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Article

해양 미세조류 *Dunaliella tertiolecta*에서 철 공급을 포함한 다중스트레스 인자가 세포성장 및 지질생산에 미치는 영향

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Effects of Multiple Stress Factors Including Iron Supply on Cell Growth and Lipid Accumulation in Marine Microalga *Dunaliella tertiolecta*

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초 록

해양 미세조류 *Dunaliella tertiolecta*에서 바이오디젤 원료인 지질생산을 위하여 철 함량 변화 및 빛 공급과 CO_2 공급에 의한 다중스트레스 인자의 조합이 세포성장 및 지질함량의 변화에 미치는 영향을 조사하였다. 1차 스트레스 인자로 정상보다 높거나 부족한 철 함량 조건이 지질 합성을 유도할 수 있음을 확인하였다. 2차 스트레스 인자로 빛 또는 CO_2 공급이 제한될 때 지질함량이 증가하였지만 오랜 시간 배양할 때 세포성장이 감소하는 단점이 있었다. 이와 같이 스트레스 조건에서 세포의 성장과 지질생산이 서로 다른 경향을 보이면 단일 배양기에서 지질생산성을 높이기 어려우므로, 세포성장과 지질생산을 분리한 2단계 배양 전략을 적용하였다. 1단계 배양에서는 성장 위주의 조건으로 고농도배양을 얻은 후, 2단계에서 지질생산을 유도하는 스트레스 조건을 부여하는 것이다. 암소 조건이 다른 조건에 비해 세포농도 감소폭이 작고 지질함량이 높아졌기 때문에, 세포 2 g/L의 고농도로 접종한 2단계에서 5X 철 농도(3.25 mg/L as Fe) 및 암소 조건을 사용하여 12 h의 짧은 배양을 통하여 1.44 g/L/d의 높은 지질생산성을 얻을 수 있었다.

Abstract

Changes in the cell growth and lipid accumulation of marine microalga *Dunaliella tertiolecta* were investigated in response to the combination of different stress factors including the variation of iron supply as a primary stress factor and different options in light irradiation and CO_2 supply as a secondary stress factor. High or limited Fe conditions could act as a stress for lipid synthesis. As a secondary stress factor, non-CO_2 condition was good for lipid accumulation, but the overall cell growth was sacrificed significantly after a long-time cultivation. Dark condition as a secondary stress factor also favored lipid accumulation and the extent of cell density reduction at the early period in the dark was small compared to other stress conditions. The two-stage cultivation strategy was necessary to maximize lipid production because tendencies of the cell growth and lipid content were not identical under the chosen stress condition. The first stage was for preparing a high cell density under the normal growth-favoring condition and the second stage was the stress condition to induce lipid accumulation in a short time. The short-term (12 h) incubation under the 5X Fe (3.25 mg/L) and dark conditions resulted in the best lipid productivity of 1.44 g/L/d providing 2 g/L inoculum at the second stage.

Keywords: *Dunaliella tertiolecta, lipid production, stress factors, iron dose, two-stage process*

1. Introduction

Microalgae have received growing interest as a potential biofuel feedstock. Microalgae can grow whole year through both outdoor and indoor cultivation, and thus oil production goes beyond the yield of the terrestrial oil crops. The fast growth rate and oil content between

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20-50% of their dry weight are other advantages of selecting microalgae for biofuel production. Despite the necessity of their growth in aqueous media, the freshwater demand of microalgae cultivation is less than that of terrestrial crops since municipal wastewater or brackish water can be utilized for microalgal cultivation unless nutrients like nitrogen and phosphorus are limited[1].

Microalgae produce ATP and NADPH during photosynthesis which serve as energy carrier and reducing power for CO_2 fixation. Conversion of CO_2 into glyceraldehyde-3-phosphate leads to the synthesis of energy storage compounds like lipid and carbohydrate. Generally the productions of lipid and carbohydrate compete with each other, and the type of energy storage compound production is species or strain specific[2-4]. For microalgae with high lipid content as trigacylglycerides, biodiesel (BD) production is advantageous, while bioethanol production is recommended when carbohydrate content is high.

