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Overview of microalgae and cyanobacteria-based biostimulants produced from wastewater and CO_2 streams towards sustainable agriculture: A review

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ABSTRACT

For a long time, marine macroalgae (seaweeds) have been used to produce commercial biostimulants in order to ensure both productivity and quality of agricultural crops under abiotic stress. With similar biological properties, microalgae have slowly attracted the scientific community and the biostimulant industry, in particular because of their ability to be cultivated on non-arable lands with high biomass productivity all year long. Moreover, the recent strategies of culturing these photosynthetic microorganisms using wastewater and CO_2 opens the possibility to produce large quantity of biomass at moderate costs while integrating local and circular economy approaches. This paper aims to provide a state of the art review on the development of microalgae and cyanobacteria based biostimulants, focusing on the different cultivation, extraction and application techniques available in the literature. Emphasis will be placed on microalgae and cyanobacteria cultivation using liquid and gaseous effluents as well as emerging green-extraction approaches, taking in consideration the actual European regulatory framework.

1. Introduction

One of the direct consequences of significant world population growth (EIT Food, 2021) is the need of augmentation (double) the yield of agricultural crops and their quality (Kumar et al., 2019). Nevertheless, the rapid increase in global temperatures, caused by human-induced climate change (IPCC, 2019) and consequent massive drought in the extratropical region of the northern hemisphere (Copenhagen Center for Disaster Research, 2022) as well as the lack of water, are currently affecting the agriculture, the production of crops and livestock products for the feed supply chain. For decades, synthetic fertilizers have been widely used to improve the production and quality of food crops, but their over usage directly contributes to water, soil and air pollution (Kumar et al., 2019). Moreover, these environmentally-hazardous consequences do not only occur at the time of their application, but start from their production (Wu et al., 2021), where toxic chemical gases (NH_4^+, CO_2, CH_4 etc.) are released, by directly polluting the air (Kumar et al., 2019). Even if these fertilizers are applied under ideal conditions, plants only assimilate up to 50% of the initial quantity. Regarding the other half, from 2% to 20% volatilises, 15–25% reacts with organic compounds in the soil, and the rest is interfering with the surface area around the plant (Shaviv and Mikkelsen, 1993).

As far as water pollution is concerned, large amounts of nitrogen (N) applied to crops can reach the groundwater causing eutrophication. Water eutrophication can result in the extinction of aquatic life, the spread of invasive species, and a loss of recreational opportunities owing to foul odours and dirty water (Kumar et al., 2019). Regarding the soil pollution, the overuse of synthetic fertilizers can cause soil acidification and soil crust, decreasing the amount of organic matter, humus, and beneficial organisms in the soil and stunts plant growth (Bisht et al., 2020). It can also change the pH of the soil, increase pests, and even cause the release of greenhouse gases (GHGs) through the action of the microorganisms in the soil. Once applied to soil, microbial denitrification and nitrification processes are responsible for the production of a large quantity of nitrous oxide (N_2O), one of the most important GHGs

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(Yoon et al., 2019). Severe air pollution is caused by nitrogen fertilizer, which excessive usage emits nitrogen oxides (NO, N_2O, and NO_2) (Chai et al., 2019). In addition, after being deposited in the atmosphere and oxidized to produce nitric acid and sulphuric acids, ammonia that has been volatilized or released from fertilized land becomes acid rain (Shaviv and Mikkelsen, 1993). Moreover, during the production of nitrogenous fertilizer, GHGs including CO_2, CH_4 and N_2O are produced and consequently, polluting the air.

In this context, and according to the United Nations statements in the context of the European Green Deal (United Nations, 2023), it is vital to move towards a climate-smart agriculture (CSA) system by enhancing the yield and growth of crops, as well as their resistance to different stresses such as lack of water (EIT Food, 2021). In the same way, the Food and Agriculture Organization (FAO) put in place a Strategic Framework 2022–2031 in order to improve and protect agrifood systems according to four main pillars (reffers as "Four Betters"): "better production, better nutrition, better environment and better life for all, leaving no one behind" (FAO, 2023).

In the present review, the state of the art on microalgae and cyanobacteria-based biostimulants as one innovative and sustainable alternative for climate-smart agriculture (CSA) system is presented and discussed.

Biostimulants are defined by the European Biostimulant Industry Council (EBIC) as “a substance or microorganism that, when applied to seeds, plants, or on the rhizosphere, stimulates natural processes to enhance or benefit nutrient uptake, nutrient use efficiency, tolerance to abiotic stress, or crop quality and yield” (EBIC, 2019). The current European Union (EU) regulation differentiates between microbial plant biostimulants (CFP6-A) and non-microbial plant biostimulants (CFP6-B). In particular, biostimulants from microalgae extracts refer to the second group, CFP6-B, containing elements from the component material category 2 (CMC 2 – plants, parts of plants or vegetal extracts). Non-microbial plant biostimulants comprise macroalgae extracts, humic substances and protein hydrolysates (Kapoore et al., 2021). It is important to highlight that optimum plant biostimulants should be not only long-lasting and effective, but also made from bioactive molecules capable of promoting the closing of nutrient loops in agriculture (Ajeng et al., 2022).

As highlighted by Ricci et al., (Ricci et al., 2019) the term “biostimulant” refers to the function but not to the chemical nature of the molecule. Thus, biostimulants, biocontrol agents and biofertilizers shoud be carefully dissociated (du Jardin, 2015). Biocontrol agents for plant diseases are often fungal or bacterial strains that play a vital role in suppressing plant-pathogenic organisms (Thambugala et al., 2020). Biofertilizers can contain living microorganisms that colonize the rhizosphere or plant interior when applied to the seed, plant surface, or soil, improving growth, yield, and improving the supply or availability of primary nutrients to the host plant (Adoko et al., 2021). While biofertilizers are applied in higher doses, biostimulants are applied in low concentrations. The idea is to optimise the minimum amount of fertilizers used thanks to the potentiating effect of biostimulants.

Algae have the capacity to produce bioactive molecules with biostimulant potential for plants as phytohormones, oligosaccharides and phenolic compounds (Dmytryk and Chojnacka, 2018). Seaweeds have been used for a long time in the biostimulant industry (Nanda et al., 2022), with some commercially available products such as Phyter®, Kelpak®, Seamac®, and Plagron®. Even though, producing and extracting these molecules from microalgae and cyanobacteria present certain advantages as the control of the environment in which the microalgae grown, so it is not limited by seasonal variations (Boukhari et al., 2020), and avoiding potential water contamination (Martini et al., 2021). Also, microalgae are easier to cultivate and present high photosynthetic conversion efficiency and fast grow on land that would otherwise be unsuitable for other types of food crops (Kapoore et al., 2021; Navarro-López et al., 2020a; Ferreira Carraro et al., 2022).

Apart from the previous advantages microalgae and cyanobacteria can play an essential role in the sustainability and circular bioeconomy agenda (Ajeng et al., 2022), as they are mostly photosynthetic microorganisms – thus capable of sequestering CO_2 (Xu et al., 2023) and they present great ability to grow in environments that may be hostile to other type of life as waste effluents, removing its nutrients (Abdelfattah et al., 2023; Calicioglu and Demirer, 2022) while biosynthesising high-value molecules (bioactive molecules), as phycobiliproteins, exopolysaccharides, proteins, vitamins, polyphenols or polyunsaturated fatty acids (PUFAs) (Levasseur et al., 2020), presenting bioactive and biodegradable nature (Tan et al., 2021; Ajeng et al., 2022)).

In particular, and in view of the need for a more efficient sustainable agriculture, these bioactive molecules can have biostimulant effect for a sustainable agriculture as presented in Fig. 1 and in more detail in Section 3 (Table 1) of this review.

The production of different types of biostimulants from microalgae and cyanobacteria grown in wastewaters and adding CO_2 will be discussed closing the loop in a circular economy strategy, in agreement with the principles advocated in the Farm to Fork Strategy stated by the European Commission, making food systems fair, healthy and environmentally-friendly (European Comission, 2020) and taking into account the strictness of the difference between biostimulants and biofertilizers. The most commonly used green-extraction techniques and other novel approaches for a green-extraction will also be discussed, as well as the application of biostimulants directly on plants and how they influence different types of plant response to various stresses. Finally, the actual biostimulants market and regulation will be described. Microalgae and cyanobacteria are altogether abbreviated in this review as microalgae.

