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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 (0.22 ppm), humic acid (20 ppm), kelp extract (250 ppm), methanol (500 ppm), ferric chloride (3.2 ppm), putrescine (0.09 ppm), spermidine (1.5 ppm) were prescreened for their impact on growth and chlorophyll for the green alga—*Chlorella sorokiniana. C. sorokiniana* responded best to phytohormones in the auxin family, particularly NAA. Thereafter, two studies were conducted on combinations of phytohormones to compare blends from within the auxin family as well as against other families. These treatments were $NAA_{5\ ppm}$+$PAA_{30\ ppm}$, $NAA_{2.5\ ppm}$+$PAA_{15\ ppm}$, $NAA_{5\ ppm}$+$IBA_{10\ ppm}$, $NAA_{5\ ppm}$+$GA3_{10\ ppm}$, $NAA_{5\ ppm}$+$ZT_{1\ ppm}$, 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 effect of biochemical stimulants were also measured on metabolites such as chlorophyll, protein, and lipids in *C. 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

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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 gasses, 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 [3–8]. 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* genuses [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, 12–16]. 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} day^{-1} [17]. This translates to approximately 18 to 36 dry t ha^{-1} $year^{-1}$. Increasing algal productivity from 21 g m^{-2} day^{-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

C. 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 °C and 100±10 μmol m^{-2} s^{-1} light intensity provided by cool white fluorescent (6500K) 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 days 5 and 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 days 5 and 10 where the day 5 sampling represents 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 2 and 3. After inoculation, the flasks were incubated for 10 days in the growth chamber. Because initial cell

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]
		1.3×10^{-4}	Increased growth by 59% in *Nostoc muscorum*	[24]
		1.3×10^{-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	7×10^{-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 *C. sorokiniana* for each of the 10 day experiments.

Biochemical stimulants	Type of stimulant	Concentration (ppm)
Experiment I		
Phenylacetic acid	Auxin	30
Indole butyric acid	Auxin	10
Napthalene acetic acid	Auxin	5
Gibberellic acid	Gibberellin	10
Zeatin	Cytokinin	0.002
Thidiazuron	Cytokinin	0.22
Humic acid	Humate	20
Kelp extract	Plant extract	250
Methanol	Chemical	500
Ferric chloride	Micronutrient	3.2
Experiment II		
Putrescine	Polyamine	0.09
Spermidine	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

densities, resulting from addition of inocula, can have a significant impact on measured productivity over time, comparisons across experiments with different initial cell densities are reported as increases in productivity relative to the control within that experiment.

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 °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 5,000 rpm for 10 min, 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:

$$\text{Chlorophyll } a\left(\mu\text{g ml}^{-1}\right) = 16.29 \times \text{OD}_{665} - 8.54 \times \text{OD}_{652}$$

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 °C with hexane as solvent. Bags were then transferred to a forced-air oven and dried at 60 °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 (Fig. 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} day^{-1} compared to 0.018 g L^{-1} day^{-1} in the control (no biostimulants) and showed a 133% increase in biomass productivity on day 10 (Fig. 1a). 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 days 5 and 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 days 5 and 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 and TDZ, 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. 1a). The results showed substantial increases in chlorophyll *a* for

