Biochemical Stimulation of Microalgae for Enhancing Biomass Productivity

by

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(Under the Direction of Keshav Das)

ABSTRACT

The influence of 12 biochemical stimulants, namely 2-phenylacetic acid (PAA; 30 ppm), indole-3 butyric acid (IBA; 10 ppm), 1- naphthaleneacetic acid (NAA; 2.5, 5 and 10 ppm), gibberellic acid (GA3, 10 ppm), zeatin (ZT; 0.002 ppm), thidiazuron (TDZ; 0.22 ppm), humic acid (HA; 20 ppm), kelp extract (KE; 250 ppm), methanol (MeOH; 500 ppm), ferric chloride ($FeCl_3$; 3.2 ppm), putrescine (PU; 0.09 ppm), spermidine (SPD; 1.5 ppm) were prescreened for their influence on growth and metabolites for the green alga- *Chlorella sorokiniana. C. sorokiniana* responded best to phytohormones in the auxin family, particularly NAA. Combinations of phytohormones were studied which compared blends from within the auxin family as well as against other families. The following study investigated the impact on biomass and chlorophyll productivity by comparing the delivery method of one of the top performing compounds shortlisted from prior research, the synthetic auxin naphthalene acetic-acid (NAA), solubilized by ethanol or methanol. This treatment was applied to on the green alga, *Chlorella sorokiniana,* as well as a mixed consortium that includes *C. sorokiniana* along with two other wild-isolated green algae, *Scenedesmus bijuga* and *Chlorella minutissima.* It was found that the use of ethanol to dissolve NAA was the most effective to boost the biomass productivity of *C. sorokiniana,*

whereas, the mixed consortia did not demonstrate a dramatic beneficial response. The most effective treatment, $EtOH_{500ppm}$+NAA_{5ppm}, along with two other NAA concentrations ($NAA_{2.5ppm}$ and NAA_{5ppm}) were then applied to six diverse species of microalgae to determine if the treatment dosage was effective for other freshwater and marine green algae, cyanobacteria, coccolithophore and diatoms. The use of ethanol and NAA at a combined dosage of $EtOH_{500ppm+}NAA_{5ppm}$ was found to generate the highest biomass productivity for each of the species which responded positively to the treatments. If scalable, NAA and ethanol may have the potential to lower production costs by increasing biomass yields for commercial microalgae cultivation.

INDEX WORDS: Plant growth regulators, Phytohormones, Auxins; naphthalene acetic acid; Bioenergy; Biofuels; Biomass; Biostimulants; Microalgae; Phytohormones; *Chlorella sorokiniana; Haematococcus pluvialis; Phaeodactylum tricornutum; Pleurochrysis carterae; Dunaliella bardawil; Nostoc species.*

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

Introduction

The production of microalgae for renewable biomass production is gaining international recognition because algae are a sustainable, high-productivity feedstock for fuels, feeds, fibers, and value added products. It is well known that microalgae are capable of some of the highest growth rates and photosynthetic conversion rates in nature. In terms of biofuel production, algae are preferred to terrestrial crops due to their environmental robustness, and ability to grow in diverse water sources, including freshwater, brackish water, saltwater as well as many types of wastewaters from both agricultural and industrial sources (1, 2). The use of microalgae has a long history of use as a bioremediation agent in oxidation ponds at wastewater treatment facilities (3). The coupling of wastewater remediation with renewable biomass production offers significant advantages over growing food crops for energy applications (4). However, there many challenges to overcome for making microalgae-based biomass production commercially viable.

There is great commercial potential of algae biomass production for producing renewable energy and material production due to the biomass productivities ranging from 9 to 18 tons ha^{-1} yr^{-1} (5) on the low end, to 158 tons ha^{-1} yr^{-1} (6) . However, the costs associated with the construction of the cultivation system and operation are considered a major hurdle to overcome to achieve these productivities The economic feasibility The projected cost of generating renewable algal biomass has been estimated between \$1,300 ton^{-1} (7) to \$3,000 ton^{-1} for a outdoor facility producing 100 tons per year (2). By the dosage of a small but potent amount of

phytohormones, it may be possible to lower the production costs by increasing biomass productivities.

One of the primary areas of active research is the enhancement of biomass productivities for robust strains. There are many limitations that prevent algae from sustaining extremely high growth rates, such as light limitation and nutrient limitation, particularly at high cell densities. However, at lower cell concentrations where these constraints are not as pronounced, researchers are looking at ways to manipulate the photosynthetic apparatus (8), or genetically modify them to sustain high growth rates while accumulating triglycerides as storage lipids (9). The aim of this research was to investigate alternatives to genetic modification by applying growth promoting substances, such as auxins, gibberellins, cytokinins, plant extracts, micronutrients, and solvents at dosages found in the literature to be bioactive and beneficial to plant or algae growth.

For the research presented here, a group of ten compounds were selected from a literature review and these were first tested against a species from the prolific family of algae, *Chlorella.* The strain chosen for these initial experiments was *Chlorella sorokiniana* due to its fast growth rate, wide temperature tolerance, and ability to thrive in wastewaters. The strain was used to evaluate the impact of the group of growth promoters and to further examine whether these compounds are synergistic with each other to concoct an optimal biostimulant treatment. The first manuscript (Chapter 2) addressed three key questions: [1] which compound was most effective at increasing biomass productivity; [2] can a combination of the two superior performing biochemical stimulants from the same family of phytohormones act in a synergistic manner to stimulate growth further; [3] can different families of phytohormones be combined to improve biomass productivities in a synergistic manner?

Results reported in the first manuscript included treatments and combination of treatments that dramatically enhanced (insert here the % ranges of increase in growth relative to the control) the biomass productivity of *C. sorokiniana* and therefore the top performing compound was used for further studies. The second manuscript presented here reports results of the top performing compound, naphthalene acetic acid (NAA), and the impact of the solvent used (Ethanol versus Methanol) to dissolve the substance on growth and chlorophyll contents in C. sorokiniana. The most effective treatment was then assayed against 5 strains of microalgae, and a cyanobacterium to determine whether the positive impact on biomass productivity was universal or specific to *C. sorokiniana.* The strains used in this research were selected based on commercial applicability, diverse phylogenetic characteristics, high growth rate, and ability to grow in wastewater. The selection was assisted through previous research published on screening many of these strains against industrial wastewaters (10). The biomass productivity and chlorophyll productivity was analyzed for a range of NAA concentrations on each strain selected.

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CHAPTER 2

Effect of biochemical stimulants on biomass productivity and metabolite content of the microalga, *Chlorella sorokiniana*

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Abstract

The influence of 12 biochemical stimulants, namely 2-phenylacetic acid (PAA; 30 ppm), indole-3 butyric acid (IBA; 10 ppm), 1- naphthaleneacetic acid (NAA; 2.5, 5 and 10 ppm), gibberellic acid (GA3, 10 ppm), zeatin (ZT; 0.002 ppm), thidiazuron (TDZ; 0.22 ppm), humic acid (HA; 20 ppm), kelp extract (KE; 250 ppm), methanol (MeOH; 500 ppm), ferric chloride ($FeCl_3$; 3.2 ppm), putrescine (PU; 0.09 ppm), spermidine (SPD; 1.5 ppm) were prescreened for their influence on growth and metabolites for the green alga- *Chlorella sorokiniana. C. sorokiniana* responded best to phytohormones in the auxin family, particularly NAA. Combinations of phytohormones were studied which compared blends from within the auxin family as well as against other families. These treatments are $NAA_{5\ ppm}$+ $PAA_{30\ ppm}$, $NAA_{2.5\ ppm}$+ $PAA_{15\ ppm}$, $NAA_{5\ ppm}$+ $IBA_{10\ ppm}$, $NAA_{5\ ppm}$+ $GA3_{10\ ppm}$, $NAA_{5\ ppm}$+ $ZT_{1\ ppm}$ and $NAA_{5\ ppm}$+ $GA3_{10\ ppm}$+ $ZT_{1\ ppm}$. Combinations of NAA with other auxins did not have synergistic or antagonistic effects on the growth. However, combinations of compounds from different phytohormone families, such as $NAA_{5\ ppm}$+$GA3_{10\ ppm}$+ $ZT_{1\ ppm}$ dramatically increased the biomass productivity by 170% over the control followed by the treatments: $NAA_{5\ ppm}$+ $GA3_{10\ ppm}$ (138%), $NAA_{5\ ppm}$+ $ZT_{1\ ppm}$ (136%) and $NAA_{5\ ppm}$ (133%). The additional costs of combining multiple treatments may not exceed much more dramatic increases in growth over the single NAA_{5ppm} treatment. The effect of biochemical stimulants were also measured on metabolites such as chlorophyll, protein and lipids in *Chlorella sorokiniana.* Renewed interest in microalgae for biotechnology and biofuel applications may warrant the use of biochemical stimulants for cost reduction in large-scale cultivation through increased biomass productivity.

Keywords: Auxins; Bioenergy; Biofuels; Biomass; Biostimulants; Microalgae; Phytohormones.

Introduction

The passion for carbon neutral and carbon negative fuels has led many research teams to explore the potential of microalgae for biofuel and bioenergy applications. Microalgae are an attractive option as a feedstock for biofuel relative to terrestrial crops because they grow fast, can produce large quantities of lipids, carbohydrates and proteins, can grow in poor quality waters, can utilize carbon dioxide from sources such as industrial flue gases, and can remove pollutants from industrial, agricultural and municipal wastewaters (1). Most previous efforts to increase algae biomass productivities have focused only on strain selection, and supplementation of nutrients such as nitrogen, phosphorus and CO_2. Apart from natural selection, genetic engineering modalities can be used for the enhancement or manipulation of biological systems. Metabolic engineering and synthetic biology are gaining attention due to their potential to enhance living systems especially microbes for medical, agricultural, industrial and environmental applications (2). However, genetic manipulation leads to inheritable changes in a species that might affect the ecosystem adversely when used for environmental and agricultural applications. Attempts to improve microalgal biomass productivity using alternative means such as phytohormones and micronutrients has been reported a few times since the 1930's (3, 4, 5, 6, 7, 8). Although contemporary research on phytohormone action remains almost completely focused on the higher plants, there are a few studies devoted to auxins, in green algae from *Chlorella* and *Scenedesmus* genus (9, 10). Studies with *Chlorella* species show that use of natural and synthetic auxins, as well as their precursors, have considerable stimulating effects on algal growth and composition (11).

Earlier studies indicate that biochemical stimulants such as phytohormones, plant extracts, polyamines and chemicals offer significant potential to enhance microalgae productivity

(3, 5, 12, 13, 14, 15, 16). The average biomass productivity reported in the literature for conventional commercial-scale open pond systems are in the range of 8.5 – 21 g m^{-2} d^{-1}(17). This translates to approximately 18 to 36 dry t ha^{-1} $year^{-1}$. Increasing algal productivity from 21 g m^{-2} d^{-1} to a higher level can reduce the cost of biomass production and increase the economic viability of biomass production from algae. Thus the goal of this research was to first prescreen 12 biochemical stimulants categorized as phytohormones, plant extracts, polyamines and micronutrients and their combinations on biomass and chlorophyll productivity of the alga-*Chlorella sorokiniana* which was used as a model organism. Additional experiments were performed to identify whether combinations of the most effective compounds from the same and from different families would have any synergistic effect by measuring both biomass and chlorophyll growth parameters as well as compositional content, such as protein and lipids. By identifying potential biostimulants and their combinations which can enhance biomass productivity, it may be possible to lower production costs to increase the profitability of industries producing algae for food, feed, biomaterials, nutraceutical and pharmaceutical applications.

Materials and Methods

Strain and culture maintenance

Chlorella sorokiniana (UTEX 2805) was obtained from UTEX Culture Collections and maintained in BG11 growth medium (18). The pH of the BG11 culture medium was adjusted to 7.5±0.2 before inoculation and the alga was maintained in a temperature controlled growth chamber at 25±1^{o}C and 100±10 μmoles m^{-2} s^{-1} light intensity provided by cool white fluorescent (6500 K) T-8 bulbs with light:dark cycles of 12:12 h .

Selection of the biochemical stimulants

Biochemical stimulants were short listed on the basis of a literature survey, where the top performers were selected for each categorical type of growth promoters (Table 1). Samples of 1-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), gibberellic acid-3 (GA3) and kelp extract (KE) were obtained from Super-Grow Plant Care, Montreal, Canada (www.super-grow.biz). TerraVive humic acid liquid (HA) was used with a total organic acid content of 16% with a 50/50 humic to fulvic acid ratio and was obtained from Natural Environment Systems, LLC, Dallas, TX, USA (www.naturalenviro.com). The trans-isomer of zeatin (ZT) and thidiazuron (TDZ) was obtained from bioWORLD, GeneLinx International, Inc, Ohio, USA. Ferric chloride (FeCl) and methanol (MeOH) were obtained from Fisher Scientific, Pittsburgh, PA, USA. 2-phenylacetic acid (PAA), putrescine (PU), and spermidine (SPD) were obtained from Sigma-Aldrich, St. Louis, MO, USA.

Experimental conditions

All the experiments were conducted in 250 mL Erlenmeyer flasks with 100 mL BG11 growth medium supplemented with the biochemical stimulants to be tested. Growth studies were performed in a temperature controlled growth chamber as mentioned earlier. For the purpose of screening, previously reported dosages that demonstrated growth enhancing effects were used.

The research presented in this paper is a compilation of three experiments that were performed with *C. sorokiniana.* The first preliminary screening experiment was conducted using ten individual biochemical stimulants for a 10 day static culture growth study with *C.sorokiniana*. This preliminary experiment was followed by a 10 day static culture growth study using the most productive auxins from the first screening experiment along with amines, namely putrescine and spermidine. Individual amines such as putrescine and spermidine along with mixtures of the top performing auxins on day 5 and day 10 were evaluated in this

experiment for their effect on the growth of *C. sorokiniana.* The third experiment was conducted to evaluate NAA at a higher concentration and its combination with IBA, GA3 and ZT. Details of treatments used in all the three experiments are summarized in Table 2. In all experiments, the cultures were sampled on day 5 and day 10 based upon previous data (not shown) which indicates that day 5 sampling will represent initial exponential phase while the day 10 sampling represents the culture entering the late exponential phase. Each treatment was performed in triplicates, and the parameters measured were given as the mean with respective standard deviations for each set of triplicates shown in the figures.

Known quantity of each biostimulant was dissolved in 500 µL of ethanol and 500 µL of deionized water to obtain the desired concentrations mentioned in Table 2, whereas. HA, KE and FeCl, were dissolved in deionized water only. The addition of only 500 µL of ethanol did not appear to be toxic to algal growth productivities. All biostimulants were filter sterilized using a 0.22 µm Whatman syringe filter and then added to the sterilized BG11 growth medium aseptically. Each biochemical stimulant was added to the growth medium alone or in combination as per the treatment listed in Table 2. Exponentially growing culture of *C. sorokiniana* was used as the inoculum with an initial cell concentration of 0.01 g L^{-1} for the first preliminary screening experiment, and 0.08 g L^{-1} for the experiments auxin combinations and multi-hormone experiments. After inoculation, the flasks were incubated for 10 days in the growth chamber.

Analyses

Biomass was determined by filtering 25 mL of algal culture through a preweighed Whatman GF/C filter (4.7 cm diameter; 1.2 µm pore size). The filter was washed with 10 mL of 0.65 M ammonium formate solution to remove excess salts and dried overnight at 60 ^{o}C in a hot

air oven. Dried filter with biomass was cooled in a desiccator and weighed again to estimate the final dry weight. For chlorophyll *a* estimation, 10 mL of homogenized algal culture was centrifuged at 5000 rpm for 10 minutes and the algal pellet was exhaustively extracted with hot methanol (95% v/v) until it was colorless. The amount of chlorophyll *a* extracted in the methanol was determined spectrophotometrically according to the method described by Porra et al (12) using the following equation:

$$\text{Chlorophyll } a \text{ } (\mu g \text{ } mL^{-1}) = 16.29 \times OD_{665} - 8.54 \times OD_{652}$$

The ultimate analysis of 2 mg of dry algal sample was performed using a LECO CHNS932 analyzer to estimate the nitrogen content of the biomass. Measured percentage values of nitrogen were multiplied with the nitrogen-to-protein conversion factor of 6.25 to estimate the protein content. Lipid content was measured gravimetrically with an Ankom XT10 automated extraction system using hexane as solvent (19). The same filters used for the biomass measurements (from 25 mL of culture) were used for the lipid estimation as they provided the final dry weight (W_1). The filters were then placed into Ankom XT4 extraction bags and sealed with the impulse sealer. After drying, the extraction bags were held in a resealable plastic bag with desiccant material while each individual bag was removed and carefully weighed (W_2) to obtain the dry weight before extraction. Extraction bags were then placed into the Ankom extractor and extraction was performed for 2 h at 105^{o}C with hexane as solvent. Bags were then transferred to a forced-air oven and dried at 60^{o}C overnight, then cooled in a dessicator and weighed (W_3). The following equation was used to calculate the lipid content of algal samples:

$$\text{Lipid } \% = (W_2 - W_3) / W_1 \times 100$$

Results and Discussion

The preliminary screening showed that seven of the 10 treatments had marked increase in productivity compared to the control (Figure 1a). The least effective treatment (Kelp Extract) had an inhibitory impact and reduced productivity by 44%. This decrease is possibly due to the increased turbidity of the medium affecting light penetration or presence of molecules that might be inhibitory to freshwater algae. The best performing compound was $NAA_{5\ ppm}$ which recorded a biomass productivity of 0.042 g L^{-1} d^{-1} compared to 0.018 g L^{-1} d^{-1} in the control (no biostimulants) and showed a 133% increase in biomass productivity on day 10 (Fig. 1 a). Data collected on day 5 indicate NAA did not have a higher impact in the first 5 days and recorded only a 64% increase in biomass production over the control during that period. This could be a result of a longer acclimatization phase required by the algal cells. Higher biomass productivity exhibited by NAA treatment between day 5 and day 10 could be due to prolonged exponential phase resulting in net increase in biomass productivity over the 10 days tested. It has been reported that auxins suppress the process of oxidation and degeneration of chlorophylls and carotenoids thus delaying algal senescence (9).

