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Phytohormones for enhanced biomass production of marine microalgae

A Subramanian & Imelda Joseph*

ICAR-Central Marine Fisheries Research Institute, Post Box No. 1603, Ernakulam North, P.O., Kochi, Kerala – 682 018, India

*[E-mail: imeldajoseph@gmail.com]

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Microalgae, abundant in essential nutrients like vitamins, proteins, lipids, and pigments, hold immense potential for diverse applications spanning food, biofuels, pharmaceuticals, and environmental solutions. Present study focuses assessment of cell multiplication in microalgae *Chlorella vulgaris*, *Nannochloropsis salina* and *Isochrysis galbana*, widely used in aquaculture hatcheries with Indole Acetic Acid (IAA), Gibberellic Acid (GA), and zeatin at 1.0 mg/L, 0.1 mg/L, and 0.01 mg/L in 16 days culture period. The study results revealed that the microalgal biomass can be increased by inducing phytohormones at different concentrations. With zeatin, the cell count observed in *C. vulgaris* and *N. salina* was more than that in control at all three concentrations. For *I. galbana*, except in 1.0 mg/L, the cell count was higher than that in control. *Chlorella vulgaris* attained the maximum cell density at 0.1 mg/L of zeatin (2.5×10^5 cells/ml) and with GA, the cell count attained was highest at 0.01 mg/L (2.25×10^5 cells/ml). *Nannochloropsis salina* also showed higher cell count than the control at all three concentrations of GA. *Isochrysis galbana* showed 1.43×10^5 & 0.61×10^5 cells/ml cell count with 0.01 mg/L of GA. The maximum cell count recorded for *C. vulgaris* was 2.29×10^5 cells/ml at 0.01 mg/L of IAA. Similarly, *N. salina* showed the highest growth in 0.01 mg/L of IAA (2.16×10^5 cells/ml), and the growth was lower than in control at 1 mg/L (1.25×10^5 cells/ml). *Isochrysis galbana* recorded higher cell count than in control (0.57×10^5 cells/ml) in 0.1 mg/L (1.9×10^5 cells/ml) and 0.01 mg/L concentrations of IAA. Study suggests the possibility and potential of phytohormones in the enhanced production of microalgae for various applications.

[**Keywords**: *Chlorella vulgaris*, GA, IAA, *Isochrysis galbana*, *Nannochloropsis salina*, Phytohormones, Zeatin]

Introduction

Microalgae have gained considerable importance in recent years due to their diverse applications in aquaculture hatcheries, nutraceutical development, biofuel production, and essential biomolecule synthesis like β-carotene, astaxanthin, and polyunsaturated fatty acids (PUFA)[1]. Their remarkable diversity is reflected in the existence of several million species, surpassing the number of higher plants by far[2]. Microalgae play a pivotal role in the global ecosystem as they constitute more than 70 % of the world's biomass and form the foundation of the food chain[3]. Microalgae's ease of cultivation, adaptability to diverse environments, rapid biomass accumulation, and high lipid content underscore their immense potential for applications ranging from biofuel production to environmental remediation[4,5]. They are crucial in the world's primary productivity, responsible for nearly half of the annual conversion of carbon dioxide into organic matter and production of oxygen[6,7]. This inherent ability to produce oxygen, coupled with their potential to serve as sources for biofuels, bioactive pharmaceutical compounds, and sustainable food ingredients, positions microalgae as renewable and cost-effective resources with a positive impact on the environment[8].

Microalgae play a major role in aquaculture by serving as essential live feeds for bivalve molluscs during all growth stages and for the early development of various aquatic organisms like abalone, crustaceans, and certain fish species[9]. Their nutritional value and ability to support larval nutrition make them indispensable in aquaculture industry. Moreover, microalgae exhibit significant potential beyond aquaculture, as they have the ability to produce essential compounds such as polyunsaturated fatty acid oils beneficial for newborns[10], pigments serving as nutritional supplements for humans[11], and a diverse range of vitamins, proteins, carbohydrates, fatty acids, enzymes, pigments, and bioactive substances with medicinal properties[12]. This makes microalgae a subject of great interest in biotechnology, with applications in animal feed, human nutrition, pharmaceuticals, and nutraceuticals contributing significantly to their global production[13]. The demand for microalgal feed in commercial fish seed production is significant, necessitating high algal

biomass in larval rearing systems to ensure a continuous supply of microalgae as feed and maintain green water systems in the rearing tanks[14]. At various stages in the life cycle of most cultured marine organisms, marine microalgae play a crucial role as a primary food source[15]. This dependence on microalgae makes algal biomass a highly sought-after resource in the fish-food and aquaculture markets, and it is anticipated that there will be a significant increase in the demand for the algal industry in the future, leading to substantial revenue[16].

