Effects of Plant Growth Substances on Biomass and Lipid Production in Diatoms

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Abstract—**The effects of plant growth stimulating substances like naphthalene-acetic acid (NAA), Gibberellic acid 3 (GA_3) and 6-benzylaminopurine (6-BA), or plant growth inhibiting substances like abscisic Acid (ABA) and [(2-chloroethyl) trimethylammonium chloride] (CCC), or their combinations, on biomass and lipid production in *Pinnularia gibba var. linearis* and *Chaetoceros gracilis* were studied. When applied at day 1 (stage I), treatments with NAA, GA_3 and 6-BA or their combinations enhanced biomass production but reduced lipid contents. The total lipids, however, were higher in these treatments than that in the control. 6-BA was more effective in stimulating total lipid production. No difference was found among the treatments with NAA, GA_3 or their combination. Adding NAA or GA_3 to 6-BA did not enhance biomass or lipid production compared with 6-BA alone. When applied at day 4 (stage II), growth stimulating substances increased biomass production compared with the control but did not affect total lipid production. Less biomass was obtained when the treatments were applied at day 4 compared with that applied at day 1. Treatments with growth inhibitors like ABA, CCC or ABA + CCC at day 1 reduced biomass production and total lipids, although lipid contents were increased by the treatments. When treated with ABA, CCC and ABA + CCC at day 4 , however, they did not reduce biomass production while increased lipid contents and total lipids.**

Index Terms—**Plant growth regulator biomass lipids micro algae diatoms.**

I. Introduction

Oil or lipids produced by algae has been used as an important source for biodiesel production [1], [2]. To achieve optimal oil production in algae, different approaches have been tested and used, which includes (1) Select species of algae with desirable characteristics of growth, robustness, and high lipid production; (2) Develop ideal cultural conditions that could maximize lipid production; (3) Identify metabolic pathways and key enzymes or genes that involved in lipid biosynthesis; and (4) Create new species based on the understanding on the key enzymes and genes by using molecular biology and technology.

Algae are autotrophic organisms and photosynthetic like plants [3]. It has been suggested that algae might have similar biochemical systems as higher plants and their growth and development is regulated by endogenously produced growth substances [4]-[7] and minerals [8]-[10]. How exogenous plant hormones or growth regulators affect algae growth and lipid production, however, is not clear.

Manuscript received May 18, 2013. This work was supported in part by the Natural Science Foundation of Shandong Province, China.

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Early study [9], [10] showed that the growth of diatoms like *Pinnularia gibba var. linearis* or *Chaetoceros gracilis* had two phases, a log phase (about 3 days in culture, stage I) with a linear increase in biomass production and a stable phase with no significant increase in biomass (stage II). How exogenous growth substances affect biomass and lipid production during these two stages, however, has not been studied. Here we report our study on this approach using *Pinnularia gibba var. linearis* and *Chaetoceros gracilis.*

II. Materials and Methods

A. Strain of Pinnularia gibba var. linearis and Chaetoceros gracilis

Strain of *Pinnularia gibba var. linearis* and *Chaetoceros gracilis* were collected from the seashore 40 mile north of Seattle. A strain with higher neutral lipid production rate was separated and prepared for experiment as described earlier [9], [10].

B. Cultural Conditions

Basic solution: Strain of *Pinnularia gibba var. linearis* or *Chaetoceros gracilis* was cultured in a basic sea water solution at 25℃, 12D/12L light period with a light intensity at 3500-4000lx except indicated. The cultural solution contained: sea water, $NaHCO_3$ 0.15g/L, $Na_2SiO_3(9H_2O)$0.20g/L, $NaNO_3$1.00g/L, KH_2PO_4 0.02g/L, Vitamin B_1 2.7mg/L, and Vitamin B_{12} 1.5μg/L.

C. Treatments

1) Growth promoting solution

Strain was incubated in basic solution with NAA, GA_3, 6-BA, NAA + GA_3, NAA + 6-BA, GA_3 + 6-BA, and NAA + GA_3 + 6-BA respectively. For each regulator, a concentration range from 1 to 20 μmol/L was tested and the optimum concentrations were used for comparison, which were: NAA 15 μmol/L, GA_3 10 μmol/L, and 6-BA 10 μmol/L. For the combinations, half of the optimum concentration was used in two-regulator combinations and one third was used in the three-regulator combination.

2) Growth inhibiting solution

Strain was incubated in basic solution with ABA,CCC and ABA + CCC, respectively. For each regulator, a concentration range from 10 to 200 μmol/L was tested and the optimum concentration was ABA 15 μmol/L and CCC 50 μmol/L. For the combination, half of the optimum concentration was used for each inhibitor.

