

Influence of Organic Carbon Sources on Growth and Lipid Content of Marine Green Alga *Dunaliella tertiolecta*

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(Received 19 December 2014, Revised 28 December 2014, Accepted 29 December 2014)

Abstract This study investigated the potential use of various organic carbon sources (glucose, glycerol and acetate) and different concentrations of CO₂ for culturing marine microalga *Dunaliella tertiolecta*. Cell growth and lipid production were monitored under heterotrophic, mixotrophic and photoautotrophic modes of cultivation. *D. tertiolecta* showed the ability to grow under mixotrophic (acetate and glucose), heterotrophic (glucose) and photoautotrophic condition under high CO₂ concentration (15%). With all the organic carbon sources (glucose, glycerol and acetate) tested in this study, 1-5% acetate enhanced cell growth rate and lipid content, while higher concentrations of acetate (10% and 15%) were inhibitory and resulted in cell death.

Keywords : *Dunaliella tertiolecta*, organic carbons, mixotrophic, heterotrophic, lipid

Introduction

Microalgae are one of the prospective feedstock for biofuels as their capability to convert CO₂ into carbon-rich lipids or carbohydrates and lesser demand of cultivation area than agricultural energy crops [12]. Microalgae can grow photoautotrophically basically using inorganic carbons and solar energy, and some species are able to utilize organic carbons mixotrophically or heterotrophically [26,36,39]. High-density cultivation of biomass up to several grams per liter is possible in a small space using appropriate bioreactors, and their photosynthesis efficiency is higher than that of terrestrial plants.

The classical photoautotrophic culture is difficult to reach a high density of microalgae biomass due to the

limited mass transfer of dissolved CO₂ and light penetration in broth [5,6]. Although many CO₂ sources are gratuitous, it is not easy to connect the CO₂ pipeline to microalgae reactor unless the location of the source is close enough. To overcome the light penetration issue, microalgae that are able to grow heterotrophically by using organic carbon sources such as sugars or organic acids without light were studied widely [35,36]. Mixotrophic growth, which combines the use of an organic nutrient and light as energy sources, takes advantages of both phototrophy and heterotrophy and therefore has the potential to attain high biomass concentration while maintaining a high content of the valuable products in the microalgal biomass [25]. However, the cost of organic carbon (usually glucose) is usually high compared with all the necessary nutrients [21]. The cost

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of glucose could contribute about 80% of the total cost of growth medium, making mixotrophic algae cultivation economically unfeasible [20].

Biodiesel is produced using neutral lipids (triglycerides) or fatty acids through trans-esterification with monoalkyl alcohol like methanol and a series of purification steps. Glycerol is produced as a byproduct of trans-esterification [34]. In general, 10 gallons of crude glycerol is generated for every 100 gallons of biodiesel produced [8,9]. Acetate can also be used as carbon source in microalgae mixotrophic cultures, and the incorporation of acetate is a process dependent on both anabolic and catabolic metabolism [4]. Therefore, searching inexpensive carbon sources like process by-product that can be used for algal growth is important in scaling-up of heterotrophic or mixotrophic algal cultivation.

Dunaliella tertiolecta is a unicellular marine green alga (Chlorophyceae) that can be cultivated with inorganic nutrients present in artificial sea water along with light. It has relatively high growth rate, high lipid content, and high contents of biodegradable biomass [7]. Previous studies had shown that lipid contents in *Dunaliella* could be enhanced by salinity and nitrate stress as well as by combining auxin with salt stress [2,23,33]. However, studies done on the effect of organic carbon sources on growth and lipid content in this species are limited. This research set out to investigate the influence of different organic carbons on growth and the production of lipid in *D. tertiolecta*.