2. Biostimulants from microalgae and cyanobacteria

2.1. Molecules of interests

Microalgae are today widely used in agriculture mainly because they contain many interesting bioactive molecules for crops, including those with biostimulant effects as phytohormones (Alvarez et al., 2021), polysaccharides or phenolic compounds (Gonçalves, 2021). These molecules are interesting because in some cases, as for phytohormones, they are structurally identical in microalgae and higher plants, and they are biosynthesized using relatively conserved biosynthetic pathways (Lu and Xu, 2015). The use of these microorganisms in agriculture is gaining more focus nowadays, and new products are currently emerging in the agriculture market (Fernández et al., 2021). For instance, *Scenedesmus* or *Chlorella* microalgae species are well known for their ability to produce phytohormones suitable for various crops (Lu and Xu, 2015).

The cultivation of these microalgae for biostimulants has been considered as costly. Nevertheless, it is possible to reduce the cost of the culture medium while contributing to circular economy and the reduction of GHGs. This can be possible by using waste effluents as nutrients source for microalgae (Carneiro et al., 2021a; Ferreira et al., 2021; Navarro-López et al., 2020a; Ranglová et al., 2021; Viegas et al., 2021; Zapata et al., 2021). These questions regarding the use of wastewater and flue CO_2 will be discussed in detail in Section 3.

Different types of molecules can be extracted from microalgae and cyanobacteria cells and referred as biostimulants: (i) phytohormones (e.g., auxin, abscisic acid (ABA), cytokinin, ethylene, gibberellins (Lu and Xu, 2015) and jasmonic acid (Li et al., 2022; Zapata et al., 2021); (ii) polysaccharides and exopolysaccharides (EPS) mainly heteropolysaccharides formed of xylose, glucose and galactose (López-Sánchez et al., 2022; Morais et al., 2022); (iii) proteins hydrolysates and amino acids (Rupawalla et al., 2022); (iv) humic acid-like substances (Behera et al., 2021); and (v) vitamins (Gitau et al., 2022). Microalgae and cyanobacteria growth curves have generally six phases when referring to a batch culture: (i) lag phase, where they adapt to the new medium; (ii) exponential phase in which they grow and reproduce themselves, they are not limited by the quantity of nutrients or light intensity; (iii) linear

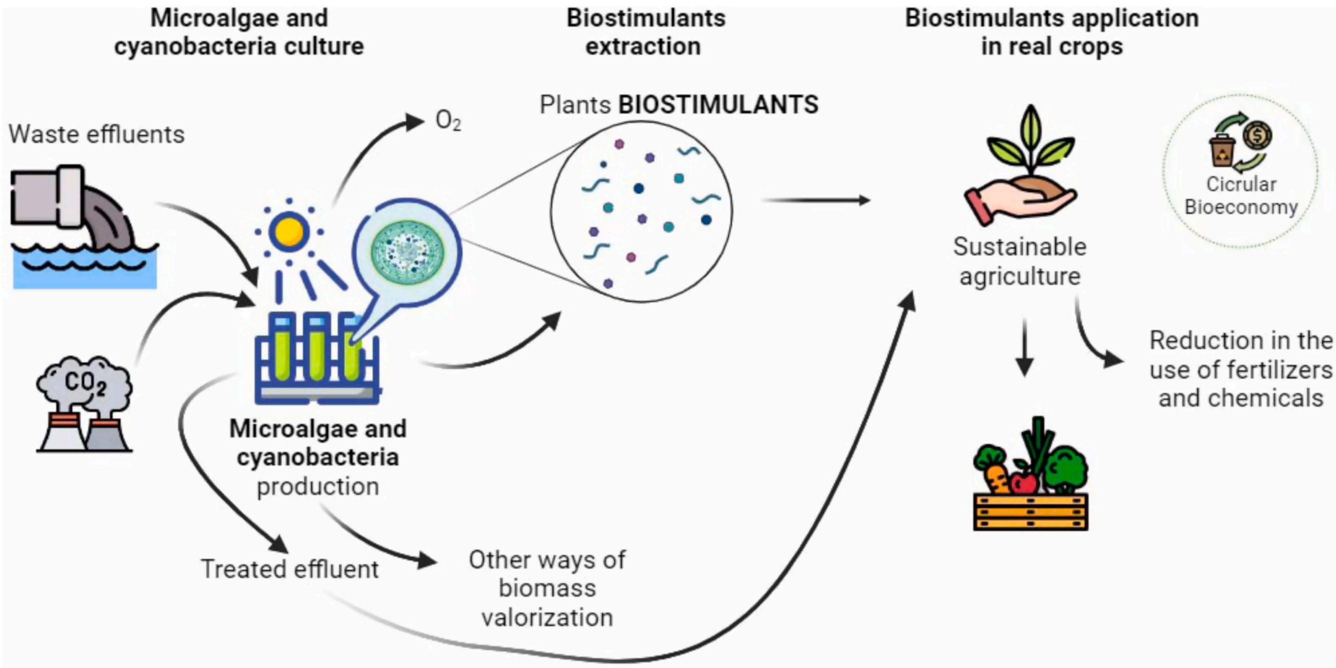

Fig. 1. Circular economy principles of the production, extraction and utilization of microalgae- and cyanobacteria-based biostimulants produced from waste streams and flue CO_2 for a sustainable agriculture.

phase, here microalgae and cyanobacteria biomass accumulates; (iv) declining growth phase, where a reduction of cell division is observed; (v) stationary phase comes when growth rate arrives to zero; and (vi) death phase, at this phase biomass concentration declines very rapidly (Lee et al., 2015). Interestingly, these bioactive molecules are produced in different phases of the microalgae and cyanobacteria growth curve. For instance, in *Chlorella vulgaris* and *Arthrospira platensis* the auxin IAA (Indole-3-acetic acid) is produced in stationary phase nevertheless, total cytokinins are higher in early exponential phase (Tan et al., 2021).

2.2. Biostimulants effects on plants

Different experimental researches have shown the direct effect of certain biostimulants from microalgae on plant germination, growth, production yield and defence (Fig. 2).

Rupawalla et al. (2022) determined the effectiveness of phytohormones extracted from the microalgae *Chlorococcum* sp., such as the cytokinin Zeatin or gibberellins (GA_{20} and GA_{29}) on *Spinacia oleracea* germination by measuring the germination and green cotyledon improvement indexes. As reported by Khan et al., cytokinins increase cell division and seed germination (Khan, 1971) through the activation of mitotic cell division (Yang et al., 2021) and the deletion of inhibitors present in seeds (Sharma et al., 2022), respectively. In another work, the application of *Arthrospira* sp. and *Scenedesmus* sp. microalgae hydrolysates with high content of phytohormones was studied on *Petunia x hybrida* plant (Plaza et al., 2018). It has been shown that after foliar application of these extracts accelerated plant growth, leading to a reduced time of flowering and a higher rates of root, leaf and shoot development. Mazepa et al. (2021) demonstrated that *Desmodesmus subspicatus* (Chodat) microalgae extract applied to tomato plant (*Solanum lycopersicum*) increased the length of its root and improved its germination. They characterized between the biostimulants glycosides and zeatin (cytokinin). The co-authors also verified that different concentrations of the extract (0.5, 1.0, 1.5 and 2.0 $g.L^{-1}$) have different effects on the plant phenotype, being 1.5 $g.L^{-1}$ the most significant concentration for increasing tomato hypocotyl volume, since the concentration at which biostimulants (such as phytohormones or polysaccharides) are applied can be decisive in carrying out their function (Davies, 1995; Rachidi et al., 2020; Tan et al., 2021), since once reached a threshold dose, it can reduce its effect, as can be seen in the graphs of several researches (K. Supraja et al., 2020; Mazepa et al., 2021). In 2020, Rachidi et al. (2020) concluded that polysaccharide extracts of *Arthrospira platensis*, *Dunaliella salina* and *Porphyridium* sp., acted as biostimulants in tomato plant (*Solanum lycopersicum*) with positive effects on morphological parameters such as shoot size and root dry weight. The authors also highlighted that the polysaccharide extract of *Porphorydium* sp., at a dosage of 1 $mg.mL^{-1}$, had the greatest effect on biochemical and enzymatic parameters of the plant. Furthermore, it is important to note that the effect of each phytohormone does not only depend on its dose, but also on the concentration of the other phytohormones. This is due to hormonal crosstalk (Khan et al., 2020), i.e., the interaction between the nature of the phytohormones and their ratios, which have direct phenotypic effects on the plant.