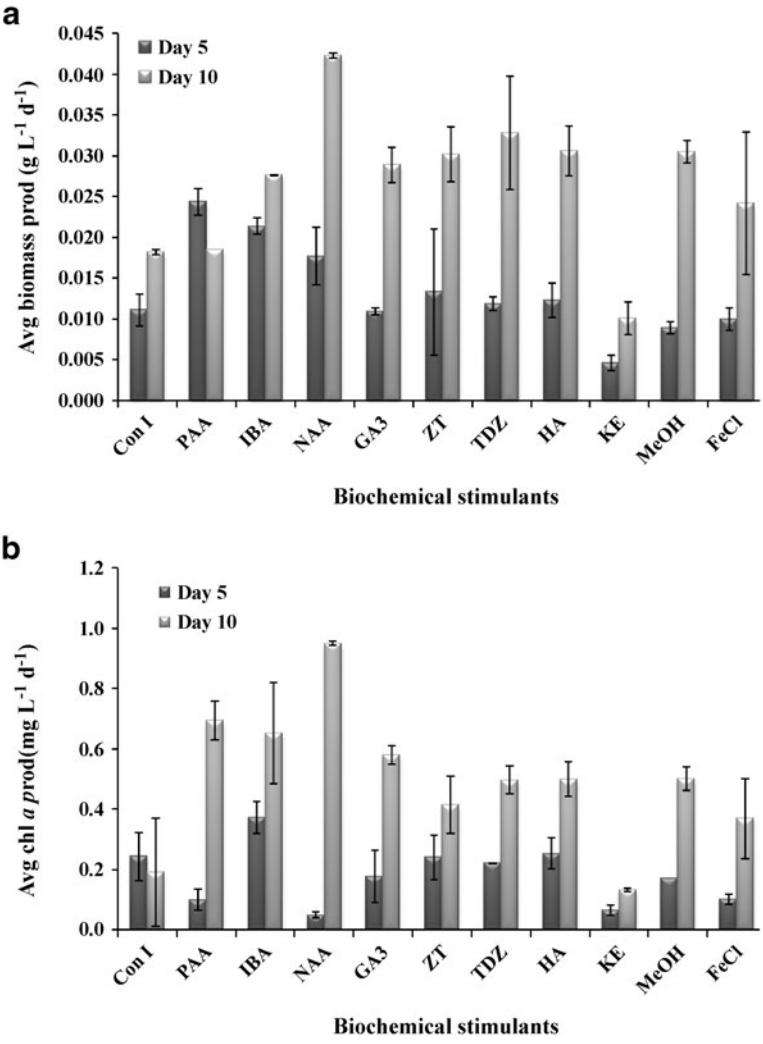

Fig. 1 Results from preliminary biochemical stimulant prescreen **a** increase in biomass productivity compared and **b** increase in chlorophyll a productivity for days 0–5 and 6–10. *Con I* control from experiment 1; *PAA* phenylacetic $acid_{30\ ppm}$; *IBA* indole butyric $acid_{10\ ppm}$; *NAA* 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

the auxin group on the final sampling day 10. The highest increase in chlorophyll *a* productivity was exhibited by NAA between days 5 and 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* productivity 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 days 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. This model proposes that auxins at high concentrations increase the activity of 1-aminocyclopropane-1-carboxylic acid 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. Figure 2a, b shows 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 5 days. However, it was only 36%, 16%, and 28% higher than the control over 10 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 days 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