3) Application time treatments

Growth promoting or inhibiting solutions were prepared separately as above and added to the incubation solution

either at day 1 (stage I), or at day 4 (end of log phase in growth, stage II), respectively. When algae was need to transfer from one solution to another, the algae was washed with sea water first, then added to the new incubation solution.

All treatments were carried out in a self-made 10 L-bioreactor and inoculates were incubated at 25℃ with 12D/12L light period and a light intensity of 3500-4000lx except specifically indicated. Each treatment had 3 replicates. Samples were harvested after 7 days incubation, centrifuged at 6000 r/min for 10 min, freeze dried, and stored in a freezer for further use.

D. Biomass Measurement

The incubation solution was mixed thoroughly, 100 mL of the solution was centrifuged 6000 r/min., supernatant was discarded and the precipitate was washed with distilled water twice, centrifuged at 6000 r/min. for 10 min., dried in an oven at 80℃ for 5 hr., placed in a desiccator for 10 hr. and weighted. The biomass of P*innularia gibba var. linearis* and *Chaetoceros gracilis* was represented as dry mass (mg D.W./L).

E. Neutral Lipid, Chrysolaminarin and Pectin Measurement

Neutral lipids were measured as described by Roesller [11]. Chrysolaminarin measurement was carried out by the method of Beattie *et al.* [8] and the method of Meijer *et al.* [12] was used to measure pectin.

Data were subjected to analysis of variance (ANOVA) using the SAS Statistical Software (SAS Institute Inc. NC, USA).

III. Results

A. Effects of NAA, GA_3 and 6-BA or Their Combinations on Biomass and Total Lipid Production When Applied at Day 1

Compared to the control, NAA , GA_3 and 6-BA or their combinations enhanced biomass production, reduced lipid contents, but increased total lipid production in *Pinnularia gibba var. linearis* (Table I). Cultures containing 6-BA had the highest level of biomass and total lipid among all the treatments. No differences were found among NAA, GA_3 and their combination. Adding GA_3 or NAA to 6-BA did not increase biomass or total lipid production compared with 6-BA alone. Results from *Chaetoceros gracilis* showed similar pattern (Table II).

B. Effects of Application Time of NAA, GA3or 6-BA on Biomass and Total Lipid Production When Applied At Day 4

When applied at day 4(stage II), all treatments stimulated biomass production,reduced lipid contents, but did not affect total lipid production compared with the control in both *Pinnularia gibba var. linearis* (Table III) and *Chaetoceros gracilis*(Table IV). There was no difference among all the treatments on both biomass production and lipid contents. Compared with treatments at day 1 (Table I and II), however, treatments at day 4 were less effective on stimulating biomass production in both *Pinnularia gibba var. linearis* and *Chaetoceros gracilis.*

TABLE I: Effects of NAA, GA_3 and 6-BA or Their Combinations Applied at Day One on Biomass and Lipid Production in *Pinnularia gibba var. linearis*

Treatments	Biomass (mg D.W/L)	Lipids Content (mg/g D.W)	Total lipid (mg/L)
Control	37.5 c	386 a	14.5 c
NAA	58.9 b	326 b	19.2 b
GA_3	66.5 ab	298 b	19.8 b
6-BA	71.7 a	338 b	24.2 a
NAA + GA_3	60.2 b	319 b	19.2 b
GA_3 + 6-BA	69.2 a	331 b	22.9 a
NAA + 6-BA	68.1 a	327 b	22.2 a
NAA + GA_3 + 6-BA	72.1 a	332 b	23.9 a
ANOVA	*	*	*

*: significant difference at $p \leq 0.05$ level. ns: not significant.

TABLE II: Effects of NAA, GA_3 and 6-BA or Their Combinations Applied at Day One on Biomass and Lipid Production in *Chaetoceros gracilis*

Treatments	Biomass (mg D.W/L)	Lipids Content (mg/g D.W)	Total lipid (mg/L)
Control	36.8 c	418 a	15.4 c
NAA	50.1 b	333 b	18.9 b
GA_3	61.4 ab	314 b	16.7 b
6-BA	73.1 a	321 b	23.5 a
NAA + GA_3	56.7 b	335 b	20.0 b
GA_3 + 6-BA	66.8 a	318 b	21.2 a
NAA + 6-BA	69.5 a	309 b	21.5 a
NAA + GA_3 + 6-BA	70.9 a	328 b	23.3 a
ANOVA	*	*	*

*: significant difference at $p \leq 0.05$ level. ns: not significant.

