Effects of gas compositions on the productivity of biomass and intra-cellular fatty acids in a green alga *Chlorococcum littorale* under the photoautotrophic conditions

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Abstract

The effects of gas composition on the productivity of biomass and intra-cellular fatty acids were investigated in semi-batch cultures of a green alga *Chlorococcum littorale* (*C. littorale*) with gas flowing at 100 ml/min under the conditions of temperature of 22 ^{o}C and light intensity of 4200 lx. Growth rates were enhanced and total fatty acids contents increased by flowing an O_2-free gas mixture of 2% CO_2 and 98% N_2. This suggested that the removal of dissolved oxygen lead to promotions of both growth and fatty acids production. It was also found that fatty acids tended to be accumulated under nitrogen deficient conditions in the culture medium based on artificial sea water. Under these photoautotrophic conditions without oxygen in the gas mixtures, the productivity of fatty acids by photosynthesis were more than the oil palm seed used as a main resource of biodeisel, which implies the feasibility of algal oil for biodiesel.

Introduction

Microalgae are potential resources for converting CO_2 using photosynthesis to high value products like starch, protein, lipids and pigments [1]. Fast growth as seen in the water bloom [2] and good adaptability to environment [3] are attractive features of microalgae for making huge variety of metabolites in a short term (several days), although most of land plants usually grow with long term (more than several months). Further, compared to other multiple biomass resources, microalgae have an another advantage of culturing in closed system with less susceptible to climates, seasons and contaminations of other livings and harmful substances [4]. Considering the crisis of starvation on a worldwide scale and the energy depletion in the future, microalgae have an alternative potential as food [5], supplement [6] and bioenergy resources [7]-[10].

In view of microalgae as bioenergy resources, not only starch accumulated as the stored energy but also fatty acids in the additional role of membrane constitutes possess application possibilities. Taking a green alga *Chlorella* in open pond cultures for instance, fatty acids are contained at approximately 10 wt% in dry weight [11]. Because production of biomass in *Chlorella* is generally in the range of 7 to 85 t ha^{-1} year^{-1} depending on climates, seasons and locations [12]-[13], production of fatty acids results in the range of 0.7 to 8.5 t ha^{-1} year^{-1}. These values almost corresponds with 4 t ha^{-1} year^{-1} of oil palm seed as a main biodiesel resource, that contains 20 wt % of fatty acids on average [14]. As several reports suggested that

microalgal cultures in closed system enhanced growth rates and increased yields of biomass and metabolites [15]-[16], further fatty acids production from microalgae might be promising with closed cultures.

To establish closed culture system, aeration with controlling gas composition is one of important operation factors. A gas component of CO_2 whose effects on algal growth and metabolism have been investigated for recent years as the global warming issue has been growing. Although growth in most species of microalgae is promoted by adding CO_2 in the range from 1 to 5 % in the air, high CO_2 tolerant species of microalgae, that can grow under dozens of CO_2 concentrations, have been found in recent researches [17]-[18]. In the lipid metabolism, it has been also reported that CO_2 affects on contents and compositions of fatty acids [19]-[20], however, effects of CO_2 on lipid metabolism is insufficient knowledge and still issue of debate. In addition, these reports experimentally conducted under the air supplied with CO_2 at desired level, which may suggest that increasing CO_2 concentration in the air brings about a decrease in nitrogen and oxygen concentration. Although nitrogen gas is inactive in green algae, oxygen is expected to have affections on growth and lipid metabolism reported in few researches [21]-[22].

In this work, we primarily aimed to discuss the effect of gas composition on the algal growth and lipid metabolism. In the each experiment, the gas composition was regulated by mixing pure gas components of CO_2, nitrogen and oxygen. For the productivity of fatty acids, the growth rates and mass contents of total fatty acids were examined. A green alga *Chlorococcum littorale* (*C. littorale*) was chosen as a model because *C. littorale* can grow fast under extremely high CO_2 concentration below 70 %(v/v) [23]. From obtained data, we also discussed the feasibility of a biodiesel resource as this strain.

Experimental

The strain used for all experiments was a green unicellular alga *Chlorococcum littorale* (MBIC 10280) gifted by Marine Biotechnology Institute Co., Ltd.. Culture medium for this strain was based on the Daigo IMK medium (Nihon-Seiyaku Co., Ltd.) that was adjusted with seawater made by adding the artificial seawater Daigo SP (Nihon-Seiyaku Co., Ltd.) in the distilled water [24].

