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Comparison of plant biostimulating properties of *Chlorella sorokiniana* biomass produced in batch and semi-continuous systems supplemented with pig manure or acetate

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ABSTRACT

Microalgae-derived biostimulants provide an eco-friendly biotechnology for improving crop productivity. The strategy of circular economy includes reducing biomass production costs of new and robust microalgae strains grown in nutrient-rich wastewater and mixotrophic culture where media is enriched with organic carbon. In this study, *Chlorella sorokiniana* was grown in 100 l bioreactors under sub-optimal conditions in a greenhouse. A combination of batch and semi-continuous cultivation was used to investigate the growth, plant hormone and biostimulating effect of biomass grown in diluted pig manure and in nutrient medium supplemented with Na-acetate. *C. sorokiniana* tolerated the low light (sum of PAR 0.99 ± 0.18 mol/photons/(m^2/day)) and temperature (3.7–23.7° C) conditions to maintain a positive growth rate and daily biomass productivity (up to 149 mg/l/day and 69 mg/l/day dry matter production in pig manure and Na-acetate supplemented cultures respectively). The protein and lipid content was significantly higher in the biomass generated in batch culture and dilute pig manure (1.4x higher protein and 2x higher lipid) compared to the Na-acetate enriched culture. Auxins indole-3-acetic acid (IAA) and 2-oxindole-3-acetic acid (oxIAA) and salicylic acid (SA) were present in the biomass with significantly higher auxin content in the biomass generated using pig manure (> 350 pmol/g DW IAA and > 84 pmol/g DW oxIAA) compared to cultures enriched with Na-acetate and batch cultures (< 200 pmol/g DW IAA and < 27 pmol/g DW oxIAA). No abscisic acid and jasmonates were detected. All samples had plant biostimulating activity measured in the mungbean rooting bioassay with the Na-acetate supplemented biomass eliciting higher rooting activity (equivalent to 1–2 mg/l IBA) compared to the pig manure (equivalent to 0.5–1 mg/l IBA) and batch culture (equivalent to water control) generated biomass. Thus *C. sorokiniana* MACC-728 is a robust new strain for biotechnology, tolerating low light and temperature conditions. The strain can adapt to alternative nutrient (pig manure) and carbon (acetate) sources with the generated biomass having a high auxin concentration and plant biostimulating activity detected with the mungbean rooting bioassay.

Abbreviations: ABA, abscisic acid; ANOVA, analysis of variance; C, carbon; COD, chemical oxygen demand; GA, gibberellin; IAA, indole-3-acetic acid; IAA-Asp, indole-3-acetyl aspartate; IAA-Glu, indole-3-acetyl glutamate; IBA, indole-3-butyric acid; JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; P, phosphorus; MACC, Mosonmagyaróvár Algal Culture Collection; N, nitrogen; OPDA, *cis*-12-oxo-phytodienoic acid; OxIAA, 2-oxindole-3-acetic acid; PAR, photosynthetically active radiation; QY, maximum quantum yield of photosystem II; SA, salicylic acid; SD, standard deviation.

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1. Introduction

Microalgae have potential to be developed as natural eco-friendly biofertilizers and biostimulants for agriculture. Application of microalgae biomass improves soil fertility, enhances plant growth and yield and modulates stress responses in a wide range of vegetable and cereal crops (Bello et al., 2021; Colla and Rouphael, 2020; Kapoore et al., 2021). The beneficial effects of microalgae biostimulants are attributed to primary metabolites (proteins, lipids and carbohydrates) and a range of secondary compounds such as phytohormones, polyamines, vitamins, phenolics, terpenoids and osmolytes (proline, glycine, betaine; Colla and Rouphael, 2020). These compounds act as elicitor molecules to increase the synthesis of compounds such as proteins, vitamins and phytohormones that are responsible for plant growth and stress responses (Kapoore et al., 2021; Tan et al.; 2021).

Large volumes of high quality biomass needs to be produced to ensure sufficient quantities are available for commercial microalgae-based biostimulant production. Cultivation costs need to be kept to a minimum to make this an economically viable technology. One large financial input in cultivating microalgae is supplying sufficient nutrients to ensure high biomass generation. Integration of wastewater resources into the cultivation of microalgae as an alternative, cheaper nutrient source provides a sustainable circular economy where nutrients are recycled, thereby reducing the costs of biomass production with an additional benefit of reducing the environmental pollutant load (Carneiro et al., 2021; Ge et al., 2017; Kothari et al., 2023). Wastewater from different agricultural, municipal and industrial sources can be used as a nutrient source but will vary in nutrient composition (Ge et al., 2017; Kapoore et al., 2021) and potential contaminants (Parmar et al., 2023). Each source of wastewater needs to be tested with the microalga strain of interest to ensure its suitability as an alternative nutrient source. Examples of microalgae cultivated on wastewater include *Chlorella vulgaris* and *Scenedesmus acutus* in municipal wastewater (Carneiro et al., 2021); *Scenedesmus obliquus* and *Spirulina* sp. in brewery wastewater (Navarro-López et al., 2020a; Pereira et al., 2022); marine microalgae *Chlorella marina*, *Nannochloropsis marina*, *Dunaliella salina* and *Thalassiosira* sp. in high salinity tannery effluent (Nambukrishnan and Singaram, 2022); *Chlorella sorokiniana*, *S. obliquus* and *Ankistrodesmus falcatus* in aquaculture wastewater (Ansari et al., 2017) and *Chlorella vulgaris* in slaughterhouse wastewater (Kothari et al., 2023). Pig manure contains a high nitrogen and phosphorus content as well as an organic acid component of volatile fatty acids (e.g. acetic acid and propionic acid) which make it a potentially feasible source of wastewater for microalgae cultivation (Montero et al., 2018). *Chlorococcum* sp. and *S. obliquus* have been successfully grown in diluted pig manure (Montero et al., 2018; Navarro-López et al., 2020b) and addition of pig manure to Bolds Basal medium increased the growth of three *Scenedesmus* species (Kim et al., 2007).