In contrast to the above, a 118% increase in biomass productivity in the first 5 days was observed in a related auxin, $PAA_{30\ ppm}$, but the productivity declined thereafter resulting in a 10 day average on par with the control. This result suggests a possible effect of PAA in shortening initial lag period before initiation of cell division. The third auxin used in the experiment $IBA_{10\ ppm}$, recorded an increase in biomass production over control measured at day 5 and day 10 of 91% and 56%, respectively. Treatments TDZ, HA, MeOH, ZT and GA3 recorded 83%, 72%, 69%, 67% and 61% increase in average productivity over 10 days relative to control. The average biomass productivity over days 0 to 5 showed an increase of 18% for ZT, and 9 % for

both TDZ and HA, while GA3 was the same as the control. Except PAA, IBA and NAA, no other treatment showed more than 50% increase in average biomass productivity over the first 5 days.

The non auxin phytohormones, such as the cytokinin compound ZT andTDZ, demonstrated substantial increase in productivity relative to the control over 10 days which was better than PAA. It should be noted that both these treatments (ZT and TDZ) had very low dosage. Uneven dissolution during preparation could have rendered ZT not as effective as NAA. From the biomass data, the auxins such as NAA and IBA were most effective for enhancing growth (Figs. 1 a). The results showed substantial increases in chlorophyll *a* for the auxin group on the final sampling day 10. The highest increase in chlorophyll *a* productivity was exhibited by NAA between day 5 and day 10 attaining a 395% increase over the control. In comparison, treatments PAA, IBA and GA3 showed an increase of 262%, 240% and 203%, respectively (Fig. 1b). Increase in chlorophyll *a* content observed in the treatments with MeOH, HA and TDZ were all approximately 160%. Interestingly, IBA was the only treatment that showed substantial increase of approximately 55% in chlorophyll *a* over 5 days relative to control. Surprisingly the strongest inhibition was found in NAA showing a 79% decrease relative to control in the first 5 days. However, NAA recorded substantial increase in chlorophyll *a* content over the 10 days, indicating a significant increase in growth rate and chlorophyll synthesis between day 5 and 10.

Upon examining the comparison of changes in biomass and chlorophyll *a*, the auxins demonstrated an interesting phenomenon on day 5. IBA seems to preferentially increase chlorophyll *a* synthesis; whereas, the other auxin treatments (i.e. NAA and PAA) showed a significantly lower chlorophyll *a* synthesis than control, while simultaneously recording substantially higher biomass production over the first 5 days. With the auxin treatments there

apparently exists some mechanism that can reduce pigment production while promoting significant increases in biomass productivity. Grossmann (20) proposed that both natural and synthetic auxins induce the phytohormone ethylene, which in turn triggers biosynthesis of another plant hormone abscisic acid (ABA). This model proposes that auxins at high concentrations increase the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, the key regulatory enzyme in ethylene biosynthesis. Production of significant amounts of ethylene might lead to the degradation of photosynthetic pigments (21). Hence, the strong inhibition observed during the first 5 days of growth in the treatments with $NAA_{5\ ppm}$ and $PAA_{30\ ppm}$ could be a dose dependent response which was overcome after adaptation of the cells. Biomass with less pigment is attractive because in downstream processing of algal biomass, chlorophyll pigments are known to interfere with lipid extraction and biodiesel conversion. Hence the biochemical stimulant identified here that simultaneously leads to higher biomass production and lower pigment production is a useful contribution to advancing biofuel applications of microalgae.

Auxins have a stimulative effect on reactions of bonding CO_2 to 1,5-biphosphoribulose and photosynthetic phosphorylation (8). The increase in intensity of photosynthesis reactions correlates well with higher contents of chlorophylls. Several authors indicate that low concentrations of synthetic auxins (2,4-D, NAA, PAA) stimulate the photosynthetic rate and chlorophylls as well as carotenoids synthesis in green algae *Chlorella pyrenoidosa*, *Scenedesmus acuminatus* and *Scenedesmus quadricauda*. A possible explanation for this and the differential response observed in auxins sensitivity of *C. sorokiniana* in the present study is that the variety and content of auxin receptor proteins within the cells differ in different species, as auxins act as a signal substance in eukaryotic algae (6).

Bradley and Cheney (22) suggested that auxins be combined with cytokinins to enhance growth of cultured seaweed cells. They found that zeatin, phenylacetic acid, and naphthalene acetic acid can stimulate growth alone or in combination with other plant growth regulators. Figures 2 a & b show the effect of amines (putrescine, spermidine) and auxins (naphthalene acetic acid and phenylacetic acid) and their combinations as biostimulants of biomass productivity and chl *a* content in experiment II. The treatments with $NAA_{5\ ppm}$+ $PAA_{30\ ppm}$, $NAA_{2.5\ ppm}$+ $PAA_{15\ ppm}$ and $NAA_{2.5\ ppm}$ showed 104%, 72% and 64% increase in average biomass productivity over the first five days. However, it was only 36%, 16% and 28% higher than the control over the tens days. This suggests a tendency of auxins to stimulate biomass growth by reducing generation time of *C. sorokiniana* thus contributing to greatly reducing the initial lag phase. The treatment with $NAA_{5\ ppm}$+ $PAA_{30\ ppm}$ recorded 114% and 87% increase in average chlorophyll *a* content over the 5 and 10 day growth period, respectively when compared to the control. Although $NAA_{2.5\ ppm}$+ $PAA_{15\ ppm}$ showed 66% and 74% increase in chl *a* content over control on day 5 and 10, respectively, its performance was only marginally better than $NAA_{2.5ppm}$ over the 10 day period. Despite the intermediate boosts in biomass productivity on day 5, this effect tapered off and productivity decreased by day 10 and the final biomass density was not substantially different from single auxin treatments. The results suggest that the auxin combinations did not result in any statistically significant antagonistic, additive or synergistic effect over the entire growth period which is in agreement with previously reported results by Vance (15) who used combinations of three phytohormones, namely IAA, GA and kinetin with *Chlorella pyrenoidosa*.

Diamines and polyamines such as putrescine and spermidine are specific regulators of cellular and metabolic processes which can stimulate active transport of metabolites, and affect

the functioning of enzymes and ion pumps in the cellular membranes; they also stimulate the photosynthetic process (12). Polyamines participate with a common mechanism in the regulation of the photosynthetic apparatus during photoadaptation and acclimation to high-CO_2 concentrations. Logothetis et al. (23) reported that the addition of putrescine to cultures grown in high-CO_2 atmospheres enhanced biomass production by increasing active reaction center density and chl *a/b* ratio and reducing the size of LHCII. In contrast to the above, exogenously supplied putrescine in cultures grown in low CO_2 atmospheres did not result in significant differences in the structure of the photosynthetic apparatus and biomass production. In the current study, the treatments with putrescine recorded only 17% increase in average biomass productivity on at the 10^{th} day over control which is in agreement with the earlier findings reported for low-CO_2 grown cultures. Czerpack et al. (12) opined that polyamines used in the range of 10^{-6} to 10^{-4} M (0.8 to 8.8 ppm) stimulate the growth of *Chlorella vulgaris*. In their study, the most stimulating influence on metabolism was found when using spermidine and putrecine at a concentration of 10^{-4} M (14.5 ppm). However, in the current study, treatments with putrescine recorded a marginal increase in average biomass productivity (0.092 g L^{-1} d^{-1}) over 10 days relative to control (0.082 g L^{-1} d-1), whereas, the other polyamine spermidine recorded lower biomass productivity (0.074 g L^{-1} d^{-1}) than the control.

The combination of stimulatnts $NAA_{5\ ppm}$+ $GA3_{10\ ppm}$+ $ZT_{1\ ppm}$ recorded the highest biomass productivity (0.143 g L^{-1} d^{-1}) and showed 170% increase over control (0.053 g L^{-1} d^{-1}) on day 10 (Figs. 3 a). The treatments with $NAA_{5\ ppm}$+ $GA3_{10\ ppm}$, $NAA_{5\ ppm}$+ $ZT_{1\ ppm}$ and $NAA_{5\ ppm}$+ $IBA_{10\ ppm}$ showed 138% , 136% and 75% increase in average biomass productivity relative to control over 10 days. However, the average biomass productivity in the first five days in all the combinations showed only 27-33% increase over control. Similarly all NAA combinations

with GA3, IBA and ZT showed marginal or no increase in chl *a* content over the first five days. Combinations NAA $_{5\ ppm}$+ GA3$_{10\ ppm}$, NAA $_{5\ ppm}$+ ZT$_{1\ ppm}$ and NAA$_{5\ ppm}$+ GA3$_{10\ ppm}$+ ZT$_{1\ ppm}$ recorded 109%, 108% and 35% increase in chl *a* content over control in the first 10 days (Fig. 3b). In contrast to the behavior when auxins alone were combined with either GA3 and ZT, the combination of three biostimulants, i.e NAA$_{5\ ppm}$+ GA3$_{10\ ppm}$+ ZT$_{1\ ppm}$, showed a much smaller increase in chl *a* content compared to the increase in biomass over the first 10 days.

The addition of seconday auxins, such as PAA$_{30\ ppm}$ or IBA$_{10\ ppm}$, to NAA did not increase the final day biomass productivity beyond what NAA$_{10\ ppm}$ demonstrated indicating that there is no significant advantage in combining these other two auxins to NAA; whereas, combining GA3$_{10\ ppm}$ (0.126 g L^{-1} d^{-1}) , ZT $_{1\ ppm}$ (0.125 g L-1 d-1) , a gibberellin and cytokinin respectively, did enhance the biomass productivity substantially over NAA$_{10\ ppm}$ (0.093 g L^{-1} d^{-1}).

NAA in all combinations with GA3, IBA and ZT showed only marginal increase in average biomass productivity between day 0 and 5 relative to control whereas the increase was significant between day 5 and 10. The same trend was observed for chl *a* productivity except the treatments NAA + GA3 for day 5 and NAA + IBA for day 10. In general the NAA treatment with IBA showed strong inhibition on chl *a* synthesis. The rate of increase in biomass productivity drastically reduced between day 5 and 10 in all the NAA treatments in combination with PAA indicating the role of PAA in shortening the lag period to enhance biomass productivity within a short cultivation period. Treatment NAA$_{5\ ppm}$ and NAA$_{10\ ppm}$ treatments showed substantial increase in chl *a* productivity between day 5 and 10.

Figure 4 shows the effect of biochemical stimulants on the protein and lipid content of *C. sorokiniana.* Treatments NAA$_{5\ ppm}$+ IBA$_{10\ ppm}$, NAA$_{5\ ppm}$+ ZT$_{1\ ppm}$ and NAA $_{5\ ppm}$+ GA3$_{10\ ppm}$+ ZT$_{1\ ppm}$ showed 19 to 20% increase in protein content whereas NAA$_{5\ ppm}$+ GA3$_{10\ ppm}$ recorded

only 7% increase in proteins. An increase in protein, carbohydrate and lipid content in algae is generally observed in algal cells in response to stress induced by temperature, depletion of nutrients such as nitrogen and phosphorus from the growth medium and salinity. The results in figure 4 show comparisons of lipid and protein content results on the effect of combined dosages performed in experiments 2 and 3. In the present study, the treatments did not show any significant increase or decrease in lipid and protein content relative to the control. The lipid content was approximately 5 to 7% of the total biomass for all treatments. These results indicate no major change in biochemical composition of *C. sorokiniana* resulting from the use of the biostimulants studied here.

Experiments in this study were conducted in static batch cultures which indicated that the biochemical stimulants such as auxins, gibberelins and cytokinins individually and in combination stimulated microalgal growth and doubled the biomass production compared to the untreated cells and indeed have a role in controlling growth and development of algae. The challenges encountered and envisioned for the use of biostimulants for various commercial applications are [1] developing blends of biostimulants that enhances the metabolite productivities and yields, i.e. predominately stimulating lipids, carbohydrates, proteins and pigment synthesis [2] developing universal mixtures of biochemical stimulants for various species of algae (fresh water and marine forms) to deliver an optimal dose for maximum stimulatory effect [3] preventing bacterial and fungal contamination in the growth medium due to addition of biochemical stimulants and [4] reducing the cost of biochemical stimulants for large-scale algae cultivation by optimizing the dose.

The best case scenario for practical application and simplicity is the NAA treatment at 5 ppm concentration, which recorded a 2.3 times increase in biomass productivity. For a

commercial-scale production system the average biomass productivity for raceway ponds is 30 t ha^{-1} with a production cost of \$150,000 ha^{-1}, based upon the estimated cost of \$135 kg^{-1} for the product in bulk. The requirement per hectare is 7.4 kg of NAA for a 5 ppm concentration translating to a cost of approximately \$1,000 ha^{-1}. If the effect is scalable, then an investment of \$1,000 ha^{-1} could more than double the biomass productivity at an additional 0.5% of the production cost, which could reduce production costs from \$5,000/ton to \$2,516/ton.

Conclusion

The treatment using a combination of $NAA_{5\ ppm}$+ $GA3_{10\ ppm}$+ $ZT_{1\ ppm}$ recorded highest average biomass productivity followed by $NAA_{5\ ppm}$+ $GA3_{10\ ppm}$, $NAA_{5\ ppm}$+ $ZT_{1\ ppm}$ and $NAA_{5\ ppm}$ over the ten days of growth. These treatments showed approximately 2.3 to 2.7 times increase in biomass productivity over control. Treatment $PAA_{30\ ppm}$ recorded the highest biomass productivity followed by NAA_{5ppm}+ $PAA_{30\ ppm}$ and $IBA_{10\ ppm}$ over the first 5 days, indicating a shortened lag period as the time required for initiation of cell division was reduced significantly. This study suggests that phytohormones can prolong the exponential growth and shorten initial lag. However, this response may be dependent on the dose, combination of biochemical stimulants, CO_2 supply and the strain. The initial algal concentration was found to influence the treatments and stimulation of growth. The initial algal concentration was 0.01 g L^{-1} for experiment 1, while it was 0.08 g L^{-1} for experiments 2 and 3, respectively. More studies will be necessary to elucidate the impact of the inoculum density as this may play an important role in regulating algal growth response to the addition of biochemical stimulants. The experiments were conducted using a nitrogen rich nutrient medium (BG 11) with limited carbon supply. However, future experiments will be conducted evaluating biochemical stimulation with

supplemented carbon and nutrient deprivation, such as bubbling CO_2 at various concentrations and N-limited conditions, respectively. Our preliminary studies may lead to developing a range of ideal mixtures of various biochemical stimulants for enhancing biomass productivity and various high value products such as lipids, proteins, carbohydrates and nutraceutical compounds such as beta-carotene and astaxanthin. However, more studies are required to optimize the dosages and combinations to enhance biomass production in fresh water and marine algae. This technology, if proven effective at large scale, will have wider applications for wastewater treatment, carbon cycling, biofuel, bioenergy and biotechnological applications in the future.

Acknowledgements

We gratefully acknowledge the support of the U.S. Department of Energy and State of Georgia that funded this project as part of the Biorefining and Carbon Cycling research program.