Materials and Methods

Microalga and seed culture

Marine microalga *Dunaliella tertiolecta* (UTEX# LB999) was cultivated in sterilized f/2 medium [11] containing 75 mg/L of NaNO₃, 4.32 mg/L of NaH₂PO₄, 0.023 mg/L of ZnSO₄·7H₂O, 0.217 mg/L of MnSO₄·5H₂O, 0.0073 mg/L of Na₂MoO₄·2H₂O, 0.014 mg/L of CoSO₄·7H₂O, 0.0068 mg/L of CuCl₂·2H₂O, 4.6 mg/L of Fe(NH₄)₂(SO₄)₂·6H₂O, 4.4 mg/L of Na₂EDTA·2H₂O and vitamins, supplemented with artificial seawater

(MBL) having 24.72 g/L of NaCl, 0.67 g/L of KCl, 1.36 g/L of CaCl₂·2H₂O, 4.66 g/L of MgCl₂·6H₂O, 6.29 g/L of MgSO₄·7H₂O, 0.18 g/L of NaHCO₃ and 0.606 g/L of Tris-HCl. Initially 100 mL of f/2 medium in 250 mL flask was used for cell growth in shaking incubator under light intensity (80–100 μmol/m²/s) and 25°C. When the cells reached the stationary phase as determined by optical density (OD) at 680 nm, they were shifted to bubble-column photobioreactor.

Cultivation

Cells were first grown photoautotrophically using 1 L of f/2 medium [10] at 25°C in a bubble-column photobioreactor (ID, 6.5 cm; height, 37 cm) [30]. The reactor was supplied with filtered air using 0.2 μm PTFE membrane at a rate of 0.2 vvm (volume to volume per minute) with 2% CO₂. Continuous light was supplied to cells using white fluorescent lights with light intensity of 100 μmol/m²/s. Photoautotrophic cultivation with normal nutrition (stage 1) was stopped after cell density reached 1.5–2.0 g/L. Cells were recovered through centrifugation at 3000 rpm for 5 min and by removing the spent medium. Collected cells were thoroughly washed several times with nitrogen-deleted f/2 medium. As the second stage of culture, these cells were suspended in new media with different concentrations of glucose, glycerol, sodium acetate and CO₂, under light or dark condition to monitor the growth and lipid production.

Lipid extraction and quantification

Modified Bligh and Dyer method was used for total lipids extraction [3]. Suspended sample (40 mL) of second stage culture was harvested using centrifugation at 3000 rpm for 5 min. Harvested sample was resuspended in 7.6 mL of chloroform/methanol/water (1/2/0.8, v/v/v). Then, sonication was done for 1 min at 100W and 20 kHz (VCX 130, Sonics & Materials Inc., CT, USA) and the mixture was vortexed for 30 s. In order to make the final ratio of chloroform/methanol/water (1/1/0.9, v/v/v), chloroform (2 mL) and water (2 mL) were added and the mixture was vortexed again for 30s. Centrifugation of the solution was carried out at 3000

rpm for 5 min and the bottom layer of solution was transferred to new tube. The same procedure of extraction was repeated using the upper layer but with half of solvent used previously. The separated bottom layer (chloroform fraction) was combined and evaporated for 24 h in a drying oven at 80°C. The total lipid contents were expressed as the % of dry cell weight (DCW).

Analytical methods

Cell concentration was determined regularly after dilution by measuring optical density at wavelength of 680 nm (OD_{680}) using UV/VIS spectrophotometer (DR-4000U, Hach, USA). For measuring dry cell weight (DCW), 5 mL of microalgae biomass was filtered using cellulose acetate membrane filter (0.7 μ m pore size, 47 mm in diameter, Whatman, UK). The filter was dried in oven at 80°C for 12 h and then transferred to desiccator until the weight was invariant. For converting OD_{680} values into biomass, 1.0 unit of OD_{680} equals 0.96 of DCW g/L approximately. Reading multi-wavelength UV absorbance according to the standard method was used for nitrate determination [1].