The most economical option for large-scale cultivation is outdoor or greenhouse systems where environmental conditions are not controlled, thereby reducing the energy input (Sánchez-Zurano et al., 2021). Microalgae grown outdoors or in greenhouses will be exposed to abiotic stresses due to sub- and supra-optimal conditions, including low light conditions. Microalgae can either grow photoautotrophically where they utilize inorganic C (e.g. CO_2 or HCO_3^-) via photosynthesis or heterotrophically where they utilize organic C (e.g. glucose, acetate or glycerol) dissolved in the media via aerobic respiration (Nur and Buma, 2019). Mixotrophic cultures utilize both organic and inorganic C substrates simultaneously. Generally, mixotrophic growth rates are higher than photoautotrophic and heterotrophic growth rates in light-limiting conditions (Nirmalakhandan et al., 2019) as the microalgae fix atmospheric CO_2 via photosynthesis during the day and use heterotrophic metabolism to fix C during both diurnal and dark respiration (Ge et al., 2017). Shifting from a photoautotrophic system that relies solely on light as an energy source to a mixotrophic system is a possible strategy for cultivating microalgae in low light conditions as can occur in greenhouse and outdoor settings due unfavourable weather conditions.

Environmental fluctuations affect not only the growth rate and productivity but also the biomass quality (Borowitzka, 2016; Ge et al., 2017). Microalgae have a high degree of metabolic plasticity and modify their biochemical pathways in response to culture conditions (Liang et al., 2018). Similarly, trophic conditions (photoautotrophic vs mixotrophic) either up-regulate or down-regulate various metabolic pathways which trigger the accumulation of different primary and secondary metabolites (Liang et al., 2018; Nirmalakhandan et al., 2019). These induced metabolic changes either stimulate or reduce the synthesis of specific metabolites, including phytohormones. Microalgae synthesize a range of phytohormones including auxins, cytokinins, gibberellins (GAs), abscisic acid (ABA), brassinosteroids, jasmonates and salicylic acid (SA; Stirk and van Staden, 2020). There is evidence that phytohormones act as signalling molecules in microalgae to regulate their growth and stress responses (Stirk and van Staden, 2020). They are synthesized in response to both internal (e.g. cell division, growth phase, accumulation of other phytohormones) and external factors (e.g. light, temperature, N levels, C sources, pH; Tan et al., 2021; Žižková et al., 2017). Cultivation of microalgae in outdoor systems could potentially influence not only the primary metabolite content but also the secondary metabolite and phytohormone content as the microalgae adapt to the prevailing conditions. This could potentially alter the quality of the biomass in terms of its effectiveness when used as a biostimulant.

While the effect of stresses on primary metabolism has been extensively researched, the effect of stress on secondary metabolite content is not as well studied. Most studies investigating cultivation of microalgae in wastewater have been conducted under controlled laboratory conditions (Ge et al., 2017; Sánchez-Zurano et al., 2021) focusing on the production of macromolecules for biofuel and animal feed (Nur and Buma, 2019). Light and nutrient depletion are both growth limiting factors which can be managed by the mode of cultivation, namely batch vs semi-continuous cultivation (Borowitzka, 2016). The aim of the present study was to compare the effect of two strategies, namely the use of wastewater (pig manure) as a nutrient source and mixotrophic cultivation (Na-acetate as an organic C source) on growth, macromolecule and hormone content in semi-continuous cultures of *Chlorella sorokiniana* grown under sub-optimal (low light and temperatures) environmental conditions in a greenhouse. The growth experiment was carried out in simple, closed photobioreactors similar to those used for biomass production and research work with microalgae. The plant biostimulating potential of the biomass was assessed in the mungbean rooting assay.

2. Materials and methods

2.1. Experimental microalga and inoculum preparation

Chlorella sorokiniana MACC-728 (Mosonmagyaróvár Algal Culture Collection) was selected as the experimental microalga based on its high growth rate, good protein and potentially high lipid productivity as well as its biostimulating activity. The identity of the microalgae was determined by marker gene sequencing using algal genomic DNA purified from cultures based on single algal colonies on plates. Both 16S rDNA and 18S rDNA/ITS marker sequencing confirmed that the MACC-728 isolate was *C. sorokiniana* (Wu et al., 2001). The microalga was inoculated from an agar culture into two 500 ml Erlenmeyer flasks containing 250 ml complete Tamiya liquid medium (Kuznjecov and Vladimirova, 1964). The cultures were grown at 25 ± 2 °C in a 12:12 h light:dark photoperiod and illuminated from below with 130 μmol photons/(m^2 s) light intensity and aerated continuously. The sterile air (1.33 vvm 20 l/h per flask) was enriched with 1.5% CO_2 during the light phase of growth. After 7 days, the suspension cultures were used to inoculate four 500 ml flasks, each containing 250 ml N-limited Tamiya liquid medium (140 mg/l N; 20% N). The starting density of the cultures was 10 mg DW/l. The cultures were maintained in the described conditions for a

further 7 days, then combined and used to inoculate twelve culture tubes containing 2 l N-limited (20% N) Tamiya liquid medium. The starting algal dry matter content of the cultures was 30 mg/l. The cultures were aerated with 1.5% CO_2-enriched air between 7 am and 7 pm, and incubated under greenhouse conditions for 7 days. Then the cultures were combined and used as inoculum for the 100 l bioreactor experiments conducted under greenhouse conditions. Culture density was calculated by dry weight measurement (Ördög et al., 2012).

2.2. Medium additive preparation

Non-stored, fresh pig manure was provided by Agro-Milch Ltd (Lázi, Hungary). Filtration was necessary to decrease the total suspended solids (TSS) content that causes turbidity, thereby reducing the light penetration (Ge et al., 2017). The pig manure was filtered with a Bauer separator (Bauer Hungary Ltd, Szolnok, Hungary) which can process up to 12–14 m^3 h with screen baskets having an opening of 0.25–0.5. The main characteristics of the resulting filtrate were 1081 ± 20 mg/l total suspended solids (TSS), 1057 ± 12 mg/l total Kjeldahl nitrogen, 908 ± 20 mg/l NH_3-N and 43 ± 9 mg/ total P, 35 ± 7 mg/l dissolved reactive phosphorus (PO_4-P) and the chemical oxygen demand (COD) was 7098 ± 27 mg/l.

The pig manure filtrate was then diluted to 40% (2 l filtrate made up to 5 l with tap water) for the growth experiment. This means that on days 5–8 and days 12–15 where 5 l suspension culture was harvested daily from each bioreactor and replaced with 5 l diluted pig manure, 2% non-diluted filtered pig manure was added to 100 l suspension culture. This daily addition of microalgal-available nutrients added with the pig manure were 18.6 mg/l NH_4-N and 0.7 mg/l PO_4-P.