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Table 1. Effect of various biochemical stimulants on algae

Biostimulant	Type	Dosage (ppm)	Effect	Reference
Phenylacetic Acid	Auxin	30	Increased growth by 261% in *Chlorella vulgaris*	3
		$1.3x10^{-4}$	Increased growth by 59% in *Nostoc muscorum*	24
		$1.3x10^{-3}$	Increased growth by 48% in *Tolypothrix tenuis*	24
Indole-butryic Acid	Auxin	6.7	Increased growth by 166% in *Chlorella vulgaris*	3
		0.1	Increased growth by 55% in *Acacia mangium*	25
Naphthalene acetic Acid	Auxin	3.3	Increased growth by 172% in *Chlorella vulgaris*	3
Gibberellic Acid	Gibberellin	5	Carbohydrate content increased by 95% in wheat seedlings	4
		5	Increased growth by 14% in wheat seedlings	4
		100	Stem length of soybeans increased by 300% over control	26
		100	Increased dry biomass in pinto bean tops by 35%	26
		200	458% increase in glucose content in barley endosperm	27
Zeatin	Cytokinin	0.5	539% increase in fresh weight in radish cotyledon	28
		0.002	115% increase in cell number in *Chlorella vulgaris*	29
Thidiazuron	Cytokinin	$7x10^{-4}$	300% increase in growth of soybean callus	30
		0.22	86% increase in growth of radish cotyledon	30
Humic acid	Extract	60	Increased chlorophyll content by 86% in *Botrydium sp.*	5
		4	Increased growth by 1,500% in *Chlorella sp.*	31

Kelp extract	Extract	1	85% increase in dry weight and enhanced mineral uptake in *Secale cereale*	13
		2	Enhanced plant yield, dry weight, and germination in swiss chard	14
		20	Increased plant yield by 19-133% in *Fragaria vesca*	32
		20	Increased plant yield by 44% in *Fragaria vesca*	33
Methanol	Solvent	500	340% increase in growth after 40 h in *Scenedesmus obliquus*	34
		500	Enhanced photosynthesis by 100% after 24 h in *Scenedesmus obliquus*	35
		50	One time single dose increased growth rate by 480% in *Chlorella minutissima*	36
		5	Split application in daily doses increased growth rate by 720% in *Chlorella minutissima*	36
Ferric Chloride	Micronutrient	3.2	625% increase in lipid content of *Chlorella vulgaris*	7
Putrescine	Polyamine	0.9	Increased growth by 50% in *Chlorella vulgaris*	12
		0.09	Increased growth by 69% in *Acacia mangium*	25
		0.044	Increased growth by 60% in *Dunaliella primolecta*	16
		0.044	Increased Chlorophyll *a* by 176% in *Dunaliella primolecta*	16
		0.026	Increased growth by 67% in tomato dry weight	25
		0.07	Increased growth by 50% in Tomato	25
Spermidine	Diamine	0.07	Increased growth by 42% in *Dunaliella primolecta*	16
		0.07	Increased Chlorophyll *a* by 290% in *Dunaliella primolecta*	16

Table 2. Biochemical stimulants and dosages used on *Chlorella sorokiniana* for each of the 10 day experiments

Biochemical stimulants	Type of Stimulant	Concentration (ppm)
Experiment I		
Phenylacetic acid (PAA)	Auxin	30
Indole butyric acid (IBA)	Auxin	10
Napthalene acetic acid (NAA)	Auxin	5
Gibberellic acid (GA3)	Gibberellin	10
Zeatin (ZT)	Cytokinin	0.002
Thidiazuron (TDZ)	Cytokinin	0.22
Humic acid (HA)	Humate	20
Kelp extract (KE)	Plant Extract	250
Methanol (MeOH)	Chemical	500
Ferric chloride ($FeCl_3$)	Micronutrient	3.2
Experiment II		
Putrescine (Pu)	Polyamine	0.09
Spermidine (SPD)	Diamine	1.5
$NAA_{2.5}$	Auxin	2.5
NAA_5+ PAA_{30}	Auxin	5 + 30
$NAA_{2.5}$+ PAA_{15}	Auxin	2.5 + 15
Experiment III		
NAA_{10}	Auxin	10
NAA_5+IBA_{10}	Auxin	5 + 10
NAA_5+$GA3_{10}$	Auxin+Gibberellin	5 + 10
NAA_5+$ZT_{.002}$	Auxin+Cytokinin	5 + 1
NAA_5+GA3+$ZT_{.002}$	Auxin+Gibberellin+Cytokinin	5 + 10 + 1

Legends for Figures

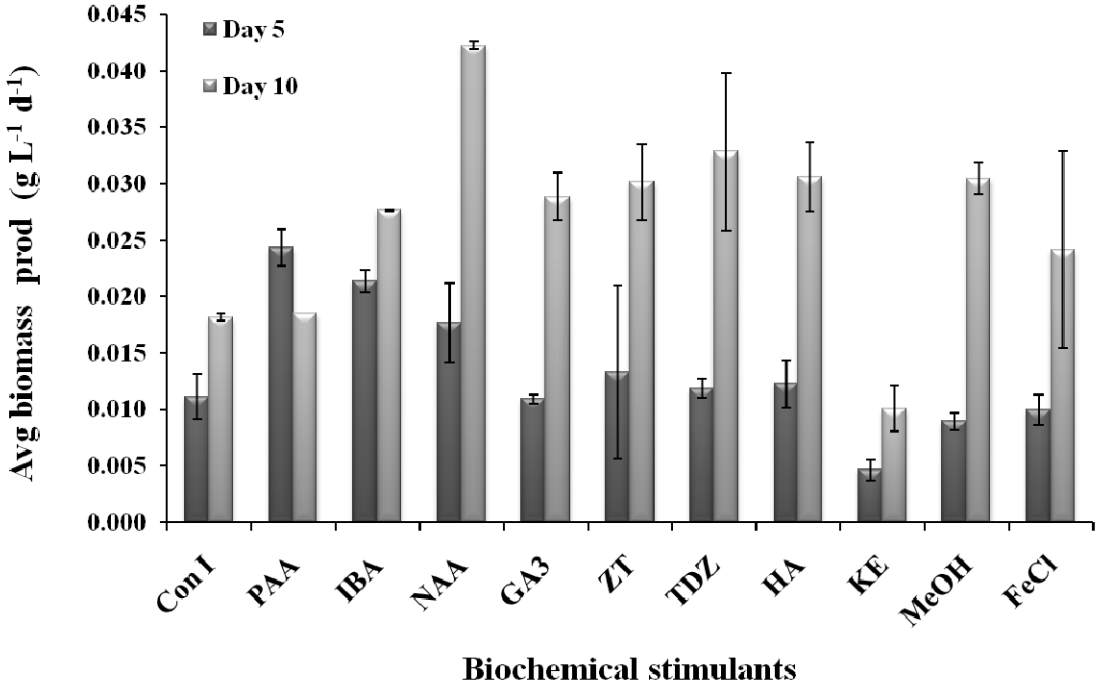
Fig. 1 a & b. Results from preliminary biochemical stimulant prescreen a) Increase in biomass productivity compared to control and b) Increase in Chl a productivity compared to control. *PAA* - Phenylacetic acid $_{30\ ppm}$; *IBA*- Indole butyric acid $_{10\ ppm}$; *NAA* - Naphthaleneacetic acid $_{5\ ppm}$; *GA3* - Gibberellic acid $_{10\ ppm}$; *ZT*- Zeatin $_{0.002\ ppm}$; *TDZ* - Thidiazuron $_{0.22\ ppm}$; *HA* - Humic acid $_{20\ ppm}$; KE - Kelp extract $_{250\ ppm}$; MeOH – $_{Methanol\ 500\ ppm}$; *FeCl* - Ferric chloride $_{3.2\ ppm}$. All the data reported as means ± standard deviation of triplicates.

Fig. 2 a & b. Effect of various polyamines, auxins and their combinations on a) average biomass productivity and b) average chl *a* over 5 days and 10 days of algal growth. *Con*- Control (BG11 medium without any biochemical stimulants); *PU* – Putrescine $_{0.09\ ppm}$; *SPD* – Spermidine $_{1.5\ ppm}$; *NAA2.5* - $NAA_{2.5\ ppm}$; *NAA5+PAA30* - $NAA_{5\ ppm}$ + $PAA_{30\ ppm}$; *NAA2.5+PAA15* - $NAA_{2.5\ ppm}$ + $PAA_{15\ ppm}$. All the data reported as means ± standard deviation of triplicates.

Fig. 3 a & b. Effect of auxin combinations and auxin with cytokinin and gibberellin combinations on a) average biomass productivity and b) average chl *a* over 5 days and 10 days of algal growth. *Con*- Control (BG11 medium without any biochemical stimulants); *NAA10* - $NAA_{10\ ppm}$; *NAA+GA3*- $NAA_{5\ ppm}$ + $GA3_{10\ ppm}$; *NAA+IBA* - $NAA_{5\ ppm}$ + $IBA_{10\ ppm}$; *NAA+ZT* - $NAA_{5\ ppm}$ + $ZT_{1\ ppm}$; *NAA+GA3+ZT* - $NAA_{5\ ppm}$ + $GA3_{10\ ppm}$ + $ZT_{1\ ppm}$. All the data reported as means ± standard deviation of triplicates.

Fig. 4. Effect of various biochemical stimulants and their combinations on lipid and protein content of the algae on the 10^{th} day of growth. *CON II* – Control from experiment II ; *CON III* – Control from experiment III; *PU* – Putrescine $_{0.09\ ppm}$; *SPD* – Spermidine $_{1.5\ ppm}$; *NAA2.5* - $NAA_{2.5\ ppm}$; *NAA5+PAA30* - NAA $_{5\ ppm}$ + $PAA_{30\ ppm}$; *NAA2.5+PAA15* - $NAA_{2.5\ ppm}$ + $PAA_{15\ ppm}$; *NAA10* - $NAA_{10\ ppm}$; NAA+GA3 - $NAA_{5\ ppm}$ + $GA3_{10\ ppm}$; *NAA+IBA* - *NAA* $_{5\ ppm}$ + IBA $_{10\ ppm}$; *NAA+ZT* - $NAA_{5\ ppm}$ + $ZT_{1\ ppm}$; *NAA+GA3+ZT* - $NAA_{5\ ppm}$ + $GA3_{10\ ppm}$ + $ZT_{1\ ppm}$. All the data reported as means ± standard deviation of triplicates.

Fig. 5. Effect of various biochemical stimulants and their combinations on increases in biomass density compared to their respective controls for day 5 and day 10. *PU* – Putrescine $_{0.09\ ppm}$; Spermidine – SPD $_{1.5\ ppm;}$ *NAA2.5* - $NAA_{2.5\ ppm}$; *NAA5+PAA30* - NAA $_{5\ ppm}$ + $PAA_{30\ ppm}$; *NAA2.5+PAA15* - $NAA_{2.5\ ppm}$ + $PAA_{15\ ppm}$; *NAA10* - $NAA_{10\ ppm}$; NAA+GA3 - $NAA_{5\ ppm}$ + $GA3_{10\ ppm}$; *NAA+IBA* - *NAA* $_{5\ ppm}$ + IBA $_{10\ ppm}$; *NAA+ZT* - $NAA_{5\ ppm}$ + $ZT_{1\ ppm}$; *NAA+GA3+ZT* - $NAA_{5\ ppm}$ + $GA3_{10\ ppm}$ + $ZT_{1\ ppm}$. All the data reported as means ± standard deviation of triplicates.



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b)

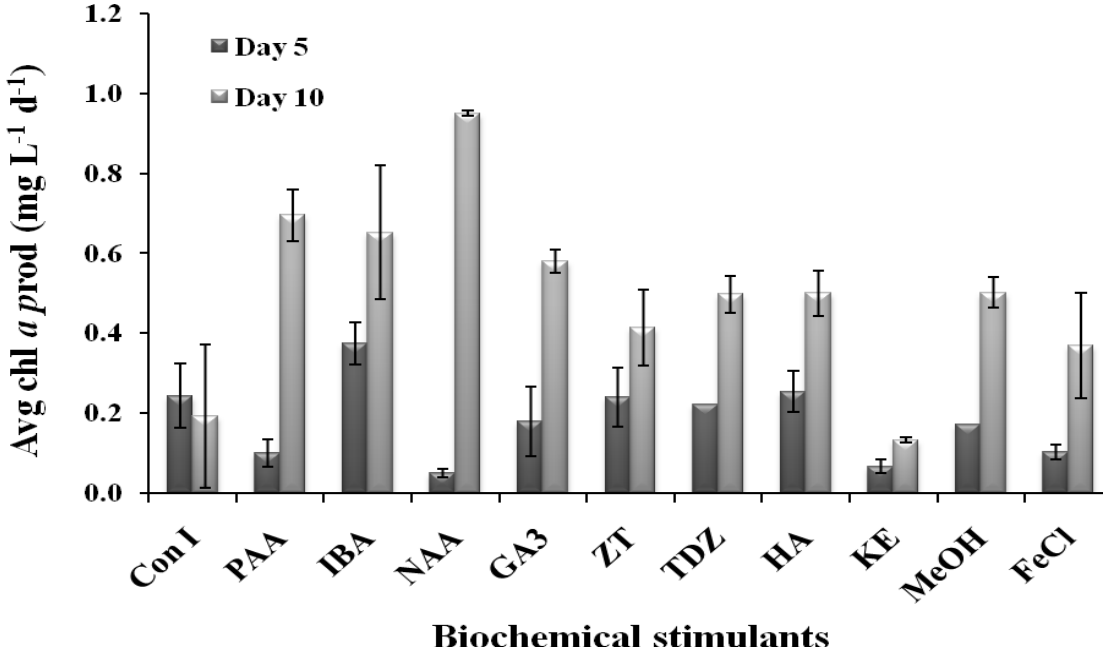
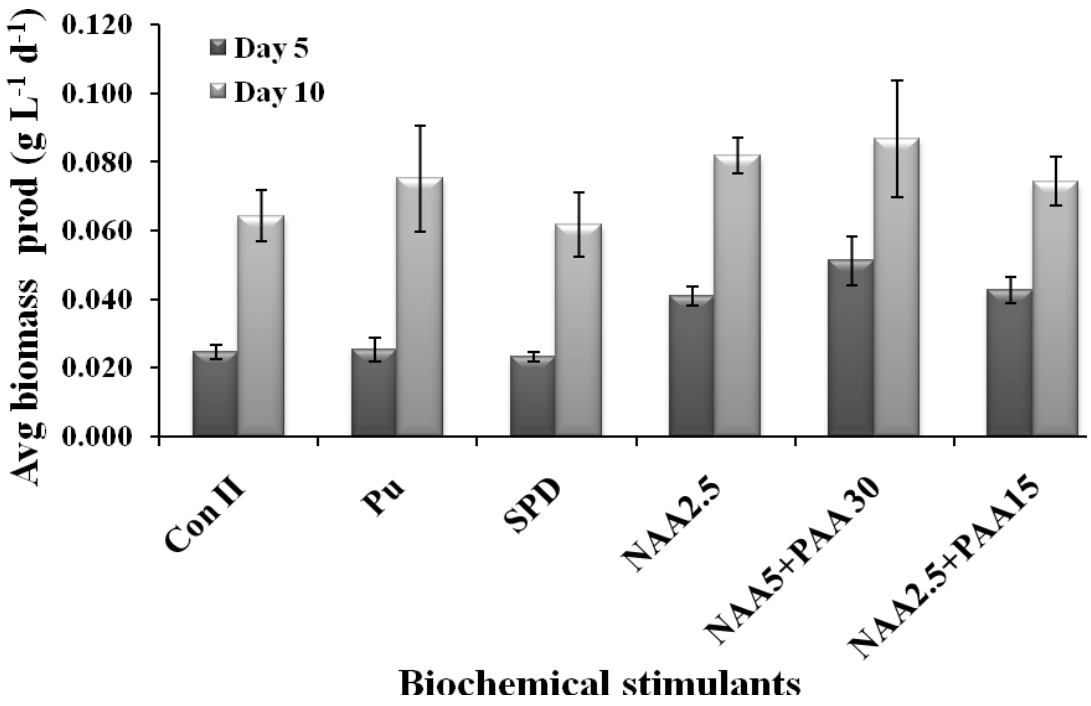


