high light exposure, to store energy and protect themselves from photoinhibition [4,6].

In order to determine nutrient availability, carbon utilization, and effects of light intensity on photosynthetic activity and lipids synthesis in each layer of the biofilms, analyses of elemental contents (C, N, P), photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and lutein), and fatty acid compositions were conducted and listed in Table 2. The distribution of elemental contents (C, N, and P) of biofilms showed a similar tendency under all medium conditions as all biofilms showed higher carbon, and lower nitrogen and phosphorous contents in the top layer. The gradient in the carbon content of biofilm indicates that carbon fixation was more significant in the top layer due to higher photosynthetic activity with easier access to the ambient air and light energy. Sufficient light supply also has a positive impact on CO_2 fixation capacity, which changes the biochemical composition of photoautotrophic microalgae [19,20]. In the meantime, nitrogen and phosphorous contents of the biofilms were lower in the top as they were supplied from the bottom, thus diffusional limitation must have occurred in the upper part of the biofilm. Under all nitrogen-depleted conditions (ND, NPD, and AND), nitrogen contents in the bottom of biofilms were around 2–3 %, which is similar to the nitrogen content in the top layer of the biofilms under nitrogen-sufficient conditions (ANR and PD), indicating that top layers were under nitrogen depleted condition even with an adequate supply of nitrogen.

The increase in pigment contents over the depth of biofilms is also evidently supporting that cells in the top were experiencing stress from high light exposure while those in the bottom were lacking light exposure due to degradation of light penetration across the biofilm. Chlorophyll *a* to b ratios were also higher in the top as chlorophyll *b* is responsible for light harvesting and its content decreases more significantly than chlorophyll *a* under excessive light exposure [21,22]. The high light stress in the top layer, therefore, restricted cell growth and induced lipids synthesis. On the other hand, the reduction of pigmentation in the cells in the upper layer allows more penetration of the light to the cells underneath, keeping cells in the middle growing under a healthier condition [23]. This was also reported as a muted effect, where the cells in the middle of biofilm are under the ideal condition where light and nutrients are all sufficiently supplied [24]. Among different medium conditions, nitrogen-depleted conditions have negatively affected overall pigment contents. Nitrogen-depleted condition is reported to decrease pigment content of algal cells as they break down chlorophyll *a* and b as an internal source of nitrogen for the synthesis of proteins and nucleic acids [22]. Lutein contents, a xanthophyll in the photosynthetic system that functions as light-harvesting pigments, likewise decreased in the top and increased in the bottom as its function [25,26]. The lutein content in the bottom of the biofilm under the ANR condition was around 0.7 wt%, as high as the optimum lutein content of *Ettlia* sp. YC001 found in the previous study (~0.8 %), suggesting a promising application for high lutein production as a by-product if the upper layer of the biofilm is separately harvested for biofuel application [27]. Fatty acids compositions across the biofilm also evidently support the high lipid accumulation in the top layer of the biofilm as it shows higher mono-unsaturated fatty acids (MUFA) contents in the top of the biofilm in response to the enhanced neutral lipid synthesis. Higher contents of polyunsaturated fatty acids (PUFA) in the bottom of the biofilm responding to the development of a photosynthetic membrane in order to acclimate to low light conditions [4,28,29].

In summary, the result of compositional analyses of elements, pigments, and fatty acids indicated that the top layer of the biofilm had higher lipid accumulation in response to stress from nutrient depletion and high light stress with more readily accessible CO_2 regardless of medium types. Due to its natural lipid induction at the top layer of the biofilm, sufficient nutrient supply for the optimum biomass growth also allowed the highest lipids productivity, outperforming those with nutrient starvation steps.

4. Conclusion

The present study has identified that the lipid production of *Ettlia* sp. YC001 through the attached cultivation under sufficient nutrient supply outperforms the processes with nutrient starvation steps due to its natural lipid accumulation in the upper layer of the biofilms. The unnecessity of the nutrient starvation strategy for high lipid production will benefit to keep the process simple and operating costs low. Furthermore, the study provides a potential for further improvement in lipid productivity through a more precise harvesting technique to separate the upper layer of the biofilm for biofuel production.

CRediT authorship contribution statement

Sungwhan Kim: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Minsik Kim:** Conceptualization, Investigation, Validation, Writing – review & editing. **Yong Keun Chang:** Project administration, Funding acquisition, Writing – review & editing. **Donghyun Kim:** Conceptualization, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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