Research

Biostimulants from Algae Biomass Uncovered: Phytohormones Determination and Plant Growth Stimulation

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Abstract:
Microalgae are increasingly recognized as a valuable source of bioactive compounds, including phytohormones, which play crucial roles in regulating plant growth and development. This study investigates the potential use of microalgae biomass as natural biostimulant for sustainable agriculture, where focus on boosting crop resilience and productivity is needed. While most previous studies of the microalgae phytohormones production have been limited to laboratory-scale experiments using expensive synthetic growth media, this research advances the field by utilizing pilot-scale systems for algae biomass cultivation in real municipal wastewater (WW), which represents an alternative, cost-effective growth medium. Microalgae cultures were grown under controlled laboratory conditions and in a pilot high-rate algae pond (HRAP), and specific phytohormones in selected microalgae cultures were quantified. Biomass samples were prepared using various methods (fresh utilisation, freezing, drying), with lyophilization being identified as more effective for preserving biostimulative activity, compared to frozen and thawed biomass, which was found to be less suitable. Preliminary tests, performed with commercial phytotoxkit liquid samples, confirmed biostimulatory effects of algae biomass on roots growth of white mustard (*Sinapis alba*). Phytohormone profiling, conducted using LC-MS/MS, revealed up to 30-fold higher levels of auxin indole-3-acetic acid (IAA) in pilot-scale cultures compared to laboratory-grown biomass. These findings highlight the potential for scaling up microalgal cultivation to produce phytohormone-enriched biomass. Understanding how different growth conditions influence phytohormone production in microalgae is essential for developing large-scale application guidelines. Variability in biomass composition due to fluctuating wastewater characteristics necessitates further research, as well as the development of optimized application strategies to enhance biostimulatory efficacy.

Keywords: High-rate Algae Pond; Microalgae Cultivation; Phytohormones Screening; Plant Growth Promoters

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1. Introduction

1.1. Microalgae as a source of phytohormones

Microalgae are a diverse group of photosynthetic microorganisms, including both eukaryotic and prokaryotic organisms, cyanobacteria (Dineshkumar et al., 2017; Singh & Saxena, 2015). Algal biomass is gaining attention due to its many beneficial properties and its importance across various industries, including cosmetics, food (nutritional supplements, animal feed), as well as to produce pigments and other bioactive compounds which are valuable in medicine and the pharmaceutical industry (Basheer et al., 2020; Castro et al., 2023; Eze et al., 2023). Some species of microalgae have ability to produce polysaccharides, antimicrobial compounds and other metabolites, such as phytohormones, which are well known for their biostimulatory properties and can positively influence plant growth, development, and resilience under stress conditions (Gonçalves, 2021; Guo et al., 2020; Haak et al., 2017; Liu & Ruan, 2022). Auxins and cytokinins play a critical role in plant cell division and elongation, while gibberellins regulate seed germination and plant growth, and abscisic acid acts as a stress-related hormone under adverse conditions (Stirk et al., 2014; Wang et al., 2021). The use of phytohormones derived from microalgae offers a promising sustainable solution in agriculture by reducing the need for synthetic fertilizers, thereby contributing to a decrease in their negative environmental impact (Kapoore et al., 2021; Miranda et al., 2024; Oancea et al., 2013).

