FEDERAL UNIVERSITY OF RIO GRANDE
COLLEGE OF CHEMISTRY AND FOOD
POSTGRADUATE PROGRAM IN FOOD ENGINEERING AND SCIENCE

BIOECONOMY IN THE BREWING INDUSTRY: MICROALGAL CULTIVATION STRATEGIES FOR EFFLUENT VALORIZATION INTO AGRICULTURAL APPLICATIONS

JENYFER DE ALMEIDA CONCEIÇÃO

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Rio Grande, RS
2026

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JENYFER DE ALMEIDA CONCEIÇÃO

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in Food Engineering and Science

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“It always seems impossible until it’s done.”
— Nelson Mandela

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CHAPTER I

(Resumo, Abstract, Introdução, Introduction, and Objectives)

RESUMO

A indústria cervejeira gera efluentes com elevadas concentrações de nitrogênio e fósforo que, quando lançados sem tratamento adequado, podem ocasionar impactos ambientais como a eutrofização. Nesse contexto, as microalgas têm surgido como promissora plataforma biotecnológica capaz de integrar o tratamento de efluentes à produção de biomassa e à valorização de recursos no âmbito da bioeconomia circular. Com base nessa abordagem, a presente tese investigou o cultivo de *Chlorella fusca* LEB 111 e *Spirulina* sp. LEB 18 em efluente cervejeiro, avaliando o desempenho do processo, a composição da biomassa e estratégias de valorização em quatro estudos complementares. O primeiro estudo, uma revisão de literatura, sistematizou o conhecimento atual sobre o cultivo de microalgas em efluente cervejeiro, abordando o desempenho da biorremediação, a composição da biomassa, as aplicações agronômicas e as perspectivas de comercialização, identificando o potencial de substituição de insumos convencionais, bem como restrições regulatórias e de escalabilidade para implementação em larga escala. No segundo estudo, *Chlorella fusca* LEB 111 foi cultivada em regime semicontínuo com concentrações de efluente variando de 25% a 100%. A produtividade de biomassa atingiu 52,3 mg L^{-1} d^{-1}, com perfil bioquímico equilibrado em 100% de efluente. Os custos de produção foram reduzidos de €84 kg^{-1} em meio sintético para €18 kg^{-1} em efluente puro, indicando, em nível laboratorial, uma redução estimada nos custos de cultivo ao se utilizar o efluente cervejeiro como meio alternativo. O terceiro estudo avaliou o potencial bioestimulante de ambas as espécies cultivadas em efluente cervejeiro. O perfil fitohormonal confirmou a presença de citocininas, giberelinas, auxinas, ácido salicílico, ácido jasmônico e ácido abscísico nos extratos. Os ensaios agronômicos demonstraram que o extrato de *Spirulina* sp. aumentou a germinação da cevada em 58% e dobrou o comprimento radicular, enquanto o extrato de *Chlorella fusca* manteve a massa fresca da alface 46,5% acima do controle sob estresse hídrico severo, com aumento de 25% na taxa de transporte de elétrons. Esses resultados indicam que o efluente cervejeiro atua como um fator metabólico que influencia a biossíntese fitohormonal nas microalgas e, consequentemente, a eficácia bioestimulante. O quarto estudo investigou a integração do biochar de caroço de açaí como estratégia de intensificação de processo no cultivo de *Chlorella fusca* LEB 111. A adição de biochar modulou a dinâmica de nutrientes e melhorou a estabilidade do cultivo, com remoção de carbono total superior a 84,0% e remoção de fosfato variando de 78,0% a 86,8%. O teor de lipídios aumentou de 27,9% para 45,6% com adição de 0,5 g L^{-1} de biochar. A caracterização do biochar confirmou a adsorção de nitrogênio e a retenção de matéria orgânica, corroborando seu papel como componente multifuncional no sistema. Em conjunto, esses resultados demonstram que o efluente cervejeiro pode ser convertido em um meio de cultivo estratégico capaz de suportar múltiplas rotas de valorização, contribuindo para o desenvolvimento de sistemas integrados para a produção circular de biomassa microalgal e bioinsumos agrícolas.

Palavras-chave: Biochar. Bioestimulantes. Efluente Cervejeiro. Fitohormônios. Microalgas. Recuperação De Recursos.

ABSTRACT

The brewing industry generates effluents with high concentrations of nitrogen and phosphorus that, when discharged without adequate treatment, can cause environmental impacts such as eutrophication. In this context, microalgae have emerged as a promising biotechnological platform capable of integrating effluent treatment with biomass production and resource valorization within the circular bioeconomy. Based on this approach, the present thesis investigated the cultivation of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 in brewery effluent, evaluating process performance, biomass composition, and valorization strategies across four complementary studies. The first study, a literature review, systematized current knowledge on microalgal cultivation in brewery effluent, addressing bioremediation performance, biomass composition, agronomic applications, and commercialization perspectives, identifying the potential for replacing conventional inputs as well as regulatory and scalability constraints for large-scale implementation. In the second study, *Chlorella fusca* LEB 111 was cultivated under semicontinuous regime with effluent concentrations ranging from 25% to 100%. Biomass productivity reached 52.3 mg L^{-1} d^{-1}, with a balanced biochemical profile at 100% effluent. Production costs were reduced from €84 kg^{-1} in synthetic medium to €18 kg^{-1} in pure effluent, indicating, at laboratory scale, an estimated reduction in cultivation costs when using brewery effluent as an alternative medium. The third study evaluated the biostimulant potential of both species cultivated in brewery effluent. The phytohormonal profile confirmed the presence of cytokinins, gibberellins, auxins, salicylic acid, jasmonic acid, and abscisic acid in the extracts. Agronomic assays demonstrated that the *Spirulina* sp. extract increased barley germination by 58% and doubled root length, while the *Chlorella fusca* extract maintained lettuce fresh mass 46.5% above the control under severe water stress, with a 25% increase in the electron transport rate. These results indicate that brewery effluent acts as a metabolic factor influencing phytohormone biosynthesis in microalgae and, consequently, biostimulant efficacy. The fourth study investigated the integration of açaí seed biochar as a process intensification strategy in the cultivation of *Chlorella fusca* LEB 111. Biochar addition modulated nutrient dynamics and improved cultivation stability, with total carbon removal exceeding 84.0% and phosphate removal ranging from 78.0% to 86.8%. Lipid content increased from 27.9% to 45.6% with the addition of 0.5 g L^{-1} of biochar. Biochar characterization confirmed nitrogen adsorption and organic matter retention, corroborating its role as a multifunctional component in the system. Taken together, these results demonstrate that brewery effluent can be converted into a strategic cultivation medium capable of supporting multiple valorization routes, contributing to the development of integrated systems for circular microalgal biomass production and agricultural bioinputs.

Keywords: Biochar. Biostimulants. Brewery Wastewater. Microalgae. Phytohormones. Resource Recovery.

1. INTRODUCTION

The brewing industry is one of the most water-intensive food sectors, consuming between 3 and 10 L of water per liter of beer produced, generating effluents with chemical oxygen demand (COD) typically ranging from 2,000 to 6,000 mg L^{-1}, and nitrogen and phosphorus concentrations that can reach up to 120 mg L^{-1} and 30 mg L^{-1}, respectively (Amenorfenyo *et al.*, 2019; Olajire, 2020). Globally, the brewing sector comprises more than 19,000 breweries, of which approximately 94% are classified as craft breweries, and Brazil stands out as the third largest producer worldwide, contributing significantly to employment and economic activity (Dias *et al.*, 2023). Despite this growth, the sector faces important challenges related to waste management, since effluents inadequately treated may contribute to environmental impacts such as eutrophication (Malone; Newton, 2020).

In this context, microalgae have been increasingly investigated as a biotechnological platform capable of integrating wastewater treatment with biomass production. These photosynthetic microorganisms are able to assimilate nutrients and organic compounds under a wide range of conditions, enabling their cultivation in wastewater-based systems (Acién Fernández; Gómez-Serrano; Fernández-Sevilla, 2018; Morais, Michele Greque de *et al.*, 2018). Compared to conventional synthetic media, the use of residual streams significantly reduces operational costs while improving process sustainability (Dragone, 2022).

Beyond their role in nutrient removal, microalgae are capable of producing a wide range of biocompounds of industrial interest. Their biomass can be used as a feedstock for multiple applications, including biofuels, biochemicals, and agricultural inputs. In particular, the presence of bioactive compounds such as phytohormones, amino acids, and antioxidants enables the use of microalgal biomass as a bioinput in agriculture, contributing to plant development and stress tolerance (Braun; Colla, 2023; Miranda *et al.*, 2024).

The integration of wastewater-based cultivation with biomass valorization is aligned with the concept of microalgal biorefinery, in which different process streams are converted into value-added products. Within a circular bioeconomy perspective, industrial effluents are no longer considered solely as waste, but as strategic resources capable of supporting biomass production and enabling the development of more sustainable and decentralized production systems (Moreira, Juliana Botelho *et al.*, 2023). This approach contributes to reducing treatment costs while valorizing industrial residues within integrated production chains (Pessôa *et al.*, 2021).

Despite the growing body of evidence supporting microalgal cultivation in brewery effluents, significant knowledge gaps remain. Most studies have been conducted under batch

conditions and with a single microalgal species, limiting the understanding of process dynamics under semicontinuous operation and mixed-species comparisons. Furthermore, the biostimulant potential of biomass produced in brewery wastewater remains poorly characterized, and the role of auxiliary materials such as biochar as process intensification agents in complex industrial media has been scarcely explored. The integration of these dimensions (cultivation performance, biomass valorization, and agricultural application) within a unified bioeconomy framework has not been systematically addressed.

Advancing toward this integrated model, however, requires the optimization of cultivation strategies to improve process performance, system stability, and biomass composition. Operational modes and nutrient availability are well-recognized drivers of microalgal productivity. In addition, the incorporation of auxiliary materials such as biochar has emerged as a process intensification strategy with potential to modulate nutrient dynamics, adsorb inhibitory compounds, and influence biomass biochemical composition. This approach is particularly relevant in complex culture media such as industrial wastewater (Morais, M. G. de *et al.*, 2024; Rehman *et al.*, 2024).

The Laboratory of Biochemical Engineering (LEB) has been developing research in microalgal biotechnology since 1996, with a focus on microalgal biorefinery since 2005. The group has conducted studies involving the use of industrial residues as substrates for microalgal cultivation (Costa *et al.*, 2002), including wastewater (Bezerra *et al.*, 2020; Cruz; da Rosa; Costa, 2024), combustion gases (Braga *et al.*, 2019), and agro-industrial by-products such as açaí seeds for biochar production (Zaparoli *et al.*, 2023). More recent studies have explored the application of microalgae cultivated in agro-industrial effluents, such as dairy wastewater, as well as the development of bio-based products including biostimulants and biofertilizers derived from microalgal biomass (Cruz *et al.*, 2023).

Building on this background, the present thesis investigates the cultivation of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 in brewery wastewater as an integrated strategy for biomass production and resource valorization within a bioeconomy framework. Specifically, this work evaluates cultivation performance and production costs under semicontinuous mode, assesses the biostimulant potential of the resulting biomass on seed germination and plant stress tolerance, investigates the role of açaí seed-derived biochar as a process intensification agent, and synthesizes current knowledge through a review, collectively addressing the transition from industrial effluent treatment toward the circular production of agricultural bioinputs.

INTRODUÇÃO

A indústria cervejeira é um dos setores alimentícios mais intensivos em consumo de água, consumindo entre 3 e 10 L de água por litro de cerveja produzida, gerando efluentes com demanda química de oxigênio (DQO) tipicamente variando de 2.000 a 6.000 mg L^{-1}, e concentrações de nitrogênio e fósforo que podem atingir até 120 mg L^{-1} e 30 mg L^{-1}, respectivamente (Amenorfenyo et al., 2019; Olajire, 2020). Globalmente, o setor cervejeiro compreende mais de 19.000 cervejarias, das quais aproximadamente 94% são classificadas como cervejarias artesanais, e o Brasil se destaca como o terceiro maior produtor mundial, contribuindo significativamente para o emprego e a atividade econômica (Dias et al., 2023). Apesar desse crescimento, o setor enfrenta importantes desafios relacionados ao gerenciamento de resíduos, uma vez que efluentes tratados de forma inadequada podem contribuir para impactos ambientais como a eutrofização (Malone; Newton, 2020).

Nesse contexto, as microalgas têm sido cada vez mais investigadas como uma plataforma biotecnológica capaz de integrar o tratamento de efluentes com a produção de biomassa. Esses microrganismos fotossintéticos são capazes de assimilar nutrientes e compostos orgânicos sob uma ampla gama de condições, permitindo seu cultivo em sistemas baseados em águas residuárias (Acién Fernández; Gómez-Serrano; Fernández-Sevilla, 2018; Morais, Michele Greque de et al., 2018). Comparado aos meios sintéticos convencionais, o uso de correntes residuais reduz significativamente os custos operacionais ao mesmo tempo em que melhora a sustentabilidade do processo (Dragone, 2022).

Além de seu papel na remoção de nutrientes, as microalgas são capazes de produzir uma ampla gama de biocompostos de interesse industrial. Sua biomassa pode ser utilizada como matéria-prima para múltiplas aplicações, incluindo biocombustíveis, bioquímicos e insumos agrícolas. Em particular, a presença de compostos bioativos como fitohormônios, aminoácidos e antioxidantes possibilita o uso da biomassa microalgal como bioinsumo na agricultura, contribuindo para o desenvolvimento das plantas e a tolerância ao estresse (Braun; Colla, 2023; Miranda et al., 2024).

A integração do cultivo baseado em águas residuárias com a valorização da biomassa está alinhada com o conceito de biorrefinaria microalgal, no qual diferentes correntes de processo são convertidas em produtos de valor agregado. Dentro de uma perspectiva de bioeconomia circular, os efluentes industriais deixam de ser considerados apenas como resíduos, passando a ser vistos como recursos estratégicos capazes de sustentar a produção de biomassa e possibilitar o desenvolvimento de sistemas de produção mais sustentáveis e descentralizados (Moreira, Juliana Botelho et al., 2023). Essa abordagem contribui para a

redução dos custos de tratamento ao mesmo tempo em que valoriza resíduos industriais dentro de cadeias produtivas integradas (Pessôa et al., 2021).

Apesar do crescente corpo de evidências que sustenta o cultivo de microalgas em efluentes cervejeiros, permanecem lacunas de conhecimento significativas. A maioria dos estudos foi conduzida em condições de batelada e com uma única espécie microalgal, limitando a compreensão da dinâmica do processo sob operação semicontínua e comparações entre múltiplas espécies. Além disso, o potencial bioestimulante da biomassa produzida em águas residuárias cervejeiras permanece pouco caracterizado, e o papel de materiais auxiliares como o biochar como agentes de intensificação de processo em meios industriais complexos foi pouco explorado. A integração dessas dimensões (desempenho do cultivo, valorização da biomassa e aplicação agrícola) dentro de uma estrutura unificada de bioeconomia ainda não foi sistematicamente abordada.

Avançar em direção a esse modelo integrado, no entanto, requer a otimização de estratégias de cultivo para melhorar o desempenho do processo, a estabilidade do sistema e a composição da biomassa. Os modos operacionais e a disponibilidade de nutrientes são fatores reconhecidamente determinantes da produtividade microalgal. Além disso, a incorporação de materiais auxiliares como o biochar tem emergido como uma estratégia de intensificação de processo com potencial para modular a dinâmica de nutrientes, adsorver compostos inibitórios e influenciar a composição bioquímica da biomassa. Essa abordagem é particularmente relevante em meios de cultivo complexos, como as águas residuárias industriais (Morais, M. G. de et al., 2024; Rehman et al., 2024).

O Laboratório de Engenharia Bioquímica (LEB) desenvolve pesquisas em biotecnologia de microalgas desde 1996, com foco em biorrefinaria microalgal desde 2005. O grupo conduziu estudos envolvendo o uso de resíduos industriais como substratos para o cultivo de microalgas (Costa et al., 2002), incluindo águas residuárias (Bezerra et al., 2020; Cruz; da Rosa; Costa, 2024), gases de combustão (Braga et al., 2019), e subprodutos agroindustriais como sementes de açaí para produção de biochar (Zaparoli et al., 2023). Estudos mais recentes exploraram a aplicação de microalgas cultivadas em efluentes agroindustriais, como o soro de laticínios, bem como o desenvolvimento de produtos bio-based, incluindo bioestimulantes e biofertilizantes derivados da biomassa microalgal (Cruz et al., 2023).

Com base nesse contexto, a presente tese investiga o cultivo de *Chlorella fusca* LEB 111 e *Spirulina* sp. LEB 18 em águas residuárias cervejeiras como estratégia integrada de produção de biomassa e valorização de recursos dentro de uma estrutura de bioeconomia. Especificamente, este trabalho avalia o desempenho do cultivo e os custos de produção em

modo semicontínuo, avalia o potencial bioestimulante da biomassa resultante sobre a germinação de sementes e a tolerância ao estresse das plantas, investiga o papel do biochar derivado de sementes de açaí como agente de intensificação de processo, e sintetiza o conhecimento atual por meio de uma revisão, abordando de forma conjunta a transição do tratamento de efluentes industriais para a produção circular de bioinsumos agrícolas.

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

This work aims to establish microalgal cultivation strategies in brewery wastewater, considering process performance, biomass composition, and resource valorization within a bioeconomy framework.

2.2 SPECIFIC OBJECTIVES

- To analyze the state of the art on microalgal cultivation in brewery wastewater and its applications, with focus on biomass production, agricultural use, and resource valorization within the bioeconomy context;
- To evaluate the cultivation of *Chlorella fusca* LEB 111 in brewery wastewater under semicontinuous mode, with emphasis on biomass productivity, biochemical composition, and production cost;
- To investigate the biostimulant potential of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 biomasses produced in brewery wastewater, through the assessment of seed germination, root development, and plant responses under drought stress;
- To evaluate the effect of açaí seed-derived biochar as a process intensification strategy in the cultivation of *Chlorella fusca* LEB 111 in brewery wastewater, considering microalgal growth, nutrient removal, and biomass composition.

CHAPTER II

(Literature Review)

3 LITERATURE REVIEW

3.1 BIOECONOMY AND WASTE VALORIZATION IN THE BREWING INDUSTRY

Industrial development has played a fundamental role in economic growth and technological advancement. However, it has also intensified waste generation and environmental impacts, increasing the demand for sustainable approaches that reconcile production with resource efficiency (Oláh *et al.*, 2020). In this context, the bioeconomy has been increasingly recognized as a key strategy to promote sustainable development by integrating biological resources and processes into industrial systems. This approach supports the transition from linear production models to more circular systems, in which waste streams are converted into value-added products (Moreira, Juliana Botelho *et al.*, 2023).

Within this perspective, industrial residues are no longer viewed solely as environmental liabilities but rather as potential resources that can be reintegrated into productive cycles. The concept of waste valorization is therefore central to the bioeconomy, aiming to reduce environmental impacts while generating economic value (Rossi, D. *et al.*, 2025). Among industrial sectors, the brewing industry stands out for its high water consumption and the resulting large volumes of wastewater.

The brewing industry is characterized by intensive water use and the generation of significant volumes of wastewater, reinforcing the need for sustainable management strategies (Driessen; Vereijken, 2003; Olajire, 2020). This wastewater is characterized by a high organic load and the presence of nutrients such as nitrogen and phosphorus, which, if improperly managed, may contribute to environmental issues such as eutrophication (Ashraf; Ramamurthy; Rene, 2021).

The composition of brewery wastewater can vary significantly depending on factors such as the type of beer produced, raw materials used, and operational conditions. For instance, streams originating from fermentation and filtration stages typically contain high organic loads, while cleaning operations contribute to larger volumes with lower concentrations of organic matter but may include chemical residues (Arantes *et al.*, 2017). This variability represents a challenge for conventional treatment processes, reinforcing the need for alternative and more flexible approaches.

In addition to the environmental implications associated with wastewater discharge, the high nutrient and organic content of brewery effluents represent an opportunity for resource recovery. From a bioeconomy perspective, these effluents can be considered alternative cultivation media capable of supporting biological processes, reducing the need for freshwater

and synthetic nutrients. This approach not only mitigates environmental impacts but also contributes to the development of integrated systems in which waste streams are converted into valuable products (Marchão; Lopes, 2017; Miotti *et al.*, 2024; Schagerl *et al.*, 2022).

Thus, the valorization of brewery wastewater aligns with the principles of circularity and sustainability, enabling the integration of industrial processes with biological systems. In this context, the integration of microalgal cultivation with brewery wastewater represents a promising approach within the bioeconomy framework, enabling simultaneous resource recovery and biomass production (Silva *et al.*, 2025).

3.2 MICROALGAE AS A BIOTECHNOLOGICAL PLATFORM

Microalgae are photosynthetic microorganisms that can be either prokaryotic or eukaryotic, typically unicellular and capable of rapid growth under a wide range of environmental conditions. Through photosynthesis, they play a fundamental role in oxygen production and carbon assimilation, contributing to global biogeochemical cycles (Grobbelaar, 2013). Due to their metabolic versatility, microalgae can grow under autotrophic, heterotrophic, or mixotrophic conditions, allowing their cultivation in diverse environments and enhancing their applicability in biotechnological processes.

The biochemical composition of microalgal biomass is primarily composed of carbohydrates, proteins, and lipids, although their relative proportions vary depending on the species and cultivation conditions (Becker, 1994). This compositional variability enables the production of a wide range of compounds of industrial interest. For example, *Spirulina* sp. LEB 18 can presents 11.1% carbohydrates, 64.4% proteins, and 15.9% lipids, whereas *Chlorella fusca* LEB 111 can presents 23.5% carbohydrates, 50.5% proteins, and 26.7% lipids (Cruz *et al.*, 2023).

These macromolecules can be used as raw materials for different applications. Proteins and pigments exhibit antioxidant, anti-inflammatory, and bioactive properties, making them suitable for pharmaceutical and nutraceutical applications (Zhuang *et al.*, 2022). Carbohydrates can serve as substrates for bioethanol production (Silvello *et al.*, 2022), while lipids can be converted into biodiesel (Vyas *et al.*, 2022) or used in the development of nanomaterials and biopolymers (Cassuriaga *et al.*, 2023).

Studies have demonstrated the potential of microalgae in integrated processes combining biomass production and resource recovery. Rana *et al.* reported the use of *Chlorella* sp. S5 for the treatment of agricultural runoff, achieving nitrate and phosphate removals of 95%

and 91%, respectively, while simultaneously producing biomass suitable for biofuel production. Similarly, Khan *et al.* (2019) investigated a microalgal biorefinery approach using *Chlorella minutissima*, *Scenedesmus* spp., and *Nostoc muscorum*, demonstrating the feasibility of coupling wastewater treatment with biodiesel and biofertilizer production. These studies highlight the potential of microalgae in integrated systems focused on both environmental and industrial applications.

In addition to energy-related applications, microalgae have gained increasing attention in the agricultural sector. Microalgal biomass and extracts contain bioactive compounds such as phytohormones, amino acids, and polysaccharides, which can promote plant growth, improve nutrient uptake, and enhance tolerance to abiotic stress (Braun; Colla, 2023; Miranda *et al.*, 2024). Studies have reported improvements in seed germination, root development, and biomass production following the application of microalgal-derived products, reinforcing their potential as sustainable bioinputs (Álvarez-González *et al.*, 2022b).

From a physiological and ecological perspective, microalgae are naturally found in diverse aquatic environments and can adapt to a wide range of conditions, including variations in light intensity, temperature, pH, and salinity. Species of *Spirulina* and *Chlorella* are among the most studied due to their high productivity and robustness under different cultivation conditions. These microorganisms have been reported to tolerate environmental stresses such as high pH, salinity, and nutrient limitation, while maintaining significant biomass productivity and biochemical accumulation (Bezerra *et al.*, 2023; Braga *et al.*, 2019; Leal *et al.*, 2026).

Overall, the combination of rapid growth, metabolic flexibility, and diverse biochemical composition positions microalgae as a versatile platform for the development of sustainable biotechnological processes. Their ability to produce compounds of commercial interest and to be cultivated under different operational conditions makes them particularly suitable for integration into systems focused on resource efficiency and waste valorization, as further discussed in the following sections.

3.3 MICROALGAL CULTIVATION IN WASTEWATER AND PROCESS STRATEGIES

3.3.1 Microalgal Cultivation in Wastewater

The use of wastewater as a cultivation medium for microalgae has emerged as a promising strategy for sustainable bioprocesses. Wastewaters are typically rich in organic matter and nutrients such as nitrogen and phosphorus, which are essential for microalgal growth (Eldiehy *et al.*, 2022; Morais, M. G. de *et al.*, 2018). This composition enables the partial or

complete replacement of conventional synthetic culture media, significantly reducing production costs and resource demand (Kumar, A. K. *et al.*, 2020). In this context, the use of wastewater represents not only an alternative cultivation strategy but also an opportunity to integrate resource recovery into biomass production systems.

Among different types of industrial effluents, brewery wastewater has attracted considerable attention due to its high organic load and nutrient content. The brewing industry generates large volumes of wastewater throughout its production process, mainly from fermentation, filtration, and cleaning operations. These effluents are characterized by elevated concentrations of chemical oxygen demand (COD), biochemical oxygen demand (BOD), nitrogen, and phosphorus, making them suitable substrates for microalgal cultivation (Arantes *et al.*, 2017; Olajire, 2020). This nutrient-rich composition provides favorable conditions for microalgal growth, while also enabling the recovery of valuable compounds from waste streams.

The composition of brewery wastewater varies depending on the production stage and operational conditions, with fermentation and filtration streams presenting high organic loads and cleaning operations generating larger volumes with lower organic concentrations but potential chemical residues (Arantes *et al.*, 2017). While such variability poses challenges for conventional treatment systems, it can be advantageous for biological processes, as microalgae are capable of metabolizing a diverse range of substrates. By utilizing available nutrients and organic compounds for growth, microalgae enable the integration of biomass production with resource recovery, reducing dependence on freshwater and synthetic inputs while minimizing the environmental impacts associated with effluent discharge (Álvarez-González; Castro; *et al.*, 2025). In particular, brewery wastewater may support mixotrophic cultivation, a metabolic mode in which microalgae simultaneously assimilate inorganic carbon through photosynthesis and organic carbon from the medium, potentially enhancing both biomass productivity and biochemical accumulation compared to strictly autotrophic conditions (Dragone, 2022).

Studies have demonstrated the feasibility of microalgal cultivation in wastewater systems. Schagerl *et al.* (2022) reported the cultivation of microalgae in brewery wastewater with up to 95% COD reduction, highlighting the efficiency of this approach at laboratory scale. Similarly, Song *et al.* (2020) achieved biomass concentrations of approximately 1.02 g L^{-1} after 10 d of cultivation of *Scenedesmus* sp., along with significant removal of nitrogen (75.96%), phosphorus (95.71%), and COD (73.66%). These results reinforce the potential of microalgal systems to simultaneously support biomass production and nutrient recovery, demonstrating their applicability in integrated and sustainable processes.

Overall, the use of wastewater as a culture medium represents a key approach for improving the sustainability of microalgal processes. By enabling the integration of waste streams into productive systems, this strategy aligns with the principles of bioeconomy and waste valorization, while also providing the foundation for the development of optimized cultivation strategies, as discussed in the following section (Pessôa *et al.*, 2021). Nevertheless, the variability of wastewater composition, the presence of inhibitory compounds, and operational constraints continue to limit large-scale implementation, reinforcing the need for more robust and adaptable process strategies (Acién-Fernández *et al.*, 2012; Acién Fernández; Gómez-Serrano; Fernández-Sevilla, 2018).

3.3.2 Operational Strategies for Microalgal Cultivation

Microalgal cultivation can be performed under different operational strategies, including batch, fed-batch, semicontinuous, and continuous modes. The selection of the cultivation strategy plays a critical role in determining biomass productivity, nutrient utilization, and biochemical composition, directly influencing the overall performance and feasibility of the process (Schmidell *et al.*, 2001).

Batch cultivation is the most commonly used approach at the laboratory scale due to its simplicity and ease of operation. In this mode, nutrients are supplied at the beginning of the process, and microalgal growth follows a typical pattern consisting of lag, exponential, and stationary phases. Although batch systems are suitable for controlled studies, they often present limitations related to nutrient depletion and reduced productivity over time (Dragone, 2022).

Fed-batch cultivation represents an intermediate strategy in which nutrients are added during the process to prolong the exponential growth phase and enhance biomass accumulation. Rosa, Morais, and Costa (2019) demonstrated that the addition of monoethanolamine (MEA) in fed-batch cultivation of *Chlorella fusca* LEB 111 resulted in increased dissolved inorganic carbon availability and improved protein and lipid productivity. These results highlight the potential of controlled nutrient addition to modulate metabolic pathways and improve process performance.

Semicontinuous cultivation has been widely explored as a strategy to increase productivity and maintain stable growth conditions. In this approach, part of the culture medium is periodically removed and replaced with fresh medium, allowing for sustained nutrient availability and light penetration. Ramírez-López, Perales-Vela, and Fernández-Linares (2019) reported that semicontinuous cultivation of *Chlorella vulgaris* with medium renewal rates of

15–20% every three days improved biomass productivity by enhancing light distribution and nutrient availability. Similarly, Rosa, Morais, and Costa (2022) demonstrated that semicontinuous cultivation of *Chlorella fusca* LEB 111 enabled higher lipid accumulation when combined with appropriate medium renewal strategies.

Continuous cultivation offers the potential for maximum productivity under steady-state conditions, as nutrients are continuously supplied and biomass is simultaneously harvested. However, despite its theoretical advantages, continuous systems require precise control of operational parameters and are more susceptible to contamination and system instability, which limits their widespread application, particularly at smaller scales (Schmidell *et al.*, 2001).

In addition to the cultivation mode, reactor configuration also plays a significant role in microalgal production. Photobioreactors provide controlled conditions of light, temperature, and mixing, enabling higher biomass concentrations and process reproducibility (Deprá *et al.*, 2019). Among them, tubular and airlift photobioreactors are commonly used for their efficient mass transfer and light utilization. In contrast, open systems such as raceway ponds are widely applied at large scale due to their lower operational costs, despite presenting limitations in terms of environmental control (Masojídek *et al.*, 2023).

The choice of operational strategy is therefore closely linked to the desired process outcomes. Different cultivation modes and reactor configurations can significantly influence not only biomass productivity but also the biochemical composition of microalgae. For instance, nutrient limitation, light availability, and operational conditions can shift metabolic pathways toward lipid or carbohydrate accumulation, depending on the process design (Becker, 1994).

Overall, the optimization of microalgal cultivation strategies is essential to enhance process efficiency and ensure scalability. The selection and combination of appropriate operational modes and reactor configurations enable the development of more robust and productive systems, particularly when applied to complex media such as industrial wastewater. These aspects are fundamental for advancing microalgal technologies toward practical and economically viable applications (Costa *et al.*, 2019). However, the translation of these strategies to large-scale systems remains limited by operational complexity and cost constraints.

3.4 PROCESS INTENSIFICATION AND BIOMASS VALORIZATION

3.4.1 Process Intensification Strategies

Process intensification has gained increasing relevance in microalgal biotechnology as a strategy to overcome limitations associated with light distribution, nutrient availability, and mass transfer, particularly in systems based on industrial wastewater. Recent advances in microalgae-based processes have emphasized the need for integrated and efficient approaches capable of enhancing biomass productivity while maintaining system stability and scalability (Patle; Gautam; Kumar, 2023).

In this context, process intensification strategies have evolved from conventional operational optimization toward more complex and integrated solutions, including reactor design improvements, nutrient modulation, and the incorporation of functional materials (Kumar, V.; Nigam, 2012). According to recent studies, intensification in microalgal systems is not limited to increasing productivity but also involves improving resource efficiency and enabling the coupling of biomass production with value-added applications (Minhas *et al.*, 2026).