The accumulation of energy storage compounds in microalgae can be enhanced by altering cultivation conditions[5]. Previous studies had shown that nutrient starvation[6] and salinity change[7] can increase lipid production in some microalgae species. Similarly, some stressful conditions like abnormal ranges of nutrients and metallic compounds can also influence their lipid or carbohydrate synthesis behaviors[5].

Iron is an important metal ion for microalgae in that it plays a major role in various metabolic functions like photosynthetic electron transport, respiratory chain, nitrate and nitrite reduction, and sulfate reduction[8]. Iron limitation significantly decreases photosynthetic electron transfer, resulting in less NADPH formation. Iron deficiency can also lead to reduction in photosynthesis and oxygen consumption rates, which ultimately decreases microalgal biomass productivity[9,10]. Contrarily, some studies showed that, by increasing the concentration of bio-available iron in the culture medium, it was possible to increase microalgal growth rate and lipid content simultaneously[11].

Dunaliella tertiolecta is a unicellular green alga which has a potential for scaled-up biofuel production using sea water[12]. It is known that the lipid content of *D. tertiolecta* increases in response to external stimuli such as salt stress, nitrogen depletion, etc[13,14]. Also the starvation of certain component (which is a stress) can trigger its consumption and lipid synthesis when cells return to normal condition again. Although there are studies on the influence of iron on cell growth and biodiesel production with *D. tertiolecta*[15], the reports on the influence of multiple stress factors including iron stress and others on the accumulation of energy storage compounds are limited. In this study, the changes in cell growth and lipid accumulation were investigated in response to the combination of different stress factors including the variation of iron supply as a primary stress factor and the different options in light irradiation and CO_2 supply as a second stress factor.

2. Materials and Methods

2.1. Microalgae and culture medium

Dunaliella tertiolecta (UTEX LB999) was obtained from the culture collection of the University of Texas, Austin, TX, USA. For cell culturing, sterilized F/2 medium, which contained 75 mg/L of $NaNO_3$ as a nitrogen source, 4.32 mg/L of NaH_2PO_4 as a phosphorous source and 4.6 mg/L of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ as an iron source, in artificial seawater (MBL) was used[16]. The original F medium is a common enriched seawater-based medium designed for the culture of coastal marine diatoms[16], and F/2 medium is the modified one to use in general marine microalgae cultivation by reducing the original concentration of F medium by half. Seed culture was first grown in 100 mL of F/2 medium in a flask under light intensity of 80-100 μmol/m^2/s at 25 ℃. After cells reached late exponential phase as determined by optical density (OD) at 680 nm, they were shifted to bubble-column photobioreactor for experiments.

2.2. Experimental setup

2.2.1. Photobioreactor

In a bubble-column photobioreactor (ID, 6.5 cm; height, 37 cm), cells were grown photoautotrophically in 1 L F/2 medium at 25 ℃. Filtered air using 0.2 μm PTFE membrane was supplied to the reactor at the rate of 0.2 vvm with 2% CO_2. Continuous light irradiation (100 μmol/m^2/s) was supplied to reactors using white fluorescent lamps. Light intensity meter (LI-COR, LI-250A, USA) was used for the measurement of light intensity. Photoautotrophic cultivation in normal F/2 medium (Stage 1) was carried out to obtain algal biomass to be used in main experiments.