To verify if biostimulants play a role in plant's defence, different trials should be carried out under stress conditions for the plant. Some of these stresses can be: nutrient deficit or excess of pollutants, high salinity or lack of water. The last one is of particular importance in the current climatic context, as explained above.

2.2.1. Effect under salt stress

Salinity stress negatively affects plant growth through chlorophyll degradation and low osmotic potential, leading to stomata closure, which reduces CO_2 fixation, increases both photorespiration and production of reactive oxygen species (ROS) (Mutale-joan et al., 2021). In this context, different studies have been carried out under salinity stress in order to improve plants tolerance and productivity. For instance, Abd El-Baky et al. (2010) demonstrated an improvement of the antioxidant availability and the protein content in wheat (*Triticum aestivum*) grains harvested from plants previously cultivated under seawater stress with microalgae water extracts. Experimental results indicated that *Chlorella ellipsoida* and *Spirulina maxima* microalgae extracts improved the overall plant salinity tolerance as compared to standard plant growth enhancers (ascorbic acid and benzyladenine). Also, Guzmán-Murillo et al. (2013) concluded that the negative effects of salt stress on the red pepper (*Capsicum annuum* L.) were reduced by adding polysaccharide extracts from microalgae *Dunaliella salina* and *Phneodnctylum tricornutum* to the

Table 1
State of the art of microalgae and cyanobacteria strains grown in wastewater for the production of different biostimulants. Plants varieties on which they were applied and the resulted effect on them.

	Microalgae or cyanobacteria culture				Biostimulants	Effect of biostimulants on plants development			
Type	Specie	Wastewater type and treatment	CO2 addition in the culture	Biomass productivity	Characterized biostimulant	Plant varieties tested	Method and concentration of biostimulants application	Caused effect on plants	References
Microalgae	*Tetradesmus obliquus*	Piggery wastewater Phases separation + dilutions (df 1, 2, 5, 10 and 20)	-	31.6 $mg.L^{-1}.d^{-1}$	Not specified	Cucumber, barley, wheat, soybean, watercress and tomato	In seeds germination (0.5 $g.L^{-1}$)	Increased germination Root elongation	(Ferreira et al., 2021)
	Chlorella protothecoides	Piggery wastewater Phases separation + dilutions (df 1, 2, 5, 10 and 20)	-	36.8 ± 7.9 $mg.L^{-1}.d^{-1}$	32.7% carbohydrate	Cucumber, barley, wheat, soybean watercress	In seeds germination (0.5 $g.L^{-1}$)	Increased germination Root elongation	(Ferreira et al., 2021)
	Chlorella vulgaris	Piggery wastewater Phases separation + dilutions (df 1, 2, 5, 10 and 20)	-	22.4 ± 3.9 $mg.L^{-1}.d^{-1}$	Not specified	Cucumber, wheat, soybean, watercress and tomato	In seeds germination (0.5 $g.L^{-1}$)	Increased germination Root elongation	(Ferreira et al., 2021)
	Chlorella vulgaris	Poultry wastewater pretreated with biomass ash	-	Batch: 76.2 $mg.L^{-1}.d^{-1}$ Semi-continuous: 194 mg $mg.L^{-1}.d^{-1}$	Not specified	Wheat and watercress	In seeds germination different concentrations	At 0.2 $g.L^{-1}$ improved germination	(Viegas et al., 2021)
	Tetradesmus obliquus	Poultry wastewaterpretreated with biomass ash	-	Batch: 94.9 $mg.L^{-1}.d^{-1}$ Semi-continuous: 245 $mg.L^{-1}.d^{-1}$	Not specified	Wheat and watercress	In seeds germination different concentrations	At 0.2 $g.L^{-1}$ little improvement of germination	(Viegas et al., 2021)
	Chlorella vulgaris	Municipal wastewater Centrifugation	CO_2 addition for maintaining pH (8.0 ± 0.2)	3.1 and 2.4 g $DW.L^{-1}$	Auxin-like activity: not observed Cytokinin-like activity: not observed	Watercress	In seeds (0.5 and 2 mg $DW.mL^{-1}$)	Not studied	(Ranglová et al., 2021)
	Chlorella vulgaris	Municipal wastewater Centrifugation	CO_2 addition for maintaining pH (8.0 ± 0.2)	-	-	Wheat	Seed application (10 g $DW.L^{-1}$)	No effect	(Carneiro et al., 2021a)
	Scenedesmus acutus	Municipal wastewater Centrifugation	CO_2 addition for maintaining pH (8.0 ± 0.2)	-	-	Wheat	Seed application (10 g $DW.L^{-1}$)	No effect	(Carneiro et al., 2021b)
Cyanobacteria	*Spirulina platensis*	Dairy wastewater Filtration, nutrient supply and pH adjustement	-	-	Auxins: IAA, PAA Salicylic acid Jasmonic acid Giberelins: GA1, GA4	-	-	-	(Zapata et al., 2021)
	Spirulina platensis	Cheese whey wastewater Filtration, nutrient supply and pH adjustement	-	-	Auxins: IAA Salicylic acid 1-Aminocyclopropane-1-carboxylic acid Abscisic acid Giberelins: GA4	-	-	-	(Zapata et al., 2021)
	Synechoscistis sp.	Piggery wastewater Phases separation + dilutions (df 1, 2, 5, 10 and 20)	-	23.7 ± 2.6 $mg.L^{-1}.d^{-1}$	47.3% proteins	Cucumber, barley, wheat, soybean and watercress	In seeds germination (0.5 $g.L^{-1}$)	Increased germination Root elongation	(Ferreira et al., 2021)