Among these approaches, the use of auxiliary materials has emerged as a promising pathway for enhancing microalgal cultivation performance. Materials such as biochar, activated carbon, and other porous substrates can act as adsorbents, nutrient reservoirs, and structural supports, modifying the physicochemical environment of the culture and influencing microalgal growth dynamics (Comitre *et al.*, 2021; Shenk *et al.*, 2022).

Biochar has received particular attention due to its physicochemical properties, including high surface area, porosity, and the presence of functional groups that enable interactions with both organic and inorganic compounds. It is typically produced through the thermochemical conversion of biomass under oxygen-limited conditions, resulting in a carbon-rich material with strong adsorption capacity (Shackley *et al.*, 2016). Recent advances in biochar production from microalgae and other biomass sources have highlighted its potential for environmental and biotechnological applications, including wastewater treatment and integrated cultivation systems (Ghosh *et al.*, 2024).

When applied to microalgal cultivation systems, biochar can play multiple roles. It can enhance nutrient retention, particularly nitrogen and phosphorus, improving nutrient availability and uptake efficiency. Additionally, biochar has been reported to adsorb inhibitory compounds present in wastewater (Huang, X. *et al.*, 2020), contributing to increased culture stability and reduced stress conditions. These effects are particularly relevant in wastewater-based systems, where variability in composition can negatively impact microalgal performance.

Beyond its role in nutrient dynamics, biochar can also influence microalgal metabolism and biomass composition. Studies have demonstrated that the presence of biochar can alter

metabolic pathways, promoting the accumulation of specific compounds such as lipids, depending on the cultivation conditions (Morais, M. G. de *et al.*, 2024). Furthermore, integrated systems combining biochar and microalgae have shown synergistic effects, where biochar acts both as a biosorbent and as a facilitator of microalgal growth, improving overall system efficiency (Rehman *et al.*, 2024).

Despite these advantages, the performance of biochar-based intensification strategies depends on several factors, including feedstock type, pyrolysis conditions, particle size, and concentration applied in the system. These parameters directly affect the surface chemistry and interaction mechanisms, making system-specific optimization essential for achieving consistent results (Mustapha; Isa, 2025). These approaches are especially relevant in wastewater-based processes, where system complexity requires adaptable and resilient solutions. In this context, the incorporation of biochar into microalgal systems not only enhances process performance but also contributes to the development of integrated and sustainable biotechnological platforms.

3.4.2 Biomass and By-Product Valorization in Microalgal Systems

As discussed in Section 3.2, the biochemical composition of microalgal biomass underpins its wide range of applications. In this context, biomass valorization is strongly dependent on cultivation conditions and process design, which regulate metabolic pathways and determine the accumulation of compounds of interest. Variations in nutrient availability, light intensity, and operational strategies directly influence metabolic responses, enabling the selective accumulation of lipids, carbohydrates, and bioactive molecules (Becker, 1994). This relationship between cultivation strategy and biomass composition is particularly relevant in systems based on industrial effluents, where medium composition plays a key role in shaping metabolic responses (Silva, A. L. e *et al.*, 2025).

In the context of agricultural applications, it is important to distinguish between biofertilizers and biostimulants. Biofertilizers are formulations containing living microorganisms that enhance nutrient availability through mechanisms such as biological nitrogen fixation, phosphorus solubilization, and rhizosphere support (Alori; Glick; Babalola, 2017; Nosheen; Ajmal; Song, 2021; Yadav; Yadav, 2024). Biostimulants, in contrast, are non-nutrient substances or microbial products that modulate plant physiology, improving nutrient use efficiency, stress tolerance, and crop quality when applied at low doses (Quille *et al.*, 2025).

Microalgal biomass and extracts can function as biostimulants, given their content of bioactive compounds capable of influencing plant growth and stress responses.

The agricultural applicability of microalgal biomass is closely linked to its phytohormonal and bioactive composition, which can be directly influenced by cultivation conditions, including the nature of the growth medium. Microalgal extracts have been shown to contain a diverse array of plant growth regulators, including cytokinins (trans-zeatin, zeatin riboside, isopentenyl adenine), gibberellins (GA1, GA3, GA4), auxins (indole-3-acetic acid), salicylic acid, jasmonic acid, and abscisic acid. These compounds play central roles in regulating seed germination, root architecture, and plant responses to abiotic stress (Braun; Colla, 2023; Miranda *et al.*, 2024). When applied as biostimulants, these extracts have demonstrated agronomic effects including enhanced germination rates, increased root elongation, improved shoot biomass accumulation, and sustained photosynthetic activity under drought conditions. Crops such as barley (*Hordeum vulgare* L.) and lettuce (*Lactuca sativa* L.) have been reported as responsive models for evaluating these effects, highlighting the potential of wastewater-grown microalgal biomass to support plant development and stress tolerance under field-relevant conditions (Álvarez-González *et al.*, 2022a; Morillas-España *et al.*, 2024). Importantly, the composition of the cultivation medium, including industrial effluents such as brewery wastewater, may act as a metabolic driver influencing phytohormonal biosynthesis in microalgae, thereby modulating the biostimulant potential of the resulting biomass (Conceição, Jenyfer de Almeida; *et al.*, 2026). Rather than representing a single-product system, microalgal processes enable the generation of multiple value streams depending on cultivation and downstream strategies.

Beyond biomass valorization, increasing attention has been given to the utilization of by-products and auxiliary materials associated with microalgal cultivation systems. Among these, biochar has emerged not only as a process intensification agent but also as a potential value-added product. Due to its physicochemical properties, including high porosity, large surface area, and nutrient retention capacity, biochar has been widely investigated for environmental and agricultural applications (Sridhar; Manian, 2025). When derived from biomass residues, such as açaí seeds, biochar represents an additional pathway for waste valorization within the system. Its application in soil has been associated with improvements in nutrient retention, water holding capacity, and microbial activity, contributing to enhanced plant growth and soil quality (Zaparoli *et al.*, 2023). These characteristics position biochar as a complementary product that can extend the value chain of microalgal-based processes.

From a systems perspective, the integration of microalgal cultivation with waste-derived

materials enables the development of more complex and sustainable production schemes. In such systems, industrial effluents can be used as cultivation media, supporting biomass production, while process additives such as biochar can improve cultivation performance and subsequently be redirected to agricultural applications. This integrated approach allows multiple outputs to be generated from a single system, increasing overall process efficiency and resource utilization (Uggetti *et al.*, 2018).

In addition, the potential reuse of treated water from microalgal cultivation systems represents another dimension of resource recovery. Although the degree of treatment depends on operational conditions, the reduction of nutrient and organic load may enable its reuse in agricultural or industrial applications, contributing to water conservation strategies (Akdag *et al.*, 2026).

These interconnected pathways illustrate how microalgal systems can be positioned within a bioeconomy framework. Rather than focusing solely on biomass production, these systems enable the simultaneous valorization of multiple streams, including biomass, by-products, and treated water (Morillas-España *et al.*, 2024). This integrated perspective reinforces the role of microalgae as a platform for sustainable and circular biotechnological processes, highlighting the transition from single-product systems toward multi-output bioprocesses.

Overall, the valorization of biomass and associated products represents a key component in advancing microalgal technologies toward practical applications. By enabling the conversion of low-cost or waste-derived inputs into valuable outputs, these systems contribute to the development of integrated processes aligned with bioeconomy principles and circular resource use (Kumar, A. K. *et al.*, 2020). Despite the potential of integrated systems, challenges related to process standardization, scalability, and regulatory frameworks still limit their widespread application.

In this context, the development of integrated approaches combining microalgal cultivation and agricultural applications has also led to technological innovations, including the formulation of bio-based products derived from wastewater-grown microalgae, as proposed in recent patent developments derived from this research (Conceição, Jenyfer de Almeida; *et al.*, 2026).

CHAPTER III

(Research Development)

RESEARCH DEVELOPMENT

The present thesis was structured into four complementary studies developed throughout the doctoral period, aligned with the research lines of microalgal biotechnology, resource valorization, and biorefinery. The studies were primarily conducted at the Laboratory of Biochemical Engineering (LEB), Federal University of Rio Grande (FURG), with additional contributions developed during an international research mobility period.

The first study consisted of a literature review addressing the use of microalgae cultivated in brewery wastewater, focusing on biomass production, biochemical composition, agronomic applications, and commercialization aspects. This study aimed to establish the current state of the art and identify knowledge gaps related to the integration of wastewater-based cultivation and biomass valorization, providing the theoretical foundation for the experimental studies that followed.

Building on this framework, the second study evaluated the cultivation of *Chlorella fusca* LEB 111 in brewery wastewater under semicontinuous conditions, investigating the effects of effluent concentration on biomass productivity, biochemical composition, and production cost. This study was conducted prior to the level upgrade from master's to doctoral degree, a transition that expanded the research scope and allowed for the development of the subsequent studies presented in this thesis, contributing to the assessment of brewery wastewater as an economically viable and sustainable alternative cultivation medium.

The third study investigated the biostimulant potential of microalgal biomass produced in brewery wastewater, representing the transition from biomass production toward functional biomass, defined here as biomass with demonstrated capacity to modulate plant physiological responses. The effects of *Chlorella fusca* LEB 111 and Spirulina sp. LEB 18 extracts were evaluated on seed germination, root development, and plant responses under drought stress. This study was predominantly developed during an international academic mobility at the Universitat Politècnica de Catalunya (UPC), contributing to the integration of agronomic validation and biochemical characterization within the thesis framework.

The fourth study focused on process intensification through the integration of açaí seed-derived biochar in the cultivation of *Chlorella fusca* LEB 111. The effects of biochar on growth performance, nutrient dynamics, biomass composition, and biochar properties were evaluated, aiming to understand its role as a nutrient-modulating, adsorptive, and process-stabilizing component in wastewater-based microalgal systems. This study was developed within the

framework of the CHARMI2 project, in collaboration with the Universitat Politècnica de Catalunya (UPC).

Together, these studies contribute to the development of an integrated approach in which brewery wastewater is used as a cultivation medium to produce microalgal biomass and value-added products, collectively addressing the transition from industrial effluent treatment toward the circular production of agricultural bioinputs.

This thesis resulted in four scientific articles and one patent, as summarized below:

- **Article 1:** Turning Brewery Effluents into Agricultural Inputs: The Potential of Microalgal Biofertilizers and Biostimulants
- **Article 2:** Exploiting Brewery Wastewater for the Sustainable Cultivation of *Chlorella fusca*: Biomass Productivity, Biochemical Composition, and Production Cost
- **Article 3:** Biostimulant Effects of Microalgae Cultivated in Brewery Effluent on Seed Germination and Plant Growth
- **Article 4:** Effects of Açaí Seed-Derived Biochar on Growth, Biomass Properties, and System Performance in Brewery Wastewater Cultivation of *Chlorella fusca* LEB 111
- **Patent:** Processo de Obtenção de Biomassa de *Chlorella fusca* LEB 111 e *Spirulina* sp. LEB 18 Cultivadas em Efluente Cervejeiro e Seu Uso Como Bioestimulante e Biofertilizante / Process for Obtaining Biomass of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 Cultivated in Brewery Wastewater and Its Use as Biostimulant and Biofertilizer

ARTICLE I

TURNING BREWERY EFFLUENTS INTO AGRICULTURAL INPUTS: THE POTENTIAL OF MICROALGAL BIOFERTILIZERS AND BIOSTIMULANTS

ABSTRACT

Microalgae cultivated in brewery wastewater have emerged as a sustainable strategy to couple effluent remediation with the production of agricultural bioinputs. Brewery effluents contain high organic loads and variable nutrient concentrations, requiring effective treatment to mitigate environmental impacts. Studies show that microalgae efficiently remove nitrogen, phosphorus, and organic matter while generating biomass enriched with proteins, carbohydrates, pigments, phytohormones, and antioxidant compounds. This biochemical profile supports the use of wastewater-grown biomass as a biofertilizer or biostimulant, with documented benefits for germination, root development, nutrient uptake, photosynthetic performance, and plant tolerance to drought and salinity. Agronomic data indicate that biomass derived from brewery wastewater can partially or fully replace mineral fertilizers during early crop development, including in barley, a crop central to the brewing chain. Compared with conventional inputs, microalgal biomass offers combined nutrient provision and physiological stimulation, representing an alternative to both mineral fertilizers and seaweed-based biostimulants. Despite these advances, large-scale adoption faces regulatory, technological, and market constraints. Heterogeneous definitions and registration pathways hinder commercialization, while fluctuations in wastewater composition affect biomass consistency. Emerging innovations, including encapsulation technologies, nano-enabled formulations, and integrated biorefineries, may improve stability, efficacy, and scalability. Within a circular bioeconomy perspective, brewery wastewater provides a strategic cultivation medium that enables decentralized production, reduces treatment costs, and valorizes industrial residues. This review synthesizes current knowledge on remediation performance, biomass composition, agronomic responses, and commercialization prospects, highlighting the potential of brewery wastewater–grown microalgae to support sustainable agriculture and resource recovery.

1. INTRODUCTION

The brewing industry generates large volumes of effluents with high organic loads, requiring treatment technologies capable of handling constant fluctuations in effluent composition while maintaining the economic viability of the process (Amenorfenyo *et al.*, 2019). There are more than 19,000 breweries worldwide, of which 94% are craft breweries

(Dias *et al.*, 2023). Microbreweries, although essential to the sector's growth, face significant challenges in waste management due to limited space, high energy consumption, and the costs associated with effluent treatment (Colpo; Funck; Martins, 2022; Veleva *et al.*, 2024). In addition, inadequate effluent disposal can cause eutrophication and severe environmental impacts (Malone and Newton 2020), underscoring the need for sustainable solutions.

Microalgae have been widely studied as a promising alternative for effluent bioremediation due to their capacity to remove nutrients and reduce eutrophication processes (Costa; Cruz; Rosa, 2021). These photosynthetic organisms metabolize nutrients under adverse conditions and produce biomass rich in compounds of interest, which makes cultivation in wastewater economically viable compared to synthetic culture media (Morais, M. G. de *et al.*, 2018). Several studies demonstrate that integrating effluent treatment with microalgal production can simultaneously reduce pollutant loads and generate high-value biomass (Álvarez-González; Castro; *et al.*, 2025; Cruz; da Rosa; Costa, 2024; Silva, P. H. B. de S. *et al.*, 2025).

Other highlights of microalgal cultivation include the ability to remove nutrients from wastewater and to biofix carbon dioxide (CO_2), a process that reduces greenhouse gas emissions while sustaining cell growth (Cheah *et al.*, 2015; Morais, M. G. de; Costa, 2007). When cultivated in industrial effluents, such as brewery wastewater, these characteristics complement each other and reinforce the concept of circular economy by simultaneously enabling the valorization of liquid waste, CO_2 biofixation, and the production of high-value biomass. The resulting biomass can be used within the microalgal biorefinery concept, as it is rich in bioactive compounds, such as amino acids, polysaccharides, antioxidants, and phytohormones, which present recognized potential as biofertilizers and biostimulants (Braun; Colla, 2023; Pessôa *et al.*, 2021).

In this context, an opportunity emerges for the brewing sector: the cultivation of microalgae in brewery wastewater for subsequent application of the biomass as a biofertilizer or biostimulant in the crops that comprise the brewery production chain, such as barley and hops. This strategy integrates waste remediation, nutrient recovery, CO_2 capture, and the supply of agricultural inputs in a circular manner, closing the loop between production, treatment, valorization, and return to the production chain (Chabili *et al.*, 2024; Silva, A. L. e *et al.*, 2025).

Microalgal biofertilizers and biostimulants have shown potential to improve plant performance across different cultivation conditions, owing to the diversity of bioactive metabolites in their biomass. Microalgal extracts contain phytohormones, amino acids, peptides, sulfated polysaccharides, and antioxidants that modulate plant development,

including root architecture, cell division and elongation, seed germination, and stomatal regulation (Rupawalla *et al.*, 2022; Sun *et al.*, 2024). Recent studies show consistent benefits of these biostimulants in germination assays, tolerance to salinity and water deficit, maintenance of photosynthetic efficiency (ΦPSII, ETR), higher stomatal conductance, and biomass retention, as demonstrated by Vangenechten et al. (2025). Furthermore, there is evidence that microalgal biomass cultivated in wastewater can act as an effective biofertilizer, increasing the growth and nutritional content of vegetables (Álvarez-González; Serrano; *et al.*, 2025), while extracts obtained from cultures in industrial effluents promote greater germination and seedling vigor (Ruales *et al.*, 2025). Despite recent advances, few reviews specifically and integratively address the use of microalgae cultivated in brewery wastewater as a source of biofertilizers and biostimulants, articulating remediation, biomass composition, and agronomic performance in a circular economy context.

While previous reviews have focused primarily on bioremediation performance and biomass productivity in brewery wastewater systems, the present work extends this scope by critically addressing the agricultural applicability of the produced biomass, including its comparison with conventional commercial inputs, the regulatory constraints governing its use, and the market perspectives for its valorization. These dimensions collectively constitute the distinctive contribution of this review toward advancing the field from process-oriented knowledge to application-ready frameworks. The objective of this review is to analyze the use of brewery effluents in microalgal cultivation, with a focus on remediation, biofertilizer and biostimulant production, and perspectives for agricultural application within the circular bioeconomy.

2. MICROALGAE IN BREWERY WASTEWATER REMEDIATION

2.1 INDUSTRIAL WASTEWATER

Industrial liquid effluent refers to wastewater generated by productive activities, primarily from areas that transform raw materials into finished products (CONSEMA, 2017). This effluent is nutrient-rich, posing a risk of eutrophication in water bodies (Malone; Newton, 2020).

The primary objective of wastewater treatment is to improve water quality, enabling reuse or minimizing environmental impact when discharged into natural ecosystems. In addition to reducing contamination below regulatory limits, treatment processes provide

alternative sources of freshwater, such as river maintenance during droughts and aquifer recharge, thereby supporting the sustainability of groundwater supplies. Treated water is also used in agroforestry systems and aquaculture, enhancing river quality and ecosystem health while alleviating pressure on conventional water resources (Ahmed, J.; Thakur; Goyal, 2021).

Jones et al. (2021) conducted a global assessment of wastewater production, collection, treatment, and reuse. The study estimates that approximately 359.4 billion m³ of wastewater are generated annually worldwide, with an average of 49.0 m³ per person. Of this total, around 225.6 billion m³ are collected, and 188.1 billion m³ are treated, corresponding to 63% and 52% of the total generated, respectively. Moreover, about 84% of the collected wastewater undergoes effective treatment. Wastewater reuse is estimated at 40.7 billion m³ per year, which represents approximately 11% of the total volume produced and 22% of the treated volume. Meanwhile, 147.4 billion m³ of treated wastewater are discharged into the environment, compared to 171.3 billion m³ released without any treatment.

In this context, several regulations govern the discharge of effluents, such as the European Union Council Directive 91/271/EEC, which addresses the collection, treatment, and discharge of urban wastewater in non-sensitive areas (García-Cambero *et al.*, 2019). Therefore, pretreatment is required before effluent release, and one promising alternative is the cultivation of microalgae in wastewater (Cruz *et al.*, 2023).

The ability of microalgae to metabolize toxic components has been investigated as a bioremediation method for both industrial and domestic effluents (Álvarez-González; Castro; *et al.*, 2025; Biswas *et al.*, 2021; Costa; Cruz; Rosa, 2021; Kumar, A. K. *et al.*, 2020). According to Kumar et al. (2020), the initial goal of cultivating microalgae in wastewater was to reduce biomass production costs, but this concept has since expanded. The integration of microalgae cultivation with wastewater yields remarkable results, including eliminating freshwater use, enabling low-cost effluent bioremediation, and generating algal biomass suitable for biofuel, bioenergy, and animal feed production. It also opens possibilities for the development of other high-value products.

Recent advances have demonstrated the feasibility of integrating microalgae-based wastewater treatment with the production of biostimulants and subsequent agricultural use. Morillas-España et al. (2024) evaluated an 80 m² raceway reactor operated with a microalgae–bacteria consortium treating urban wastewater and compared its performance with an identical system supplied with freshwater and commercial fertilizers. The wastewater-based system achieved efficient nutrient removal, with average nitrogen removal rates of 1.35 g $m^{-2} \cdot day^{-1}$, of which 60–95% corresponded to assimilation into biomass, and phosphorus removal ranging

from 0.15 to 0.68 g $m^{-2} \cdot day^{-1}$. The treated effluent supported the hydroponic cultivation of zucchini seedlings, increasing root dry weight and stem diameter compared with water alone, and the produced biomass showed potential as a feedstock for biostimulant formulations. These findings reinforce the applicability of microalgae-based wastewater treatment as a multifunctional platform that simultaneously enables nutrient recovery, biomass generation, and agricultural valorization.

According to Acién Fernández et al. (2018), using microalgae for wastewater treatment can reduce energy consumption by up to 50% compared to conventional methods, while enabling the recovery of up to 90% of the nutrients present in the effluent. Among various types of effluents, brewery wastewater contains high concentrations of organic compounds that can significantly contribute to environmental pollution (Amenorfenyo *et al.*, 2019; Silva, P. H. B. de S. *et al.*, 2025).

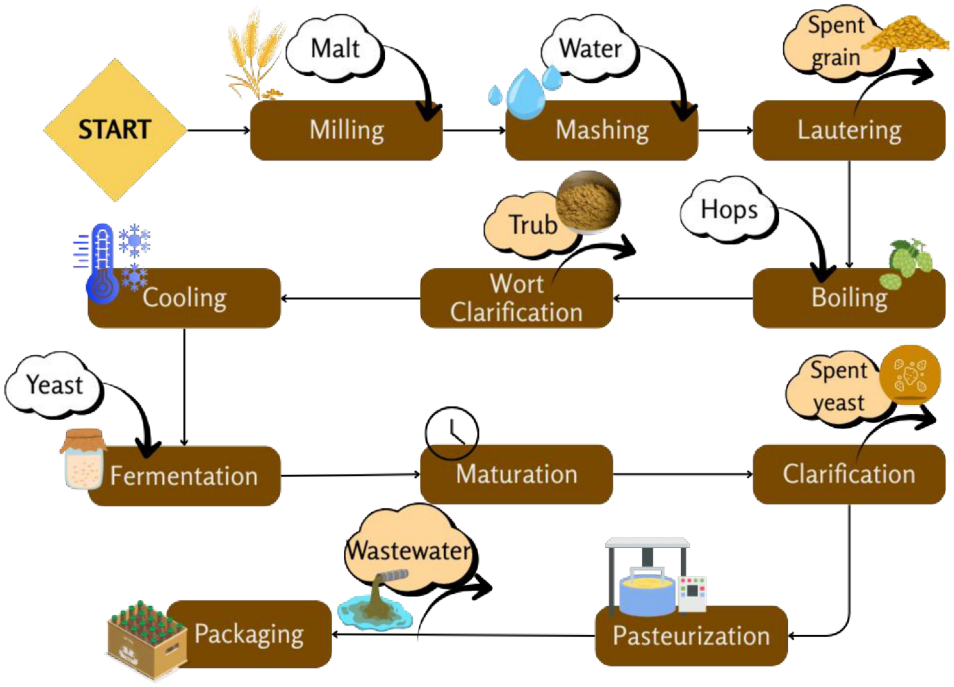
2.2 BREWERY WASTEWATER

Beer is the most widely consumed low-alcohol beverage in many countries every year. The brewing industry is a globally significant sector with substantial economic importance, generating enormous annual revenues. Global beer consumption reached 187.9 million kiloliters in 2023. China remained the largest beer-consuming country for the 21st consecutive year, and Asia accounted for 32.0% of global consumption, followed by Europe (25.8%) and Central/South America (19.7%) (Kirin Holdings Company, 2024).

Water is a fundamental component of beer production, accounting for 90–95% of the beverage's total mass. It is used in various stages of the brewing process, including cleaning operations. Water consumption varies greatly depending on factors such as the type and size of the brewery, packaging and cleaning methods, and other factors. Efficient breweries may use between 4 and 7 liters of water per liter of beer produced. Additionally, water is used for heating, cooling, and equipment cleaning. However, brewing operations also generate considerable wastewater, with estimates ranging from 3 to 10 liters of effluent per liter of beer produced (Driessen; Vereijken, 2003; Olajire, 2020).

Beer production involves multiple stages, culminating in yeast fermenting sugars from malt into alcohol and carbon dioxide. Proper management of these wastes can yield economic benefits and help protect the environment from pollution caused by excessive accumulation (Rachwał *et al.*, 2020). The brewing process and its associated waste generation are illustrated in Figure 1.

Figure 1 - Schematic representation of the brewing process and the generation of residues at each production stage. Adapted from Rachwał et al. (2020)



According to Ferreira et al. (2011), the composition of brewery effluents varies depending on the type of beer produced, the ingredients used (such as yeast, malt, and corn), and the efficiency of equipment cleaning processes. An example of this variability can be observed in two distinct stages of the brewing process. The first occurs during fermentation and filtration, which generate approximately 3% of the total effluent volume but account for nearly 97% of the total organic load, including malt and barley residues. The second stage involves bottle washing, which produces a large volume of effluent with a low organic load, although it may contain chemical residues (Arantes *et al.*, 2017). Table 1 provides an overview of the approximate composition of this effluent, including values for Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), total Nitrogen (N), and total Phosphorus (P).

Table 1 – Physicochemical Parameters (mg L^{-1}) of Brewery Wastewater Reported in the Literature. *Information not reported by the authors. Adapted from Arantes et al. (2017)

COD	BOD	N	P	Reference
2000-6000	1200-3600	25-80	10-50	Simate et al., 2011
6000	2350	90	0.5	Sinbuathong et al. 2015
2000	1877	22	21	Enitan et al. 2015
226-4000	*	22.78-69	1.15-18.45	Silva et al. 2025b
1250-20000	1200-36000	0.0196-80	10-50	Ashraf et al. 2021

The byproducts of this wastewater, such as spent grains from fermentation, are among the main contributors to environmental pollution when discharged as effluents. Therefore, the treatment of brewery wastewater using chlorophyte microalgae has been studied as an efficient and promising alternative (Amenorfenyo *et al.*, 2019).

Han et al. (2021) investigated the purification efficiency of integrated brewery wastewater treatment via co-cultivation of Scenedesmus sp. 336 and Chlorella sorokiniana UTEX1602 for CO_2 fixation. The authors concluded that microalgae enhanced the bioremediation performance for brewery effluents. They reported reductions of N, P, and COD by up to 90.57%, 97.37%, and 78.83%, respectively. Additionally, they identified the accumulation of high-value biocompounds, including 16.05 mg L^{-1} of chlorophyll, 9.57 mg L^{-1} of carotenoids, 30.42 mg L^{-1} of carbohydrates, 20.82 mg L^{-1} d^{-1} of lipids, and 142.12 mg L^{-1} of proteins.

Song et al. (2020) studied the purification of brewery effluents and CO_2 fixation through microalgal cultivation of *Scenedesmus sp.* 336. After 10 days of cultivation, biomass production reached 1.02 g L^{-1}, with removal rates of 75.96% for total nitrogen, 95.71% for total phosphorus, and 73.66% for COD in the brewery effluent. The authors suggested that simultaneous effluent purification and CO_2 fixation can be achieved through proper strain selection and cultivation condition optimization.

Silva, Arthur Lima *et al.* (2025) assessed the bioremediation of brewery wastewater through pilot-scale cultivation of *Arthrospira* sp. at a brewery facility. Using an inoculum concentration of 0.5 g L^{-1}, the authors scaled up lab tests to a 300 L open-raceway pond operated semi-continuously for 100 days, reaching an average biomass productivity of 10.68 g m^{-2} day^{-1}. The system achieved nutrient removal efficiencies of 56.8% for nitrate, 100% for ammonium, and 78.7% for sulfate. Preliminary biofertilization tests showed that replacing 50% or 100% of a commercial mineral fertilizer with raw *Arthrospira* biomass improved barley growth by about 60% compared to using the fertilizer alone, demonstrating the potential of wastewater-grown biomass as a biofertilizer in circular production systems.

3. PRODUCTION OF BIOFERTILIZERS AND BIOSTIMULANTS FROM MICROALGAL BIOMASS

Microalgal biomass cultivated in brewery wastewater has gained attention as a potential agricultural input due to its content of proteins, polysaccharides, lipids, and phytohormones such as auxins, gibberellins, abscisic acid, and cytokinins (O'Rourke; Gaffney; Murphy, 2016;

Raposo *et al.*, 2012). These compounds are associated with plant growth promotion, nutrient uptake, and stress tolerance, giving the biomass both biostimulant and biofertilizer potential. Because cultivation conditions and wastewater characteristics strongly influence the biochemical profile of microalgae, biomass composition can vary significantly across effluent types (Papadopoulos *et al.*, 2022; Yirgu *et al.*, 2023).

Studies using domestic, municipal, and industrial wastewaters have consistently shown positive agronomic effects (including enhanced germination, early vigor, and nutrient use) supporting the feasibility of wastewater-grown microalgae as sustainable agricultural inputs (Cruz *et al.*, 2023). These systems supply a nutrient environment that shapes the biochemical profile of the resulting biomass and influences agronomic responses such as germination, biomass accumulation, and stress tolerance. Overall, the literature indicates that wastewater-grown biomass can partially replace conventional mineral inputs and improve crop performance without compromising safety (Alling; Funk; Gentili, 2023).

Li et al. (2021) reported that *Phaseolus vulgaris* seeds responded positively to extracts of *Chlorella pyrenoidosa* enriched with selenium and cultivated in domestic wastewater, resulting in higher germination rates and increased productivity. Similar results were reported by Álvarez-González *et al.* (2022), who tested microalgae cultivated in municipal wastewater as a fertilizer for basil. Treatments using microalgal biomass alone or in combination with inorganic fertilizer increased leaf dry weight compared with inorganic fertilizer alone, with distinct effects on root morphology and nutrient accumulation. The same authors later (Álvarez-González; Uggetti; *et al.*, 2023) demonstrated that applying wastewater-grown biomass to lettuce did not increase the transfer of heavy metals or CECs to edible tissues, indicating agronomic safety.

Additional studies confirm the potential of microalgae cultivated in animal-industry wastewaters and show that even exposure to pharmaceutical contaminants does not preclude biostimulant activity. Dagnaisser et al. (2024) assessed *Arthrospira platensis* cultivated in dairy cattle wastewater as a nitrogen-rich biofertilizer for arugula (*Eruca vesicaria*). The microalgal treatment produced plants with a Dickson Quality Index comparable to urea-fertilized plants, confirming its agronomic potential. Morillas-España et al. (2025) cultivated *Scenedesmus almeriensis* in wastewater containing pharmaceutical contaminants. They found that the resulting biomass retained biostimulant activity in germination and root-induction assays, demonstrating its applicability even under contaminant exposure.

The biochemical composition of microalgae grown in brewery wastewater reflects the effluent's high nutrient content, particularly its elevated organic load and nitrogen and

phosphorus levels (Silva *et al.*, 2025). These conditions typically stimulate the accumulation of pigments, proteins, carbohydrates, and bioactive metabolites linked to biostimulant activity. This composition is relevant because it underpins the agronomic effects observed in subsequent applications to crops (Navarro-López *et al.*, 2020).