The potential of phytohormones to enhance the biosynthesis of high-value products in microalgae holds promise for bioprospecting strategies[17]. Phytohormones exhibit versatility, and their functions depend on their concentrations, localisation in plant tissues, and interactions with different hormone groups[18]. In the context of aquaculture hatcheries, phytohormones can boost microalgae production[19]. Phytohormone treatments have shown significant effects on microalgae, with auxins[20], brassinosteroids[21], jasmonates and cytokinins[6] being among them, leading to increased chlorophyll and carotenoid concentrations. Auxins and cytokinins are phytohormones that enhance photosynthetic efficiency in microalgae[22]. The objectives of the present study are to determine the effects of phytohormones *viz:* indole acetic acid, gibberellic acid, and zeatin on the growth and biomass increase of three commonly used microalgae in mariculture: *Chlorella vulgaris*, *Nannochloropsis salina*, and *Isochrysis galbana* under laboratory conditions.

Materials and Methods

Algal stock culture

The study utilised three microalgae species *viz. Isochrysis galbana*, *Nannochloropsis salina*, and *Chlorella vulgaris*. The initial cultures of these microalgae were obtained from the algal culture collection of the Central Marine Fisheries Research Institute in Cochin. The stock culture of each microalgae was maintained separately in 10 ml culture tubes, ensuring axenic conditions for their growth and preservation during the study. Axenisation of the algae was accomplished using the method described by Droop[23]. Algal cultures were maintained in Guillard and Ryther's modified F/2 Medium[24].

Determination of the effect of phytohormones on algal growth

Phytohormone preparation

Three phytohormones employed in the study were: 1) Zeatin, 2) Gibberellic acid (GA3), and 3) Indole acetic acid (IAA). The phytohormones were sourced from Merck, India (Mumbai).

To prepare a 1 mg/mL stock solution, added 100 mg of the desired phytohormone to a 100 mL volumetric flask. Added 2 – 5 mL of solvent to dissolve the powder. Once completely dissolved, the volume was adjusted with Milli-Q water. Continuous stirring of the solution was done while adding the water to keep the material in solution. The stock solution was stored under refrigeration. Added 1.0 mL of the stock solution to 1 litre of medium to obtain a final concentration of 1.0 mg/L of the phytohormone in the culture medium.

$$\textit{Volume of stock solution} = (\textit{Desired hormone conc.} \times \textit{Medium volume}) / \textit{Stock solution conc.}$$

Each phytohormone was tested at three different concentrations: 1.0 mg/L, 0.1 mg/L, and 0.01 mg/L.

Experimental setup

The experiment involved three sets of triplicate 250 ml culture flasks, with each flask containing 90 ml of fresh F/2 medium. To each set of triplicate flasks, 10 ml of each of the 1.0 mg/L, 0.1 mg/L, and 0.01 mg/L concentrations of Zeatin, GA3, or IAA was added individually, along with 3 ml of the stock culture (3×10^4 cells/ml) of *C. vulgaris*, *N. salina*, or *I. galbana*, respectively.

A triplicate culture was maintained under identical conditions without the addition of IAA, zeatin, or gibberellic acid, and this untreated group served as the control for the experiment. All the culture flasks were kept without aeration at 25 °C with 18 h illumination using 1000 lux fluorescent lamps.

Estimation of microalgal biomass

The microalgal growth was assessed by measuring the Optical Density (OD) of *C. vulgaris* and *N. salina* at 680 nm and *I. galbana* at 650 nm using a UV/Vis spectrophotometer. Optical density measurements were taken every 48 h throughout the experiment. Individual graphs were plotted using the obtained values for all three microalgae under different treatment conditions to analyse their growth patterns. The cell numbers were further determined using the formula:

$$\textit{Cell number (cells/ml)} = (12.813 \times OD - 0.0782) \times 10^4$$

Biomass estimation was performed by measuring OD, and cell counting was also conducted for the control.