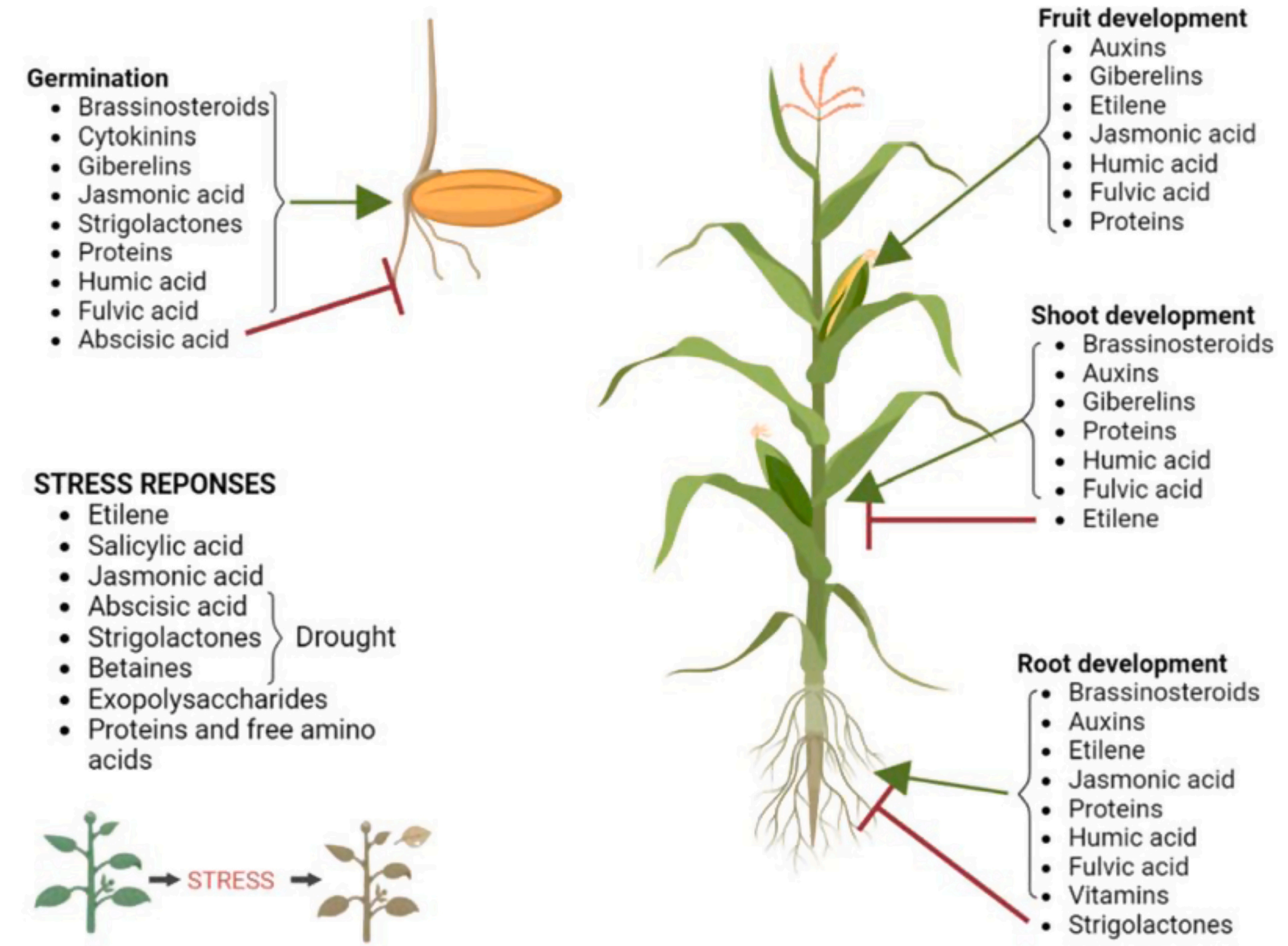

Fig. 2. Non-exhaustive list of biostimulant molecules extracted from microalgae and their associated effects on plant development. The green and red arrows refer to the promoting and inhibiting effects of the different biostimulants on seed germination; fruit development; shoot and root developments and stress responses.

seeds. In 2021, Mutale-joan et al. (2021) demonstrated that an extract constituted of *Dunaliela salina* + *Chlorella ellipsoidea* + *Arthrospira maxima* + *Aphanothece* sp. concentrated at 5% stimulates salt stress tolerance response, improves nutrient uptake and growth of tomato plants (*Solanum lycopersicum* L.).

2.2.2. *Effect under drought stress*

The drought stress affects plants at the biochemical, cellular, and molecular levels. In drought condition, stress-related hormones are mobilized, osmolytes are produced, stress-protective proteins such as LEA (Late Embryogenesis Abundance) are accumulated, and free proline concentration increases in the plant as a defence mechanisms (Goñi et al., 2018; Ördög et al., 2021). Methods for defending against water stress include the production of a robust root system, the generation of epidermal wax, the shedding of older leaves, the management of stomatal closure to prevent dehydration, the modification of photosynthetic performance, the inhibition of cell proliferation, or the induction of senescence (Yang et al., 2021). Therefore, biostimulants can help plants grow under water stress, i.e., water deficit (Fernandes et al., 2022; Petropoulos et al., 2020), nevertheless the mechanism by which they promote plant defence against drought is not yet well studied, but it is reported to be due to the enhancement of nutrient uptake from the soil and the improvement of osmotic adjustment in the plant (Rezaei--Chiyaneh et al., 2023).

For example, Oancea et al. (2013) studied the application of extracts containing phytohormones, osmoprotectants, free amino acids and soluble carbohydrates from the microalgae *Nannochloris* sp. to tomato plant (*Lycopersicum esculentum* cv.). The morphological parameters like height, root length, number and surface area of leaves of the plant measured during the study showed a better development of the plants to which these extracts were applied by sprying the leaves at the 2nd and the 29th day after replanting. In another study, Kusvuran (2021) used extracts from *Chlorella vulgaris* 5% concentrate on broccoli plants (*Brassica oleracea*) grown with 25% water compared to the control (100%). Protection against oxidative stress was demonstrated by measuring the activity of different antioxidant enzymes: superoxide dismutase, catalase, glutathione reductase and ascorbate peroxidase that increased 32%, 150%, 80% and 76% respectively when applying the concentrate with respect to the control.

3. Coupling biostimulants production and effluents valorisation

3.1. *Residual nutrients and organic carbon from wastewater*

Growing microalgae in wastewater involves some challenges to overcome, however this strategy remains a suitable alternative to reduce fresh water expenses and valorize residual nutrients, largely explored in the literature (Ahmed et al., 2022; Jia and Yuan, 2016). As microalgae and cyanobacteria are able to grow in many different environments, wastewaters can be used as culture medium, as they are rich in nutrients as nitrogen (N), phosphorus (P) and other macro and micronutrients (Fernandes et al., 2020; Kaloudas et al., 2021). Some examples of these effluents are: (i) digestate from anaerobic digestion (AD) (Navarro-López et al., 2020a); (ii) piggery wastewater (Ferreira et al., 2021); (iii) municipal wastewater (Bellver et al., 2023); (iv) aquaculture wastewater (Guo et al., 2013); and (iv) cheese whey wastewater (Zapata et al., 2021), among others.

For cultivating microalgae and/or cyanobacteria in wastewater there exist some important considerations to take in account that include: (i) economic feasibility; (ii) microalgae selection; (iii) effluent sterilization; (iv) suspended particulates in murky wastewater; (v) microalgae harvesting; (vi) variable light and temperature conditions; (vii) and a long retention time (Sharma et al., 2022). Another bottleneck would be the easy volatilization of the ammonium depending on the pH and the acclimation time (Fernandes et al., 2022). It is also difficult to stablish the proper C/N and P/N ratio that have direct influence on the grow of microalgae. Moreover, questions regarding biological contaminants may be also important, as some phytoplankton-lytic bacteria, specific viruses, foreign algae or other bacteria can appear in the culture (Chong et al., 2022). However, several physicochemical techniques offer the opportunity to reduce the overall toxicity of the effluents, including filtration (Rude et al., 2022), dilution (Rajagopal et al., 2021), or adsorption (Sánchez-Quintero et al., 2023), to name just a few examples. The selection of the appropriate microalgae or cyanobacteria strain (or consortium) is also an important criterion to consider when developing a microalgae-based wastewater treatment process aiming to produce biostimulants. Indeed, the photosynthetic microorganisms should be carefully selected in order to ensure a sufficient biomass production as well as an efficient uptake of residual nutrients while accumulating the molecules of interests.

Table 1 displays the state of the art of microalgae and cyanobacteria strains grown in wastewater (with or without the presence of CO_2)for the production of different biostimulants. Ferreira et al., (Ferreira et al., 2021) studied that *Chlorella vulgaris* grown in piggery wastewater has beneficial potential for cucumber (*Cucumis sativus*) seed germination as well as for root and shoot growth in tomato (*Licopersicon esculentum*), soybean (*Glycine max*) and wheat (*Triticum aestivum*), when applying the microalgae in a suspension of 0.5 $g.L^{-1}$. Another study did not show any biostimulant effect, in terms of seed germination for the cress (*Lepidium sativum*), when *Chlorella vulgaris* microalgae strain was grown in municipal wastewater (Ranglová et al., 2021). However, the authors noticed a positive biostimulant effect when using the microalgae strain previously cultivated in synthetic culture media (BG11) rather than wastewater. According to the authors, the low biostimulating activity observed in the case of *C. vulgaris* strain grown in wastewater could be due to the presence of inhibiting substances present in the effluent. Morillas-España et al. (2022) noticed phytohormone activities (gibbereline, auxins and cytokinins – like-activities) of *Chlorella vulgaris* extracts on watercress (*Lepidium sativum* L.) seeds, adventitious soybean (*Glycine max* L.) and excised cucumber (*Cucumis sativus* L.). Viegas et al. (2021) concluded that the microalgae *Chlorella vulgaris* grown in poultry wastewater increased the germination index of wheat (*Triticum aestivum*) from 100% to 147%, as compared to the control performed using distilled water. In another recent study, Álvarez-González et al., demonstrated the biostimulant effect of extracts from different microalgae strains (*Synechocystis, Phormidium* and *Scenedesmus)* cultivated in urban wastewater (Álvarez-González et al., 2023)

In most of the studies, the different biostimulant molecules present in the algae-based extracts are not always precisely identified, which makes difficult to attribute one or several plant responses to a specific biostimulant. In addition, the majority of the agronomic assays are performed in the absence of abiotic stress. Until now, a few number of scientific studies have been carried out on the extraction and utilization of microalgae-based biostimulants where microalgae were previously cultivated in wastewater. According to the literature, it has been observed that the use of these microorganisms cultured in wastewater and used as biofertilizers is much more common than their use and study as biostimulants (Alvarez et al., 2021).