Studies with *Scenedesmus sp.*, *Chlorella sorokiniana*, and *Arthrospira* sp. cultivated in brewery wastewater consistently show increases in chlorophylls, carotenoids, carbohydrates, and proteins, alongside reductions in organic and inorganic pollutants (Han, Xiaoxuan *et al.*, 2021; Song *et al.*, 2020a). Papadopoulos et al. (2022) cultivated *Arthrospira platensis* in brewery wastewater and reported the highest biomass yield (950 mg L^{-1}) when NaCl and $NaHCO_3$ were added under a 16:8 light–dark cycle. Carbohydrate and lipid contents reached ~34% and 19%, respectively. Yap et al. (2022) optimized *Haematococcus pluvialis* cultivation using synthetic brewery wastewater. They found that cultures at 50% BW under red light and pH 7.5 yielded the highest biomass and protein content, with detectable 45–60 kDa protein bands indicative of RuBisCO and glycoproteins. Yirgu et al. (2023) cultivated *Scenedesmus* sp. in sterilized and non-sterilized anaerobically digested brewery wastewater. Non-sterilized cultures yielded more biomass, while sterilized ones accumulated more lipids (14.79% DW). COD, TN, NH_4^+-N, and TP removal efficiencies met national discharge standards except for TP.

Biomass produced under these conditions, therefore, combines environmental remediation with biochemical attributes of agronomic interest, reinforcing the relevance of brewery wastewater as a suitable medium for both treatment and bioinput production. Pozzobon et al. (2024) cultivated *Chlorella vulgaris* in diluted brewery wastewater supplemented with micronutrients and obtained biomass with 24–27% lipids, 26–36% carbohydrates, and 32–34% proteins. Barati et al. (2025) grew *Arthrospira platensis* in brewery effluent and showed that red light enhanced biomass productivity (0.249 g L^{-1} day^{-1}) and phycocyanin production (223.9 mg g^{-1} day^{-1}).

The agronomic performance of microalgal biomass cultivated in brewery wastewater has been evaluated in germination assays, plant growth studies, and preliminary fertilization trials (Miotti *et al.*, 2024). These studies collectively indicate that brewery effluent-grown microalgae can enhance seedling vigor, promote root development, and contribute to nutrient supply, supporting their use as biofertilizers or biostimulants under a circular-economy approach. Ferreira et al. (2019) evaluated *Scenedesmus obliquus* biomass cultivated in brewery wastewater in germination assays with wheat and barley. Biomass pellets promoted higher germination index and vigor, particularly in barley (FGP 100%, GI 85), in both filter paper and

soil assays. Navarro-López et al. (2020) tested BWW-grown *S. obliquus* biomass in three hormone-like bioassays. Extracts without enzymatic hydrolysis often showed more potent effects, including gibberellin-like activity (GI 139.1%), auxin-like activity (167.9% root induction), and cytokinin-like cell expansion (287.5%). More recently, Silva et al. (2025a) reported that *Arthrospira sp.* cultivated at pilot scale in untreated brewery wastewater improved barley growth by approximately 60% when replacing 50–100% of mineral fertilizer, demonstrating a direct agronomic benefit within the brewing value chain. Collectively, these results indicate that brewery wastewater-derived microalgal biomass can serve as an effective agricultural input, contributing to both effluent remediation and the production of biofertilizers aligned with circular-economy principles.

4. COMPARISON WITH COMMERCIAL FERTILIZERS AND BIOSTIMULANTS

4.1 OVERVIEW OF COMMERCIAL FERTILIZERS, BIOFERTILIZERS, AND BIOSTIMULANTS

Modern agriculture still depends on synthetic mineral fertilizers to sustain high yields amid population growth and changing diets, with global food demand projected to exceed 3000 kcal per person per day by 2080 (Alexandratos; Bruinsma, 2012; Gu; Andreev; Dupre, 2021). While increased fertilizer application usually translates into higher productivity, low nutrient use efficiency (NUE) leads to nutrient losses to the environment, contributing to eutrophication, biodiversity loss, and greenhouse gas emissions, as well as economic losses for farmers (Langholtz *et al.*, 2021; Menegat; Ledo; Tirado, 2022). In this context, improving NUE has become a central goal in agronomy and underpins the search for complementary technologies that can reduce reliance on conventional fertilizers without compromising yields (Quille *et al.*, 2025).

Biofertilizers and plant biostimulants have emerged as key tools in this transition. Biofertilizers are typically defined as formulations containing living microorganisms that enhance nutrient availability and uptake, contribute to biological nitrogen fixation, solubilize or mobilize phosphorus and other nutrients, or support rhizosphere processes relevant to plant nutrition (Alori; Glick; Babalola, 2017; Nosheen; Ajmal; Song, 2021; Yadav; Yadav, 2024). Biostimulants, in turn, are usually non-nutrient substances or microbial products that modulate plant physiology, improving nutrient use efficiency, stress tolerance, and crop quality when applied at low doses (Quille *et al.*, 2025). Recent work shows that biostimulants from diverse sources can increase macro- and micronutrient use efficiency across several crops, suggesting

that their integration with conventional fertilization strategies may allow reductions in fertilizer inputs while maintaining productivity (Brito-Lopez *et al.*, 2025; Dasgan *et al.*, 2023).

At the commercial level, the biofertilizer and biostimulant sectors have grown rapidly in response to environmental concerns, policy incentives, and consumer demand for more sustainable food systems. Market analyses indicate that the global biofertilizer market, although still smaller than the synthetic fertilizer sector, is expanding at double-digit growth rates. More recent projections place the value at USD 2.8 billion in 2022 with expectations of reaching USD 5.2 billion by 2028 (Kumar, D. *et al.*, 2024), while similar growth is anticipated for biostimulant products (Peter; Amalraj; Talluri, 2020). These markets are commonly segmented by microorganism type, crop type, formulation, mode of application, and region, reflecting the diversification of products and target environments (Joshi; Gauraha, 2022; Kumar, D. *et al.*, 2024).

Beyond microbe-based inoculants, there is also increasing interest in biofertilizers derived from residual materials, such as digestate and biomass ash, as part of broader circular bioeconomy strategies. Studies with tomato (Cruz *et al.*, 2023) and other vegetable (İkiz *et al.*, 2024; Lyalina *et al.*, 2023) crops have shown that well-formulated digestate- and ash-based biofertilizers can wholly or partially replace standard mineral fertilizers, provided that application rates and C:N ratios are adequately managed and that heavy metal contents remain within regulatory limits. These products illustrate how nutrient-rich wastes can be transformed into commercial fertilizers, contributing simultaneously to waste management, nutrient recycling, and reduced dependence on finite mineral resources (Przygocka-Cyna *et al.*, 2021).

Despite these advances, the commercialization of biofertilizers and biostimulants still faces several challenges. Field performance is often variable due to differences in soil conditions, climate, and management practices, and there is a need for more standardized protocols for strain selection, formulation, and quality control (Hemati *et al.*, 2021). Regulatory frameworks remain fragmented across countries, with differing definitions, registration requirements, and product categories, which can slow market entry and complicate international trade (Peter; Amalraj; Talluri, 2020). In addition, competition with established distribution networks for mineral fertilizers and limited farmer awareness of the benefits of biofertilizers and biostimulants continue to constrain adoption (Joshi; Gauraha, 2022; Kumar, D. *et al.*, 2024). On the other hand, emerging approaches such as rhizosphere engineering, multi-strain and multi-trait formulations, genetic improvement of microbial strains, and nanotechnology are seen as promising avenues to increase product efficacy and robustness under field conditions (Yadav; Yadav, 2024).

Within this evolving landscape, microalgal biomass produced in wastewater, such as brewery effluents, can be viewed as a potential new class of biofertilizer or biostimulant product (Silva e *et al.*, 2025). Its positioning relative to existing commercial inputs depends not only on its nutrient and bioactive compound profile but also on its ability to meet agronomic, regulatory, and market expectations that have emerged around current biofertilizer and biostimulant technologies (Kumar *et al.*, 2024). Within this context, microalgal biomass enters the landscape not as a competing product, but as a hybrid input combining attributes of both fertilizers and biostimulants.

4.2 KEY DIFFERENCES IN COMPOSITION AND MODE OF ACTION

Commercial fertilizers, microbe-based biofertilizers, and microalgal biomass differ fundamentally in their composition and in how they influence plant performance. Mineral fertilizers supply nutrients directly in inorganic, readily available forms, primarily N, P, and K, and their agronomic effect depends mainly on matching application rates with crop demand to avoid nutrient losses and environmental impacts (Adebanjo-Aina; Oludoye, 2025). In contrast, microbial biofertilizers act through biological processes such as nitrogen fixation, phosphate solubilization, and nutrient mobilization in the rhizosphere, relying on the activity and survival of living microorganisms after field application (Alori; Glick; Babalola, 2017; Nosheen; Ajmal; Song, 2021). Their effectiveness is therefore shaped by soil conditions, microbial competition, and the compatibility between inoculated strains and the target crop (Yadav; Yadav, 2024).

Microalgal biomass, particularly when produced in nutrient-rich wastewaters, presents a distinct profile (Hasnain *et al.*, 2023). In addition to supplying organic forms of nitrogen, phosphorus, carbon, and micronutrients, microalgae accumulate proteins, lipids, carbohydrates, pigments, and a wide range of bioactive molecules, including phytohormones and antioxidant compounds (Papadopoulos *et al.*, 2022; Pozzobon; Lemaire; Lagirarde, 2024). These compounds can influence plant physiology through mechanisms typically associated with plant biostimulants, such as modulation of root architecture, enhancement of nutrient use efficiency, and increased tolerance to abiotic stress (Álvarez-González; Serrano; *et al.*, 2025). Unlike microbe-based biofertilizers, microalgal biomass does not depend on microbial survival in the soil, its activity results from its biochemical composition rather than from colonization or metabolic activity of living cells (Rupawalla *et al.*, 2022).

These differences also shape the expected agronomic outcomes. Mineral fertilizers provide rapid increases in nutrient availability but do not directly influence plant physiological pathways related to stress resilience or nutrient uptake efficiency (Okba *et al.*, 2025). Microbial biofertilizers can improve nutrient acquisition but often show variable results under field conditions due to environmental sensitivity (Schenk *et al.*, 2024). Microalgal biomass, by contrast, offers combined nutrient provisioning and biostimulant effects, particularly when rich in amino acids, phytohormones, or antioxidant compounds, which may improve early plant development even at low application rates (Navarro-López *et al.*, 2020). When cultivated in wastewater, microalgae also integrate nutrient recycling into the production chain, differentiating them from inputs derived from mineral extraction or industrial fermentation (Álvarez-González *et al.*, 2022a).

4.3 AGRONOMIC PERFORMANCE: MICROALGAL BIOMASS VS. COMMERCIAL INPUTS

Agronomic responses to microalgal biomass often differ from those achieved with mineral fertilizers and commercial biostimulants because their effects are not solely nutrient-driven. Mineral fertilizers typically promote rapid increases in biomass accumulation and yield by providing nutrients in an immediately available form (Xie *et al.*, 2025). However, these responses may plateau when nutrient uptake becomes limited by root development, soil constraints, or abiotic stress. Commercial biostimulants, frequently based on seaweed extracts, humic substances, or microbial metabolites, tend to elicit improvements in germination, root architecture, or stress tolerance, but results can vary depending on formulation, crop species, and environmental conditions (Braun; Colla, 2023; Rouphael; Colla, 2020).

Microalgal biomass has shown agronomic effects that combine nutrient supply with physiological stimulation. Studies using biomass derived from wastewater report improvements in seed germination, root elongation, and early seedling vigor, often comparable to or exceeding those achieved with commercial products. Navarro-López et al. (2020) observed enhanced root growth and chlorophyll retention in lettuce seedlings treated with *Chlorella*-based extracts. Ferreira et al. (2019) reported increases in nutrient content and biomass accumulation in horticultural crops fertilized with microalgal biomass cultivated in aquaculture wastewater. More recently, Silva et al. (2025a) demonstrated that replacing 50%–100% of a mineral

fertilizer with raw *Arthrospira* biomass cultivated in brewery effluent accelerated barley growth by approximately 60% compared to the mineral fertilizer alone.

Commercial seaweed-based products, which are widely used as benchmarks in biostimulant studies, typically improve germination and early root development due to their auxin, cytokinin, and polysaccharide content (Murmu; Sarma; Sarkar, 2025). While microalgal biomass contains similar classes of compounds, comparative trials indicate that microalgae may achieve responses comparable to or higher than those at equivalent or lower application rates, particularly when biomass is rich in proteins, amino acids, or phytohormones (Quille *et al.*, 2025; Saha *et al.*, 2022).

Another aspect is performance under stress. Several studies document that microalgal extracts alleviate drought, salinity, and oxidative stress by enhancing photosynthetic efficiency (ΦPSII), antioxidant activity, and stomatal conductance (Sun *et al.*, 2024; Vangenechten; De Coninck; Ceusters, 2025). These effects align with or surpass those reported for commercial biostimulants, mainly when microalgae are cultivated in nutrient-rich wastewaters that favor the accumulation of compatible solutes, pigments, and stress-modulating compounds (Pozzobon; Lemaire; Lagirarde, 2024).

4.4 ADVANTAGES, LIMITATIONS, AND APPLICATION POTENTIAL

Microalgal biomass cultivated in brewery wastewater offers several advantages over conventional fertilizers and commercial biostimulants. A significant strength is the dual function of biomass: it provides essential nutrients such as nitrogen, phosphorus, and micronutrients, while also supplying bioactive metabolites that modulate root development, seed germination, photosynthetic efficiency, and stress tolerance (Silva, A. L. e *et al.*, 2025; Silva, P. H. B. de S. *et al.*, 2025). When integrated into circular systems, wastewater-based microalgal production also contributes to nutrient recovery and CO_2 mitigation, reducing the environmental footprint associated with both wastewater treatment and agricultural fertilization (Morillas-España *et al.*, 2024; Pessôa *et al.*, 2021). These aspects highlight a potential competitive advantage over mineral fertilizers, whose effects are strictly nutrient-driven, and over seaweed-based products, which depend on biomass harvested from marine ecosystems (Dasgan *et al.*, 2023).

Despite these benefits, some limitations remain. Microalgal production depends on stable environmental conditions, appropriate strain selection, and operational robustness, which can be challenging when using industrial effluents with fluctuating compositions (Bai; Xu; Dai,

2025). Biomass harvesting and downstream processing also represent cost-intensive steps, although advances such as membrane-assisted concentration or direct biomass application without drying have reduced these barriers (Acién-Fernández *et al.*, 2012). Variability in biochemical composition is another challenge, as nutrient and metabolite profiles reflect both the microalgal species and the characteristics of the wastewater used as culture medium. Consequently, standardization of formulations and quality control will be essential for broader adoption in the agricultural sector (Arioli *et al.*, 2024; Brito-Lopez *et al.*, 2025).

The application potential of brewery wastewater–grown biomass aligns well with current trends in sustainable agriculture. Experimental evidence suggests that microalgal biomass can partially or fully replace mineral fertilizers during early crop development, particularly when used as a seed treatment, foliar spray, or soil amendment (Álvarez-González; Castro; *et al.*, 2025; Silva, A. L. e *et al.*, 2025). Its effects on stress mitigation, including drought and salinity tolerance, further expand the range of possible uses in regions facing climate-related constraints (Vangenechten; De Coninck; Ceusters, 2025). However, large-scale adoption will require regulatory frameworks that define product categories, quality standards, and safety parameters (Acién-Fernández *et al.*, 2012). The recent implementation of the EU Fertilising Products Regulation -Regulation (EU) 2019/1009- (European Union, 2019), which formally recognizes microalgae as potential biostimulant inputs, indicates a growing international interest and offers a pathway for future commercialization.

5. PERSPECTIVES AND CHALLENGES IN AGRICULTURAL APPLICATIONS

Microalgae cultivated in brewery wastewater occupy an emerging position within the broader landscape of sustainable bioinputs, but their translation into agricultural practice depends on overcoming scientific, regulatory, and market constraints. At the regulatory level, the global biofertilizers and biostimulants sector continues to face fragmented definitions, heterogeneous registration pathways, and inconsistencies in product categorization across countries (Nosratabad; Khoshru, 2024). These differences influence approval timelines, quality standards, and commercialization routes, and they pose additional challenges for microalgal products, which often share characteristics with both microbial inoculants and biostimulants derived from complex organic matrices. As highlighted by recent analyses of microbial biofertilizers, the lack of unified regulatory frameworks contributes to variable field

performance. It limits farmer confidence, underscoring the need for harmonized standards and more robust quality control systems (Fadiji *et al.*, 2024).

From a technological standpoint, optimizing microalgal biomass for agricultural use requires advances beyond just biomass production. Studies on the thermochemical valorization of agricultural residues suggest that integrated biorefineries capable of recovering nutrients, producing biochar, and generating bioactive compounds may offer pathways to enhance the stability, safety, and agronomic value of microalgal formulations (Paudel *et al.*, 2024). Similarly, innovations in formulation science, such as polymer encapsulation, fluid-bed drying, and improved carrier materials, have been shown to enhance microbial survival, modulate release profiles, and strengthen the resilience of biofertilizers under real field conditions (Nosratabad; Khoshru, 2024). These advances are increasingly relevant to microalgal biomass, where variability in biochemical composition and storage stability remain operational challenges. Emerging nano-enabled fertilizers and nano-biofertilizers also represent a promising frontier, offering greater nutrient-delivery precision and lower environmental impacts than conventional products (Soni *et al.*, 2024). Although still in early development, nanoformulations could be adapted to stabilize microalgal metabolites, improve their bioavailability, or enhance their efficacy under abiotic stress.

Environmental and climatic constraints add a further layer of complexity. Research on cyanobacteria-based biofertilizers in arid regions shows that strains adapted to high temperatures, salinity, and water scarcity can help build soil structure, increase nutrient bioavailability, and improve plant tolerance to stress (Bibi *et al.*, 2024). These findings indicate that microalgal products may be particularly suitable for agricultural systems exposed to climate extremes, mainly when the biomass is derived from wastewaters enriched in compatible solutes or stress-related metabolites. However, scaling such applications will require long-term field trials across different soil types, climatic zones, and cropping systems. Most of the available studies remain short-term and laboratory-based, a trend also observed in broader biomass valorization research (Paudel *et al.*, 2024; Ronga *et al.*, 2019).

Market perspectives for microalgal bioinputs are influenced by the rapid expansion of the global biofertilizer sector, projected to exceed USD 5 billion by 2030 (FBI, 2023). Yet the industry remains constrained by inconsistent field efficacy, limited farmer adoption, and competition with well-established fertilizer distribution networks. Overcoming these barriers will require integrated strategies that combine technical improvements with cost reductions, clear agronomic recommendations, and greater alignment with circular-economy policies (Miranda *et al.*, 2024). Brewery wastewater cultivation presents a particular opportunity in this

context: it enables decentralized production near industrial sites, reduces treatment costs for breweries, and generates biomass with both nutritional and biostimulant potential. Aligning these advantages with appropriate regulatory frameworks, quality assurance protocols, and scalable harvesting technologies may position microalgal biomass as a competitive input in sustainable agriculture (Silva *et al.*, 2025).

6. CONCLUSION

Microalgae cultivated in brewery wastewater represent a promising, multifunctional strategy that couples effluent remediation with the production of nutrient-rich, bioactive biomass for agricultural use, offering competitive advantages over conventional fertilizers and commercial biostimulants. Current evidence demonstrates that brewery effluent–grown microalgae can enhance germination, root development, nutrient uptake, and stress tolerance while simultaneously reducing environmental impacts and operational costs for breweries. However, transforming these advances into market-ready products will require harmonized regulatory frameworks, standardized quality-control systems, and technological improvements in biomass stabilization, formulation, and large-scale cultivation. As circular-economy initiatives expand globally, integrating brewery wastewater valorization with microalgal bioproduct development presents a unique opportunity to create sustainable agricultural inputs, close resource loops, and advance climate-resilient food production systems.

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ARTICLE II

EXPLOITING BREWERY WASTEWATER FOR THE SUSTAINABLE CULTIVATION OF *Chlorella fusca*: BIOMASS PRODUCTIVITY, BIOCHEMICAL COMPOSITION, AND PRODUCTION COST

CONCEIÇÃO, Jenyfer de Almeida; MORAIS, Michele Greque de; MORAIS, Etiele Greque de; COSTA, Jorge Alberto Vieira. Exploiting brewery wastewater for the sustainable cultivation of *Chlorella fusca*: Biomass productivity, biochemical composition, and production cost. **Journal of Applied Phycology**, 26 Feb. 2026. DOI: https://doi.org/10.1007/s10811-026-03796-x.

ABSTRACT

Beer is the fifth most consumed beverage worldwide, and its production demands large volumes of water, generating 3–10 L of effluent per liter of beer produced. This wastewater often requires municipal management, representing high operational costs and environmental concerns, particularly for microbreweries with limited infrastructure. The use of microalgae in brewery effluent offers a sustainable alternative for nutrient recovery, biomass generation, and integration into circular bioeconomy strategies. This study evaluated the growth performance and biochemical composition of *Chlorella fusca* LEB 111 cultivated in brewery effluent under semicontinuous operation, aiming to reduce production costs while maintaining biomass quality. Cultures grew in 1.8 L photobioreactors with effluent concentrations of 25%, 50%, 75%, and 100%, compared to a BG-11 control. The 100% effluent assay achieved a biomass concentration of 0.77 g L^{-1} and productivity of 52.34 mg L^{-1} d^{-1}, with a balanced biochemical profile of 39.96% proteins, 31.64% carbohydrates, and 28.89% lipids. Notably, the highest protein content was obtained in EA75 during the first cycle (46.20%), the maximum carbohydrate accumulation occurred in EA75 during the second cycle (35.59%), and the highest lipid content was recorded in EA50 during the third cycle (40.61%). Cost analysis revealed a substantial reduction in biomass production expenses, from over €84 kg^{-1} in the control to €18 kg^{-1} in the undiluted effluent assay. These results advance knowledge on the adaptive biochemical responses of *C. fusca* under nutrient-limited wastewater conditions, providing a framework for sustainable algal cultivation and biomass valorization from nutrient-limited industrial effluents.

Keywords: Algal Biorefinery; Circular Economy; Semicontinuous cultivation; Microalgae-based Bioremediation; Wastewater Valorization; Techno-economic analysis.

1. INTRODUCTION

Currently, there are over 19,000 breweries worldwide, with 94% classified as craft breweries, defined as having fewer than 30 employees or an annual production below 5,000 hectoliters. Brazil ranks as the third largest global beer producer, trailing only the United States and China, employing 2.7 million people and contributing 1.6% to the national GDP (Dias *et al.*, 2023). The expansion of microbreweries is a key factor in this growth. However,

these breweries face considerable waste management challenges due to space limitations, high energy consumption, and inadequate processing resources (Arranz *et al.*, 2021). The brewing industry is one of the largest industrial water users, requiring between 3 to 10 liters of water per liter of beer produced. This activity generates substantial volumes of wastewater typically requiring treatment at municipal facilities, imposing significant waste burdens and high operational costs on brewers (Olajire, 2020). Improper discharge contributes to eutrophication in surrounding areas (Malone; Newton, 2020). Brewery effluent varies with the type of beer produced, ingredients like yeast, malt, corn, and the efficiency of equipment cleaning processes. For example, fermentation and filtration generate about 3% of the total effluent volume but contain nearly 97% of the organic load, including malt and barley husks, while rinsing stages produce most of the volume but have a low organic content, possibly containing chemicals (Arantes *et al.*, 2017).

These effluents exhibit high biochemical oxygen demand (BOD: 1200–3600 mg L^{-1}) and chemical oxygen demand (COD: 2000–6000 mg L^{-1}), but low nutrient concentrations, nitrogen (25–80 mg L^{-1}) and phosphorus (10–50 mg L^{-1}), unlike standard media such as BG 11 (Ferreira, A. *et al.*, 2019). Integrating microalgae cultivation with brewery effluent treatment exemplifies the circular economy model. This integration not only recovers essential nutrients from wastewater but also reduces freshwater usage and carbon emissions, ultimately producing valuable microalgal biomass for various sustainable applications such as biofuels, biostimulants, and animal feed (Pessôa *et al.*, 2021). This synergy reinforces the circular economy concept, converting industrial waste into resources through algae-based biorefineries. In this context, the use of microbrewery effluents to produce biofertilizers for barley and malt has gained attention, since the process allows extraction of biopolymers and reuse of treated water for industrial cleaning or crop irrigation. Such integrated systems enhance sustainability and resource efficiency, strengthening the circular bioeconomy.

Nagarajan et al. (2020) studied the circular bioeconomy perspective on resource recovery from different effluents using microalgal cultures. They concluded that integrating microalgae into existing treatment facilities can drive resource-based economic growth. In this context, Silva et al. (2025) cultivated *Arthrospira* sp. in untreated brewery wastewater at pilot scale, achieving 10.68 ± 5.13 g m^{-2} day^{-1} of biomass productivity and removing up to 100% of ammonium. The biomass was tested as a biofertilizer for barley, promoting ~60% faster growth compared to commercial fertilizer. While promising, such studies lack detailed economic analyses, particularly under semi-continuous conditions. To assess the viability of

microbrewery wastewater cultivation, cost analysis must consider cultivation expenses, medium price, and biomass yield.

Previous studies have explored the integration of microalgae and brewery effluents for biotechnological applications. Song et al. (2020) investigated the purification of brewery wastewater and CO_2 fixation using different microalgae strains, emphasizing the production of value-added ingredients, while Wang et al. (2022) assessed the potential of *Chlorella sp.* for wastewater treatment coupled with the generation of bioproducts such as lipids and carbohydrates. However, none of these studies provided detailed economic evaluations under semicontinuous regimes. This gap underscores the originality of the present work, which combines operational efficiency with cost analysis in cultivating *Chlorella fusca* LEB 111 in brewery effluent. The aim of this work was to evaluate the potential of using brewery effluent as an alternative cultivation medium for *Chlorella fusca* LEB 111, focusing on its impact on growth and biomass composition to reduce costs and enhance production efficiency. This is the first study to analyze the cost of *Chlorella fusca* cultivation in brewery effluent under semi-continuous conditions, addressing a novel and underexplored area in algal biotechnology.

2 MATERIALS AND METHODS

2.1 MICROALGAE AND CULTURE MEDIUM

In this study, the *Chlorella fusca* LEB 111 strain (also reported as *Desmodesmus abundans*; Hegewald, 2000), isolated from the ash sedimentation ponds of the Presidente Médici Thermal Power Plant (UTPM) in Candiota, Rio Grande do Sul, Brazil (latitude 24°36’13’’ S and longitude 52°32’43’’ W), was used. The strain was obtained from the strain bank of the Laboratory of Biochemical Engineering (FURG). The strain identity was previously confirmed by morphological and molecular methods at the same institution. The *C. fusca* LEB 111 inoculum was maintained in BG-11 culture medium (Rippka *et al.*, 1979) under controlled conditions of temperature (30 °C), illumination (60 μmol photons m^{-2} s^{-1}), photoperiod (12 h light/12 h dark), and continuous agitation promoted by compressed air injection. For control assays, the microalgae were maintained in BG 11 medium. Brewery effluent from a microbrewery in the southern region of Rio Grande do Sul was used, derived from the washing of the fermentation tank after brewing pilsner beer. This effluent was filtered (pore ⌀= 11 μm) to remove suspended solid particles before being stored at -20 °C until use (Schulze *et al.*, 2017). The effluent exhibited the following characteristics: Chemical Oxygen Demand (COD)

of 948.8 mg L^{-1}, Biochemical Oxygen Demand (BOD) of 368.3 mg L^{-1}, nitrogen concentration of 5.1 mg L^{-1}, and phosphorus concentration of 2.7 mg L^{-1}.

2.2 CULTURE CONDITIONS

Chlorella fusca LEB 111 was cultured in 2.0 L Erlenmeyer flask (working volume of 1.8 L), operated in semicontinuous mode, in duplicate, with a medium renewal rate (RR) of 40%, as previously described by Moreira et al. (2016). The assays were initiated with an initial biomass concentration of 0.2 g L^{-1}, achieved by inoculating cultures previously grown in BG-11 medium into the experimental media (BG-11 or brewery effluent at the respective dilutions), adjusting the inoculum volume to ensure the same starting biomass concentration in all assays. The cultures were maintained at 30 °C, under a 12 h light/12 h dark photoperiod and an irradiance of 60 $\mu mol_{photons}$ m^{-2} s^{-1}. Semicontinuous operation was conducted over three consecutive cultivation cycles. At the end of each cycle, 40% of the culture volume was withdrawn and replaced with fresh medium, maintaining the same effluent concentration or BG-11 medium, depending on the assay. Harvests were performed when the cultures reached the fourth day of the stationary growth phase in each cycle.

Continuous aeration was supplied to all cultures by compressed air injection, ensuring mixing and gas exchange. No mechanical agitation was applied during the experiments. The same aeration and operational conditions were applied to both the control cultures (BG-11 medium) and the brewery effluent-based assays to ensure comparability among treatments. No additional CO_2 supplementation was provided during cultivation.

Variations in brewery effluent concentrations were tested as follows: 25% effluent and 75% distilled water (EA25), 50% effluent and 50% distilled water (EA50), 75% effluent and 25% distilled water (EA75), and 100% effluent (E100). A control assay was also conducted with a standard BG 11 medium for comparison purposes.

2.3 ANALYTICAL DETERMINATIONS

2.3.1 Biomass concentration and pH

The cell concentration and pH of the cultures was assessed daily. The biomass concentration of the microalgae was determined using a spectrophotometer (Shimadzu UV/VIS UVmini-1240, Japan) at 670 nm. A standard curve was previously established, which correlates the absorbances at this wavelength with the dry mass of successive dilutions of the microalgae inoculum (Costa *et al.*, 2002). Although OD_{750} is often preferred to avoid pigment interference,

OD_{670} was validated in this study for its strong correlation with biomass concentration under the tested conditions. The pH of the cultures was determined by direct measurement with a digital pHmeter (Mettler Toledo FiveGo™, Switzerland).

2.3.2 Microalgal growth

The maximum biomass concentration (X_{max}, g L^{-1}) in each assay is the highest value obtained from the calculation of microalgal biomass. The volumetric biomass productivity (P_x, g L^{-1} d^{-1}) was calculated using Equation 1, where X_t is the biomass concentration (g L^{-1}) at time t (d) and X_0 is the biomass concentration (g L^{-1}) at initial time t_0 (d) (Bailey; Ollis, 1986). For each cultivation cycle, the maximum volumetric biomass productivity (P_{max}, mg L^{-1} d^{-1}) was defined as the highest daily productivity value observed during the cycle. The reported P_{max} values correspond to the average of the maximum daily productivities obtained from duplicate assays.

$$P_x = \frac{X_t - X_0}{t - t_0} \quad (1)$$

The maximum specific growth rate (μ_{max}, d^{-1}) was measured by linear regression of the logarithmic growth phase, from a linearized biomass concentration profile (Ln X) versus time (d), where the slope of this line was taken as μ_{max}. The generation time (t_g) was determined during the exponential growth phase of the microalga, calculated according to Equation 2 (Bailey; Ollis, 1986).

$$t_g = \frac{\ln(2)}{\mu_{max}} \quad (2)$$

2.3.3 Analysis of Biomass and Biomolecules Production Costs

Many factors, including energy, harvesting, and labor, contribute to the cost of producing biomass and bioproducts. In this study, apart from using effluent, no other cultivation modifications were made. Therefore, the average cultivation cost was set at €13.00 per kg of biomass produced (Llamas *et al.*, 2021), which includes energy expenses (e.g., lighting, equipment use, and heating), materials, and labor costs, excluding the cost of the cultivation medium. The cost of the effluent was set to €0.00 since it is a byproduct of the brewing process. The price of the culture medium (BG-11) was estimated based on reagent prices obtained from commercial laboratory suppliers in Brazil, reflecting typical costs under small-scale laboratory

conditions. The cost of producing microalgal biomass and biomolecules was calculated using the equation described by Park *et al.*, (2013): Cost (€ g^{-1}) = (A + B) / C, where A is the fixed price of a microalgal cultivation (€ L^{-1}), B is the price of the effluent or culture medium (€ L^{-1}), and C is the final concentration of biomass or biomolecules produced at the end of cultivation (g L^{-1}) (Park *et al.*, 2013; Silveira *et al.*, 2023).