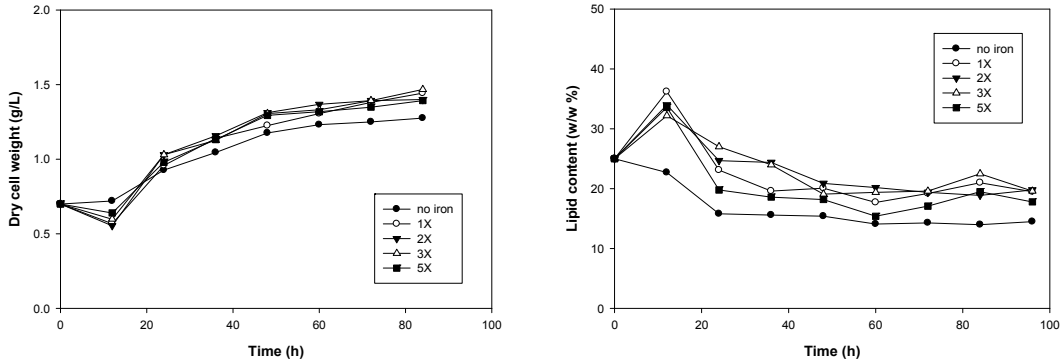
2.2.2. Two-stage cultivation

Actively grown cells were harvested by centrifugation after the Stage 1 cultivation was stopped when cell density reached 1.5-2.0 g/L. Collected cells were washed several times with sterilized artificial sea water before exposing them to new culture condition. In order to investigate the effects of different iron concentrations and other stress factors on cell growth and the production of lipid, the washed cells were suspended in a new photobioreactor with the medium containing different concentrations of ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$) and secondary stress condition (Stage 2). The basis of Fe concentration was 0.65 mg/L (1X) as in normal F/2 medium. X numbers in Figures and Tables represent the multiplication of Fe concentration compared to the normal F/2 medium with 0.65 mg/L as Fe (1X). 5X means 3.25 mg/L as Fe. The cells were then exposed to different iron concentrations (Stage 2) with/without CO_2 supply and under light/dark conditions, to monitor the changes in cell growth rate and cellular lipid content as time passed in new environments.

2.3. Analytical procedures

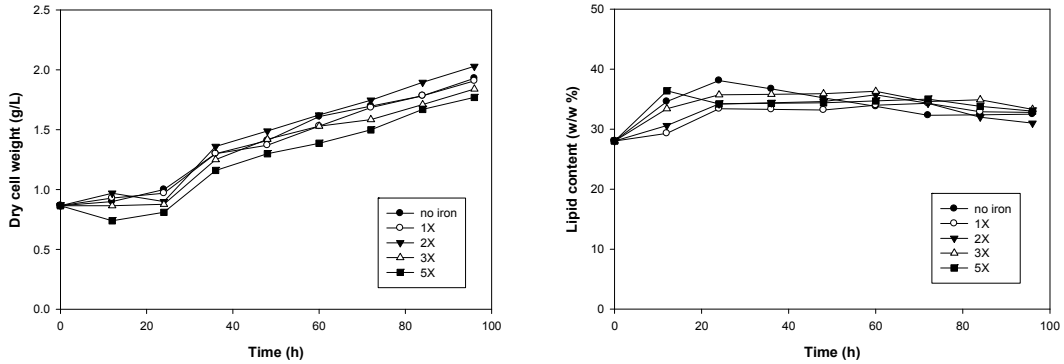
2.3.1. Biomass concentration

The cell concentration was determined regularly by measuring optical density at 680 nm (OD_{680}) using UV/VIS spectrophotometer (DR-4000U, Hach, USA). Dry cell weight (DCW) was measured using 5 mL of filtered microalgae biomass using acetate membrane filter (0.7 μm pore size, 47 mm in diameter, Whatman, UK). The filter was dried in a dry oven at 80 ℃ for 12 h after which it was transferred to the desiccators until the weight was invariant. The weight of dry blank fil-



(a) (b)

Figure 1. Effects of iron starvation on (a) cell growth and (b) lipid content. Cells were exposed to new media with different Fe concentrations, after culturing in Fe-starved condition for 2 days. X numbers represent the multiplication of Fe concentration compared to normal F/2 medium with 0.65 mg/L as Fe (1X). 5X means 3.25 mg/L as Fe.



(a) (b)

Figure 2. Effects of different iron dosage on (a) cell growth and (b) lipid content. Cells were exposed to new media with different Fe concentrations, after preparing in normal F/2 medium with 0.65 mg/L as Fe (1X).

ter was subtracted from the filter with cells to obtain the dry cell weight of the microalgae. According to the correlation between optical density and dry cell weight (DCW), 1.0 OD_{680} was approximately equivalent to 0.96 DCW g/L.