TABLE III: Effects of NAA, GA_3 and 6-BA or Their Combinations Applied at Day Four on Biomass and Lipid Production in *Pinnularia gibba var. linearis*

Treatments	Biomass (mg D.W/L)	Lipids Content (mg/g D.W)	Total lipid (mg/L)
Control	35.5 b	382 a	13.6 a
NAA	47.2 a	279 b	13.2 a
GA_3	45.6 a	269 b	12.3 a
6-BA	47.7 a	291 b	13.9 a
NAA + GA_3	43.6 a	276 b	12.0 a
GA_3 + 6-BA	44.3 a	284 b	12.6 a
NAA + 6-BA	45.2 a	290 b	13.1 a
NAA + GA_3 + 6-BA	42.8 a	282 b	12.1 a
ANOVA	*	*	ns

*: significant difference at $p \leqslant 0.05$ level. ns: not significant.

TABLE IV: Effects of NAA, GA_3 and 6-BA or Their Combinations Applied at Day One on Biomass and Lipid Production in *Chaetoceros gracilis*

Treatments	Biomass (mg D.W/L)	Lipids Content (mg/g D.W)	Total lipid (mg/L)
Control	34.3 b	431 a	14.8 a
NAA	43.2 a	305 b	13.2 a
GA_3	41.7 a	325 b	13.6 a
6-BA	45.2 a	321 b	14.5 a
NAA + GA_3	41.6 a	323 b	13.4 a
GA_3 + 6-BA	46.1 a	331 b	15.3 a
NAA + 6-BA	44.8 a	333 b	14.9 a
NAA + GA_3 + 6-BA	45.3 a	318 b	14.4 a
ANOVA	*	*	ns

*: significant difference at $p \leqslant 0.05$ level. ns: not significant.

C. Effects of Growth Inhibitors on Biomass and Total Lipid Production When Applied at Day 1

When applied at Day 1(stage I), no difference in biomass and lipid content was found among treatments with ABA, CCC or their combination (Table 5). All treatments reduced biomass production, increased lipid contents compared with the control. Total lipids in these treatments, however, were similar to the control. Trends were similar between *Pinnularia gibba var. linearis* and *Chaetoceros gracilis* (Table 6).

D. Effects of Growth Inhibitors on Biomass and Total Lipid Production When Applied at Day 4

When applied at day 4(stage II), no difference was found among treatments with ABA, CCC and their combinations in *Pinnularia gibba var. linearis* . Therefore, pooled data were used for comparison between regulators applied at day 1 and day 4. Inhibitors applied at day 1 (stage I) reduced biomass and total lipid production, although their lipid contents were higher (Table II). When applied at day 4 (stage II), however, inhibitors did not affect biomass production, but increased lipid contents and total lipids compared to the control. Inhibitors applied at day 4 significantly increased total lipid production compared with inhibitors applied at day 1. Results from *Chaetoceros gracilis* showed similar pattern (Data not shown).

E. Effects of a Combination of Growth Stimulators and Inhibitors on Biomass and Total Lipid Production

When 6-BA applied at day 1 and ABA or CCC applied at day 4, treatments increased biomass production, lipid contents, and total lipid production compared with both control or 6-BA, ABA, CCC alone in *Pinnularia gibba var. linearis* (Table VIII). No difference was found among treatments between ABA and CCC. The combination of 6-BA applied at day 1 plus ABA (or CCC) at day 4 produced maximum total lipids. Results from *Chaetoceros gracilis* showed similar pattern (Data not shown).

TABLE V: EFFECTS OF ABA, CCC AND THEIR COMBINATIONS APPLIED AT DAY 1 ON BIOMASS AND LIPID PRODUCTION IN *PINNULARIA GIBBA VAR. LINEARIS*

Treatments	Biomass (mg D.W/L)	Lipids Content (mg/g D.W)	Total lipid (mg/L)
Control	35.2 a	365 b	15.4 a
ABA	16.9 b	473 a	8.0 b
CCC	19.8 b	437 a	8.7 b
ABA + CCC	17.7 b	468 a	8.3 b
ANOVA	*	*	*

*: significant difference at p≤0.05 level. ns: not significant.

TABLE VI: EFFECTS OF ABA, CCC AND THEIR COMBINATIONS APPLIED AT DAY 1 ON BIOMASS AND LIPID PRODUCTION IN *CHAETOCEROS GRACILIS*

Treatments	Biomass (mg D.W/L)	Lipids Content (mg/g D.W)	Total lipid (mg/L)
Control	42.1 a	419 b	17.6 a
ABA	20.3 b	468 a	9.5 b
CCC	22.7 b	472 a	10.7 b
ABA + CCC	18.2 b	481 a	8.8 b
ANOVA	*	*	*

*: significant difference at p≤0.05 level. ns: not significant.