3.2. CO_2 and flue gases

In recent years, excessive emissions of GHGs have considerably increased atmospheric CO_2 concentrations up to 409 ppm, causing major global warming and climate change (Chen et al., 2022). CO_2 concentration in the atmosphere ranged from 0.03% to 0.06% (v/v), but CO_2 concentration in flue gas are around 15% (v/v) (Brilman et al., 2013). To address CO_2-induced global warming, several carbon capture, storage, and utilization systems have been proposed, particulary for the conversion of CO_2 into useful compounds (e.g., alcohols, organic acids, polymers, building materials, value biomass, etc.) and fuels (e.g., CH_4, CO, H_2, hydrocarbons, etc.) (Chen et al., 2022). Though being a promising technology, the physical technique of carbon capture and sequestration has significant drawbacks, including high running costs due to its high energy demand (Ighalo et al., 2022).

Microalgae and cyanobacteria are photosynthetic organisms that can fix CO_2 in their autotrophic and mixotrophic metabolisms, depending on the cutlure media (Table 1). In comparison to the various options for CO_2 capture and use discussed above, the employment of these microorganisms may be a sustainable and environmentally friendly approach for cost-effective carbon capture, particularly in terms of economic rewards and environmental implications. Given the current low efficiency and capability of CO_2-capture and utilization, more efficient and environmentally friendly solutions must be developed in order to achieve the economic and environmental balance required for the long-term and permanent elimination of excess CO_2 and its consequences (Chen et al., 2022). There are also several options of biological-based carbon capture. Microalgae and cyanobacteria have a potential CO_2 fixation rate of 440–550 $g.m^{-2}.d^{-1}$ (equivalent to 1800 $tons.hm^{-2}.year^{-1}$), which is approximately 60 times greater than the forest carbon sink (Wu et al., 2023). *Chlorella vulgaris* and *Anabaena* sp. for example, may fix CO_2 at rates of 6.24 $g.L^{-1}.d^{-1}$, respectively (Ighalo et al., 2022). As a matter of fact, it has been reported that microalgae =can collect up to 180 tons of CO_2 while generating 100 tons of microalgae biomass using either natural or artificial light (Converti et al., 2009).

Selection of specific microalgae and cyanobacteria species is critical to the effectiveness of CO_2 bioconversion for biomass generation. CO_2 tolerance is considered while selecting microalgae species. *Scenedesmus* sp. has strong CO_2 tolerance, allowing it to thrive at CO_2 concentrations ranging from 10% to 20% (v/v). Some reported strains as *Chlorella* sp. can thrive at 40% CO_2 or *Nannochloropsis* sp. grew at a rate of 58% when grown in 15% (v/v) CO_2 (Ighalo et al., 2022).

Additionally, CO_2 injection in microalgae culture is also interesting because it can vary their biomass composition depending on the volume of CO_2 supplemented to the culture (Barati et al., 2022). In particular, Beigbeder and Lavoie, (Beigbeder and Lavoie, 2022) observed a variation in the amount of carbohydrates in *Parachlorella kessleri* when varying the percentage of CO_2 supplementation (0.04%, 2.5%, 5.0% and 10.0%), reaching a maximum of 43.2 $mg.L^{-1}.d^{-1}$ by injecting 5.0% CO_2. In another study, an increase in carbohydrate production (23.1% $\pm$ 0.7) by *Chlorella vulgaris* was observed when increasing CO_2 from 0.03% to 10% (Yadav et al., 2019). This is relevant in terms of biostimulant production for sustainable agriculture, as carbohydrate extracts from microalgae may have biostimulant potential in plants (Chanda et al., 2019).

3.3. Potential risks

The Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019, stated the main parameters to verify when producing and marketing plant biostimulants. According to this document, several threshold values are given in term of heavy metal (Pb, Zn, As, etc.) and microbial (*Salmonella* spp., *Escherichia coli* , Vibrio spp., etc.) concentrations (Table 2). However, several tools and techniques are available to prevent the risk of biological contamination and/or heavy metals accumulation when cultivating microalgae using waste effluents, as described in the next section.

Contamination in microalgae culture is sometimes difficult to control, especially when working with wastewater, where microorganisms such as bacteria can be transfered to the cultivation media and compete with microalgae cells (Di Caprio, 2020). The risk of biological contamination can be largely reduced by using pretreatment techniques such as sterilization and membrane filtration, which also has an economical impact on the overall treatment process. Some analytical tools can be used to identify biological contaminants in these cultures, such as plate counting method and flow cytometry (Di Caprio, 2020) as well as qPCR for DNA analysis (Delanka-Pedige et al., 2019). Contamination by bacteria in wastewaters in the liquid AD digestate is inevitable and the risk of the presence of *Salmonella* or *E. coli* is increased when using manure as feedstock for AD (Chong et al., 2022).

Concerning the presence of metals, it can also be a concern when cultivating microalgae or cyanobacteria with liquid effluents more specially with industrial effluents, depending on the industrial activity (Vandana et al., 2023). Nevertheless, using municipal wastewaters (Álvarez-González et al., 2023) livestock effluents or AD liquid digestate it can be reduced. As far as AD of agricultural residues is concerned, it does not contain normally high concentrations of heavy metals (Demirel et al., 2013; He et al., 2022; Kupper et al., 2014). To measure these elements, an option would be to prepare the samples for characterisation by ICP-OES (inductively coupled plasma-optical emission spectroscopy) (Kupper et al., 2014) or to use Synchrotron radiation nano-X-ray fluorescence (SR-nXRF) to localize and quantify metal elements inside microalgae cells (Leonardo et al., 2014).

As the biostimulants extracts of microalgae correspond to the CFP6-B classification in the legislation, special attention must be paid to *Salmonella* and *E. coli*. Therefore, as complete biomass is not used, but their extracts, the presence of these bacteria and metals is avoidable.

Table 2
Threshold concentration of the different pollutants allowed by the Regulation (EU) 2019/1009 of the European parliament and of the council of 5 June 2019 for the commercial acceptation by type of biostimulant (European Parliament, 2019).

Type of biostimulant	Pollutant	Threshold concentration
General	Cadmium (cd)	1.5 mg.kg DM^{b-1}
	Hexavalent chromium (Cr VI)	2 mg.kg DM^{b-1}
	Lead (Pb)	120 mg.kg DM^{b-1}
	Mercury (Hg)	1 mg.kg DM^{b-1}
	Nickel (Ni)	50 mg.kg DM^{b-1}
	Inorganic arsenic (As)	40 mg.kg DM^{b-1}
	Copper (Cu)	600 mg.kg DM^{b-1}
	Zinc (Zn)	1500 mg.kg DM^{b-1}
Microbial plant biostimulant (CFP 6 A)	*Salmonella* spp.	Absence in 25 g or 25 mL
	Escherichia coli	Absence in 1 g or 1 mL
	Listeria monocytogenes	Absence in 25 g or 25 mL
	Vibrio spp.	Absence in 25 g or 25 mL
	Shigella spp.	Absence in 25 g or 25 mL
	Staphylococcus aureus	Absence in 25 g or 25 mL
	Enterococcaceae	10 CFU[a].g^{-1}
	Anaerobic plate count (exception: when the biostimulant is an aerobic bacteria)	10^5 CFU[a]. g^{-1} or mL^1
	Yeast and mould (exception: when the biostimulant is a fungus)	1000 CFU[a].g^1 or mL^1
Non-microbial plant biostimulant (CFP6 B)	*Salmonella* spp.	Absence in 25 g or 25 mL
	Escherichia coli or Enterococcaceae	1000 in 1 g or 1 mL

[a] CFU: Colony-forming unit.