Results and Discussion

Effect of glucose on cell growth and lipid content

Heterotrophic cultivation for microalgae is defined as the cultivation with organic carbons without the light. Glucose is one of the most easily assimilable organic carbon sources not only for heterotrophic bacteria but also for the growth of many microalgae species [5,6]. Figure 1a shows the growth of *D. tertiolecta* in heterotrophic cultivation with glucose in the dark and that best cell growth rate was achieved when *Dunaliella tertiolecta* was grown under heterotrophic condition on 1~5% glucose. On the other hand, lipid contents decreased in the presence of all glucose concentrations (Figure 1b). Similarly to the present study, previous studies have shown that heterotrophic growth of cells on glucose resulted in decrease of lipid content although glucose addition has significant effect on cell growth for some microalgae strains [21]. There was no merit in heterotrophic cultivation of

the present strain of *D. tertiolecta* using glucose.

Figure 2 shows mixotrophic cultivation of *D. tertiolecta* in the presence of light and glucose. Best cell growth was achieved in cultures supplied with low glucose concentration, i.e., 1% and 5% (Figure 2a). Cell growth became slow at 10% and 15% glucose condition. Although lipid content was not influenced by different glucose concentrations, overall lipid contents were slightly decreased as time passed in all cases (Figure 2b). It was also reported that, under mixotrophic growth conditions, cell growth was increased significantly under low glucose level (1% and 2%) as compared with high glucose level [21]. The present tendency of lipid was in accordance with previous studies where no large increase of lipid content was observed [22]. However, the rise in lipid yield was possible due to the increased biomass. In the present study, it was concluded for *D. tertiolecta* that mixotrophic culture is more advantageous than heterotrophic culture in terms of biomass productivity and lipid contents.

Effect of glycerol on mixotrophic cell growth and lipid content

During transesterification reaction between triglyceride and alcohol in the production of biodiesel, glycerol is produced as a byproduct [31]. Therefore, if glycerol can be used as an organic carbon for mixotrophic cultivation, it would be beneficial in the reduction of organic waste generation and in obtaining organic carbon gratuitously.

Figure 3 shows the results of glycerol addition in mixotrophic culture of *D. tertiolecta*. Although 1~5% glycerol resulted in increased biomass but the extent was not high (Figure 3a). It was reported that biomass productivity in *C. protothecoides* was increased when glycerol was used as carbon source [38]. When glycerol was used for cyanobacterium *Spirulina platensis*, lipids and pigments production was increased [24]. Similarly, when marine microalga *Schizochytrium limacinum* SR21 was grown on glycerol, docosahexaenoic acid (DHA) and other lipids were produced [27]. Here, lipid contents were not enhanced under glycerol variation (Figure 3b), which implies that glycerol effect on lipid is species dependent.