The total biomass and biomolecules production (B_{total}) was calculated considering the cumulative biomass obtained over the three cultivation cycles, according to Equation 3:

$$B_{total} = \frac{(BC_1 \times 0.7) + (BC_2 \times 0.7) + (BC_3 \times 1.8)}{3.2} \quad (3)$$

Where BC_1, BC_2, and BC_3 represent the biomass concentrations obtained at the end of the first, second, and third cultivation cycles, respectively. The coefficients applied to each cycle correspond to the effective harvested volumes during semicontinuous operation.

2.3.4 Biomass harvesting and characterization

At the end of the experiments, the biomass was centrifuged (Hitachi Himac CR-GIII, Tokyo, Japan) at 15,200 × g for 15 min at 20 °C. The recovered biomass was washed with distilled water to remove residual medium components and salts that could interfere with colorimetric determinations of proteins, carbohydrates, and lipids, and centrifuged again under the same conditions. The resulting biomass was then frozen at −80 °C for 48 h, lyophilized, and stored at −20 °C for later characterization.

Biomass composition was analyzed after all cultivation stages. For carbohydrate and protein determination, aqueous extracts were prepared using 10 mg of lyophilized microalgal biomass suspended in 20 mL of distilled water. The samples were subjected to ultrasonic disruption using an ultrasonic probe (Cole Parmer CPX 130, Illinois, USA) for 10 min, with alternating cycles of 1 min on and 1 min off, to promote cell disruption and release of intracellular compounds.

Total carbohydrate concentration was determined using the phenol–sulfuric acid method, with glucose as the standard (Dubois *et al.*, 1956). Protein concentration was quantified according to the colorimetric method proposed by Lowry et al. (1951), following prior thermal and alkaline hydrolysis of the extract to solubilize cellular proteins, using bovine serum albumin as the standard. Total lipid content was determined using the colorimetric method described by Marsh and Weinstein (1966), based on solvent extraction from lyophilized biomass, employing chloroform and methanol as extraction solvents and tripalmitin for calibration. Biochemical

analyses were performed in triplicate for each experimental duplicate, resulting in six analytical replicates per condition (n = 6).

2.3.5 Statistical analyses

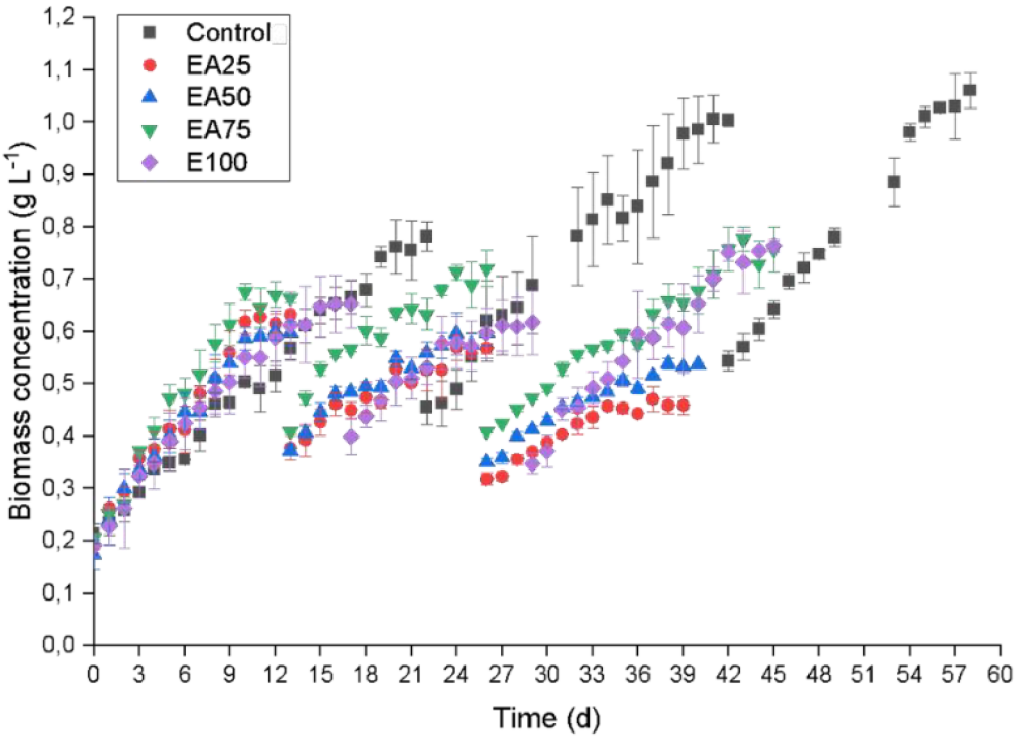
All analyses were performed in triplicate for each experimental duplicate, resulting in six analytical replicates per condition (n = 6), and results are expressed as mean ± standard deviation (SD). Before statistical analysis, data were evaluated for normality and homogeneity of variances. Normality was assessed using the Shapiro–Wilk test, and homoscedasticity was verified using Levene's test. The results were assessed using analysis of variance (ANOVA), followed by Tukey's test for comparing mean values at a 95% confidence level. Statistical analyses were performed using the Statistica 7 software (StatSoft Inc., USA).

3 RESULTS

3.1 MICROALGAL GROWTH KINETICS

The growth profiles of *Chlorella fusca* LEB 111 (Figure 1) in assays with different effluent concentrations and control were similar up to the 9th day of cultivation. From this point, the cultures with lower effluent concentrations (EA25, EA50, and EA75) reached the stationary growth phase and consequently proceeded to the first harvest on the 12th day, concluding Cycle 1. The cultures with 100% effluent (E100) continued until the 17th day for the first harvest, while the control only reached the 4th day of the stationary phase on the 22nd day.

Figure 1 - Growth curve of *Chlorella fusca* LEB 111 cultured using different concentrations of brewery effluent (0%, 25%, 50%, 75% and 100%) in semicontinuous mode. Error bars represent the standard deviation of duplicate cultivations (n = 2).



In the second cycle, the EA25, EA50, and EA75 assays lasted 13 days until the second cut, while the E100 assay lasted 12 days. The control extended over 20 days. A slight increase in error bars was observed in the control during this cycle, which may be linked to the addition of fresh medium and differences in culture agitation. In the third and final cycle, EA25 and EA50 have lasted for 14 days, while EA75 19 days, and E100 for 16 days, the same as the control. Considering the total duration of each assay, the control had the longest operation time (58 days), followed by EA75 and E100 (45 days), EA50 (40 days), and EA25 (39 days).

Over the three cycles (Table 1), the control assay consistently showed the highest maximum biomass concentrations (X_{max}), reaching 1.06 g L^{-1} in the third cycle ($p < 0.05$). However, a progressive decline was observed in biomass productivity (from 69.52 to 43.12 mg L^{-1} d^{-1}), specific growth rate (from 0.20 to 0.11 d^{-1}), and an increase in generation time (from 3.43 to 6.41 days).

Table 1 - Maximum biomass concentration (X_{max}, g L^{-1}), maximum biomass productivity (P_{max}, mg $L^{-1}d^{-1}$), maximum specifc growth rate (μ_{max}, d^{-1}), and generation time (tg, d) of *Chlorella fusca* LEB 111 in brewery effluent and semicontinuous mode. Different lowercase letters in the same column indicate significant differences ($p \leq 0.05$) between cycles for the same parameter. Data are presented as the mean±standard deviation of duplicates.

	Sample	X_{max}	P_{max}	μ_{max}	tg
Cycle 1	Control	$0.78^{a} \pm 0.03$	$31.89^{c} \pm 1.01$	$0.20^{a} \pm 0.02$	$3.49^{a} \pm 0.43$
	EA25	$0.64^{b} \pm 0.01$	$70.72^{a} \pm 6.57$	$0.22^{a} \pm 0.04$	$3.15^{a} \pm 0.58$
	EA50	$0.60^{b} \pm 0.02$	$70.04^{a} \pm 7.10$	$0.12^{a,b} \pm 0.01$	$5.96^{a,b} \pm 1.39$
	EA75	$0.68^{a,b} \pm 0.02$	$55.93^{b} \pm 3.35$	$0.08^{b} \pm 0.01$	$8.47^{b} \pm 1.57$
	E100	$0.66^{b} \pm 0.05$	$46.98^{b} \pm 4.32$	$0.12^{a,b} \pm 0.01$	$5.76^{a,b} \pm 1.23$
	Sample	X_{max}	P_{max}	μ_{max}	tg
Cycle 2	Control	$1.02^{a} \pm 0.03$	$44.93^{b} \pm 4.13$	$0.07^{c} \pm 0.01$	$10.17^{b} \pm 0.99$
	EA25	$0.57^{c} \pm 0.02$	$27.81^{b} \pm 0.75$	$0.09^{a,b,c} \pm <0.01$	$7.99^{a,b} \pm 0.18$
	EA50	$0.60^{b,c} \pm 0.03$	$37.83^{b} \pm 3.42$	$0.10^{a} \pm 0.01$	$6.69^{a} \pm 0.46$
	EA75	$0.72^{b} \pm 0.03$	$65.23^{a} \pm 6.75$	$0.08^{b,c} \pm <0.01$	$9.00^{a,b} \pm 0.51$
	E100	$0.62^{b,c} \pm 0.06$	$42.77^{b} \pm 4.60$	$0.10^{a,b} \pm 0.01$	$7.02^{a} \pm 0.71$
	Sample	X_{max}	P_{max}	μ_{max}	tg
Cycle 3	Control	$1.06^{a} \pm 0.03$	$37.99^{b} \pm 1.22$	$0.05^{c} \pm <0.01$	$14.91^{c} \pm 0.70$
	EA25	$0.47^{c} \pm 0.02$	$19.59^{d} \pm 1.23$	$0.06^{b,c} \pm <0.01$	$12.59^{b} \pm 0.90$
	EA50	$0.54^{c} \pm 0.01$	$24.19^{c} \pm 0.68$	$0.05^{b,c} \pm <0.01$	$13.36^{b,c} \pm 0.15$
	EA75	$0.78^{b} \pm 0.02$	$25.75^{c} \pm 0.20$	$0.13^{a} \pm <0.01$	$5.26^{a} \pm 0.17$
	E100	$0.77^{b} \pm 0.01$	$52.34^{a} \pm 0.94$	$0.06^{b} \pm <0.01$	$11.71^{b} \pm 0.34$

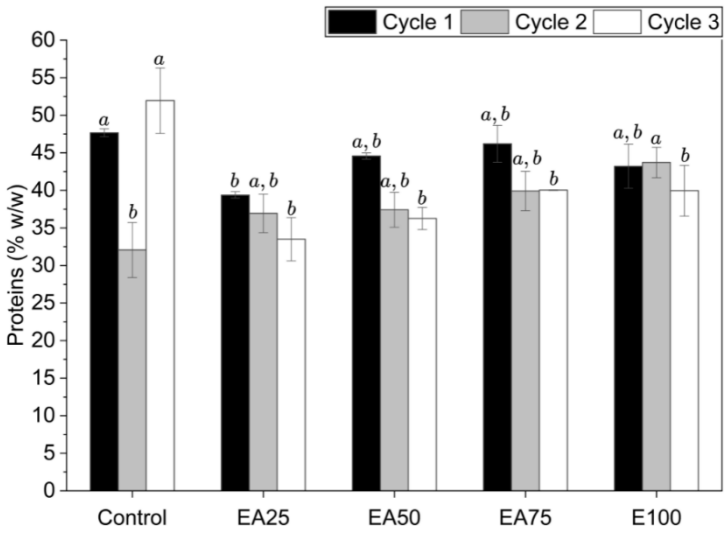
In the EA25 assay, biomass concentration increased to 0.57 g L^{-1} by the second cycle, but P_{max}, initially high (70.72 mg L^{-1} d^{-1}), decreased over time ($p < 0.05$), indicating that the low effluent concentration did not support stable growth. In contrast, EA50 showed moderate improvement in growth parameters, while EA75 exhibited consistent adaptation, with a P_{max} of 65.23 mg L^{-1} d^{-1} in the second cycle ($p < 0.05$). E100 demonstrated the greatest stability across the cycles, achieving a P_{max} of 52.34 mg L^{-1} d^{-1} in the third cycle ($p < 0.05$).

3.2 BIOMASS COMPOSITION

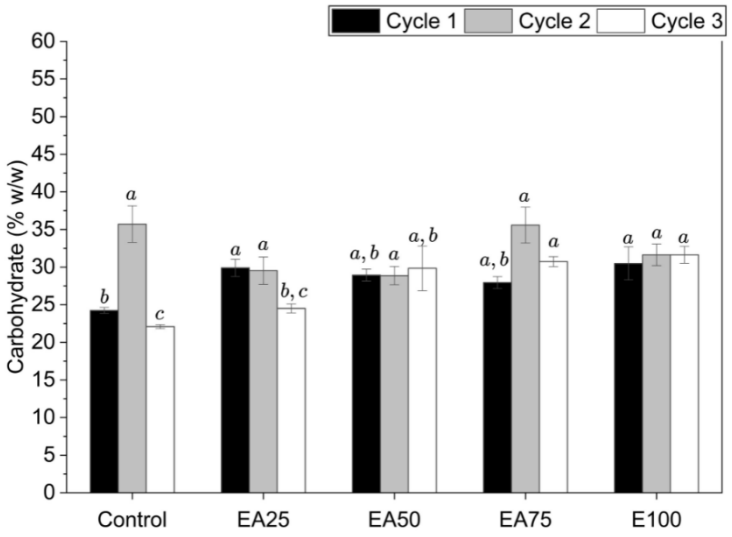
Chlorella fusca LEB 111 biomass was evaluated for its composition. The biomass compositions started to show difference from the second cycle onward (Figure 2). The control assay stood out in protein production (Figure 2a) throughout the cycles, reaching 51.95 ± 4.36% w/w in the last cycle ($p < 0.05$), while showing the lowest values for carbohydrates (Figure 2b) and lipids (Figure 2c) by the end of cultivation. The assays with lower effluent concentrations, particularly EA50, exhibited high lipid contents ($p < 0.05$), with 40.36 ± 0.11% and 40.61 ± 1.97% w/w in the second and third cycles, respectively.

Figure 2 - Protein (a), carbohydrate (b), and lipid (c) contents determined in *Chlorella fusca* LEB 111 cultured in brewery effluent in semicontinuous mode. Different lowercase letters indicate statistically significant differences among cultivation cycles for the same treatment ($p < 0.05$). Error bars represent the standard deviation of duplicate independent cultivations, with each biological replicate analysed in triplicate.

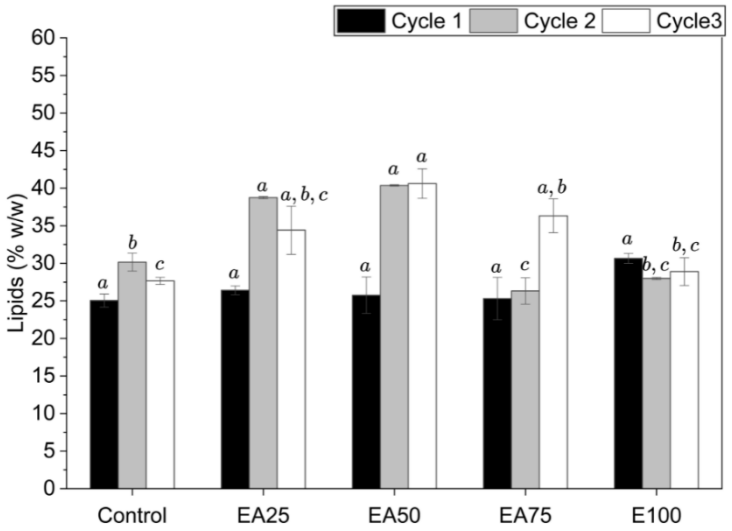
(a)



(b)



(c)



The EA25 assay, with the lowest nutrient availability, had the least protein but stood out in producing reserve compounds like carbohydrates compared to the control. The EA75 and E100 assays improve in carbohydrate production ($p < 0.05$), reaching 30.74 ± 0.66% and 31.64 ± 1.12% w/w in the last cycle, respectively. The 100% effluent assay remained stable throughout the cycles ($p > 0.05$), with minor variations, indicating that the microalgae adapted well to the raw effluent.

3.3 ANALYSIS OF BIOMASS AND BIOPRODUCTS PRODUCTION COSTS

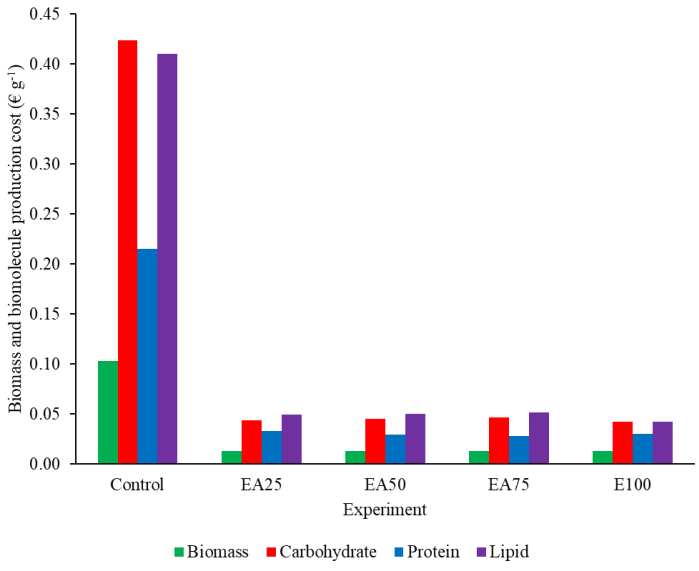
The use of brewery effluent in microalgal cultivation primarily aims to reduce the costs associated with biomass and bioproducts obtention. To provide an indicative comparison of production costs associated with the use of brewery effluent, a simplified process cost value

was established. Based on these data, we adjusted the cost to 13 € kg^{-1} for all assays conducted in this work, proportional to the amount of biomass produced in each experiment. This adjustment was made to reflect the specific experimental conditions of the present study, aligning with the economic methodology presented in Llamas et al. (2021). Additionally, the cost of the standard BG 11 cultivation medium was estimated at 0.07 € per liter. Furthermore, the cost of brewery effluent was set at 0.00 €, as it is a byproduct of the brewing process. In some cases, effluents can even have a negative cost, as companies may pay for their disposal.

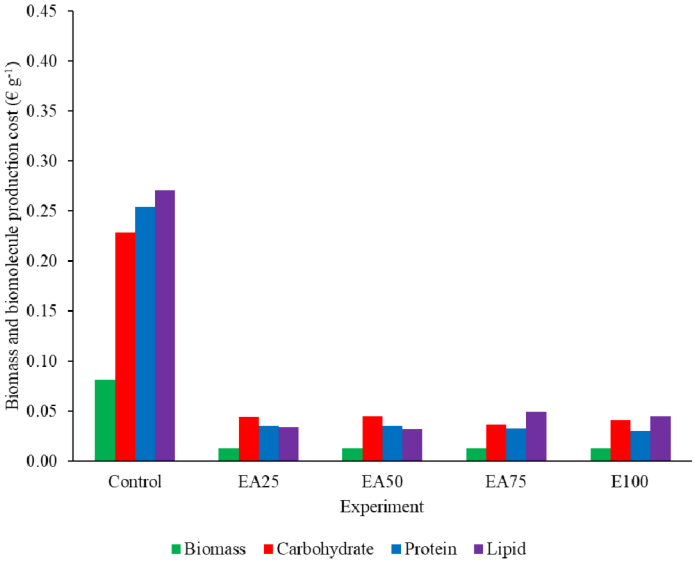
When examining costs for both biomass and biomolecules, the control assay recorded the highest values in all cycles (Figure 3). Across the three cycles, assays using brewery effluent significantly reduced production costs compared to the control. In Cycle 1, biomass production costs were reduced by up to 87%, while carbohydrate production costs decreased by as much as 90%. Among the effluent-based assays, EA75 exhibited the greatest reduction in protein production costs (87%), while E100 presented the highest reduction in lipid production costs (90%). Similar trends were observed in Cycles 2 and 3, with E100 consistently showing the highest savings, particularly in carbohydrate and lipid production, demonstrating improved cost-effectiveness under these cultivation conditions.

Figure 3 - Cost of production of biomass and biomolecules in cultivations with and without brewery effluent in (a) Cycle 1 (b) Cycle 2 (c) Cycle 3 (d) Total culture

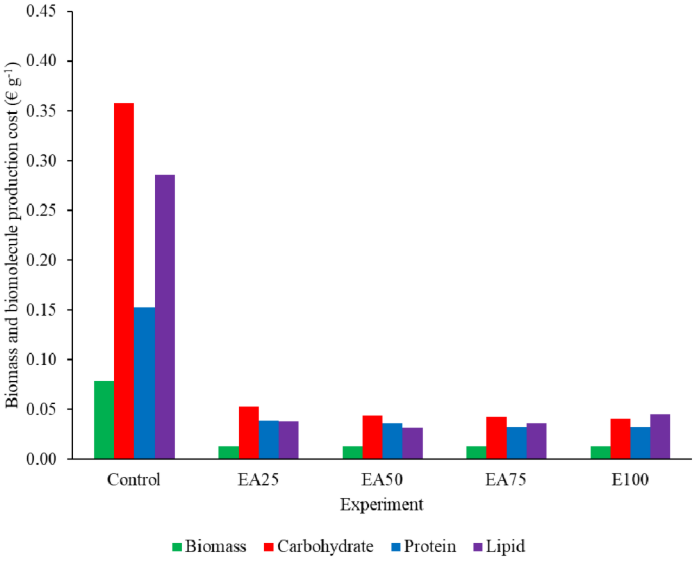
(a)



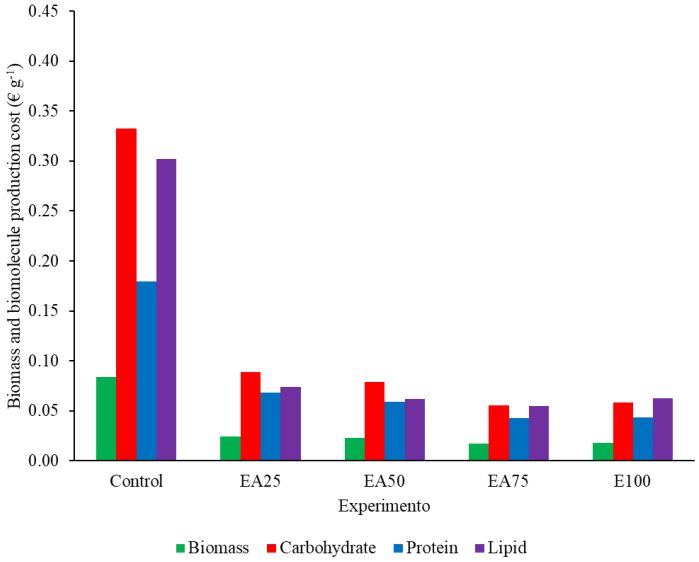
(b)


0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Biomass and biomolecule production cost (€ g-1)
Control
EA25
EA50
EA75
E100
Experiment
Biomass
Carbohydrate
Protein
Lipid

(c)


0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Biomass and biomolecule production cost (€ g-1)
Control
EA25
EA50
EA75
E100
Experiment
Biomass
Carbohydrate
Protein
Lipid

(d)



To provide an overall evaluation, the total production calculation for both biomass and biomolecules was conducted, summing up the biomass recovered during harvests and the final biomass at the last cut (Fig. 3d). In Table 2, we can observe that the control assay presented the highest final biomass concentration (0.990 g L^{-1}), highlighting the potential of effluent-based cultivation to reduce production costs at laboratory scale. Among the assays with effluent addition, EA75 showed the highest biomass production (0.745 g L^{-1}), closely followed by E100 (0.713 g L^{-1}), suggesting that effluent concentrations of 75% to 100% can sustain biomass production levels comparable to the control under the experimental conditions tested. EA25 had the lowest biomass concentration (0.529 g L^{-1}).

Table 2 - Cost of production of biomass and biomolecules in cultivations with and without brewery effluent

	Control	EA25	EA50	EA75	E100
Final biomass (g L^{-1})	0.990	0.529	0.566	0.745	0.713
Protein (g L^{-1})	0.463	0.191	0.220	0.306	0.301
Carbohydrate (g L^{-1})	0.250	0.146	0.164	0.233	0.223
Lipid (g L^{-1})	0.275	0.175	0.209	0.236	0.207
Biomass cost (€ g $biomass^{-1}$)	0.084	0.025	0.023	0.017	0.018
Protein cost (€ g $protein^{-1}$)	0.179	0.068	0.059	0.043	0.043
Carbohydrate cost (€ g $carbohydrate^{-1}$)	0.332	0.089	0.079	0.056	0.058
Lipid cost (€ g $lipid^{-1}$)	0.302	0.074	0.062	0.055	0.063

In all the analyzed compounds (proteins, carbohydrates, lipids), the control showed the highest quantities, reflecting the high nutrient availability of the standard cultivation medium, with protein, carbohydrate, and lipid concentrations of 0.463 g L^{-1}, 0.250 g L^{-1}, and 0.275 g L^{-}

^{1}, respectively. EA75 and E100 stood out, presenting carbohydrate concentrations of 0.233 g L^{-1} and 0.223 g L^{-1}. The production costs of biomolecules in the effluent assays show a significant reduction, reaching €0.017 per g of biomass, €0.043 per g of proteins, and €0.056 per g of carbohydrates in EA75, indicating a potential reduction in production costs under these cultivation conditions.

4 DISCUSSION

4.1 MICROALGAL GROWTH KINETICS

The differences observed in growth profiles among assays can be attributed to nutrient availability among the cultures: the higher the nutrient/effluent concentration, the longer it takes to reach the stationary phase (Carvalho *et al.*, 2018). The control, with the highest nutrient supply, therefore took the longest to reach this phase. Song et al., (2020) evaluated the growth of *Chlorella* sp. UTEX1602 and *Chlorella* sp. L166 in brewery effluent and observed a shorter stationary phase compared to the control. The authors correlated these results with differences in medium composition and nutrient limitation in the effluent. A shorter time to reach the stationary phase allows faster accumulation of energy storage compounds.

Microalgae biomass composition varies according to the growth phase. During the stationary phase, microalgae tend to produce more energy storage molecules, such as lipids and carbohydrates, which are of commercial interest (Yaakob *et al.*, 2021). Chi et al., (2022) studied the growth of *Chlorella* sp. in logarithmic and stationary phases to assess lipid production impacts. They found that biomass harvested in the stationary phase has a higher lipid content, approximately 24% (w/w of dry biomass), compared to 20% (w/w of dry biomass) in the logarithmic cultures. During the stationary phase, nutrient concentration in the medium is lower compared to the logarithmic phase, leading to nutrient deprivation or limitation. Consequently, *Chlorella* sp. accumulates lipids. The effect of semi-continuous cultivation to enhance productivity is demonstrated in the literature. Das et al. (2025) implemented a semi-continuous cultivation strategy for *Chlorella sorokiniana*, achieving biomass yields 4.35 times higher than batch systems. Similarly, Bai et al. (2025) optimized semi-continuous conditions for *C. vulgaris*, obtaining 2.6-fold increases in biomass compared to batch cultures. These results align with the trends observed in our study, which semi-continuous system promoted stable biomass production, particularly in the E100 and EA75 assays.

The decline in productivity observed in the control assay across successive cycles may be associated with the fixed 40% medium renewal rate applied in the semicontinuous regime.

Under these conditions, biomass growth in the control cultures may not have been sufficient to compensate for biomass removal during each renewal step, resulting in partial washout and cumulative productivity losses across cycles. Similar effects have been reported in semicontinuous microalgal systems operated at fixed dilution rates (Tan et al., 2018). In contrast, effluent-based assays maintained productivity stability, suggesting a higher capacity for biomass recovery between cycles.

Cassuriaga et al. (2023) studied the cultivation of *Chlorella fusca* LEB 111 under nitrogen limitation with the addition of organic carbon (xylose). They observed a decline in productivity (P_{max}) in the control over the cycles, which did not occur in assays with organic carbon. This effect was attributed to mixotrophic metabolism, allowing the microalgae to partially rely on organic carbon sources and thereby mitigate the negative effects of nutrient limitation on growth. Similar behavior was observed in the present study, where cultures grown in brewery effluent exhibited greater productivity stability across cycles. This stability may be associated with the presence of organic carbon in the effluent, which supports metabolic flexibility under nutrient-limited conditions, as observed in assays with higher effluent concentrations (EA75 and E100).

Low nitrogen availability, as found in brewery effluent (Ferreira, A. *et al.*, 2019), tends to reduce the content and integrity of thylakoid membranes in microalgal cells, as reported under nitrogen-limited conditions (Kumari *et al.*, 2021). This activates acyl hydrolase, which promotes the hydrolysis of phospholipids, leading to a reduction in biomass concentration (Yaakob *et al.*, 2021). However, this limitation did not impact the productivity of *Chlorella fusca* LEB 111, possibly due to the availability of organic carbon in the effluent, which supports mixotrophic metabolism and partially offsets the effects of nitrogen limitation on growth. These results reinforce that brewery effluent adequately meets the nutritional needs of *C. fusca* LEB 111, functioning as a viable substitute for the standard cultivation medium. Although the control culture exhibited higher biomass concentrations, the effluent assays outperformed in other kinetic growth parameters, showing greater stability of growth and biomass productivity across successive cultivation cycles under nutrient-limited and semicontinuous conditions. This behavior reflects the capacity of microalgae to maintain physiological performance under fluctuating nutrient availability, a feature commonly associated with metabolic flexibility in wastewater-based and mixotrophic cultivation systems (Cassuriaga *et al.*, 2023; Tan *et al.*, 2018).

Overall, the performance of *Chlorella fusca* LEB 111 under semicontinuous cultivation cannot be explained by nutrient availability alone. The interaction between fixed medium

renewal (40%), progressive biomass withdrawal across cycles, nitrogen limitation, and the presence of organic carbon in the brewery effluent collectively influenced growth kinetics, productivity stability, and biomass composition. While the control culture benefited from higher nutrient availability, it was more sensitive to productivity losses across cycles. In contrast, effluent-based assays exhibited greater metabolic flexibility, likely supported by mixotrophic metabolism, which contributed to more stable productivity under semicontinuous operation.

4.2 BIOMASS COMPOSITION

The control assay maintained optimal growth conditions without experiencing nutrient stress, which explains its consistently higher protein levels. In contrast, assays with lower nutrient availability highlighted *Chlorella fusca* LEB 111's capacity to accumulate energy reserves under nutritional stress. Low nitrogen levels in the effluent directly affected the production of microalgal proteins (Arutselvan *et al.*, 2022). The increase in carbohydrate and lipid fractions, accompanied by a decrease in proteins, is a typical response to nutrient scarcity (Niccolai *et al.*, 2019). However, in the present study, biomass composition was not governed by nutrient availability alone. The semi-continuous cultivation regime, together with progressive biomass withdrawal across cycles and the presence of organic carbon in the effluent, influenced metabolic regulation and carbon allocation, contributing to the compositional patterns observed.

Limiting essential nutrients like nitrogen (N) or phosphorus (P), as observed in EA25 and EA50 assays, can induce triacylglycerol (TAG) accumulation and cause chloroplast damage, resulting in reduced photosynthesis and chlorosis. Fatty acid synthesis in chloroplasts involves the enzyme acetyl CoA carboxylase (ACCase), essential for lipid biosynthesis. Under stress, the expression of genes related to TAG synthesis serves as a protective mechanism, where high initial photosynthetic capacity supports greater lipid yield in microalgae (Kumari *et al.*, 2021).

Tan et al., (2018) investigated the semi-continuous cultivation of *Chlorella vulgaris* using chicken waste as a substrate and a renewal rate of 30% (v/v). The results showed lipid contents between 26% and 37% w/w, lower than those obtained in this study. This difference can be explained by the contrasting nutrient profiles: chicken residue is rich in nitrogen and phosphorus, which favor protein synthesis and reduce lipid accumulation, whereas brewery

effluent is nutrient-limited, creating stress conditions that redirect carbon flux towards lipid storage.