4. Biostimulants green-extraction techniques

Being able to convert biomass into high-value marketable molecules is known as biorefining (González-Gloria et al., 2021). In the present case, one of the most important and challenging steps is the downstream process, which consists on the extraction and purification of the molecules of interest from microalgae or cyanobacteria for biostimulants. As these processes can be hazardous for the integrity of these molecules and inhibit the desired effect, several studies on optimisation of these approaches have been done (Chew et al., 2017; Yildirim, 2022).

Extracting these biostimulant compounds from microalgae or cyanobacteria is not direct. The cell wall of these microorganisms is tough and rigid mainly due to cellulosic structure and the presence of glycoproteins and other carbohdydrates (Jothibasu et al., 2022). These properties depend on the strain, some of them have high molecular weight biopolymers that make extraction difficult (Stirk et al., 2020). Thus, disrupting the cell wall by doing a pre-treatment of the biomass has been shown to greatly increase extraction yields. Several disruptive methods to breakdown the microalgae cell wall are displayed in Table 3, with a specific focus on biostimulant molecules. Mechanical or physical techniques, such as sonication, high-pressure homogenisation (HPH), mechanical breaking with mortar and liquid nitrogen or with glass beads and vibration methods have been widely investigated in the litterature. For instance, Stirk et al. (2020) demonstrated by microscopy that cells were not affected by HPH technique following a freeze-drying step; whereas sonication and bead-milling treatments disrupted 10–20% and 70–80% of the intial cells, respectively. However, plasma membrane permeability was affected by freeze-drying, although it was increased by applying sonication afterwards. In another study, Navarro-López et al. (2023) compared the degree of hydrolysis of *Scenedesmus* sp. proteins using HPH and ultrasonication pre-treatments as compared to a non-treated control. The authors indicated that the highest degree of hydrolysis occurred with disruption by HPH (54.5%), followed by disruption by ultrasound (39.3%) and ending with cells that did not undergo disruption (8.8%).

So far, chemical and biological methods have been less investigated as pre-treatment of microalgae biomass in the field of biostimulant (Navarro-López et al., 2020a). As highlighted by (Chiaiese et al., 2018), enzymatic pretreatment of microalgae are gaining popularity since this biological method allows a gentle cell-wall disruption without affecting the level of bioactive compounds present in the algae extracts.

Regarding the extraction approaches of bioactive and high-value molecules in microalgae and cyanobacteria, several extraction techniques can be highlighted, either preceded by a cell disruption phase or applied on whole cells (Corrêa et al., 2021). As an alternative to the use of conventional organic solvents, and in the context of a more sustainable chemistry, one of the most studied liquid polymers is polyethylene glycol (PEG), a biodegradable polymer widely used in aqueous two-phase systems (Clarke et al., 2018; Hoffmann, 2022). Supercritical fluid extraction (SFE) is also emerging as an efficient and suitable technique for the green extraction of compounds interest from microalgae, instead of conventionnal organic solvents (Bahadar et al., 2015; Crampon et al., 2011; Manjare and Dhingra, 2019). In terms of extracting microalgae and cyanobacteria biomolecules, SFE have been extensively used for the extraction of PUFAs, pigments and vitamin, in which the most used solvent is sc-CO_2, pure or associated with some other co-solvent as ethanol, methanol or limonene (Corrêa et al., 2021). In addition, Navarro-López et al., (Navarro-López et al., 2023) demonstrated that water has the capacity to extract almost twice as many phenols compared to ethanol or acetone in *Scenedesmus almeriensis* at 25ºC. In this field, a new type of green solvents, deep-eutectic solvents (DES), such as choline chloride-ethylene glycol, are starting to be more

Table 3
Type of pre-treatment and extraction techniques for biostimulants extraction from microalgae and cyanobacteria.

Type	Microalgae or cyanobacteria	Pretreatment	Extraction technique	Extracted molecule	Reference
Microalgae	*Chlorococcum* sp., *Micractinium pusillum, Scenedesmus* sp., *Chlorella* sp.	Sonication and centrifugation	Extraction solvent (80% acetonitrile, 1% acetic acid)	Cytokinins, Gibberellins, Auxins and Abscisic acid	(Rupawalla et al., 2022)
	Scenedesmus sp.	High Pressure homogenization	Enzymatic hydrolisis (proteases)	Ethylene, Cytokinins, Gibberellins, Auxsins, ABA, Salicilic and Jasmonic acids	(Plaza et al., 2018)
	Pheneodnctylum tricornutum	Glass beads	-	Intracellular polysaccharides	(Guzmán-Murillo et al., 2013)
	Nannochloris sp.	High pressure homogenization	Mix of lytic enzyme of *Trichoderma harzianum* (Glucanex) + Ultrasonication	Proteins, carbohydrates, beatines and cytokinins	(Oancea et al., 2013)
	Chamydomonas reinhardtii, Chlorella sp.	Centrifugation + mortar with liquid nitrogen + Dilution	-	Exopolysaccharides and auxins	(Gitau et al., 2022)
	Tetradesmus obliquus, Chlorella protothecoides, Chlorella vulgaris, Neochloris oleoabundans	-	-	Proteins, carbohydrates	(Ferreira et al., 2021)
	Chlorella vulgaris	Mortar crashing + Sonication + Centrifugation	-	Carbohydrates, proteins	(Suchithra et al., 2022)
	Chlorella vulgaris, Chlorella protothecoides, Tetradesmus obliquus	Vibratory sieve shaker + dilution + filtration	-	Proteins, carbohydrates	(Viegas et al., 2021)
	Chlamydomonas reinhardtii, Chlorella sorokiniana	Glass beads + centrifugation + freeze-drying	-	-	(Martini et al., 2021)
	Desmodesmus subspicatus	Freeze-drying	Dialysis	Glycosides, sulfolipids	(Mazepa et al., 2021)
	Chlorella vulgaris	Freeze-drying	-	Cytokinin, auxins	(Ranglová et al., 2021)
	Chlorella sp., *Scenedesmus* sp.	Mortar and pestle + ultrasonication + centrifugation	-	Proteins	(Supraja et al., 2020)
	Chlorella vulgaris, Chlorella sp., *Chlamydopodium fusiforme*	Sonication	-	-	(Morillas-España et al., 2022)
	Dunaliella salina MS002, *Porphyridium* sp.	High temperature	Absolute ethanol	Polysaccharides	(Rachidi et al., 2020)
	Dunaliella salina, Chlorella elipsoide,	Liquid nitrogen + sulphuric acid + high temperature	Autoclaving	-	(Mutale-joan et al., 2021)
	Chlorella vulgaris	Freeze-drying	Organic solvents	-	(Kusvuran, 2021)
	Nostoc piscinale	Freeze-drying + sonication	-	-	(Ördög et al., 2021)
Cyanobacteria	*Arthrospira platensis*	High Pressure homogenization	Enzymatic hydrolisis (proteases)	Ethylene, Cytokinins, Gibberellins, Auxins, ABA, Salicylic and Jasmonic acids	(Plaza et al., 2018)
	Synechocystis sp., *Nostoc* sp.	-	-	Proteins, carbohydrates	(Ferreira et al., 2021)
	Spirulina sp., *Synechocystis* sp.	Mortar and pestle + ultrasonication + centrifugation	-	Proteins	(Supraja et al., 2020)
	Arthrospira platensis	High temperature	Absolute ethanol	Polysaccharides	(Rachidi et al., 2020)
	Aphanotece sp., *Arthrospira máxima*	Liquid nitrogen + sulphuric acid + high temperature	Autoclaving	-	(Mutale-joan et al., 2021)

widely studied (Moreno Martínez et al., 2022). DES are a mixture of compounds that have a lower melting point together than their separate constituents. Their constituents can be diverse: amino acids, sugars, polyols and carboxylic acids (Song et al., 2023). They are considered as green solvents with affordable costs, stable, effective dispersants, non-toxic, biodegradable and do not react with water (Abed M. et al., 2022). Due to their solubilising capacity, inorganic, organic and polymeric substances can be extracted from microalgae (Moreno Martínez et al., 2022). Along the same lines and with the aim of improving the sustainability of extraction, research is beginning to be carried out on how to make these solvents more sustainable, giving rise to Natural DES (NADES), nonetheless further studies are still needed on the matter (Fan et al., 2022; Mehariya et al., 2021).

