SCREENING OF EFFICIENT GROWTH MODULATORS FOR TASK-SPECIFIC ENHANCEMENTS IN MICROALGAL CULTIVATION

M.Sc. Thesis

By
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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY INDORE
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A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree
of
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by
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INDIAN INSTITUTE OF TECHNOLOGY INDORE
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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **SCREENING OF EFFICIENT GROWTH MODULATORS FOR TASK-SPECIFIC ENHANCEMENTS IN MICROALGAL CULTIVATION** in the partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from August 2021 to May 2023 under the supervision of Dr. Kiran Bala, Associate Professor, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Vaibha 09/05/2023

Signature of the student with date
VAIBHAV CHOUHAN

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

15/5/23

Signature of the Supervisor of
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Date: 15/5/23

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Vaibhav Chouhan

Dedicated to my beloved family

Abstract

Microalgae are an abundant source of value-added metabolites and have emerged as a promising feedstock in food, medicine, and bioenergy sectors. However, low biomass productivity and metabolite content are major neck-bottle problems in commercializing microalgal-based bio-products. The conventional and genetic engineering tactics to improve microalgal productivity remain challenging due to compromised growth and high maintenance costs. To overcome these issues, the concept of efficient chemical stimulants has emerged as a topic of intense research in the microalgal cultivation domain. In this study, 55 chemicals with different physiological roles have been screened using *Scenedesmus* sp. as a target. A two-phase screening was performed to identify the best chemical stimulants; phase I was performed with 96-well culture plates. Chemicals showing increased growth were further studied in phase II. Phase II screening was carried out in 250 mL culture volume. Chemicals such as benzoic acid (BA), salicylic acid (SA), and folic acid (FA) enhanced the biomass and/or biochemical portfolio of *Scenedesmus* sp. Cells treated with BA have 14.61 % (18.8 ± 0.5 mg L^{-1} d^{-1}) higher biomass productivity, 120.53 % (4.3 ± 1.3 µg L^{-1} d^{-1}) higher tocopherol productivity, and 16.59 % (218.1 ± 15.4 mg g^{-1}) higher lipid content, with biomass concentration reaching up to 338.5 ± 10.1 mg L^{-1} compared to control (295.4 ± 18.2 mg L^{-1}). Similarly, FA also enhanced biomass and tocopherol productivities, and lipid content by 10.13 % (18.0 ± 0.7 mg L^{-1} d^{-1}), 81.80 % (3.6 ± 1.5 µg L^{-1} d^{-1}), and 25.57 %

(234.9 ± 16.7 mg g^{-1}), respectively compared to control. SA (1000 nM) and IAA-treated cells have enhanced tocopherol and biomass productivity. Among all modulators, only OAA enhanced the carbohydrate content and carotenoid productivity by 9.38 % and 16.5 %, respectively. Auxins (such as IAA, IBA, and IPA) treated cells have higher lipid productivity than the control. Additionally, 3-HDA and kinetin also enhanced the tocopherol productivity by 83 % and 89%, respectively. Chemical stimulants act in a dose-dependent manner and target specific metabolic pathways altering the production and accumulation of specific cellular metabolites. Hence, the emergence of chemical modulators-based media engineering tactics is a practical and sustainable approach for the development of cost-effective algal bio-refineries in various sectors.

LIST OF PUBLICATIONS

1. Ghosh, T., **Chouhan, V**., Bala, K., Task – specific growth modulators for enhancing microalgal growth and metabolite productivity. (Manuscript under preparation)
2. Ghosh, T., **Chouhan, V**., Bala, K., Evaluation of the antibiotics as growth modulators for α-tocopherol enhancement in microalgae: An alternative media engineering strategy. (Manuscript under preparation)

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ACRONYMS

PUFA	Polyunsaturated fatty acids
TAG	Triacyl glycerides
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
ALA	α-linolenic acid
CVD	Cardiovascular diseases
LDL	Low density lipoproteins
ECM	Extracellular matrix
BHA	Butylated hydroxyl anisole
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
GA_3	Gibberellin A_3
bkt	β-carotene ketolase
VFA	Volatile fatty acids
ROS	Reactive oxygen species
EGCG	Epigallocatechin gallate
DA6	Diethyl aminoethyl hexanoate
BNOA	2-Napthoxyacetic acid
OAA	Oxaloacetic acid
AA	Ascorbic acid
SA	Salicylic acid
SB	Sodium butyrate
3-HDA	3-hydroxydecanoic acid
FA	Folic acid
BA	Benzoic acid
cAMP	Cyclic adenosine monophosphate
PG	Propyl gallate
SPV	Sulfo-phospho-vanillin
BSA	Bovine serum albumin

CHAPTER 1

Introduction

Microalgae are an extremely diverse group of photosynthetic organisms that can grow under a wide range of habitats and utilize light energy, organic and inorganic nutrients to produce biomass and a wide array of metabolites such as pigments, lipids, polysaccharides, vitamins, proteins, and other value-added products. They have evolved to thrive under stressful environments assisted by the production of various biomolecules (specifically secondary metabolites) which have drawn interest from the nutraceutical, cosmeceutical, bioenergy, and pharmaceutical industries (Khan et al., 2018; X. Yu et al., 2015).

1.1. Microalgal metabolites and their application in various sectors

Microalgae are recognized as a sustainable, renewable, and economical source of biofuels, medicinal and nutritional products. Their application in different sectors has been well recognized at a commercial scale (Khan et al., 2018).

Algal biomass has now been recognized as a third-generation feedstock for the manufacture of biofuels (biodiesel and bioethanol), which have been advocated as a better alternative to non-renewable fossil fuels. Biofuels have higher oxygen and low sulphur levels compared to conventional petroleum-based fuels. The neutral lipids (TAGs) accumulated by microalgae can be converted to biodiesel, the commercialization of which has already been started (Franz et al., 2013; Jeon et al., 2017; Khan et al.,

2018). Microalgae based biofuel is a promising candidate due to its high annual yield (2500 gallons per acre), carbon neutrality, non-toxicity (Jiang et al., 2015). The microalgal biomass is enriched with carbohydrates, the fermentation of which can produce bioethanol (Jeon et al., 2017). Hence, algae with higher lipid (oleaginous) and carbohydrate content are suitable for biofuel generation. Furthermore, red biotechnology has grown in prominence due to the adverse effects of conventionally produced synthetic drugs. Microalgae-derived bioproducts have been well-recognized for antimicrobial, anti-inflammatory, antioxidant, anticancer, and anti-aging activities (M. U. et al., 2019). These bioactive substances are now available in the market in different forms (Jeon et al., 2017). PUFAs like EPA, DHA, and ALA (*Linolenic Acid - an Overview | ScienceDirect Topics*, n.d.) have high therapeutic values for decreasing the risk of CVDs, asthma, Alzheimer's, and other autoimmune diseases. They are also important in retinal and brain development in newborns. Carotenoids, the primary role of which is photoprotection and ROS quenching, are accumulated at a low level in the microalgal cells under normal conditions. Carotenoids such as -carotene, astaxanthin, fucoxanthin, and canthaxanthin accumulate at high levels under stressful circumstances and have been well-recognized for their antioxidant and anti-inflammatory, antihyperglycemic, and antihyperlipidemic effects. Astaxanthin has the potential to cross the blood-brain barrier and has a therapeutic role in neurodegenerative diseases (M. U. et al., 2019). Vitamins like pro-vitamin A and vitamins B, C, D, and E are present in microalgae and are reported to have antioxidant and anticarcinogenic properties.

Vitamin E (tocopherols and tocotrienols) specifically are effective free radical scavengers and play an important role in the prevention of lipid peroxidation, LDL oxidation, vascular damage and have other chemoprotective roles (Galasso et al., 2019).

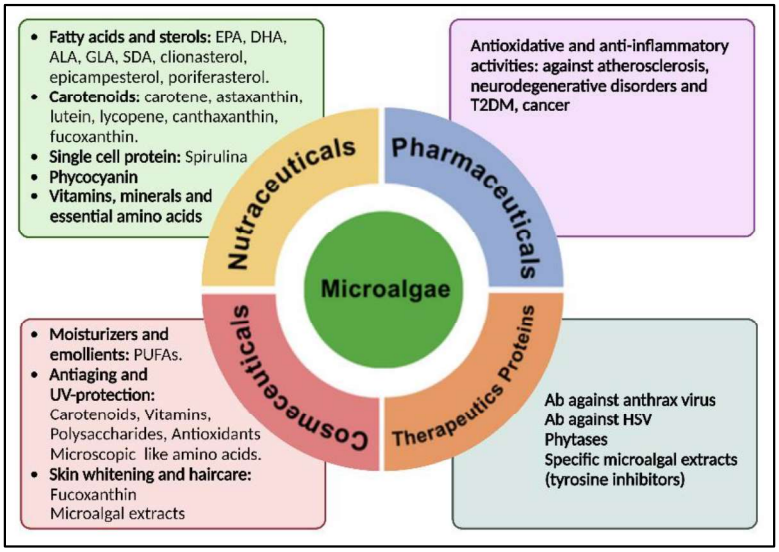


Figure 1.1. Microalgae-based products and their utility in different bio-refinery sectors (M. U. et al., 2019)

The concept of the nutritional role of microalgae is not new, as many of them are approved as GRAS (Generally Regarded as Safe) for human usage. Microalgae act as a superior source of single-cell proteins containing all essential amino acids and PUFAs (which are not synthesized by mammals) which are necessary for the overall development of humans (M. U. et al., 2019), reducing their dependence on plant-and animal-based products thus preventing their exploitation.

Due to the well-defined importance of microalgal extracts, the interest of the cosmetic industry in the usage of algal-based products has increased, they have been used in skin

and hair care formulations. Microalgal proteins, PUFAs, and other essential fatty acids are well-used in skin moisturizers due to their efficient water restoration capacity. UV radiation is the major reason for skin aging due to ROS production in the epidermal layer followed by ECM destruction. Vitamins, carotenoids, and polysaccharides have been used to treat UV-induced skin damage and in the regeneration of cells by increasing the production of ECM components (M. U. et al., 2019). Vitamin E is a renowned additive in hair formulations with proven antioxidant effects on the scalp (Keen & Hassan, 2016).

1.2. Problem statement and approaches

Microalgal cells are reported to have high photosynthetic and carbon sequestering efficiency, they lack vascular tissues and are generally more suited for economical procurement of value-added products than plant cells. Nevertheless, microalgal cultivation is the crucial yet expensive step that determines the quality and quantity of products (Dao et al., 2018). When cultivated under normal conditions, algae often produce bioactive metabolites in small amounts. Stresses (physical or chemical) are reportedly shown to have a significant impact on derailing the carbon flux to accumulate useful bioproducts (Ren et al., 2021). The traditional approach of promoting the accumulation of highly valuable bioproducts involves abiotic stresses including high irradiance and salinity, exposure to heavy metals, and change in light intensity; however, these methods have their own downsides (Wang et al., 2021). Media engineering strategies arose as an

alternative approach to redirect regular metabolic activity by influencing the productivity of targeted metabolites in a non-gene disruptive way (Paliwal & Jutur, 2021). The use of nutrient limitation and two step cultivation strategies are reported to have enhanced lipid, carbohydrate, proteins and other value added products but at the cost of compromised growth, which impedes their commercial applicability (Ram et al., 2019). In an alternative approach, studies have suggested the use of small organic molecules to improve the overall biomass and biochemical productivity of microalgae (Franz et al., 2013). These task specific chemicals, termed as modulators, act as metabolic triggers even at minuscule concentrations by modulating the metabolic pathways resulting in up- and down-regulation of specific metabolites (Paliwal & Jutur, 2021). Studies have also shown that use of chemical modulators in microalgal cultures to increase productivity is beneficial and practical at a commercial level (Franz et al., 2013).

CHAPTER 2

Literature review

2.1. Modulators – Potential biochemical stimulators

The application of chemicals in media optimization is a scalable and sustainable approach to address the cost-effective microalga cultivation at a commercial level (Parsaeimehr et al., 2017). The advantageous aspect of this tactic includes the fact that it does not necessitate knowledge of genetic background of gene targets and pathways involved in production of bioproducts (Franz et al., 2013; Paliwal & Jutur, 2021; X. Yu et al., 2015).

The concept of modulators was first introduced by Franz *et al* (Franz et al., 2013), who studied the effect of bioactive chemicals (selected based on the previous reports on mammals and yeast) on different green microalgae; the study reported that chemicals such as cAMP, forskolin, BHA, and propyl gallate at their respective optimum concentrations showed increased lipid productivity (> 50 %), among which BHA and propyl gallate can be used for large-scale cultivation, owing to their low cost. The exogenous application of non-neuronal acetylcholine on *Chlorella* sp. and *Scenedesmus* sp. at minuscule concentration showed positive effect on various metabolites productivity (Paliwal & Jutur, 2021; Parsaeimehr et al., 2015). Phytohormones serve as signalling molecules that control several aspects of development and growth and are normally active at low concentrations (Park et al., 2013). Parsaeimehr *et al.* (Parsaeimehr et al., 2017) reported increased biomass, lipid content, and ALA production using phytohormones such as

kinetin, IBA, and methyl jasmonate. A study by (Lu et al., 2010) suggested the role of stress hormones in increased carbon flux towards carotenogenesis.

Indomethacin, an analog of IAA at concentration of 100 nM increased the biomass, cell division, nucleic acid, pigments and protein content in *C. vulgaris* (Piotrowska et al., 2008). Antibiotics, such as erythromycin, when used at low concentrations, can increase the production of pigments (Zhang et al., 2021). A consolidated list representing the reported dose-dependent effects of different modulators on different algal species has been shown in Table 2.1.

2.2. Regulatory mechanisms of modulators

Broadly, the modulators regulate the enzyme activity, gene expression, and the antioxidant system of the cells. In addition, they also act as metabolic precursors in biosynthetic pathways of metabolites, directing the carbon flux towards their production. Methyl jasmonate and GA_3 influence the expression of bkt genes, which convert β-carotene to canthaxanthin in the carotenogenesis pathway (Lu et al., 2010). The analysis of the mRNA transcript showed that high transcript levels do not always correlate with increased metabolite production, there are other key regulatory pathways that interact during metabolite production. Some chemicals act as ROS stimulants; their production initiate an antioxidant signalling pathway that leads to the production of various bioproducts. Salicylic acid and methyl jasmonate at low concentrations, 100 µM and 10 µM, increase ROS-induced astaxanthin production, respectively (X. Yu et al., 2015) (Figure 2.1). Some chemicals such as pyruvate serve as a precursor for

isopentenyl pyrophosphate which itself acts as a precursor for carotenogenesis. Citric acid and malic acid can be converted to pyruvate and hence have a similar effect on microalgae. The conversion of malic acid to pyruvate increase NADPH production which results in increased lipid synthesis (X. Yu et al., 2015).