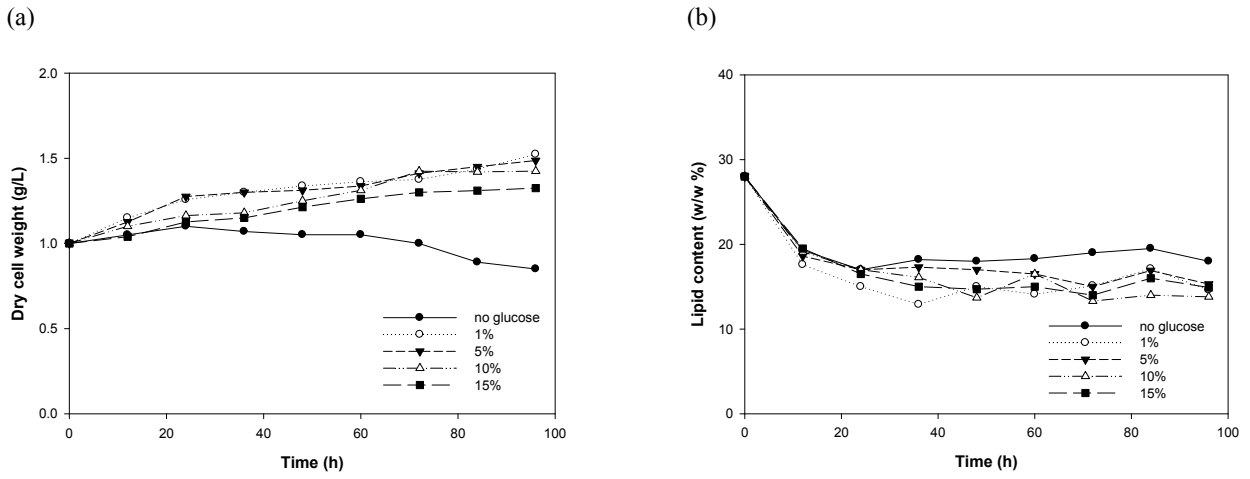


Figure 1. Effect of glucose in dark on (a) cell growth and (b) lipid content

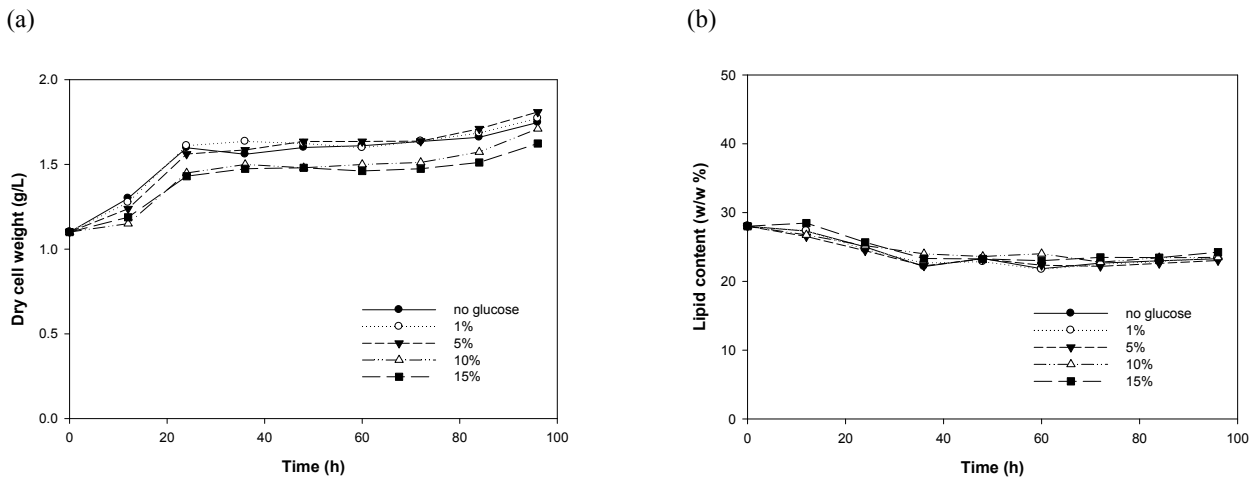


Figure 2. Effect of glucose in the presence of light on (a) cell growth and (b) lipid content