Figure 1 a & b



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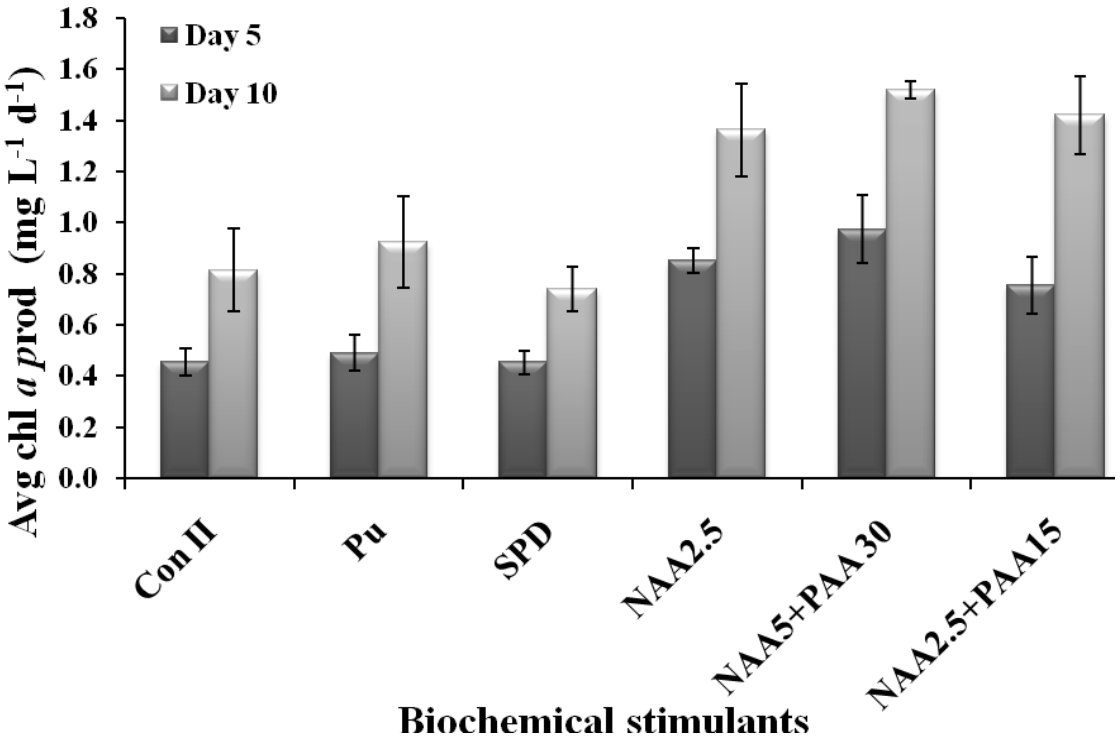
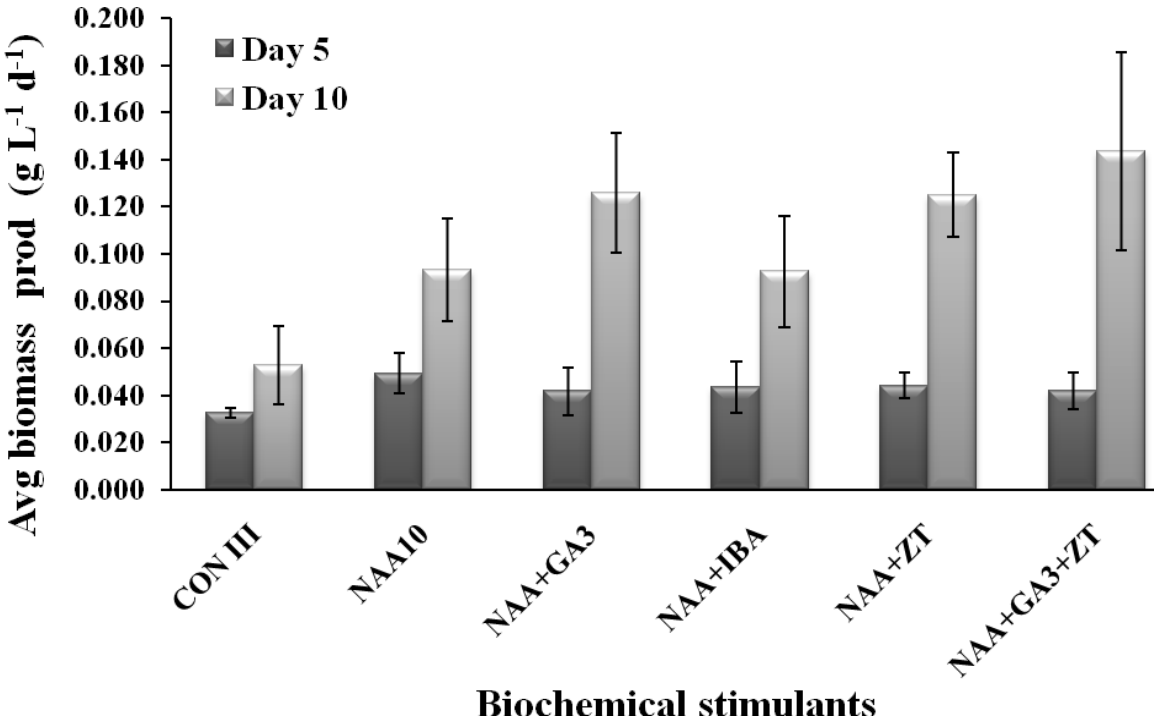


Figure 2 a & b

a)



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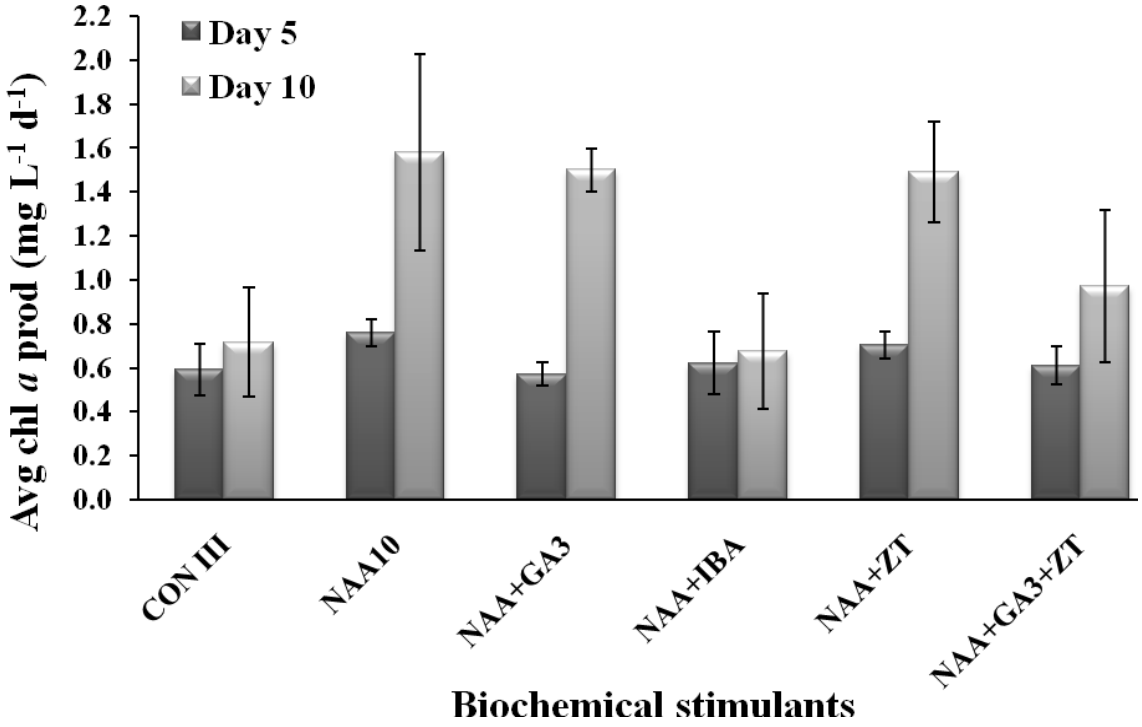

Figure 3 a & b

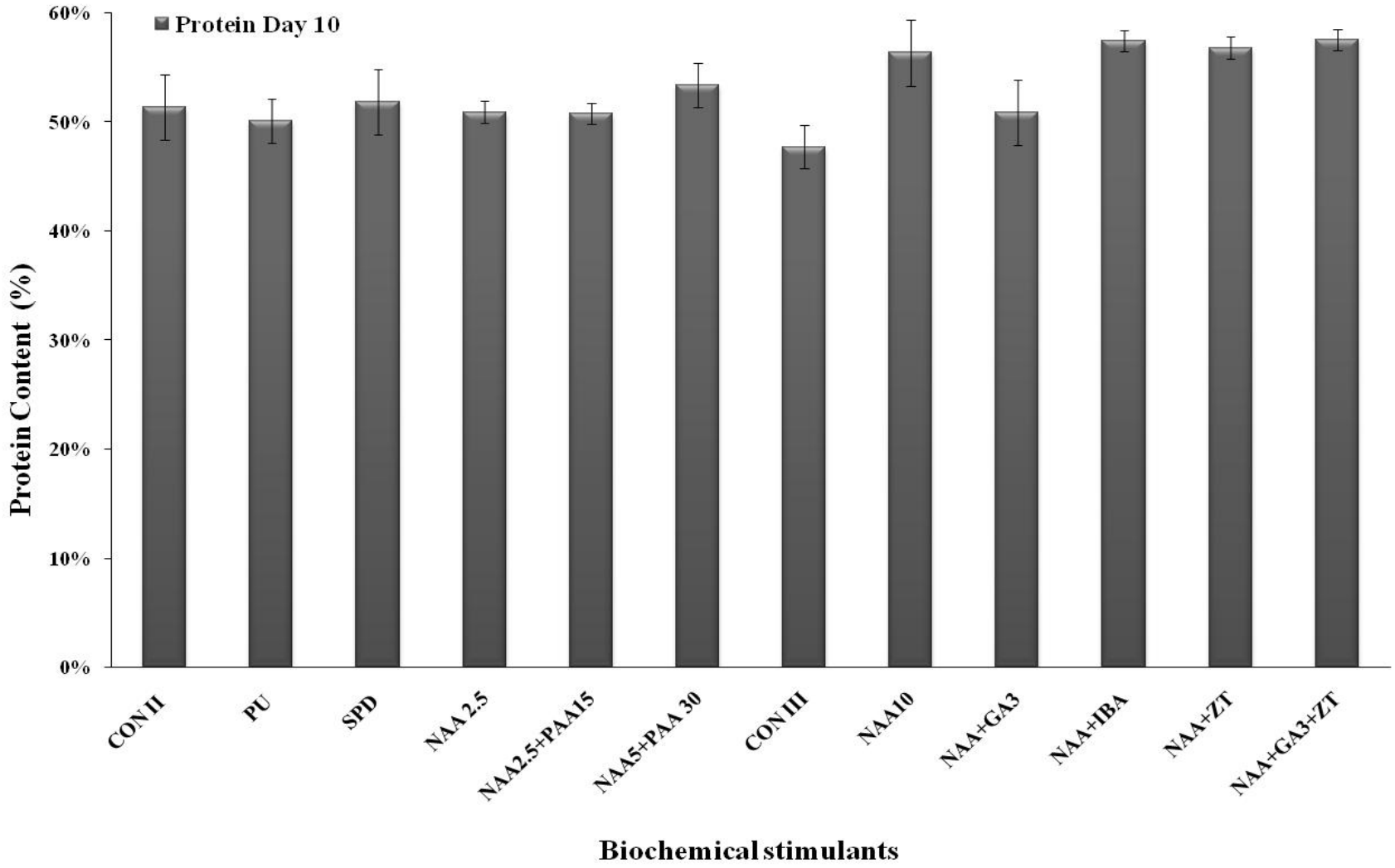
Protein Day 10
60%
50%
40%
30%
20%
10%
0%
Protein Content (%)
CON II
PU
SPD
NAA 2.5
NAA2.5+PAA15
NAA5+PAA 30
CON III
NAA10
NAA+GA3
NAA+IBA
NAA+ZT
NAA+GA3+ZT
Biochemical stimulants

Figure 4a

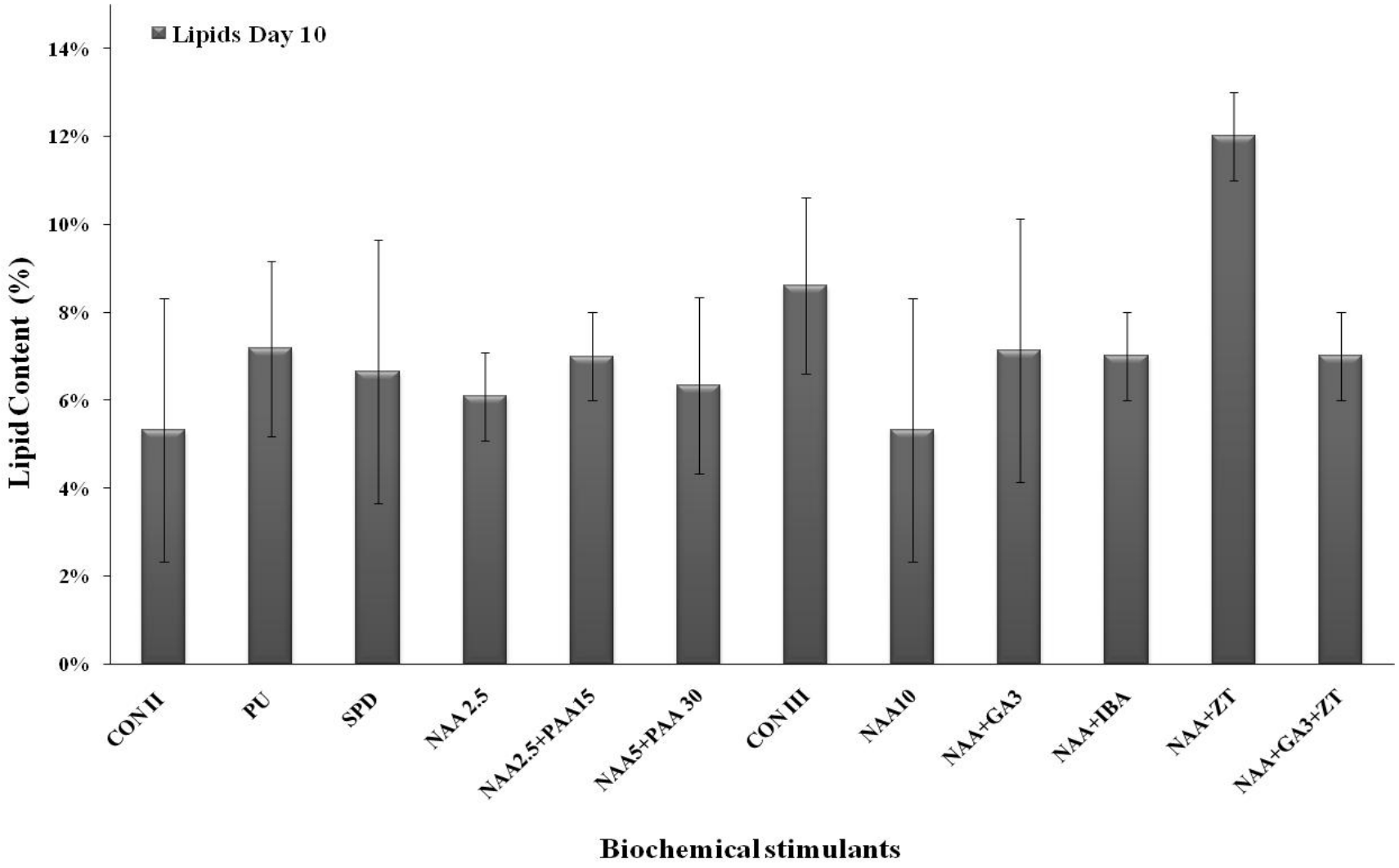
Lipids Day 10
14%
12%
10%
8%
6%
4%
2%
0%
Lipid Content (%)
CON II
PU
SPD
NAA 2.5
NAA2.5+PAA15
NAA5+PAA 30
CON III
NAA10
NAA+GA3
NAA+IBA
NAA+ZT
NAA+GA3+ZT
Biochemical stimulants

Figure 4

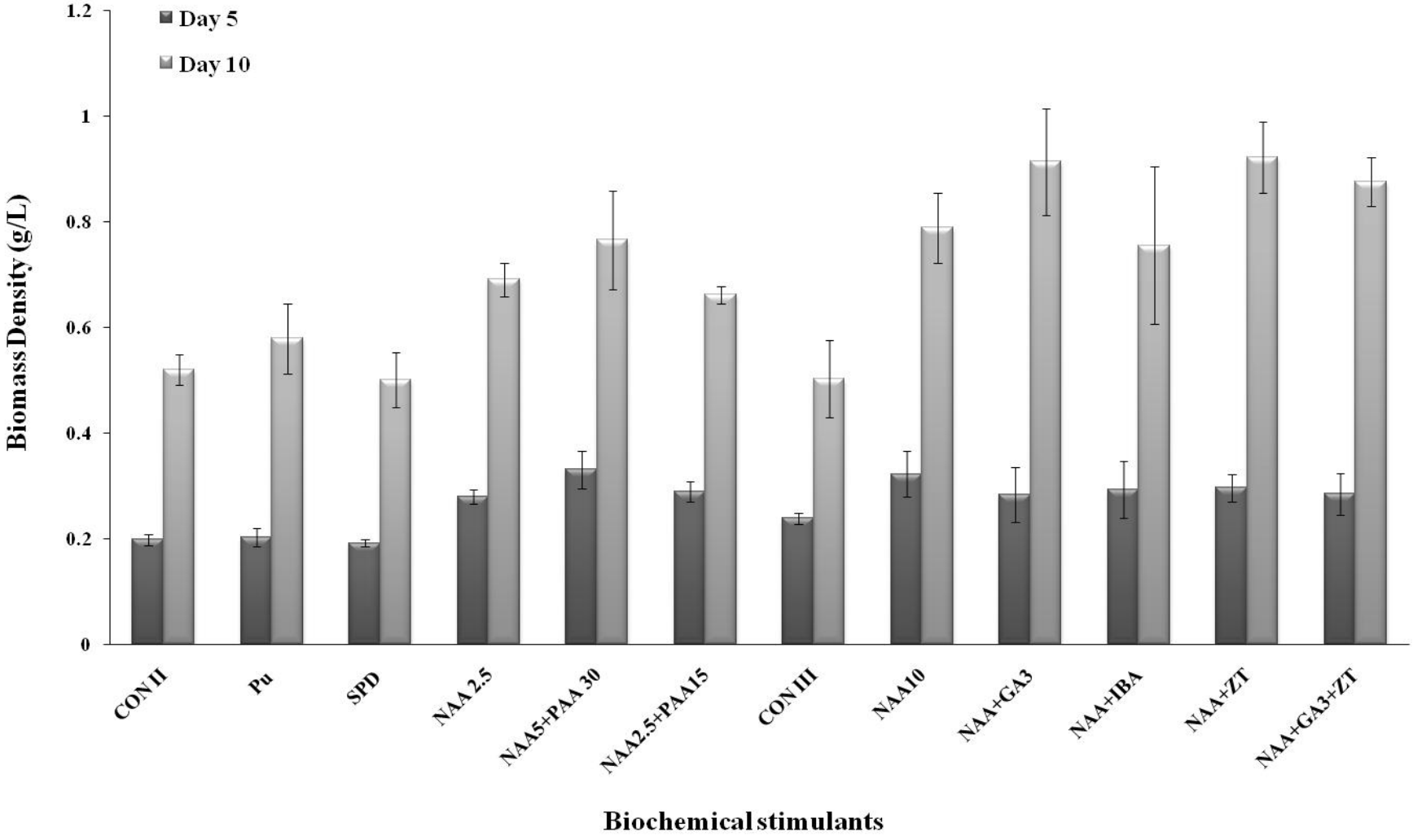
Day 5
Day 10
Biomass Density (g/L)
1.2
1
0.8
0.6
0.4
0.2
0
CON II
Pu
SPD
NAA 2.5
NAA5+PAA 30
NAA2.5+PAA15
CON III
NAA10
NAA+GA3
NAA+IBA
NAA+ZT
NAA+GA3+ZT
Biochemical stimulants

Figure 5

CHAPTER 3

The effect of naphthalene-acetic acid on biomass productivity and chlorophyll content on green algae, coccolithophore, diatom and cyanobacterium cultures.