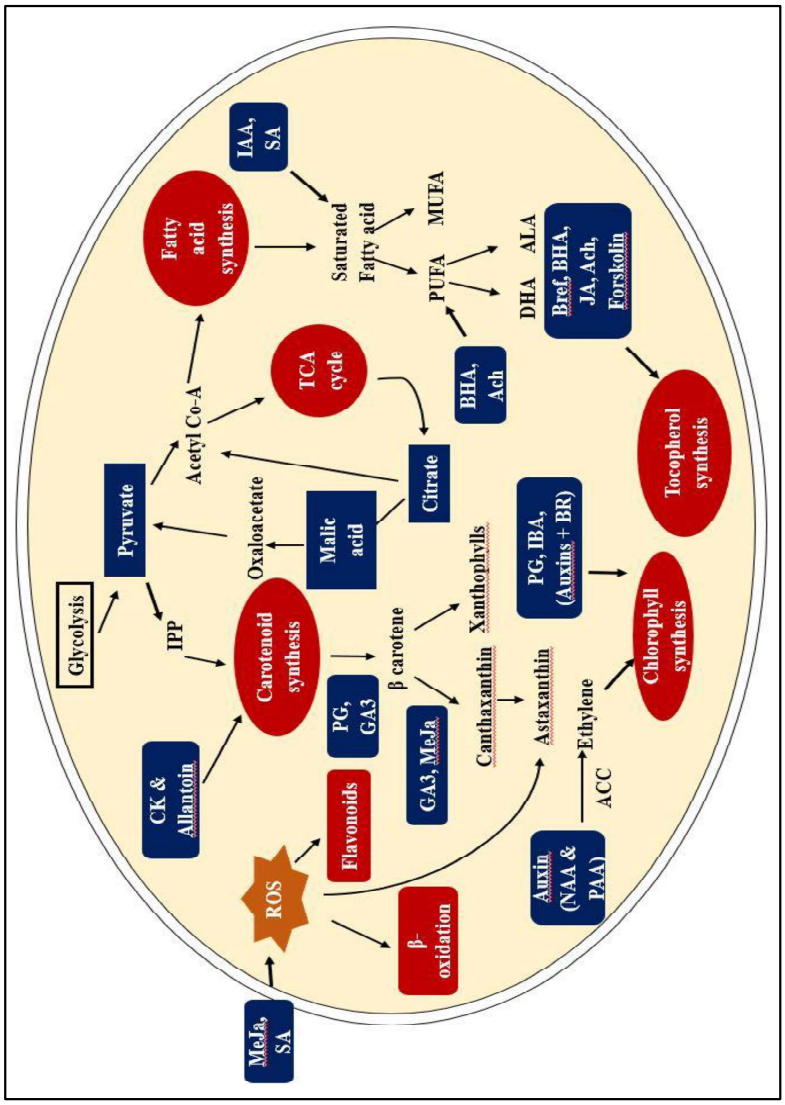


Figure 2.1. Overview of mechanisms of different chemicals on microalgae. The modulators are abbreviated as MeJa = Methyl jasmonate, CK = Cytokinin, PG = Propyl gallate, GA = Gibberellic acid, IBA = Indole butyric acid, PAA = Phenylacetic acid, Bref = Brefeldin, SA = Salicylic acid,

Ach = Acetylcholine, BHA = Butylated hydroxyl anisole, JA = Jasmonic acid, BR = Brassinosteroids, IAA = Indole acetic acid, IPP = Isopentenyl pyrophosphate, PUFA = Polyunsaturated fatty acids, MUFA = Monounsaturated fatty acid, ACC = Amino cyclopropane carboxylic acid synthase, NAA = Naphthalene acetic acid, DHA = Docosahexaenoic acid and ALA = α-linolenic acid.

Table 2.1. Dose-dependent effect of modulators on different algae.				
Modulators	**Organisms**	**Concentration**	**Effects**	**Refs.**
Forskolin	*N. oculata*	4 nM	Increased lipid content	Franz et al., 2013
	S.dimorphus	100 nM	Increased carbohydrate, protein, carotene productivity	Paliwal & Jutur, 2021
Orlistat	*Nannochloropsis* sp.	40 nM	Increased biomass and lipid productivity	Franz et al., 2013
BHA	*Chlorella protothecoides*	277.4 nM	Increased ALA content	Parsaeimehr et al., 2017
	N. salina	4 nM	Increased lipid content	Franz et al., 2013

Continued

	S. dimorphus	100 nM	Increased biomass, carotene, and tocopherol productivity	Paliwal & Jutur, 2021
PG	*N. salina*	40 nM	Increased lipid content	Franz et al., 2013
	S. dimorphus	100 nM	Increased β-carotene, Zeaxanthin and lutein productivity	Paliwal & Jutur, 2021
	D. bardawil	1 mM	Increased carbohydrate, lipid, β-carotene productivity	Einali, 2018
JA	*S. dimorphus*	100 nM	Increased biomass, β-carotene, Zeaxanthin and lutein	Paliwal & Jutur, 2021
	Schizochytrium sp.	95 µM	Increased lipid accumulation	Wang et al., 2018
BNOA	*Schizochytrium* sp.	9.8 µM	Increased lipid accumulation	Wang et al., 2018
Acetylcholine	*C. sorokiniana*	34 nM	Increased ALA content, biomass and lipid producivity	Parsaeimehr et al., 2015
	S. dimorphus	100 nM	Increased biomass, tocopherol and carotenes productivity	Paliwal & Jutur, 2021

Continued

Methyl Jasmonate	*C. protothecoides*	22.3 µM	Increased total lipids and ALA content	Parsaeimehr et al., 2017
	H. pluvialis	9.5 µM – 95 µM	Increased astaxanthin content	Lu et al., 2010
GA_3	*H. pluvialis*	5.8 µM – 58 µM	Increased astaxanthin content	Lu et al., 2010
	C. pyrenoidosa	58 µM	Increased lipid productivity	Han et al., 2018
Indomethacin	*C. vulgaris*	100 nM	Increased carbohydrates, lipid, protein and pigment content	Piotrowska et al., 2008
DA6	*C. ellipsoidea* and *S. quadricauda*	100 nM	Increased biomass and lipid content	Jiang et al., 2015
Erythromycin	*Raphidocelis subcapitata*	< 41 nM	Increased biomass, chlorophyll, and carotenoids	Zhang et al., 2021

Continued

Brefeldin	*S. dimorphus*	100 nM	Increased zeaxanthin, tocopherol and β-carotene level	Paliwal & Jutur, 2021
	C. reinhardtii	267.5 nM	Increased TAG accumulation	Kim et al., 2013
Calliterpenone	*Synechocystis* sp.	10 µM	Increased growth, carbohydrate, and lipid content	Patel et al., 2014
2,4- EBR	*H. pluvialis*	52 µM - 104 µM	Increased astaxanthin production	Gao et al., 2013
EGCG	*S. dimorphus*	100 nM	Increased canthaxanthin and tocopherol production	Paliwal & Jutur, 2021
IAA	*Scenedesmus* sp.	5.7 µM	Increased MUFAs and biomass productivity	Dao et al., 2018
IBA	*C. protothecoides*	9.8 µM	Increased biomass and lipid content	Parsaeimehr et al., 2017
Kinetin	*C. protothecoides*	4.6 µM	Increased ALA content	Parsaeimehr et al., 2017

CHAPTER 3

Hypothesis and Objectives

3.1. Hypothesis

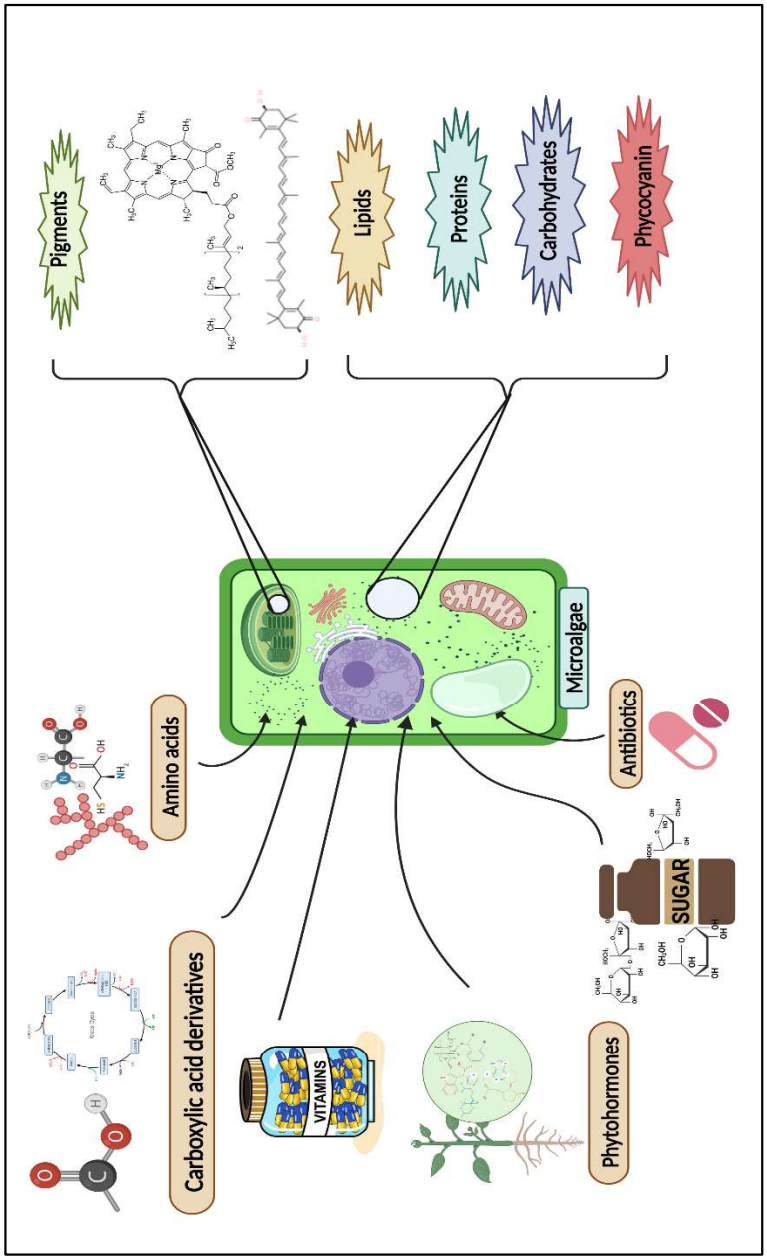


Figure 3.1. Schematic representation of hypothesis of the study

The available literature strongly supports the fact that low concentration of selected modulators can have significant effects on microalgal metabolism and their application in microalgal cultivation at large scale (Franz et al., 2013;Paliwal & Jutur, 2021). This study aims at developing a two-phase screening of chemicals (belonging to different classes) on the selected microalga, *Scenedesmus* sp. The classes (Figure 3.1) selected consists of compounds which can broadly serve as metabolite precursors (carboxylic acids) (Paliwal & Jutur, 2021), growth enhancers (phytohormones, vitamins and amino acids), organic carbon sources (sugars and their derivatives), and stress-mediators (antibiotics (Zhang et al., 2021), some phytohormones (Han et al., 2018), terpenes (Franz et al., 2013; Paliwal & Jutur, 2021) and phenols) for microalgae. These compounds are hypothesized to have a significant influence on growth and production of different value-added metabolites ranging from antioxidants (including enzymes), lipids, carbohydrates, proteins and many more.

3.2. Significance of the study

To scale-up the modulator based media engineering strategy to a biorefinery level, it is crucial to look into the cost of bioactive chemicals which can be used for microalgal cultivation. This study aimed at finding an efficient solution from commonly available chemicals which can positively alter the overall growth of microalgal cultivation. Moreover, the microalgal candidate we have selected has certain advantageous facts such as: high growth rate, high lipid content with better fatty acid profile and a great source of value added molecules (Bhalamurugan et al., 2018; Ishaq et

al., 2016; Soares et al., 2018). Additionally, the fact that this species is indigenous to Indore, Madhya Pradesh, makes it easier to apply the results of our study to large-scale or outdoor cultivation.

3.3. Objectives of the study

To sum up the aforementioned rationale, the major objective of this study was:

"Studying the effect of chemical modulators on growth and biochemical portfolio of *Scenedesmus* sp."

To accomplish this objective, we have divided our study into minor objectives which were as follows:

1. Pilot-scale screening of diverse chemicals with *Scenedesmus* sp. using 96-well microplates (Phase I).
2. Scale up studies in 500 mL culture flask with selected chemicals supplementation (Phase II).
3. Biochemical characterization of algal biomass obtained from scale up studies.

CHAPTER 4

Materials and Methodology

4.1. Microalgal cultivation and maintenance

The freshwater microalga, *Scenedesmus* sp. used in present work, was isolated indigenously from domestic wastewater collected from Lal Bagh region of Indore, Madhya Pradesh (22.69883 °N, 75.84815 ˚E) and was cultivated in BG-11 (Kashyap et al., 2021). Its growth was maintained in controlled temperature (27 ± 2 ˚C) and light condition (3000 ± 500 lux) with 12 : 12 hours photoperiod. A two-stage cultivation strategy was applied throughout the study; the cells were initially grown in flask containing 250 mL BG-11 with an initial absorbance (750 nm) of 0.2 ± 0.05. This seed culture (Figure 4.1) was then grown for 10 days, and the culture was further used for all subsequent experiments.

Components of BG11: Sodium nitrate (1.5 g L^{-1}), Dipotassium hydrogen phosphate (31 mg L^{-1}), Magnesium sulphate (0.036 g L^{-1}), Calcium chloride dihydrate (0.036 g L^{-1}), Sodium carbonate (0.02 g L^{-1}), Disodium magnesium EDTA (0.001 g L^{-1}), Citric acid (0.006 g L^{-1}), Ferric ammonium citrate (0.006 g L^{-1}) and trace elements (1 mL L^{-1}).

The trace elements stock consisted of boric acid (2.86 g L^{-1}), manganese chloride tetrahydrate (1.81 g L^{-1}), zinc sulphate heptahydrate (222 mg L^{-1}), sodium molybdate dihydrate (390 mg L^{-1}), copper sulphate pentahydrate (79 mg L^{-1}), and cobalt nitrate hexahydrate (49.4 mg L^{-1}).

Figure 4.1. Mother culture of *Scenedesmus* sp.

4.2. Chemicals

Fifty-five analytical grade chemicals, purchased from Sigma-Aldrich (USA) of HiMedia (Mumbai, India), have been used for this study (Table 4.1.)

Table 4.1. List of chemical modulators used in the study with their assigned codes and batch.

BATCH 1	
Chemical	**Code**
Succinic acid	M1
Shikimic acid	M2
Oxaloacetic acid	M3
Citric acid	M4
Erythromycin	M5
Ectoine	M6
Biotin	M7
Riboflavin	M8
Thiamine hydrochloride	M9

Cyanocobalamin	M10
Ascorbic acid	M11
BATCH 2	
Chemical	**Code**
Maltose monohydrate	M12
Rhamnose monohydrate	M13
D-glucuronic acid	M14
D-glucose	M15
D-galactose	M16
D-fructose	M17
D-xylose	M18
D-mannose	M19
D-arabinose	M20
Glucosamine hydrochloride	M21
Galacturonic acid monohydrate	M22
BATCH 3	
Chemical	**Code**
Fucose	M23
Betaine	M24
Glycine	M25
L-glutamic acid	M26
Tetracycline hydrate	M27
Rifampicin	M28
Indole propionic acid	M29
Indole acetic acid	M30
Indole butyric acid	M31
Salicylic acid	M32
Gibberellic acid	M33
BATCH 4	
Chemical	**Code**

L-proline	M34
L-isoleucine	M35
L-phenylalanine	M36
2,3-dihydroxybenzoic acid	M37
β-ionone	M38
Myo-inositol	M39
L-malic acid	M40
Ethyl lactate	M41
DL-lactic acid	M42
Sodium butyrate	M43
Crotonic acid	M44
BATCH 5	
Chemical	**Code**
Kinetin	M45
Folic acid	M46
2,6-dichlorophenolindophenol sodium salt hydrate	M47
Diacetyl monoxime	M48
Phenylmethylsulfonyl fluoride	M49
Gallic acid	M50
Sodium decanoate	M51
Ethyl caprylate	M52
Sodium dodecanoate	M53
3-hydroxydecanoic acid	M54
Benzoic acid	M55

4.3. Experimental conditions and set-up

The microalgal cells from the seed culture were grown in BG-11 supplemented with the appropriate modulator.