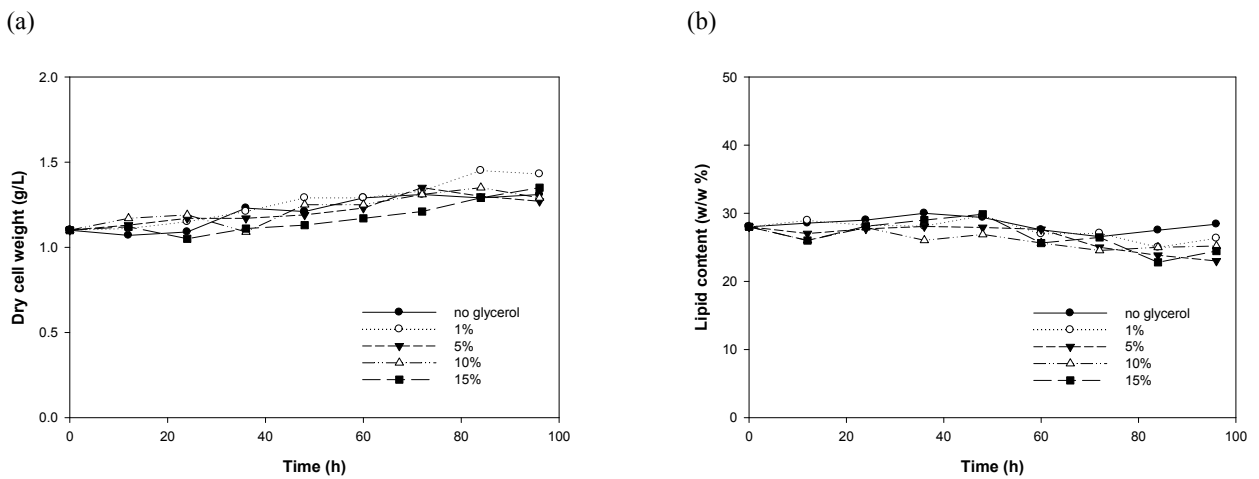


Figure 3. Effect of glycerol in the presence of light on (a) cell growth and (b) lipid content

Effect of acetate on mixotrophic cell growth and lipid content

Figure 4 shows the effect of acetate as mixotrophic carbon source in the presence of light. Best cell growth was achieved in case of 5% acetate concentration (Figure 4a). Higher concentration of sodium acetate at 10 and 15% resulted in high growth rate at early stage of experiment, but this high concentration of sodium acetate resulted in intoxication of algal cells. The color of the algal cells was bleached and eventually the cells died within 24 h. Other studies also showed that the use of sodium acetate is problematic because its effect on microalgae is concentration dependent [13]. Acetate concentrations above 1 g/L or less may cause growth inhibition due to algae intoxication [17]. In Figure 4b, lipid content increased by 5% within 12 h in the presence of 1% sodium acetate, which is similar to other studies where the growth and lipid accumulation was enhanced under lower concentrations of acetate [28].

Mixotrophic operation is capable of utilizing both organic as well as inorganic carbon sources, resulting in high biomass productivity in growth phase [29]. In general, mixotrophic cultures are advantageous to obtain higher biomass productivity due to growth-stimulating effects of light [16]. Photochemical reactions can enhance ATP formation and organic carbon metabolism in mixotrophic mode [37].

Photoautotrophic culture with continuous CO₂ supply

Photoautotrophic cultivation of *D. tertiolecta* was carried out as a reference and results are shown in Figure 5, where 15% CO₂ was best for obtaining maximum growth (Figure 5a). This result is somewhat different from other studies in that the optimal condition for maximum growth rate of *D. tertiolecta* was under 6% CO₂ or 10% CO₂ [17, 32]. Since the growth rate may depend on species chosen, gas supply rate and other multiple factors, it is difficult to generalize optimal CO₂ levels. Certain microalgae species/strains have ability to adapt to environmental change [14]. The lipid contents were almost similar under different CO₂ concentrations except that lipid content was maintained relatively high for a long time under 15% CO₂ condition (Figure 5b). A slight decrease in lipid content was observed under all CO₂ concentrations except 15% CO₂ case. Even though the achieved biomass productivity and lipid contents in autotrophic culture (Figure 5) looked better than those in mixotrophic or heterotrophic cultures (Figures 1 to 4), direct comparison is not possible because organic carbon concentrations were initial values, while CO₂ was supplied continuously in autotrophic experiments.