These extraction techniques of biostimulants are dependent on the chemical nature of the searched molecule and sustainable chemistry. For extracting lipids: ultrasonic extraction, microwave assisted extraction, supercritical CO_2 and electroporation (Chew et al., 2017; Hernández et al., 2014); proteins: supercritical-CO_2 extraction is a good alternative to organic solvents (Mazzelli et al., 2022); polysaccharides: extraction is difficult and has not been studied much, among them, exopolysaccharides are the most widely used due to their ease of extraction. Some of the methods proposed for the extraction of exopolysaccharides (EPS) at the moment are alcohol precipitation, tangential microfiltration or hot water, which is also used to extract intracellular or cell wall polysaccharides (Caetano et al., 2022). In some studies (Table 3), biostimulant extracts are made with the cells disrupted and diluted, without a specific extraction step after cell disruption.

It is important to pay attention to the storage conditions of the bioactive molecules contained in microalgae and cyanobacteria. Certain factors, as storage time or temperature can affect, in some cases (i.e.

Chlorella vulgaris), to the compound's integrity (Stirk et al., 2021). To ensure the stability of the final product, drying of the biomass after harvesting and dewatering should be considered (Nitsos et al., 2020). To obtain dry biomass, the best alternative is the freeze-drying method, which does not damage the biomolecules of interest in microalgae or cyanobacteria. Even if this technique is highly energy consuming, it allows to maintain and to preserve the microalgae cells. Other options such as sun drying can be catastrophic for the integrity of the biomolecules of interest (Ansari et al., 2018). Drying the microalgae before the extraction process of their biomolecules considerably increases the extraction efficiency (Nitsos et al., 2020).

After extraction of plant biostimulant molecules from microalgae and cyanobacteria, the characterization of the molecules of interest has been possible thanks to different analytical techniques namely high-performance liquid chromatography (HPLC) and mass spectrometry (MS), gas chromatography (GC), ultraperformance liquid chromatography (UPLC), thermogravimetric analysis (TGA), Fourier-Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) as it is shown in Table 4.

5. Application biostimulants extracts in plants

Different techniques can be used to apply microalgae-based biostimulant extracts on plants, as listed in Table 5. Other factors such as timing and the concentration of biostimulants applied also have an influence in their effect on the plant development and production yield (Parmar et al., 2023).

5.1. Diversity of applications techniques

Different application techniques of biostimulants already exist including: (i) soil drench or drip fertigation;(ii) foliar spray application and (iii) seed treatment (Colla and Rouphael, 2020), which refers to three different processes: seed priming, seed coating and seed dipping (Parmar et al., 2023).

The most appropriate application technique for a better development of plants is not yet clearly determined. Suchithra et al. (Suchithra et al., 2022) concluded that soil application of *Chlorella vulgaris* extracts had more effects than foliar spray application in tomato plant (*Solanum lycopersicum*). On the other hand, Supraja et al., (Supraja et al., 2020) observed that the application of microalgae extracts produced from a consortium of microalgae (consisting of *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp. and *Synechocystis* sp.) on seeds was more effective than the foliar spray in terms of growth rate of the tomato plant. Direct application of polysaccharides extracted from *A. platensis*, *D. salina* and *Porphorydium* sp. to the soil presented a significant effect on the increase of the leaves, nodes and shoot dry weight of tomato plant (Rachidi et al., 2020). Despite the studies mentioned above, the study provided by Kusvuran, Kusvuran (2021) concluded that foliar application of *Chlorella vulgaris* extracts (diluted at 1%, 3% or 5% (v/v)) in fenugreek plants successfully increased the plant height, leaves and branches numbers, as compared to the control leaves, which were only sprayed with water. Other authors investigated the possibility of adding to the microalgae-based biostimulants some wetting agents for a better adhesion to the leaf, as Trend 90 or ethoxylated isodecyl alcohol (0.1%) in order to improve the effect of biostimulants during foliar application (Ördög et al., 2021). These kind of molecules induce the establishment of a uniform film on the surface of plants, which can enhance the produt adhesion and promote the penetration of the active substance into plant tissues (Nexles Europe, 2023, p. 90).

Table 4
Examples of characterization techniques for specific plants biostimulants.

Biomolecule	Technique	Reference
Auxins	HPLC-MS; GC-MS; UPLC-MS/MS	(Stirk and van Staden, 2020; Álvarez-González et al., 2023)
Cytokinins	HPLC-MS/MS; HPLC-MS; UPLC-MS/MS; LC-MS/MS	(Stirk and van Staden, 2020; Álvarez-González et al., 2023)
Giberellines	UPLC-MS/MS	(Stirk and van Staden, 2020; Álvarez-González et al., 2023)
Abscisic Acid	GC-MS; ELISA; HPLC; GC; UPLC-MS/MS; LC-MS/MS	(Stirk and van Staden, 2020; Álvarez-González et al., 2023)
Brassinosteroids	GC-SIM-MS; UPLC-MS/MS; UHPLC-MS/MS	(Stirk and van Staden, 2020)
Salicylic Acid	HPLC; UPLC-MS/MS	(Navarro-López et al., 2020a; Álvarez-González et al., 2023)
Pigments (carotenoids)	HPLC	(Pereira et al., 2015)
Humic and Fulvic Acids	TGA; FTIR; NMR	(Allevato et al., 2022; Stoica et al., 2019; Zhao et al., 2023)

5.2. Impact of application time and concentration

Regarding the timing of application of the biostimulants (Caradonia et al., 2022), their frequency of application and concentration, there is no clear consensus in the literature, partly because the nature of these molecules is varied. The technique of application of the biostimulants is determined by the crops specific needs, such as nutrients supplementation, micronutrient enrichment, or disease control (Parmar et al., 2023).

Specifically for the timing and frequency of biostimulants from microalgae or cyanobacteria application, as explained by Caradonia et al. (Caradonia et al., 2022) in their review on biostimulants for potato plants, some authors concluded that the best period to apply the biostimulants is the 30th or 60th day after planting; however, in their study they also named another research which has found that the best time to apply them is the second week period after planting.

Concerning the microalgal/cyanobacterial biostimulant concentration on seeds, it is reported that less than 1 $g.L^{-1}$ is necessary, since this concentration can have toxic effects leading to a reduction of the germination rate in the plants (Navarro-López et al., 2020b). In fact, in other research studies on seed application, extracts of *Chlorella vulgaris* at 0.3 $g.L^{-1}$ presented a positive effect on the germination index of tomato, as well as extracts of *Scenedesmus obliquus* at a concentration of 0.1 and 0.3 $g.L^{-1}$ (Alling et al., 2023). Nevertheless, EL Arroussi (2016) sprayed plants with polysaccharides extracted from *Artrhospira* sp. (at a concentration of 3 $g.L^{-1}$) on tomato leaves, which resulted in an increase in root weight.

In the different examples, it is difficult to find in the literature a logical pattern of concentration, timing and frequency of application of biostimulants extracted from microalgae and cyanobacteria on plants. These factors can vary depending on the variety of plant and type of biomolecule applied as a biostimulant (Navarro-López et al., 2020b) as well as the need to be covered on the plant (Parmar et al., 2023).

6. Biostimulants market and regulation

The global market for biostimulants has expanded considerably in recent years (Gupta et al., 2023). Europe represents the largest market for biostimulants (Liebig et al., 2019). The global market for non-microbial biostimulants (including biostimulants from microalgae) was estimated at USD 2830 million in 2022 and with a cumulative aggregated growth rate of 11.3–11.6% (EBIC, 2021). It is projected to reach USD 6.2 billion and a CARG of 11.8% for 2028 (Gupta et al., 2023).