4.3.1. Phase I - Screening using 96-well microplate

The initial screening was done in 96-well microplates (Tarsons) using different modulators at five different final concentrations (20, 50, 100, 500, and 1000 nM). The modulators were divided into 5 batches of 11 chemicals each, grouped randomly (Fig. 4.2). The initial absorbance was adjusted to 0.075 ± 0.01 (Victor Nivo^{TM} Multimode Plate Reader, PerkinElmer, USA). BG11 was taken as the control. The perimeter wells were filled with sterilised deionised water to reduce the edge effect and the plates were sealed with paraffin.

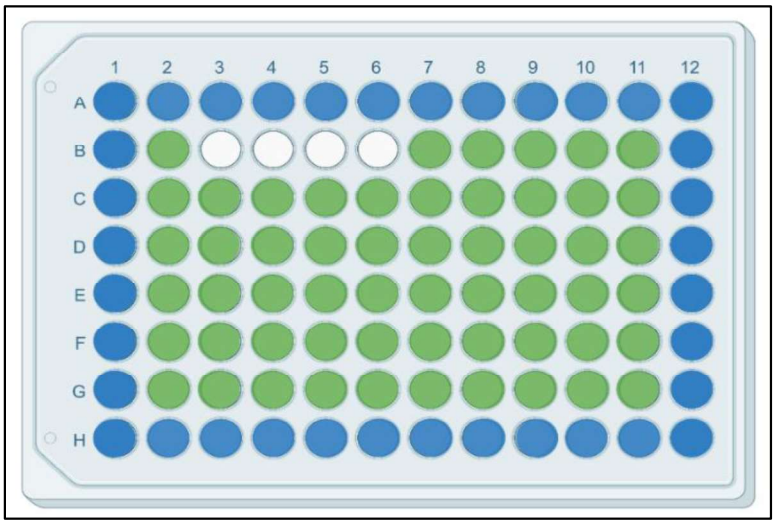


Figure 4.2. Schematic representation of plate design for Phase 1 screening. *Blue – deionised water, Green – experimental wells and White – blank.

4.3.2. Phase II – Screening in 250 mL batch culture

The compounds with their optimal concentration were selected for further validation at 250 mL scale. The initial OD was kept at 0.15 ± 0.04 and the growth conditions were maintained as mentioned in Section 4.1. Biomass

concentration, and pigments were analysed every 3rd day while the biochemical composition and tocopherol content were monitored on completion of the experiment. All the experiments were conducted in triplicates.

4.4. Determination of biomass concentration

Microalgal growth was monitored by recording the absorbance every 3rd day (A_{750}). The absorbance was then used for computing biomass concentration by using OD vs. dry weight curve:

$$A_{750} = 0.03*(\text{DCW, mg } L^{-1}) + 0.1396;\ R^2 = 0.99 \quad (1)$$

4.5. Determination of pigments

1 mL of culture was centrifuged (10,000g, 10 min, 25 °C, Centrifuge 5430R, Eppendorf, Germany); the supernatant was discarded, and the pellet was washed with 1 mL distilled water. After re-centrifuging, the pellet was resuspended in 1 mL 99.9 % methanol and was vortexed well, followed by incubation at 45 ˚C for 24 hours in the dark (Pancha et al., 2014). The absorbance spectra were collected in the wavelength range of 400 - 800 nm (UV/VIS Lambda 365, PerkinElmer, USA) using a 1 cm pathlength quartz cuvette The pigment content was then calculated by using following equations (Lichtenthaler, 1987):

$$\text{Chlorophyll a } (\mu g\ mL^{-1}) = 16.72*A_{665.2} - 9.16*A_{652.4} \quad (2)$$

$$\text{Chlorophyll b } (\mu g\ mL^{-1}) = 34.09*A_{652.4} - 15.28*A_{665.2} \quad (3)$$

$$\text{Carotenoids } (\mu g\ mL^{-1}) = (1000*A_{470} - 1.63*\text{Chl a} - 104.9*\text{Chl b})/221 \quad (4)$$

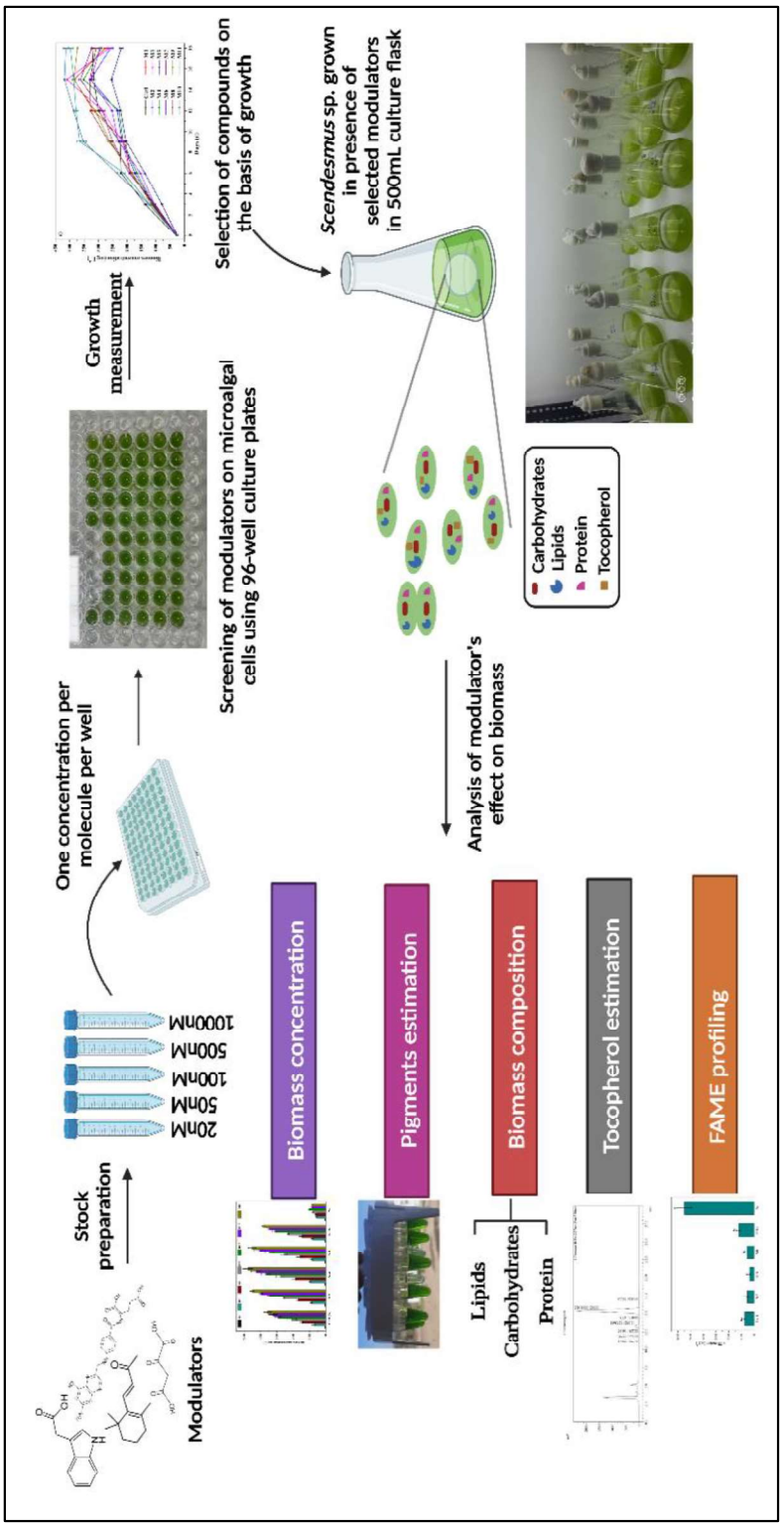


Figure 4.3. Schematic representation of experimental setup used for the study

4.6. Biochemical composition

4.6.1. Total lipid estimation

The Sulpho-phospho-vanillin (SPV) method was used for estimation of total lipids. SPV reagent was prepared by adding 600 mg vanillin in 10 mL absolute ethanol, followed by addition of 90 mL deionised water and 400 mL of orthophosphoric acid (85 % w/v) with constant stirring. 1 mL culture was centrifuged in a microcentrifuge tube (10,000g, 10 min, 25 °C) and the resultant pellet was washed with distilled water twice. It was further resuspended in 20 µL distilled water and 400 µL concentrated sulphuric acid (98 % w/v) followed by an incubation at 100 ˚C for 10 min. The reaction mixture was then ice cooled for 5 min and 1 mL of freshly prepared SPV reagent was added to it. The mixture was incubated at 37 ˚C for 15 min in the dark in a shaker incubator (220 rpm). Afterwards, the mixture was centrifuged (10,000g, 10 min, 25 °C) and the absorbance was recorded at 530 nm (Anand et al., 2023; Mishra et al., 2014). **Lipids were quantified using a standard curve prepared using triolein (0 – 50 µg, R^2 = 0.98).**

$$A_{530} = 0.0256*(\text{triolein, } \mu g) - 0.2622 \quad (5)$$

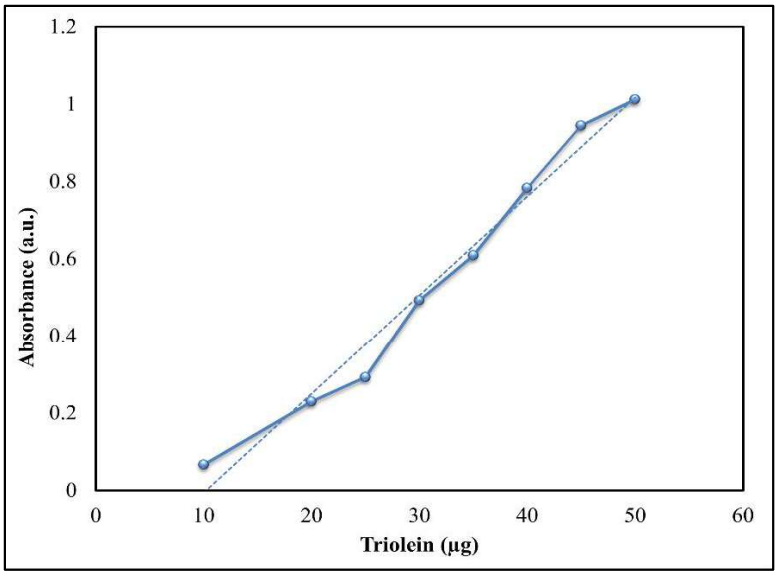


Figure 4.4. Standard plot of triolein for total lipid estimation

4.6.2. Carbohydrate estimation

The anthrone test was used for estimation of carbohydrates. Anthrone reagent was prepared by adding 100 mg anthrone in 100 mL of 75 % conc. H_2SO_4 (98 % w/v), followed by addition of 1 g thiourea. 1 mL culture was pelleted down (10,000g, 10 min, 25 °C) and the biomass was washed twice with distilled water. After re-centrifugation, it was resuspended in 250 µL distilled water and 1 mL of freshly prepared anthrone reagent followed by an incubation at 100 ˚C for 15 min. The reaction mixture was then ice-cooled for 5 min and centrifuged (10,000g, 10 min, 25 °C) and the absorbance of the supernatant was recorded at 620 nm(Roe, 1955). **Total carbohydrates were quantified using a standard curve prepared using glucose (0 – 50 µg, R^2 = 0.99).**

$$A_{620} = 0.0238*(\text{glucose, µg}) + 0.0205 \quad (6)$$

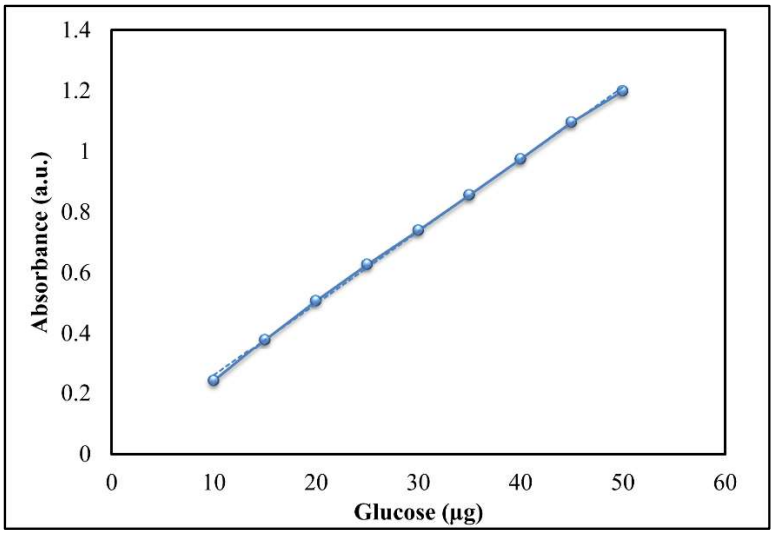


Figure 4.5. Standard plot of glucose for carbohydrate estimation.

4.6.3. Protein estimation

The modified micro-biuret method has been used for the estimation of proteins (Itzhaki & Gill, 1964; Rausch, 1981). This method involves two reagents: Reagent I was prepared by adding 1 N NaOH in 25 % methanol and Reagent II was prepared by adding 0.21 % copper sulphate in 30 % NaoH. 1 mL culture was pelleted down (10,000g, 10 min, 25 °C) and the biomass was washed twice with distilled water. After re-centrifugation, it was resuspended in 1 mL reagent I followed by an incubation at 80 ˚C for 20 min and was ice-cooled for 5 min. The suspension was centrifuged (10,000g, 10 min, 25 °C) and the supernatant was retrieved, followed by addition of reagent II in a proportion of 2 : 1, respectively. The mixture was incubated for 15 min and the absorbance of the sample was recorded a 310 nm. **Total proteins were quantified suing a standard curve prepared using BSA (0-300 µg, R^2 = 0.99).**

$$A_{310} = 0.0016*(BSA, \mu g) + 0.0038 \quad (7)$$

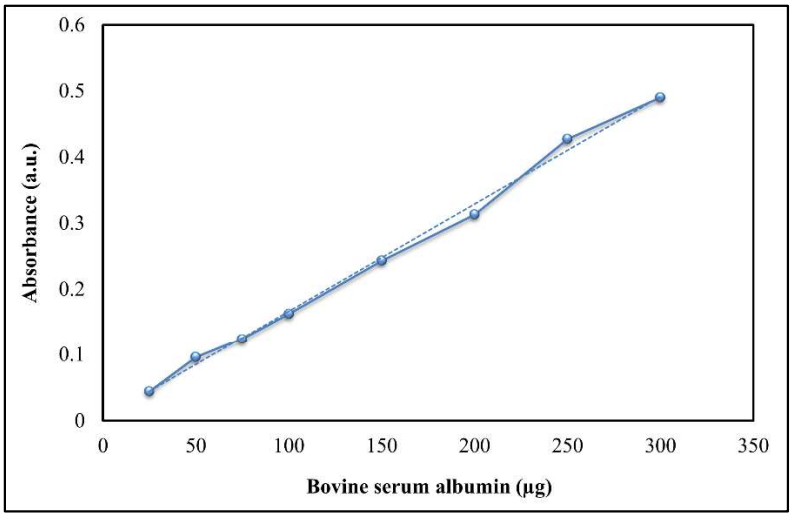


Figure 4.6. Standard plot of BSA for protein estimation.

