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Initial population density plays a vital role to enhance biodiesel productivity of *Tetraselmis* sp. under reciprocal nitrogen concentration

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ABSTRACT

This study reveal the combined effect of initial population density (IPD) (0.1, 0.5 and 1.0 g L^{-1}) and nitrogen (0 N-nil nitrogen; 1 N-optimized concentration of nitrogen; 2 N–doubling of optimized nitrogen concentration) on growth, biomass, lipid and fatty acid content of the oceanic microalga *Tetraselmis* sp. were tested. The experiment was carried out in multi-room light-emitting diode chamber for two weeks with continuous illumination of light such as 14:10 h L:D of photoperiod (PP) and 150 μmol m^{-2} s^{-1} of photosynthetic photon flux intensity (PPFI). The maximum optical density (1.15 abs), cells concentration (253 × 10^4 cells mL^{-1}), chlorophyll 'a' (2.68 mg L^{-1}) and fatty acids content were found in the combination of 0.5 g L^{-1} initial population density and 1 N, whereas the highest biomass (0.95 g L^{-1}) was noticed in 1 g L^{-1} initial population density and 2 N, and total lipid (48 ± 1.14%) was high in 0.5 g L^{-1} initial population density and 2 N combination.

1. Introduction

Microalga are one of the interesting candidates that can switch biotic and abiotic factors to valuable products (Chen et al., 2012), and they can be utilized for various applications such as cosmetics, pharmaceuticals, aquaculture, biofuel production and bioremediation too (Rasdi and Qin, 2015; Dinesh Kumar et al., 2015, 2016a,b). Mostly large scale production of microalga has been carried out by raceway pond or photobioreactor in which several factors can affect and alter the composition of microalga, such as temperature, light, nutrients and pH while operating large scale production (Panyakampol et al., 2015). Many studies revealed that the nutrient starvation is one of the main factors while focusing on biomass and lipid production in the field of biofuel production and aquaculture (Rasdi et al., 2015; Panyakampol et al., 2015; Kamalanathan et al., 2016; Negi et al., 2016). It is known that, a most number of microalgal species have a practice to accumulate large amount of lipids while facing environmental stress like starvation of nitrogen (Breuer et al., 2012) and those lipids made by form of triglycerides (TAGs) which served as carbon and energy storage bank (Hu et al., 2008).

Nowadays, biofuel energy and aqua feed requirement are increased enormously due to over population. Mass culture of microalgae almost fulfills their requirement of microalgal, but their cost of production is exceeding to limit (Ratledge and Cohen, 2008). Therefore, it is essential to reduce cost of production by altering and developing the modern design and operation such as improving irradiation conditions using natural light source for photosynthesis, reducing the usage of nutrients and developing low cost medium to replace high cost chemicals (Norsker et al., 2011; Dinesh Kumar et al., 2015, 2016a,b). Previous researchers have summarized that the optimum inoculation can allow the sustainable light penetration to the culture chamber and light availability for every individual cells can improve the biomass, lipid and astaxanthin accumulation (Wang et al., 2013). Planktonic microalga *Tetraselmis* sp. served as lipid bank for biofuel and animal production industry, compared to other species. *Tetraselmis* cell size was large (usually grow 10 μm long × 14 μm wide) and its lipid storage capacity was high (Dinesh Kumar et al., 2018). An appreciable number of investigations were made on culture of *Tetraselmis* sp. under nutrients

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depletion (Ji et al., 2011; Kim et al., 2016) and initial inoculum or biomass density (Lopez-Elias et al., 2011; Michels et al., 2014) for enhancing biomass and lipid accumulation. However, still no studies has not been available in the field of combining effect of nitrogen and initial population density on the biomass and lipid production of microalgae. In order to capitalize biomass and lipid production in *Tetraselmis* sp., an optimal nitrogen concentration and initial population density must be determined.

We studied that an optimal combination of nitrogen on culture medium and initial biomass density on the inoculum for maximum biomass and lipid productivity can be achieved by oceanic microalga *Tetraselmis* sp. Here, we provide a detailed experimental study of the combined effect of nitrogen and initial population density on biomass and lipid accumulation of oceanic microalga *Tetraselmis* sp. at the end of experiment in batch culture. During the experiment, optical density, cell density, biomass production, chlorophyll ‘a’, lipid and fatty acid methyl esters were estimated as a function of nitrogen concentration ranging from 0 N, 1 N, 2 N and IPD ranging from 0.1, 0.5, 1.0 g L^{-1}.

2. Materials and methods

2.1. Strain and culture conditions

Oceanic microalga *Tetraselmis* sp. was obtained from Center for Marine Bioenergy Consortium, Inha University, Seoul, South Korea. The stock culture of *Tetraselmis* sp. was maintained in multi room light-emitting diode (LED) chamber (HST-120LE-4, Hanbaek St. Co., South Korea) fertilized with Walne medium (Walne, 1970) at 25 °C of temperature, 12L:12D of photoperiod (PP) and 150 μmol $m^{-2} s^{-1}$ of photosynthetic photon flux intensity (PPFI) in 250 mL of round bottom conical flask containing 200 mL of sterilized sea water fertilized with Walne medium, the total indoor culture method was followed according to Perumal et al. (2014). After 5–8 d, the maximum exponential phase was obtained.