2.3. Greenhouse trial

The experiment ran from 11 to 27 November 2020 in a greenhouse at the Széchenyi István University, Mosonmagyaróvár, Hungary (47°52′N; 17°16′E). There were two 100 l non-sterile, closed flat panel photobioreactors constructed from a polyethylene tube enclosed in a wire mesh and metal frame (3 m long x 3 cm wide x 1 m high; Aremu et al., 2015). *C. sorokiniana* inoculum (100 mg/l DW) was added to modified Tamiya medium containing 140 mg/l N (20% N). Cultures grew for 5 days as batch cultures. Treatments commenced on day 5 and continued for the duration of the experiment (16 days). For the semi-continuous cultures, 5 l cell suspension was harvested daily from each bioreactor. The media was replaced with either 5 l tap water and 50 g Na-acetate to give a concentration of 500 mg/l Na-acetate (Bioreactor 1) or 2 l pig manure + 3 l tap water (Bioreactor 2). On day 9, 5 l cell suspension was removed from both bioreactors and replaced with 5 l modified Tamiya medium (20% N; Table 1). Cultures were aerated with 1% CO_2-enriched air during the day during the initial batch culture phase (days 0–5). During the semi-continuous culture phase following the treatments (days 5–16), cultures were continuously aerated with compressed air at 100 l/min (Busch Mink MM 1104 BP01, Busch Productions GmbH, Maulburg, Germany).

Environmental conditions in the greenhouse and suspension cultures were monitored for the duration of the experiment. Incoming radiation and sunshine duration was measured at the surface of the bioreactor and the temperature of the suspension cultures were continuously measured every 5 min. The following meteorological elements were used to characterize the environmental conditions - average temperature, minimum temperature and maximum temperature in the algal suspension and daily mean of sunshine duration, sum of photosynthetically active radiation (PAR) and maximum of PAR in the greenhouse. Temperature elements were quantified using the temperature sensor EcoStation BTP-06/SP/hun/v1.1 (Boreas Ltd, Érd, Hungary). Radiation elements were measured using the radiation intensity and sunshine duration sensor EcoStation BIS-06/PAR/hun/v1.2 (Boreas Ltd, Érd, Hungary).

The maximum quantum yield (QY) of photosystem II (F_v/F_m) was measured in the early afternoon to monitor the physiological state of the cultures (PSI AquaPen, Photon Systems Instruments, Drásov, Czech Republic). The pH and the electric conductivity (mS/ cm) of the samples was measured at each harvest time to evaluate the CO_2-supply for the algae and the total ionic content of the suspension, respectively.

Table 1
Experimental design outlining treatments with removal of 5 l cell suspension and addition of Na-acetate (Bioreactor 1) or dilute pig manure (Bioreactor 2) over the course of the experiment for *Chlorella sorokiniana* MACC-728 grown in 100 l bioreactors in a greenhouse in November 2020. Nitrogen concentrations in the Tamiya media were adjusted to 140 mg/l N (20% N). Daily addition of filtered pig manure provided 18.6 mg/l NH_4-N and 0.7 mg/l PO_4-P. Cultures were aerated with 1% CO_2-enriched air from days 0–5 (batch culture) and with compressed air thereafter (semi-continuous culture).

Day	Supplements	
	Bioreactor 1	Bioreactor 2
Day 0	100 l Tamiya media (20% N)	100 l Tamiya media (20% N)
Day 1	-	-
Day 2	-	-
Day 3	-	-
Day 4	-	-
Day 5	50 g Na-acetate + 5 l tap water	2 l pig manure + 3 l tap water
Day 6	50 g Na-acetate + 5 l tap water	2 l pig manure + 3 l tap water
Day 7	50 g Na-acetate + 5 l tap water	2 l pig manure + 3 l tap water
Day 8	50 g Na-acetate + 5 l tap water	2 l pig manure + 3 l tap water
Day 9	5 l Tamiya media (20% N)	5 l Tamiya media (20% N)
Day 10	-	-
Day 11	-	-
Day 12	50 g Na-acetate + 5 l tap water	2 l pig manure + 3 l tap water
Day 13	50 g Na-acetate + 5 l tap water	2 l pig manure + 3 l tap water
Day 14	50 g Na-acetate + 5 l tap water	2 l pig manure + 3 l tap water
Day 15	50 g Na-acetate + 5 l tap water	2 l pig manure + 3 l tap water
Day 16	Experiment ended	Experiment ended

- no treatment

Biomass from both bioreactors harvested on day 5 prior to treatments was combined (batch cultures) and served as the control (growth in synthetic medium). Biomass from each bioreactor harvested on consecutive days where the treatment was the same were combined (days 6+7+8 and days 13+14+15). The combined samples were stored at −19 °C until required for chemical analysis and bioassays.

2.4. Macromolecule quantification

Crude protein content in the *C. sorokiniana* biomass was quantified using a standard Kjeldahl method as previously described (Ördög et al., 2012). Lipid content was determined by hydrolysis with 3 M HCl at 95–100 °C for 1.5 h followed by sequential solvent elution using methanol, hexane and diethyl ether as previously described (Ördög et al., 2012). Carbohydrate content quantification was based on the Luff-Schoorl titration method as outlined in the MSZ 5830/26 Hungarian Standard as previously described (Ördög et al., 2016). All analyses were performed in triplicate.

2.5. Plant hormone analysis

Endogenous levels of jasmonates (jasmonic acid (JA), jasmonoyl-L-isoleucine (JA-Ile) and *cis*-12-oxo-phytodienoic acid (OPDA)), auxins (indole-3-acetic acid (IAA), 2-oxindole-3-acetic acid (oxIAA), indole-3-acetyl glutamate (IAA-Glu) and indole-3-acetyl aspartate (IAA-Asp)), ABA and SA were quantified in the samples following the methods previously described by Floková et al. (2014) with modifications. Briefly, the phytohormones were extracted using an acidified aqueous solution of methanol (1 mol/l formic acid in 10% MeOH/H_2O v/v). Appropriate stable isotope-labelled standards were added to the samples prior to purification to validate the LC-MS method as follows: 10 pmol of [$^{13}C_6$]IAA, [$^{13}C_6$]oxIAA, [$^{13}C_6$]IAA-Glu, [$^{13}C_6$]IAA-Asp, [$^{2}H_6$]JA, [$^{2}H_5$]OPDA and [$^{2}H_6$]ABA; 20 pmol of [$^{2}H_4$]SA and 5 pmol of [$^{2}H_2$]JA-Ile

(Olchemim Ltd, Czech Republic) per sample. The extracts were purified using Oasis HLB columns (30 mg/1 ml, Waters), analytes were eluted using 80% MeOH and gently evaporated to dryness under a stream of nitrogen. The samples were analyzed by ultra-performance liquid chromatography (Acquity UPLC® System, Waters, Mitford, MA, USA) coupled to a triple quadrupole mass spectrometer (Xevo™ TQ-S MS, Waters MS Technologies, Manchester, UK) equipped with an electrospray interface. The concentrations of the identified hormones were calculated by the standard isotope dilution method (Masslynx 4.1 software, Waters, MA, USA). All analyses were performed in triplicate.