Statistical analyses of the data were conducted using IBM SPSS Statistics software. Significance

of the results and differences among various treatments were evaluated at a level of $p < 0.05$ by comparing mean values for triplicate sets of data through Two-way analysis of variance (ANOVA). The data are represented in tables as Mean±SD (Standard Deviation). The statistical significance of the experimental designs was determined using the *F*-test for ANOVA.

Results

Zeatin-based cell growth of microalgae

The addition of zeatin at concentrations 1.0 mg/L, 0.1 mg/L, and 0.01 mg/L in the culture medium has significantly improved the cell growth of *C. vulgaris*, *N. salina*, and *I. galbana* over 16 days. The final cell count of microalgae at different levels of zeatin and in control is given in Table 1. The effect of zeatin on the cell count of the three microalgal strains over 16 days of the experiment is shown in Figure 1. After 16 days, a cell count of 2.5×10^5 cells/ml was recorded for *C. vulgaris* in 0.1 mg/L concentration and it was only 0.83×10^5 cells/ml in control. *Chlorella vulgaris* showed high cell concentration at all three concentration levels of zeatin. Similarly for *N. salina*, the cell counts were significantly ($p < 0.05$) higher than that in the control in all three concentrations of zeatin. The maximum cell count of 2.53×10^5 cells/ml was recorded for *I. galbana* using 1 mg/L of zeatin. The cell count recorded in 0.1 mg/L, and 0.01 mg/L of zeatin were 1.15×10^5 and 1.16×10^5, respectively, for *I. galbana*. The maximum cell count observed in the control was only 1.15×10^5 after 16 days. The ANOVA indicated significant ($p < 0.05$) cell growth for all three microalgal strains, compared to control on the use of zeatin in the culture medium.

Table 1 — Final cell count of microalgae after 16 days in control and in treatments with phytohormones (Zeatin, GA and IAA)

Sl. No	Microalgae	Control	Phytohormone conc.		
			1.0 mg/L	0.1 mg/L	0.01 mg/L
	Zeatin				
1	*C. vulgaris* (cells/ml)×10^5	0.83	1.8	2.5	1.9
2	*N. salina* (cells/ml)×10^5	1.15	2.53	1.9	1.3
3	*I. galbana* (cells/ml)×10^5	0.63	0.31	1.15	1.16
	Gibberellic acid				
4	*C. vulgaris* (cells/ml)×10^5	1.49	1.37	0.21	2.25
5	*N. salina* (cells/ml)×10^5	1.91	1.73	1.69	2.34
6	*I. galbana* (cells/ml)×10^5	0.61	0.99	1.18	1.43
	Indole acetic acid				
7	*C. vulgaris* (cells/ml)×10^5	1.57	1.79	1.98	2.29
8	*N. salina* (cells/ml)×10^5	1.25	1.12	1.71	2.16
9	*I. galbana* (cells/ml)×10^5	0.57	0.55	1.9	1.13

Gibberellic Acid (GA) based cell growth of microalgae

The growth and biomass increase of the three microalgae *C. vulgaris*, *N. salina* and *I. galbana* in the presence of GA at 1.0 mg/L, 0.1 mg/L, and 0.01 mg/L concentrations were recorded. After 16 days of experiment, the microalgae showed an increase in their growth compared to the control. The final cell count of microalgae in different levels of GA and in control are given in Table 1. The effect of GA on the cell count of the three microalgal strains over 16 days of the experiment is shown in Figure 2. The highest growth was recorded for *C. vulgaris* at 0.01 mg/L concentration, with a cell count of 2.25×10^5 cells/ml,