4.7. Fatty acid methyl ester (FAME) profiling

The FAME analysis was performed according to method described in (Paliwal & Jutur, 2021). Transesterification was done by resuspending pellet of 5 mL culture (centrifuged at 10,000g, 10 min) in 300 µL of 2 % H_2SO_4-methanol followed by an incubation at 80 ˚C for 2 h with intermittent vortexing. The mixture was then cooled down and 300 µL of 0.9 % NaCl and 300 µL of n-hexane was added, followed by continuous vortexing for 5 min. The mixture was then centrifuged, and the hexane layer was collected for quantification through gas chromatography (Agilent 7890B, Agilent, USA) using CP9080 Select Biodiesel GC capillary wide-bore column (30 m x 0.32 mm x 0.25 µm) (Agilent, USA) (Figure 4.7). Helium was used as carrier gas having flow rate of 1 mL min^{-1}. The eluted FAME was detected using a flame ionization detector (FID). The split ratio and injection volume was kept at 6 : 1 and 2 µL, respectively. The oven temperature was programmed to maintain an initial

temperature of 60 °C for 2 min, then increase at a rate of 10 °C min^{-1} to 150 °C and then further increase to 230 °C at a ramp of 20 °C min^{-1}. The inlet and detector temperatures were maintained at 250 °C and 280 °C, respectively. The inlet, column, and septum purge flow rates were 10-, 1-, and 3 ml min^{-1}, respectively.

Figure 4.7. Agilent 7890B GC system with FID for FAME profiling (Algal EcoTechnology and Sustainability group, BSBE IIT Indore)

4.8. Tocopherol estimation

The extraction and estimation of tocopherol was done using method described in (Paliwal & Jutur, 2021). Extraction was done by bead (425 - 600 µm, acid – washed, Merck Sigma, India) vortexing known amount of biomass using 500 µL of absolute ethanol for 10 min. Following extraction, 500 µL of n-hexane along with 200 µL of deionized water was added. The mixture was briefly vortexed for better phase separation.

The mixture was then centrifuged, and hexane layer was retrieved and dried under stream of nitrogen. The dry extract was resuspended in 200 µL of dichloromethane and methanol (2 : 1). The samples were then used for analysis through Prominence-i, LC-2030C 3D PLUS HPLC system (Shimadzu Corporation, Japan) (Figure 4.8.) using C_{18} column (Ascentis C18, 15 cm x 4.6 mm, 5 µm particle size, Supelco Analytical, USA) maintained at temperature 25 ˚C. The mobile phase used to run samples was a binary solvent system of Solvent A - acetonitrile and Solvent B - methanol in isocratic mode with 60 : 40, and flow rate of 0.6 mL min^{-1}. Fluorescence detector set at excitation and emission wavelength of 297 nm and 330 nm, was used to detect tocopherol, which was identified and quantified by using calibration curve prepared using tocopherol standard (Sigma-Aldrich, USA).

Figure 4.7. Prominence-i, LC-2030C 3D PLUS HPLC equipped with a fluorescence detector (RF-20A) (Central Instrumentation Facility, BSBE, IIT Indore)

CHAPTER 5

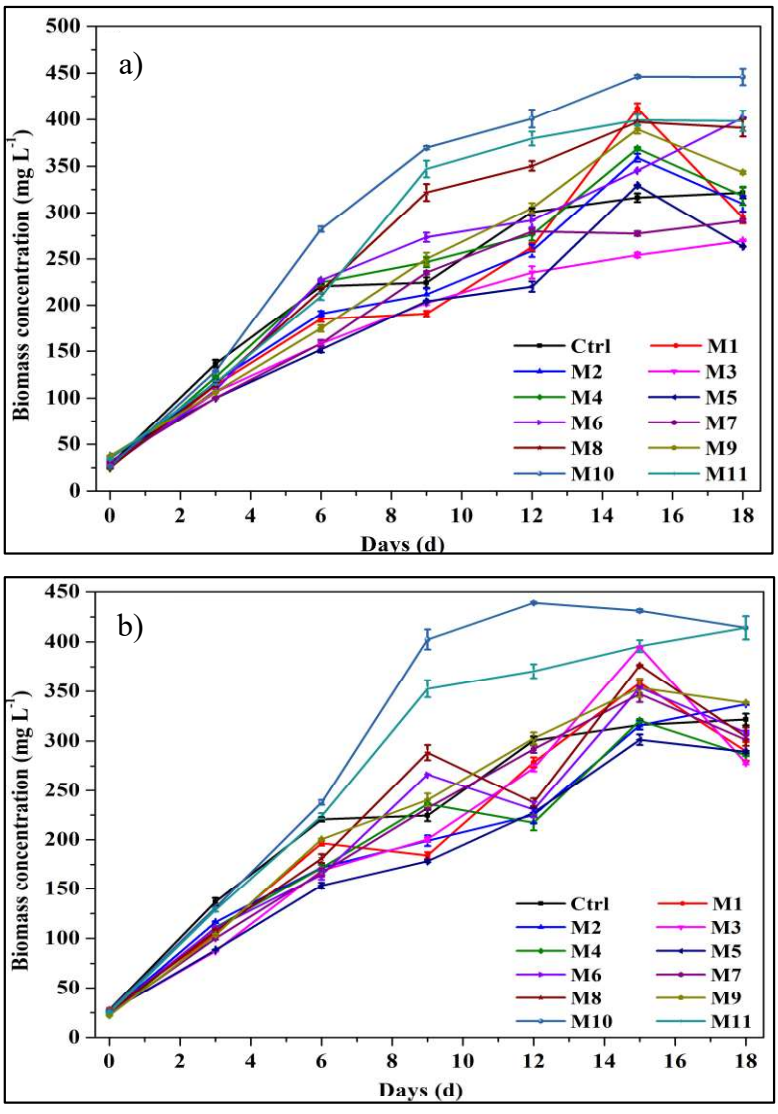
Results and Discussion

5.1. Microplate screening assay: Optimization of concentration of modulators

Cultivation cost contributes significantly in enhancing the cost of a bioprocess (Dao et al., 2018). The evaluation of nutritional and environmental parameters is an essential step for determining the overall economic feasibility of a biorefinery (Kenari et al., 2011). The media engineering approach has been recognised by many studies for enhancing microalgal growth and directing the metabolic pathways towards the production of value-added metabolites (Franz et al., 2013; Lu et al., 2010; Paliwal & Jutur, 2021). In this study, we have designed a two-phase screening experiment to identify chemical triggers with their effective concentration. The phase I screening consists of experiments carried out in 96-well microplates with 55 different chemicals at 5 concentrations, on the indigenous microalga, *Scenedesmus* sp. The microplate assay has allowed us to simultaneously monitor the growth of 56 culture conditions (including control) at a time. A total of 276 such culture treatments have been screened in phase I. The results demonstrated the effect of chemicals on the growth and biomass concentration of microalgae at different concentration. The results have been discussed separately for each batch (Table 4.1).

For batch 1, *Scenedesmus* sp. treated with M3 (oxaloacetic acid) at 50 nM (394.6 ± 0.5 mg L^{-1}) and 1000 nM (412.1 ±

3.1 mg L^{-1}) recorded better growth compared to control till 15th day, after which the biomass concentration decreased. Treatment with M6 (ectoine) positively affected growth at 20 nM (402 ± 0.3 mg L^{-1}) till 18th day as compared to control (321.6 ± 5.8 mg L^{-1}), but when the concentration was increased to 1000 nM, there was no proportionate increase in growth. High biomass concentration was observed with M10 (cyanocobalamin) and M11 (ascorbic acid) at concentration of 50 nM (414.1 ± 11.5 mg L^{-1} and 414.1 ± 11.7 mg L^{-1}, respectively). Increased M11 showed higher biomass concentration (Figure 5.1)



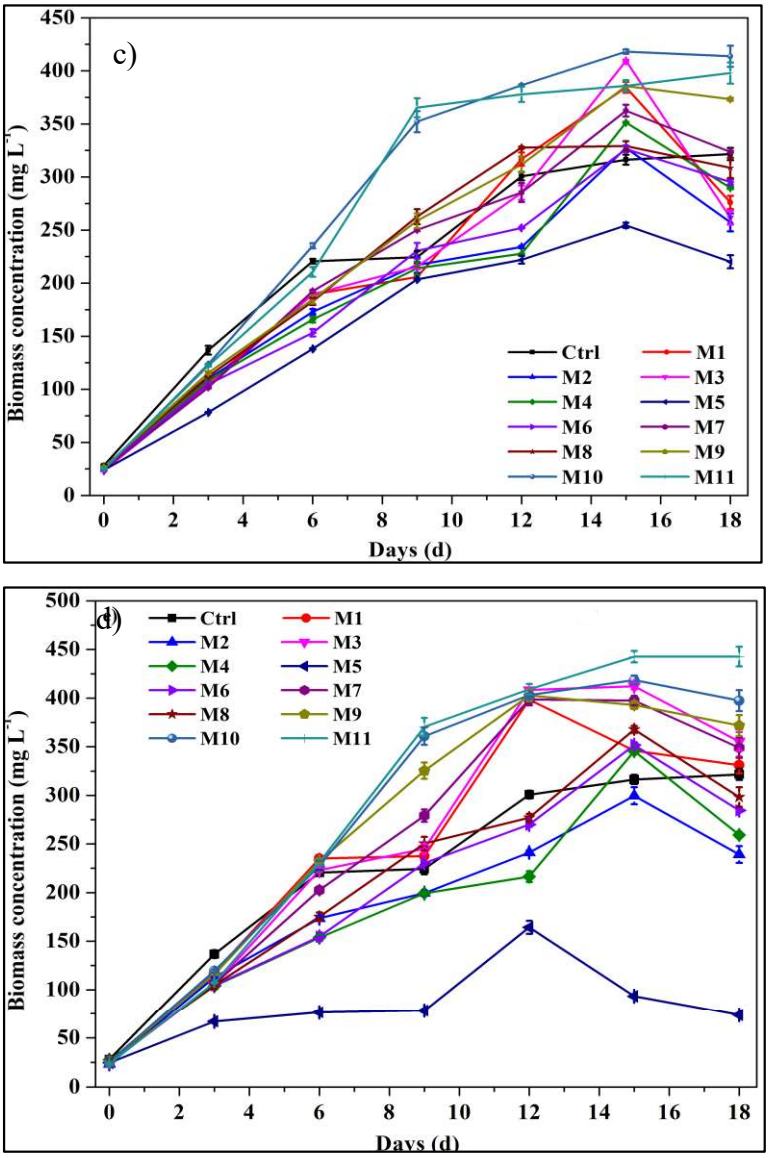
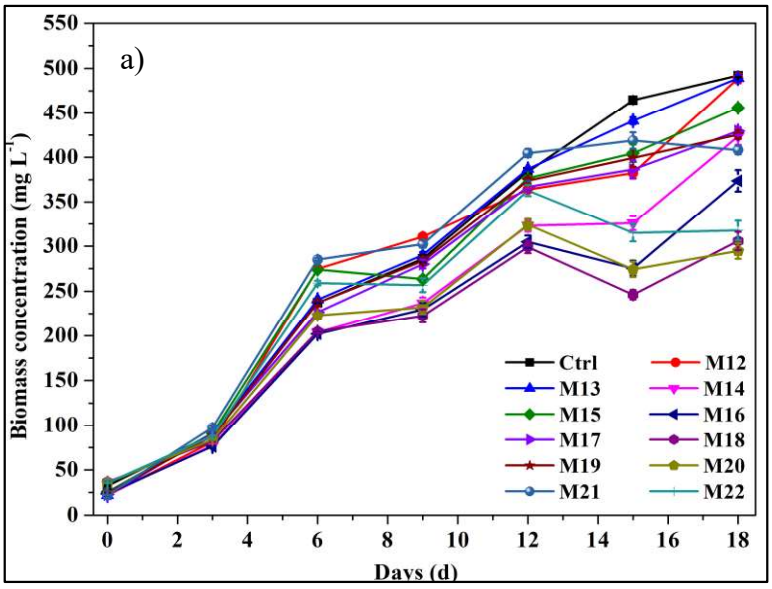
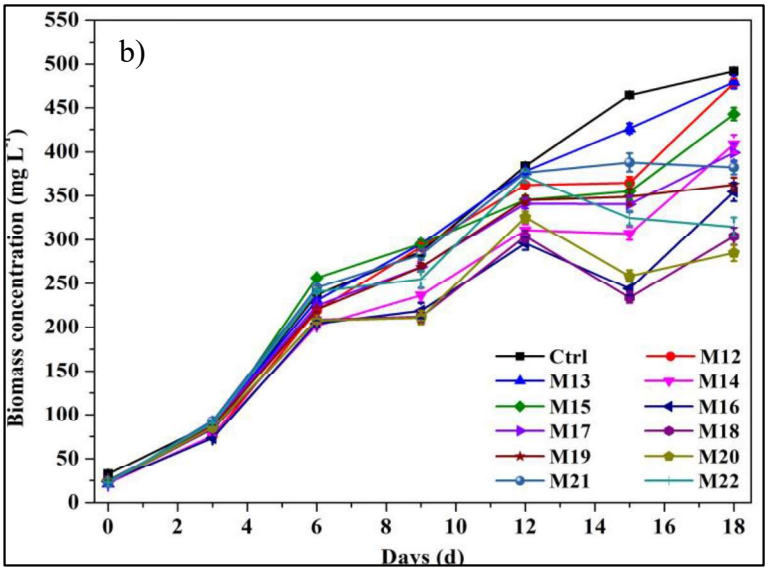


Figure 5.1. Dose-dependent effects of different chemical modulators (Batch 1) on the growth of *Scenedesmus* sp. compared to control. **a) 20 nM, b) 50 nM, c) 100 nM and d) 1000 nM.** The data points are represented as mean ± SD of triplicates (n = 3). The codes assigned for modulators are tabulated in Table 4.1.

