Research

Production of Plant Biostimulants from Microalgae Grown on Biogas Digestate and Tests on Selected Crops

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Abstract:
This study investigates the potential of microalgae-based biostimulants cultivated on the liquid part of anaerobic digestate as a sustainable solution for modern agricultural chal-lenges. The liquid part of anaerobic digestate, a nutrient-rich by-product of biogas pro-duction, is used as a growth medium for the microalgae *Scenedesmus* sp. to illustrate the principles of circular bioeconomy by converting organic waste into valuable agricultural products. The microalgae *Scenedesmus* sp. were cultivated in open raceway ponds, har-vested using sedimentation, microfiltration or centrifugation, and further dried in venti-lated cabinet at 35°C. Laboratory germination tests and field trials were conducted to eval-uate the efficacy of the produced algae as plant biostimulant. In laboratory tests microal-gae-derived biostimulants significantly improved seed germination and root develop-ment of garden cress at all concentrations, Proso millet gave positive response to 1 and 5 g DM/L, however cabbage showed no response. Foliar applications on Proso millet in the field trial further highlighted its potential to improve crop yields stability across the field after but not increased millet grain yield. This research highlights the viability of integrat-ing microalgae cultivation with waste management systems to produce biostimulants that can improve crop production while supporting sustainable agriculture.

Keywords: Circular bioeconomy; Sustainable agriculture; Anaerobic digestate; Nutrient utilization; Microalgae,;Plant biostimulant

1. Introduction

Microalgae-based biostimulants are emerging as innovative tools in agriculture to address critical challenges posed mainly by climate change, such as drought, extreme temperatures, irregular rainfall and soil degradation. These biostimulants are known to promote seed germination, enhance root system development and improve nutrient utilization, thereby increasing plant resilience to environmental stresses (Van Oosten et al., 2017). The biostimulant potential of microalgae is strongly influenced by algae growing conditions, with the availability of nutrients and environmental stresses playing a crucial role in determining their biochemical composition and efficacy (Petkov et al., 2009). A sustainable approach to the production of microalgae-based biostimulants involves the cultivation of microalgae on the liquid fraction of anaerobic digestate, a nutrient-rich by-product of biogas production (Barzee et al., 2022). Biogas plant digestate, derived from the microbial decomposition of organic waste, is an excellent example of the principles of the circular economy, transforming agricultural and industrial waste streams into valuable inputs for crop production. The liquid fraction of the digestate, which is produced after mechanical separation of the solid components, is rich in nitrogen, potassium, calcium, sodium, and trace elements, making it a cost-effective and nutrient-rich medium for the cultivation of microalgae such as *Scenedesmus* sp. (Resman et al., 2021). The use of anaerobic digestate as a growth medium offers several advantages. It supports efficient nutrient utilization by microalgae, which promotes the production of secondary metabolites, including amino acids, polysaccharides and other bioactive compounds that are critical for their biostimulatory properties (Hossain Sani et al., 2024). In addition, this approach reduces reliance on mineral fertilizers, which production is energy-intensive and has negative environmental externalities, further contributing to the sustainability of agricultural practices. Biostimulants from microalgae grown on digestate have been shown to improve plant resilience by promoting seed germination, stimulating root development and increasing plant tolerance to abiotic stress factors such as drought and salinity (Bulgari et al., 2014). Their application also benefits soil health by increasing microbial diversity, which is all in line with the principles of sustainable agriculture and regenerative farming systems (Win et al. 2018; Gonçalves et al., 2023). This study investigates the cultivation of microalgae in growing media containing water solution of anaerobic digestate as a sole source of nutrients and evaluates the effectiveness of the resulting algae biomass as plant biostimulants in laboratory and field trials. By integrating waste management and agricultural innovation, this approach offers a scalable and environmentally friendly solution to improve crop productivity and soil health, thus contributing to global sustainability goals.