2.3.2. Lipid analysis

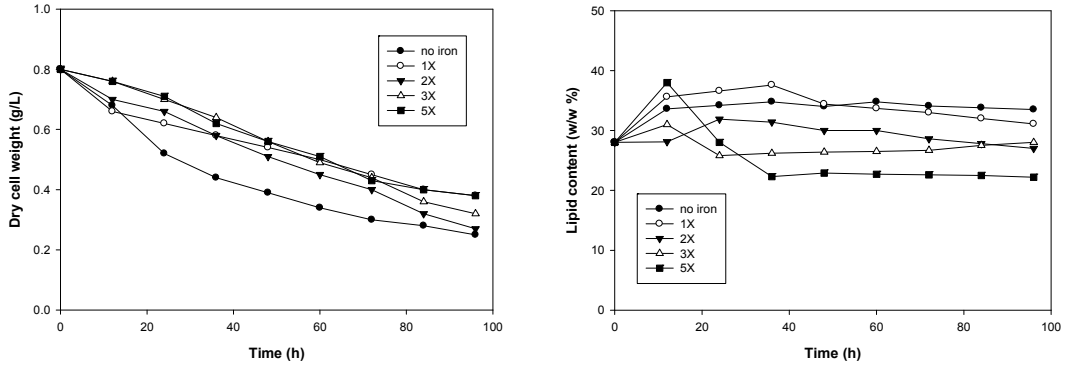
Modified Bligh and Dyer method[17] was used for the extraction of cellular lipids. Suspended cells (40 mL) from Stage 2 were harvested using centrifugation (3000 rpm for 5 min), and then re-suspended in 7.6 mL of chloroform/methanol/water (1/2/0.8, v/v/v). The mixture was then sonicated at 100W and 20 kHz for 1 min (VCX 130, Sonics & Materials Inc., CT, USA) and vortexed for 30 s. The final ratio of chloroform/methanol/water was adjusted to 1/1/0.9 (v/v/v) by adding chloroform (2 mL) and water, and the mixture was vortexed again for 30 s. The resulting mixture was centrifuged at 3000 rpm for 5 min and bottom layer (chloroform) was collected. The same extraction procedure was repeated using the upper layer. The separated and combined bottom layer (chloroform) was evaporated at 80 ℃ for 24 h in a drying oven and weighed. The total lipid contents were expressed as the % of dry cell weight (DCW).

3. Results and Discussion

3.1. Effects of iron starvation

Iron is one of the most vital metallic elements which are necessary for the microalgal growth, as it plays major roles in nitrogen consumption, photochemistry, enzymatic reactions, and chlorophyll synthesis[9], and iron starvation can influence photosynthetic activity, oxygen consumption, and cell growth[10,18]. It is known that exposing to iron sufficient condition after a period of iron starvation may trigger certain metabolic synthesis or biochemical activity. In order to see the effect of iron starvation on the cell growth and lipid synthesis, *D. tertiolecta* cells were grown for two days in iron-starved condition and then exposed to various different concentrations of iron.

Figure 1 shows the cell growth and the time-course changes in lipid content. Cell density as DCW decreased slightly at the beginning in all cultures except the culture with no iron, but then DCW rapidly increased after 12 h (Figure 1a). This initial decrease of cell growth was probably because cells needed some time to adjust themselves to new environment with different iron concentration. Cellular lipid content decreased gradually when iron was still limited, but increased when cells exposed to iron-existing environments (Figure 1b). The highest



(a) (b)

Figure 3. Effects of different iron dosage on (a) cell growth and (b) lipid content. Cells were exposed to new conditions with different Fe concentrations in dark, after preparing in normal F/2 medium with 0.65 mg/L as Fe (1X) with irradiation.

lipid content reached at 12 h when iron existed, and then decreased gradually again. This variation of lipid contents implied that the change from iron starvation to iron sufficient condition is acting as a stress for induction of lipid synthesis temporarily, but is not lasting for a longer time. It is considered that acclimating cell metabolisms to new iron sufficient condition after sudden change could be a stress for a while. Although it is species dependent, it has been reported that a high iron concentration increased total lipid of *C. vulgaris*[8]. Similar results were found in the study conducted with *Scenedesmus* species, where the lipid content increased also when the initial iron concentration was increased[19].