In batch 2, the control has the highest biomass concentration (491.9 ± 1.38 mg L^{-1}) compared to other 55 culture treatments. Similar biomass concentration was observed

with M12 (maltose monohydrate) and M13 (rhamnose monohydrate) at 20 nM concentration (488.9 ± 5.4 mg L^{-1} and 489.1 ± 3.7 mg L^{-1}, respectively), but when the concentration was increased to 1000 nM, a decrease in biomass concentration was observed. Additionally, it was observed that sugars and their derivatives are not very efficient in increasing microalgal biomass at such low concentrations, compared to control (BG11) which is more effective (Figure 5.2.). This is possibly due to the fact that sugars naturally present in the cells at concentrations in the µM (micromolar) range. Consequently, minute sugar concentration in the growth medium apparently do not have any additional effect on the growth or biomass, which is best illustrated by the observation that BG11 performs better than any of the sugars tested.




b)
Biomass concentration (mg L-1)
Days (d)
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M12
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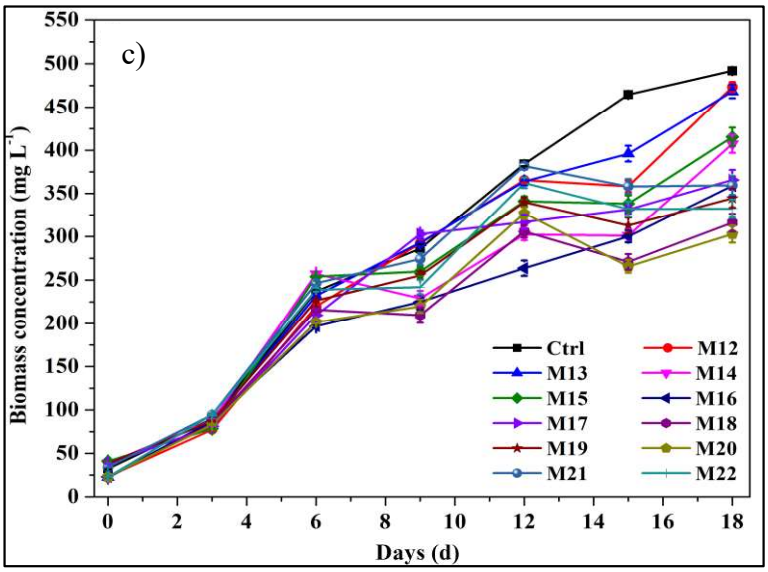
M14
M15
M16
M17
M18
M19
M20
M21
M22


c)
Biomass concentration (mg L-1)
Days (d)
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M12
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M14
M15
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M18
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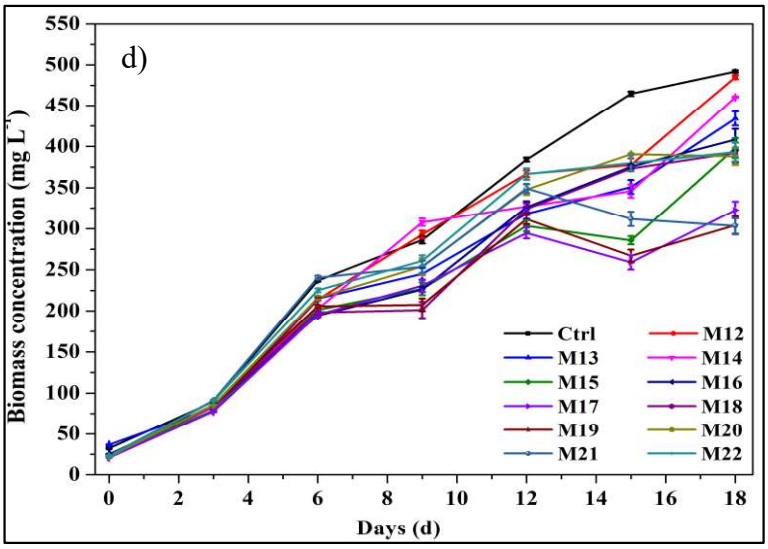
M22


d)
Biomass concentration (mg L-1)
Days (d)
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M12
M13
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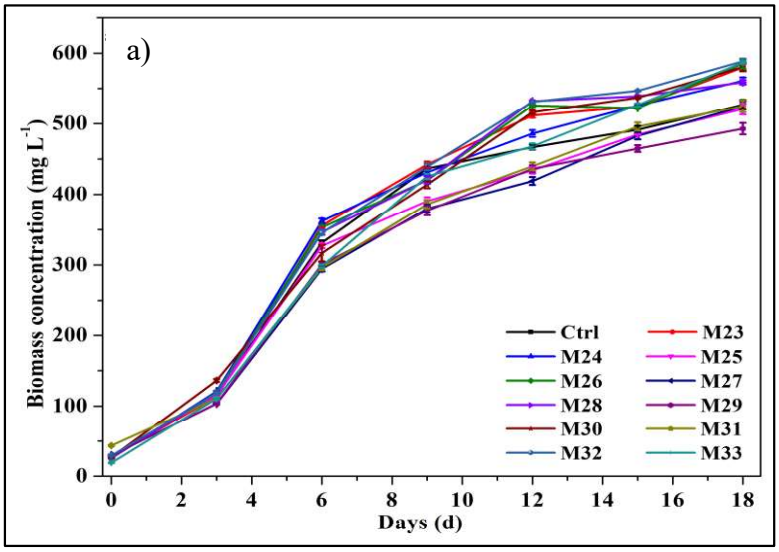
M15
M16
M17
M18
M19
M20
M21
M22

Figure 5.2. Dose-dependent effects of different chemical modulators (Batch 2) on the growth of *Scenedesmus* sp. compared to control. **a) 20 nM, b) 50 nM, c) 100 nM and d) 1000 nM.** The data points are represented as mean ± SD of triplicates (n = 3). The codes assigned for modulators are tabulated in Table 4.1.

In batch 3, M23 (fucose), M30 (IAA) and M33 (GA_3) at 20 nM showed high biomass concentration (579.9 ± 4.3 mg L^{-1}, 580.4 ± 6.0 mg L^{-1} and 586.6 ± 1.5 mg L^{-1}, respectively) compared to control (527.1 ± 6.7 mg L^{-1}). In case of M30 the increasing concentration resulted in decreased growth and even lower than control at 1000 nM concentration (474.2 ± 7.5 mg L^{-1}). Studies have reported that IAA at concentrations of 100 nM or higher have an inhibitory effect on microalgal growth (Kozlova et al., 2017; Noble et al., 2014; Paliwal & Jutur, 2021) while some studies have shown IAA to be effective till 100 μM concentration. The possible reason for such differences lies in the physiological and growth parameters of the microalgal strain (Kozlova et al., 2017). The stimulating effect of M29 (IPA) and M31 (IBA) was observed at 100 nM concentration (564.4 ± 7.7 mg L^{-1} and 545.5 ± 2.0 mg L^{-1}, respectively). Moreover, treatment with M32 (SA) showed increased biomass concentration at all the selected concentrations, reaching 632.2 ± 6.7 mg L^{-1} at 1000 nM, i.e., 20 % higher than control (Figure 5.3).


a)
Biomass concentration (mg L-1)
Days (d)
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M23
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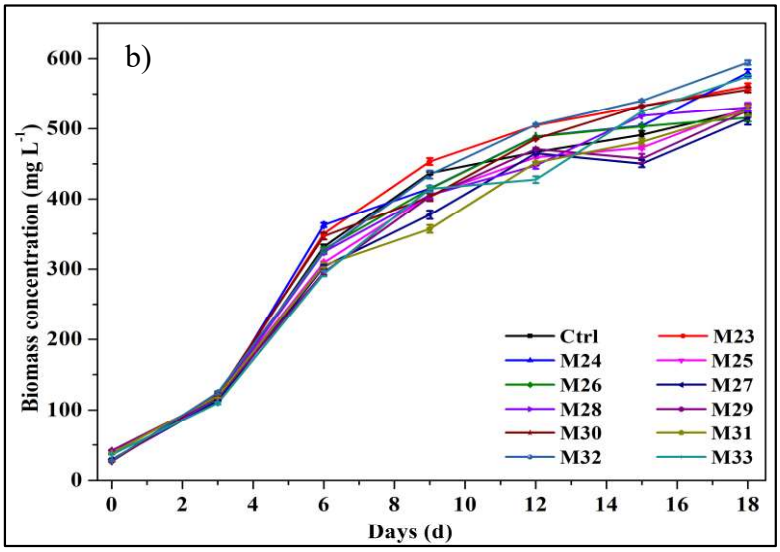
b)
Biomass concentration (mg L-1)
Days (d)
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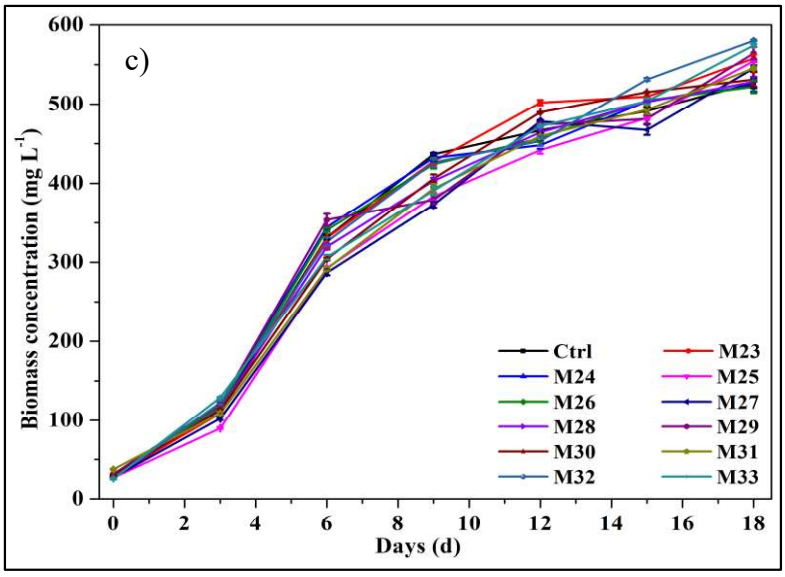
M33


c)
Biomass concentration (mg L-1)
Days (d)
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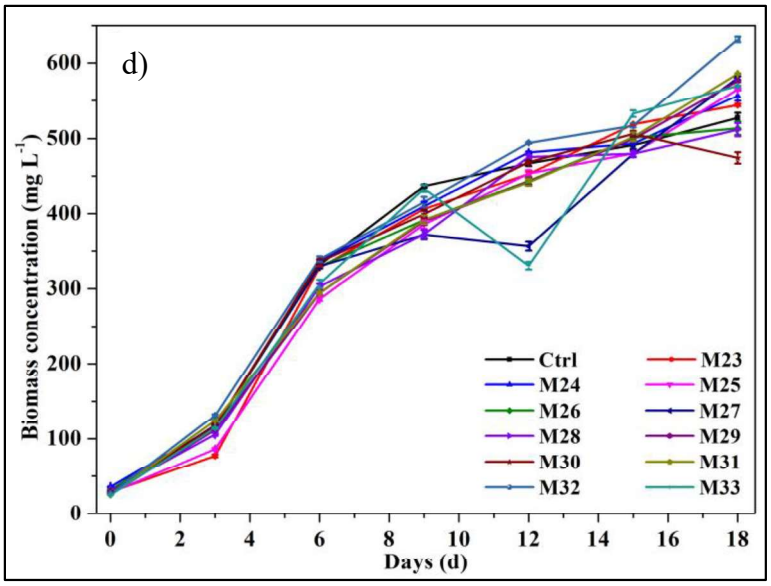
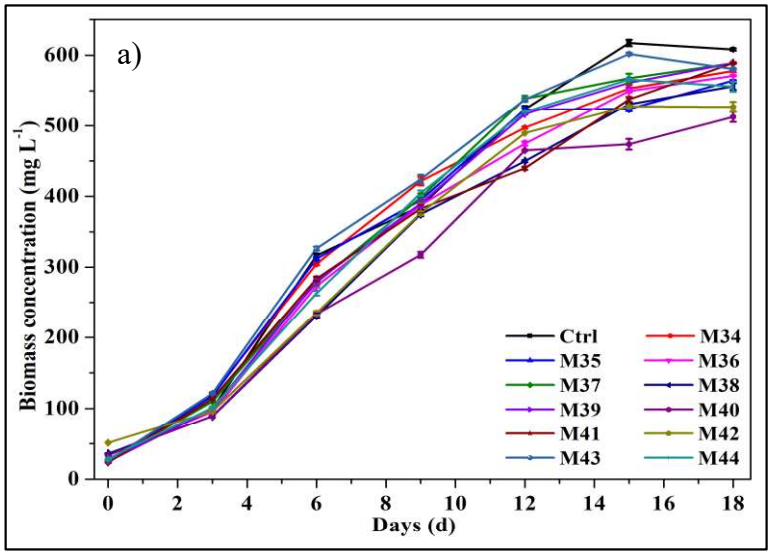


Figure 5.3. Dose-dependent effects of different chemical modulators (Batch 3) on the growth of *Scenedesmus* sp. compared to control. **a) 20 nM, b) 50 nM, c) 100 nM and d) 1000 nM.** The data points are represented as mean ± SD of triplicates (n = 3). The codes assigned for modulators are tabulated in Table 4.1.

In batch 4, the highest biomass concentration was observed in case of control (607.9 ± 1.8 mg L^{-1}). The two potential modulator candidates selected from this batch were M38 (β-ionone) and M43 (sodium butyrate) having biomass concentration of 585.00 ± 3.6 mg L^{-1} and 580.6 ± 1.0 mg L^{-1} at 1000 nM and 20 nM concentration respectively, which were still lower compared to control. The possible reasons could be the growth regulatory and antioxidant activities of β-ionone (Paparella et al., 2021) and the increased lipid and protein content in microalgae due to application of VFAs, compared to sugars in culture media (Patel et al., 2022; Su et al., 2021).