Microalgae require specific growth conditions, including appropriate light, temperature, pH, nutrients concentration, and the presence of CO_2, which increases production costs, cultivation, harvesting, and processing costs, particularly for biomass with high valuable compounds that often necessitates optimized growth conditions, expensive synthetic media, or complex bioreactors (Amaro et al., 2023; La Bella et al., 2022; Menegazzo & Fonseca, 2019). This requires the development of innovative, energy-efficient, and cost-effective technologies to enable sustainable production of high-quality algae biomass and its easy integration into agricultural practices (Barsanti & Gualtieri, 2018; Do et al., 2021; Parmar et al., 2023).
Microalgae are capable of thriving in diverse environments, including mixotrophically in wastewater systems (Najdenski et al., 2013; Orfield et al., 2014; Singh & Saxena, 2015). They can be cultivated auto/hetero/mixotrophically either in closed (photo/fermentation) bioreactors or open systems, such as high-rate algae ponds (HRAPs) (Borowiak & Krzywonos, 2022; Muhammad et al., 2020; Tran et al., 2020). Their ability to assimilate nutrients from wastewater (WW) while producing valuable biostimulatory compounds underscores potential for agricultural application and environmental remediation, increasing sustainability (Chew et al., 2017; Kumar et al., 2024). While WW has shown potential for microalgae producing biostimulants, the variability in its composition and specific microbial consortium could affect consistency in phytohormones production (Chai et al., 2024; Li et al., 2024; Oruganti et al., 2022). Production of phytohormones (types and levels) varies among different microalgal and bacterial strains as well as on factors in microorganisms, such as cultivation method, light, and stress factors (Mujtaba & Lee, 2016; Wang et al., 2021). Stirk & van Staden (2020) demonstrated that phytohormone production is closely linked to algal growth and the cell cycle, with different types and concentrations of hormones produced at various growth stages. In actively growing cells, auxins and cytokinins appear to regulate the cell cycle, gibberellins are associated with cell elongation, and abscisic acid functions as a stress hormone (Stirk et al., 2014). Therefore, standardize cultivation parameters are needed and further research focuses should include exploring the production of phytohormones by selected microalgae strains under varying culture conditions, with an emphasis on their application in agriculture (Liang et al., 2009; Magalhães et al., 2024; Mohsenpour et al., 2021).

1.2. Phytohormones and biostimulatory screening of microalgae

Specific methods are required for effective extraction, purification and quantification of target phytohormones in microalgae (Do et al., 2021). Unlike other intracellular active substances, phytohormones encompass many different types with varying structural charac-

teristics, numerous derivatives or metabolites. Additionally, phytohormones are not stable, and they are present in low concentrations in microalgae, therefore sensitive, simple, and rapid processes are needed for determination of phytohormones (Wang et al., 2021). Appropriate techniques for determining endogenous phytohormones are gas chromatography (GC) or high-performance liquid chromatography (HPLC), and for more complex samples, liquid chromatography coupled with mass spectrometry (LC-MS) has been determined as suitable (Zhou et al., 2003; Hoyerová et al., 2006). Form of LC, coupled with tandem mass spectrometry (LC-MS/MS), is an advanced form of LC-MS analysis and is extensively used in targeted analysis, where specific compounds are selected for quantification or identification. This makes it ideal for comprehensive coverage of nearly all known plant hormones and meeting the high accuracy demands of researchers (Creative Proteomics). GC is a commonly used method offering even greater precision and reliability (Birkemeyer et al., 2003; Wang et al., 2021).

Quantification of phytohormones in microalgae samples with LC-MS/MS, along with potential biostimulatory screening of biomass, could provide a comprehensive approach to understanding phytohormone production in microalgae for process optimizations, enabling scalable applications in sustainable agriculture (Nephali et al., 2020; Stirk & van Staden, 2020; Wang et al., 2021). Seedling growth tests are widely used to assess the biological activity of various substances, which include bioactive substances in microalgae that could influence plant growth and development (Godlewska et al., 2016; Rogovska et al, 2012). White mustard (*Sinapis alba*) seeds are common in such tests because of their fast germination, high sensitivity and uniformity (Pannacci et al., 2013).

Most research of microalgae producing phytohormones remains confined to laboratory-scale studies using synthetic media, leaving critical gaps in understanding their performance in real-world conditions (Abdelfattah et al., 2023; Chakraborty et al., 2023; Villaró-Cos et al., 2024). The research aim of this study was to examine production of phytohormones and biostimulatory potential of microalgae, cultivated under controlled laboratory conditions, either in sterile or semi-sterile growth medium, and on pilot scale in HRAP; an open system demonstrating secondary WW treatment. We hypothesize that from microalgae, grown on HRAP, higher concentration of bi-ostimulants can be gained compared to the lab scale.