2. Material and Methods

The biogas plant digestate used in this study was derived from the anaerobic digestion (AD) of food production residues (KOTO d.o.o.). The process is thermophilic (55-55.5°C) with separate hydrolysis at pH 3.5-4 and digester reactors at pH 7.8-8.0 and thus differs from conventional mesophilic processes. Mixing is continuous. Regulation of pH is only minimally regulated; soda is occasionally added (e.g. $NaHCO_3$, Na_2CO_3) (Lavrič, 2019). The system in this study includes a screw press for digestate separation to liquid and solid part. The solid digestate is transported for drying and the liquid digestate which is rich in essential nutrients such as nitrogen, phosphorus, potassium and trace elements can be used for microalgae production. For the growth of the green microalgae *Scenedesmus* sp. (dominant *Scenedesmus quadricauda*) a liquid part of the separated digestate was used as a source of nutrients (Table 1). The addition of digestate is regulated based on the basis of the limit values of the indicative parameters defined in previous experiments: pH 6-8, electrical conductivity EC 1000-2000 μS cm^{-1}, NH_4-N 50-150 mg L^{-1}, NO_3-N 5-50 mg L−1, NO_2-N <1 mg L^{-1}, optical density OD680 from 1 to 1.5 (Resman et al., 2023). The input of digestate is about 4 to 10 L per 1000 L of growing medium per day, depending on the cultivated microalgae and intended product. The nutrient content of the digestate and the produced microalgae biomass was analyzed, including parameters such as electrical conductivity (EC), dry matter (DM), organic carbon (Corg), nitrogen (N), phosphorus (P), potassium (K), and the C:N ratio. Additionally, the levels of micronutrients and potentially toxic elements in microalgae grown on digestate (Pb, Cd, Zn, Cr, Cu, Ni, Hg, As) were measured and compared to regulatory limits.

The microalgae cultivation on digestate took place in an unheated greenhouse with a double PE foil roof at the Center for Algal Technologies of the Biotechnical Faculty, University of Ljubljana. The microalgae were grown in open race-way ponds, which were heated only at the bottom to prevent freezing in the winter. The effective production period was approximately 240-280 days per year. The average daily biomass production was 11±1.5 g/m² at temperature between 10 and 30 °C under optimal conditions, from March to October. The microalgae were harvested using sedimentation (for 24 hours) and later filtration of the sedentary part with a vibro-microfiltration unit (Vibro-Lab 3500, SANI Membranes A/S, DK) to obtain ca. 7.5 % DM and/or centrifugation (rpm 12 000; max. rcf 27 500; T = 15°C; 10 min; V = 250 mL) to achieve a dry matter content of approximately 15 %. The obtained algae paste could be used fresh or dried at 35 °C and ground into a fine powder. Obtained powder was diluted with water to a concentration suitable for application as plant biostimulant. The biostimulants were tested for their effectiveness in promoting seed germination, root development and crop yield. Germination tests (Figure 1) were carried out in in-vitro tests with Proso millet, garden cress and cabbage seeds, with treatments including different concentrations of the biostimulant (1 g DM/L, 5 g DM/L, 10 g DM/L, 25 g DM/L, 50 g DM/L) and deionised water as control. The second germination tests were repeated with Proso millet treated with microalgae *Scenedesmus* sp. biostimulant at different concentrations (1 g DM/L, 5 g DM/L, 50 g DM/L) and with growth medium after *Scenedesmus* sp. cultivation (V-BF). Again, water was used as a control. The average root length and germination index were measured and analyzed using the ImageJ software.

$$GI\ [\%] = \frac{(\text{no. germinated seeds} * \text{root lenght})}{(\text{no. germinates seeds in control} * \text{root lenght in control})}\ \text{x}\ \ 100$$

Figure 1. Visual example of germination test (left) and evaluation of roots length with ImageJ app (right)

The field trials were conducted at the Biotechnical Faculty in Ljubljana, where Proso millet (*Panicum miliaceum*, variety 'Sonček') was sown (on June 20, 2024), 125 kg seeds/ha. The soil is eutric-brown, pseudo-gleyic, texture sandy-clay-loam to clay-loam, with a pH of about 7. In 2024, a basic fertilization with 138 kg N/ha NPK 15-15-15 was applied, followed by 75 kg N/ha KAN 27% in the BBCH 16 phase. Four treatments were studied: control (untreated seeds and no foliar application; NS-NF), biostimulant applied to seeds (AS-NF), biostimulant applied foliarly (NS-AF), twice during the growing season (first in the development phase of BBCH 59; on August 7, 2024, and the second time in the phase of BBCH 69; on August 9, 2024) (Ventura et al., 2020), and biostimulant applied to seeds and foliarly (AS-FA), also twice during growing season. The concentration of microalgae biostimulant for the seed application was 0,5 kg/ha and for the foliar application a concentration of 1 g microalgae DM preparation/L of water (for each application a dose of 300 L/ha). The effectiveness of the biostimulation was evaluated by measuring root depth (at BBCH 13; July 3, 2024), fresh biomass, air-dry biomass and yield. Plants were harvested (on September 4, 2024) within a 1 m x 1 m frame in three replicates for each treatment for the purposes of crop analysis. We weighed the produced biomass and grain (drying of the plants was carried out in a ventilation drying cabinet at 40 °C to a constant mass). The data obtained from the germination tests and field trials were statistically analyzed using the R statistical language (R Commander).