a)
Biomass concentration (mg L-1)
Days (d)
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M36
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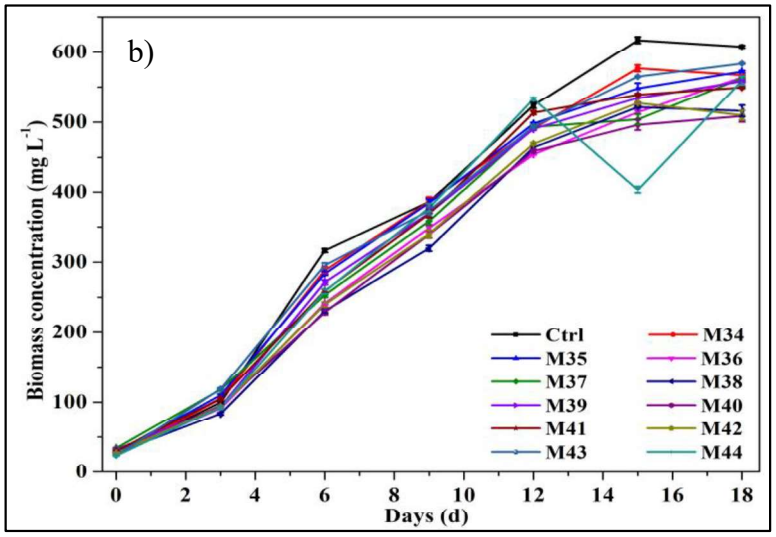
M43
M44


b)
Biomass concentration (mg L-1)
Days (d)
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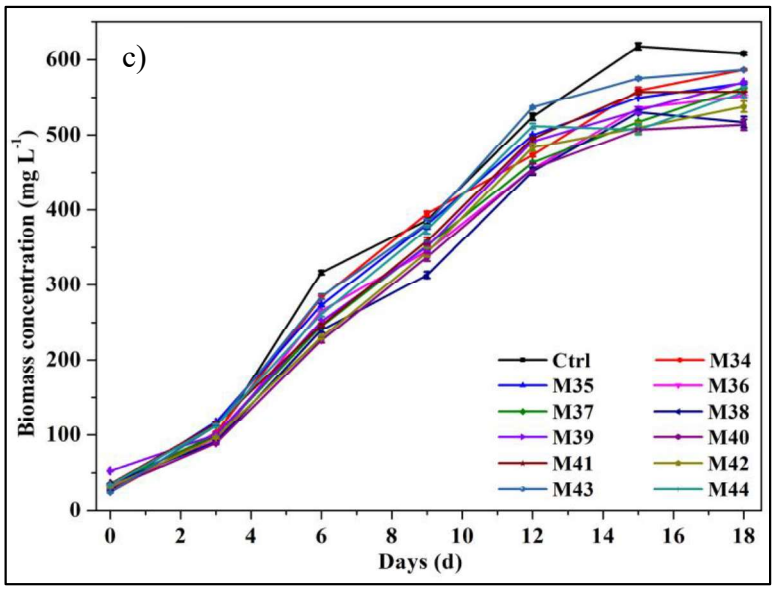
c)
Biomass concentration (mg L-1)
Days (d)
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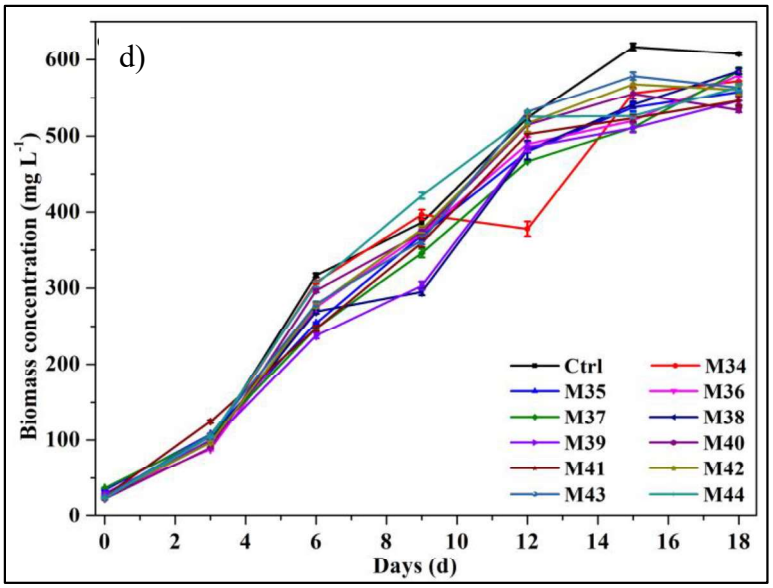
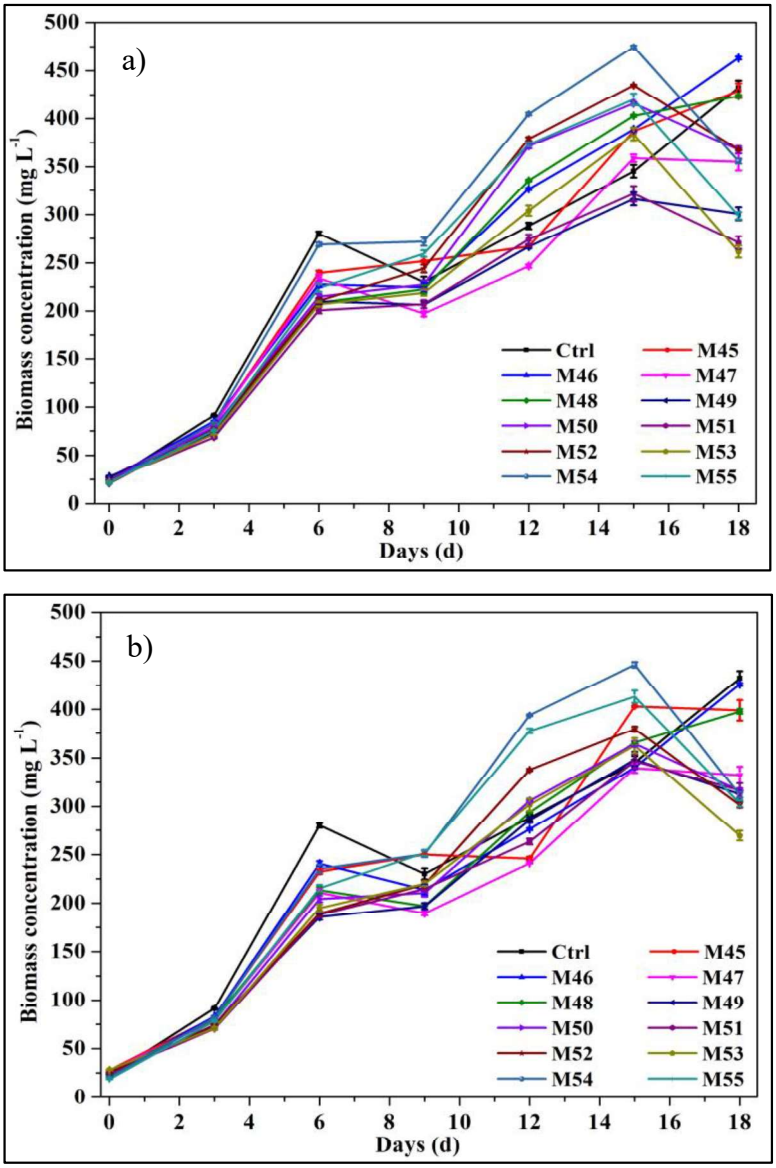


Figure 5.4. Dose-dependent effects of different chemical modulators (Batch 4) on the growth of *Scenedesmus* sp. compared to control. **a) 20 nM, b) 50 nM, c) 100 nM and d) 1000 nM.** The data points are represented as mean ± SD of triplicates (n = 3). The codes assigned for modulators are tabulated in Table 4.1.

In batch 5, M45 (kinetin) weakly enhanced the biomass yield at concentrations lower than 100 nM but have positive effect on growth with increasing concentrations, which has been supported by the studies suggesting stimulatory effects of cytokinins on photosynthetic and cell division processes of microalgae (Park et al., 2013). M46 (FA) showed weaker growth enhancement at 20 nM concentration, followed by decreased growth, even lower than control at concentrations > 20 nM. The role of folic acid in improving lipid accumulation has been described earlier, but its effect on biomass has not been studied yet (Li et al., 2019). M54 (3-HDA) also showed a high biomass yield at 20 nM. 3-HDA is a medium-chain fatty acid and can act as a substrate for

the generation of acetyl-CoA via the β-oxidation pathway; its effect on microalgal metabolic profile has not been described yet. M55 (BA) showed increased biomass yield at all concentration with slight differences in values, but higher than control. The 100 nM was selected as mid-point concentration to study the effect of M55 on microalgae.

The graphs representing the effect of modulators (M1-M55) at 500 nM concentration on the growth of *Scenedesmus* sp. are included in supporting information (Figure A1).



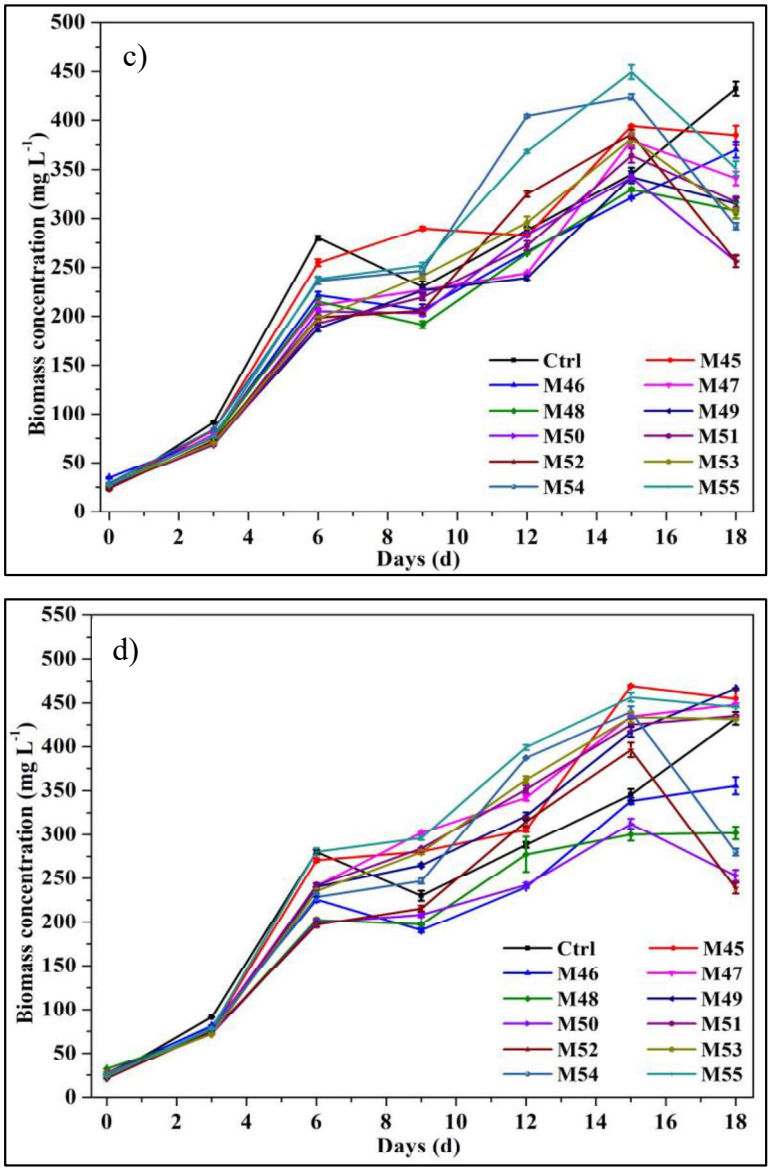


Figure 5.5. Dose-dependent effects of different chemical modulators (Batch 5) on the growth of *Scenedesmus* sp. compared to control. **a) 20 nM, b) 50 nM, c) 100 nM and d) 1000 nM.** The data points are represented as mean ± SD of triplicates (n = 3). The codes assigned for modulators are tabulated in Table 4.2.

5.2. Batch culture screening: Translation of effect of selected modulators in larger cultures

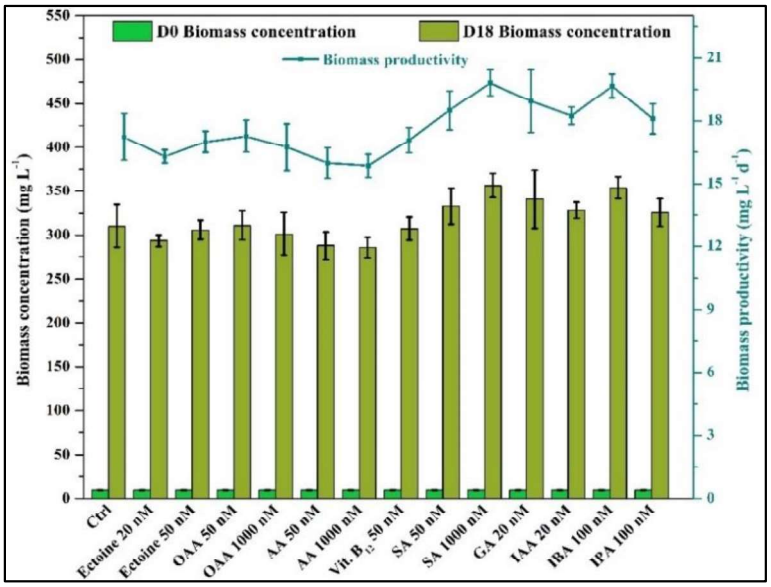
As reported in earlier studies, modulators are effective at a specific concentration and their effect may vary from species to species (Paliwal & Jutur, 2021). For this reason, the optimal concentration of selected chemicals was then screened in 250 mL culture to confirm their effectiveness at large-scale cultivation. The chemicals used for phase II screening were: ectoine 20 nM, ectoine 50 nM, OAA 50 nM, OAA 1000 nM, AA 50 nM, AA 1000 nM, Vit B_{12} 50 nM, SA 50 nM, SA 1000 nM, GA_3 20 nM, IAA 20 nM, IBA 100 nM and IPA 100 nM, fucose 20 nM, β-ionone 20 nM, β-ionone 1000 nM, SB 20 nM, kinetin 100 nM, kinetin 1000 nM, 3-HDA 20 nM, FA 20 nM and BA 100 nM. The chemicals were grouped into two batches: Batch 1 and Batch 2, for screening in batch cultures. The culture treatments were then analysed for biomass and pigment productivity, biochemical composition, and tocopherol productivity.

5.2.1. Effect of chemicals on biomass productivity

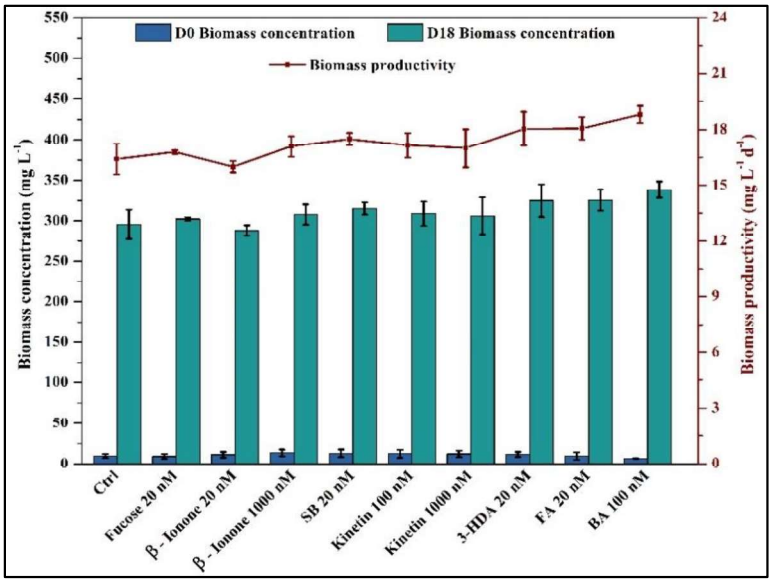
The effect of different chemicals treatments on the biomass yield and productivity of *Scenedesmus* sp. is represented in Figure 5.6. Similar to phase I screening, the experiments were carried out for 18 days. The results indicated that the chemicals influence the growth profile of microalgal cultures in three possible ways: stimulatory, inhibitory, or no effect. The controls from two batches have biomass productivity of 17.2 ± 1.4 mg L^{-1} d^{-1} and 16.4 ± 1.0 mg L^{-1} d^{-1} (Fig 5.6. A and B). The highest biomass productivity has been reported with SA 1000 nM (19.8 ± 0.8 mg L^{-1} d^{-1}).