Therefore, the biostimulatory effects of microalgae biomass prepared in various ways were investigated, and a direct effect on seed germination and early growth of *S. alba* was observed. Different samples of biomass were lyophilized (freeze-dried) for phytohormones quantification conducting LC-MS/MS, as described below.

2. Material and Methods

2.1. Microalgae culturing, harvesting and biomass processing

Chlorella vulgaris cultures were grown under controlled laboratory conditions, at the room temperature, under fluorescent 4000 K (FLUORA, Osram, Germany) and LED lights (65 μmol/m^{2}s). Cultivation was performed in sterile 2 L Erlenmeyer flasks placed on a magnetic stirrer, containing 1.2 L of Bold's Basal Medium (BBM; Phytotechlab) (**Figure 1a**), and in a 30 L photobioreactor (PBR) operating under semi-sterile conditions, with stirrer and CO_2 addition, containing supplemented BBM with 0.34 g/L $NaNO_3$. The PBR maintained a working volume of 27 L (**Figure 1b**).

Mature cultures were harvested by centrifugation, washed with sterile water to remove residual medium, and processed either by oven-drying at 45 °C or lyophilization. The dried biomass was stored in airtight, light-protected containers at room temperature until further analysis. For quantitative phytohormone analysis, approximately 100 mg of lyophilized biomass was needed. Samples were labeled "F, W, D, L" to indicate the preparation steps: fresh, washed, dried, and lyophilized biomass, respectively.

Preliminary tests were performed to confirm biostimulatory effect of algae biomass on *Sinapis alba* (*S. alba*) seeds; various samples from PBR, including fresh biomass, dried, lyophilized or few weeks old from the fridge (labeled ''O'') were analyzed (**Figure 1**).

Mixed culture of *Chlorella*, *Scenedesmus*, and *Pseudopediastrum* species (**Figure 1c**) was obtained from the pilot HRAP in the beginning of September 2024, with real municipal WW (following primary treatment) used as the growth medium.
Biomass was harvested from the sedimentation tank, without washing, and either directly lyophilized or subjected to a freeze-thaw cycle before lyophilization. The processed samples were stored at room temperature in airtight, light-protected containers until further analysis. Samples were labeled ''F, FR+T, L'' to indicate the preparation steps: fresh, previously freeze-thawed, and lyophilized, respectively (**Figure 1**). The biostimulatory effect of selected algae biomass from the pilot HRAP on *S. alba* root growth has not yet been confirmed.