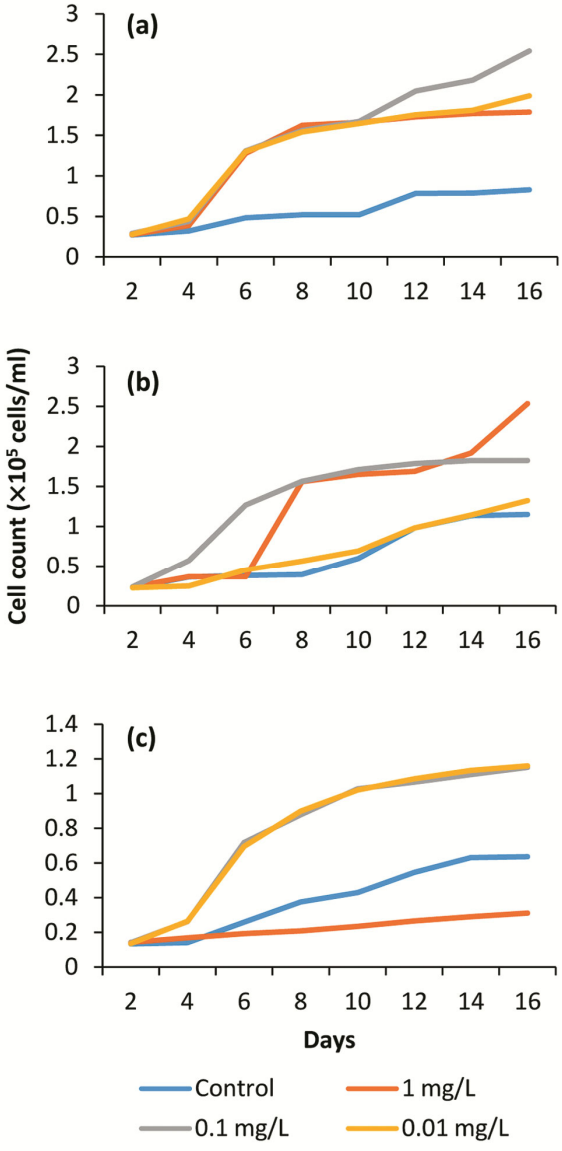

Fig. 1 — Cell count variation of a) *C. vulgaris*, b) *N. salina* and c) *I. galbana* in control and on treatment with zeatin

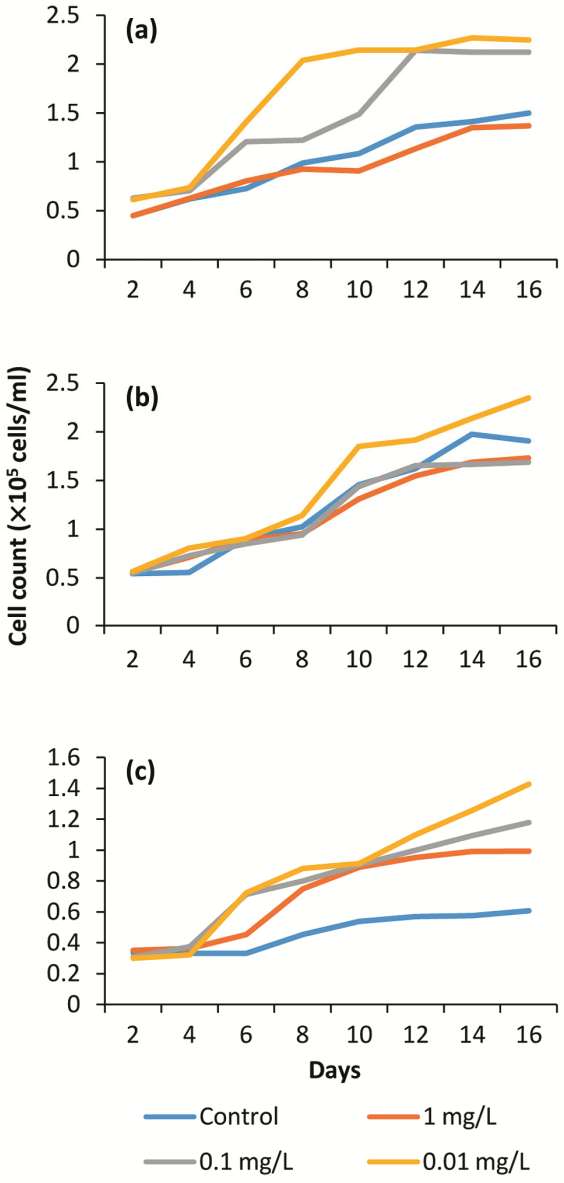

Fig. 2 — Cell count variation of a) *C. vulgaris*, b) *N. salina* and c) *I. galbana* in control and on treatment with GA