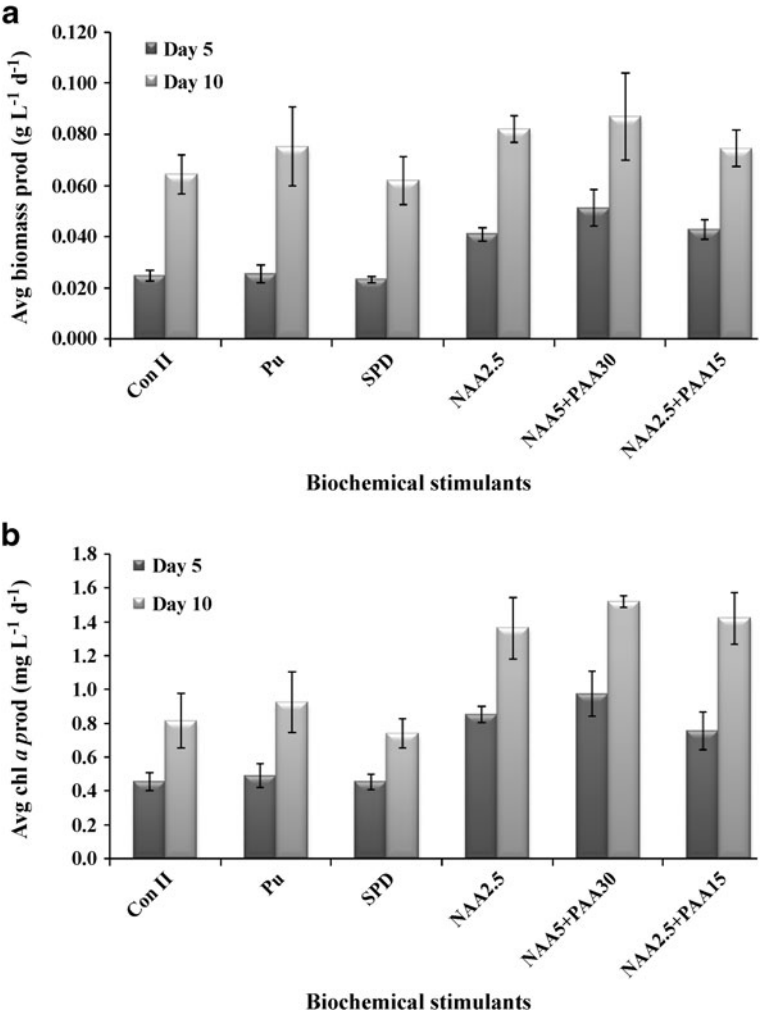

Fig. 2 Results from experiment 2 on the effect of various polyamines, auxins, and their combinations on **a** average biomass productivity and **b** average chlorophyll *a* for days 0–5 and 6–10 of algal growth. *Con II* control from experiment 2; *PU* putrescine $_{0.09\ ppm}$; *SPD* spermidine$_{1.5\ ppm}$; *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

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 *C. 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 10th 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} day^{-1}) over 10 days relative to control (0.082 g L^{-1} day¯1), whereas the other polyamine spermidine recorded lower biomass productivity (0.074 g L^{-1} day^{-1}) than the control.

The combination of stimulants $NAA_{5\ ppm}$+$GA3_{10\ ppm}$+$ZT_{1\ ppm}$ recorded the highest biomass productivity (0.143 g L^{-1} day^{-1}) and showed 170% increase over control (0.053 g L^{-1} day^{-1}) on day 10 (Fig. 3a). 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 5 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 5 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} day^{-1}), $ZT_{1\ ppm}$ (0.125 g L^{-1} day^{-1}), a gibberellin and cytokinin, respectively, did enhance the biomass productivity over $NAA_{10\ ppm}$ (0.093 g L^{-1} day^{-1}).

NAA in all combinations with GA3, IBA, and ZT showed only marginal increase in average biomass productivity between days 0 and 5 relative to control whereas the increase was significant between days 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 days 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 days 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,