Next, iron variation experiments were performed on *D. tertiolecta* cells without prior iron starvation stage. Seed culture was prepared under the medium with normal Fe concentration, 0.65 mg/L as Fe (1X), as seen in Materials and Methods. Figure 2a shows that cells grew faster than previous Fe-starved experiments shown in Figure 1a, with lesser duration of adjusting period. DCWs increased significantly after 24 h in all cultures. Maximum increase was in case of 2X Fe although growth rates were similar for all cases (Figure 2a), while 5X Fe case showed the lowest. Lipid contents increased as time passed (Figure 2b) and the increased level was maintained longer than Fe-starved experiments shown in Figure 1b. Among the tested Fe concentrations, 5X Fe resulted in the highest lipid increase quickly after 12 h, and no Fe case did after 24 h (Figure 2b). 3X Fe case maintained the increased lipid level stably for the whole cultivation period. The results in Figure 2 implied that the abnormally high Fe environment such as 5X Fe could be a stress for cell growth and then induce lipid synthesis. Also it is seen that the sudden change from normal Fe condition to Fe starvation (no iron case) also triggered lipid accumulation. It is known that an exceedingly high Fe condition can inhibit normal cell metabolism due to radical-mediated oxidative stress[20,21].

By comparing Figures 1 and 2, it was concluded that the iron starvation was not having a significant advantage in triggering the induction of lipid synthesis when compared with non-starved normal cultivation in terms of cell growth rate and lipid productivity in the current microalgal species *D. tertiolecta*.

3.2. Effect of iron in dark condition

Previous experiments on iron variation were performed in the presence of light which was the normal photoautotrophic condition, and resulted in the enhancement of both biomass and lipid (Figure 2). Because the shortage of light may reduce photosynthesis activity of microalgae, dark condition can act as a stress to induce lipid synthesis [5,22] and thus a similar set of experiments of iron variation was performed in dark with cells prepared in the normal 1X Fe concentration with 100 μmol/m^2/s of continuous irradiation.

In the absence of light, DCW gradually decreased indeed in all cultures (Figure 3a). These results have been expected because the reduced photosynthesis activity without the light influences cell survival negatively. The growth inhibition was most severe in no Fe case. Cellular lipid contents (Figure 3b) increased moderately in cultures with 0-2X Fe cases, but decreased in 3X and 5X cases after 24 h, although a very quick initial induction was observed in 5X Fe case at 12 h. By comparing Figures 2 and 3, iron variation resulted in the similar extent of lipid content enhancement under light and dark condition. However, the biomass was significantly reduced in the absence of light, which showed that light is important in maintaining microalgae viability and therefore dark condition could not be used as an induction stress for lipid synthesis because lipid productivity must sacrifice.