In order to introduce biostimulants on the market, it is important to agree on what can be classified as a biostimulant. For this, EBIC proposes some criteria to follow when establishing plant biostimulant declarations. For placing these products in the European market, they have to attempt to the EU definition (demonstrating its effect), so enhancing nutrient use efficiency, abiotic stress tolerance, crop quality characteristics, or limited nutrient availability in the soil and rhizosphere.

Table 5
Different microalgal or cyanobacterial biostimulant application techniques in different varieties of plants.

Type	Microalgae or cyanobacteria strain	Extracted molecules	Technique of application of the biostimulant in plants	Plant varieties	Reference
Microalgae	*Chlorococcum* sp., *Micractinium pusillum*, *Scenedesmus* sp., *Chlorella* sp.	Phytohormones	Seed application	Spinach	(Rupawalla et al., 2022)
	Scenedesmus sp.	Phytohormones	Foliar application	*Petunia x hybrida*	(Plaza et al., 2018)
	Chlorella ellipsoida	-	Seed application	Wheat	(Abd El-Baky et al., 2010)
	Pheneodnctylum tricornutum	Intracellular polysaccharides	Seed application	Bell pepper	(Guzmán-Murillo et al., 2013)
	Nannochloris sp.	Proteins, carbohydrates, betaines and cytokinins	Foliar application	Tomato	(Oancea et al., 2013)
	Chamydomonas reinhardtii, *Chlorella* sp.	Exopolysaccharides and auxins	Soil and Foliar application	Tomato	(Gitau et al., 2022)
	Tetradesmus obliquus, Chlorella protothecoides, Chlorella vulgaris, Neochloris oleoabundans	Proteins, carbohydrates	Seed application	Cucumber, barley, wheat, soybean, watercress and tomato	(Ferreira et al., 2021)
	Chlorella vulgaris	Carbohydrates, proteins	Foliar application	Tomato	(Suchithra et al., 2022)
	Chlorella vulgaris, Chlorella protothecoides, Tetradesmus obliquus	Proteins, carbohydrates	Seed application	Wheat	(Viegas et al., 2021)
	Chlamydomonas reinhardtii, Chlorella sorokiniana	-	Seed application	Maize	(Martini et al., 2021)
	Desmodesmus subspicatus	Glycosides, sulfolipids	Seed application	Tomato	(Mazepa et al., 2021)
	Chlorella vulgaris	Cytokinin, auxins	Seed application	Watercress	(Ranglová et al., 2021)
	Chlorella sp., *Scenedesmus* sp.	Proteins	Seed and foliar applications	Tomato	(K.Supraja et al., 2020)
	Chlorella vulgaris, Chlorella sp., *Chlamydopodium fusiforme*	-	Seed application	Watercress, soybean, cucumber and wheat	(Morillas-España et al., 2022)
	Dunaliella salina, *Porphyridium* sp.	Polysaccharides	Soil application	Tomato	(Rachidi et al., 2020)
	Dunaliella salina, Chlorella elipsoide,	-	Seed application	Tomato	(Mutale-joan et al., 2021)
	Chlorella vulgaris	-	Soil and foliar applications	Brocoli	(Kusvuran, 2021)
	Nostoc piscinale	-	Foliar application	Maize	(Ördög et al., 2021)
Cyanobacteria	*Arthrospira platensis*	Phytohormones	Foliar application	*Petunia x hybrida*	(Plaza et al., 2018)
	Spirulina maxima	-	Seed application	Wheat	(Abd El-Baky et al., 2010)
	Synechocystis sp., *Nostoc* sp.	Proteins, carbohydrates	Seed application	Cucumber, barley, wheat, soybean, watercress and tomato	(Ferreira et al., 2021)
	Spirulina sp., *Synechocystis* sp.	Proteins	Seed and foliar applications	Tomato	(K.Supraja et al., 2020)
	Arthrospira platensis	Polysaccharides	Soil application	Tomato	(Rachidi et al., 2020)
	Aphanotece sp., *Arthrospira maxima*	-	Seed application	Tomato	(Mutale-joan et al., 2021)

Moreover, to be considered in the European market experimental data under specific study conditions are required, and the support of the effect of the biostimulant or the different used techniques by the scientific literature can reinforce the request. The effect of the biostimulant does not have to be guaranteed under all growing conditions (Ricci et al., 2019).

Regarding the regulation, as mentioned before in section 1.2 of this review, the new EU Regulation n°2019/1009 (from July 2022) gives a precise definition of biostimulants and thereby delineates a border with Plant Protection Products, thanks to the efforts of EBIC and the European Commission. In this regulation biostimulants are referred as CPF6: CPF6-A for microbial and CPF6-B for non-microbial. Microalgal extracts would enter in the second group (CPF6-B) from the component material category 2 (CMC 2 – plants, parts of plants or vegetal extracts). For this particular class, microalgal extracts (included in group CFP6-B, CMC2), according to European legislation, only extracts that have undergone one of the following processes are allowed: (i) cutting; (ii) grinding; (iii) milling; (iv) sieving; (v) sifting; (vi) centrifugation; (vii) pressing; (viii) drying; (ix) frost treatment; (x) freeze-drying; (xi) extraction with water and (xii) supercritical CO_2 extraction (European Parliament, 2019). In the case of microalgae themselves, for the moment, they are not listed in the regulation as microbials, and, as only the high-interest molecules contained in the microalgae are applied, they cannot be considered as microbial biostimulants either. If the complete biomass of the microalgae were applied, the essential macronutrients for the plant would also be applied by this biomass, thus interfering its fertilizer activity with its biostimulant activity. In 2022, Hendriksen (2022) asked for modifications in the regulation at microbiological level, since the study concluded that more scientific rigor is required in the regulation, as the microorganisms classification is ambiguous and it does not include some microorganisms.

It is also relevant to note that biostimulants extracted specifically from cyanobacteria are not yet included in European legislation and cannot still be commercialised in Europe. Nonetheless, this regulation has the potential to change and these extracts could be included in the near future. Similarly, DES and NADES are also not yet included as possible techniques to extract biostimulants from microalgae, but it is expected that international legislation will progress along with research in the domain.

Since 16th July 2022, there are two alternatives for carrying biostimulants on the market. They can be followed either under national regulation or EU harmonized marketing process. In the first case, the biostimulant will be marketable in a Member State but not in other states. For asking to place the product in other Member State the principle of mutual recognition in accordance with Regulation (EU) 2019/515 have to be used. This process depends on the country where the recognition is asked. Nevertheless, if the EU harmonized marketing process for biostimulants is followed, once a biostimulant has received an EU-type certificate, the marketer will be allowed to apply the CE mark, and the product will then have access to the whole EU market (Liebig et al., 2019). In addition to all this, it is important to take into consideration what was discussed in Section 3.3 (Table 2) on the permitted contamination thresholds for biostimulants to be measured in extracts in order to be placed on the EU biostimulant market.

7. Conclusions

It can be concluded that the introduction of biostimulants from microalgae and cyanobacteria on the market can be a key factor in achieving a sustainable agriculture. For this, different aspects need to move forward at the same time like research and legislation at the international level. Extracting biostimulants from microalgae and cyanobacteria grown in wastewater and flue CO_2, is a strategy that contributes directly to a circular economy, and will be important in the coming years due to climatic conditions and the different stresses that crops will be subjected to. Even so, control and vigilance have to be taken on possible contaminations and thresholds not to be exceeded (Table 3) in order to be able to place these products on the market.

Furthermore, in the area of green extraction, new and attractive possibilities for biostimulants extraction from microalgae and cyanobacteria are emerging. For instance, the use of deep eutectic solvents (DES) and Natural-DES (NADES) could be considered as interesting alternatives to widening the field of green extraction approaches.

In the face of the coming climate adversities, far from fighting them in the long term by using environmentally damaging products, it is necessary to look for sustainable tools that contribute to a global and long-term improvement of the overall situation, where biostimulants extracted from microalgae and cyanobacteria have a very promising role.

CRediT authorship contribution statement

Ángela Sánchez-Quintero: Conceptualization; Formal analysis, Writing – original draft. **Jean-Baptiste Beigbeder**: Writing – review & editing, Supervision, Funding acquisition, Project administration. **Susana C.M. Fernandes**: Writing – review & editing, Supervision, Funding acquisition, Project administration.

Data availability

No data was used for the research described in the article.

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