Further IBA, GA and BA also showed stimulatory effect on microalgal growth enhancing the productivity, 19.6 ± 0.7 mg L^{-1} d^{-1}, 18.9 ± 1.8 mg L^{-1} d^{-1} and 18.8 ± 0.5 mg L^{-1} d^{-1}. Additionally, SA 50 nM, IAA, IPA, FA, 3-HDA, kinetin 100 nM, kinetin 1000 nM, SB, β-ionone 1000 nM, and fucose were also shown to increase the productivity of *Scenedesmus* sp. In contrast, ectoine, OAA, AA, vit. B_{12}, and β-ionone 20 nM showed weaker inhibitory effects. AA, a well-known antioxidant, plays a key role in maintaining the redox state of cell. Their role in promoting the plant growth at various developmental stage has been well studied (Gallie, 2013). Moreover, few reports have suggested the positive effect of ascorbate (at mM concentration) on microalgal biomass. However, we observed the inhibitory effect of ascorbate on microalgal biomass content. The plausible explanation could be the differences in the selected concentration of ascorbate and species-to-species variability. The application of vitamin B_{12} showed no growth enhancement; further studies with higher concentrations are needed to be done to decipher its role in microalgae. The exogenous supply of OAA had no obvious effect on biomass content during cultivation; moreover, OAA at 1000 nM showed inhibitory effects on microalgal growth which is in accordance with a study suggesting the promotion of respiration and inhibition of photosynthesis by OAA (W. Yu et al., 2022). SA and BA are important signalling molecules in microalgae; they are involved in the upregulation of glycolytic enzymes resulting in increased carbon assimilation, and in this study the SA (1000 nM) and BA both increased the productivity by ~ 15 % (Fu et al., 2021). The effect of auxins on microalgal growth was reported to be in order: IBA (100 nM) > IAA (20

nM) > IPA (100 nM). Kinetin was reported to have higher growth enhancing capability at low concentration (100 nM) compared with higher concentration (1000 nM). 3-HDA is an aliphatic fatty acid and can enter the β-oxidation pathway and produce acetyl-CoA, which can increase the carbon flux into TCA cycle, resulting in increased metabolic rate.



(A)



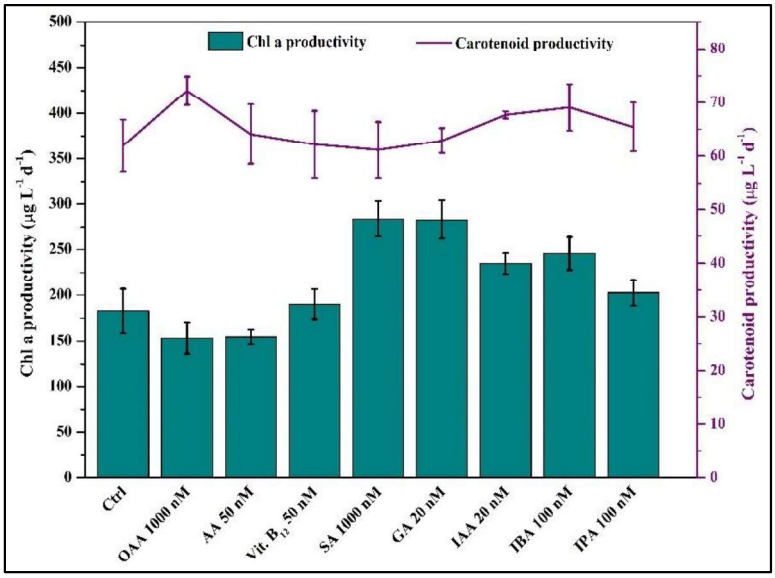
(B)

Figure 5.6. Effect of different modulators on the biomass yield and productivity of *Scenedesmus* sp. after 18 days. **(A) Batch 1 (B) Batch 2.** The data points are represented as mean ± SD of triplicates (n = 3).

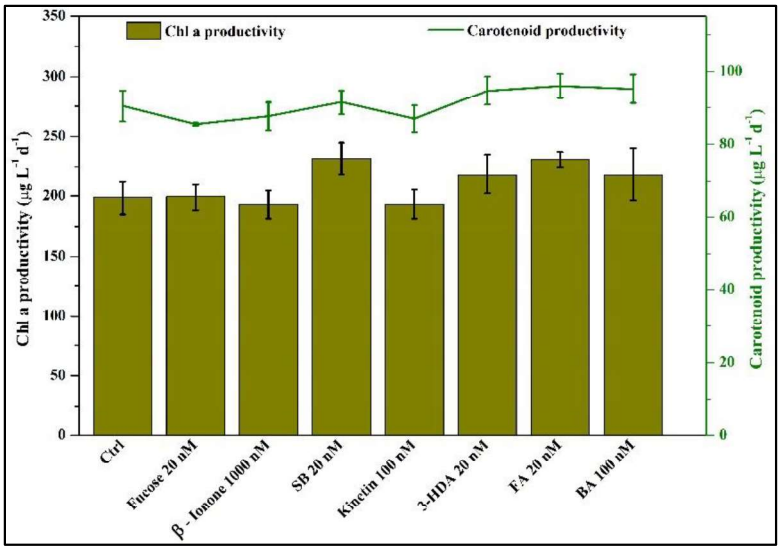
5.6.2. Effect of chemicals on pigments productivity

Photosynthetic pigments content was measured to study the effect of chemical treatments on the photosynthetic efficiency and overall growth of microalgal cells. Similar to biomass productivty, we also observed differences in pigment productivity after supplementation by modulators. Chlorophyll a (Chl a) content in SA at 50 nM and 1000 nM (268.6 ± 24.9 μg L^{-1} d^{-1} and 283.7 ± 19.4 μg L^{-1} d^{-1}, respectively), GA (283.1 ± 37.0 μg L^{-1} d^{-1}), IAA (234.8 ± 11.2 μg L^{-1} d^{-1}), IBA (245.7 ± 17.9 μg L^{-1} d^{-1}), IPA (202.5 ± 14.2 μg L^{-1} d^{-1}), SB (231.6 ± 13.2 μg L^{-1} d^{-1}), 3-HDA (218.4 ± 16.2 μg L^{-1} d^{-1}), FA (230.7 ± 6.2 μg L^{-1} d^{-1}), BA (218.2 ± 21.9 μg L^{-1} d^{-1}), and Vit. B_{12} (190.3 ± 16.3 μg L^{-1} d^{-1}) was higher in comparison to control: batch 1 (182.8 ± 24.1 μg L^{-1} d^{-1}) and batch 2 (210.9 ± 19.1 μg L^{-1} d^{-1}). However, ectoine, OAA, AA, fucose, β-ionone and kinetin showed decrease in Chl a content. In addition, Chl b showed a similar trend in case of batch 1, but in batch 2 the control had the highest Chl b productivity (110.0 ± 8.7 μg L^{-1} d^{-1}) (Table A1 and A2). The carotenoid (ct) productivity in OAA 1000 nM and ectoine 20 nM was reported to be highest with increment of 16.5 % and 13.2 % respectively, compared to control. The carotenoids to total chlorophyll ratio is related with stress response in microalgae (Pancha et al., 2014); OAA 1000 nM and kinetin 1000 nM enhanced carotenoid to total chlorophyll ratio. As discussed earlier, OAA had no growth

promoting effect at 1000 nM concentration but showed high carotenoid productivity, suggestive of re-routing of carbon flux towards carotenogenesis; the results reported are similar to the role of OAA in astaxanthin accumulation in *H. pluvialis* (W. Yu et al., 2022). Kinetin at both the concentrations (100 and 1000 nM) showed decreased chlorophyll which points towards its inhibitory activity on cell division, while its positive stimulation on biomass productivity decreased with increasing concentrations; it is contradictory to results reported in previous study (Udayan et al., 2018). The results showed the varying effects of chemicals on pigment productivity. Figure 5.7 represents the effect of some selected modulators on pigment productivity, the full data set is tabulated in Table A1 and A2.



(A)



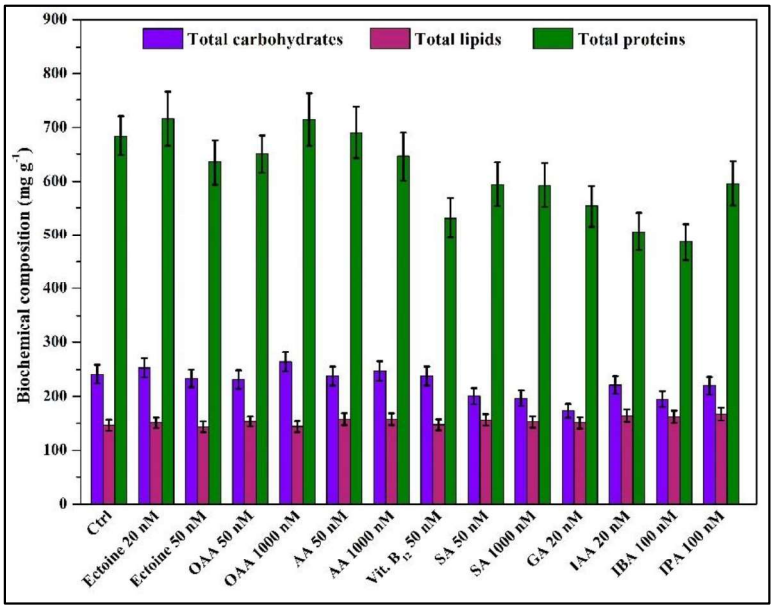
(B)

Figure 5.7. Effect of different modulators on the biomass yield and productivity of *Scenedesmus* sp. after 18 days. **(A) Batch 1 (B) Batch 2.** The data points are represented as mean ± SD of triplicates (n = 3).

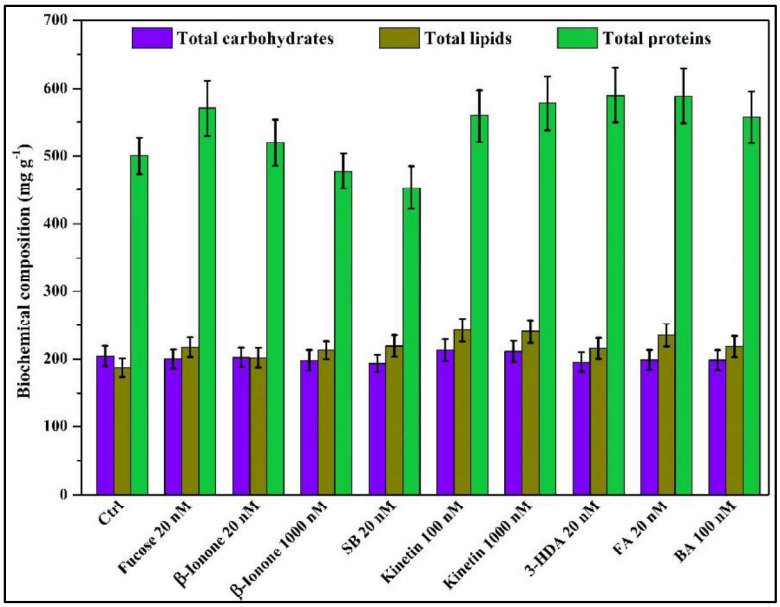
5.6.3. Effect of chemicals on biochemical composition

The changes in biochemical profile of *Scenedesmus* sp. in the presence of different chemical treatments were observed (Figure 5.8). *Scenedesmus* sp. maintained a high level of protein in all the treatments. However, in a few cases, protein content was enhanced such as fucose (570.5 ± 41.1 mg g^{-1}), β-ionone 20 nM (519.7 ± 33.8 mg g^{-1}), kinetin 100 nM (559.4 ± 38.6 mg g^{-1}), kinetin 1000 nM (577.9 ± 39.9 mg g^{-1}), 3-HDA (590.1 ± 40.7 mg g^{-1}), FA (588.8 ± 40.6 mg g^{-1}), and BA (557.5 ± 38.5 mg g^{-1}) compared to control (500.6 ± 26.5 mg g^{-1}). The FA and 3-HDA treatments showed an 18 % increment in protein content. We also observed differences in lipid content in various treatments. IAA, IBA, and IPA showed increased lipid content by ~ 12 - 14 %,

which is lower than what was reported by (Lin et al., 2018), possible due to the high concentration (mM - µM) of phytohormones. GA and SA had no promoting effects on lipid production. Moreover, kinetin increased the lipid content by 30 % at 100 nM concentration. Folic acid has been reported earlier to increase lipid synthesis in microalgae by increasing the NADPH pool of cells and an important donor of the C-unit for the generation of malonyl CoA (Li et al., 2019); we have reported 26 % increase in lipid content with FA treatment. Fucose, BA, 3-HDA and SB also enhanced lipid content by 15 - 17 %, than control. However, carbohydrate content in all treatments showed not much difference than control in our study.



(A)



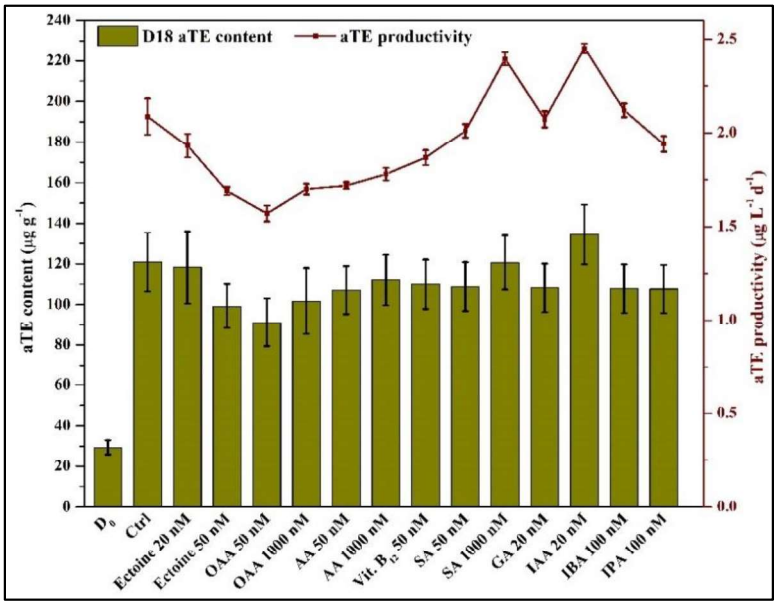
(B)

Figure 5.8. Effect of different modulators on productivities of lipid, carbohydrates and proteins in *Scenedesmus* sp. after 18 days. **(A) Batch 1 (B) Batch 2**. The data points are represented as mean ± SD of triplicates (n = 3).