Hunt, R.W., Chinnasamy, S., Das, K.C. To be submitted to *Applied Biochemistry and Biotechnology.*

Abstract

The application of biochemical stimulants to enhance biomass and metabolite productivity is being investigated here and may be a simpler approach to achieve our goals of higher productivity and lower costs than methods such as genetic modification. The research builds on prior work screening various biochemical stimulants representing different types of plant growth regulators with the green alga, *Chlorella sorokiniana.* Here, we report the impact on biomass and chlorophyll productivity by comparing the delivery method of a previously identified superior stimulant, the synthetic auxin naphthalene acetic-acid (NAA), solubilized in ethanol or methanol. Algae evaluated included the green alga, *Chlorella sorokiniana,* as well as a mixed consortium that includes *C. sorokiniana* along with two other wild-isolated green algae, *Scenedesmus bijuga* and *Chlorella minutissima.* It was found that NAA in ethanol was more effective in enhancing biomass productivity of *C. sorokiniana.* However, no differences were observed with the mixed consortia. The most effective treatment from this step, $EtOH_{500ppm}$+NAA_{5ppm}, along with two other NAA concentrations ($NAA_{2.5ppm}$ and NAA_{10ppm}) were then applied to six diverse species of microalgae to determine if the treatment dosage was effective for other freshwater and marine green algae, cyanobacteria, coccolithophore and diatoms. It was found that three of the species bioassayed, *P carterae, C. sorokiniana* and *H. pluvialis* exhibited a substantial boost in biomass productivity over the 10-day growth period. The use of ethanol and NAA at a combined dosage of $EtOH_{500ppm+}NAA_{5ppm}$ was found to generate the highest biomass productivity for each of the species that responded positively to the treatments. If scalable, NAA and ethanol may have the potential to lower production costs by increasing biomass yields for commercial microalgae cultivation.

Keywords: Plant growth regulators, Phytohormones, Auxins; naphthalene acetic acid; Bioenergy; Biofuels; Biomass; Biostimulants; Microalgae; Phytohormones; *Chlorella sorokiniana; Haematococcus pluvialis; Phaeodactylum tricornutum; Pleurochrysis carterae; Dunaliella bardawil; Nostoc.*

Introduction

The production of microalgae on a commercial scale for bioremediation of wastewaters and production of bioenergy, and biomaterials is being pursued with great interest in the recent past. One of the key bottlenecks with commercial microalgae production for biofuels is the high cost of production that is estimated in excess of \$3,000 ton^{-1} today (2). One way to reduce cost is by increasing biomass productivity within the production cycle. Use of biochemical stimulants can be an effective way to achieve this because they are relatively low cost, easy to incorporate, and can have significant productivity enhancements. Past studies have shown that the auxin family of phytohormones is particularly effective at inducing a growth response from plants and algae. The research presented here builds upon prior results from biochemical stimulation studies that investigated many types of growth promoters ranging from phytohormones to elemental micronutrients with the green alga, *Chlorella sorokiniana* (11). That study identified the synthetic auxin, naphthalene-acetic acid (NAA), as the most effective growth promoter at a concentration ranging from 2.5-10 ppm with the green alga, *C. sorokiniana*.

Research in the field of plant hormones began in the late 1800's and over the course of 50 years scientists identified several compounds responsible for affecting plant growth and development. It was observed that plant hormones were active at small concentrations, usually in the nanomolar range, although sometimes even at lower picomolar concentrations. The identification of many auxins, particularly Indole-acetic acid (IAA), as plant growth regulator occurred in 1933 (12). One of the other auxins identified was naphthalene acetamide (NAM) and it was thought that the molecularly similar synthetic compound, naphthalene acetic acid (NAA), may be able to induce growth response in plants as well. Mitchell and Stewart compared the effect of these two compounds and found that the synthetic auxin, NAA at a concentration of 5

ppm, stimulated the top growth of bean plants, and further that NAA was associated with cellular proliferation of various tissues in the stem (13). The same year, Brannon and Bartsch began investigating the impact of various natural and synthetic auxins on the growth of green algae and found that the application of naphthalene-acetic acid dissolved in ethanol at a concentration of 3.3 ppm increased the growth of *Chlorella vulgaris* by 172% compared to control. Additionally, it was observed that the naturally-occurring auxin, phenyl-acetic acid at a concentration of 30 ppm, increased growth in *C. vulgaris* by 261% over control (14). Decades of research has shown that auxins are involved in the regulation of cell division, cell growth, apical dominance, responses to directional stimuli and fruit setting. However, it is known that not all responses to auxin are stimulatory and auxins can become inhibitory at higher concentrations (12).

The synthetic auxins, such as NAA, act by increasing the endogenous IAA concentrations either by promoting new synthesis or by inhibiting IAA conjugation or breakdown (12). Among the synthetic auxins, NAA is commonly used at relatively low concentrations to elicit auxin-type responses in cell growth, cell division, fruit setting, rooting etc., and are found to be relatively stable in plant tissues and are metabolized very slowly in plant tissues (12). Recent research has discovered the existence of mutant phenotypes that exhibit an altered response to auxins in terrestrial plants, such as tomato, soybean and tobacco. The genes identified provide genetic evidence that the encoded proteins of several *Aux/IAA* genes are involved and regulate distinct aspects of the auxin response. However, other genes exist which are related to physiological responses from the presence of auxins. These genes encode proteins that are related to known auxin functions, such as cell wall loosening, ethylene biosynthesis, production of proteins associated with the cell walls, production of calcium binding proteins that modulate activities of protein kinases or phosphatases, and induction of cell cycle regulatory proteins (12).

It is recognized that the research on the effect of plant growth regulators with algae lags far behind work with other terrestrial plants. Traditionally, plant hormones and synthetic plant growth regulators are used as valuable research tools to elucidate physiological responses of plants or to probe biochemical control mechanisms. However, their use could also be extended to the field of algae production to enhance the potential viability of commercial applications of algae-based renewable biomass production (15). By identifying optimal stimulant dosages that enhances biomass productivity in commercially attractive strains of microalgae to enhance biomass productivity, it may be possible to lower production costs to increase the profitability of industries producing algae for food, feed, fuel, biomaterials, nutraceuticals and pharmaceuticals.

The research presented here evaluated whether ethanol or methanol was a more suitable solvent to dissolve the auxin, NAA, for dosing algal cultures. The use of ethanol or methanol as a solvent may introduce inhibitory effects on the algal growth, therefore a study designed to compare 500 ppm of each solvent with NAA was performed to determine which is solvent is preferred. This was conducted with a monoculture and a consortia culture containing 3 species of green algae to evaluate whether a mixed culture would respond in a similar manner as a single species. The results from this experiment established the most effective treatments combinations and were then applied to six diverse species of microalgae to determine if the treatment dosage was effective for other freshwater and marine green algae, cyanobacteria, coccolithophore and diatoms. These species were selected for this multispecies screening for various reasons. The freshwater green alga, *C. sorokiniana*, was selected because of its use in prior experiments as being a fast growing strain that thrives in wastewater conditions. It has also been shown to contain considerable amounts of lutein (16). The freshwater green alga, *Haematococcus pluvialis*, was selected due to the high value pigment, astaxanthin, it produces in the highest

concentrations found in nature (17). The marine diatom, *Phaeodactylum tricornutum*, was selected to diversify the study to include diatoms, since they are known to have high oil content, and because this specific strain is well studied in the literature (18). The marine coccolithophore, *Pleurochrysis carterae* (also known as, *Cricosphaera carterae*), was selected due to its high oil content, its ability to grow on wastewater and dominate outdoor raceway cultivation (10, 19). The halotolerant green alga, *Dunaliella bardawil* (also known as, *Dunaliella salina*), was selected due to its commercially viable cultivation for beta-carotene and high glycerol content (20). The wild isolated cyanobacterium, *Nostoc sp.*, was selected as a nitrogen fixing organism that showed promise for cultivation in wastewater (10).

Materials and Methods

Strain and culture maintenance

The freshwater green alga, *Chlorella sorokiniana* (UTEX 2805) and *Haematococcus pluvialis* (UTEX 2505), were obtained from the UTEX Culture Collections, while the mixed consortia which was comprised of *Chlorella sorokiniana* (UTEX 2805), *Chlorella minutissima* (wild isolate) and *Scenedesmus bijuga* (wild isolate) was created from previously isolated organisms in our laboratory (10) and maintained in BG11 growth medium using the method described by Stanier et al. (13). The diatom, *Phaeodactylum tricornutum* (UTEX 640) and coccolithophore, *Pleurochrysis carterae* (UTEX LB 1014), were obtained from UTEX Culture Collections and maintained in a modified BG11 saline growth medium that was comprised of standard BG11 with the addition of Oceanic marine salt mix (Oceanic Systems, Dallas, TX USA) at a concentration of 35 g/L. The hypersaline green alga, *Dunaliella bardawil* (UTEX LB 2538), was obtained from UTEX Culture Collections and maintained in the modified BG11

saline growth medium which was further supplemented with additional NaCl (23 g/L) and $MgCl_2$ (5 g/L) for a hypersaline media. The The cyanobacteria, *Nostoc species,* were previously isolated in our laboratory from a carpet industry wastewater (10) and maintained in a nitrogen-free BG11 growth media. The pH of all BG11 culture mediums were adjusted to 7.5±0.2 before inoculation and the algae were maintained in a temperature controlled growth chamber at 25±1^{o}C and 100±10 μmoles m^{-2} s^{-1} light intensity provided by cool white fluorescent (6500 K) T-8 bulbs with light:dark cycles of 12:12 h .

Experimental conditions and experimental plan

The experimental conditions and protocol used for these studies were the same as those reported by Hunt et al (11). The biochemical stimulant, 1-naphthalene acetic acid (NAA), was used for all treatments and dosages were selected based upon the experimental results from previous investigations (11). The pure compound of NAA was obtained from Super-Grow Plant Care, Montreal, Canada (www.super-grow.biz). The solvents, ethyl alcohol and methanol were both supplied by Thermo Fisher Scientific Inc, Waltham, MA, All experiments were conducted in 250 mL Erlenmeyer flasks with 100 mL BG11 growth medium supplemented with the biochemical stimulants to be tested. Growth studies were performed in a temperature controlled growth chamber as previously mentioned. For the purpose of screening, previously reported dosages that demonstrated growth enhancing effects were used (11). The experimental results presented here are the result of two 10-day static flask experiments each investigating a different aspect of the biochemical stimulant treatments.

The first experiment evaluated the impact of the solvent used to dissolve NAA, by comparing ethanol (EtOH) and methanol (MeOH) at 500 ppm as the solvent containing NAA at

5ppm ($EtOH_{500ppm}$+NAA_{5ppm}, $MeOH_{500ppm}$ +NAA_{5ppm}). Each treatment was concocted with a known quantity of biostimulant dissolved in 500 µL of ethanol and added to 1 liter of the respective BG11 growth media for a final concentration of 500 ppm for ethanol and the desired concentrations of NAA and were autoclaved for sterility at 121˚C at 15 PSI. These treatments were evaluated for their impact on growth and chlorophyll concentration of *C. sorokiniana* and the mixed algal consortia described earlier.

The second experiment was a multispecies study investigating the impact of three dosages of NAA ($EtOH_{500ppm}$+$NAA_{2.5ppm}$, $EtOH_{500ppm}$+NAA_{5ppm}, $EtOH_{500ppm}$+NAA_{10ppm}) using ethanol as the solvent (500 ppm). An additional delayed dosage treatment was added to *C. sorokiniana*, where three previously un-dosed *C. sorokiniana* cultures were removed from the growth chamber and a filter-sterilized dosage of $EtOH_{500ppm}$+NAA_{5ppm} was applied to each flask and returned to the growth chamber for the remaining five days of growth. The goal was to determine whether previously identified effects were more universally applicable for other species. The study investigated the two freshwater green algae *C. sorokiniana* and *H. pluvialis,* the hypersaline green alga *D. bardawil* , the saltwater diatom *P. tricornutum*, the coccolithophore *P. carterae, ,* and the cyanobacteria, *Nostoc sp* for their response to NAA.

All media preparations were autoclaved after adding the biochemical stimuluant. An exponentially growing culture of *C. sorokiniana* at a cell concentration of 0.06 g L^{-1} was used as inoculum in experiment I, while exponentially growing cultures at cell densities of 0.07 g L^{-1} (*C. sorokiniana),* 0.26 g L^{-1} (*H. pluvialis*), 0.26 g L^{-1} (*P. tricornutum*), 0.32 g L^{-1} (*P.carterae*), 0.33 g L^{-1} (*D. bardwil*), and 0.05 g L^{-1} (*Nostoc sp.*) were used in experiment II. After inoculation, the flasks were incubated for 10 days in the growth chamber. Details of culture and treatment dosages used in both experiments are summarized in Table 1.

Cultures were sampled on day 5 and day 10, where day 5 sampling represented initial exponential phase while the day 10 sampling represented the culture entering the late exponential phase. The productivity values presented in this manuscript were calculated by taking the biomass density (g/L) on the sampling day (day 5 and day 10) and dividing by 5, which represents the number of days between sampling, to provide productivity values (g/L-d) between day 0 and day 5 and day 5 and day 10, respectively. Each treatment was performed in triplicate, and the parameters measured are reported as the mean with respective standard. Due to variations in initial cell densities resulting from the addition of inocula, which can have a significant impact on measured productivity over time, comparisons across species with different initial cell densities are reported as increases in productivity relative to their respective control within each experiment. The data for both experiments was analyzed using SAS for ANOVA and Tukey analysis comparing the phytohormone treatments with each individual day. The Tukey analysis was evaluated at the $P< 0.05$ confidence interval.

Analyses

Biomass was determined by filtering 25 mL of algal culture through a preweighed Whatman GF/C filter (4.7 cm diameter; 1.2 μm pore size). The filter was washed with 10 mL of 0.65 M ammonium formate solution to remove excess salts and dried overnight at 60 ^{o}C in forced air oven. The dried filter with biomass was cooled in a desiccator and weighed again to estimate the final dry weight. For chlorophyll *a* estimation, 10 mL of homogenized algal culture was centrifuged at 5000 rpm for 10 minutes and the algal pellet was exhaustively extracted with hot methanol (95% v/v) until it was colorless. The amount of chlorophyll *a* extracted in the methanol was determined spectrophotometrically according to the method described by Porra et al (21) using the following equation:

$$\text{Chlorophyll } a \text{ } (\mu g \text{ } mL^{-1}) = 16.29 \times OD_{665} - 8.54 \times OD_{652}$$

Results and Discussion:

Experiment 1: Comparison of ethanol or methanol as solvent for biochemical stimulant

Results from *Chlorella sorokiniana*

The specific solvent used for dissolving NAA_{5ppm} was found to dramatically affect the growth and chlorophyll dynamics of both *C. sorokiniana* and the mixed consortia (Figure 1a and 1b). The highest biomass density attained by *C. sorokiniana* was (0.576 g L^{-1}) by the treatment of $EtOH_{500ppm}$+NAA_{5ppm} representing a 120% increase versus the control (0.263 g L^{-1}) over the 10 day experiment. These results are similar to the results that were previously reported for a mixture of NAA in EtOH (1).

It was found that the biomass productivity between day 0 and 5 with ethanol alone ($EtOH_{500ppm}$) was statistically the same as the treatment $EtOH_{500ppm}$+NAA_{5ppm} ($p< 0.05$) and induced almost the same increase in biomass productivity of 0.042 g L^{-1} d^{-1} and was found to be approximately 150% over control (Figure 1a). This suggests that the initial boost in growth seen in both treatments may be the result of the ethanol during the initial lag and early exponential phases of *C. sorokiniana*. In the second phase of growth (days 5 to 10) biomass productivity of the $EtOH_{500ppm}$ treatment reduced near the control productivity and the difference was not statistically significant, whereas the $EtOH_{500ppm}$+NAA_{5ppm} treatment was statistically different from ethanol alone ($p< 0.05$) and exhibited a second boost in growth productivity of 0.062 g L^{-1} d^{-1} representing 138% higher than control, where the control was at 0.026 g L^{-1} d^{-1}. The decrease in biomass productivity in the $EtOH_{500ppm}$ treatment and the boost in productivity for

$EtOH_{500ppm}$+NAA_{5ppm} after day 5 gave the $EtOH_{500ppm}$+NAA_{5ppm} treatment the highest overall growth for *C. sorokiniana* compared to the other treatments.