2.2. Culture medium, IPD preparation and experimental conditions

In order to examine the combined effect of nitrogen variation on medium and initial population density on biomass and lipid production from *Tetraselmis* sp. nitrogen replication such as 0 N, 1 N and 2 N used. Walne medium was used and its composition was provided in Table 1.

Table 1
Medium composition for experiment.

Components (g)	1N	2N	0N
(A) Macro nutrients			
$NaNO_3$	100	200	–
$FeCl_3{\cdot}6H_20$	1.3	1.3	1.3
$MnCl_2$ $4H_2$	0.36	0.36	0.36
H_3BO_3	33.6	33.6	33.6
EDTA	45.0	45.0	45.0
$NaH_2PO_4{\cdot}2H_2O$	20.0	20.0	20.0
Dissolved in 1000 ml of DW			
(B) Trace metal solution			
$ZnCl_2$	4.2	4.2	4.2
$CoCl_2$ $6H_2O$	4.0	4.0	4.0
$(NH_4)6Mo7O_2$ $4H_2O$	1.8	1.8	1.8
$CuSO_4$ $5H_2O$	4.0	4.0	4.0
Dissolved in 1000 ml of DW			
(C) Vitamin solution			
Cyanocobalamin	0.01	0.01	0.01
Thiamine HCl	0.01	0.01	0.01
Biotin	0.0002	0.0002	0.0002
Dissolved in 100 ml of DW			

Each solution A, B and C dissolved 1, 0.5, 0.1 ml dissolved in 1 l of sea water respectively.

Preparation of different initial population density was done according to Dinesh Kumar et al. (2015). In brief *Tetraselmis* sp. was harvested at exponential growth phase and centrifuged at 5000 ×*g* for 10 min by using centrifuge (Rotina 48R, Hettich, South Korea). After that, the algal pellets were washed thrice by sterilized sea water and re-suspended in different volumes of sterilized seawater to prepare three levels of cell density: 0.1, 0.5 and 1.0 g L^{-1}.

The prepared inoculums was transferred to 500 mL of round bottom conical flasks (Byrex, South Korea) filled with 400 mL of sterilized sea water and flask was placed in orbital shaker (Fine PCR, SH30). The experiment was carried out in multi room for 14 days with photoperiod and illumination 14:10 h L:D and 150 μmol $m^{-2} s^{-1}$. During experiment, optical density, cell density, biomass, chlorophyll ‘a’ were analyzed for once in a two days and at end of the experiment total lipid and FAME content were analyzed.

2.3. Estimation of growth

Tetraselmis sp. growth was observed by assaying the absorbance of sampled at 680 nm (OD680) using a UV visible Spectrophotometer (3200, X-MA, Human Corporation, South Korea). The cell concentration was estimated by counting cells using hemocytometer with proper dilution.

2.4. Estimation of biomass

Biomass was estimated every two days by the gravimetric method according to Richmond et al. (2003) with minor modifications. In brief, 10 mL of *Tetraselmis* sp. culture filtered onto a pre-weighed (W_0) glass fiber (GF/C) filter paper (pore size 0.45 μm) and the filter paper was dried over at 100 °C for 24 h. The weight of algal cells along with filter paper was estimated (W_1). The biomass concentration of *Tetraselmis* sp. was calculated as per the following equation:

$$\mathrm{Biomass(gL-1)} = \frac{\mathrm{W1-W0}}{(10/1000)}$$

2.5. Estimation of chlorophyll ‘a’

The chlorophyll ‘a’ was measured according to Wellburn (1994) with minor modification. In short, 1 mL of sample was centrifuged at 5000 ×*g* for 10 min, after that supernatants were discarded and 1 mL of methanol added into the pellets. Then the sample was incubated at 60 °C for 50 min in water bath and lysates was removed by centrifuging sample over 5000 ×*g* for 6 min. The sample absorbance was measured at 470, 653, and 666 nm chlorophyll ‘a’ was calculated using the following formula:

$$\mathrm{Chl\ a} = 15.65\ A666 - 7.34\ A653$$

A_{666}, A_{653}, are optical density values of respective nanometers in spectrophotometer.

2.6. Estimation of total lipid

Total lipid content was estimated by Bligh and Dyer (1959) method with slight modification. Briefly, 100 mg of lyophilized algal biomass was homogenized by mortar and pestle after adding 15 mL of chloroform:methanol (2:1) mixture. The homogenized algal sample was filtered by no. 1 Whatman filter paper and these extraction and filtration were continued up to three cycle. By using hexane, mortar-pestle and filter paper were washed and all the filtrates were shifted to round bottom flask through sodium sulfate for removing moisture content. Then the solvent was transferred to rotary evaporator for evaporation. After completing evaporation process, the lipid was re-dissolved with 10 mL of hexane and shifted to dry pre-weighed test tube. After evaporation of hexane by using evaporator, the lipid content was estimated