The alga was grown axenically as a semi-batch culture in a 300 ml flask with aeration. The total volume of culture medium was 210 ml including the pre-culture volume of 10 ml, and thus the initial cell concentration was set at ca. 1.5 mg/L. The flask was placed on the clear acrylic board in the air bath where temperature was controlled in the range from 16 to 22 ^{o}C within the accuracy of ± 0.5 ^{o}C. Fluorescent light was set at the bottom of the air bath and exposed at the light intensity of 4200 lx.

The semi-batch culture with aeration was conducted in a flask with rubber plug possessing two vents to aerate. The gas compositions were volumetrically adjusted by supplying N_2 (99.9 %), O_2 (99.7 %) and CO_2 (99.95 %) made by Nippon Sanso without further purification. Concentration of CO_2 in the gas mixture was adjusted in the range of 2 % to 5 % (v/v) with dilution gas of nitrogen or oxygen for each experiment. The flow gas was filtered and bubbled in the medium from the grass tube with internal diameter of 2 mm. The flow rate was constant at 100 ml/min in the each experiment and pH of culture medium was measured by pH electrode

(Yokogawa, model pH 82).

Growth rates were measured with changes in the optical density at 750 nm using a ultraviolet-visible spectrophotometer (JASCO, V-570). Cell concentration (g/L) at a given cultivation time were determined by the relation of available OD_{750} to the cell dry weight.

To analyze the culture medium, the ion chromatography was used. After small sample was collected at the given cultivation time, negative ions of nitrate, phosphate, sulfate and halogen in the filtered sample were separated through the column (IC NI-424, Shodex) with diameter of 4.6 mm and length of 100 mm. Eluting solvent, whose composition was 18 mM of 4-hydroxybenzoic acid, 2.8 mM of Bis-Tris, 2mM of phenylboronic acid and 0.005 mM of trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, was flown at 1.0 ml/min. Separated ions were detected by the conductometric detector (CD-5, Shodex). Column temperature was controlled at 45 ^{o}C.

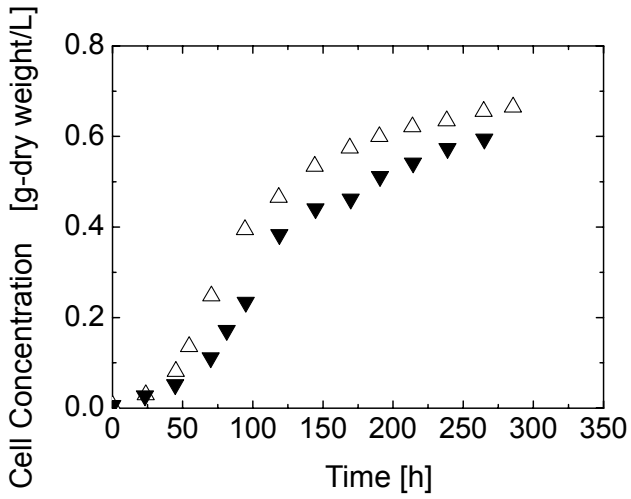

Fig.1 Cell concentrations with time at 22 ^{o}C and the light intensity of 4200 lx. Composition of CO_2 in gas mixtures at 2% with dilution gas of N_2 (upper triangles) or O_2 (lower triangles).

Cells were harvested and collected by centrifugation at 4800 rpm. After freeze dry, approximately 10.0 mg of sample was trans-esterified with 2 ml of ethanol including 5 wt% hydrogen chloride at 85 ^{o}C for the reaction time of 3 h. After cooling to room temperature, petroleum ether was added and the resulting upper phase (petroleum ether rich phase) was collected in a vacuum flask. The solvent was removed by evaporation under reduced pressure or a steam of N_2. The resulting ethyl esters were analyzed by gas chromatography equipped with the flame ionization detector (Hewlett Packard, 5890 series II) using n-pentadecanoic acid ethyl ester as an internal standard.

Results and discussion

The effects of gas composition on growth rates for *C. liitorale*

Figure 1 shows how the cell concentration changes with incubation time depending on gas compositions at temperature of 22 ^{o}C and intensity of illumination of 4200 lx. In each experiment, CO_2 concentration was fixed at 2 % (v/v) with flowing rate of 100 ml/min. Cell concentrations were exponentially increased with time in the initial stage below the cell concentration of ca. 0.4 g/L for each experiment. After that, the slopes changed and rates increased slowly. In the typical growth curve of microalgae, lag, logarithmic growth, linear growth, decay, stationary and death phase occurs in this order. In Fig. 1, such a typical growth curve until decay phase except for lag phase was also observed in the *C. littorale* growth for each experiment. On the other hand, growth rates with dilution gas of nitrogen was faster than that with oxygen. This slow growth rates with oxygen flowing was probably due to the

photorespiration occurring with photosynthesis [25]. Therefore, it is important for the effective algal growth and photosynthesis to keep dissolved oxygen in the culture medium as low as possible.