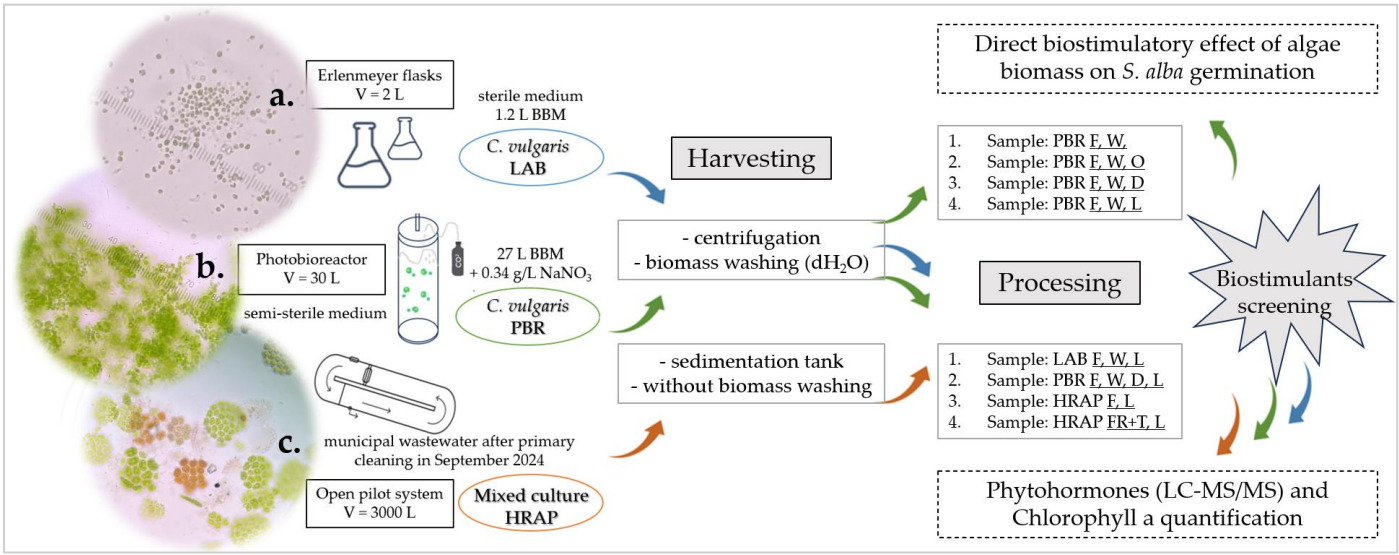

Figure 1. Microalgae culturing, harvesting and biomass processing; **a.** *C. vulgaris* grown in sterile laboratory conditions (LAB), **b.** *C. vulgaris* grown in semi-sterile laboratory conditions (PBR), **c.** mixed algae-bacteria culture grown in open pilot system (HRAP). **F** = fresh, **W** = washed, **O** = few weeks in the fridge (old), **D** = dried at 45 °C, **L** = lyophilized, **FR+T** = freezed and thawed.

2.2. *Phytohormones quantification in microalgae and biostimulatory potential of biomass*

Selected 4 microalgal samples were analyzed for quantification of specific phytohormones using LC-MS/MS (AB Sciex QTRAP 6500, Waters Xevo TQ-S) at the Creative Proteomics (USA). For quantification of phytohormones, approximately 100 mg of lyophilized biomass was needed. The obtained data were normalized against the company's internal standards to account for experimental variation and differences in hormone extraction and ionization efficiency.
Chlorophyll A (Chl A) content (mg/g) was determined using a spectroscopic method (UV-VIS) according to the modified method by Lichtenthaler & Buschmann (2001). Briefly, a 0.1% suspension of lyophilized biomass sample (w/w), mixed with water (dH2O) was prepared. Chl A was extracted by the following procedure: centrifugation at 6500 rpm for 25 min, discarding the supernatant, addition of 95% ethanol (8 mL), heating at 60°C for 1 hour, centrifugation at 4500 rpm for 10 min, measurement of absorbance at 649 nm, 664 nm and 750 nm. Concentration of Chl A in mg/g was calculated according to **Eq. (1)**,

$$Chl\ A = (13.36 \times (A_{664} - A_{750}) - 5.19 \times (A_{649} - A_{750})) \times (V_{EtOH}/(V_{sample} \times l_{cuvette})) \quad . \quad (1)$$

Eq.(1) incorporates coefficients (13.36 and 5.19) to accurately quantify chlorophyll A (Chl A) in mg/g. Additionally, the calculation adjusts for the volume of solvent (V_{EtOH}), the sample volume (V_{sample}), and the cuvette path length (l) to standardize the result.

Phytotoxkit liquid bioassays (Microbiotests) were conducted using *Sinapis alba* seeds to evaluate fresh (F), aged (O), dried (D), and lyophilized (L) samples from PBR. A 10% dry matter buffer mixture was prepared, then algae solutions were applied to test plates at 0.5% and 5% concentration prepared in final 20 mL, where 6 seeds were placed per half

plate, and distilled water was used as the control. After 3 days, root growth was determined with ImageJ tool (available for downloading online), and the germination index (GI) was calculated (**Eq. (2)**). GI of >120% indicated biostimulation, while GI <80% suggested phytotoxicity.

$$GI\ [\%] = ((RL \times GR \text{ of sample})/(RL \times GR \text{ of control})) \times 10 \quad . \quad (2)$$

Eq.(2) accounts for average root elongation (RL) and germination rate (GR), providing a comprehensive determination of the biostimulatory or phytotoxic potential of the tested biomass. Germination rate (GR) was determined as the percentage of germinated seeds relative to the total number of tested seeds.