2.6. Plant growth stimulating activity

The mungbean rooting bioassay was used to assess the plant biostimulating activity of the samples (Crouch and van Staden, 1991). Briefly, mungbeans seeds (*Vigna radiata*) were germinated in moist vermiculite at 26 ± 1 °C in 16:8 h light:dark photoperiod and 120 µmol photons/(m^2 s) light intensity. On day 10, uniform mung bean cuttings (12 cm stem length) with two fully opened leaves were placed in prepared microalgae and phytohormone solutions for 6 h, then rinsed and transferred to clean vials containing water. There were six cuttings per vial and three vials per solution (18 cuttings in total per extract). Water extracts of the microalgae biomass (3 mg/ml) were prepared on the day of the pulse treatment by sonication for 15 min (Stirk et al., 2020). A range of concentrations (0.5–50 mg/l) of indole-3-butyric acid (IBA) and ABA were included in the assay and well as combination solutions (2+1 mg/l and 2+2 mg/l IBA+ABA) for comparative purposes. Distilled water was included as a control. The number of roots were recorded 10 days after the pulse treatment.

2.7. Statistical analysis

Two-sample t-tests were conducted on samples harvested on the same day from the two bioreactors to compare the effects of the treatments on dry weight and daily biomass production. The effects of the treatments on the macromolecule, phytohormone content and rooting in the mungbean assay were analysed using a randomized block design with three replications. When ANOVA was significant, Duncan's multiple range test was performed for comparisons. P values greater than 0.05 were considered as not significant. The rooting response to a range of IBA and ABA concentrations as well as the *C. sorokiniana* samples were analysed by one-way analysis of variance and Duncan's multiple range test ($P \leq 0.05$). Regression analysis was performed to show the relationship between IBA concentration and root number. Pearson's correlation between eight parameters encompassing growth parameters (daily biomass productivity), macromolecules (proteins, lipids and carbohydrates), hormones (indole-3-acetic acid (IAA), 2-oxindole-3-acetic acid (oxIAA) and SA) and rooting activity (number of roots) were performed to reveal relationships between these factors. All the statistical analyses were performed with Genstat 19th Edition (https://www.genstat.co.uk).

3. Results

3.1. Growth of C. sorokiniana cultures conducted under greenhouse conditions

Environmental conditions in the greenhouse were sub-optimal for *C. sorokiniana* compared to when it is grown under laboratory conditions. There were low temperatures (below 24 °C) and limited light for the duration of the current experiment (Table 2).

There was initially slow growth in the batch cultures (day 0–5) with low daily dry matter production (Fig. 1A and B). *C. sorokiniana* growth increased in the semi-continuous cultures in response to pig manure or Na-acetate supplementation with similar DW in both bioreactors at the end of the experiment (day 16). In the Na-acetate supplemented culture, dry weight increased steadily over time and daily dry matter productivity was constant from day 7–14 and declined thereafter (Fig. 1A and B). The pig manure supplemented culture had faster growth and higher daily dry matter production from day 6–10 compared to the Na-acetate supplemented culture. However, growth initially declined with the addition of Tamiya media (day 10–12), then increased with the addition of more pig manure (day 13–15) and then remained stationary until day 16 (Fig. 1A and B). The two sample t-test showed that addition of 2 L pig manure or Na-acetate had no significant effect on dry weight ($P = 0.240$ and $P = 0.672$) and daily dry matter production ($P = 0.223$ and 0.326) on days 6–8 and days 13–15 respectively.

QY values for *C. sorokiniana* were similar in both bioreactors regardless of the treatment and ranged from 0.63 to 0.66 F_v/F_m

Table 2
Meteorological conditions in the greenhouse during the experiment. Results are presented as mean ± SD for the duration of the experiment. Light parameters were measured at the bioreactor surface and the temperature of the algal suspension was measured.

Environmental parameter	11–27 November 2020
Average temperature (° C)	12.0 ± 1.0
Average minimum temperature (° C)	8.7 ± 0.9
Average maximum temperature (° C)	15.5 ± 1.2
Temperature range (° C)	3.7–23.7
Daily mean of sunshine duration (h)	0.3 ± 0.1
Sum of PAR (mol photons/m^2/day)	0.99 ± 0.18
Maximum PAR (µmol photons/m^2/day)	177.2 ± 45.6

PAR photosynthetically active radiation

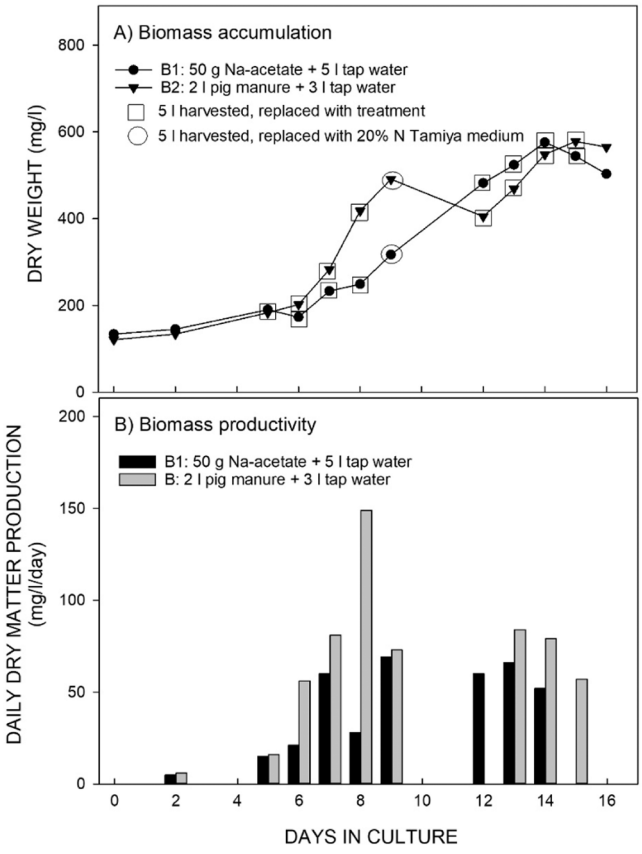

Fig. 1. Growth measured as A) dry weight of biomass and B) daily productivity of batch and semi-continuous *Chlorella sorokiniana* cultures grown in 100 l bioreactors under greenhouse conditions in November 2020. Cultures were supplemented with either Na-acetate (Bioreactor 1) or dilute pig manure (Bioreactor 2).