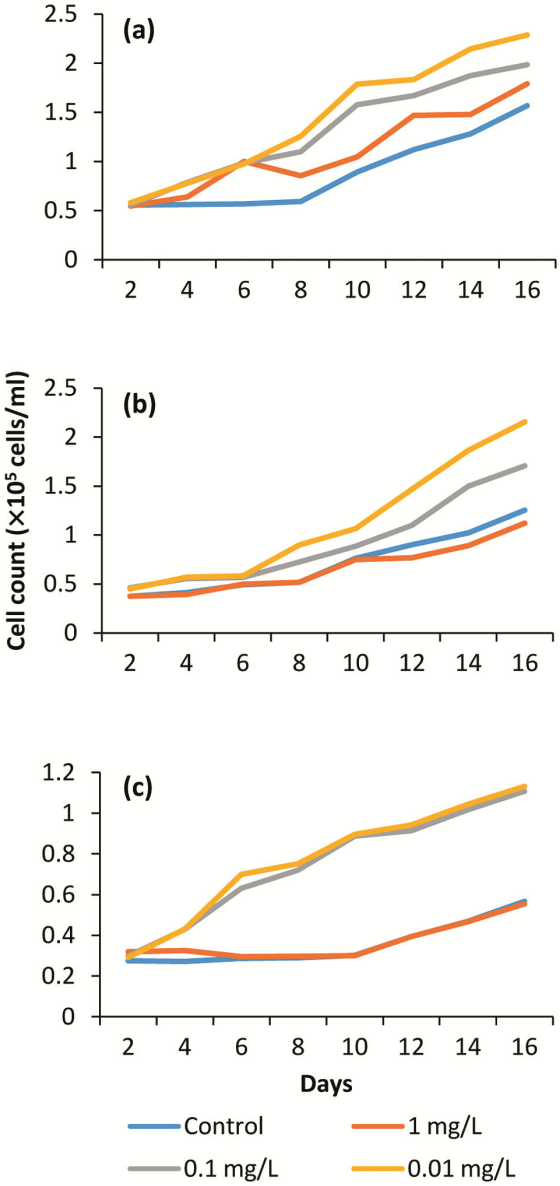

Fig. 3 — Cell count variation of a) *C. vulgaris*, b) *N. salina* and c) *I. galbana* in control and on treatment with IAA

but the cell count in the control was higher than that in 1.0 mg/L and 0.1 mg/L of GA. At 0.1 mg/L GA, *N. salina* also demonstrated an increase in cell count (2.34×10^5 cells/ml). For *I. galbana* using GA, the maximum count of 1.43×10^5 cells/ml was recorded in 0.01 mg/L.

Indole Acetic Acid (IAA) based cell growth of microalgae

The effect of IAA on cell count of microalgae *C. vulgaris*, *N. salina* and *I. galbana* at 1.0 mg/L, 0.1 mg/L, and 0.01 mg/L concentrations were recorded. The final cell count in different levels of IAA and in control are given in Table 1. The effect of IAA on the cell count of the three microalgal strains over 16 days of the experiment is shown in Figure 3. The maximum cell count recorded using IAA for *C. vulgaris* was in 0.01 mg/L concentration (2.29×10^5 cells/ml). For control, the maximum cell count recorded after 16 days was 1.57×10^5 cells/ml for *C. vulgaris*. *Nannochloropsis salina* recorded the highest count in 0.1 mg/L (1.71×10^5 cells/ml) and 0.01 mg/L (2.16×10^5 cells/ml) concentrations, and the growth was lower than in control with 1 mg/L concentration. The cell count in control was 1.25×

10^5 cells/ml. *Isochrysis galbana* showed enhanced growth in 0.1 mg/L, and 0.01 mg/L concentrations. At the end of the experiment for *I. galbana*, at 0.1 mg/L the highest cell count of 1.9×10^5 cells/ml was obtained and in the control, it was only 0.57×10^5 cells/ml.

Statistical analysis of the data showed *F* values for microalgae, hormone, and algae-hormone interactions as 17.2, $p < .001$ (algae); 116.6, $p < .001$ (hormone), and 2.6, $p = .035$ (algae and hormone interaction), respectively. The interaction between microalgae and hormones was significant ($p < 0.05$), and was able to deduce that these phytohormones have resulted in an increase in microalgae biomass production at specific concentration levels.

