

Integrated Mixotrophic Cultivation Strategy for *Limnospira* (*Spirulina*) *Platensis* using Vinegar Wastewater: Productivity Enhancement through Controlled Organic Supplementation and Salinity Optimization

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Agro-industrial wastewaters rich in organic compounds represent both an environmental liability and a potential resource for sustainable bioprocesses. This study evaluated a cultivation strategy for *Limnospira* (*Spirulina*) *platensis* based on controlled mixotrophic supplementation with vinegar wastewater and salinity optimization to enhance biomass production while maintaining culture stability. Vinegar supplementation significantly improved growth compared to the control (Jourdan medium), with biomass concentrations ranging from 3.32 to 6.30 g · L⁻¹ within four days, versus 2.42 g · L⁻¹ in autotrophic conditions. The effect of NaCl under mixotrophy revealed a clear interaction between salinity and organic carbon availability, with optimal performance (5.85 g · L⁻¹) at 1 g · L⁻¹ NaCl. These results demonstrate that controlled mixotrophic supplementation defines an operational window that enhances biomass productivity without compromising physiological stability.

1. Introduction

The increasing production of wastewaters (WWs) represents an important environmental concern due to their high organic load and potential impact on aquatic ecosystems. However, these effluents can also be exploited as a valuable resource for sustainable biotechnological processes, particularly for microalgal cultivation (Cosenza et al., 2026; Lutzu et al., 2020). Many agro-industrial WWs contain significant amounts of organic carbon, nitrogen, phosphorus and trace elements that can support algal growth while simultaneously enabling WWs treatment (Lutzu et al., 2024; Miotti et al., 2022). For this reason, the integration of microalgal cultivation with WW valorization is gaining increasing attention as a strategy within the circular bioeconomy (Cavallini et al., 2024). Microalgae and cyanobacteria can activate different metabolic pathways depending on the availability of inorganic and organic carbon (Concas et al. 2013). Under mixotrophic conditions, the simultaneous use of photosynthesis and organic carbon assimilation can significantly enhance biomass productivity compared to purely autotrophic cultivation. Several studies have shown that organic substrates present in WWs can stimulate algal growth by providing additional energy sources and metabolic intermediates (Baraldi et al., 2025; Cosenza et al., 2026). Among agro-industrial effluents, vinegar production generates liquid residues rich in biodegradable organic compounds such as residual sugars, organic acids and fermentation by-products, which may represent a potential carbon source for microalgal cultivation (Sousa et al., 2025). However, their direct use is often limited by factors such as acidic pH, nutrient imbalance and osmotic stress, which can negatively affect microalgal growth (Gao et al., 2023). *Limnospira* (*Spirulina*) *platensis* is a filamentous cyanobacterium widely cultivated at

industrial scale due to its high nutritional value, rapid growth and tolerance to alkaline environments (Rizzoli et al., 2025). Its metabolic flexibility makes it a promising candidate for cultivation strategies based on the integration of organic substrates with mineral media (Di Stasi et al., 2026).

Despite the increasing interest in mixotrophic cultivation using agro-industrial WWs, most studies have focused on the direct use of effluents or on single-factor optimization, often neglecting the combined effects of process control strategies and environmental parameters such as salinity.

In particular, the lack of controlled supplementation approaches limits the stability, reproducibility, and scalability of these systems. In this context, the present study proposes an integrated and process-oriented *L. platensis* cultivation strategy based on controlled mixotrophic supplementation of vinegar-derived WW within a stabilized mineral medium, and salinity optimization to investigate the interaction between organic carbon availability and osmotic stress.

Unlike conventional approaches based on direct WW utilization, this strategy enables the decoupling of biomass growth and metabolic activation, allowing the identification of an operational window in which organic input enhances biomass productivity without compromising physiological stability.

2. Material and methods

2.1 Biological material and inoculum preparation

Experiments were carried out using a *L. platensis* strain SAG 21.99 obtained from the Sammlung Culture Collection at the University of Göttingen, Germany. Biomass used for inoculation was collected during the exponential growth phase to ensure high metabolic activity. Initial biomass concentration was standardized using a previously established relationship between optical density and dry weight.

All cultures were inoculated at an initial concentration of $0.3 \text{ g} \cdot \text{L}^{-1}$ (dry weight). Two agro-industrial effluents derived from vinegar production were evaluated as potential substrates: a vinegar wastewater (V) and a mixed vinegar–must effluent (VM). Because both streams presented strongly acidic conditions (approximately pH 3), the pH was adjusted with 1 M NaOH to near-neutral values (pH 7.6 for V and pH 7.8 for VM). After adjustment, the effluents were sterilized by autoclaving (121°C , 0.1 MPa, 20 min).