Similary, Morais et al. (2022) reported that microalgae cultivated in nitrogen-rich tertiary urban wastewater had low lipid contents (5.43–12.8%) but higher protein fractions (23.2–36.3%). This pattern reinforces the influence of nitrogen availability on biomass composition. In contrast to these nitrogen-rich wastewater conditions, the nitrogen-limited profile of brewery effluent in our study favored lipid accumulation (up to 40.61%) while still sustaining balanced protein and carbohydrate levels, evidencing the metabolic plasticity of *Chlorella fusca* under different wastewater compositions.

4.3 ANALYSIS OF BIOMASS AND BIOPRODUCTS PRODUCTION COSTS

According to Llamas et al., (2021), the average cost of microalgal cultivation was estimated to be approximately 12.4 € per kg of biomass produced. This value was obtained through a sensitivity analysis conducted to identify the main factors influencing biomass production costs, considering the use of closed photobioreactors on a laboratory scale. The study indicated that the main cost factors are labor and equipment depreciation.

Although this study focused on closed photobioreactors due to their controlled conditions and practicality in brewery environments, it is important to acknowledge that open raceway ponds offer significant economic advantages. These systems have reduced operational costs, particularly in sunny climates where natural light can replace artificial lighting (Masojídek *et al.*, 2023). However, photobioreactors provide greater control over contamination risks, a critical factor in maintaining biomass quality, which justifies their choice in this work (Paladino; Neviani, 2021).

The cost of brewery effluent was set at 0.00 €, as it is a byproduct of the brewing process. In some cases, effluents can even have a negative cost, as companies may pay for their disposal. If this is the case for the target brewery, the process becomes even more economically attractive (Verhuelsdonk; Glas; Parlar, 2021), further enhancing the viability of integrating brewery effluent into microalgal cultivation. This highlights an additional potential advantage of this approach in reducing overall production costs.

This efficiency in the production of microalgal compounds, in addition to biomass, suggests the potential applicability of brewery effluent within a microalgae biorefinery concept. Using brewery effluent can reduce production costs for these compounds and produce bioproducts, such as advanced biofuels, which require significant amounts of carbohydrates

and lipids, produced in this study (DeRose *et al.*, 2019). Other bioproducts produced through biorefinery processes include biopolymers (Morais, M. G. de *et al.*, 2023), biofertilizers (Costa; Cruz; Rosa, 2021), and pigments (Russell; Rodriguez; Yaseen, 2022).

Rossi et al. (2023) explored the use of digestate wastewater for microalgae cultivation in a microalgae-bacteria consortium, containing mainly *Chlorella*, *Scenedesmus*, and *Chlamydomonas* spp. Their findings confirm that these practices can reduce operational costs while treating effluents and producing value-added biomass. The study highlights the ability of microalgae to remove nutrients such as nitrogen and phosphorus, which are crucial for reducing costs in wastewater treatment systems. Aggarwal and Remya, (2022) conducted a study on the production of next-generation biofuels from microalgae with simultaneous wastewater treatment, examining various types of wastewater, including industrial and municipal. Focusing on green algae and cyanobacteria, the study observed that process variables influenced both the yield and production costs of the biofuel, concluding that microalgae cultivated in wastewater represents a promising alternative to address the growing energy demand.

The cost analysis indicates that utilizing brewery effluent can be a cost-effective strategy for producing biomass, lipids, carbohydrates, and proteins, thereby contributing to a sustainable and economically viable biorefinery operation within the framework of a circular economy. Due to the high volume of effluent produced, many more cycles can be conducted to produce biomass at a much lower cost. This approach reduces reliance on expensive resources and promotes efficient use of industrial byproducts, aligning with circular economy principles. Considering that the biomass production cost reported in closed photobioreactors exceeds 20 € kg^{-1} (Llamas *et al.*, 2021), the estimated biomass production cost obtained in this study was approximately 18 € kg^{-1} when cultivated in 100% brewery effluent. Acién et al., (2012) emphasize that open raceway ponds in large-scale operations can further reduce costs, achieving values as low as 3 € kg^{-1}. These findings support the potential economic relevance of effluent-based microalgal cultivation, while acknowledging that comprehensive techno-economic assessments are required to validate large-scale feasibility.

Stirk et al. (2024) highlighted the potential of *Chlorella sorokiniana* biomass as a plant biostimulant when cultivated under semi-continuous and mixotrophic conditions. In their study, cultures grown in diluted pig manure achieved daily biomass productivities of up to 149 mg L^{-1} day^{-1}, even under suboptimal greenhouse conditions with low light (PAR = 0.99 ± 0.18 mol photons m^{-2} day^{-1}) and variable temperatures (3.7–23.7 °C). Biomass derived from pig manure cultures exhibited auxin concentrations above 350 pmol g^{-1} DW for IAA and 84 pmol g^{-1} DW for oxIAA, considerably higher than those observed in Na-acetate enriched

and batch cultures. Notably, biostimulant activity was confirmed via mungbean rooting assays, where the biomass elicited effects equivalent to 1–2 mg L^{-1} of IBA in Na-acetate cultures and 0.5–1 mg L^{-1} in pig manure systems, surpassing the batch culture controls. These findings support the application of nutrient-rich effluents and semicontinuous systems not only for cost-effective cultivation, but also for enhancing the bioactivity of the resulting biomass for agricultural use.

5 CONCLUSION

This study demonstrated that brewery effluent is a viable alternative medium for *Chlorella fusca* LEB 111 cultivation under semi-continuous conditions, effectively supporting growth and modulating biomass composition. Higher effluent concentrations, especially EA75 and E100, enabled stable productivity over time and improved accumulation of reserve compounds, such as lipids (up to 40.36%) and carbohydrates, while lower concentrations induced stress and productivity loss. Although the control assay reached the highest biomass concentration (1.06 g L^{-1}), it showed a progressive decline in growth efficiency. The effluent assays favored metabolic adaptation and resource conversion, with EA75 standing out for its biochemical performance and E100 for its growth stability. Economically, production costs were reduced by up to 79%, reaching €18 kg^{-1} in the 100% effluent assay. These findings confirm that brewery effluent not only meets the nutritional requirements of *C. fusca* but also enhances process sustainability and economic feasibility, supporting its integration into circular bioeconomy strategies.

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ARTICLE III

BIOSTIMULANT EFFECTS OF MICROALGAE CULTIVATED IN BREWERY EFFLUENT ON SEED GERMINATION AND PLANT GROWTH

ABSTRACT

Brewery wastewater is a low-cost medium for microalgal cultivation. Microalgal extracts contain bioactive compounds that influence plant development and responses to stress, enabling their use in the production of biostimulants. This study aimed to evaluate the biostimulant potential of *Chlorella fusca* and *Spirulina* sp. extracts, cultivated in brewery wastewater, on seed germination and root development of barley and lettuce, as well as on the physiological and growth response of lettuce under drought stress following foliar application. Both microalgal species grew effectively in the effluent, with *Spirulina* sp. reaching high productivity (69.9 mg L^{-1} d^{-1}). Beyond biomass growth performance, this work demonstrates for the first time the integration of brewery wastewater remediation, targeted phytohormonal production, and agronomic validation in a single approach. On the one hand, *Spirulina* sp. extract increased barley germination by 58% and doubled root length compared to the control without biostimulant. On the other hand, under severe drought stress, *Chlorella* performed better and treated lettuce maintained biomass levels comparable to well-watered plants, with fresh weight 46.5% higher than the drought-stressed control and sustained photosynthetic activity (25% increase in electron transport rate). These results demonstrate that brewery wastewater can act not only as a low-cost cultivation medium but also as a metabolic factor influencing phytohormonal biosynthesis in algae and, consequently, the biostimulant effects observed in plants. By establishing a direct link between wastewater-grown microalgae, hormone profiles, and crop physiological responses, this study provides functional evidence for the conversion of brewery effluents into effective biostimulant inputs with agronomic relevance.

Keywords: Circular economy; Foliar application; Phytohormones; Sustainable agriculture; Waste valorization.

1 INTRODUCTION

The transition towards circular bioeconomy models has intensified interest in the recovery and valorization of organic waste streams as sources of nutrients and bio-based products (Leong *et al.*, 2021). In this context, industrial sectors such as brewing generate large volumes of nutrient-rich effluents that pose environmental and treatment challenges, while simultaneously offering opportunities for resource recovery (Rubert *et al.*, 2026). According to Olajire (2020), the brewing sector produces up to 7–10 liters of effluent per liter of beer,

highlighting its environmental burden when not properly managed. However, these effluents contain carbon, nitrogen and trace elements capable of sustaining microalgal growth, thus transforming a liability into a potential resource. Previous studies have reported successful cultivation of microalgae in brewery wastewater (Conceição, Jenyfer de Almeida *et al.*, 2026; Papadopoulos *et al.*, 2022; Silva, A. L. e *et al.*, 2025)**,** confirming its suitability as a low-cost medium. Within this framework, microalgae-based systems offer a dual benefit by combining wastewater remediation with the sustainable production of high-value bioproducts. Among these, microalgae-derived biostimulants stand out due to their biochemical richness and multifunctional roles in modulating plant physiological responses.

Microalgae-derived biostimulants supply a suite of bioactive molecules that modulate plant growth and stress responses through complementary mechanisms. Their phytohormonal composition regulates root system architecture, cell division and elongation, seed germination and stomatal behavior, thereby influencing water use efficiency under drought (Rupawalla *et al.*, 2022). As highlighted by Sun et al. (2024), amino acids and small peptides act as osmoprotectants and metabolic precursors that sustain carbon–nitrogen balance and support antioxidant defenses. Consistent benefits of microalgal biostimulants have been reported in seed priming, salinity and drought tolerance assays, and nutrient efficiency trials, including higher germination and radicle elongation, maintenance of photosynthetic performance (e.g., ΦPSII and ETR), enhanced stomatal conductance, and maintenance of plant biomass under stress, as described by Vangenechten et al. (2025). Álvarez-González et al. (2025a) demonstrated that microalgal biomass grown in domestic wastewater could be used as a biostimulant to improve lettuce cultivation, while Ruales et al. (2025) showed that *Scenedesmus* sp. cultivated in industrial wastewaters produced biostimulant extracts that enhanced germination and seedling development of leafy crops. Together, these studies highlight the feasibility of producing biostimulants from waste-grown microalgae and their effectiveness in improving plant performance under stress.

Despite these advances, several knowledge gaps remain. Efficacy is dose- and matrix-dependent, varying with species, extraction method, and application mode (seed soaking vs. foliar), which underscores the need for chemically resolved profiles to guide use. Although numerous studies demonstrate that microalgal extracts mitigate salinity and water deficit and enhance germination (Fiorentino *et al.*, 2025), Santos et al. (2025) emphasize that few investigations explicitly connect hormonal profiling with both targeted bioassays and whole-plant physiological and agronomic assessments. This lack of integration is even more evident for biomass cultivated in industrial effluents, where hormonal composition, germination

performance, and plant physiological responses under drought have rarely been analyzed together. Addressing these interconnected gaps, the present study proposes an integrated framework that couples wastewater valorization, biochemical characterization, and agronomic validation using *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18.

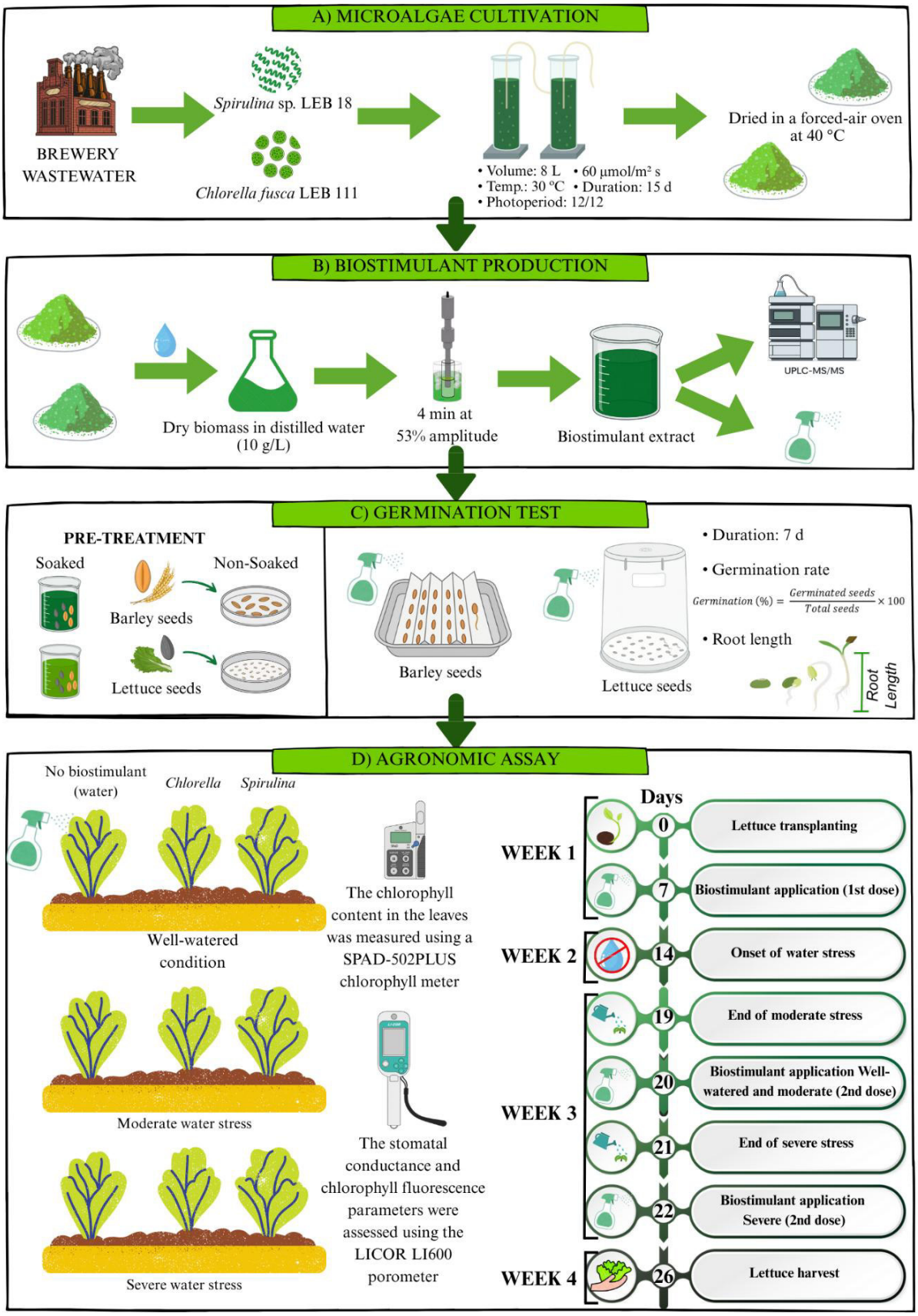
Barley (*Hordeum vulgare* L.) and lettuce (*Lactuca sativa* L.) were selected as complementary model species to evaluate the biostimulant effects of wastewater-grown microalgae. Barley has historical and economic significance as a cereal crop in the brewing industry, and seed priming studies have demonstrated that algal extracts can enhance its germination and vigor under stress conditions (Alharbi *et al.*, 2023; Alling; Funk; Gentili, 2023)**.** Lettuce, in turn, is a widely used model in plant physiology due to its short cycle, ease of cultivation, and high sensitivity to environmental conditions. As highlighted by Bella et al. (2021), lettuce is particularly responsive to bioactive compounds, making it suitable for germination bioassays and for assessing physiological parameters under water deficit. In germination assays, seeds are often subjected either to direct application or to pre-soaking treatments, which could facilitate the rapid uptake of bioactive compounds and improve germination and seedling vigor; such effects have been investigated by previous studies evaluating the potential of hydration as a complementary biostimulant strategy (Sorour *et al.*, 2021). Its commercial importance as a leafy vegetable further supports its use in agronomic trials. Together, these species allow for a dual perspective: barley connecting microalgal biostimulants to the brewing chain, and lettuce serving both as a sensitive model for physiological responses and as a crop of agronomic relevance under drought stress.

Therefore, this study aimed to evaluate the biostimulant potential of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 extracts, cultivated in brewery wastewater, on seed germination and root development of barley (*Hordeum vulgare* L.) and lettuce (*Lactuca sativa* L.), as well as on the physiological performance of lettuce under drought stress.

2 MATERIAL AND METHODS

The methodological steps involved in the experimental design are summarized in Figure 1.

Figure 1 - Schematic representation of the experimental workflow



2.1 MICROALGAE CULTIVATION

The cultivation of two microalgae species, *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18, was carried out using brewery wastewater collected from the cleaning of fermentation tanks after Pilsen-type beer production in a local microbrewery (Rio Grande, RS, Brazil) (COD: 948.8 mg L^{-1}; BOD: 368.3 mg L^{-1}; total N: 5.1 mg L^{-1}; total P: 2.7 mg L^{-1}; pH: 11,92). The strains were obtained from the microalgae culture collection of the Laboratory of Biochemical Engineering at the Federal University of Rio Grande (FURG). Batch cultures were conducted in duplicate using vertical tubular photobioreactors (8 L working volume) under controlled conditions (temperature of 30 °C, 12:12 h light/dark photoperiod, and light intensity of 60 $\mu mol_{photons} m^{-2} s^{-1}$) and an initial biomass concentration of 0.2 g L^{-1}. The cultivation was conducted for 15 days, with continuous aeration provided by filtrated compressed air (Cruz; da Rosa; Costa, 2024).

Microalgal growth was monitored daily by optical density at 670 nm (UVmini-1240, Shimadzu UV/VIS, Japan). A standard curve was previously established to correlate absorbance values with dry biomass concentration (Costa *et al.*, 2002).

The maximum biomass concentration (X_{max}, g L^{-1}) was defined as the highest value recorded during the assay. Volumetric biomass productivity (P_x, g L^{-1} d^{-1}) was calculated according to Equation 1, where X_t is the biomass concentration (g L^{-1}) at time t (days), and X_0 is the initial concentration at time t_0 (Bailey; Ollis, 1986).

$$P_x = \frac{X_t - X_0}{t - t_0} \quad (1)$$

The maximum specific growth rate (μ_{max}, d^{-1}) was measured by linear regression of the logarithmic growth phase, from a linearized biomass concentration profile (Ln X) versus time (days), where the slope of this line was taken as μ_{max}. The generation time (tg) was determined during the exponential growth phase of the microalgae, calculated according to the equation 2 (Bailey; Ollis, 1986).

$$t_g = \frac{\ln(2)}{\mu_{max}} \quad (2)$$

At the end of the cultivation period, the biomass was harvested by centrifugation, dried in a forced-air oven at 40 °C, and stored at –20 °C until use.

2.2 BIOSTIMULANT EXTRACTION AND HORMONAL PROFILE ANALYSIS

Biostimulant extracts were prepared by suspending the oven-dried microalgal biomass in distilled water at a concentration of 10 g L^{-1}. The suspension was subjected to sonication for 4 minutes at 53% amplitude using an ultrasonic processor (Cole Parmer CPX 130 – Illinois, USA), following the protocol described by Morillas-España et al. (2022), to promote cell disruption and metabolite release. The resulting extract was used for germination and agronomic assays.

For hormonal characterization, the dried microalgal biomass was quantified according to the method proposed by Balcke et al. (2012). A total of 50 mg of dried biomass was extracted with methanol containing an isotope-labeled internal standard and centrifuged. The supernatant was purified using solid-phase extraction (SPE) and analyzed by ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) (Agilent Technologies, USA). Quantified compounds included cytokinins (trans-zeatin, zeatin riboside, isopentenyl adenine), gibberellins (GA1, GA3, GA4), auxins (indole-3-acetic acid, IAA), salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA)(Cao *et al.*, 2020).

2.3 GERMINATION ASSAYS

Germination tests were conducted using barley (*Hordeum vulgare*) and lettuce (*Lactuca sativa*) seeds, following standard methodologies recommended for each plant species. For barley seeds, filter paper was folded into four parts, with five seeds placed in each fold, totaling 20 seeds per replicate. For lettuce seeds, the test was carried out using circular filter paper moistened and placed inside Petri dishes, with 20 seeds per dish. The dishes were covered with perforated lids to allow gas exchange and minimize evaporation.

Seeds from both species were subjected to two conditions: with and without pre-soaking. Pre-soaking was performed 24 h before the start of the experiment by immersing the seeds in 10 mL of the respective treatment solution (biostimulant from *Chlorella fusca* LEB 111, biostimulant from *Spirulina* sp. LEB 18, or distilled water for the control). The seeds remained submerged until the setup of the germination tests. For the non-soaked seeds, the same volumes of the respective solutions were applied directly at the beginning of the test, maintaining an approximate ratio of 10 mL per group of 20 seeds.

Each combination of treatment (type of biostimulant or control) and condition (with or without pre-soaking) were tested with 20 seeds, totaling 120 seeds per species (60 soaked and 60 non-soaked). The germination test was conducted over a period of 7 days, with daily monitoring.

Throughout the experimental period, germination rate (eq. 3) was evaluated to assess the germinative performance of the seeds under the different treatments applied.

$$Germination\ (\%) = \frac{Germinated\ seeds}{Total\ seeds} \times 100 \quad (3)$$

At the end of the germination assay (i.e. 7 days) root length was measured on each seedling.

2.4 AGRONOMIC ASSAY

Lactuca sativa (lettuce) nursery plant with 3 to 4 leaves were transplanted into 400 cm^3 pots filled with a homogeneous mixture of 50% soil and 50% perlite, adjusted with calcium carbonate at a rate of 3 g L^{-1} to correct the pH. After transplanting, the pots were maintained in a growth chamber at 25 °C, under a 16/8 h light/dark photoperiod. The experiment was conducted under specific water stress conditions, comprising three treatments: a control with regular irrigation (50 mL of Hoagland nutrient solution applied daily), a moderate drought treatment in which irrigation was withheld for 5 consecutive days, and a severe drought treatment in which irrigation was withheld for 7 consecutive days. The experimental design involved 90 pots containing Lactuca sativa, divided into three groups according to these irrigation regimes. Each group consisted of 30 pots, equally distributed among three treatments, with 10 pots each: control (biostimulant or distilled water application), application of *Chlorella fusca* LEB 111 biostimulant, and application of *Spirulina* sp. LEB 18 biostimulant (Figure 1). The microalgal biostimulants were applied by foliar spraying at two time points: before and after the stress period.

2.5 PHYSIOLOGICAL AND BIOMETRIC ANALYSES

Throughout the experiment, leaf chlorophyll content was measured using a SPAD-502PLUS chlorophyll meter (Konica Minolta Inc., Japan), In addition, stomatal conductance and chlorophyll fluorescence parameters were assessed using the LICOR LI600 porometer (LI-COR Biosciences, USA). Among the parameters obtained with the LICOR device, the apparent transpiration rate (*E_apparent*) was measured to indicate water vapor loss through the leaf surface. and stomatal conductance to water vapor (gs_w) was evaluated to determine the degree of stomatal opening. The effective quantum yield of photosystem II (*PhiPS2*) was also determined, providing information on the efficiency of photochemical energy conversion under

light-adapted conditions. Additionally, the electron transport rate (*ETR*) was measured, representing the rate of electron flow through PSII and serving as an indicator of the photosynthetic performance of the plants.

At the end of the assay, the lettuce plants were harvested, carefully removed from the pots, and weighed individually to determine their fresh weight.

2.6 STATISTICAL ANALYSES

All analyses were performed in triplicates. The results were assessed using analysis of variance (ANOVA), followed by Tukey's test for comparing mean values at a 90% confidence level. Statistical analyses were performed using the Statistica 7 software.

3 RESULTS AND DISCUSSION

3.1 MICROALGAL GROWTH PERFORMANCE

Spirulina sp. LEB 18 reached higher maximum biomass concentration (0.76 g L^{-1}; $p < 0.10$) and productivity (69.86 mg L^{-1} d^{-1}; $p < 0.10$) than *Chlorella fusca* LEB 111 (0.52 g L^{-1}; 46.34 mg L^{-1} d^{-1}). The maximum specific growth rate (μ_{max}) was also superior for *Spirulina* (0.31 d^{-1}; $p < 0.10$), whereas *Chlorella* presented μ_{max} of 0.16 d^{-1}. Generation time (tg) was approximately half in *Spirulina* (2.24 d; $p < 0.10$) compared to *Chlorella* (4.24 d).

The superior performance of *Spirulina* is consistent with the physiology of cyanobacteria. *Spirulina* exhibits a capacity to assimilate bicarbonate efficiently, high carboxysome activity, and tolerance to fluctuations in pH and salinity, enabling rapid metabolic adjustment in alkaline and carbon-rich environments (Cogne; Gros; Dussap, 2003; Li, P. *et al.*, 2023; Liu *et al.*, 2025). In contrast, *Chlorella* exhibits a metabolism more dependent on inorganic nitrogen availability, a nutrient limited in brewery effluents, which may slow biomass accumulation under such conditions (Ansari *et al.*, 2025; Huang, Y. *et al.*, 2021; Tavares *et al.*, 2023). Under nitrogen-restricted environments, *Chlorella* also tends to experience physiological stress, which can redirect metabolic fluxes toward alternative compounds such as phenolics, amino-acid derivatives and stress-related phytohormones, potentially altering growth efficiency (Das *et al.*, 2025). The observed kinetic differences between the two strains therefore reflect their distinct physiological strategies and nutrient requirements.

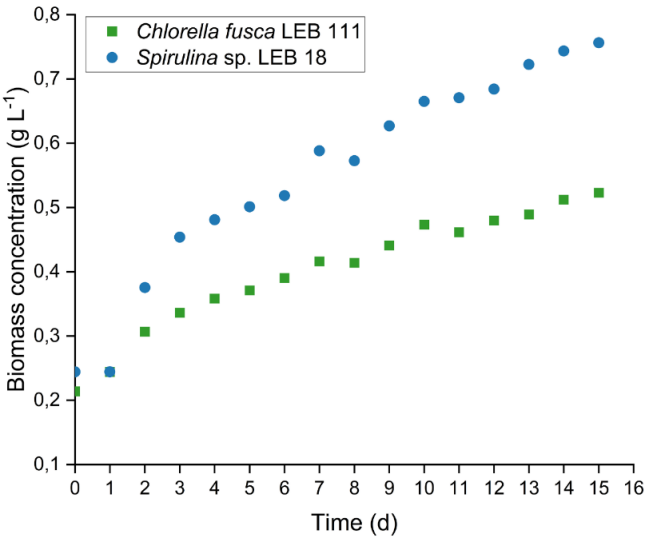
Studies in the literature report similar trends. Barati et al. (2025) achieved specific growth rates of 0.29 d^{-1} for *Arthrospira platensis* cultivated in brewery wastewater under

optimized light and CO_2 supply conditions, values similar to this study. Silva et al. (2025) reported average productivities of 60 mg L^{-1} d^{-1} in pilot-scale *Arthrospira* cultivation using brewery effluent. Cruz et al. (2024) evaluated the growth kinetics of *Spirulina* sp. LEB 18 and *Chlorella fusca* LEB 111 under standard conditions and also reported clear physiological differences between the two species. *Chlorella* exhibited a μ_{max} of 0.16 d^{-1} and a tg of 4.43 d, whereas *Spirulina* reached higher μmax values (0.29 d^{-1}) and a shorter tg (2.44 d).

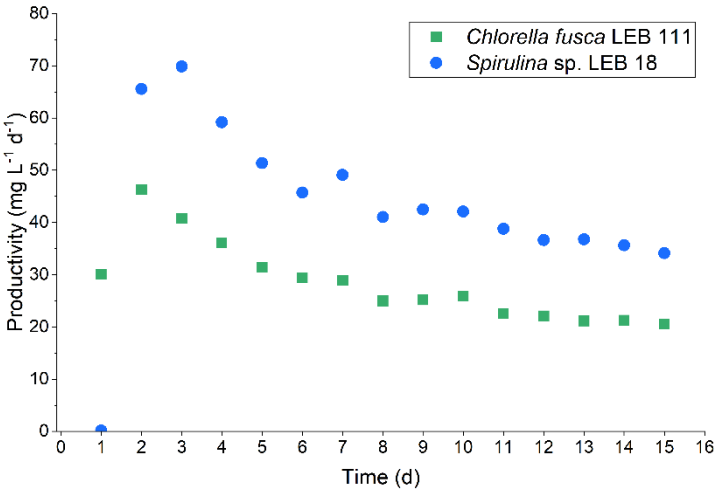
The growth curves (Figure 2a) show that both microalgae displayed positive and continuous biomass accumulation throughout the 15 d cultivation period. *Spirulina* presented a short lag phase (approximately 24 h), followed by an exponential increase between days 1 and 3, whereas *Chlorella* entered exponential growth earlier but with a lower slope and reduced final biomass. Neither microalga reached the stationary phase during this period; however, *Chlorella* showed an incipient trend toward stabilization, suggesting the onset of nutrient depletion and the transition out of exponential growth. In general, phytohormone biosynthesis in microalgae intensifies during the late exponential and stationary phases, when nutrient availability decreases and secondary metabolism is enhanced. Auxins and cytokinins typically accumulate at the transition between exponential and stationary phases (Ahmed, M.; Stal; Hasnain, 2010; Žižková *et al.*, 2017).

Figure 2 - Growth profile (a), daily biomass productivity (b), and pH variation (c) of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 cultivated in brewery wastewater over the cultivation period. Values represent means ± SD (n = 2).

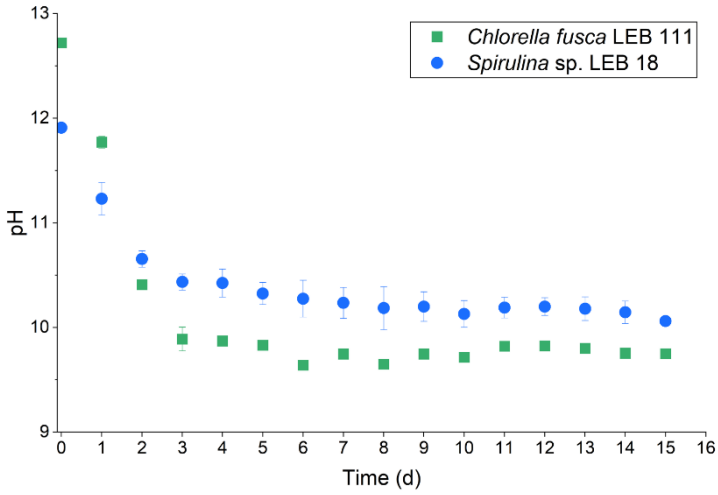
(a)



(b)



(c)



The lag phase observed in *Spirulina* sp. LEB 18 may be related to its cyanobacterial nature, which often requires more time to adjust its metabolic machinery to organic-rich environments such as brewery wastewater (Papadopoulos *et al.*, 2020). The composition of brewery wastewater, typically low in nitrogen and phosphorus but rich in organic carbon, influences growth dynamics (Simate *et al.*, 2011). In this study, the relatively balanced C:N:P ratio and low turbidity (total N: 5.1 mg L^{-1}; total P: 2.7 mg L^{-1}) likely favored efficient light penetration and nutrient uptake.

Maximum biomass productivity for both strains occurred between days 2 and 3, coinciding with the exponential growth phase (Figure 2a,b). During this period, a marked decrease in pH was observed, from initial alkaline values above 12 to approximately 10 (Figure 2c). This early acidification is commonly reported in microalgal cultures grown in organic-rich

wastewaters and is associated with the oxidation of organic carbon, respiratory CO_2 release and microbial activity, processes that reduce alkalinity and facilitate metabolic activation and biomass accumulation (Morais, E. G. de *et al.*, 2024). After this initial adjustment, pH values stabilized above 10, reflecting the onset of intense photosynthetic activity and bicarbonate uptake, particularly in Spirulina, which exhibited higher productivity under these alkaline conditions.