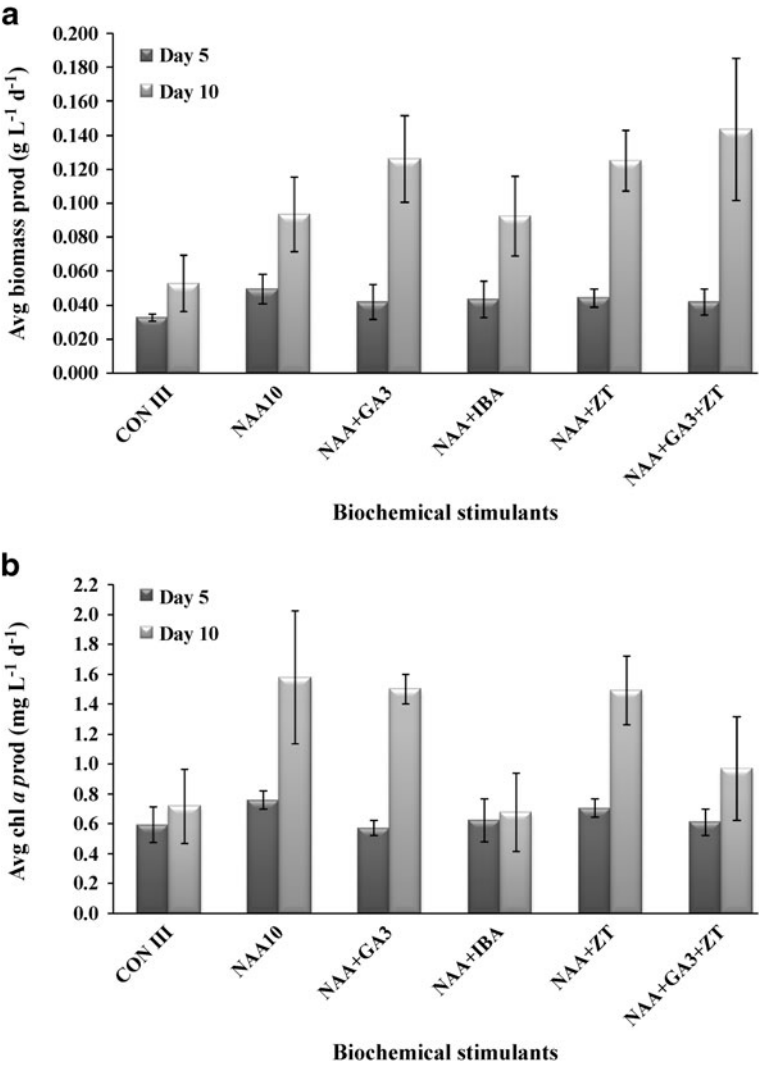

Fig. 3 Results from experiment 3 on the effect of auxin combinations and auxin with cytokinin and gibberellin combinations on **a** average biomass productivity and **b** average chlorophyll *a* for days 0–5 and 6–10 of algal growth. *CON III* control from experiment 3; *NAA10* $NAA_{10\ ppm}$; *NAA+GA3* $NAA_{5\ ppm}+GA3_{10\ ppm}$; *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

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 Fig. 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.

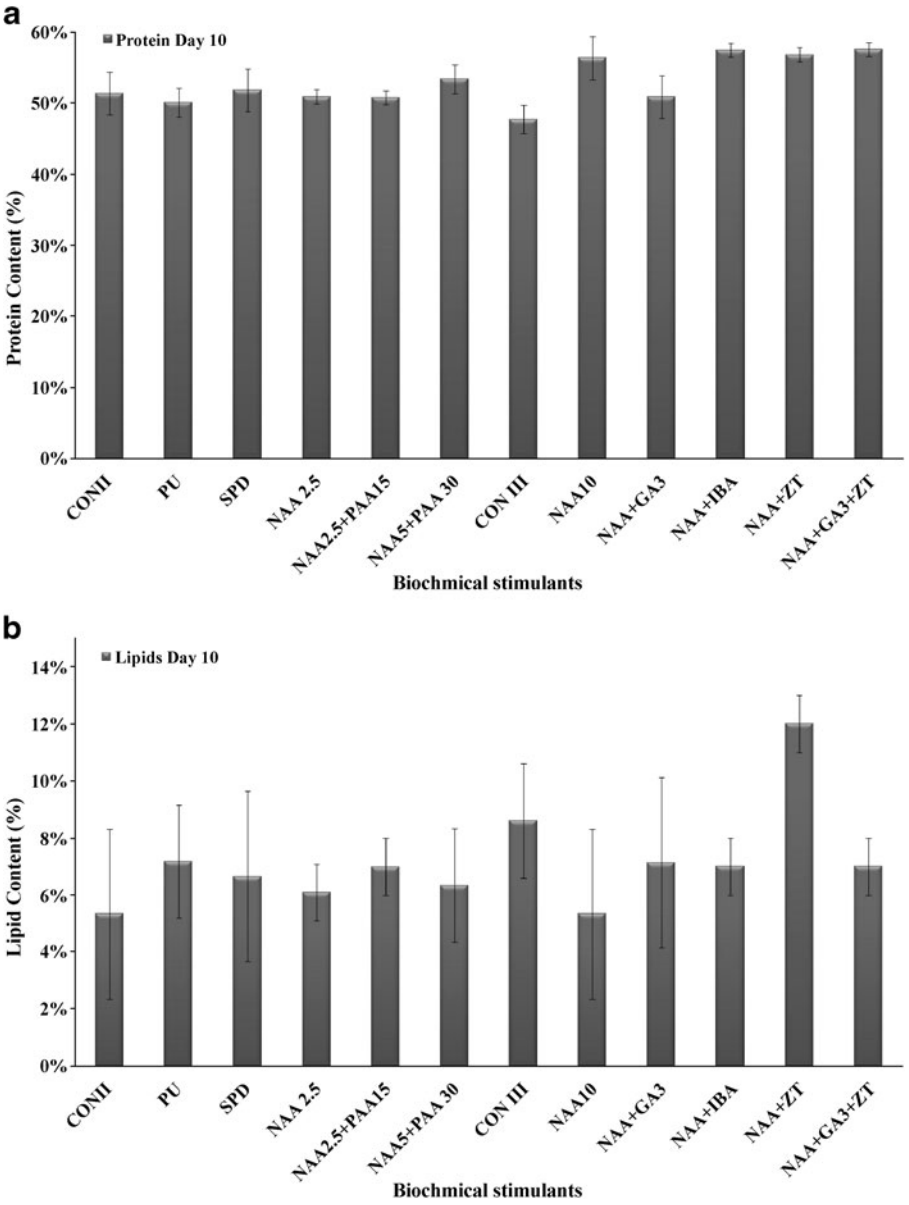

Fig. 4 Effect of various biochemical stimulants and their combinations from experiments 2 and 3 on lipid and protein content of the algae on the 10th day of growth. *CON II* control from experiment 2; *CON III* control from experiment 3; *PU* putrescine$_{0.09\ ppm}$; *SPD* spermidine$_{1.5\ ppm}$; *NAA2.5* $NAA_{2.5\ ppm}$; *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