Discussion

Three common plant hormones, *i.e.* zeatin, gibberellic acid, and IAA, were used to examine their effect on the cell count of *C. vulgaris*, *N. salina* and *I. galbana*. The results suggest the potential of these phytohormones to enhance microalgal cell multiplication and biomass production. Application of zeatin, gibberellic acid and IAA at 0.1 mg/L and 0.01 mg/L in microalgal culture medium was found to be effective in cell multiplication at a faster pace for *C. vulgaris*, *N. salina* and *I. galbana*. Phytohormone technology can help to lower microalgae biomass production costs by promoting rapid growth, improving nutrient uptake, enhancing stress tolerance and by optimising harvesting efficiency. Integrating phytohormones into cultivation processes offers potential for scalable and cost-effective microalgae production for various applications[19]. In the current study, zeatin, had the greatest impact, followed by GA and IAA in algal biomass production. Zeatin-treated *C. vulgaris* a steady increase in cell count, especially at the concentration of 0.1 mg/L (Fig. 1). Zeatin supplementation can play a crucial role in promoting cell growth, division, and potentially lipid accumulation in microalgae cultures by influencing nitrogen metabolism and other physiological processes[25]. Sivaramakrishnan & Incharoensakdi[26] have reported that the maximum biomass of *C. vulgaris* was obtained by adding 0.1 mg/L of zeatin to the growing medium. Present research also demonstrated that supplementation of zeatin at 0.1 mg/L led to a significant ($p < 0.001$) increase in the cell count of *C. vulgaris*, with a maximum of 2.5×10^5 cells/ml. It has also been reported that zeatin increased the growth and accumulation of photosynthetic pigments in *C. vulgaris*[20] and *D. salina*[27]. The most important phytohormone used for increasing biomass and lipids under nitrogen stress in algal cells is Zeatin[23].

It is well documented that GA is a major phytohormone that regulates a variety of microalgal functions, including cell growth[28]. The results of current investigation show that adding GA resulted in growth in the three microalgal strains. Whereas at higher concentrations (at 1.0 mg/L and 0.1 mg/L), reduced growth was observed for *N. salina*. However, it has been confirmed that the microalgal strains can grow better in the presence of gibberellic acid at varying concentrations. In the case of *C. vulgaris*, the results by Madani *et al.*[29] showed that both 4 mg/L and 0.1 mg/L concentrations of GA improved cell growth and biomass. Gibberellic acid at a concentration of 4 mg/L significantly enhanced cell growth in *I. galbana*, with 2 and 4 mg/L being identified as the most beneficial doses for promoting growth, while 6 mg/L had an inhibitory effect on biomass[29]. *Chlorella vulgaris* cells treated with gibberellic acid had recorded a similar rise in biomass content[27]. Doses of phytohormones above 1 mg/L were not used in the present study because all three microalgae selected are widely utilised in aquaculture, suspecting any harmful effect for cultured organisms in higher concentrations.

Indole Acetic Acid (IAA) demonstrates promising potential in the advancement of efficient microalgal cultivation and has been reported to enhance growth by increasing chlorophyll content, consequently boosting photosynthetic activity in *Scenedesmus* sp.[30]. In the current study, all three microalgae showed the biomass increase with the supplementation of IAA (Fig. 3). *Chlorella vulgaris*, *N. salina* and *I. gabana* showed positive responses to using IAA at specific concentration levels. The effect of IAA on *C. vulgaris* was demonstrated by Bajguz & Piotrowska-Niczyporuk[31], and they recorded that a dosage of 5×10^3 M increased the number of cells by 1.8-fold compared to the control. Similarly, Salama *et al.*[32] reported that after 6 days of cultivation, IAA increased the cell density of *Scenedesmus obliquus* by 1.9 times compared to the control. The concentration of IAA that had the greatest effect on cell growth was 100 nM, which increased cell density by 2.3-fold after 10 days[33].

In addition to cell growth and biomass production, treatments with phytohormones can also boost the

amount of primary and secondary metabolites in microalgae. The application of phytohormones at minimal levels to microalgae is not expected to cause any safety concerns; however, cytotoxicity testing would be required to ensure compliance with food safety requirements. But, in the case of biofuel production and other non-edible purposes, using phytohormones will be safe and will not pose any safety issues[19].

Conclusion

The present study showed that phytohormones like zeatin, GA or IAA can effectively be used at low concentrations to improve microalgal growth and multiplication in aquaculture. The findings of this study thus offer a simple and practical method for large-scale microalgal production in aquaculture.

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Conflict of Interest

The authors have no competing or conflicts of interest to declare.

Author Contributions

AS: Culture experiments in the laboratory, data collection and drafting of the manuscript. IJ: Original concept and manuscript preparation.

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