3.2 PHYTOHORMONAL COMPOSITION

The hormonal profile of the biomass cultivated in brewery wastewater (Table 1) detected auxins (IAA), cytokinins (tZ, ZR and iP), gibberellins (GA_1, GA_3 and GA_4), ethylene precursors (ACC) and stress-related hormones (SA, MLT, ABA and JA) in both *Chlorella fusca* LEB 111 and *Spirulina* LEB 18. The total hormone content reached 0.433 mg g^{-1} in *Chlorella* and 0.408 mg g^{-1} in *Spirulina*, showing that both strains accumulated similar hormone levels on a dry-weight basis. Even with similar total hormone contents, each microalga exhibited a distinct hormonal composition, conferring different functional properties to the biomass when applied as a biostimulant (Tripathi; Srivastava, 2025).

Table 1 - Concentrations of endogenous phytohormones (ng g-1) in *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 biomass cultivated in brewery effluent

Hormones	(ng g^{-1})	*Chlorella fusca* LEB 111	*Spirulina* sp. LEB 18
Ethylene Precursor	ACC	15,930	15,905
Cytokinins	tZ	107,815	48,436
	ZR	56,199	50,607
	iP	250,710	289,363
		414,724	*388,406*
Gibberellins	GA_1	72.82	292.12
	GA_3	6.65	1,153.49
	GA_4	37.94	40.10
		117.41	*1,485.71*
Auxin	IAA	1,296	2,159
Other hormones	SA	411.82	196.42
	MLT	93.35	11.35
	ABA	NF	NF
	JA	278.37	61.91

ACC refers to 1-aminocyclopropane-1-carboxylic acid; tZ is trans-zeatin; ZR is zeatin riboside; iP stands for isopentenyl adenine; GA1, GA3, and GA4 are gibberellins A1, A3, and A4, respectively; IAA is indole-3-acetic acid;

MLT is melatonin; SA stands for salicylic acid; ABA is abscisic acid; and JA refers to jasmonic acid. NF refers to Not Found. For cytokinins and gibberellins, the values in italics correspond to the sum of individual compounds within each group.

From a biochemical perspective, the phytohormones detected in both microalgae originate from distinct metabolic pathways that respond differently to environmental conditions. Growth-related hormones such as cytokinins, auxins and gibberellins are generally associated with primary metabolism, cell division and chloroplast development (Chen *et al.*, 2024; Lu *et al.*, 2014; Werner; Schmülling, 2009), whereas salicylic acid and jasmonic acid are more closely linked to stress perception and acclimation responses, particularly under oxidative and alkaline conditions (Arnao *et al.*, 2023; Awad; Vega-Estévez; Griffiths, 2020; Umar; Ahmad; Xu, 2025). Tryptophan-dependent synthesis of IAA and melatonin, as well as the activation of stress-related hormonal pathways, has been reported as a common response of microalgae to variable environmental conditions (Huang, J.; Yue; Hu, 2014; Kang *et al.*, 2025; Zhao, 2012). This functional differentiation among hormone classes helps explain how distinct physiological strategies may emerge from similar total hormone contents, depending on the relative allocation among growth-promoting and stress-related compounds.

Cytokinins were the dominant group in both species (approximately 4.15×10^5 ng g^{-1} in *Chlorella* and 3.88×10^5 ng g^{-1} in *Spirulina*), particularly isopentenyl adenine (iP) and zeatin-type cytokinins (250,710 ng g^{-1} iP and 107,815 ng g^{-1} tZ in *Chlorella fusca* LEB 111), followed by auxins and gibberellins. Although both microalgae showed high cytokinin contents, their profiles differed: *Spirulina* exhibited higher levels of iP (289,363 ng g^{-1}), a cytokinin commonly associated with cell proliferation and early growth processes, whereas *Chlorella* accumulated higher trans-zeatin concentrations, a hormone frequently linked to stress signaling and physiological adjustment (Miranda *et al.*, 2024).

Auxin (IAA) contents were high in both microalgal biomasses; however, *Spirulina* sp. LEB 18 accumulated approximately twice the IAA concentration observed in *Chlorella fusca* LEB 111 (2,159 and 1,296 ng g^{-1}, respectively).Cruz et al. (2023), who cultivated *Spirulina* sp. LEB 18 under standard synthetic medium, reported IAA levels of 1.94 nmol g^{-1} (≈340 ng g^{-1}). Rupawalla et al. (2022) also quantified lower IAA levels (216 ng g^{-1}) in *Chlorella* grown in synthetic medium. These comparisons demonstrate that IAA accumulation is sensitive to cultivation conditions rather than being an intrinsic species trait alone. The presence of residual organic carbon and micronutrients in brewery effluent likely modulated the C:N:P balance and stimulated tryptophan-dependent IAA pathways (Álvarez-González; Greque de Morais; *et al.*, 2023; Bajguz; Piotrowska, 2009). The alkaline initial pH may have further activated oxidative

stress-related auxin pathways in both strains (Stirk, W. A. *et al.*, 2014). The higher IAA content in *Spirulina* agrees with its higher specific growth rate and shorter generation time in brewery wastewater, since auxins act synergistically with cytokinins to promote cell division, and biomass accumulation (Žižková *et al.*, 2017).

Chlorella fusca LEB 111 accumulated higher levels of salicylic acid (411.8 ng g^{-1}) and jasmonic acid (278.4 ng g^{-1}), hormones classically associated with stress perception and acclimation responses (Balcke *et al.*, 2012; Tripathi; Srivastava, 2025). This hormonal profile is consistent with an adaptive response to the alkaline conditions of the brewery wastewater used in this study (initial pH ≈ 11.9). Elevated pH and nutrient imbalance are recognized abiotic stressors for microalgae, capable of inducing oxidative stress and triggering salicylic acid biosynthesis to mitigate cellular damage through antioxidant regulation, while jasmonic acid is commonly linked to growth restraint and metabolic reprogramming, promoting resource redistribution under unfavorable conditions (Han, Xingfeng *et al.*, 2018; Stirk, Wendy A.; van Staden, 2022). Accordingly, the higher SA and JA levels observed in *Chlorella* are consistent with its slower growth and earlier tendency toward growth limitation in brewery effluent, suggesting a stronger stress burden under alkaline and chemically complex conditions.

In contrast, *Spirulina* sp. LEB 18 exhibited higher concentrations of growth-promoting gibberellins, particularly GA_1 (292.1 ng g^{-1}) and GA_3 (1,153.5 ng g^{-1}), hormones directly involved in seed germination, reserve mobilization and cell elongation (Chen *et al.*, 2024). This contrasting hormonal allocation reflects distinct physiological strategies between the two microalgae: while *Chlorella* preferentially invests in stress-related signaling pathways under alkaline stress, *Spirulina* directs a greater fraction of its metabolic resources toward terpenoid hormones associated with rapid cell division and biomass accumulation, in agreement with its superior growth performance and productivity in brewery wastewater (Chen *et al.*, 2024; Stirk, W. A. *et al.*, 2014).

Comparisons with other wastewater-based cultivation systems support this interpretation. Álvarez-González et al. (2025b) reported that total phytohormone levels in *Scenedesmus* can vary by more than two orders of magnitude depending on cultivation scale and environmental conditions, ranging from about 100 ng g^{-1} in indoor cultures to 36,236 ng g^{-1} in outdoor raceway ponds. Bayona-Morcillo et al. (2020) quantified around 3,000 ng g^{-1} of total hormones in hydrolysates of *Arthrospira platensis* from commercial production. Altogether, the contrasting hormonal profiles observed between *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 suggest distinct agronomic potentials. The higher levels of SA and JA in the *Chlorella* extract are compatible with signaling pathways typically associated with stress perception and

acclimation, supporting its possible use in priming responses against abiotic stress (Álvarez-González; Serrano; *et al.*, 2025; Ronga *et al.*, 2019). In turn, the elevated IAA and gibberellin contents in the *Spirulina* extract are in line with hormonal routes linked to seed germination and early growth promotion (Bewley; Black, 2014; Chenyin *et al.*, 2023; Rahmawati *et al.*, 2025; Reed; Bradford; Khanday, 2022), which is consistent with the germination and seedling performance observed in the bioassays in this study (Sections 3.3–3.4).

3.3 EFFECTS OF MICROALGAE BIOSTIMULANTS ON SEED GERMINATION

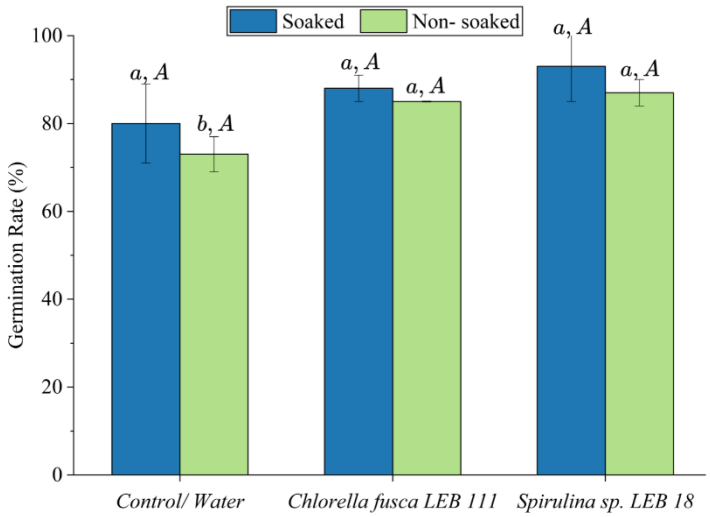
3.3.1 Germination Rate

In the non-soaked condition (Figure 3a), lettuce seeds treated with microalgal extracts showed significantly higher germination than the control. Germination reached 85 ± 2% with *Chlorella* and 87 ± 3% with *Spirulina*, compared to 73 ± 5% in water-treated seeds, representing increases of approximately 16% and 19% ($p < 0.10$). No differences were detected between soaked and non-soaked seeds within each biostimulant, indicating that soaking did not modify the response pattern in lettuce. In barley (Figura 3b), the microalgal extracts increased germination under all conditions tested. In soaked seeds, *Chlorella* increased germination from 55 ± 5% (control) to 75 ± 13%, equivalent to a 36% increase, whereas *Spirulina* reached 87 ± 3%, corresponding to a 58% increase ($p < 0.10$) compared to control. In non-soaked seeds, germination increased from 50 ± 10% (water) to 70 ± 13% with *Chlorella* (+40%) and 75 ± 5% with *Spirulina* (+50%). Only *Spirulina* showed a significant effect of soaking (87 ± 3% vs. 75 ± 5%), suggesting that the 24h priming step facilitated earlier uptake of phytoactive compounds in this case. These plant species-dependent responses reflect differences in seed anatomy and physiology. Lettuce seeds possess high permeable coats, reduced endosperm and robust cotyledons, which support rapid germination (Bewley; Black, 2014; Chenyin *et al.*, 2023; Reed; Bradford; Khanday, 2022). Consistent with this, microalgal treatments often exert limited influence on final germination percentage in lettuce but may affect subsequent seedling development stages (Díaz *et al.*, 2024). In contrast, barley germination is strongly regulated by hormonal signaling following imbibition, particularly through gibberellin-mediated activation of hydrolytic enzymes in the endosperm (McCullen; Awal; Christie, 2024).

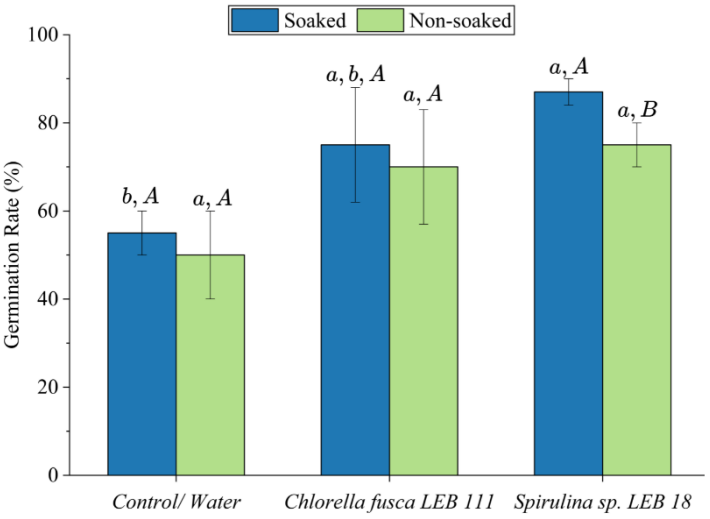
Figure 3 - Effect of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 biostimulants on germination rate (%) of lettuce (a) and barley (b) seeds under non-soaked and soaked conditions. Values represent means ± SD (n = 3). Different lowercase letters above bars indicate significant differences ($p < 0.10$) among biostimulant treatments within the same seed

condition, whereas uppercase letters indicate significant differences ($p < 0.10$) between soaked and non-soaked seeds within the same treatment.

(a)



(b)



The higher levels of GA_1 and GA_3 detected in *Spirulina* (approximately 4-fold and 173-fold higher than in *Chlorella*, respectively; Table 1) help explain its superior performance in barley, as these gibberellins regulate the transcription of genes encoding α-amylase, thereby promoting starch hydrolysis and reserve mobilization during early germination. This interpretation aligns with previous studies reporting enhanced germination and early vigor in cereals treated with microalgal extracts rich in gibberellins and auxins (Alling; Funk; Gentili, 2023; Ronga *et al.*, 2019).

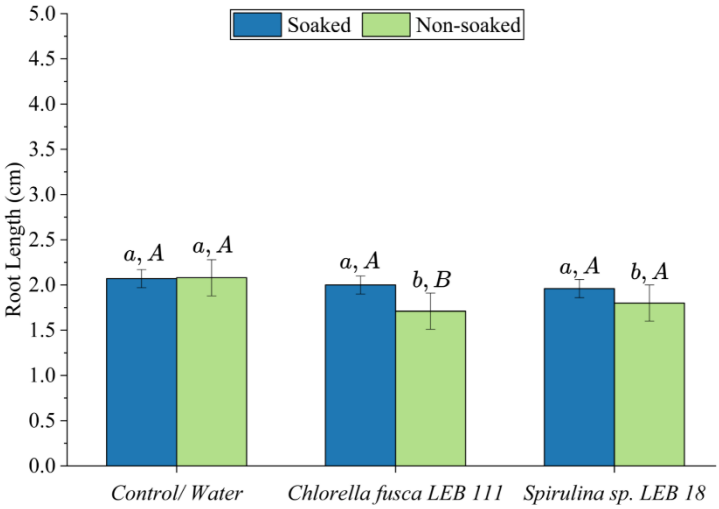
In barley, the positive effect of soaking observed for *Spirulina* may be related to earlier uptake of phytoactive compounds, enhancing GA-mediated responses during the initial hours of germination (Younis *et al.*, 2024). However, this priming effect appears to influence early metabolic activation rather than the final germination percentage, as reported in previous studies using *Spirulina*-based treatments (Rahmawati *et al.*, 2025).

3.3.2 Root Length

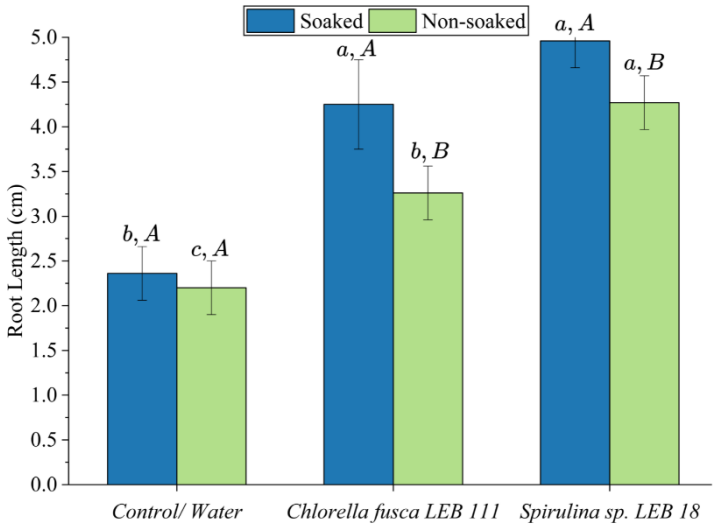
In lettuce, radicle length remained close to 2.0 cm in soaked seeds (2.07 cm for water, 2.00 cm with *Chlorella* and 1.96 cm with *Spirulina*; $p > 0.10$) (Figure 4a). In non-soaked seeds, radicle length was 1.71 cm with *Chlorella* and 1.80 cm with *Spirulina*, compared to 2.08 cm in the control, representing reductions of approximately 18% and 13%, respectively ($p < 0.10$). No differences were observed between soaked and non-soaked seeds within each treatment, indicating that direct application of the extracts to dry seeds may have produced a transient osmotic or hormonal imbalance that restricted early radicle elongation without affecting final germination. This limited response is consistent with findings from Lyalina et al. (2023), who also reported minimal changes in early root growth of lettuce treated with biostimulants, suggesting that dicotyledonous seeds with rapid water uptake may be inherently less sensitive to biochemical stimulation during the initial germination window.

Figure 4 - Effect of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 biostimulants on root length (cm) of lettuce (a) and barley (b) seeds under non-soaked and soaked conditions. Values represent means ± SD (n = 3). Different lowercase letters above bars indicate

(a)



(b)



In barley, root growth was consistently stimulated (Figure 4b). *Spirulina* produced the highest values in all conditions. For soaked seeds, radicle length increased from 2.36 cm (control) to 4.23 cm with *Chlorella* (79% increase) and 4.98 cm with *Spirulina* (111% increase). In non-soaked seeds, radicle length increased from 2.20 cm (water) to 3.26 cm with *Chlorella* (48% increase) and 4.27 cm with *Spirulina* (94% increase). The superior effect of *Spirulina* is consistent with its hormonal profile, which includes higher concentrations of GA_1, GA_3 and GA_4 (1485.71 vs 117.41 ng g^{-1} in *Chlorella*; Table 1) and IAA (2,158.8 vs. 1,296.4 ng g^{-1} in *Chlorella*; Table 1). This combination supports both reserve mobilization and cell elongation in the emerging radicle (Farooq *et al.*, 2009; Reed; Bradford; Khanday, 2022). Hamouda et al. (2022) reported similar trends in wheat, where a 2% *Spirulina* liquid extract increased germination and radicle length by 10% and 49%, respectively, compared to the control. Although their crop, concentration and extraction protocol differ from those used here, the consistent enhancement of early root growth across studies supports the view that *Spirulina*-based biostimulants exert robust promotive effects on cereals (Hamouda *et al.*, 2022).

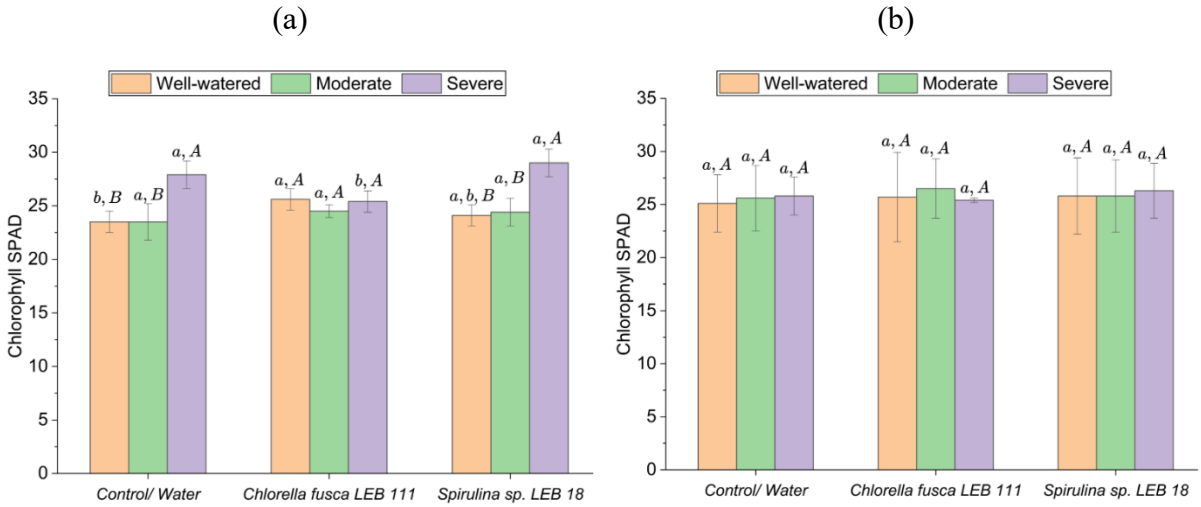
Overall, the results show clear species-dependent and extract-dependent patterns. Barley benefited substantially from the microalgal extracts, particularly *Spirulina*, whereas lettuce showed more discrete responses. These differences highlight the importance of aligning the phytohormonal composition of microalgal biomass (Section 3.2) not only with the physiological requirements of each crop, but also with the intended mode of biostimulant application. In this context, knowledge of whether growth-promoting hormones are predominantly associated with protein- or carbohydrate-related fractions (e.g., auxins and cytokinins) or with lipid-linked compounds (e.g., jasmonates) may guide the selection of

extraction strategies, enabling the targeting of specific agronomic functions such as germination enhancement or stress tolerance (Fiorentino *et al.*, 2025; Gharehbeglou *et al.*, 2024; Miranda *et al.*, 2024).

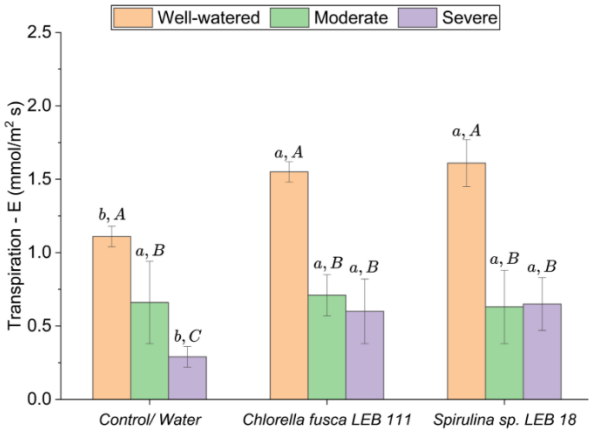
3.4 AGRONOMIC AND PHYSIOLOGICAL RESPONSES IN LETTUCE PLANTS

Physiological variables were evaluated at two key moments of the lettuce cycle: the third week (corresponding to the end of the imposed water-stress period), and the fourth week (after seven days of re-irrigation; recovery phase). At both times, irrigation regimes (i.e., well-watered, moderate stress and severe stress) were compared across the biostimulant treatments: water (control), *Chlorella fusca* LEB 111 biostimulant and *Spirulina* sp. LEB 18 biostimulant. Figure 5 presents all measured agronomic and physiological responses, assessed at week 3 (end of the stress period) and week 4 (post-recovery). Individual panels are referenced throughout the text (Figure 5a–k).

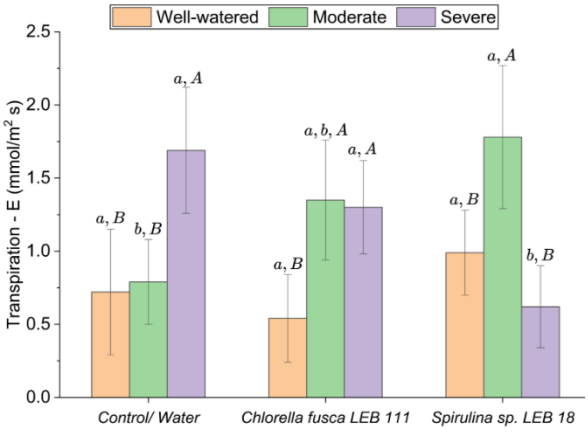
Figure 5 - Effects of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 biostimulants on lettuce physiological performance and biomass accumulation under well-watered, moderate, and severe water-stress conditions. (a,b) Chlorophyll content index (SPAD); (c,d) transpiration rate (E); (e,f) stomatal conductance to water vapour (gsw); (g,h) effective quantum yield of photosystem II (ΦPSII); (i,j) electron transport rate (ETR) measured at weeks 3 and 4; and (k) fresh weight at harvest. Values represent means ± SD (n = 10). Different lowercase letters indicate significant differences ($p < 0.10$) among biostimulant treatments within the same water-stress level, whereas uppercase letters indicate significant differences ($p < 0.10$) among water-stress levels within the same biostimulant.



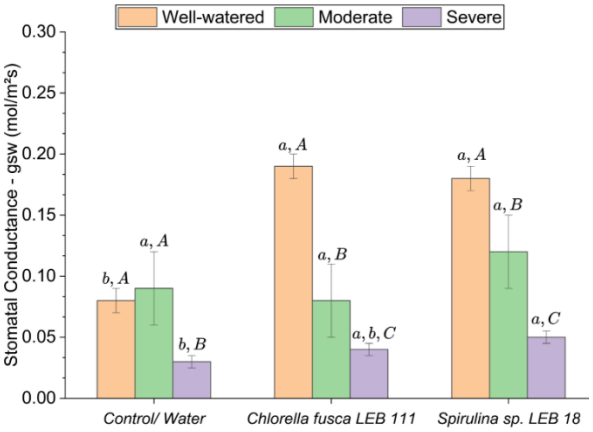
(c)


Well-watered
Moderate
Severe
Transpiration - E (mmol/m² s)
Control/ Water
Chlorella fusca LEB 111
Spirulina sp. LEB 18

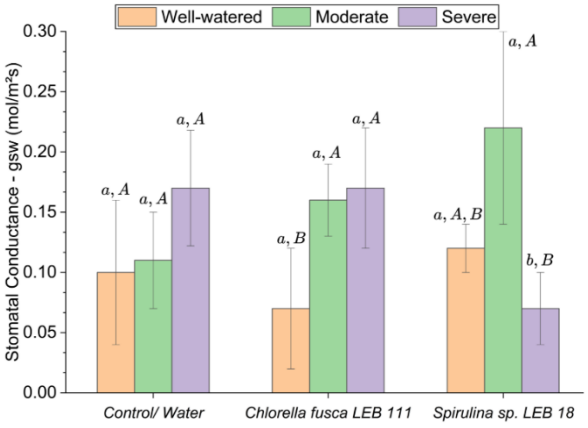
(d)


Well-watered
Moderate
Severe
Transpiration - E (mmol/m² s)
Control/ Water
Chlorella fusca LEB 111
Spirulina sp. LEB 18

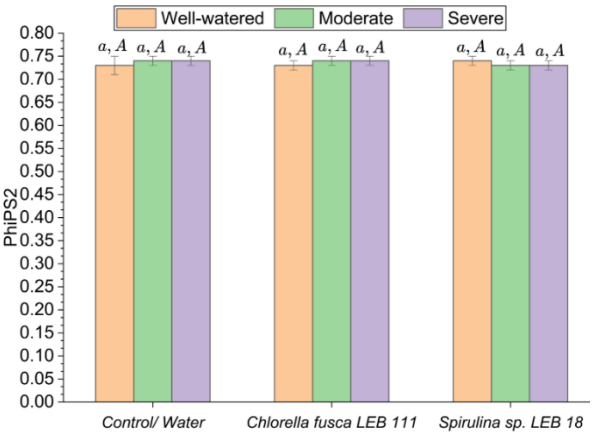
(e)


Well-watered
Moderate
Severe
Stomatal Conductance - gsw (mol/m²s)
Control/ Water
Chlorella fusca LEB 111
Spirulina sp. LEB 18

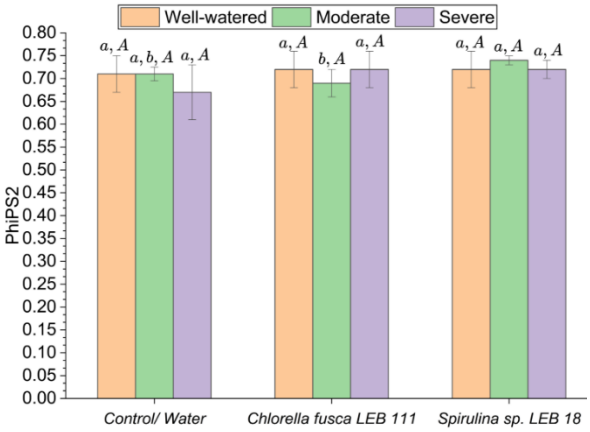
(f)


Well-watered
Moderate
Severe
Stomatal Conductance - gsw (mol/m²s)
Control/ Water
Chlorella fusca LEB 111
Spirulina sp. LEB 18

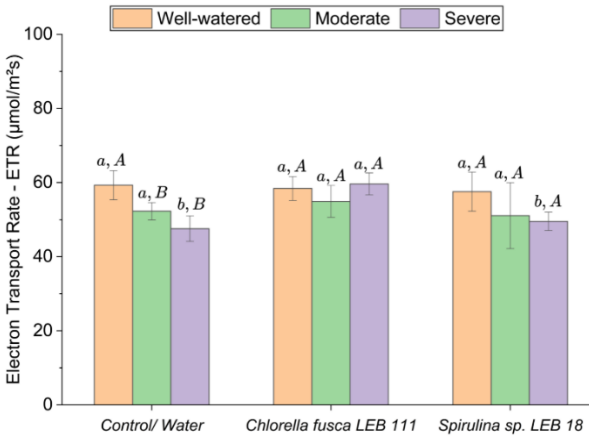
(g)


Well-watered
Moderate
Severe
PhiPS2
Control/ Water
Chlorella fusca LEB 111
Spirulina sp. LEB 18

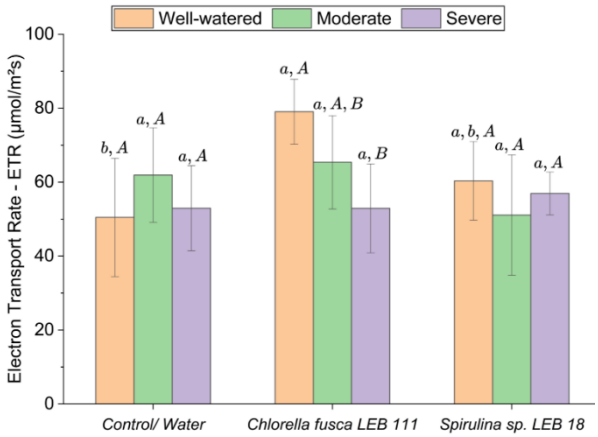
(h)


Well-watered
Moderate
Severe
PhiPS2
Control/ Water
Chlorella fusca LEB 111
Spirulina sp. LEB 18

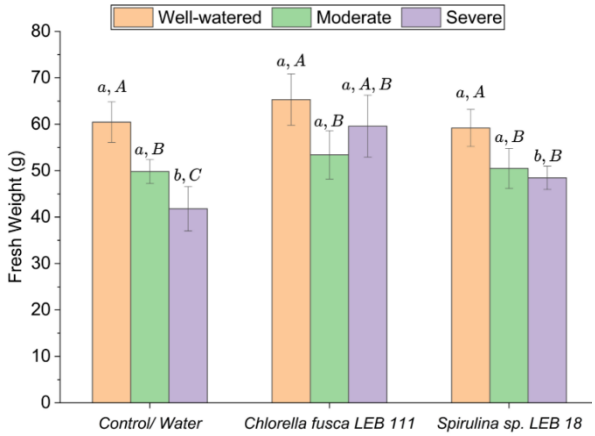
(i)



(j)



(k)



3.4.1 Chlorophyll content index (SPAD)

The chlorophyll content index (SPAD) is a non-destructive proxy that estimates chlorophyll concentration per unit of leaf area, rather than total chlorophyll content per leaf (Zhang *et al.*, 2022). In the control treatment, plants subjected to severe water stress exhibited higher SPAD values at the end of the stress period (week 3; Figure 5a) compared to moderate stress and well-watered conditions and a similar response was observed in the treatments with *Spirulina* biostimulants. This response is commonly observed under short-term drought, as tissue dehydration leads to increased tissue density and a reduction in expansive growth, resulting in higher chlorophyll concentration per unit area (Tiwari *et al.*, 2021). . In contrast, no significant variations in chlorophyll content were observed in Chlorella-treated plants under

stress conditions. This response could be attributed to the higher concentrations of JA and SA in Chlorella compared to Spirulina, which likely contributed to turgor maintenance and, thereby, sustained expansive growth (cell elongation), a process commonly associated with hormonal regulation under abiotic stress (Miranda *et al.*, 2024; Vangenechten; De Coninck; Ceusters, 2025). This interpretation is supported by the higher fresh biomass observed in *Chlorella*-treated plants (Figure 5k.