Table 3
Culture conditions and physiological factors monitored during the growth experiment with *Chlorella sorokiniana* MACC-728 conducted in 100 l bioreactors under greenhouse conditions in batch culture (Day 0–5) and in semi-continuous mode (Day 5–16) with the addition of Na-acetate (Bioreactor 1) or dilute pig manure (Bioreactor 2). Results are presented as mean ± SD.

Day	Quantum Yield (F_v/F_m)		pH		Electrical conductivity (mS/cm)	
Bioreactor	1	2	1	2	1	2
Day 5	0.64 ± 0.05	0.66 ± 0.06	6.5 ± 0.1	6.5 ± 0.1	2.85 ± 0.24	2.78 ± 0.20
Day 6	0.66 ± 0.04	0.66 ± 0.04	7.2 ± 0.1	7.0 ± 0.2	2.82 ± 0.22	2.77 ± 0.19
Day 7	0.65 ± 0.07	0.63 ± 0.05	7.8 ± 0.1	7.3 ± 0.2	2.79 ± 0.28	2.75 ± 0.17
Day 8	0.64 ± 0.04	0.63 ± 0.05	8.1 ± 0.2	7.2 ± 0.1	2.75 ± 0.17	2.69 ± 0.22
Day 13	0.64 ± 0.06	0.65 ± 0.06	8.4 ± 0.2	7.9 ± 0.2	2.55 ± 0.15	2.52 ± 0.27
Day 14	0.66 ± 0.03	0.66 ± 0.03	8.9 ± 0.2	8.2 ± 0.1	2.53 ± 0.27	2.52 ± 0.24
Day 15	0.64 ± 0.05	0.65 ± 0.05	9.0 ± 0.2	8.2 ± 0.1	2.49 ± 0.18	2.48 ± 0.17

(Table 3). Addition of Na-acetate or pig manure increased the pH of the cultures compared to the batch culture with Tamiya media (pH 6.5). The pH increased over time and was highest in the Na-acetate supplemented cultures (ranging from pH 7.2–9.0) compared to pig manure supplementation (ranging from pH 7.0–8.2; Table 3). The electrical conductivity of the cultures was stable regardless of the treatment, ranging from 2.48 to 2.85 mS/cm (Table 3).

3.2. Macromolecule content

The protein and lipid content in *C. sorokiniana* were significantly affected by the treatment with cultures treated with Na-acetate having the lowest protein and lipid content ($P \leq 0.01$; Table 4). There were significant differences in the carbohydrate ($P \leq 0.01$) content in the batch and semi-continuous cultures with the two treatments (Table 4).

3.3. Plant hormones

Two auxins, namely indole-3-acetic acid (IAA) and the catabolite 2-oxindole-3-acetic acid (oxIAA) were present in the *C. sorokiniana* samples with IAA occurring in higher amounts. The biomass produced in the pig manure treatments had a significantly higher ($P \leq 0.01$) auxin content (both IAA and oxIAA) compared to the batch cultures and Na-acetate supplemented biomass (Table 4). SA was also present in the biomass with similar concentrations in all the samples regardless of the treatment (Table 4). No ABA and jasmonates were detected in the samples.

3.4. Mungbean bioassay

IBA stimulated rooting in the mungbean bioassay with a linear regression between $\log_{10}$ IBA concentration and the number of roots. The lowest IBA concentration to elicit a significant rooting effect was 2 mg/l IBA and the highest rooting effect was achieved with 20–50 mg/l IBA (Fig. 2A). ABA (0.5–20 mg/l) had no significant effect on rooting in the mungbean assay (Fig. 2A). There were no significant differences in the rooting response between 2 mg/l IBA and combination treatments with IAA+ABA (2+1 and 2+2 mg/l IAA+ABA), suggesting that there were no interactive effects between these compounds (Fig. 2A).

The rooting response elicited with the *C. sorokiniana* biomass grown in semi-continuous cultures enriched with Na-acetate was significantly higher (equivalent to 1–2 mg/l IBA) compared to the biomass harvest from the batch culture (day 5; equivalent to the water control). The biomass grown in dilute pig manure had slightly lower biostimulatory activity (equivalent to 0.5–1 mg/l IBA; Fig. 2B) compared to the Na-acetate generated biomass.

3.5. Correlation between growth parameters, compounds and rooting activity

There was a significant negative correlation between protein content and root number ($P = 0.032$). There was a significant positive correlation between daily biomass production and oxIAA concentration ($P = 0.025$) and a weak positive correlation between daily biomass production and IAA concentration ($P = 0.058$). There was a weak positive correlation between protein content and SA concentration ($P = 0.81$). There were no other significant or weak correlations between the other eight parameters analysed (Table 5).

4. Discussion

The growth experiment was conducted in late autumn with sub-optimum light and temperature parameters (Table 2). The biomass and daily biomass productivity of *C. sorokiniana* MACC-728 was greatly reduced (<150 mg/l/day) compared to two other *C. sorokiniana* strains (MACC-438 and MACC-452) grown in batch cultures with 20% N Tamiya medium under greenhouse conditions in spring to summer (April-July 2019) where daily productivity was >400 mg/l/ day (Stirk et al., 2021). Light and temperature are important environmental factors affecting microalgae growth with light influencing photosynthesis and temperature modulating enzymatic activity (Borowitzka, 2016). There is generally a decrease in microalgae productivity in autumn and winter when grown in outdoor conditions in temperate climates. The successful cultivation of *C. sorokiniana* in a large range of environmental conditions from sub-optimal low light and temperature (present study) to super-optimal high light and temperatures over 30 ° C (Stirk et al., 2021) under greenhouse conditions indicate that this species can tolerate a wide range of light and temperature conditions and is thus a potential candidate for outdoor cultivation in temperate climates. Similarly, three cold tolerant microalgae strains (*Micractinium reisseri*, *C. vulgaris* and

Table 4
Macromolecule and phytohormone content of *Chlorella sorokiniana* MACC-728 semi-continuous cultures grown in 100 l bioreactors under greenhouse conditions with the addition of Na-acetate or dilute pig manure. Results are presented as means. Different letters within each column indicate significant differences analysed by Duncan's multiple range test ($P < 0.05$).