3. Results

The liquid part of the digestate from the KOTO biogas plant had an electrical conductivity (EC) of 14.9 mS/cm and a dry matter (DM) content of 0.74%. The phosphorus (P) content was 0.53 g/kg DM and the potassium (K) content was 6.70 g/kg DM, which are crucial for the optimal growth of microalgae like *Scenedesmus* sp. The high organic carbon (Corg) content and low Corg:N ratio further support the efficient nutrient utilization by the microalgae (Table 1). The produced microalgae biomass of *Scenedesmus* sp. grown on the liquid digestate had a Corg content of 45.0 % DM, an N content of 7.5 % DM and a C:N ratio of 6.0. The nutrient profile of the microalgae biomass showed higher levels of organic carbon and phosphorus compared to the digestate, reflecting the microalgae's ability to concentrate these nutrients (**Table 1**).

Table 1. Electrical conductivity (EC), dry matter (DM) in digestate, N, P, K and C:N value in the used liquid part digestate (KOTO) and in produced microalgae biomass *Scenedesmus* sp. (Centre of Algae Technologies, Biotechnical Faculty) grown on the liquid part of the digestate.

		Liquid part of digestate (KOTO)	*Scenedesmus* sp. (DM)
EC	[mS/cm]	14.9	/
DM	[%]	0.74	/
Corg	[% DM]	34.7	45.0
N	[% DM]	17.8	7.5
Corg:N		2.0	6.0
P	[g/kg DM]	0.53	13.0
K	[g/kg DM]	6.70	5.70

The content of potentially toxic elements in the digestate (Fuentes et al., 2004) and in the produced microalgae biomass were measured and compared with the legal limits. The levels of elements such as lead, cadmium, zinc, chromium, copper, nickel, mercury, and arsenic were all below the permissible thresholds, ensuring the safety of the biostimulants for agricultural use. Notably, the microalgae biomass had lower concentrations of these elements compared to the digestate, suggesting that the microalgae can effectively mitigate the presence of potentially harmful substances. All values of the potentially toxic elements in the digestate and in the cultivated microalgae were within the legal limits (Regulation (EU) 2019/1009) for plant biostimulants (**Table 2**).

Table 2. Values of potentially toxic elements according to Regulation (EU) 2019/1009 in the biogas plant digestate (KOTO) and produced microalgae biomass *Scenedesmus* sp. (Centre of Algae Technologies, Biotechnical Faculty) grown on the liquid part of the digestate.

		Liquid part of digestate (KOTO)	Limit values for plant biostimulant (DM)	*Scenedesmus* sp. grown on diluted digestate (DM)
Pb	[mg/kg DM]	9.20	120	3.43
Cd	[mg/kg DM]	0.70	1.5	0.13
Zn	[mg/kg DM]	277	1500	114
Cr	[mg/kg DM]	29.0	/	3.30
Cu	[mg/kg DM]	47.2	600	20.5
Ni	[mg/kg DM]	8.10	50	1.90
Hg	[mg/kg DM]	0.05	1	0.009
As	[mg/kg DM]	<0.5	40	0.50

The roots of germinated plants were on average longer with biostimulant concentration up to 5 g DM/L. Higher biostimulant concentrations had inhibitory effect. Optimal concentration depended on the plant species itself. For millet concentration of 50 g DM/L had phytotoxic effect (**Figure 2**).