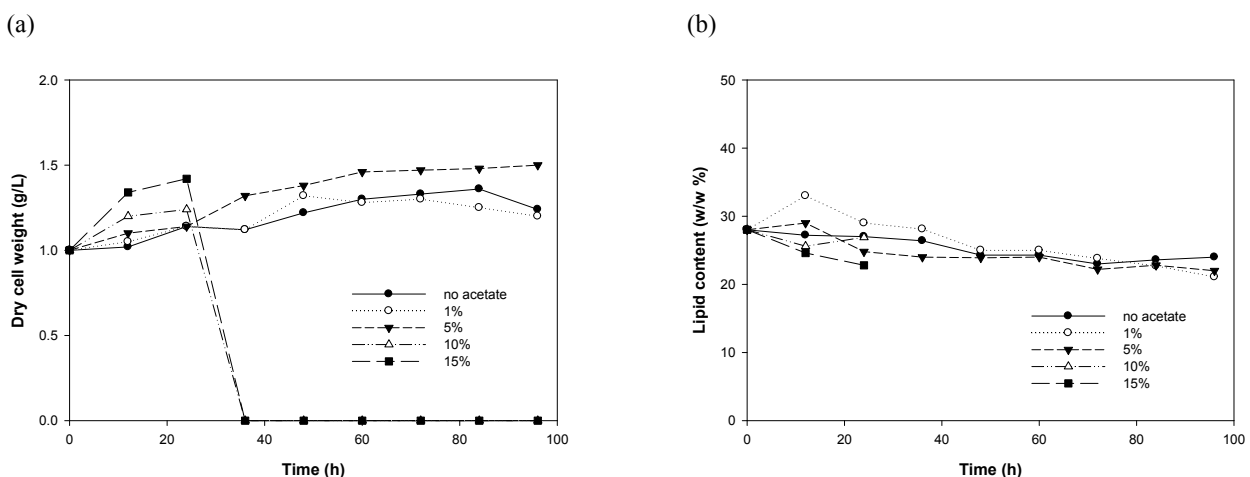


Figure 4. Effect of sodium acetate in the presence of light on (a) cell growth and (b) lipid content

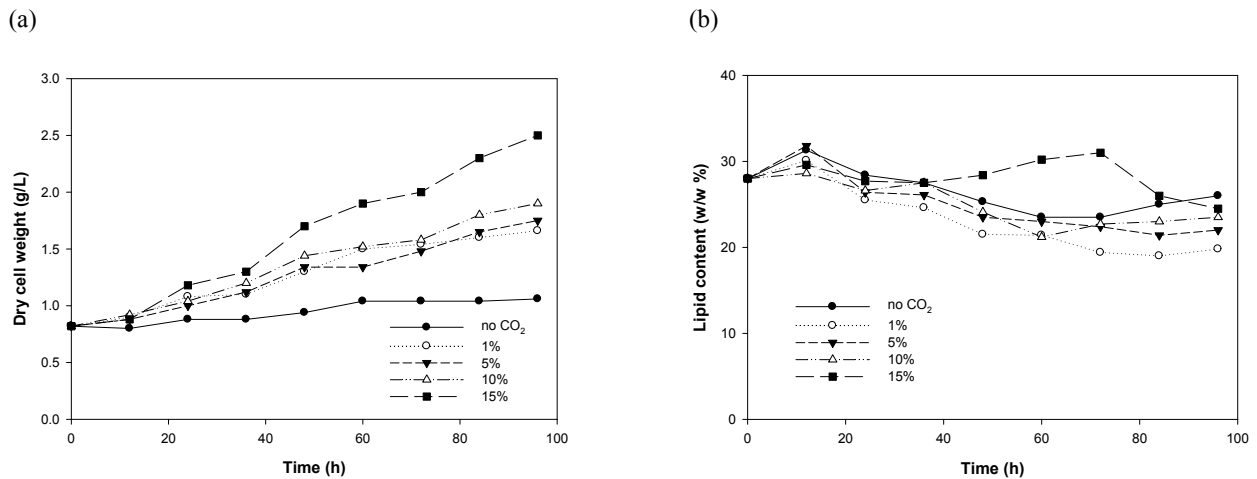


Figure 5. Effect of CO₂ in the presence of light on (a) cell growth and (b) lipid content

Conclusions

The marine alga *D. tertiolecta* had shown the abilities to grow under mixotrophic (with acetate and glucose) and heterotrophic (with glucose) conditions as well as under photoautotrophic conditions. Use of organic carbons (glucose and acetate) favored cell growth in mixotrophic cultivation, while lipid contents were not influenced significantly. Glycerol, the byproduct of biodiesel production, has no significant influence on cell growth and lipid production. Among all the organic carbon sources (glucose, glycerol and acetate) tested in this study and inorganic carbon source, 1~5% sodium acetate under mixotrophic culture resulted in the increase of both cell density and lipid content although higher concentration of acetate was inhibitory.

Acknowledgement

This research was supported by a grant from Marine Biotechnology Program funded by the Ministry of Oceans and Fisheries, Korea.

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