Treatment		Macromolecules			Phytohormones		
Media replacement	Harvest day	Protein g/100 g DW	Lipid	Carbohydrate	IAA pmol/g DW	oxIAA	SA
Pre-treatment 1% CO_2 + Tamiya medium	5*	55.6^{c}	28.0^{c}	6.2^{b}	144.3^{ab}	13.9^{a}	100.6^{b}
50 g Na-acetate + 5 l tap water	6+7+8	38.8^{a}	14.0^{a}	7.8^{c}	125.5^{a}	20.5^{ab}	64.9^{a}
50 g Na-acetate + 5 l tap water	13+14+15	37.5^{a}	13.9^{a}	20.2^{d}	199.9^{b}	26.7^{b}	55.7^{a}
2 l pig manure + 3 l tap water	6+7+8	53.8^{c}	16.8^{c}	22.1^{e}	900.7^{d}	84.6^{c}	107.0^{b}
2 l pig manure + 3 l tap water	13+14+15	50.0^{b}	39.7^{b}	2.6^{a}	357.7^{c}	110.7^{d}	63.3^{a}

* samples combined from bioreactors 1 and 2

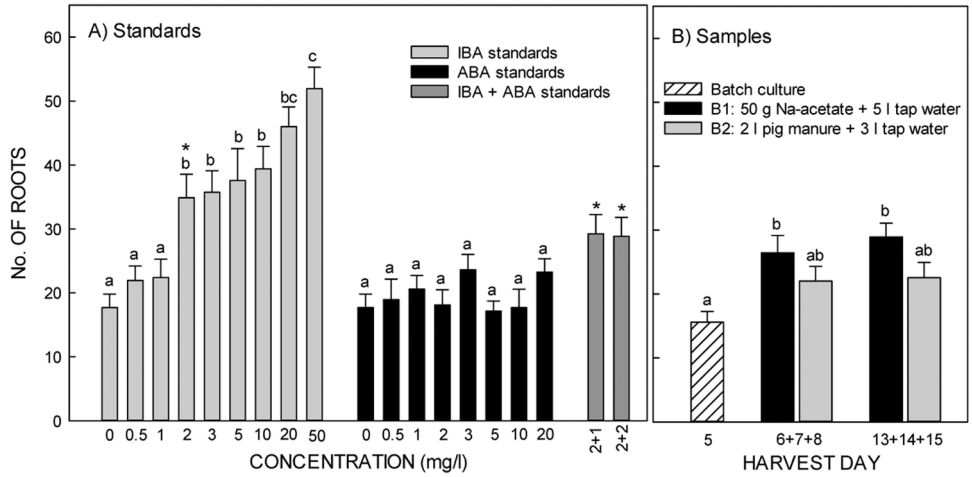

Fig. 2. Plant biostimulating activity of A) phytohormones (IBA and ABA) measured as a rooting response in the mungbean bioassay and B) rooting response of water extracts made from *Chlorella sorokiniana* grown in 100 l bioreactors under greenhouse conditions in November 2020 where the semi-continuous cultures were supplemented with either Na-acetate (Bioreactor 1) or dilute pig manure (Bioreactor 2). Results are presented as mean ± SEM (n = 18). Different letters indicate significant differences analysed by one-way analysis of variance and Duncan's multiple range test ($P < 0.05$). Comparison of the rooting response of IBA+ABA are shown by *.

Table 5
Pearson's correlation matrix representing the interrelationship between variables in *Chlorella sorokiniana* grown in 100 l bioreactors under greenhouse conditions and supplemented with Na-acetate or filtered pig manure. The correlation was followed by a two-sided test of correlations different from zero. Text in bold, italic font highlight significant correlations at the 0.05 probability level ($P < 0.05$; *) and weak correlations ($P > 0.05$; $^{+}$). Number of observations 5.

Variables		Root No.	IAA	oxIAA	SA	Daily Biomass	Protein	Lipid	Carbohydrate
Root No.	r								
	p								
IAA	r	-0.104							
	p	0.868							
oxIAA	r	-0.038	0.658						
	p	0.952	0.918						
SA	r	-0.765	0.572	0.048					
	p	0.132	0.314	0.938					
Daily Biomass	r	-0.059	***0.866***	***0.923***	0.272				
	p	0.925	***0.058***$^{+}$	***0.025****	0.658				
Protein	r	***-0.911***	0.479	0.385	***0.831***	0.460			
	p	***0.032****	0.415	0.522	***0.081***$^{+}$	0.436			
Lipid	r	-0.537	-0.064	0.563	0.008	0.334	0.557		
	p	0.350	0.918	0.324	0.990	0.583	0.329		
Carbohydrate	r	0.402	0.561	-0.051	0.205	0.294	-0.181	-0.724	
	p	0.502	0.325	0.936	0.741	0.632	0.771	0.167	

Scenedesmus rubescens) could tolerate early morning minimum temperatures of 0 °C when grown outdoors (Dahlin et al., 2018). Selection of cold-tolerant strains would improve productivity in sub-optimal conditions and extend the growing season.

The semi-continuous mode of cultivation used in the present study maintained stable culture conditions when either dilute pig manure or Na-acetate was added to the media. The pH remained within the optimum range when either diluted pig manure or Na-acetate were used as a supplement (Table 3). The optimal pH range for most microalgae is between pH 7 and 9 with more acidic or alkaline conditions limiting growth by affecting the solubility and availability of CO_2 and other essential nutrients (Juneja et al., 2013; Mohsenpour et al., 2021). The electrical conductivity of the cultures was also stable (Table 3), indicating a fairly constant total ionic content in the semi-continuous cultures. In contrast, EC values decreased in *Chlorella* sp. and *Scenedesmus* sp. batch cultures grown in dairy wastewater, indicating a reduction in nutrient load over time with EC values negatively correlated to biomass production (Labbé et al., 2017).

The semi-continuous mode of cultivation was a successful strategy for the cultivation of *C. sorokiniana* with a positive daily productivity maintained for the 16 day culture period (Fig. 1). Cell density has an impact on the light penetration in a culture due to self-shading and could be important factor affecting the growth rate in the present study due to the low light levels and short photoperiods in the colder autumn months. The semi-continuous mode of cultivation maintained less dense cultures to enable each *C. sorokiniana* cell to receive a higher average irradiance.