After seven days of re-irrigation (Figure 5b), chlorophyll content converged across all treatments and irrigation regimes, with no significant differences among them ($p > 0.10$), This was likely driven by turgor recovery in control and Spirulina-treated plants, which reversed the previously observed dehydration-induced concentration effect.

3.4.2 Transpiration rate (E) and stomatal conductance (gsw)

At the end of the stress period, transpiration declined along with increased water stress (Figure 5c) across all biostimulant treatments, primarily due to progressive stomatal closure (Figure 5e), a well-established plant response to limit water loss under water deficits (Farooq *et al.*, 2009; Ronga *et al.*, 2019).

Under well-watered conditions in week 3, plants treated with both microalgal biostimulants exhibited significantly higher transpiration and stomatal conductance compared to the control, with *Spirulina* and *Chlorella* showing transpiration rates of 1.61 ± 0.16 and 1.55 ± 0.07 mmol m^{-2} s^{-1}, respectively, compared to 1.11 ± 0.07 mmol m^{-2} s^{-1} in the control ($p < 0.10$). Similarly, gsw values were higher in biostimulant-treated plants than in water-treated plants. These differences indicate an association between biostimulant application and enhanced gas exchange capacity under non-stress conditions, rather than a direct mechanistic stimulation of stomatal opening (Miranda *et al.*, 2024).

Under moderate water stress, no significant differences in transpiration or stomatal conductance were observed among treatments. Under severe stress, however, plants treated with *Chlorella* and *Spirulina* maintained higher transpiration rates than control plants (0.60 ± 0.24 and 0.65 ± 0.18 vs. 0.29 ± 0.07 mmol m^{-2} s^{-1}, respectively; $p < 0.10$). *Spirulina*-treated plants also showed higher gsw values than the control, while *Chlorella* exhibited intermediate values. These results suggest that microalgal treatments contributed to the partial maintenance of stomatal functionality under severe drought conditions (Brito-Lopez *et al.*, 2025; Huber; Melcher; Bauerle, 2023).

During the recovery phase (week 4), transpiration and stomatal conductance values converged across treatments under well-watered conditions, indicating a general re-establishment of gas exchange following re-irrigation. Under moderate stress, *Spirulina*-treated plants exhibited higher transpiration than control plants, while *Chlorella*-treated plants showed intermediate responses. Interpretation of gas exchange patterns during the recovery phase should be made with caution, as post-drought physiological recovery often involves delayed and asynchronous responses among treatments (Huber; Melcher; Bauerle, 2023; Wang, Y. *et al.*, 2023).

3.4.3 Photosystem II (ΦPSII) and electron transport rate (ETR)

The effective quantum yield of photosystem II (ΦPSII) remained relatively stable throughout the experiment (Figure 5g,h). In week 3, ΦPSII values ranged from 0.73 to 0.74, with no significant differences among treatments or irrigation regimes ($p > 0.10$), indicating that the imposed water deficit did not measurably impair PSII photochemical efficiency at this stage.

The electron transport rate (ETR) provided additional insight into photosynthetic performance (Figure 5i,j). In week 3, no differences were observed among treatments under well-watered and moderate stress conditions ($p > 0.10$). Under severe stress, however, *Chlorella*-treated plants showed significantly higher ETR (59.64 ± 2.96 µmol m^{-2} s^{-1}) than the control (47.56 ± 3.42 µmol m^{-2} s^{-1}) and *Spirulina* (49.52 ± 2.50 µmol m^{-2} s^{-1}; $p < 0.10$). Because ΦPSII did not differ significantly among treatments, these ETR variations are most plausibly attributed to differences in incident PPFD reaching the leaf surface, which can fluctuate due to changes in leaf inclination and canopy architecture. In lettuce, leaf angle adjustments under stress are known to modify light interception and downstream photosynthetic responses, affecting ETR independently of PSII quantum yield (An *et al.*, 2022). This interpretation is consistent with the reported role of microalgal biostimulants in supporting photosynthetic stability and mitigating stress-induced limitations without directly altering PSII efficiency (Fiorentino *et al.*, 2025; Miranda *et al.*, 2024; Vangenechten; De Coninck; Ceusters, 2025).

3.4.4 Fresh Weight

As expected, well-watered plants showed the highest FW, with no significant differences among treatments ($p > 0.10$) (Figure 5k). Under moderate stress, FW values decreased relative to the well-watered condition, but no statistical differences were detected among treatments for the different biostimulants applied.

Under severe stress, plants treated with *Chlorella* reached the highest FW (59.59 ± 6.67 g), differing significantly from both control and *Spirulina* treatments ($p < 0.10$). Control plants showed the lowest FW (41.79 ± 4.78 g), while *Spirulina*-treated plants presented intermediate values (48.44 ± 2.51 g). The superior performance of *Chlorella* under severe stress is consistent with its hormonal profile (Section 3.2), characterized by elevated levels of salicylic and jasmonic acids, which may contribute to sustained growth and stress mitigation during water deficit.

Although *Spirulina* was less effective than *Chlorella* in sustaining lettuce growth under severe drought, its application still resulted in higher fresh weight values than the water-treated control, confirming its biostimulant activity under stress conditions. These biomass results align with previous studies on *Chlorella*-based biostimulants in lettuce. Puglisi et al. (2022) showed that *Chlorella vulgaris* extract increased fresh and dry weight of lettuce seedlings compared to controls. The present study extends these observations by demonstrating that *Chlorella fusca* LEB 111, cultivated in brewery wastewater, can also support biomass accumulation when water availability is limiting.

Taken together, the physiological and agronomic data indicate that *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18 exert distinct but complementary effects on lettuce under a drought–recovery cycle. *Spirulina* showed stronger influence on stomatal conductance during the peak of water deficit, whereas *Chlorella* more consistently supported chlorophyll content, electron transport and final biomass under severe stress. These contrasting patterns suggest that tailoring microalgal biostimulant formulation, for example, combining extracts from both species or adjusting their ratios, could be a promising strategy to optimise plant performance under fluctuating water availability.

4 CONCLUSIONS

This study demonstrates that brewery wastewater is a suitable low-cost medium for the cultivation of *Chlorella fusca* LEB 111 and *Spirulina* sp. LEB 18, enabling the production of microalgal biomass with agronomic functionality. Although both species grew effectively in the effluent, *Spirulina* exhibited higher biomass productivity (69.9 mg L^{-1} d^{-1}), indicating

advantages in terms of process efficiency and scalability for integrated biorefinery applications. At the biostimulant level, *Spirulina* was more effective during early plant development, promoting the highest germination rate (87%) and radicle elongation (4.98 cm) in barley, consistent with its higher gibberellin content (1,485.7 ng g^{-1}). In contrast, *Chlorella* showed superior performance under water deficit, with lettuce plants maintaining higher fresh biomass under severe drought stress (59.59 ± 6.67 g), in agreement with its enrichment in stress-related phytohormones such as jasmonic and salicylic acids. Overall, the results demonstrate distinct and complementary agronomic functions of microalgae-derived biostimulants produced from brewery wastewater. *Spirulina* combines high biomass productivity with strong seed-level responses, whereas *Chlorella* enhances drought tolerance at the whole-plant level, providing a functional basis for defining application strategies and extraction approaches within circular bioeconomy frameworks.

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ARTICLE IV

EFFECTS OF AÇAÍ SEED-DERIVED BIOCHAR ON GROWTH, BIOMASS PROPERTIES, AND SYSTEM PERFORMANCE IN BREWERY WASTEWATER CULTIVATION OF *Chlorella fusca*

ABSTRACT

The integration of waste valorization and microalgal cultivation has emerged as a promising approach for sustainable biomass production and wastewater treatment. Therefore, this study aimed to evaluate the effects of açaí seed-derived biochar on the cultivation of *Chlorella fusca* LEB 111 in synthetic brewery wastewater, focusing on microalgal growth, nutrient removal performance, biomass biochemical composition, and changes in biochar properties. Cultivations were conducted for 7 days under three conditions: control (without biochar), and with 0.5 and 1.0 g L^{-1} biochar. Growth kinetics, nutrient removal, biomass composition, and biochar characterization (TGA, SEM, and EDS) were evaluated. All treatments exhibited rapid biomass accumulation, reaching up to 1.03 g L^{-1} within 4 days. Biochar addition reduced the μ_{max} (0.49 d^{-1} in control to 0.34 and 0.40 d^{-1}), but improved culture stability, with sustained growth observed at 1.0 g L^{-1}. Total carbon removal exceeded 84% in all treatments. Ammonium removal was highest at 0.5 g L^{-1} (32.50%). Phosphate removal ranged from 77.99% to 86.82%. Biochar significantly influenced biomass composition. Lipid content increased from 27.91% (control) to 45.62% and 39.54% at 0.5 and 1.0 g L^{-1}, respectively, while carbohydrates decreased. EDS analysis confirmed nitrogen adsorption onto biochar, increasing from not detected to 7.61% and 13.50%, while TGA indicated organic matter retention (~13.9%). These results demonstrate that açaí seed-derived biochar acts as a dynamic component in microalgal systems, modulating nutrient availability and enhancing biomass valorization, highlighting its potential for integrated wastewater treatment and biorefinery applications.

Keywords: microalgal systems; effluent remediation; adsorption–desorption; nutrient dynamics; biomass valorization

1. INTRODUCTION

The increasing generation of agro-industrial residues has become a significant environmental concern, particularly due to their high organic load and nutrient content, which can lead to soil and water contamination when improperly managed. At the same time, these residues represent a valuable source of carbon and nutrients that can be redirected into biotechnological processes, aligning with the principles of circular economy and sustainable resource management (Ghosh *et al.*, 2024; Pessôa *et al.*, 2021). The integration of waste

valorization and biomass production has therefore emerged as a promising strategy to reduce environmental impacts while generating value-added products.

Among agro-industrial residues, brewery wastewater stands out due to its high volume and complex composition, typically characterized by elevated organic matter content and variable concentrations of nitrogen and phosphorus (Barati *et al.*, 2025). Although this effluent poses a pollution risk, its composition also makes it a suitable medium for microbial cultivation (Conceição et al. 2026). In parallel, the açaí processing industry generates large amounts of solid residues, mainly composed of seeds, which are often underutilized. In Brazil, particularly in the northern and northeastern regions, açaí processing generates large volumes of solid residues, since approximately 85% of the fruit corresponds to the seed, a lignocellulosic material that is often discarded irregularly, contributing to environmental management challenges such as vector-borne disease proliferation (Zaparoli *et al.*, 2023). These residues can be converted into biochar through thermochemical processes, producing a carbon-rich material with high surface area and functional groups capable of interacting with dissolved compounds in aqueous systems (Zaparoli *et al.*, 2023).

Microalgae have been investigated for wastewater treatment due to their ability to assimilate nutrients such as nitrogen and phosphorus while producing biomass with potential applications in biofuels, agriculture, and biotechnology (Álvarez-González; Greque de Morais; *et al.*, 2023; Arias; García; Uggetti, 2020; Kumar, R. *et al.*, 2022). Species such as *Chlorella fusca* LEB 111 have demonstrated high adaptability to different cultivation conditions and the ability to grow in nutrient-rich effluents (Cruz; da Rosa; Costa, 2024; Leal *et al.*, 2026). The efficiency of microalgal systems, however, is strongly dependent on nutrient availability and balance, which directly influence growth kinetics and biomass composition (Metin; Altınbaş, 2024).

In this context, biochar has attracted attention as a functional material that can be integrated into biological systems. Due to its porous structure and surface chemistry, biochar can adsorb nutrients and organic compounds, potentially influencing their availability in the medium (Laishram *et al.*, 2025). In microalgae-based systems, this property suggests a dual role for biochar, acting both as a sorbent and as a modulator of nutrient dynamics. Such interactions may affect not only nutrient removal efficiency but also microalgal growth patterns and metabolic responses (Behl *et al.*, 2019; Rehman *et al.*, 2024).

Despite the growing number of studies on microalgae cultivation in wastewater and on the application of biochar as an adsorbent material, the combined effects of biochar addition on microalgal systems remain insufficiently explored. In particular, there is a lack of understanding

regarding how biochar influences nutrient availability over time and how this affects growth kinetics, biomass composition, and system performance. Furthermore, the physicochemical changes occurring in biochar during its interaction with microalgal cultures are still poorly described. Therefore, this study aimed to evaluate the effects of açaí seed-derived biochar on the cultivation of *Chlorella fusca* LEB 111 in synthetic brewery wastewater, focusing on microalgal growth, nutrient removal performance, biomass biochemical composition, and changes in biochar properties.

2 MATERIALS AND METHODS

2.1 MICROALGAE AND CULTURE MEDIUM

In this study, the *Chlorella fusca* LEB 111 strain, isolated from the ash sedimentation ponds of the Presidente Médici Thermal Power Plant (UTPM) in Candiota, Rio Grande do Sul, Brazil (latitude 24°36'13" S and longitude 52°32'43" W), was used. The strain was obtained from the strain bank of the Laboratory of Biochemical Engineering (FURG). The strain identity was previously confirmed by morphological and molecular methods at the same institution. For control assays, the microalgae were maintained in BG 11 medium (Rippka *et al.*, 1979).

A synthetic brewery effluent was prepared according to the formulation adapted from Mata et al. (2012), containing malt extract (1.00 g L^{-1}), yeast extract (0.50 g L^{-1}), peptone (0.15 g L^{-1}), maltose (0.86 g L^{-1}), ammonium sulfate (2.20 g L^{-1}), ethanol (2.00 mL L^{-1}), NaH_2PO_4 (0.08 g L^{-1}), and Na_2HPO_4 (0.14 g L^{-1}). The initial pH of the prepared medium was 6.1. To avoid precipitation of complexes during sterilization, the medium components were autoclaved in two separate fractions. Ammonium sulfate, peptone, and yeast extract were dissolved in 500 mL of distilled water, while maltose, malt extract, and phosphate salts were dissolved in another 500 mL fraction. Both fractions were autoclaved at 121 °C for 20 min, then combined under aseptic conditions after cooling, and ethanol was added afterwards.

2.2 BIOCHAR SOURCE AND CULTIVATION CONDITIONS

The biochar used in this study was obtained from açaí seed (*Euterpe oleracea* Mart.) residue and produced according to the methodology described by Zaparoli et al. (2023). Briefly, the açaí seeds were washed, oven-dried at 60 °C for 24 h, and subjected to pyrolysis at 700 °C for 1 h under oxygen-limited conditions, using capped porcelain crucibles and a heating rate of

18 °C min^{-1}. After cooling, the material was ground, sieved to particle size <75 µm, and stored in hermetic containers at room temperature until use.

Cultivation assays were carried out in 2.0 L Erlenmeyer flask photobioreactors with a working volume of 1.8 L, consisting of 1.6 L of synthetic brewery effluent and 0.2 L of inoculum. The assays were initiated at a biomass concentration of 0.2 g L^{-1}, maintained at 30 °C, under a 12 h light/dark photoperiod and an illuminance of 60 $\mu mol_{photons}\, m^{-2}\, s^{-1}$. Continuous aeration was supplied throughout the experiments. The cultivation period lasted 7 days. Three cultivation conditions were evaluated: control, without biochar; 0.5 g L^{-1} biochar; and 1.0 g L^{-1} biochar. In addition, abiotic controls without microalgal inoculation were carried out in parallel, with and without the addition of açaí seed biochar, in order to monitor the behavior of the medium in the absence of biological growth.

2.3 ANALYTICAL DETERMINATIONS

2.3.1 Biomass concentration and pH

The cell concentration and pH of the cultures were assessed daily. The biomass concentration of the microalgae was determined using a spectrophotometer (Shimadzu UV/VIS UVmini-1240, Japan) at 670 nm. A standard curve was previously established, which correlates the absorbances at this wavelength with the dry mass of successive dilutions of the microalgae inoculum (Costa *et al.*, 2002). The pH of the cultures was determined by direct measurement with a digital pHmeter (Mettler Toledo FiveGo™, Switzerland).

2.3.2 Microalgal growth

The maximum biomass concentration (X_{max}, g L^{-1}) was defined as the highest biomass value obtained in each assay. The volumetric biomass productivity (P_x, g L^{-1} d^{-1}) was determined for each growth cycle using Equation 1, where X_t is the biomass concentration (g L^{-1}) at time t (d) and X_0 is the biomass concentration (g L^{-1}) at initial time t_0 (d) (Bailey; Ollis, 1986).

$$P_x = \frac{X_t - X_0}{t - t_0} \quad (1)$$

The maximum specific growth rate (μ_{max}, d^{-1}) was measured by linear regression of the logarithmic growth phase, from a linearized biomass concentration profile (Ln X) versus time (d), where the slope of this line was taken as μ_{max}. The generation time (t_g) was determined

during the exponential growth phase of the microalga, calculated according to the Equation 2 (Bailey; Ollis, 1986).

$$t_g = \frac{\ln(2)}{\mu_{max}} \quad (2)$$

2.3.3 Effluent characterization and removal efficiency

The effluent was characterized throughout the cultivation period by monitoring ammoniacal nitrogen (NH_4^+-N), inorganic phosphate, and total carbon (TC). Samples were collected every two days, centrifuged, and the supernatant was used for the analyses.

Ammoniacal nitrogen was determined by micro-Kjeldahl distillation without the digestion step, followed by titration with standardized hydrochloric acid (Baird; Eaton; Rice, 2017). Inorganic phosphate was determined colorimetrically by the ammonium molybdate method (Baird; Eaton; Rice, 2017). Total carbon (TC) was measured using a TOC analyzer (Shimadzu, Japan). Removal efficiency was calculated based on the initial and final concentrations of each parameter during cultivation (Ji *et al.*, 2012).

2.3.4 Biomass harvesting and characterization

At the end of the cultivation period, the biomass was centrifuged (Hitachi Himac CR-GIII, Tokyo - Japan) at 15200 x g for 15 minutes at 20°C, and stored for subsequent characterization. Biomass moisture and ash contents were determined by gravimetric methods.

Biomass composition was performed after all cultivation stages. Total carbohydrate concentration in the biomass was determined using the phenol-sulfuric method with a standard glucose curve (Dubois *et al.*, 1956). Total protein concentration was assessed via the colorimetric method developed by Lowry et al. (1951), involving prior thermal and alkaline hydrolysis of the extract, using a bovine serum albumin standard curve. Lipid concentration was measured using the Marsh and Weinstein method, employing chloroform and methanol as solvents (Marsh; Weinstein, 1966).

Protein and carbohydrate analysis were carried out on extracts prepared from 10 mg of dried microalgal biomass and 20 mL of distilled water, subjected to an ultrasonic probe for 10 minutes (Cole Parmer CPX 130 – Illinois, USA), with operating cycles of 1 minute on and 1 minute off, to release the intracellular material into the liquid medium.

2.3.5 Biochar characterization

After cultivation, the biochar was recovered by vacuum filtration using filter paper and stored under refrigeration until further analysis. The biochar was characterized before and after cultivation by thermogravimetric analysis (TGA), scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDS). Thermogravimetric analysis was performed using a Shimadzu DTG-60 instrument (Japan). Approximately 3 mg of moisture-free sample was heated up to 500 °C under a nitrogen atmosphere (30 mL min^{-1}) at a heating rate of 10 °C min^{-1} (ASTM D3850–12, 2025).

Morphological analysis was carried out using a JEOL JSM-6610 LV scanning electron microscope (JEOL Ltd., Japan). The samples were mounted on metal stubs and coated with gold prior to analysis using a sputter coater (Denton Vacuum, United States). Elemental surface composition was determined by energy-dispersive X-ray spectroscopy (EDS) coupled to the SEM. The analysis was performed under high vacuum conditions, and the elemental composition was expressed as weight percentage (wt%). The contribution of gold from the sputter coating was excluded from the quantitative analysis (ASTM E 986–04, 2017).

2.4 STATISTICAL ANALYSES

All analyses were performed in triplicates, and results are expressed as mean ± standard deviation (SD). The results were assessed using analysis of variance (ANOVA), followed by Tukey's test for comparing mean values at a 95% confidence level. Statistical analyses were performed using the Statistica 7 software (StatSoft Inc., USA).

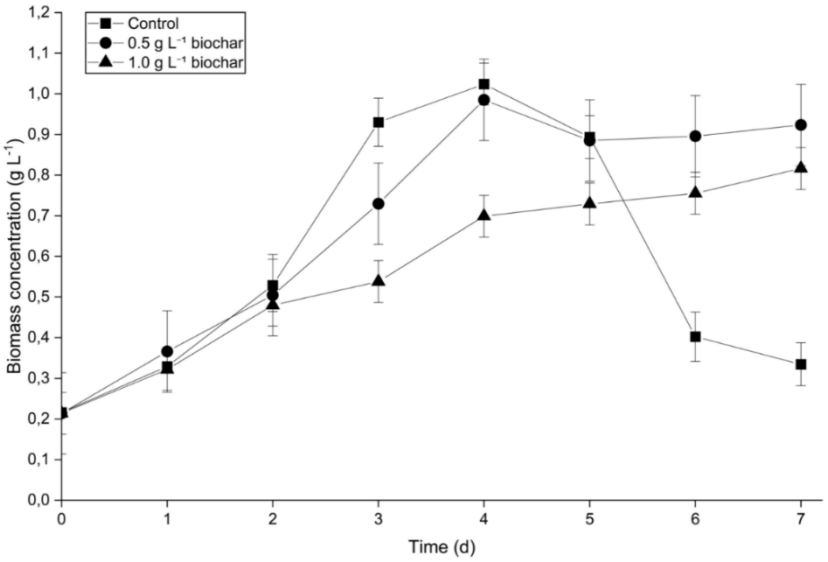
3 RESULTS AND DISCUSSION

3.1 GROWTH PERFORMANCE AND KINETIC PARAMETERS UNDER DIFFERENT BIOCHAR CONCENTRATIONS

The growth profiles of *Chlorella fusca* LEB 111 under the tested conditions are presented in Figure 1. All treatments exhibited rapid biomass accumulation during the initial cultivation period, though differences in growth dynamics emerged among them. The control and the 0.5 g L^{-1} biochar treatments reached the maximum biomass concentration (X_{max}) at day 4, whereas the 1.0 g L^{-1} treatment exhibited a delayed but sustained growth pattern, reaching its peak only at the end of the experimental period. Despite these contrasting dynamics, all

treatments achieved biomass concentrations approaching 1 g L^{-1} within a few days, a result that compares favorably with previous reports on *Chlorella* cultivation in agro-industrial effluents. Cruz et al. (2023) reported an X_{max} of 1.03 g L^{-1} for *Chlorella fusca* LEB 111 in dairy effluent after 15 days of cultivation, while Wang et al. (2022) obtained biomass concentrations of 1.77–2.03 g L^{-1} for *Chlorella* sp. in brewery wastewater only under optimized conditions involving dilution strategies, nutrient supplementation, and extended cultivation periods (15 d). The substantially shorter cultivation time required in the present study, therefore, highlights the high productivity achieved under the proposed conditions.

Figure 10 - Growth profile of *Chlorella fusca* cultivated in brewery effluent with different biochar additions: control (■), 0.5 g L^{-1} biochar (●), and 1.0 g L^{-1} biochar (▲).



This elevated productivity can be associated with the nutritional composition of the synthetic brewery wastewater, which offered two advantages over conventional culture media. First, the initial total carbon concentration (TC = 1680.43 mg C L^{-1}) is higher than typically supplied in standard media such as BG-11, where carbon is provided in limited amounts (e.g., Na_2CO_3 at 0.02 g L^{-1}). Second, nitrogen in the synthetic wastewater was predominantly in the form of ammonium (NH_4^+), a reduced nitrogen species more readily assimilated by microalgae than the nitrate provided in BG-11 (1.5 g $NaNO_3$ L^{-1}) (Metin; Altınbaş, 2024). This combination of high carbon availability and readily assimilable nitrogen likely drove the intense growth observed across all treatments. It is worth noting that, unlike real brewery wastewater, which often presents elevated organic load but comparatively low nitrogen and phosphorus concentrations (Ferreira, A. *et al.*, 2019), the synthetic medium used here created a nutrient-

rich environment closer to optimized cultivation conditions, thereby further enhancing microalgal productivity.

From a kinetic perspective (Table 1), the elevated values of maximum biomass productivity (P_{max}) and maximum specific growth rate (μ_{max}) confirm that conditions were highly favorable across all treatments. However, the specific growth rate was significantly reduced in the presence of biochar (μ_{max} = 0.34 d^{-1} and 0.40 d^{-1} for 0.5 and 1.0 g L^{-1}, respectively, vs. 0.49 d^{-1} in the control), indicating that biochar addition imposed an initial constraint on growth kinetics. This initial reduction is consistent with the known capacity of biochar to adsorb dissolved nutrients, owing to its porous structure and high surface area (Rehman *et al.*, 2024), thereby temporarily reducing nutrient bioavailability during the early exponential phase. Notably, this effect was most pronounced in the 0.5 g L^{-1} treatment (lowest μ_{max}), suggesting a non-linear relationship between biochar concentration and its initial impact on growth kinetics, a pattern potentially linked to differences in nutrient adsorption–desorption dynamics at each concentration.

Table 1 - Maximum biomass concentration (X_{max}), maximum biomass productivity (P_{max}), maximum specific growth rate (μ_{max}), and generation time (tg) of *Chlorella fusca* cultivated in brewery effluent supplemented with different biochar concentrations.

Parameter	Control	0.5 g L^{-1} Biochar	1.0 g L^{-1} Biochar
X_{max} (g L^{-1})	$1.03^{a} \pm 0.14$	$0.97^{a} \pm 0.28$	$0.82^{a} \pm 0.01$
P_{max} (mg L^{-1})	$238.07^{a} \pm 21.12$	$184.06^{a} \pm 61.86$	$145.38^{a} \pm 18.06$
μ_{max} (d^{-1})	$0.49^{a} \pm 0.01$	$0.34^{c} \pm 0.01$	$0.40^{b} \pm 0.01$
tg (d)	$1.43^{a} \pm 0.01$	$2.06^{c} \pm 0.01$	$1.73^{b} \pm 0.01$
***Δt**	0-3	1-4	0-2
****R^2**	0.99	0.99	0.99

Different lowercase letters in the same row for the same parameter indicate significant differences among the means at the 95% confidence level ($p < 0.05$). *Δt: initial time-final time of the exponential growth phase. **R2: the linear regression determination coefficient was applied in the logarithmic phase of growth. Data are presented as the mean value±standard deviation.

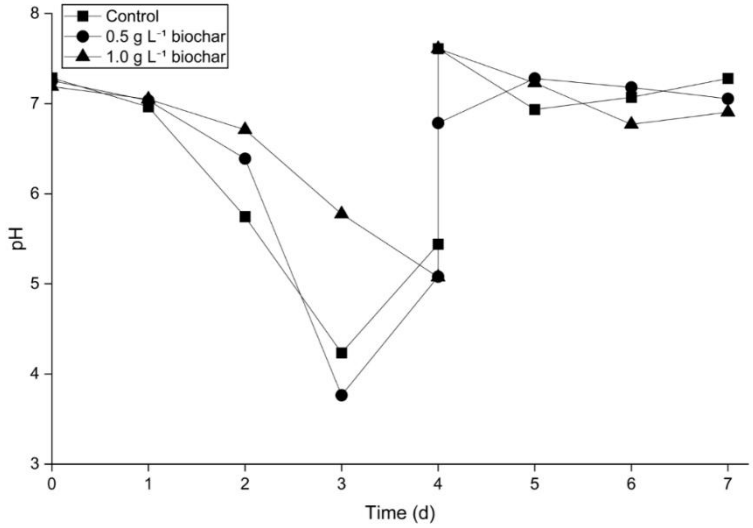
After reaching X_{max}, the control and the 0.5 g L^{-1} treatment exhibited a decline or stabilization in biomass concentration, whereas the 1.0 g L^{-1} treatment maintained continuous growth until the end of the cultivation period. This divergence reveals a dual role of biochar in the system. During the exponential phase, the adsorption of NH_4^+ by biochar reduces its immediate bioavailability, which is also reflected in the lower NH_4^+–N removal efficiency observed in the 1.0 g L^{-1} treatment (25.84%, vs. 32.50% in the 0.5 g L^{-1} treatment; Section 3.2)

but simultaneously prevents nitrogen from being fully consumed or volatilized. As cultivation progresses and external nutrient concentrations decline, the adsorbed nitrogen may be gradually released, sustaining microalgal growth beyond the point at which the control and low-biochar treatment became limited. At higher biochar loading (1.0 g L^{-1}), this buffering capacity was sufficient to extend active growth throughout the entire experimental period, whereas at 0.5 g L^{-1} the adsorption capacity was not large enough to prevent the eventual nutrient depletion responsible for the observed growth decline. Similar nutrient-buffering behaviour has been reported in microalgae–biochar co-systems, where the material acts as both an adsorbent and a slow-release nutrient regulator (Behl *et al.*, 2019; Rehman *et al.*, 2024).

The rapid biomass accumulation observed until day 4 in the control and 0.5 g L^{-1} treatments corresponds to a period of intense nutrient consumption, particularly nitrogen. During the exponential phase, cellular division and metabolic activity are intensified, creating high demand for nitrogen as a substrate for protein and nucleic acid synthesis (Metin; Altınbaş, 2024). Excess ammonium, while a preferred nitrogen source, can also exert inhibitory effects under certain conditions, particularly when its concentration remains high and is not sufficiently modulated (Metin; Altınbaş, 2024). In the control, the absence of any adsorbing matrix means that ammonium was simultaneously highly available during early growth and potentially inhibitory at elevated concentrations, while also susceptible to rapid depletion as growth proceeded, a combination that may have contributed to the observed post-peak decline.

The pH dynamics during cultivation (Fig. 2) further reflect the metabolic activity of the culture and are closely linked to the growth patterns described above. In all treatments, pH decreased markedly during the first days of cultivation, reaching values of 3.8–4.2 at day 3, a drop likely driven by the accumulation of organic acids arising from microbial metabolic activity and possibly CO_2 produced during heterotrophic or mixotrophic metabolism. This acidification occurred precisely during the period of most intense growth and nutrient uptake, reinforcing the interpretation that the culture was metabolically active. To prevent pH-induced growth inhibition and avoid contamination risks, manual corrections with 0.5 mol L^{-1} NaOH were applied from day 4 onwards, restoring pH to values between 6.5 and 7.5. After correction, pH remained relatively stable until the end of the experiment, indicating that the buffering effect of the culture medium, combined with external pH control, was sufficient to maintain suitable conditions for continued growth, particularly in the 1.0 g L^{-1} treatment.

Figure 2 - pH profile of *Chlorella fusca* cultivated in brewery effluent supplemented with different biochar concentrations.