3. Results

Among detected phytohormones were auxins, cytokinins and gibberellins (**Table 1)**. Phytohormones' levels were varying in samples; algae biomass grown in HRAP exhibited up to 30-fold higher levels of indole-3-acetic acid (IAA) compared to laboratory-grown biomass. However, these elevated levels may partially resulted from exogenous sources present in directly used biomass, and some bacteria could secrete IAA to enhance the interaction between algae and bacteria which could be the reason for the lowest content in biomass cultured under sterile laboratory conditions (Chen et al., 2019).

Drying (lyophilization) was identified as a suitable method for preserving phytohormones and biostimulant activity, but freezing and thawing affected the content of phytohormone, as well as Chl A (**Figure 2**).

Table 1. Phytohormones quantification (ng/g) conducting LC-MS/MS (Creative Proteomics, USA) in lyophilized biomass.

Phytohormones (ng/g)	Cytokinins			Gibberelins			Auxins		
/ Sample (preparation)	cZ	cZR	tZR	GA9	GA4	GA29	IAA	MethlyIAA	IAA-Trp
Chlorella vulgaris LAB (F, W, L)	0.3	1.2	0.1	/	7.3	/	7.7	0.8	/
Chlorella vulgaris PBR (F, W, D, L)	0.2	0.8	/	/	/	/	119.0	1.4	/
Mixed culture HRAP (F, L)	0.6	0.9	0.1	5.6	/	1.5	3083.2	3.6	0.4
Mixed culture HRAP (FR+T, L)	7.0	21.6	1.2	/	/	/	1356.9	24.8	/

F = fresh, W = washed, D = dried, L = lyophilized, FR+T = freezed and thawed, cZ = c-Zeatin. cZR = c-ZRiboside, tZR = t-ZRiboside, GAs = gibberelins, IAA = indole-3-acetic acid, IAA-Trp = IAA-tryptophan. Abscisic acid (ABA) was not detected.

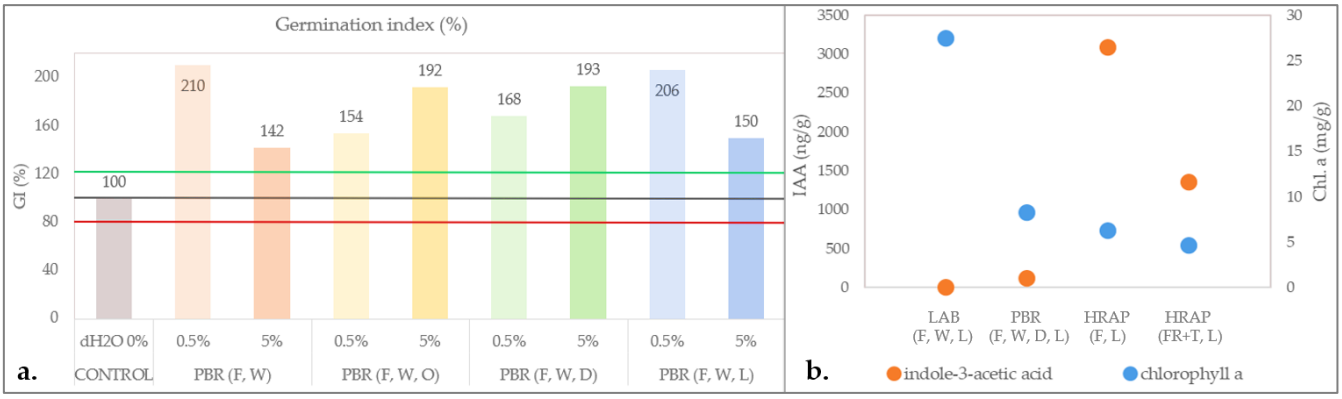

Figure 2. **a.** germination index (%) calculation and direct biostimulatory effect of algae biomass determination (>120% = biostimulatory effect, <80 = phytotoxic effect, 80-120% = no effect), **b.** indole-3-acetic acid (IAA, ng/g) and chlorophyll a (Chl. a, mg/g) content in lyophilized biomass. **F** = fresh, **W** = washed, **O** = few weeks in the fridge (old), **D** = dried at 45 °C, **L** = lyophilized, **FR+T** = freezed and thawed.