Filtered pig manure (2%) was used as a nutrient source as a potential strategy to reduce production costs. The positive growth rates indicated that *C. sorokiniana* was able to assimilate the nutrients provided by the pig manure. Wastewaters, especially agricultural wastewaters, generally have a high concentration of ammonia/nitrates, phosphates, organic compounds and suspended solids and it is necessary to dilute them to ensure favourable growth conditions (Ge et al., 2017). Additional experiments will need to be conducted in summer conditions with *C. sorokiniana* where growth rates and productivity are higher (thus more rapid nutrient depletion) in order to determine the optimum concentrations of pig manure to maintain positive growth rates in a wider range of environmental conditions. For example, growth rates of *Chlorococcum* sp. increased with increasing concentrations of pig digestate (2–8%) with a higher rate compared to the control grown in

BBM medium (Montero et al., 2018). *C. sorokiniana* was also able to utilize the C provided by Na-acetate as indicated by the positive growth rate and daily biomass productivity although growth was initially (days 6–10) lower compared to the pig manure supplemented cultures (Fig. 1). The initial slower growth rate may be due to the microalgae requiring time to adapt to the alternative organic carbon source.

The growth rate is proportional to the uptake rate of the most limiting nutrient (Juneja et al., 2013). Replacing the media in semi-continuous cultures as was done in the present experiment introduces fresh nutrients into the system (Carneiro et al., 2021; Sánchez-Zurano et al., 2021) and reduces the build-up of toxic compounds by removing and diluting the media (Nur and Buma, 2019). Wastewater may be limited in a specific nutrient and this limitation can be mitigated by supplementation with additional nutrients. In the present study, once weekly supplementation with synthetic Tamiya medium ensured that macro-and micronutrients that were potentially limiting in both Na-acetate and pig manure supplemented cultures were added to the medium to maintain a positive growth rate in *C. sorokiniana* (Fig. 1). Similarly, biomass productivity increased in batch cultures of *C. sorokiniana*, *S. obliquus* and *A. falcatus* when the aquaculture wastewater was supplemented with sodium nitrate (Ansari et al., 2017), growth and macromolecule content of *C. sorokiniana* was enhanced when a N supplement (urea) was added to municipal wastewater (Ramsundar et al., 2017) and *C. pyrenoidosa* produced higher biomass when poultry excreta leachate was combined with BG-11 media (Singh et al., 2023).

The chlorophyll fluorescence variable QY was used as a measure of physiological stress. The QY of green microalgae grown in ideal conditions is between 0.7 and 0.8 F_v/F_m with lower values indicating that the cultures are physiologically stressed (Malapascua et al., 2014). The relatively stable QY values (0.63–0.66 F_v/F_m; Table 3) in the present study indicated that *C. sorokiniana* cultivated in semi-continuous mode with diluted pig manure or Na-acetate supplementation were mildly stressed in the sub-optimal environmental conditions. In comparison, the QY decreased in batch and semi-continuous cultures of *C. vulgaris* grown under greenhouse conditions in undiluted municipal wastewater centrate due to increased shading in the denser cultures (Ranglová et al., 2021); QY decreased in *S. almeriensis* grown in wastewater in raceway ponds exposed to supra-optimal light and temperature conditions due to the high ammonia content of the wastewater and high irradiance levels causing photoinhibition (Sánchez-Zurano et al., 2021); QY values decreased in batch cultures of *C. sorokiniana*, *S. obliquus* and *A. falcatus* when grown in aquaculture wastewater due to low nutrient levels in the aquaculture wastewater (Ansari et al., 2017).

The primary metabolite content of the *C. sorokiniana* biomass was significantly influenced by the cultivation conditions. The semi-continuous cultivation system generated *C. sorokiniana* biomass with a higher protein content compared to the lipid and carbohydrate content. Biomass generated with dilute pig manure had a significantly higher protein and lipid content compared to the Na-acetate and batch cultures (Table 4) which may be due to a lower nutrient content in the mixotrophic and batch cultures. Daily addition of pig manure provided NH_4-N for algal growth which was absent with Na-acetate addition. Nutrient depletion triggers the synthesis of neutral lipids and starch with an inverse relationship to biomass productivity and protein content (Ge et al., 2017). Amino acids and peptides which are part of the protein content, have biostimulatory activity (Navarro-López et al., 2020a). It would therefore be advantageous to produce biomass with a high protein content if it is to be used as a biostimulant.

The phytohormone content in *C. sorokiniana* was also significantly influenced by the culture conditions. The only endogenous phytohormones detected in *C. sorokiniana* were auxins (IAA and oxIAA) and SA where the auuxin content was significantly higher in the biomass generated using dilute pig manure compared to the control and the Na-acetate supplemented cultures and SA content was similar in all cultures. Stress-associated phytohormones such as ABA and jasmonates were not present in detectable limits in the *C. sorokiniana* biomass (Table 4). Similarly, the only phytohormones detected in *C. sorokiniana* grown in BG11 medium under autotrophic and mixotrophic conditions were auxins and GAs with lower concentrations when grown in an outdoor raceway pond compared to laboratory generated cultures (Do et al., 2020). Cyanobacteria biomass (*Anabaena* and 2 *Nostoc* spp) generated in BG medium combined with municipal wastewater had higher auxin and cytokinin concentrations compared to the BG-generated biomass and GA and ABA were not detected (Elakbawy et al., 2021). Actively dividing and faster growing cultures have a higher endogenous auxin content (Stirk et al., 2013; Stirk et al., 2014) and the positive growth rates in the present study may account for the detection of auxin. There was a significant positive correlation between daily biomass production and oxIAA concentration in *C. sorokiniana* and a weak positive correlation between daily biomass production and IAA content (Table 5). Phytohormones levels in microalgae fluctuate in response to stress. For example, auxin and gibberellin content decreased in *C. sorokiniana* subjected to salt stress (Do et al., 2020), brassinosteroids increased in six microalgae subjected to salt stress (Stirk et al., 2018) and cytokinin biosynthesis decreased and ABA biosynthesis increased over time in N-stressed *Nannochloropsis oceanica* (Lu et al., 2014). In the present study, physiological parameters of *C. sorokiniana* such as high protein content and optimum QY values suggest that the cultures were not physiologically stressed and may explain why stress-associated phyothormones were not detected.