3.2 NUTRIENT REMOVAL EFFICIENCY FROM BREWERY WASTEWATER

The removal of nutrients from synthetic brewery wastewater during the cultivation of *Chlorella fusca* LEB 111 is summarized in Table 2. Significant reductions in total carbon (TC), ammoniacal nitrogen (NH_4^+–N), and inorganic phosphate (PO_4–P) were achieved within seven days of cultivation across all treatments, demonstrating the overall efficiency of the system over a relatively short experimental period. However, the magnitude and pattern of removal differed substantially among nutrients and across biochar concentrations, reflecting the interplay between microalgal assimilation, biochar adsorption, and initial nutrient load.

Table 2 - Initial and final concentrations and removal efficiency of total carbon (TC), ammoniacal nitrogen (NH_4^+–N), and inorganic phosphate (PO_4–P) during the cultivation of *Chlorella fusca* in brewery effluent supplemented with different biochar concentrations.

Parameter	Control	0.5 g L^{-1} biochar	1.0 g L^{-1} biochar
TC initial (mg C L^{-1})	$1680.43^a \pm 142.85$	$1680.43^a \pm 142.85$	$1680.43^a \pm 142.85$
TC final (mg C L^{-1})	$209.37^a \pm 0.80$	$264.57^b \pm 1.90$	$265.37^b \pm 1.56$
TC removal (%)	$87.45^a \pm 1.44$	$84.15^a \pm 1.75$	$84.10^a \pm 1.78$
NH_4^+–N initial (mg L^{-1})	$420.21^a \pm 2.88$	$420.21^a \pm 2.88$	$420.21^a \pm 2.88$
NH_4^+–N final (mg L^{-1})	$308.15^a \pm 8.23$	$283.64^a \pm 7.00$	$311.66^a \pm 7.00$
NH_4^+–N removal (%)	$26.67^b \pm 1.46$	$32.50^a \pm 1.20$	$25.84^b \pm 1.16$
PO_4–P initial (mg L^{-1})	$69.47^a \pm 0.27$	$69.47^a \pm 0.27$	$69.47^a \pm 0.27$
PO_4–P final (mg L^{-1})	$15.29^a \pm 0.01$	$10.27^a \pm 4.01$	$9.16^a \pm 3.74$
PO_4–P removal (%)	$77.99^a \pm 0.09$	$85.20^a \pm 5.83$	$86.82^a \pm 5.33$

Different lowercase letters in the same row indicate significant differences among the means at the 95% confidence level ($p < 0.05$). Data are presented as the mean value±standard deviation.

Total carbon removal was high and statistically equivalent across all conditions ($p > 0.05$), reaching 87.45% in the control, 84.15% in the 0.5 g L^{-1} biochar, and 84.10% in the 1.0 g L^{-1} biochar treatments. The predominant carbon removal mechanism is attributed to mixotrophic assimilation by *Chlorella fusca*, which simultaneously exploits photosynthetic CO_2 fixation and heterotrophic uptake of dissolved organic carbon, a metabolic flexibility particularly advantageous in effluent-based systems with high organic load (Kim *et al.*, 2013; Wang, X. *et al.*, 2025). Volatilization of dissolved CO_2 and low-molecular-weight organic compounds may have contributed as a secondary removal pathway, especially during the initial period of intense metabolic activity when culture pH dropped (Section 3.1). The residual TC in biochar treatments is consistent with the partial adsorption of dissolved organic carbon onto the biochar surface, which may have reduced its immediate bioavailability for microbial assimilation, an interpretation further supported by the thermogravimetric evidence of organic matter retention on biochar after cultivation (Section 3.4).

In contrast to TC, NH_4^+–N removal was comparatively low across all treatments, and significant differences among conditions were detected ($p < 0.05$). The highest removal efficiency was observed at 0.5 g L^{-1} biochar (32.50%), which differed significantly from both the control (26.67%) and the 1.0 g L^{-1} treatment (25.84%). The limited overall removal of NH_4^+–N reflects, in part, the very high initial ammonium concentration (420.21 mg L^{-1}), which substantially exceeded the assimilatory capacity of the microalgal cultures over the seven days. At such elevated concentrations, ammonium can exert inhibitory effects on microalgal metabolism (Metin; Altınbaş, 2024), potentially constraining the biological nitrogen uptake rate and contributing to the relatively modest removal efficiencies observed, a limitation intrinsic to the use of undiluted or minimally pre-treated brewery wastewater as a cultivation medium. The combination of a high initial NH_4^+–N load, potential inhibitory effects at elevated concentrations, and the relatively short cultivation period collectively explains why nitrogen removal remained partial across all treatments, even under otherwise productive growth conditions.

The superior NH_4^+–N removal observed at 0.5 g L^{-1} biochar suggests the existence of an intermediate biochar loading that optimizes the combined contribution of biological uptake and physicochemical adsorption. At this concentration, biochar may have provided sufficient adsorption capacity to complement microalgal assimilation without excessively sequestering nitrogen from the dissolved phase, thereby maintaining bioavailability for biological uptake while still contributing to overall removal. At 1.0 g L^{-1}, the greater adsorption capacity may have shifted the equilibrium towards nutrient retention on the biochar surface, reducing

biological uptake and ultimately yielding removal efficiencies comparable to or lower than the control. Although the precise mechanistic threshold between these regimes cannot be fully resolved from the available data, the pattern is consistent with dose-dependent adsorption dynamics reported for biochar in nutrient-rich aqueous systems (Rehman *et al.*, 2024). These observations also align with the growth and kinetic data discussed in Section 3.1, where the 1.0 g L^{-1} treatment showed sustained but delayed growth, indicative of a system where nitrogen was available, but at a rate governed by desorption from the biochar matrix rather than direct dissolution.

Phosphate removal was high across all treatments, ranging from 77.99% in the control to 86.82% in the 1.0 g L^{-1} biochar treatment, with no statistically significant differences among conditions ($p > 0.05$). Final PO_4–P concentrations decreased to values between 9.16 and 15.29 mg L^{-1}, confirming efficient phosphorus removal regardless of biochar addition. The higher removal efficiency of phosphate compared to ammoniacal nitrogen reflects the lower initial PO_4–P concentration (69.47 mg L^{-1}) relative to NH_4^+–N (420.21 mg L^{-1}), resulting in a more favorable ratio between microalgal uptake capacity and nutrient supply. Phosphorus removal in microalgae–biochar systems occurs through the combined action of biological assimilation for cellular biosynthesis and physicochemical adsorption onto biochar functional groups (Rehman *et al.*, 2024).

3.3 EFFECT OF BIOCHAR ON THE BIOCHEMICAL COMPOSITION OF *Chlorella fusca* BIOMASS

The biochemical composition of *Chlorella fusca* LEB 111 biomass was influenced by biochar addition (Table 3). Carbohydrate content was significantly higher in the control (24.75%) than in both biochar treatments (13.33% and 15.46% for 0.5 and 1.0 g L^{-1}, respectively; $p < 0.05$), while protein content showed a non-significant increasing trend with biochar concentration (33.27%, 36.89%, and 38.17% for control, 0.5, and 1.0 g L^{-1}, respectively; $p > 0.05$). Lipid content was elevated in biochar treatments relative to the control (45.62% and 39.54% vs. 27.91%), representing increases of 63% and 42%, respectively. Together, these results indicate a reorganization of intracellular carbon allocation in response to biochar-mediated changes in the cultivation environment, with carbon flux redirected away from carbohydrate storage and towards lipid biosynthesis (Ma *et al.*, 2022).

Table 3 - Biochemical composition of *Chlorella fusca* LEB 111 biomass cultivated in brewery effluent supplemented with different biochar concentrations.

Macromolecules	Control	0.5 g L^{-1} Biochar	1.0 g L^{-1} Biochar
Carbohydrates	$24.75^{a} \pm 1.34$	$13.33^{b} \pm 1.13$	$15.46^{b} \pm 0.04$
Proteins	$33.27^{a} \pm 1.80$	$36.89^{a} \pm 0.80$	$38.17^{a} \pm 0.50$
Lipids	$27.91^{b} \pm 1.95$	$45.62^{a} \pm 2.83$	$39.54^{a} \pm 1.73$

Different lowercase letters in the same row indicate significant differences among the means at the 95% confidence level ($p < 0.05$).

The metabolic reorientation can be explained by modification of the effective carbon-to-nitrogen (C:N) ratio experienced by the cells during cultivation. As discussed in Section 3.2, biochar adsorbed NH_4^+ from the medium, reducing its immediate bioavailability and effectively elevating the C:N ratio available to the cells relative to the control, given that total carbon in the medium remained high throughout (initial TC = 1680.43 mg C L^{-1}). An elevated C:N ratio is a well-established trigger for lipid accumulation in microalgae, as excess assimilated carbon cannot be fully incorporated into nitrogen-requiring macromolecules and is instead partitioned into triacylglycerols (TAGs) and other storage lipids (Ma *et al.*, 2022). Under such conditions, the carbon flux from central metabolic pathways is redirected toward fatty acid biosynthesis: nitrogen limitation suppresses chlorophyll and protein synthesis, disrupts respiratory complexes, and promotes the accumulation of reactive oxygen species (ROS), which collectively stimulate lipid biosynthesis as a cellular response to metabolic imbalance (Wang, L. *et al.*, 2024; Wang, X. *et al.*, 2025). The simultaneous reduction in carbohydrate content observed in biochar treatments is consistent with this model, reflecting a competitive reallocation of carbon between storage pathways, with lipid biosynthesis prevailing under the elevated C:N conditions imposed by biochar-mediated nitrogen adsorption (Ghosh *et al.*, 2024; Ma *et al.*, 2022).

The highest lipid content was observed in the 0.5 g L^{-1} treatment (45.62%), which also showed the greatest NH_4^+–N removal efficiency (32.50%) and the lowest specific growth rate (μ_{max} = 0.34 d^{-1}). This combination is mechanistically coherent: the more efficient removal of dissolved nitrogen in this treatment, coupled with the lowest growth rate, indicates that cells experienced a greater relative nitrogen deficit during the cultivation period, the condition most conducive to lipid accumulation in *Chlorella* (Wang, L. *et al.*, 2024). At 1.0 g L^{-1} biochar, although nitrogen bioavailability was also reduced by adsorption, the gradual desorption mechanism that sustained growth throughout the experimental period (Section 3.1) may have partially alleviated nitrogen limitation over time, resulting in a moderately lower but still

elevated lipid content (39.54%). In the control, the absence of any nitrogen-buffering matrix and the rapid consumption of dissolved NH_4^+ during the exponential phase were followed by growth decline, with carbon allocation shifting preferentially towards carbohydrate accumulation (24.75%), a response associated with nutrient imbalance under conditions that do not favour sustained lipid induction in *Chlorella* (Ma *et al.*, 2022; Wang, X. *et al.*, 2025).

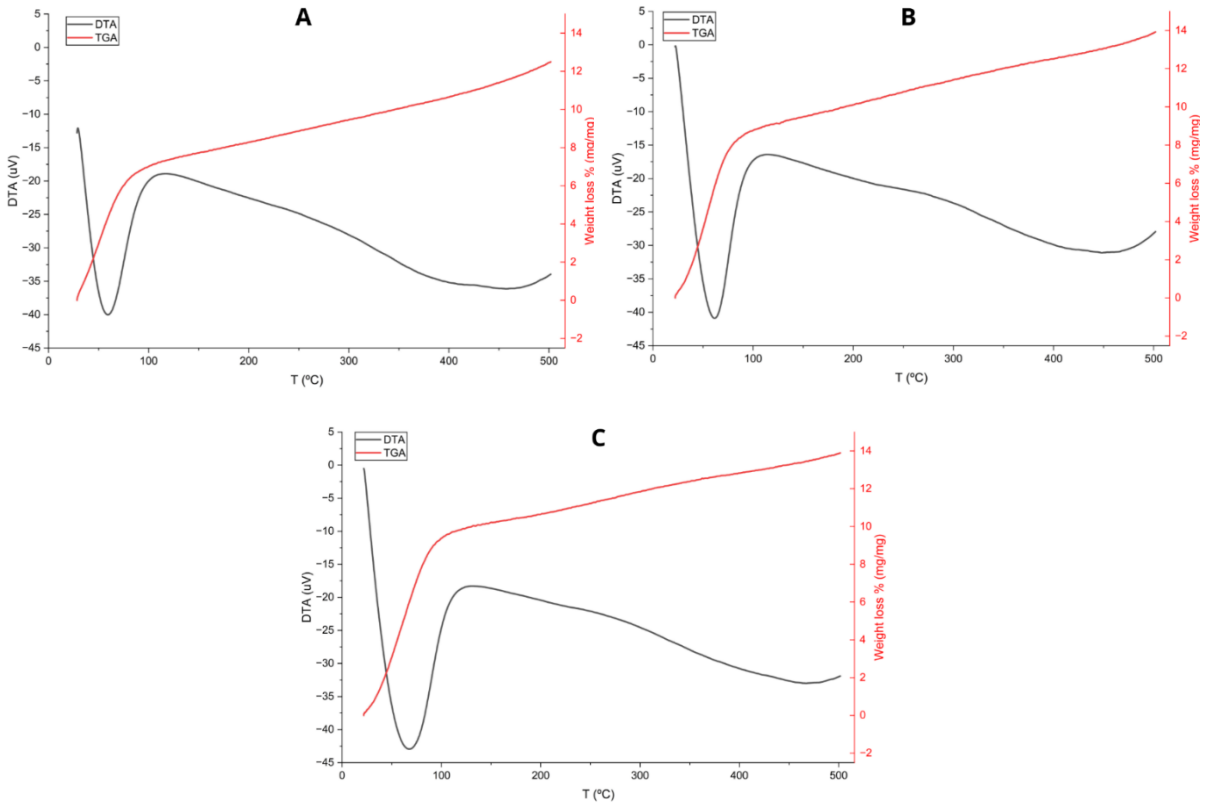
The non-significant increase in protein content with biochar addition (33.27% → 38.17%) is biologically consistent with the more stable nitrogen supply mediated by the biochar desorption mechanism, particularly in the 1.0 g L^{-1} treatment, where sustained nitrogen availability throughout the cultivation period (Section 3.1) would have supported continued amino acid synthesis. Protein accumulation in microalgae is directly coupled to nitrogen assimilation, and conditions that maintain a moderate but continuous nitrogen supply, as opposed to either excess or acute depletion, are known to favour protein biosynthesis alongside growth (Metin; Altınbaş, 2024). The absence of statistical significance likely reflects the inherent variability in biochemical composition analyses of microalgal biomass combined with the modest replication of this study, rather than a true absence of biological effect. Larger-scale studies with extended cultivation periods would be better positioned to resolve whether this protein trend is robust.

From a biotechnological perspective, the lipid contents observed in the biochar treatments are noteworthy. A lipid content of 45.62% on a dry weight basis places the 0.5 g L^{-1} treatment within the range considered technically relevant for microalgal biodiesel production and other high-value lipid-based applications (Stephy *et al.*, 2025). This result is particularly significant given that it was achieved in synthetic brewery wastewater without the application of nitrogen-starvation protocols or costly nutrient manipulation strategies typically required to induce lipid accumulation in microalgae. The addition of biochar thus simultaneously served two functions with direct biotechnological value: improving culture stability through nutrient buffering (Section 3.1) and enhancing lipid yields through modulation of the effective C:N ratio. This dual effect positions açaí seed-derived biochar as a promising low-cost additive for integrated wastewater treatment and microalgal biorefinery systems, where simultaneous nutrient removal and high-value biomass production are desirable outcomes (Ghosh *et al.*, 2024).

3.4 THERMAL CHARACTERIZATION AND SURFACE MODIFICATIONS OF BIOCHAR AFTER CULTIVATION

The thermogravimetric (TGA) and differential thermal analysis (DTA) curves of açaí seed biochar before and after cultivation with *Chlorella fusca* LEB 111 are presented in Figure 3. In all samples, the DTA curves exhibited an endothermic peak at low temperatures, associated with the evaporation of physically adsorbed water. This peak shifted to higher temperatures after cultivation, from approximately 59 °C in the fresh biochar to 61 °C and 68 °C in the samples recovered from the 0.5 and 1.0 g L^{-1} treatments, respectively, while the minimum DTA signal increased in magnitude (from ~ −40 µV to ~ −43 µV), indicating a greater energy requirement for water removal. This shift is consistent with increased surface hydrophilicity of the biochar after interaction with the cultivation medium, likely resulting from the incorporation of oxygenated functional groups or the adsorption of polar organic compounds onto the biochar surface (Laishram *et al.*, 2025). Similar modifications in biochar surface chemistry have been reported following interaction with aqueous systems rich in dissolved organic matter, where adsorbed compounds increase surface polarity and water retention capacity (Rehman *et al.*, 2024).

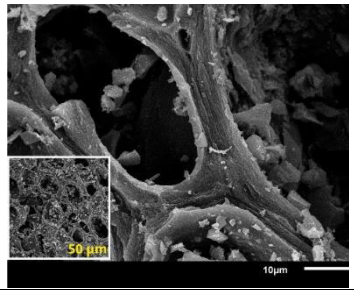
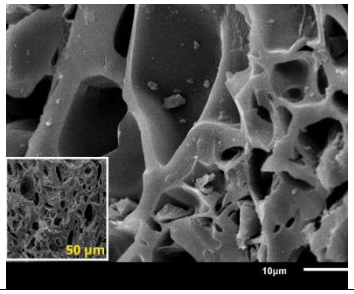
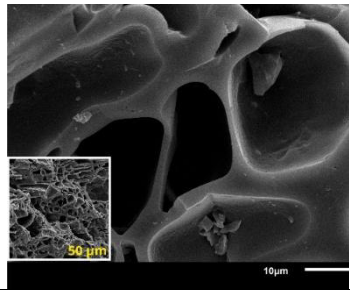
Figure 3 - TGA and DTA curves of the biochar before cultivation (A) and after cultivation in the treatments with 0.5 g L^{-1} (B) and 1.0 g L^{-1} (C).



The TGA curves revealed a gradual mass loss up to 500 °C in all samples, characteristic of biochar materials with stable aromatic carbon structures and labile surface functional groups. However, the biochar recovered after cultivation exhibited a higher total weight loss (~13.9%) than the fresh material, indicating the presence of additional thermally labile compounds acquired during cultivation. This increase in mass loss is attributed to the adsorption of organic matter during cultivation, including dissolved organic compounds from the effluent, extracellular polymeric substances (EPS) released by *Chlorella*, and potentially attached cells or cellular debris on the biochar surface, all of which decompose at temperatures below 500 °C (Mustapha; Isa, 2025; Tadesse; Huang; Zhou, 2025). The near-identical total weight losses observed for the 0.5 and 1.0 g L^{-1} treatments (13.92% and 13.89%, respectively) suggest that the adsorption capacity of the biochar reached saturation under the experimental conditions, such that increasing biochar concentration did not proportionally increase the mass of material adsorbed per unit of biochar. This observation is consistent with the nutrient removal data (Section 3.2), where the 1.0 g L^{-1} treatment did not achieve higher NH_4^+–N removal than the 0.5 g L^{-1} treatment, reinforcing the interpretation that adsorption sites were similarly occupied at both concentrations under these cultivation conditions.

The scanning electron microscopy (SEM) images presented in Table 4 reveal clear morphological differences between the fresh biochar (control) and the post-cultivation samples. The fresh biochar displayed a highly porous, heterogeneous surface with an open macroporous structure and fibrous matrix, characteristics consistent with the lignocellulosic origin of the açaí seed feedstock and typical of biochar materials with high adsorption potential (Laishram *et al.*, 2025; Zaparoli *et al.*, 2023). After cultivation, the surface of both post-cultivation samples appeared more irregular, with evidence of particulate deposits and surface coverage not observed in the fresh material, suggesting the adsorption of organic compounds from the cultivation medium onto the biochar surface during the experimental period. These morphological changes are consistent with the thermogravimetric data (Section 3.4.1), which indicated higher mass loss in post-cultivation biochar samples and supported the retention of labile organic matter on the biochar surface.

Table 4 - Scanning electron microscopy (SEM) images and elemental surface composition (EDS, weight %) of açaí seed-derived biochar before cultivation (Control) and after cultivation in treatments with 0.5 and 1.0 g L^{-1} biochar. n.d.: not detected.

	Control	0.5 g L^{-1} Biochar	1.0 g L^{-1} Biochar
Element (weight %)			
C	69.97	73.55	70.15
N	n.d	7.61	13.50
O	13.62	16.37	13.86
Mg	1.00	0.31	n.d
P	2.48	0.70	0.79
K	12.93	1.14	1.71
Ca	n.d	0.32	n.d

The elemental composition of the biochar surface before and after cultivation, determined by energy-dispersive X-ray spectroscopy (EDS), is presented in Table 4. The change observed upon cultivation was the appearance of nitrogen on the biochar surface, from not detected (n.d.) in the fresh biochar to 7.61 wt% and 13.50 wt% in the 0.5 and 1.0 g L^{-1} post-cultivation samples, respectively. This pronounced accumulation of nitrogen on the biochar surface provides evidence for the NH_4^+ adsorption mechanism proposed in Sections 3.1 and 3.2, corroborating the hypothesis that biochar sequestered nitrogen from the medium and regulated its availability throughout the cultivation period. Nitrogen adsorption onto biochar occurs primarily through electrostatic interactions between NH_4^+ and negatively charged surface functional groups (such as $–COO^-$ and $–O^-$), as well as through ion exchange mechanisms facilitated by the biochar's cation exchange capacity (Marzbali *et al.*, 2023; Shenk *et al.*, 2022). The higher nitrogen content detected at 1.0 g L^{-1} (13.50 wt%) relative to 0.5 g L^{-1} (7.61 wt%) is consistent with the greater total adsorption surface available at the higher biochar loading, although, as discussed in Section 3.2, this did not translate into higher NH_4^+–N removal from the medium, suggesting that part of the adsorbed nitrogen may have been subsequently desorbed and made available to the microalgae over time, sustaining growth beyond the period observed in the control and 0.5 g L^{-1} treatments.

The oxygen content of the biochar surface increased after cultivation (from 13.62 wt% to 16.37 and 13.86 wt% for 0.5 and 1.0 g L^{-1}, respectively), consistent with the surface hydrophilicity increase evidenced by TGA/DTA and likely reflecting the incorporation of oxygenated functional groups from adsorbed organic compounds. Additionally, a substantial

reduction in potassium content was observed (from 12.93 wt% in the fresh biochar to 1.14 and 1.71 wt% in the post-cultivation samples), suggesting that K^+ was released from the biochar matrix into the cultivation medium during the experiment. This release is consistent with the known behaviour of plant-derived biochars, which commonly contain mineral-associated potassium that is mobilized upon contact with aqueous media through ion exchange processes (Laishram *et al.*, 2025). While the contribution of this K^+ release to microalgal nutrition was not specifically quantified in this study, it represents an additional mechanism by which the biochar may have influenced the cultivation environment beyond nitrogen dynamics.

Taken together, the TGA/DTA and SEM/EDS results confirm that the açaí seed-derived biochar was not an inert additive in the cultivation system, but rather an active material that underwent measurable physicochemical modifications during interaction with the microalgal culture and the brewery wastewater matrix. The evidence for nitrogen adsorption (EDS), organic matter retention (TGA), surface hydrophilicity increase (DTA), cell adhesion (SEM), and potassium release collectively support the dual role of biochar proposed throughout this discussion: as a nutrient adsorbent that modulates the C:N ratio experienced by the microalgae, and as a structural support that facilitates microalgal attachment and may buffer cultivation conditions. These findings agree with recent studies on microalgae–biochar systems, which have highlighted the multifunctional role of biochar as simultaneously an adsorbent, a slow-release nutrient regulator, and a cell support matrix in wastewater-based cultivation (Ghosh *et al.*, 2024; Rehman *et al.*, 2024).

4 CONCLUSIONS

The results of this study demonstrate that açaí seed-derived biochar significantly influences the cultivation of *Chlorella fusca* LEB 111 in synthetic brewery wastewater, affecting growth dynamics, nutrient removal, biomass composition, and biochar properties. Although biochar addition reduced the initial specific growth rate, it improved culture stability, particularly at 1.0 g L^{-1}, where sustained growth was observed. Nutrient removal was efficient within 7 days, with total carbon removal above 84% and phosphate removal above 77%, while ammonium removal was maximized at 0.5 g L^{-1} (32.50%), indicating an optimal balance between adsorption and biological uptake. Biochar addition promoted a marked shift in biomass composition, with lipid content increasing up to 45.62%, associated with biochar-mediated changes in nitrogen availability and effective C:N ratio. Physicochemical analyses confirmed that biochar actively interacted with the cultivation system, as evidenced by nitrogen adsorption

(up to 13.50 wt%), organic matter retention, and surface modifications. Overall, the findings indicate that biochar acts as a dynamic component capable of modulating nutrient availability, influencing both microalgal metabolism and system performance. These results highlight the potential of açaí seed-derived biochar as a low-cost and multifunctional material for integrating wastewater treatment with high-value microalgal biomass production.

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CHAPTER IV

(General Conclusion, Conclusão Geral)

GENERAL CONCLUSIONS

This thesis demonstrated that brewery wastewater can be understood not only as an industrial residue, but as a strategic resource capable of supporting microalgal cultivation and enabling the production of value-added biomass within a circular bioeconomy framework. Across four complementary studies, a consistent link was established between the use of industrial effluents and the development of agricultural bioinputs, highlighting the potential for integrating these approaches.

The literature analysis showed that microalgae cultivated in brewery wastewater are capable of efficiently removing nutrients and organic matter while producing biomass enriched with bioactive compounds. At the same time, it became clear that studies integrating wastewater treatment, phytohormonal characterization, and agronomic validation are still limited. In this context, this thesis contributes by advancing this integration.

The experimental results confirmed the feasibility of using brewery wastewater as a cultivation medium. The semicontinuous cultivation of *Chlorella fusca* LEB 111 showed stable performance over multiple cycles and enabled a significant reduction in biomass production costs, indicating that replacing synthetic media with industrial effluents can be an economically viable alternative, particularly in scenarios with limited infrastructure.

In addition, the results related to the use of biomass as a biostimulant demonstrated that the cultivation medium directly influences the biochemical profile of the microalgae. The identification of phytohormones, combined with validation in assays with barley and lettuce, showed that brewery wastewater acts not only as a nutrient source but also as a factor capable of influencing microalgal metabolism and, consequently, plant responses. This relationship contributes to a more integrated understanding of cultivation conditions, biomass composition, and agricultural application.

The incorporation of açaí seed-derived biochar further expanded this approach by showing that process intensification can be combined with waste valorization. The use of biochar improved culture stability, influenced nutrient dynamics, and promoted changes in biomass composition, while also presenting potential for subsequent application in soil. These results indicate that different residual streams can be integrated within a single system, reinforcing the concept of multiple resource use.

Taken together, the results demonstrate that brewery wastewater can serve as a basis for integrated production systems, in which not only waste treatment occurs, but also the generation

of valuable products. The combination of microalgal cultivation, biomass characterization, agronomic evaluation, and process intensification contributes to the development of more sustainable processes aligned with bioeconomy principles.

Finally, although the results are promising, challenges remain regarding process standardization, scale-up, and regulatory requirements for the application of bioinputs. Future studies may focus on large-scale validation, product formulation, and the development of strategies to support the practical implementation of these systems.

CONCLUSÃO GERAL

A presente tese demonstrou que o efluente cervejeiro pode ser compreendido não apenas como um resíduo industrial, mas como um recurso estratégico capaz de sustentar o cultivo de microalgas e viabilizar a produção de biomassa com valor agregado no contexto da bioeconomia circular. Ao longo de quatro estudos complementares, foi possível estabelecer uma conexão consistente entre o aproveitamento de efluentes industriais e o desenvolvimento de bioinsumos agrícolas, evidenciando o potencial de integração entre essas abordagens.

A análise da literatura mostrou que microalgas cultivadas em efluente cervejeiro apresentam elevada capacidade de remoção de nutrientes e matéria orgânica, ao mesmo tempo em que produzem biomassa rica em compostos bioativos. No entanto, também ficou evidente que ainda são limitados os estudos que conectam, de forma integrada, o tratamento de efluentes, a caracterização fitohormonal e a validação agronômica. Nesse sentido, esta tese contribui ao avançar justamente nessa integração.

Os resultados experimentais reforçaram a viabilidade do uso do efluente cervejeiro como meio de cultivo. O cultivo de *Chlorella fusca* LEB 111 em regime semicontínuo demonstrou estabilidade ao longo dos ciclos e permitiu uma redução significativa nos custos de produção de biomassa, indicando que a substituição de meios sintéticos por efluentes industriais pode ser uma alternativa economicamente viável, especialmente em cenários com limitações de infraestrutura.

Além disso, os resultados relacionados ao uso da biomassa como bioestimulante evidenciaram que o meio de cultivo exerce influência direta sobre o perfil bioquímico da microalga. A identificação de fitohormônios e a validação em ensaios com cevada e alface demonstraram que o efluente cervejeiro não atua apenas como fonte de nutrientes, mas também como um fator capaz de influenciar o metabolismo microalgal e, consequentemente, a resposta das plantas. Essa relação contribui para uma compreensão mais integrada entre cultivo, composição da biomassa e aplicação agrícola.

A incorporação do biochar de caroço de açaí ampliou essa abordagem ao demonstrar que a intensificação do processo pode ser associada à valorização de resíduos. O uso do biochar contribuiu para a estabilidade do cultivo, influenciou a dinâmica de nutrientes e promoveu alterações na composição da biomassa, além de apresentar potencial para aplicação posterior no solo. Esses resultados indicam que diferentes correntes de resíduos podem ser integradas em um mesmo sistema, reforçando a lógica de aproveitamento múltiplo de recursos.

De forma conjunta, os resultados obtidos mostram que o efluente cervejeiro pode ser utilizado como base para sistemas integrados de produção, nos quais ocorre não apenas o tratamento do resíduo, mas também a geração de produtos de interesse. A combinação entre cultivo microalgal, caracterização da biomassa, avaliação agronômica e estratégias de intensificação contribui para o desenvolvimento de processos mais sustentáveis e alinhados com os princípios da bioeconomia.

Por fim, embora os resultados sejam promissores, ainda existem desafios relacionados à padronização dos sistemas, ao escalonamento do cultivo e às exigências regulatórias para aplicação de bioinsumos. Estudos futuros podem avançar na validação em escala maior, na formulação de produtos e na consolidação de estratégias que viabilizem a aplicação prática desses sistemas.

CHAPTER V

(Waste Generation and Disposal)

WASTE GENERATION AND DISPOSAL

The development of this study involves the generation of both chemical and biological waste, in liquid and solid forms. Liquid waste is predominantly composed of solvents used in analytical procedures, while solid waste includes plastics, paper, glass, metals (such as aluminum), and organic residues generated throughout the research activities. Waste management procedures established by the laboratory were strictly followed, considering the characteristics and potential risks associated with waste generation, collection, storage, transportation, treatment, and final disposal. These practices ensure environmentally appropriate handling and contribute to the protection of both human health and the environment.

All chemical and biological residues were properly packaged, labeled, stored, and segregated before being transferred to the waste management unit of the Federal University of Rio Grande. Subsequently, the waste was directed to a specialized company for appropriate treatment and final disposal.

SUGGESTIONS FOR FUTURE WORK

1. Investigate the cultivation of microalgae under continuous or fed-batch regimes using real microbrewery effluent, without prior dilution or physicochemical treatment, in order to obtain more realistic data on biomass productivity and biostimulant yield under industrially relevant conditions.
2. Evaluate the agronomic application of biochar derived from microalgae cultivation in brewery wastewater as a soil amendment, combined with the biostimulants produced in this thesis, reinforcing the circular economy framework proposed throughout the studies.
3. Assess the biostimulant effects of the microalgal extracts obtained in this work on other agriculturally relevant crops, such as cereals, legumes, or horticultural species, in order to broaden the understanding of their mode of action and expand their potential agronomic applications.
4. Develop stable formulations for the biostimulants produced from microalgal biomass, exploring encapsulation techniques and nanoemulsions to improve shelf life, bioavailability, and ease of field application.

CHAPTER VI

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