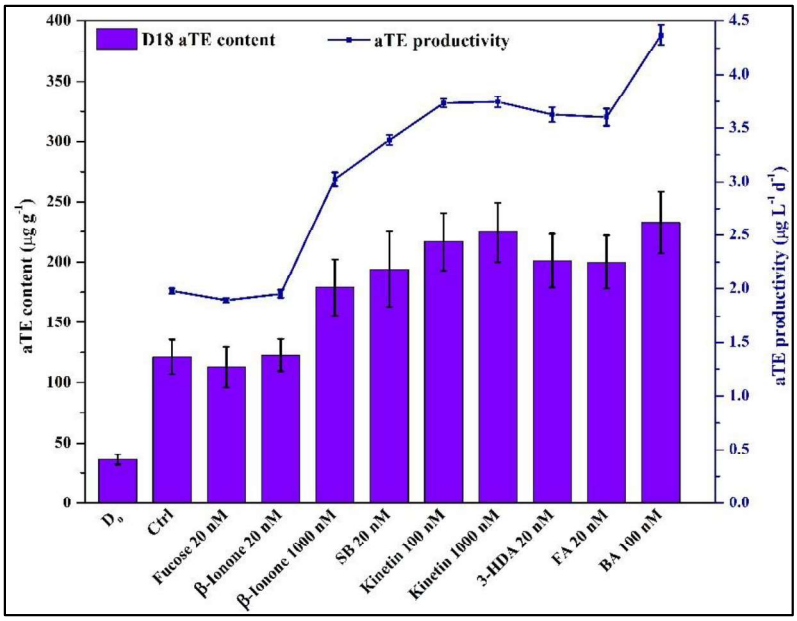
5.6.3. Effect of chemicals on α-tocopherol productivity

Tocopherol is a fat-soluble vitamin that is produced in plastids and plays a major role as an antioxidant molecule in the cellular defence system (Rizvi et al., 2014). They are generally produced as secondary metabolites and their content increases during stress or unfavorable conditions (Paliwal & Jutur, 2021). In this study, we have also studied the effect of exogenously supplied chemicals on tocopherol productivity in cells. BA (4.4 ± 1.3 µg L^{-1} d^{-1}), FA (3.6 ± 1.5 µg L^{-1} d^{-1}), 3-HDA (3.6 ± 0.4 µg L^{-1} d^{-1}), kinetin 100 nM (3.7 ± 0.6 µg L^{-1} d^{-1}), kinetin 1000 nM (3.7 ± 1.1 µg L^{-1} d^{-1}), SB (3.4 ± 0.6 µg L^{-1} d^{-1}), β-ionone (3.0 ± 1.2 µg L^{-1} d^{-1}), IAA (2.4 ± 0.1 µg L^{-1} d^{-1}), SA 1000 nM (2.4 ± 0.2 µg L^{-1} d^{-1}), and

IBA (2.1 ± 0.4 µg L^{-1} d^{-1}) showed enhanced α-TE productivity compared with control. Kinetin (both 100 nM and 1000 nM) showed similar α-TE productivity (Figure 5.9B). Studies extrapolating the mechanisms behind the activity of these chemicals in alteration of bioproducts productivity are rare, though few are available. Kinetin is reported to have an effect on expression of tocopherol biosynthetic genes in plant, *Vigna angularis* (mung bean) (Kawano, 2003). SA and IAA are signalling molecules and are involved in generation of ROS in cells (Kawano, 2003). In addition to this, the antioxidant property of SA has been reported in tobacco plant cultures where they promoted activity of antioxidant enzymes(Kim & Roh, 2014).



(A)



(B)

Figure 5.9. Effect of different modulators on yield and productivity α-Tocopherol in *Scenedesmus* sp. after 18 days. **(A) Batch 1 (B) Batch 2**. The data points are represented as mean ± SD of triplicates (n = 3).

CHAPTER 6

Conclusion and Future Perspective

Microalgae are tiny little factories of wide range of complex compounds which have been recognised as beneficial for human usage in different sectors, namely: bioenergy, nutraceuticals, pharmaceuticals, and cosmeceuticals. Nevertheless, the major barricades that comes in the way of commercialization of these bioproducts are low biomass productivity and bioactive metabolite content in the cells. To address these issues, continuous efforts have been undertaken to increase the biomass and value-added products production in microalgae. Traditional stress mediators and nutrient deprived media engineering tactics have been proven useful in increasing bioproducts accumulation but at the cost of decreased biomass productivity. In past decade, the concept of task-specific chemicals or modulators have gained much attention to improve the overall metabolic portfolio of microalgae. With time different chemicals have been identified as modulators that target specific pathways/hubs affecting the production of desired metabolites such as carotenoids, vitamins, lipids, carbohydrates, etc.

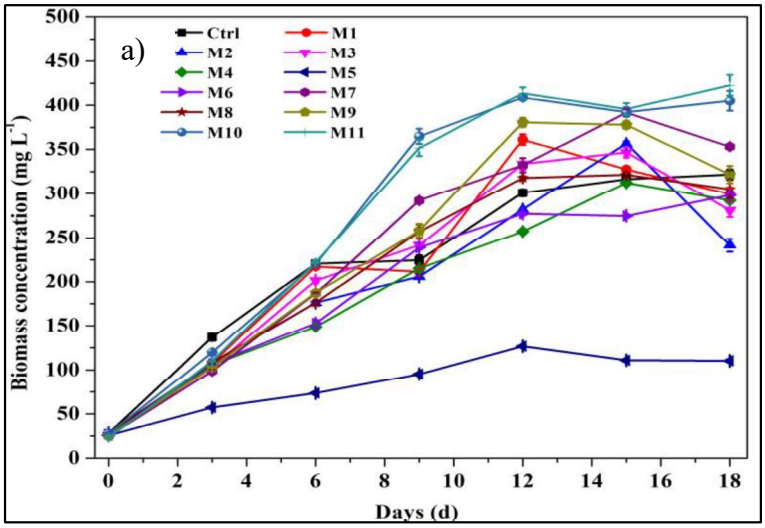
In this study, 55 chemicals from different classes of compounds were screened on indigenous freshwater green microalgae, *Scenedesmus* sp. The screening was done in two phases: firstly in 96-well culture plates and secondly in batch flask. We observed variant effect of different chemicals in enhancing different metabolites. Kinetin and FA both enhanced lipid yield and biomass productivity. OAA at 1000

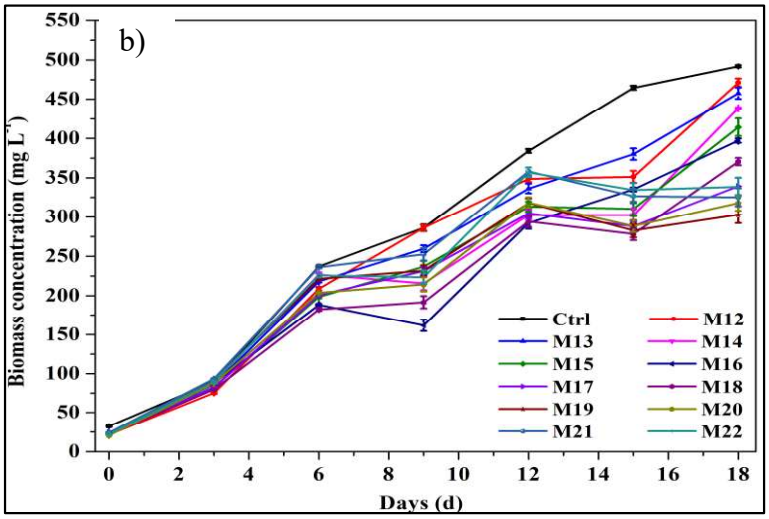
nM enhanced the carbohydrate content while other maintained it to level of control. BA and SA enhanced the biomass as well as tocopherol productivity while IAA have promoting effect on increasing the tocopherol content per gram of dry cell weight. Tocopherol is very important of our body as its deficiency is associated many health-related complication, it is also used as therapeutics for treatment of Parkinson, Alzheimer, diabetes, coronary heart diseases, etc. In our study, BA enhanced tocopherol productivity by 121% and owing to the low cost and non-toxic effects of this chemical it might be a new promising candidate for its usage potential in large scale cultivation.

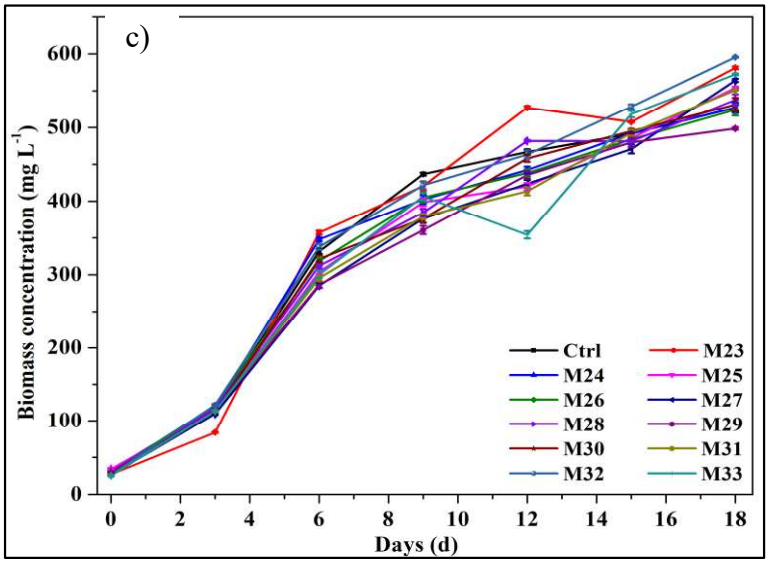
With new discoveries coming up in years for identifying new chemical stimulants for microalgal cultures, the ultimate goal is to translate its potential at large scale cultivation so as to generate sustainable and cost effective biorefinery for production of valuable products at cheaper costs. Moreover, in order to discover new chemicals and broaden their application as elicitors, its necessary to better understand the metabolic mechanism underlying cellular growth, value added metabolites production and accumulation and their mechanism of action in microalgae.

Nowadays the effect of combinatorial approach of nutrient limitation or environmental stress coupled to modulator supplementation have been studied and are reported to show better results than when done separately. Hence, investigating interactions between modulators and other stressors and their overall effect on microalgal physiology can be used to establish an efficient and cost-effective solution for value added bioproducts production.

APPENDIX-A


a)
Ctrl
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
M11
Biomass concentration (mg L-1)
Days (d)


b)
Ctrl
M12
M13
M14
M15
M16
M17
M18
M19
M20
M21
M22
Biomass concentration (mg L-1)
Days (d)


c)
Ctrl
M23
M24
M25
M26
M27
M28
M29
M30
M31
M32
M33
Biomass concentration (mg L-1)
Days (d)

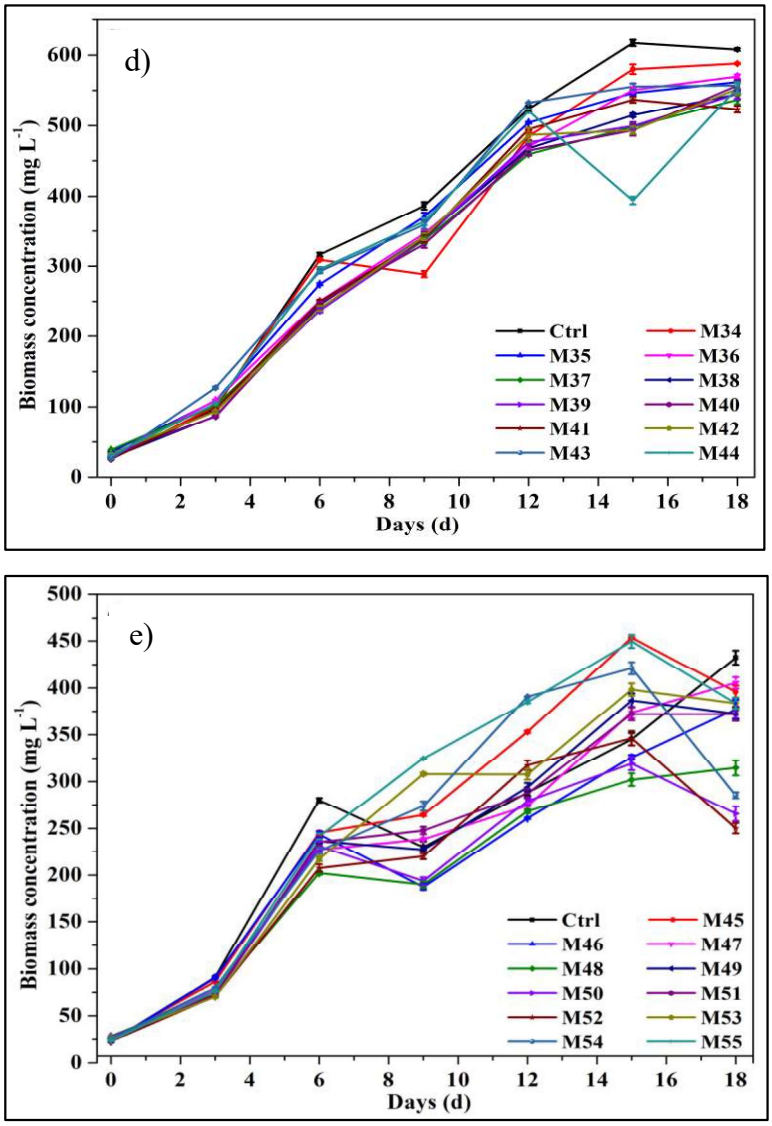


Figure A1. Effects of different chemical modulators on the growth of *Scenedesmus* sp. at 500 nM concentration. **a) Batch 1, b) Batch 2, c) Batch 3, d) Batch 4, and e) Batch 5.**

Table A1. Effect of different chemical treatments (Batch 1) on the pigment productivity of *Scenedesmus* sp.

Treatment	Chl a (µg L^{-1} d^{-1})	Ct (µg L^{-1} d^{-1})	Ct/tChl
Ctrl	182.8 ± 24.1	61.9 ± 9.9	0.24 ± 0.1
Ectoine 20 nM	174.9 ± 15.9	70.2 ± 3.8	0.26 ± 0.1
Ectoine 50 nM	178.4 ± 13.7	61.3 ± 2.1	0.22 ± 0.1
OAA 50 nM	173.4 ± 3.1	68.2 ± 4.4	0.24 ± 0.1
OAA 1000 nM	153.1 ± 17.1	72.2 ± 8.8	0.30 ± 0.1
AA 50 nM	154.4 ± 8.1	64.1 ± 5.5	0.26 ± 0.1
AA 1000 nM	144.6 ± 4.4	60.1 ± 0.8	0.26 ± 0.1
Vit. B_{12} 50 nM	190.3 ±16.2	62.2 ± 10.6	0.21 ± 0.1
SA 50 nM	268.6 ± 24.9	60.9 ± 0.6	0.13 ± 0.1
SA 1000 nM	283.7 ± 19.4	61.1 ± 8.9	0.13 ± 0.1
GA 20 nM	283.1 ± 37.1	62.9 ± 2.3	0.14±0.1
IAA 20 nM	234.8 ± 11.3	67.7 ± 0.7	0.17 ± 0.1
IBA 100 nM	245.7 ± 17.9	69.1 ± 4.3	0.17 ± 0.1
IPA 100 nM	202.5 ± 14.2	65.4 ± 4.5	0.19 ± 0.1

***Chl a = Chlorophyll a, Ct = Carotenoids, Ct/tChl = ratio of carotenoids to total chlorophyll**

Table A2. Effect of different chemical treatments (Batch 2) on the pigment productivity of *Scenedesmus* sp.

Treatments	Chl a (µg L^{-1} d^{-1})	Ct (µg L^{-1} d^{-1})	Ct/tChl
Ctrl	210.9 ± 19.1	90.5 ± 4.3	0.28 ± 0.1
Fucose 20nM	199.5 ± 11.1	85.4 ± 0.4	0.30 ± 0.1
β-ionone 20 nM	203.3 ± 40.9	85.6 ± 5.5	0.30 ± 0.1
β-ionone 1000 nM	192.9 ± 11.7	87.6 ± 3.8	0.31 ± 0.1
SB 20nM	231.6 ± 13.2	91.5 ± 3.3	0.28 ± 0.1
Kinetin 100 nM	193.1 ± 12.0	86.9 ± 3.7	0.31 ± 0.1
Kinetin 1000 nM	174.5 ± 37.9	84.5 ± 6.9	0.35 ± 0.1
3-HDA 20nM	218.4 ± 16.3	94.7 ± 3.9	0.30 ± 0.1
FA 20nM	230.7 ± 6.2	96.0 ± 3.3	0.29 ± 0.2
BA 100 nM	218.2 ± 21.9	95.2 ± 4.0	0.31 ± 0.1

***Chl a = Chlorophyll a, Ct = Carotenoids, Ct/tChl = ratio of carotenoids to total chlorophyll**

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