Variation in the metabolite composition of the *C. sorokiniana* biomass produced in the different cultures had a significant impact on the biostimulatory activity in the mungbean rooting assay. The Na-acetate generated biomass elicited the highest rooting response equivalent to 2 mg/l IBA. (Fig. 2B). Previous greenhouse grown batch cultures of two *C. sorokiniana* strains grown in spring and summer in 20% N Tamiya media had rooting activity equivalent to 0–0.5 mg/l IBA with activity increasing in older cultures (Stirk et al., 2021). The rooting activity in the present study was not linked to the protein and auxin content that was significantly higher in the pig manure generated biomass compared to the Na-acetate generated biomass (Table 4). This suggests that the biomass contained other compounds with biostimulating activity that still need to be identified. Positive rooting effects have been elicited in this assay with the phlorotannin eckol extracted from the seaweed *Ecklonia maxima* (Rengasamy et al., 2015) and karrikinolide, a smoke-derived butenolide (Jain et al., 2008). *Coccomyxa chodatii* biomass improved the growth of mercury stress wheat seedlings where the biomass generated in N and/or P-limited media was more effective than biomass grown in full-strength media. The increased activity was attributed to a higher auxin, peptide, polysaccharide flavonoid and phenolic content as well as a higher antioxidant capacity of the biomass (Dawood et al., 2023).

Previous studies describing biostimulating activities of microalgae biomass grown in wastewater have variable results. For example, *S. obliquus* produced using brewery wastewater had biostimulatory activity in four bioassays (watercress germination, mungbean rooting, cucumber chlorophyll retention and cotyledon expansion assays; Navarro-López et al., 2020a) and *Scenedesmus* sp. grown in semi-continuous mode with 10% diluted pig manure had a positive effect on watercress seed germination (Navarro-López et al., 2020b). In contrast, only *Chlorella* sp. biomass cultivated in BG-11 media elicited positive effects in the watercress germination assay, the mungbean rooting assay and the wheat chlorophyll retention assay while biomass generated in municipal wastewater had no activity (Ranglová et al., 2021). Co-culturing of *C. vulgaris* and *S. acutus* in outdoor cultures using municipal wastewater produced biomass that had no plant biostimulating activity in a seed germination assay, the mungbean rooting assay and a chlorophyll retention assay (Carneiro et al., 2021). The lack of activity in the municipal wastewater generated biomass extracts was attributed to inhibiting compounds accumulating in the biomass (Carneiro et al., 2021; Ranglová et al., 2021). The current results highlight

that diluted pig manure is a more suitable source of nutrients than synthetic media for the cultivation of *C. sorokiniana* to be used for biostimulant production. Apart from being a cheaper source of nutrients, pig-manure generated *C. sorokiniana* biomass was a more effective biostimulant, eliciting a significantly stronger rooting response in the mungbean bioassay compared to biomass generated in synthetic Tamiya medium. Shifting to mixotrophic growth also enhanced the biostimulatory activity of the *C. sorokiniana* biomass.

The present growth trial was conducted in 100 l non-sterile flat panel bioreactors to obtain results about the properties of *C. sorokiniana* related to its growth and biostimulating activities when grown in non-conventional nutrient medium. The difficulties in mass production are that closed bioreactors are expensive while open ponds are not suitable for monoalgal cultivation. Scaling-up the 100 l closed reactor (3 m long x 3 cm wide x 1 m high) used in this study to 500 l by increasing the length to 6 m and width to 8 cm is possible and may be part of a hybrid culture system. Mass culture of 10 bioreactors of 500 l can produce sufficient inoculum for mass cultivation in an open raceway pond. Maintaining the dominance of *C. sorokiniana* in open systems may be successful by regular inoculation with dense suspension of the stain harvested from closed bioreactors (Van den Berg et al., 2022). Long term biomass production of the strains in large-scale open reactors are scarce although open cultivation of microalgae in wastewater is very promising (Al-Jabri et al., 2021). *C. sorokiniana* shifted its metabolism to mixotrophic growth to adapt to the low light conditions of the present study that was conducted in late autumn, indicating that it should be able to grow in larger cultivation systems where light limitation becomes more of an issue. Additional bioassays, greenhouse and field trials with selected crops need to be conducted to confirm the plant biostimulating effect of biomass generated in the suggested hybrid system.

5. Conclusions

The strategy of semi-continuous cultivation maintained *C. sorokiniana* pilot-scale cultures in an exponential growth phase for up to 16 days when grown in a greenhouse under low light and temperature conditions. The cultures utilized nutrients provided by dilute pig manure, thereby reducing production costs. They could also adjust their metabolism to mixotrophic growth when the media was supplemented with Na-acetate, thereby overcoming low light limitations to maintain a positive daily productivity. *C. vulgaris* adjusted its metabolic pathways to these media amendments as measured by changes in primary metabolite and endogenous phytohormone content. The mixotrophic culture had a significantly lower protein and lipid content compared to batch culture in synthetic media and the pig manure supplemented culture. The pig manure supplemented culture had a significantly higher auxin content (IAA and oxIAA) compared to the batch culture and Na-acetate supplemented culture. SA content was similar regardless of the media and no ABA or jasmonates were detected in any of the cultures. Variation in metabolite composition had a significant effect on the biostimulatory properties with culture amendments enhancing the bioactivity of the biomass. Na-acetate generated biomass elicited the best rooting response in the mungbean assay, followed by the pig manure generated biomass. This activity was not correlated to the protein and IAA content which was higher in the dilute pig manure generated biomass. Thus, addition of either pig manure or Na-acetate improved the quality (and hence value) of the biomass produced for use as a plant biostimulant. The lack of correlation between IAA concentration and biostimulating effect highlights the need to use a number of bioassays to be performed to check bioactivity of the harvested biomass when developing a biostimulant product.

Thus *C. sorokiniana* is a robust strain for biotechnology and has potential to be developed as a plant biostimulant as it can utilize wastewater (pig manure) as an alternative nutrient source to synthetic medium, thereby reducing production costs and can adjust its metabolism to mixotrophic growth as a strategy to compensate for low light conditions. Both these media amendments increased the quality of the biomass with increased plant biostimulatory activity in comparison to the biomass produced in batch culture in synthetic media.

CRediT authorship contribution statement

Rétfalvi Tamás: Investigation. **Novák Ondřej:** Investigation, Resources. **Berzsenyi Zoltán:** Formal analysis. **van Staden Johannes:** Resources, Writing – review & editing. **Maróti Gergely:** Investigation, Funding acquisition, Resources. **Varga Zoltán:** Investigation. **Notterpek Jácint:** Investigation. **Široká Jitka:** Investigation. **Bálint Péter:** Investigation. **Ördög Vince:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Writing – review & editing. **Stirk Wendy:** Investigation, Writing – original draft, Writing – review & editing. **Strnad Miroslav:** Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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