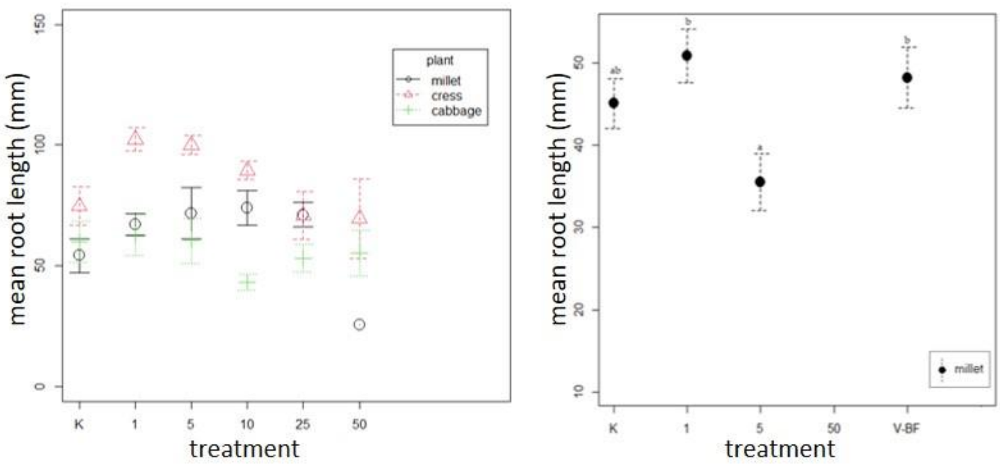

Figure 2. 1st germination tests (left figure): average **root length** of germinated plants (millet, cress and cabbage) at different treatments (1 g DM/L (**1**), 5 g DM/L (**5**), 10 g DM/L (**10**), 25 g DM/L (**25**), 50 g DM/L (**50**), control (**K**)) with standard error and 2nd germination tests (right figure): average root length of germinated Proso millet plants at different treatments (1 g DM/L (**1**), 5 g DM/L (**5**), 50 g DM/L (**50**), growth medium from *Scenedesmus* sp. (**V-BF**), control (**K**)) with standard error**.**

Additional germination tests were conducted on Proso millet seeds with different treatments of microalgae biostimulant including growth medium from *Scenedesmus* sp. This additional germination test on millet indicated that the 1 g DM/L treatment was significantly better than 5 g DM/L ($p = 0.00553$), with the best germination index (GI) of 113% (**Table 3**). Higher concentrations showed a decrease in germination and root development, indicating an optimal concentration for biostimulant efficacy at 1 g DM/L (**Table 3**).

Table 3. Average root length and germination index (**GI**) of Proso millet plants at different treatments (1 g DM/L (**1**), 5 g DM/L (**5**), 50 g DM/L (**50**), growth medium from *Scenedesmus* sp. (**V-BF**), control (**K**)) with standard error.

Treatment	Average germination [%]	Average root length [mm]	GI [%]
K	86.1	45.13	100
1	86.1	51.11	113
5	75.0	31.38	61
50	0.0	0	0
V-BF	86.1	48.90	108

Field trials were carried out with Proso millet (*Panicum miliaceum*) to evaluate the effectiveness of the biostimulant in real-world conditions. The results of biostimulant application on seeds at 0.5 kg DM/ha showed no improvement in root length at BBCH 13 (**Figure 3**). The highest plant dry biomass (dried at 35°C in a ventilated drying rack) measured at harvest was observed at foliar application (NS-FA, AS-FA) with 9.5 t/ha (the lowest at AS-NA 8.2 t/ha).

The highest average yield of dry millet grain was observed in the control (3780 kg/ha), but it showed the greatest yield variability within treatment. The lowest yield variability between repetitions was observed in the foliar application (3549 kg/ha), followed by the combined treatment with seed and foliar application (AS-FA) (3671 kg/ha). The treatment in which the biostimulant was only applied to the seed had the lowest yield (3071 kg/ha). The results indicate that the application of biostimulants can increase yield the stability across the field when applied on leaves (foliar), although the differences were not statistically significant.