In the first five days of growth, MeOH and +NAA_{5ppm} showed no statistical differences from that of the control ($p< 0.05$), however, with NAA ($MeOH_{500ppm}$ +NAA_{5ppm}) the second growth phase (days 5 to 10) was significantly higher than control ($p< 0.05$) with an increase in biomass productivity of 129% over that of the control. In contrast, the treatment with methanol alone ($MeOH_{500ppm}$) dropped in productivity to 87% below control and was found to be statistically different from all other treatments on by day 10. The final biomass density of 0.463 g L^{-1} for the $MeOH_{500ppm}$ +NAA_{5ppm} treatment, however, was less than that for $EtOH_{500ppm}$+NAA_{5ppm} (0.576 g L^{-1}). These results seem to suggest that independent of the solvent used for delivering NAA, the effectiveness of NAA appears to be in the 5-10 day period. Additionally, in contrast to methanol that had no observable effect of its own, ethanol seemed to provide a growth stimulus in the 0-5 day growth period.

Results of chlorophyll productivity of *C. sorokiniana* mirrored the biomass productivity in the case of ethanol treatments ($EtOH_{500ppm}$ and $EtOH_{500ppm}$+NAA_{5ppm}) where these treatments were significantly different than the control ($p< 0.05$). Large increases in their chlorophyll productivity by day 5 of 139% and 128 % (0.700 and 0.668 µg ml^{-1} d^{-1}) relative to control (0.293 µg ml^{-1} d^{-1}), respectively, were observed (Figure 1b). However between day 5 and 10, these two treatments showed a decrease in chlorophyll productivity of 70 to 90% below the control. There was no statistical difference between the treatments $EtOH_{500ppm}$+NAA_{5ppm}, MeOH, or $MeOH_{500ppm}$+NAA_{5ppm}, however, EtOH, $EtOH_{500ppm}$+NAA_{5ppm}, and MeOH were all statistically different from the control ($p< 0.05$). The observed chlorophyll inhibition was correlated with biomass productivity inhibition in the case of the $EtOH_{500ppm}$ treatment. However when

NAA_{5ppm} was present with ethanol, the biomass productivity increased despite low chlorophyll productivities. These results seem to suggest a mechanism of hormone-induced cell division or proliferation rather than growth stimulation from enhancing the photosynthetic efficiency or apparatus. The treatment of methanol ($MeOH_{500ppm}$ and $MeOH_{500ppm}$ +NAA_{5ppm}) on *C. sorokiniana* demonstrated a small increase in chlorophyll productivity during the first 5 days but was not statistically significant ($p< 0.05$).

Results from Mixed Consortium

The response on biomass productivity to NAA and solvents from the mixed consortia of green algae relative to control was much lower than that of *C. sorokiniana* (Figure 1a). The treatments $EtOH_{500ppm}$+NAA_{5ppm}, $MeOH_{500ppm}$+NAA_{5ppm}, and EtOH were found to be statistically different from MeOH and the control on day 5 ($p<0.05$). The treatment $EtOH_{500ppm}$ showed increase of biomass productivity to 0.049 g L^{-1} d^{-1} representing a 41% increase over the control, where the control had a value of 0.034 g L^{-1} d^{-1}. These productivities were very comparable to that of $EtOH_{500ppm}$+NAA_{5ppm} which was found to be 0.050 g L^{-1} d^{-1}. The biomass productivity for treatments containing ethanol were all reduced to the control level by day 10 (Figure 1a).

The application of methanol alone ($MeOH_{500ppm}$) had no statistical difference on biomass productivity during the first or last 5 days of growth compared to the control, while all other treatments were statistically different to the control after the first 5 days of growth ($p< 0.05$). When NAA was added ($MeOH_{500ppm}$ +NAA_{5ppm}) a 43% increase in biomass productivity (0.049 g L^{-1} d^{-1}) was observed relative to control (0.034 g L^{-1} d^{-1}). This suggests that there is a positive growth stimulation impact with NAA, however, the toxicity or inhibition of methanol at the

levels tested prevented a growth response analogous to using ethanol as a solvent. By day 10, only the treatment MeOH and $EtOH_{500ppm}$ +NAA_{5ppm} were found to be statistically different from each other ($p< 0.05$). The methanol treatment ($MeOH_{500ppm}$) showed a slight increase to 19% higher average biomass productivity, but was not statistically significant from the control ($p< 0.05$). The $MeOH_{500ppm}$ +NAA_{5ppm} was observed to be at the same biomass productivity as the control (0.075 g L^{-1} d^{-1}).

The response of chlorophyll productivities from the mixed consortium to the treatments demonstrated marginal variation by the end of day 5, where all treatments had average chlorophyll productivities less than 20% different relative to control (approximately 0.8 µg ml^{-1} d^{-1}) and were not found to be statistically different from each other ($p<0.05$). However, the inhibition of ethanol on chlorophyll synthesis becomes evident by day 10 as both treatments with ethanol ($EtOH_{500ppm}$ and $EtOH_{500ppm}$+NAA_{5ppm}) demonstrated a reduction in chlorophyll productivity of -124% and -132% compared to control, and these two treatments were statistically different from the control and $MeOH_{500ppm}$+NAA_{5ppm} , but was not different from MeOH alone ($p< 0.05$). This implies that chlorophyll concentrations for $EtOH_{500ppm}$ and $EtOH_{500ppm}$+NAA_{5ppm} at the 10^{th} day were lower than that at the 5^{th} day. These treatments were the only ones in this experiment that exhibited a negative productivity value suggesting strong chlorophyll *a* inhibition, although the biomass productivity was comparable to the control (0.075 g L^{-1} d^{-1}) during this same period. The results suggest that using these treatment combinations are not ideal for stimulating the growth of the mixed consortia tested here, and further that the use of ethanol appears to be toxic and inhibitory to chlorophyll synthesis at the 500 ppm concentration tested.

The ethanol and NAA treatments dosed in the mixed consortia demonstrated a different growth response than *C. sorokiniana.* During the first 5 days of growth, the ethanol treatments exhibited the largest increase in biomass productivity for this culture which corresponded with marginal increases in chlorophyll productivity. However by day 10, this enhancement in biomass productivity waned and subsequently demonstrated a strong inhibition in chlorophyll *a* productivity which was the highest inhibition observed in any treatment from this experiment. The cultures that experienced this inhibition had visible differences in the flasks, where the cultures had turned a light yellowish-green color compared to their control, which was dark green. Despite the decreased chlorophyll content measured in the cells, the biomass productivity and final density was very similar to their control.

This study demonstrated that using ethanol as a solvent for NAA was better than methanol for *C. sorokiniana* and that a NAA dosage of 5ppm was able to stimulate the growth response substantially above the control. The application of NAA with either solvent in the dosages examined in this study was not supportive or synergistic to enhance the biomass productivity of the mixed green algae consortia despite the inclusion of *C. sorokiniana,* which comprised 1/3rd of the consortia. This could be due to the release of toxins or inhibitory compounds, such as reactive oxygen species, if one of the other two species (*S. bijuga or C. minutissima*) had an adverse reaction to the solvent dosage.

Experiment II: Effect of ethanol and NAA on different species of algae

Results from *Chlorella sorokiniana*:

The previous investigation demonstrated that ethanol was the preferred solvent to dissolve NAA for growth stimulation. The aim of this study was to confirm these previous

results and evaluate different dosages of NAA with six different algae species. All references to NAA treatments assume an additional to $EtOH_{500ppm}$ for the delivery of NAA. During the first 5 days of growth, *C. sorokiniana* did not demonstrate any changes in biomass productivity compared to the control ($p< 0.05$) (Figure 2a,d). However, the treatments of NAA_{5ppm}, $NAA_{10\ ppm}$ and the 5-day delayed dosage NAA_{5ppm} (DD- NAA_{5ppm}) were all found to be statistically different from the control ($p< 0.05$). The growth stimulation which was observed between day 5 and 10 under the treatments of NAA_{5ppm} and $NAA_{10\ ppm}$ showed 69 and 79% increase in biomass productivity relative to control, respectively, ($p< 0.05$). This treatment exhibited the highest increase in biomass productivity of 177% (0.414 g L^{-1} d^{-1}) versus control (0.0149 g L^{-1} d^{-1}) compared to any other treatment of *C. sorokiniana* in this study. The delayed dosage samples were dosed at a cell concentration of 0.12 g L^{-1} compared to the standard treatments where NAA was present on day 0 at a cell concentration of 0.07 g L^{-1}. The difference in biomass productivity between the NAA_{5ppm} and delayed dose NAA_{5ppm} was 64% +/- 10%, whereas the difference in inoculum density was 76% +/- 6%. This suggests that future studies should investigate the potential for adding delayed dosages of EtOH+NAA after 5 days as well as extend the growth period and apply dosages at 10 days to *C. sorokiniana* and the other species that responded well to these treatments.

There was no statistically significant impact on chlorophyll productivity from the treatments using ethanol and NAA during the first 5 days with *C. sorokiniana* ($p< 0.05$). However between days 5 and 10, the chlorophyll productivity significantly increased ($p< 0.05$) in the treatments NAA_{5ppm} NAA_{10ppm}, and delayed dose NAA_{5ppm} exhibiting a 122%, 119%, 124% increase compared to the control, respectively. The increase in chlorophyll was correlated with the respective increase in biomass productivity. In all treatments the chlorophyll

productivity reached a plateau from 0.4 to 0.6 μg ml^{-1} d^{-1} by day 10, indicating that chlorophyll synthesis or inhibition is not the most critical parameter for maximizing growth when dosing with the auxin, NAA.

Results from *Haematococcus pluvialis*:

The freshwater green algae, *H. pluvialis,* showed positive responses to NAA with respect to its biomass and chlorophyll productivity over the 10-day growth period (Figure 2a,d). The first five days of growth showed no statistical difference compared to the control for all three treatments ($p<0.05$). The measured biomass productivity by day 10 showed more statistically significant differences between the treatments with NAA_{5ppm} and $NAA_{2.5ppm}$ compared to the control ($p< 0.05$). The maximum increase observed in biomass productivity was from the NAA_{5ppm} treatment with a 116% (0.0308 g L^{-1} d^{-1}) increase over control, which resulted in a final biomass density of 17% higher than control. The treatment $NAA_{2.5ppm}$ had a significant impact on growth by increasing biomass productivity by 85% (0.0263 g L^{-1} d^{-1}) compared to the control (0.0142 g L^{-1} d^{-1}). Higher concentrations of NAA (NAA_{10ppm}) did not produce further benefits and showed a tapering effect with a biomass productivity of 0.0203 g L^{-1} d^{-1} which was not statistically significant compared to control ($p< 0.05$).

The impact on average chlorophyll productivity with *H. pluvialis* appeared to have a slight negative impact of 33% and 38% decrease compared to control on day 5 for the $NAA_{2.5ppm}$ and NAA_{10ppm}, respectively, however these changes were not statistically significant ($p< 0.05$). The chlorophyll productivity was not inhibited as strongly for the NAA_{5ppm} treatment and was found to be similar to the control productivity despite the dramatic increase in biomass productivity observed by day 10. It was also noted that the color of most of the cultures treated

with EtOH+NAA were a different color than the control, which appeared as a green-orange color suggesting that this treatment may also induce stress factors which may accelerate the onset of astaxanthin production, however more detailed studies are needed to confirm this effect.

Results from *Phaeodactylum tricornutum*:

The diatom, *P. tricornutum,* exposed to EtOH+NAA showed only small variations in biomass productivity (Figure 2b,d). The NAA_{10ppm} treatment had the largest impact over the first five days showing an increase in average biomass productivity of 225% over control, but was not statistically significant ($p< 0.05$). The biomass productivity data for the day 10 data showed that the treatments $NAA_{2.5ppm}$ and NAA_{5ppm} did not have a strong impact. However, it was observed that the NAA_{10ppm} produced an opposite effect past day 5 and measured an 81% (0.0036 g L^{-1} d^{-1}) decrease in biomass productivity compared to the control (0.0185 g L^{-1} d^{-1}) (Figure 2b). The high variability observed by the control affected any statistically significance in the results obtained ($p< 0.05$).

The chlorophyll activity of *P. tricornutum* exhibited a slight depression in chlorophyll productivity for all NAA treatments, but these changes were small and not statistically significant ($p< 0.05$). Although the NAA_{5ppm} treatment showed a marginal average decrease of 22% for day 5, the day 10 samples measured a dramatic 390% (0.159 μg ml^{-1} d^{-1}) increase in chlorophyll productivity compared to the control (0.032 μg ml^{-1} d^{-1}). This enhancement was not observed with the NAA_{10ppm} that showed a highly variable increase of 53% compared to control and was not statistically significant ($p< 0.05$). In all *P. tricornutum* samples, it was noted that the chlorophyll productivity for day 5 was much higher than the day 10, suggesting that the cultures

were able to synthesize enough chlorophyll in the early exponential phase to sustain continual growth through day 10.

Results from *Pleurochrysis carterae*:

The coccolithophore, *P. carterae*, demonstrated a strong response to the EtOH+NAA treatments tested (Figure 2b,d). For all treatments, the biomass productivity by day 5 was not significantly different than control ($p< 0.05$) for the treatments $NAA_{2.5ppm}$, NAA_{5ppm} and NAA_{10ppm}. However by day 10, the treatments NAA_{5ppm} and NAA_{10ppm} were both significantly different from the control ($p<0.05$). The biomass productivity in the NAA treatments was dramatically increased by 293% (0.0454 g L^{-1} d^{-1}), 631% (0.0844 g L^{-1} d^{-1}) and 519% (0.0714 g L^{-1} d^{-1}) for $NAA_{2.5ppm}$, NAA_{5ppm} and NAA_{10ppm}, respectively compared to the control (0.0115 g L^{-1} d^{-1}). One reason that *P. carterae* had such extremely high increases in percent difference in productivities (293%-631%) was because the control culture after day 5 decreased its biomass productivity by -59%, whereas the treated cultures continue to increase their biomass productivity by (28%, 117% and 242%) for $NAA_{2.5ppm}$, NAA_{5ppm} and NAA_{10ppm}, when comparing their day 10 to their day 5 biomass productivity values. The optimal dosage of the ones tested for *P. carterae* appears to be NAA_{5ppm} because as the dosage increases or decreases the enhancement of biomass productivity decreases.

These impressive increases in biomass productivity did not directly correlate to the chlorophyll productivities. The average chlorophyll productivity was enhanced sharply during the first 5 days of growth, providing 63% (0.294 µg ml^{-1} d^{-1}), 43% (0.258 µg ml^{-1} d^{-1}) and 98% (0.358 µg ml^{-1} d^{-1}) increases over control (0.180 µg ml^{-1} d^{-1}), but due to high variation in the control and $NAA_{2.5ppm}$, none of the treatments demonstrated a statistically significant results

($p<0.05$). However, after the day 5 increase in chlorophyll productivity over control, the day 10 productivity values were similar to the control and not statistically significant.

Results from *Dunaliella bardawil*:

The halotolerant green algae, *D. bardawil* was observed to have high (Figure 2c,d). The biomass productivity during the first 5 days was mostly inhibitory showing -31%, 3% and -85% responses for $NAA_{2.5ppm}$, NAA_{5ppm} and NAA_{10ppm}, respectively, however these differences in the average biomass productivity were not statistically significant compared to the control ($p<0.05$). Interestingly, the most dramatic increase in biomass productivity was observed in the latter most inhibited samples of NAA_{10ppm}, which demonstrated a 119% (0.0270 g L^{-1} d^{-1}) increase compared to control (0.0132 g L^{-1} d^{-1}), but this difference did not meet the $P< 0.05$ confidence level.

The response of EtOH+NAA to chlorophyll productivity showed little effect for $NAA_{2.5ppm}$, while the response for NAA_{5ppm} had a 22% increase over the control by day 5, which was statistically significant ($p<0.05$). The chlorophyll productivity of NAA_{5ppm} treatment dropped to -101% compared to control by day 10, but due to higher variations, this was not significant ($p< 0.05$).

Results from *Nostoc species*:

The wild isolated cyanobacteria, *Nostoc sp.,* exhibited a response to the EtOH+NAA treatments examined (Figure 2c,d). The treatment $NAA_{2.5ppm}$ had only a marginal effect on the average biomass productivity , however the NAA_{5ppm} treatment was found to initially boost the growth by 96%, although these samples showed a high variability and were not statistically

significant ($p<0.05$). The biomass productivity of the treatment NAA_{10ppm} showed an early increase of 90% (0.0095 g L^{-1} d^{-1}) by day 5 compared to control (0.0050 g L^{-1} d^{-1}), however, that enhancement is reversed as well by day 10 which was measured to have the same biomass productivity as the control. Despite the differences in biomass productivities for day 5 and 10, none of these treatments were found to be statistically significant ($p<0.05$).