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 specific productivity from various biostimulant treatments will depend upon variables associated with culture density, as the initial inoculum, mutual shading, and nutrient availability may impact the measured growth response. Experiments 2 and 3 had an initial inoculum density of 0.08 g/L and a final day concentration from 0.5 to 0.9 g/L which is presented in Fig. 5. 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 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 in the preliminary experiment. 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.

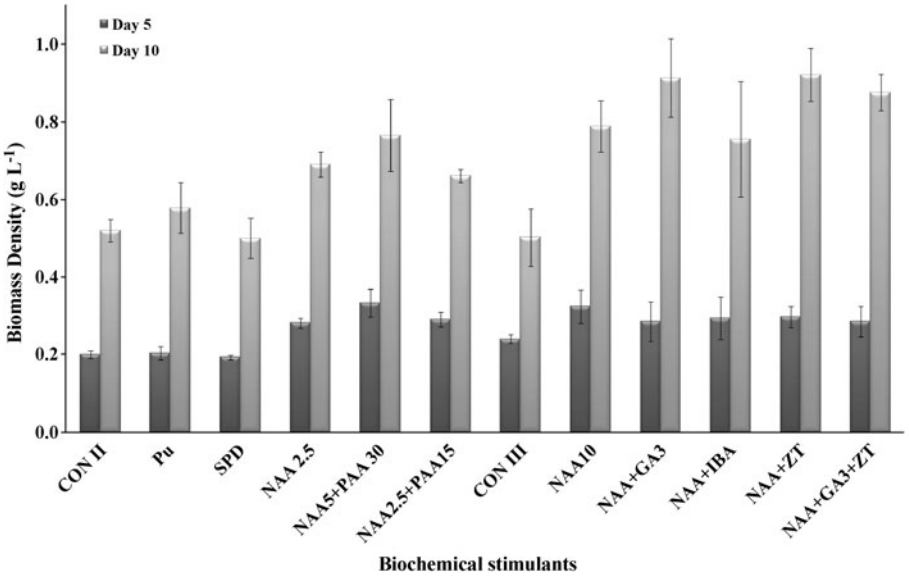

Fig. 5 Results from experiment 2 & 3 on the effect of various biochemical stimulants and their combinations on increases in biomass density for days 5 and 10. *CON II* control from experiment 2; *PAA* phenylacetic $acid_{30\ ppm}$; *IBA* indole butyric $acid_{10\ ppm}$; *NAA* naphthaleneacetic $acid_{5\ ppm}$; *GA3* gibberellic $acid_{10\ ppm}$; *ZT* $zeatin_{0.002\ ppm}$; *TDZ* $thidiazuron_{0.22\ ppm}$; *HA* humic $acid_{20\ ppm}$; *MeOH* $methanol_{500\ ppm}$; *PU* $putrescine_{0.09\ ppm}$; *NAA2.5* $NAA_{2.5\ ppm}$; *NAA5+PAA30* $NAA_{5\ ppm}$+$PAA_{30\ ppm}$; *NAA2.5+PAA15* $NAA_{2.5\ ppm}$+$PAA_{15\ ppm}$; *CON III* control from experiment 3; *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

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 10 days of growth when compared to their respective controls. These treatments showed approximately 2.3 to 2.7 times increase in biomass productivity over control. Treatment $PAA_{30\ ppm}$ recorded the largest increase in 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. More studies will be necessary for quantitative comparisons of productivity under different environmental conditions, such as inoculum density and phase, reactor configuration, nutrient availability, cell shading, etc. It is well known that these can impact the algal growth response, which limits us from reporting a universally applicable productivity for each biochemical stimulant. All of these experiments were conducted using a nitrogen rich nutrient medium (BG 11) and a carbon supply limited by diffusion into the culture medium. Thus, future studies can examine the impact of NAA with supplemental CO_2 enrichment and nitrogen-limited conditions. 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 freshwater 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.

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