TABLE VII: EFFECTS OF APPLICATION TIMING FOR GROWTH INHIBITORS ON BIOMASS AND LIPID PRODUCTION (POOLED DATA) IN *PINNULARIA GIBBA VAR. LINEARIS*

Treatments	Biomass (mg D.W/L)	Lipids Content (mg/g D.W)	Total lipid (mg/L)
Control	42.1 a	372 b	15.7 b
Inhibitor (day one)	18.1 b	459 a	8.3 c
Inhibitor (day four)	41.2 a	438 a	18.0 a
ANOVA	*	*	*

*: significant difference at p≤0.05 level. ns: not significant.

TABLE VIII: EFFECTS OF STIMULATOR AND INHIBITOR COMBINATION ON BIOMASS AND LIPID PRODUCTION (POOLED DATA) IN *PINNULARIA GIBBA VAR. LINEARIS*

Treatments	Biomass (mg D.W/L)	Lipids Content (mg/g D.W)	Total lipid (mg/L)
Control	38.4 c	387 c	14.9 c
6-BA(day 1)	69.2 a	279 d	19.3 b
ABA(day 4)	19.8 d	487 a	19.6 b
CCC(day 4)	21.2 d	479 a	19.2 b
6-BA(day 1)+ABA(day 4)	60.3 b	403 b	24.3 a
6-BA(day 1)+CCC(day 4)	58.3 b	409 b	23.8 a
ANOVA	*	*	*

*: significant difference at p≤0.05 level. ns: not significant.

IV. DISCUSSION

Plant growth substances play an important role in algae growth [4]-[6]. How these growth regulators affect lipid biosynthesis and accumulation in diatoms, however, has not been studied. Our results showed that the growth promoting (NAA, GA_3, 6-BA and their combinations) and inhibiting substances (ABA, CCC and their combination) displayed different roles on biomass and total lipid production in both *Pinnularia gibba var. linearis* and *Chaetoceros gracilis*. The growth stimulating substances enhanced biomass and total lipid production, reduced lipid contents in the treatments compared with that in the control. 6-BA was most effective in stimulating biomass production and lipid accumulation among the 3 growth promoting substances being used. The growth inhibiting substances, however, reduced biomass and total lipid production, while increased lipid contents in the treatments than that in the control.

It has been well documented that growth stimulating substances enhance cell division and enlargement, while growth inhibiting substances inhibit cell division and cell growth [4]-[6]. Our results indicate that the growth stimulating substances enhanced lipid production by increasing biomass production in diatoms. 6-BA was more effective when applied at day 1 than applied at day 4(cells start to mature), indicating early application could be used to increase biomass and lipid yields in diatom culture. Since treatment with these substances reduced lipid contents in cells, however, growth promoting substances may not involved in regulating lipid biosynthesis or accumulation directly.

On the other hand, growth inhibiting substances like ABA and CCC did not increase total lipid production(majorly because of the reduced biomass), they did enhanced lipid contents, indicating ABA or CCC treatment may involved in

regulating lipid biosynthesis or accumulation process. When applied at day 1, ABA, CCC or ABA + CCC reduced biomass and total lipid production. When applied at day 4, however, these treatments increased lipid content without affecting biomass production, resulted higher total lipid production, further confirmed that ABA or CCC may involve in regulating lipid biosynthesis or accumulation.

Since stage I is the period for active cell division and growth and stage II is the period of cell maturation in Pinnularia *gibba var. linearis* or *Chaetoceros gracilis*[9,10], it is understandable that growth promoting substances like 6-BA were more effective in increasing biomass production when applied at day 1, while applying growth inhibiting substances like ABA or CCC at day 4 were more effective in stimulating lipid production without affecting biomass production in diatoms . And the most effective treatment to stimulate total lipid production, therefore, is the combination of 6-BA and ABA (or CCC) at different stages(Table 8). While 6-BA treatment applied at day 1 produce total lipid of 19.3 mg/L, ABA treatment applied at day 4 produced total lipid of 19.6 mg/L, a combination of 6-BA at day 1 and ABA (or CCC) at day 4 treatment produced about 23-24 mg/L of total lipid, indicating a very promising approach to increase total lipid production in diatoms.

V. Summary

Growth promoting substances promote biomass production and reduced lipid content, while growth inhibiting substances reduced biomass production and increased lipid content. A balance between biomass production and lipid content is needed to achieve higher lipid production and a combination of promoting substances applied at early stage (day 1) and inhibiting substances applied at late stage (day 4) resulted higher lipid production in diatoms.

How these treatments affect enzymes and genes that involve in lipid biosynthesis and accumulation in diatoms, however, needs further investigation.

Acknowledgment

This project was supported by an oversea specialist fund from Shandong government. The authors are in great debt to Dr. Brain Smith for his support in conducting this research.

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