Next, we discussed about the slope changes in growth curve at the cell concentration of ca. 0.4 g/L as observed in Fig. 1. It is well-known that the algal growth could be controlled by CO_2 mass-transfer in the culture medium, concentration of dissolved oxygen evolved by photosynthesis, light availability and depletion of substances in the culture medium like nitrogen compounds or metal ions and so on [26].

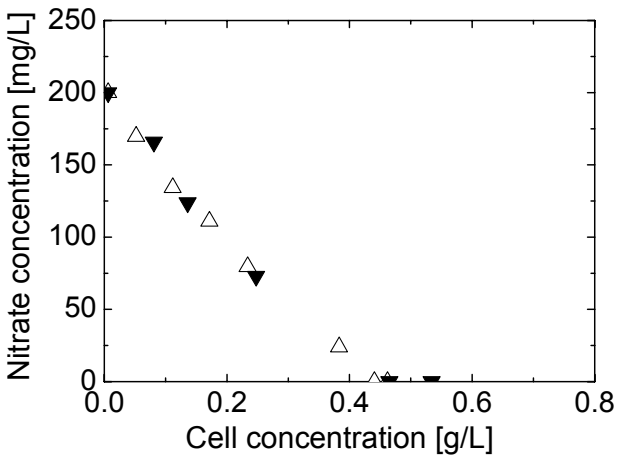

Fig.2 Nitrate concentration in the culture medium as a function of cell concentration at 22 ^{o}C and 4200 lx. CO_2 composition of 2% with 98 % N_2 (upper triangles) or 98 % O_2 (lower triangles).

For the possibility of CO_2 mass-transfer limitation, it might not be the reason because pH in the culture medium with flowing 2 % CO_2 was constant at 6.8 during the each experiment. On the other hand, it was considered from high bubbling rate of gas into the culture medium (ca. 0.5 vvm) that the possibility for the limitation with increasing dissolved oxygen evolved by photosynthesis was probably not the factor. It was supported by the independence of the oxygen concentration in the gaseous mixtures to the cell concentration where slope changes occurred (ca. 0.4 g/L). For the light availability limitation, no dependence of light intensity was observed if the intensity of illumination increased for ranging from 4,200 lx to 12,000 lx (data not shown). From these observations, that is to say, it was highly possible that the depletion of substances in the culture medium was precisely a limitation factor.

Since algal growth was significantly affected by nitrogen compounds in the culture medium [27], main substance of nitrate (ca. 200 mg/L) in the medium of this work was measured by the ion chromatography. Nitrate concentrations in the culture medium as a function of cell concentrations were shown in Figure 2. Despite of gas compositions, nitrate concentrations decreased linearly with cell concentrations in each experiment. The depletion of nitrate was occurred at the cell concentration of ca. 0.4 g/L, which was almost coincident with the value of slope changes in growth rates as shown in Fig. 1. From available data, as a consequence, the slope changes of growth rates observed in Fig.1 was probably caused by depletion of nitrate which was essentially needed for algal growth and protein production.

The effects of gas composition on fatty acids production for *C. liitorale*

Fatty acids were measured by the GC-FID. Main fatty acids contained in *C. littorale* were palmitic acid (16:0), hexadecatetraenoic acid (16:4), octadecamonoenoic acid (18:1), linoleic acid (18:2), linolenic acid (18:3) and stearidonic acid (18:4). These main components were analogous to the soybean oil, which had been utilized for biodiesel, containing 16:0, 18:1, 18:2 and 18:3 [14]. Because compositions of fatty acids changed under the conditions of temperatures, oxygen concentrations in the bubbling gas or incubation time, productivity of fatty acids were evaluated from contents of total fatty acids in dry weight.

Figure 3 shows the contents of total fatty acids as a function of cell concentrations by comparison of two bubbling gas compositions. In the experiment at CO_2 concentration of 2 % with dilution gas of nitrogen, total fatty acids were almost constant at ca. 8 wt% below the cell concentration of ca. 0.4 g/L, after that, total fatty acids increased rapidly up to 26 wt% at the cell concentrations of ca. 0.65 g/L. The cell concentration at beginning the rapid production of fatty acids (0.4 g/L) was almost coincident with the value at beginning of the nitrate depletion as observed in Fig. 2. Gordillo et al. suggested from the cultures of *Dunaliella viridis* that the main reserve lipid, triglycerides, accumulated in high amounts under 1% CO_2 and nitrogen limitation [28]. From available data in this work and literature, production of fatty acids promoted by depletion of nitrogen compounds in the medium.