Conclusion

The treatment of microalgae with NAA requires the use of a solvent to dissolve the NAA powder for dosing into liquid media. It was found that for *C. sorokiniana,* ethanol was a better solvent than methanol, which caused inhibition over the ten days of growth. For this species, it was found that ethanol at a concentration of 500 ppm was a useful growth stimulant for the first 5 days of growth, however, this effect wanes between day 5 and 10. The inclusion of NAA at a concentration of 5 ppm was found to sustain the accelerated biomass productivity through day 10 and was able to enhance productivity over two-fold. Although it was observed that NAA_{10ppm} resulted in slightly higher biomass productivity, this treatment doubled the amount of NAA applied for only a marginal increase in biomass productivity, thus the NAA_{5ppm} still may be the best option for stimulating a growth response in *C. sorokiniana.* Further, the delayed dosage of NAA_{5ppm} after 5 days of normal growth exhibited the largest impact on biomass productivity suggesting that the timing and amount of dosage are critical parameters. Methanol was inhibitory to chlorophyll synthesis for the dosages tested. Ethanol showed little impact on chlorophyll for the first 5 days, and then became inhibitory by day 10.

Although, it was determined that the combination of ethanol and NAA was ideal for *C. sorokiniana*, the response to the mixed consortia to treatments containing ethanol were observed

to have an early boost in biomass productivity, but this impact vanished by day 10 showing little enhancement over the control. Ethanol also induced inhibition of chlorophyll by day 10, which was similar to the effect observed with *C. sorokiniana.* The mixed consortia was also boosted in the early growth phase by methanol, but only when combined with NAA. The treatments tested in this study did not demonstrate substantial enhancements for the mixed consortia, future studies can investigate a reduction in the solvent concentration, which appeared to be inhibitory, while also testing different ranges of phytohormones such as NAA.

Both cultures showed that despite inhibition in chlorophyll productivity and content, this did not strongly impact growth rates, as the *C. sorokiniana* treatment with the lowest chlorophyll productivity by day 10 ($EtOH_{500ppm}$+NAA_{5ppm}) had the highest biomass productivity and final day biomass density. Likewise, the same treatment for the mixed consortia showed substantial decrease in chlorophyll productivty, yet the biomass productivity was similar to the control. The most productive treatment was $EtOH_{500ppm}$+NAA_{5ppm} and this treatment was considered as the basis for the following multispecies screening experiment.

The multispecies experiment showed that half of the cultures tested responded favorably to the treatment of ethanol and NAA in the dosages tested. The top responder was *P. carterae* which demonstrated impressive increases in biomass productivity compared to the control and had the highest final day density of any species tested. *P. carterae* has been investigated for potential commercial production of biofuels from microalgae due to its fast growth rate, high oil content and dominance in outdoor cultures.

The next species that responded best was *C. sorokiniana*, which demonstrated that even higher productivity values can be attained if the dosage is given at a delayed interval (NAA addition on the 5^{th} day only). The delayed dosage aspect needs to be investigated further to

determine what factors are involved in inducing the largest increase in biomass productivity, such as cell density, growth phase, etc. Although, this particular species of *Chlorella* responds well to these treatments, other strains should be tested such that this treatment may be applicable to large scale production of *Chlorella.*

The third species that responded well to the treatment, *H. pluvialis,* has great potential for enhancing productivity and lowering costs associated with its cultivation for the high value pigment, astaxanthin and omega-3 fatty acids. Although this increase is relatively small compared to the two other top performers, if scalable and adopted in commercial production, this could result in a substantial improvement for cultivation of this species. Although the increase was a modest 17% higher than the control, future research can optimize the dosage and timing for even greater impact.

The impact of ethanol and NAA on the other three species, *P. tricornutum, D. bardawil* and *Nostoc sp.*, did not show a statistically significant response to consider these dosages for future applications. Although each species did show some marginal effects on biomass or chlorophyll, the final day biomass densities were comparable to the control. This indicates that the EtOH+NAA treatments may be very species specific and that a wider range of dosages and solvents should be evaluated with these and other species to see if NAA can induce an increase in biomass productivity. Furthermore, there does not appear to be any correlation with freshwater or marine species being more or less susceptible because two freshwater and one marine species responded best, while two marines, and one freshwater species did not exhibit a strong response.

The results from this research shows that combining ethanol with NAA is a viable approach for enhancing biomass productivity in diverse species of microalgae, however future

studies with other species of algae not tested are needed to evaluate their response to this treatment.

Acknowledgements

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Experiment	Organism	Treatment*	Dosage (ppm)
Experiment I: Comparison of Ethanol and Methonal as a solvent for NAA	*Chlorella sorokiniana*	EtOH	500
		MeOH	500
		EtOH+NAA	500+5
		MeOH+NAA	500+5
	Mixed Consortia (*C. sorokiniana, C. minutissima, S. bijuga)*	EtOH	500
		MeOH	500
		EtOH+NAA	500+5
		MeOH+NAA	500+5
Experiment II: Effect of NAA with ethanol on the growth and chlorophyll productivities of six different algae species	*Chlorella sorokiniana*	EtOH+NAA	500+2.5
			500+5
			500+10
	Haematococcus pluvialis	EtOH+NAA	500+2.5
			500+5
			500+10
	Phaeodactylum tricornutum	EtOH+NAA	500+2.5
			500+5
			500+10
	Pleurochrysis carterae	EtOH+NAA	500+2.5
			500+5
			500+10
	Dunaliella bardawil	EtOH+NAA	500+2.5
			500+5
			500+10
	Nostoc species.	EtOH+NAA	500+2.5
			500+5
			500+10

Table 1: Experimental treatments and dosages for each culture tested. *EtOH: ethanol; MeOH: methanol; NAA: 1-naphthalene acetic acid.

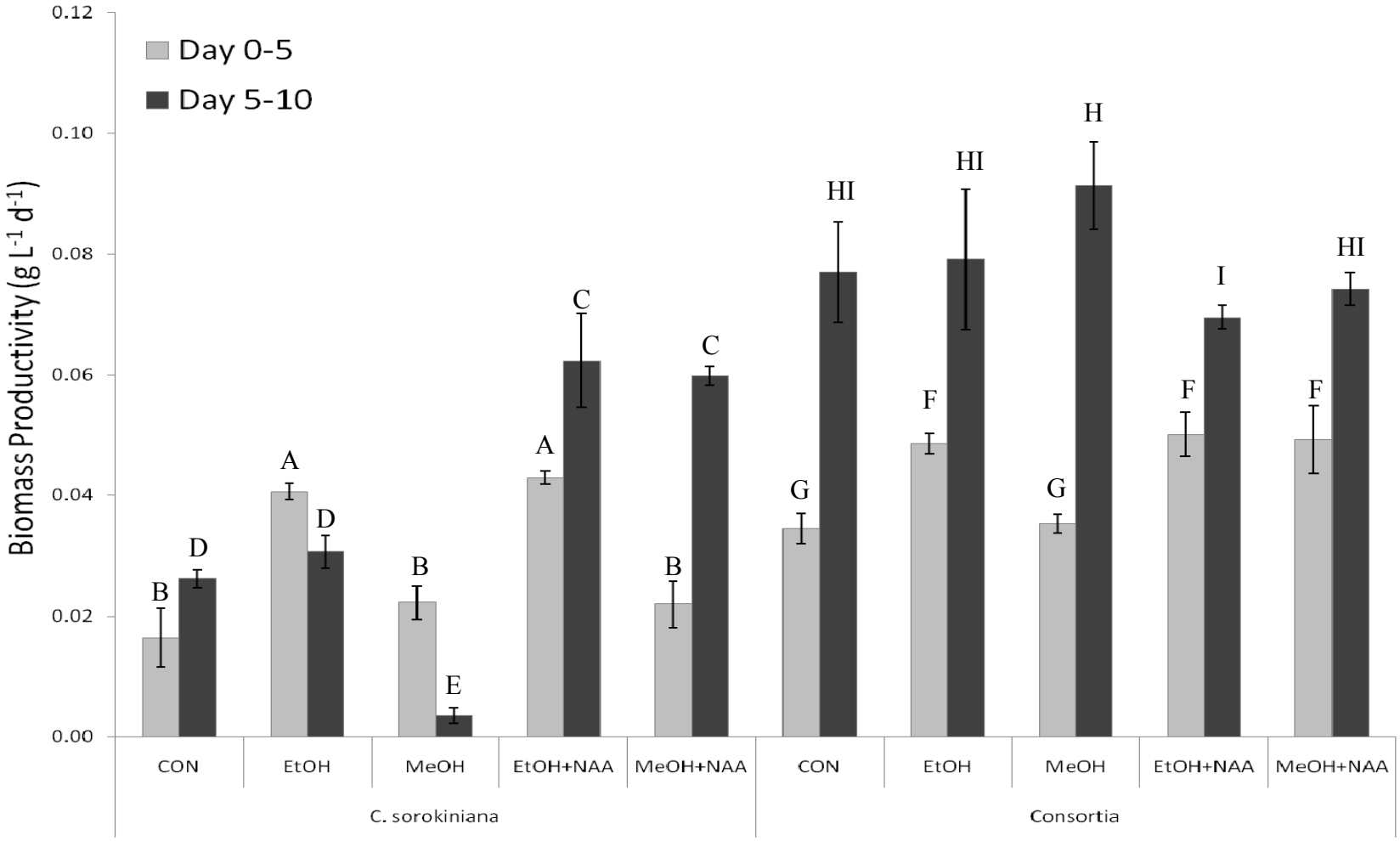

Figure 1a: Biomass productivity responses of *C. sorokiniana* (left) and Mixed Consortia (right) to solvent $_{500\ ppm}$ and NAA $_{5ppm}$. Solvents compared are methanol (MeOH) and ethanol (EtOH) either by themselves or with biochemical stimulant naphthalene acetic acid (NAA). The control (CON) did not receive any biochemical stimulant. Individual bars are means (n=3) and error bars represent two standard deviations. Day 0-5 and Day 0-10 bars represent average productivities over the first five days and between 5th and 10th day, respectively. Statistical comparison is only valid for each individual species during the same time interval.

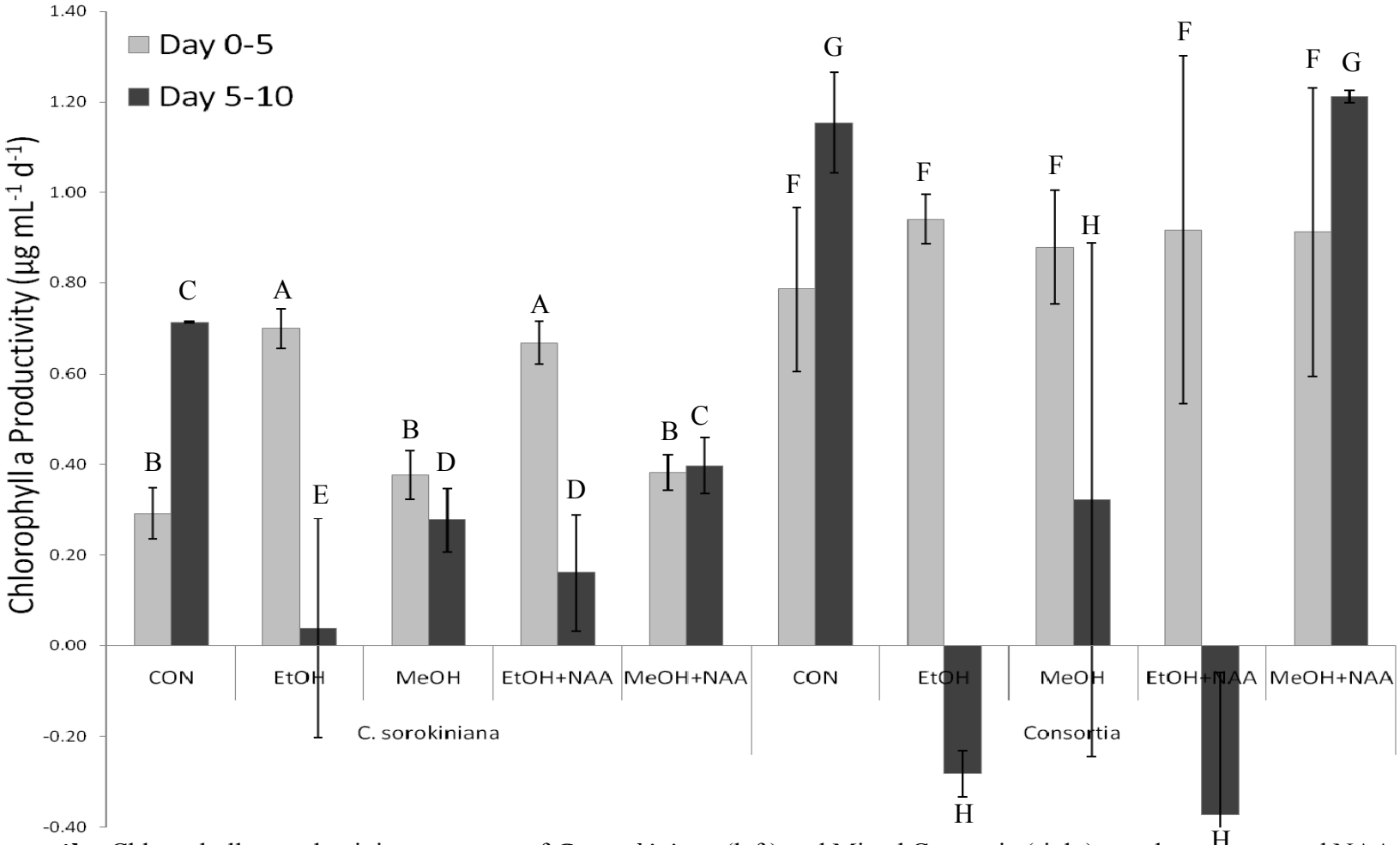

Figure 1b: Chlorophyll *a* productivity responses of *C. sorokiniana* (left) and Mixed Consortia (right) to solvent $_{500\ ppm}$ and NAA $_{5ppm}$. Solvents compared are methanol (MeOH) and ethanol (EtOH) either by themselves or with biochemical stimulant naphthalene acetic acid (NAA). The control (CON) did not receive any biochemical stimulant. Individual bars are means (n=3) and error bars represent two standard deviations. Day 0-5 and Day 0-10 bars represent average productivities over the first five days and between 5th and 10th day, respectively. Statistical comparison is only valid for each individual species during the same time interval.

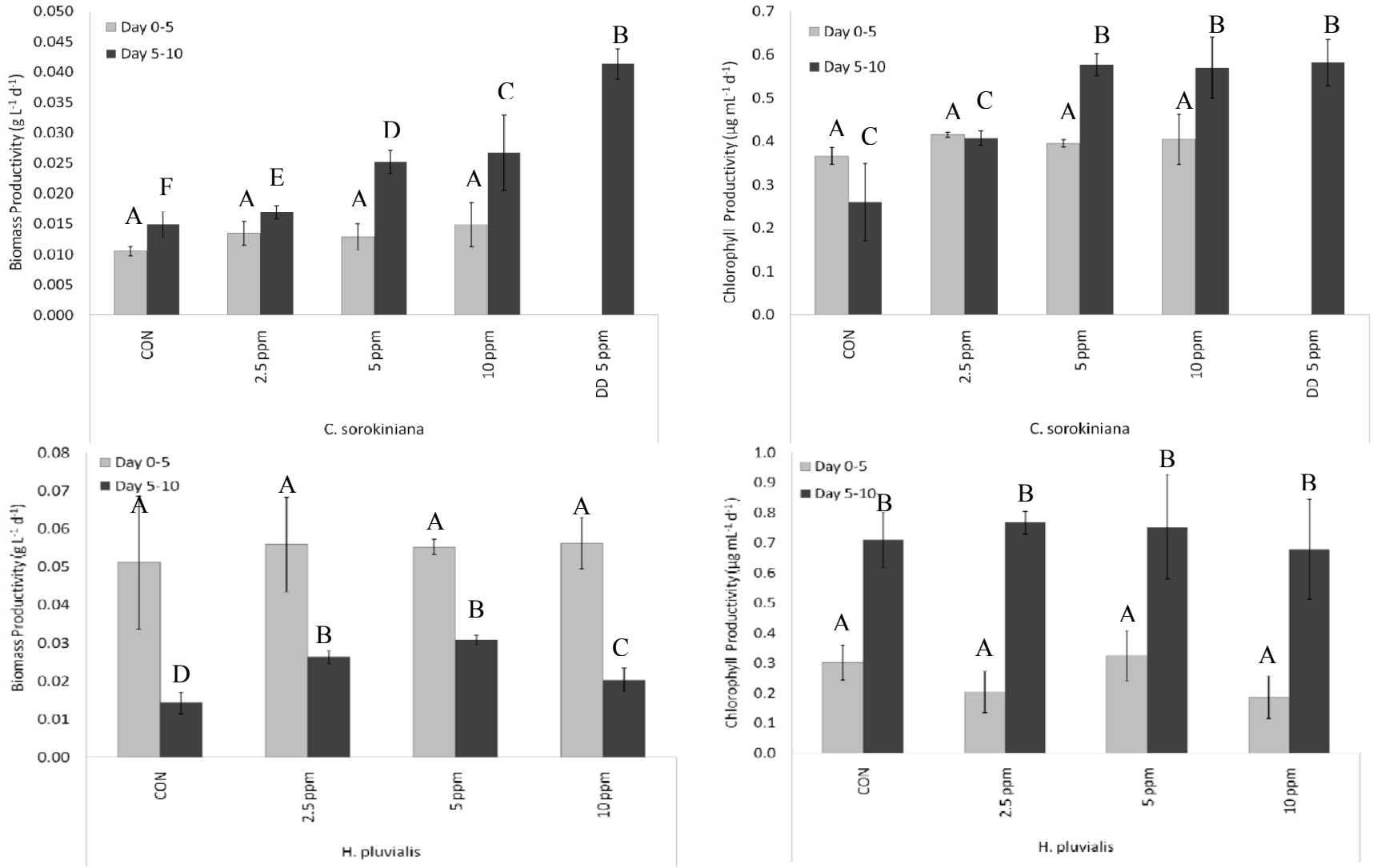

Figure 2a: Biomass and Chlorophyll *a* Productivity for *C. sorokiniana* and *H. pluvialis* to ethanol $_{500\ ppm}$ and NAA $_{2.5ppm,\ 5ppm,\ 10ppm}$. The control (CON) did not receive any biochemical stimulant. Individual bars are means (n=3) and error bars represent two standard deviations. Day 0-5 and Day 0-10 bars represent average productivities over the first five days and between 5th and 10th day, respectively. Statistical comparison is only valid for each individual species during the same time interval.