3.3. Effect of iron without CO_2

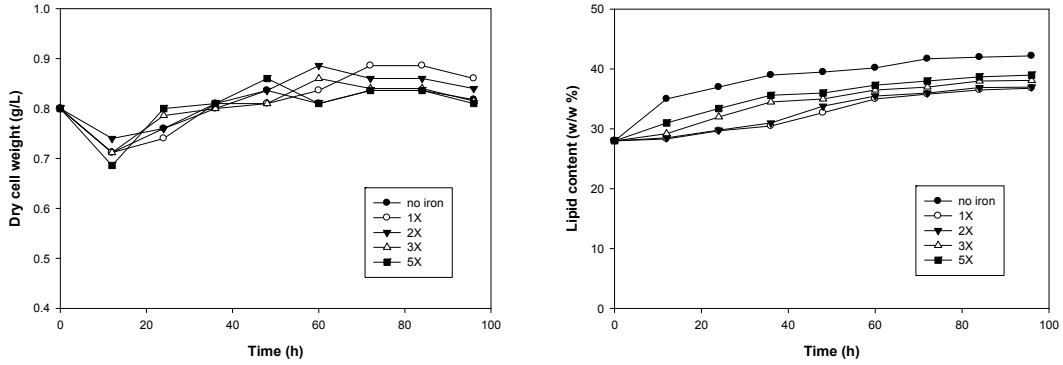
Previous iron variation experiments (Figure 2) were performed in the presence of continuous supply of 2% (v/v) CO_2 and light to maintain regular photoautotrophic metabolism. Here a set of comparative experiments were carried out in the absence of CO_2 in the light in order to see the effect of insufficient carbon source as a stress on biomass and lipid content of *D. tertiolecta*. The supply of CO_2 was stopped and the suspended mixing was maintained by sparging ambient air Therefore, the only carbon source was the bicarbonate in the initial culture medium and CO_2 (0.038% v/v) in the atmospheric air.

As shown in Figure 4a, DCW decreased slightly at the beginning, and then increased a little and showed almost stable level in all cultures. No significant increase of DCW was definitely due to the absence of continuous supply of CO_2 as a carbon source. The lipid con-

Table 1. Changes in Growth Rate, Lipid Content and Productivity Depending upon Fe Concentration and Secondary Stress Factors

Stress factor	Fe dose[a] (1X = 0.65 mg/L)	Growth rate[b] (g/L/d)	Lipid content[c] (%)	Lipid productivity (mg/L/d)
Prior Fe-starvation	3X	0.231	22.5	52.0
Normal	3X	0.344	34.6	119.0
Dark	0	-0.173	34.1	-59.0
No CO_2	0	0.012	42.0	5.0

a : the Fe dose value at which gave highest lipid content
b : the overall growth rate for 3 days
c : measured at 3 days in Stage 2



(a) (b)

Figure 4. Effects of different iron dosage on (a) cell growth and (b) lipid content. Cells were exposed to new conditions with different Fe concentrations in F/2 medium without CO_2 supply, after preparing with 0.65 mg/L as Fe (1X) under normal irradiation and CO_2 supply.

tents were gradually increased as in Figure 4b, showing 10-14% enhancement. This extent of enhancement was relatively larger than those obtained in the presence of CO_2 (Figure 2b), especially when no Fe and high Fe existed. Therefore, theses extreme Fe cases were considered as a stress to induce lipid synthesis. However, the lipid productivity, which is calculated as the multiplication of biomass concentration (mg/L) and cellular lipid content (w/w %), did not enhance accordingly because DCW was low due to the absence of CO_2 supply (Table 1).

Table 1 summarized the lipid productivity values which were obtained at different stress conditions. Here the Fe doses are the values at which resulted in the best lipid content after 3 days of cultivation in Stage 2. Based upon the overall growth rate and lipid content for 3 days, regular photoautotrophic cultivation at the elevated Fe level to 3X dose under normal irradiation and CO_2 supply resulted in the best lipid productivity as 119 mg/L/d, although cellular lipid content under stress conditions of none CO_2 was higher.

It is known that some microalgae can change lipid metabolism in response to abnormal environmental conditions. Under normal growth conditions, the assimilated carbons can go either to microalgae biomass, and lipid contents in their biomass are relatively low. When they are exposed to unfavorable environment or stress conditions, microalgae can modify their lipid biosynthetic pathways towards more formation and accumulation of neutral lipids, enabling microalgae to endure adverse conditions. Under normal growth conditions, the energy carrier and reducing power (ATP and NADPH) produced by photosynthesis are consumed in carbon fixation, resulting ADP and $NADP^+$ as electron acceptors, and in generating biomass. When major nutrients are limited, cell growth and proliferation slow down and the pool of the major electron acceptor for photosynthesis, $NADP^+$, will become depleted. Thus the surplus NADPH tends to be consumed in fatty acids biosynthesis, which is stored as triglycerides and replenishes the pool of $NADP^+$[23,24].