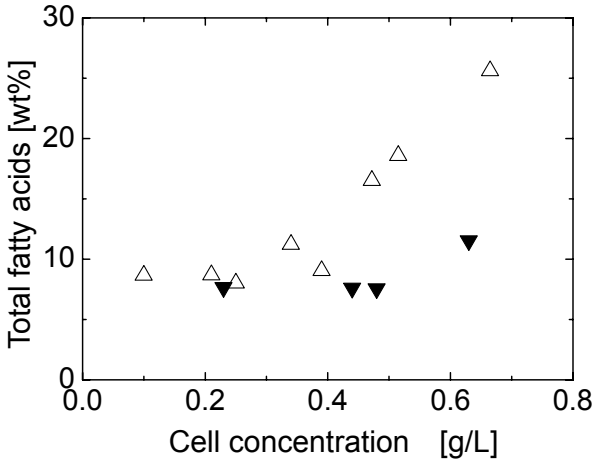

Fig.3 Content of total fatty acids in dry weight as a function of cell concentration at 22 ^oC and 4200 lx. CO_2 composition of 2% with 98% N_2 (upper triangles) or 98% O_2 (lower triangles).

On the other hand, in the experiment with dilution gas of oxygen as shown in Fig. 3, total fatty acids were almost constant at ca. 7 wt % below the cell concentration of ca. 0.5 g/L, after that, total fatty acids increased at 0.62 g/L. It is also noted that the total fatty acids with the dilution gas of oxygen were lower than those with nitrogen through the whole cultivation time. This phenomenon was possibly caused by decomposition of fatty acids with the β-oxidation under high dissolved oxygen concentrations. Therefore, it is important for the effective production of fatty acids, as well as algal growth and photosynthesis, to keep dissolved oxygen in the culture medium as low as possible .

Feasibility of biodiesel material as *C. liitorale*

From data obtained in this work, contents of total fatty acids in *C. littorale* reached 26 wt% at the cell concentration of 0.65 g/L under the conditions of temperature of 22 ^oC, light intensity of 4200 lx and CO_2 concentration of 2 % with dilution gas of nitrogen in the photoautotrophic culture. Although we conducted the experiments of low nitrate concentration in the culture medium (200 mg/L) to investigate the fatty acids metabolism, it was possible for *C. littorale* to grow up to the cell concentration of 84 g/L (over the 100-fold of this work) [24] in case of further addition of nitrate and replacing the culture medium regularly with a fresh one. It has been also reported that *C. littorale* cells exhibited a very high linear growth rate of 384 ± 30 mg of dry cells l^{-1} h^{-1} in a small-scale flat-plate photobioreactor, which corresponds to the highest CO_2 uptake rate of 0.70 g/h so far [29]. From these features and profits of *C. littorale*, this strain has a potential for huge production of biomass and metabolites.

Table 1 shows the comparison of major four resources of biodiesel [14] with the *C. littorale* investigated in this work on the ability of fatty acids production. For forur biodiesel resources, main material is the oil palm seed. Because it can be produced for tropical zone in a few times per year, the highest yield achieved in the four

resources. The other materials are produced in only one time per year, resulting in the lower yields of seed.

On the contrary, if yield of *C. littorale* was assumed to be the value of *Chlorella* in open pond cultures [12]-[13] because of possessing the analogous features, especially of green alga, biomass yield was as well or better than the other resources. Contents of fatty acids in *C. littorale* were also feasible. In open pond culture of microalgae, however, variation of productivity possibly due to the affection of climates, seasons or locations were observed in Table 1. If stable production of *C. littorale* was possible with less susceptible to environmental factors, the productivity of fatty acids would be much higher, and thus, this method might be promising for biodiesel production.

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Table 1. Comparison of major biodiesel resources [14] with *C. littorale* on the productivity of fatty acids. * Yields of biomass in *C. littorale* was assumed to be the value of *Chlorella* in open pond cultures [12]-[13]

	Oil palm seed (Palm oil)	Rape seed (Canola oil)	Soybean (Soybean oil)	Sunflower seed (Sunflower oil)	*C. littorale* This work
Yield of seed or biomass [t ha^{-1} $year^{-1}$]	20	1.4 - 2.5	1.8 - 2.3	1.4 - 2.3	7 - 85*
Fatty acids contents [wt%]	20	35	17	35	26
Productivity of fatty acids [t ha^{-1} $year^{-1}$]	4.0	0.5 - 0.9	0.3 - 0.4	0.5 - 0.8	1.8 - 22

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