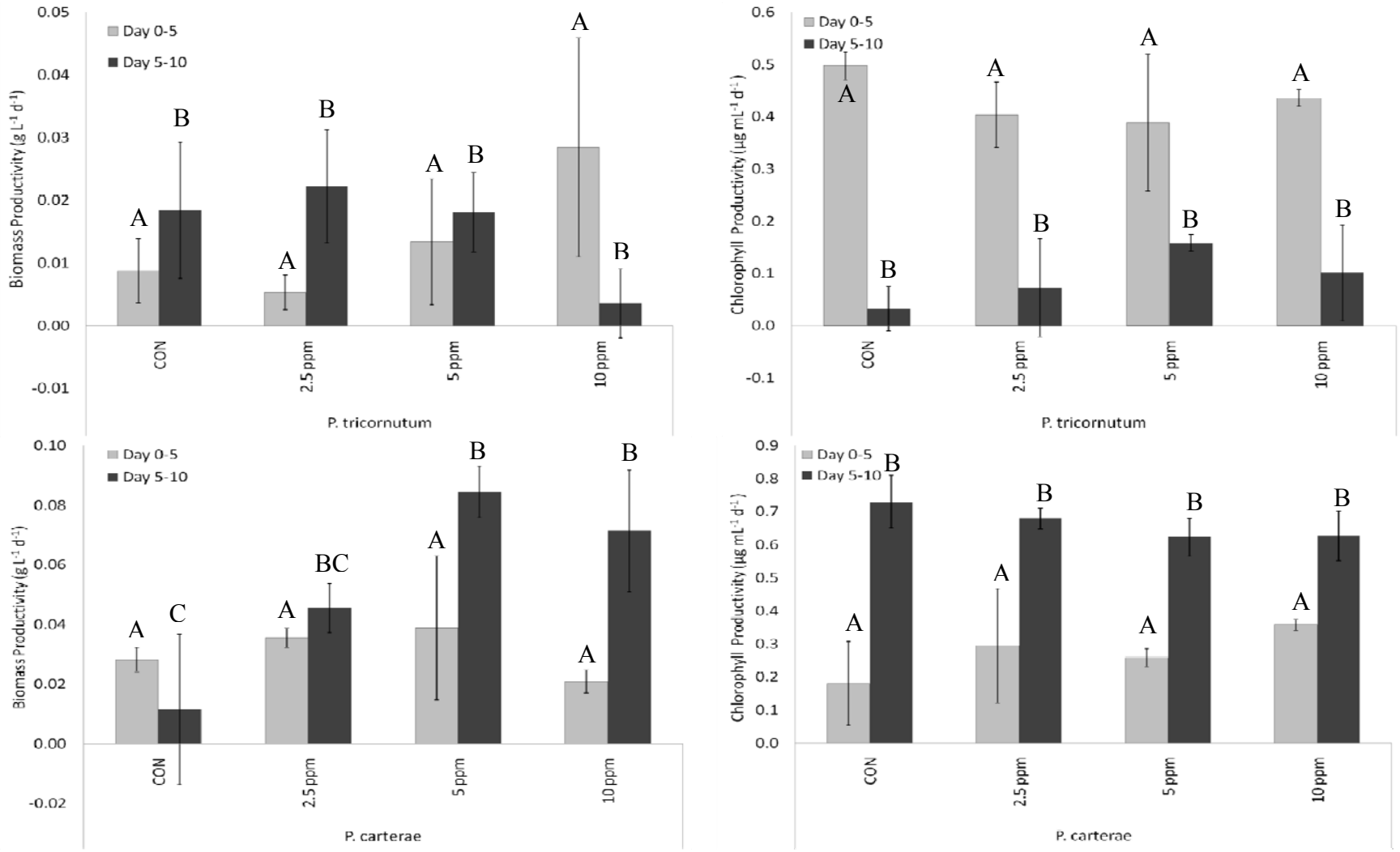

Figure 2b: Biomass and Chlorophyll *a* Productivity for *P. tricornutum* and *P. carterae* to ethanol $_{500\ ppm}$ and NAA $_{2.5ppm,\ 5ppm,\ 10ppm}$. The control (CON) did not receive any biochemical stimulant. Individual bars are means (n=3) and error bars represent two standard deviations. Day 0-5 and Day 0-10 bars represent average productivities over the first five days and between 5th and 10th day, respectively. Statistical comparison is only valid for each individual species during the same time interval.

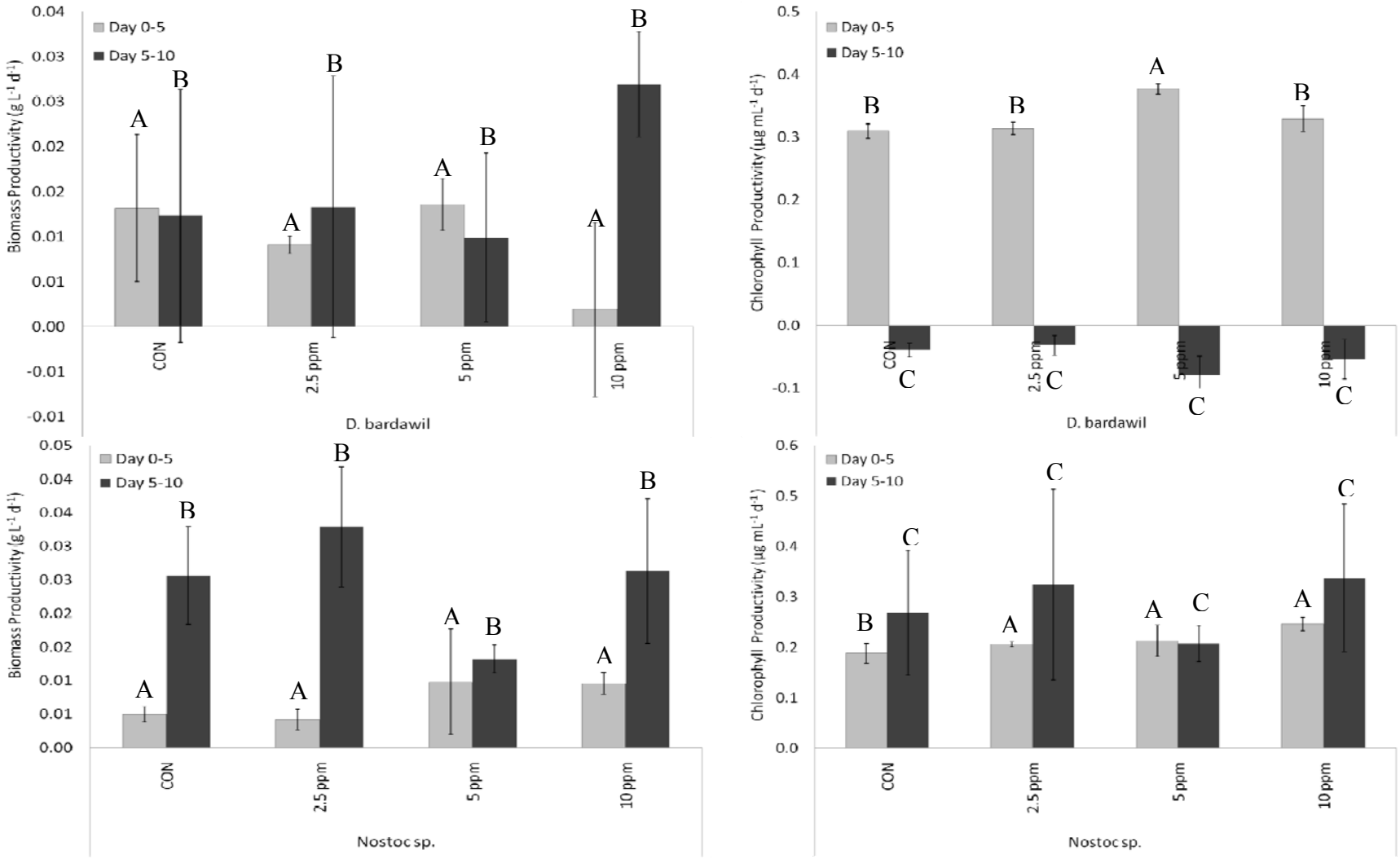

Figure 2c: Biomass and Chlorophyll *a* Productivity for *D. bardawil* and *Nostoc sp.* to ethanol $_{500\ ppm}$ and NAA $_{2.5ppm,\ 5ppm,\ 10ppm}$. The control (CON) did not receive any biochemical stimulant. Individual bars are means (n=3) and error bars represent two standard deviations. Day 0-5 and Day 0-10 bars represent average productivities over the first five days and between 5th and 10th day, respectively. Statistical comparison is only valid for each individual species during the same time interval.

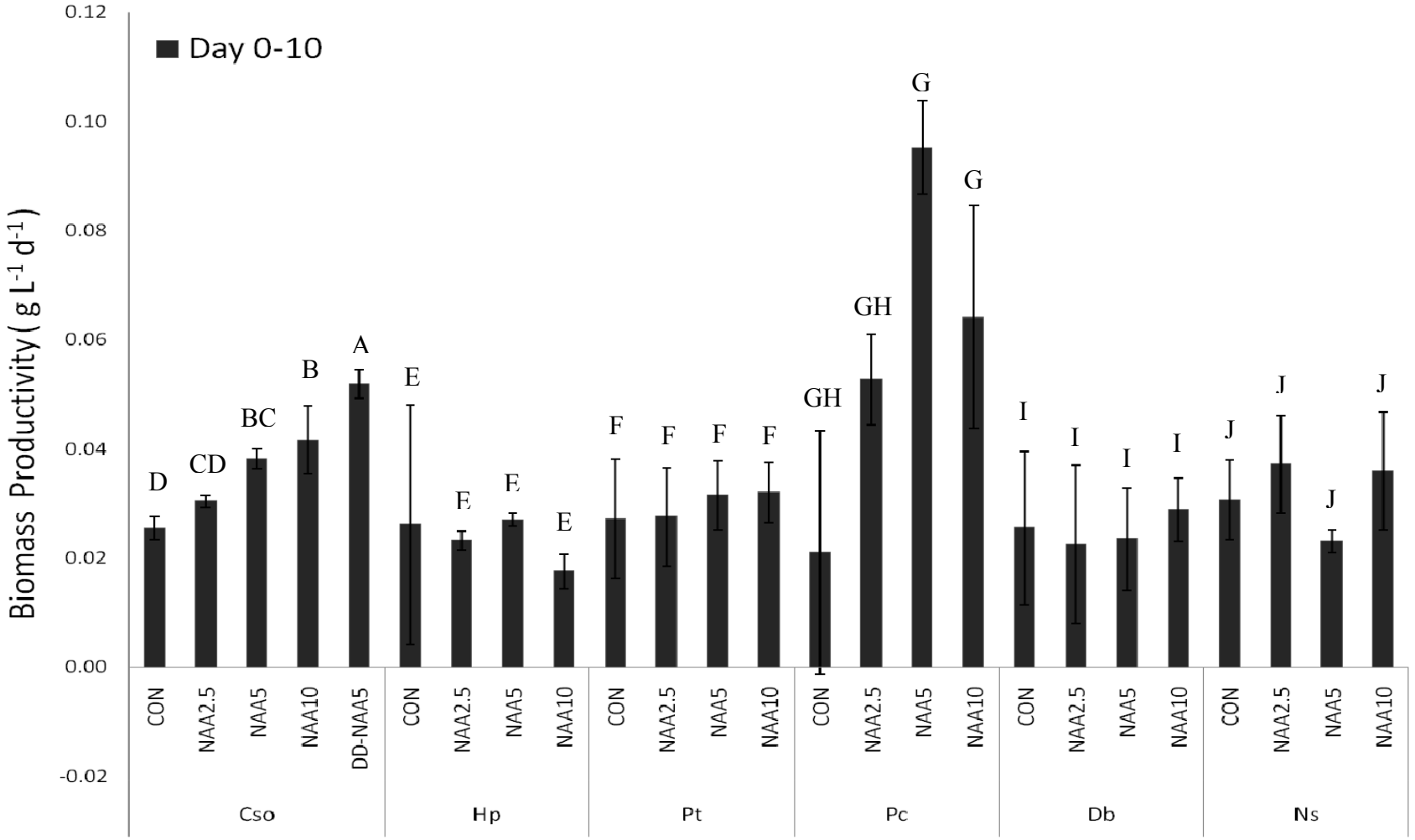

Figure 2d: Biomass Productivity between day 0 and day 10 for *C. sorokiniana*, *H. pluvialis*, *P. tricornutum*, *P. carterae*, *D. bardawil* and *Nostoc sp.* in experiment II under the treatments of ethanol $_{500\ ppm}$ and NAA $_{2.5ppm,\ 5ppm,\ 10ppm}$. The control (CON) did not receive any biochemical stimulant. Individual bars are means (n=3) and error bars represent two standard deviations.

CHAPTER 4

Conclusion

The research contribution presented here was focused on investigating various types of biochemical growth promoting compounds that have been shown in the literature to be effective at stimulating growth of plants and algae. The research effort established several bioactive agents that were capable of biostimulation of the green alga, *Chlorella sorokiniana*. The top performing compounds were found to be in the auxin family, and naphthalene-acetic acid (NAA) was observed to induce the largest increase in growth rate after the 10 day growth period in *C. sorokiniana.* The auxin, phenyl-acetic acid (PAA), was found to induce the largest increase in growth during the first five days of growth, but the combination of the two auxins, NAA and PAA, were not found to be synergistic with each other on *C. sorokiniana.* The combination of phytohormones from other families, such as gibberellins and cytokinins, did produce additional stimulation in growth over the effect of agent applied alone. This effect was most dramatic for NAA combined with gibberellic acid 3 (GA3) and zeatin (Zt), however, the increase in growth was not purely additive and the additional growth may not warrant the expensive costs associated with large scale application of GA3 and Zt to commercial microalgae cultivation. It was concluded that NAA dosed at the 5ppm level was most effective as a simple, yet effective treatment for stimulating growth in *C. sorokiniana.*

The results from the first manuscript indicated that NAA_{5ppm} was the best candidate for future exploration of biochemical stimulation with microalgae. The second manuscript

investigated the impact of the choice of solvent used to dissolve and dose the NAA treatment. Two solvents were assayed, ethanol and methanol, and their combination of NAA_{5ppm}. It was found that ethanol was a superior solvent to methanol with *C. sorokiniana*, and that the application of ethanol at a concentration of 500 ppm was effective at stimulating an increase in biomass productivity within the first five days of growth. Methanol at this concentration, was not effective as a sole agent under these conditions, however when added with NAA, the inhibitory impact was reversed and the final day growth rate of *C. sorokiniana* was higher than control, but not as high as the ethanol counterpart. Thus, a combination of ethanol and NAA at a concentration of 500 ppm and 5 ppm, respectively, were carried over into the next experiment. The final objective was to determine whether the effect of NAA in ethanol was universally applicable to any microalgae, or was this effect a special case and only effective with *C. sorokiniana.*

The multispecies experiment investigated 5 microalgae strains and one cyanobacterium, and found that three of the six species were positively impacted by the biostimulant treatment. The treatment dosages tested were taken from a range of effective dosages found in the literature, namely, $NAA_{2.5ppm}$, NAA_{5ppm}, NAA_{10ppm}. It was observed that the green alga, *C. sorokiniana* and *H. pluvialis,* and the coccolithopore, *P. carterae,* all responded best to the NAA_{5ppm} treatment exhibiting dramatic increases in biomass productivity compared to their control. The three other species had only marginal impact, or in some cases, an inhibitory impact compared to the control. There did not seem to be any correlation with the impact of this treatment with the family of organisms tested or type of media used (freshwater vs. saltwater). Thus, the treatment of NAA with ethanol does seem to be effective with other algal strains and may have potential

for use in larger scale application, however not all species may respond, and some may be negatively affected most likely from the choice of solvent used.

The results from these studies show that the use of $EtOH_{500ppm}$+NAA_{5ppm} was an effective biochemical stimulant treatment for approximately half of the cultures tested in these studies. The use of this combination should be tested on the culture of interest and further studies are needed to investigate whether this effect is scalable. If the application of such low doses of NAA is found to be an effective biostimulant, then its application can be considered as an alternative to using genetic modification for boosting algal growth for commercial microalgae cultivation.