The results in Figures 3-4 implied that moderately high concentration of Fe is advantageous in enhancing cell growth and lipid synthesis, but that lipid content may be reduced when Fe concentration is too high beyond certain range. Previous studies had also shown that the increase in growth rate and lipid content can be observed for some microalgal species, when iron concentration is within certain ranges [19,20]. One hypothesis regarding the relationship between iron dose and lipid production is that iron concentration beyond a certain range may cause an oxidative stress. This oxidative stress can arise when iron involves in the production of hydroxyl or superoxide radicals in the presence of light[21]. Although several microalgal species were able to accumulate increased amounts of storage lipids in response to such oxidative stress[25], it is noted that such radical species would be harmful to cell viability and thus the resulting biomass productivity would not increase correspondingly. Since the effect of iron on the growth and lipid production of various microalgal strains is species dependent, the necessary optimum range of iron to maximize lipid productivity should be determined for each strain separately to avoid decline in productivity.

Table 2. Comparisons of Expected Short-term (12 h) Lipid Production Depending upon Depending upon Fe Concentration and Secondary Stress Factors Assuming that 2 g/L Cell Density was Exposed to Stage 2

Stress factor	Fe dose[a] (1X = 0.65 mg/L)	Cell density[b] (g/L)	Lipid content[c] (%)	Lipid concentration (mg/L)
Prior Fe-starvation	1X	1.62	36.2	586.4
Normal	5X	1.71	36.4	622.4
Dark	5X	1.90	38.0	722.0
No CO_2	0	1.78	35.0	623.0

a: the Fe dose value at which gave highest lipid content
b: cell density at 12 h
c: measured at 12 h in Stage 2

3.4. High cell density short-term lipid production in Stage 2

Some of the results in Figures 1 to 4 showed that lipid content tended to increase temporarily at the beginning of Stage 2, as new environment, while biomass concentrations decreased in some cases. Therefore, it is recommended that the prepared inoculation cell density in Stage 1 should be as high as possible and that the Stage 2 incubation should stop properly when the lipid content was maximized, in order to achieve increased lipid production. We estimated the achievable lipid concentration providing that the transferred cell density from Stage 1 to Stage 2 was 2 g/L, with the assumption that cells are in exponential growth phase without inoculation lag. Table 2 shows that such a short-term incubation in Stage 2 under dark condition as a secondary stress produced best lipid content as 38% and lipid concentration as 722 mg/L, under 5X Fe dose (3.25 mg/L). This concentration corresponded to lipid productivity of 1.44 g/L/d using 2 g/L cell inoculation in Stage 2. None CO_2 stress under Fe-deficient condition was also better (623 mg/L) than the results of normal culture (622.4 mg/L). Therefore, it can be concluded that the two-stage cultivation strategy is necessary to maximize lipid production because the tendencies of cell growth and lipid content are not identical under the chosen stress condition and that an appropriate termination of Stage 2 is important. The first stage is to be the preparation a high cell density under normal growth-favoring condition and the second stage is the stress condition to induce lipid accumulation in a short time.

4. Conclusions

In *D. tertiolecta*, the environment change to high Fe conditions could act as a stress for lipid synthesis and the prior Fe-starvation treatment had no significant enhancement. As a secondary stress factor, none CO_2 condition was good for lipid accumulation, but overall cell growth was sacrificed after a long-time cultivation. Exposing the cells to dark condition as a secondary stress factor also favored lipid accumulation. The short-term (12 h) incubation under 5X Fe (3.25 mg/L) and dark condition enabled us to achieve the best lipid production because the extent of cell density reduction at the early period in Stage 2 in the dark was small compared to other stress conditions. The application of two-stage cultivation was proved to be a good strategy to obtain better lipid productivity if cell growth rate was reduced when stress condition was imposed to induce lipid accumulation. Since the lipid-producing response to such stress condition is relatively short, the appropriate termination of Stage 2 incubation is necessary.

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