4. Discussion

We have previously reported that freeze-thawed algal biomass was not effective as a biostimulant for *S. alba* seeds (Cepec et al., 2024). One possible explanation is the damage of the cell wall during thawing at room temperature, which could leads to the release and likely degradation of intracellular contents. The freeze-thaw principle for cell disrupting has been described, where the efficiency of cell rupture using this method depends on factors such as the freezing rate, thawing conditions, extraction solution, and algal species (Chittapun et al., 2025). Recent studies have shown that an increase in thawing temperature and duration can exacerbate the loss of photosynthetic pigments (Chen et al., 2024).

Light intensity is a major factor influencing chlorophyll content; however, culture concentration, CO_2 availability, and nutrient conditions can also play significant roles. Self-shading likely reduces the average light available to cultures in PBRs, whereas in HRAPs, chlorophyll content tends to increase with decreasing water depth. Seasonal variations and the presence of environmental pollutants further influence chlorophyll levels in microalgae (da Silva Ferreira & Sant'Anna, 2017; Kim et al., 2018; Perin et al., 2022; Sutherland et al., 2014).

Our results match others, as IAA being the most prevalent phytohormone in actively growing microalgal strains, where concentrations of cZ>tZ-type cytokinins were reported (Stirk et al., 2009; Stirk et al., 2013). Stress-responses can explain lower content of active forms and increase of precursors in freeze-thawed sample, additionally, bioactive compounds are susceptible to degradation over time, as exposing the secondary metabolites to oxygen is quickening their degradation (Stirk & van Staden, 2020; Stirk et al., 2021). Other studies have similarly indicated that growth conditions and biomass processing significantly affect the phytohormone content and biostimulant potential of biomass (Ranglová et al., 2021). For example, the phytohormone content in *Chorella sorokiniana* is highly influenced by culture conditions, however, our findings differ slightly, suggesting that phytohormone production in microalgae is both growth- and species-specific (Stirk et al., 2024). In contrast, lower auxin concentrations were detected in *C. sorokiniana* grown phototrophically in outdoor raceway ponds with synthetic growth medium compared to laboratory-grown cultures (Do et al., 2020). On the other hand, microalgal biomass cultivated in synthetic medium combined with municipal WW exhibited higher auxin and cytokinin concentrations compared to biomass grown in synthetic medium alone (Elakbawy et al., 2022).

Microalgae produce bioactive compounds under specific conditions, but the precise mechanisms and environmental factors regulating their synthesis remain insufficiently understood (Do et al., 2020; Senousy et al., 2023). Based on our results, we conclude that further research is essential to fully understand the complex interplay of factors influencing the production and stability of bioactive compounds in microalgae. Special emphasis should be placed on optimizing mixotrophic cultivation at larger scales while reducing production costs.

5. Conclusions

The findings of this study suggest that HRAP systems can be optimized for large-scale production of phytohormone-enriched biomass and freeze-drying is a reliable method for preserving the biostimulatory potential of microalgae biomass. Therefore, in further research it is necessary to standardize cultivation parameters and optimize production systems. Future studies should explore how varying culture conditions influence phytohormone synthesis in selected microalgae cultures. The focus should remain on enhancing their application as biostimulants to support plant growth and improve stress resistance, conducting pilot-scale experiments using real municipal wastewater. This approach not only offers a natural alternative to synthetic chemicals but also contributes to environmental sustainability by utilizing waste resources in biomass production. Understanding influence of growth conditions and processing methods on the production and activity of phytohormones in microalgae can guide the development of high-quality biomass for agricultural use.

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