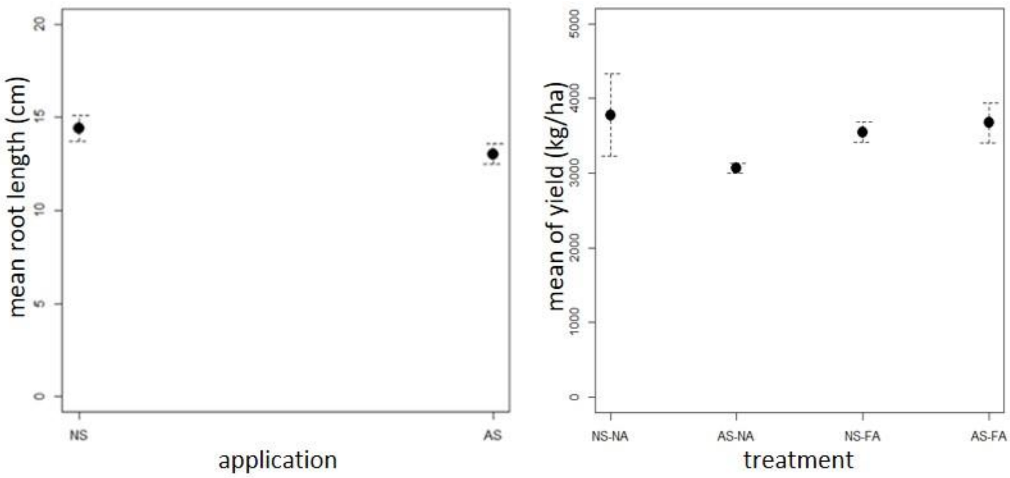

Figure 3. Field trials (Figure left): root depth [cm] of Proso millet plants in the BBCH 13 stage in untreated seed (**NS**) and in the case of seed treated with a microalgae biostimulant (**AS**) with standard error, and (Figure right): yield of dry millet grain [kg/ha] at treatments without microalgae biostimulant application (**NS-NA**), the application of a biostimulant on seed (**AS-NA**), only foliar application of the biostimulant (**NS-FA**) and the application of a biostimulant to seed and foliar (**AS-FA**) with a standard error**.**

4. Discussion

The results of this study demonstrate the potential of using anaerobic digestate as a growth medium for the cultivation of microalgae and the subsequent production of effective plant biostimulants. The nutrient analysis of the digestate and the produced microalgae biomass shows that the liquid fraction of the digestate provides a nutrient-rich environment that promotes the growth of microalgae *Scenedesmus* sp. The content of potentially toxic elements in the digestate and the produced microalgae biomass is within legal limits, ensuring the safety of the biostimulants for agricultural use. This is a crucial aspect for the widespread introduction of microalgae-based biostimulants, as it minimizes concerns about the accumulation of harmful substances in soil and plants.

The germination tests showed that microalgae-based biostimulants significantly improve seed germination and root development only at the optimal concentration which was found to be 1 g DM/L for Proso millet, cabbage and garden cress. Garden cress was the most responsive test plant, but Proso millet showed the highest susceptibility; the highest germination index and average root length for millet concentrations was at 1 g DM/L. Higher concentrations resulted in a decrease in germination and root development, suggesting that there is a threshold beyond which the biostimulant effect diminishes. This result is consistent with previous studies that have determined similar optimal concentrations for biostimulant applications (Oancea et al., 2013; Alling et al., 2023; Chovanček et al., 2023). The field trials also confirmed the effectiveness of microalgae-based biostimulants in promoting plant growth and development under real-world conditions. The biostimulant treatments did not improve yield in Proso millet, although the trend towards higher yields in treated plants with foliarly applied biostimulant suggests potential benefits of biostimulant application, so the experiments should be repeated. Current challenges and opportunities in biostimulant research emphasize the need for standardized testing protocols (Roopashree et al., 2024). Future research should focus on optimizing the cultivation conditions for microalgae in anaerobic digestate to maximize the production and efficacy of biostimulants. In addition, long-term field studies are needed to assess the different environmental conditions and stressors (Khalid et al., 2024). Exploring the potential of different microalgae species and their specific biostimulant properties could further enhance the versatility and effectiveness of this sustainable agricultural practice.

5. Conclusions

In conclusion, this study highlights the potential of microalgae-based biostimulants cultivated on anaerobic digestate as a sustainable and effective solution to address the growing agricultural challenges posed by climate change and environmental protection. The reduction in reliance on chemical fertilizers combined with the transformation of waste into agricultural resources aligns with global sustainability goals and highlights the potential of this technology to support more resilient food systems. The use of the liquid fraction of digestate, a nutrient-rich by-product of anaerobic digestion, provides an efficient and cost-effective medium for the cultivation of microalgae, for species such as *Scenedesmus* sp. The results of this study show that the digestate supports microalgae growth and enables the production of biostimulants that significantly improve germination and root development of different plants. Yield increases in the field trial with Proso millet were not statistically significant, however the stability of yield across the field was improved.

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Conflicts of Interest: The authors declare no conflict of interest.

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