2.2 Culture medium

Cultivation experiments were performed using Jourdan 100X medium (JM), a mineral formulation widely used for cyanobacterial growth. The chemical composition of medium is described detailed in Cosenza et al., (2026). The formulation provides alkaline conditions with an N:P molar ratio of approximately 12:1, while NaCl at $1 \text{ g} \cdot \text{L}^{-1}$ represents the reference salinity level. To limit bicarbonate decomposition, JM was sterilized at 105°C and 0.1 MPa for 20 min. After cooling, trace elements and a metavanadate-based antibacterial agent were added aseptically.

2.3 Cultivation conditions and experimental design

Batch cultures were grown in 250 mL Erlenmeyer flasks containing 150 mL working volume for 14 days. Each condition was tested in four biological replicates. Cultures were maintained at room temperature ($25\text{--}30^\circ\text{C}$) under a 12 h light/12 h dark photoperiod provided by 36 W T8 LED panels, with a light intensity of approximately $85 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Aeration with filtered atmospheric air ensured mixing, gas exchange and prevention of biomass sedimentation. The experimental program consisted of three sequential phases.

In the first phase, the two sterilized effluents (V and VM) were evaluated as cultivation substrates and compared with the standard JM medium. The second phase investigated mixotrophic growth by adding increasing amounts of vinegar after seven days of photoautotrophic cultivation. In the final phase, NaCl concentration in JM was modified to generate different salinity conditions, followed by the addition of the optimal vinegar concentration identified in the previous phase in order to assess the interaction between salinity and organic carbon supplementation.

2.4 Growth monitoring

Microalgal growth was monitored by measuring optical density at 680 nm using a UV–Vis spectrophotometer (model ONDA V30 SCAN – UV VIS, ZetaLab, Padua, Italy). A calibration curve correlating OD values with dry biomass concentration was previously established and used to estimate biomass throughout the cultivation experiments.

Biomass concentration as dry weight (X_{max}), specific growth rate (μ), doubling time (t_d), and biomass productivity (Q_x) were calculated according to equations reported in Rizzoli et al., (2025). Culture pH was measured using a pHmeter (HI 2210, Hanna Instruments, USA).

3. Results and discussion

3.1 Growth performance in vinegar-derived effluents

In the standard medium (JM), the cyanobacterium exhibited a typical growth pattern characterized by an exponential phase followed by stabilization, reaching a maximum biomass concentration ($X_{\max}$) of approximately $2.42 \text{ g} \cdot \text{L}^{-1}$ after 15 days of cultivation. In contrast, when the effluents were used as the sole culture medium, growth was strongly inhibited, with $X_{\max}$ of $0.56 \text{ g} \cdot \text{L}^{-1}$ in the vinegar effluent and $0.46 \text{ g} \cdot \text{L}^{-1}$ in the vinegar–must mixture. These results indicate that the effluents alone were unable to sustain efficient cyanobacterial growth under the tested conditions. The limited growth observed in the effluents can be explained by their chemical composition. Wastewaters generated during vinegar production show high variability in physicochemical characteristics (Table 1). Reported values include BOD_5 around $4.80 \text{ g} \cdot \text{L}^{-1}$ and COD up to $33.16 \text{ g} \cdot \text{L}^{-1}$, indicating a high organic carbon content. However, these effluents generally contain relatively low concentrations of essential nutrients such as total nitrogen ($\text{TN} \approx 20.50 \text{ mg} \cdot \text{L}^{-1}$) and total phosphorus ($\text{TP} \approx 7.40 \text{ mg} \cdot \text{L}^{-1}$). Moreover, they are typically characterized by acidic pH values (3.03–5.60) and moderate electrical conductivity ($1.8\text{--}2.0 \text{ mS} \cdot \text{cm}^{-1}$). Although the high BOD and COD values suggest a substantial availability of organic carbon, the limited availability of nitrogen and phosphorus represents a major constraint for microalgal growth. In addition, the acidic pH conditions are incompatible with the physiology of *L. platensis*, which is an alkaliphilic cyanobacterium that grows optimally under alkaline conditions (pH 8–10). These factors collectively explain the poor growth observed when vinegar-derived effluents were used as the sole culture medium. These observations highlight that vinegar-derived WWs, despite their high organic carbon content, cannot be directly employed as complete culture media for *L. platensis*. Instead, their effective utilization requires integration within a controlled cultivation strategy capable of compensating for nutrient limitations and physicochemical imbalances. This finding reinforces the concept that agro-industrial effluents should be considered as process inputs rather than standalone substrates, requiring appropriate operational frameworks to ensure culture stability and performance.

Table 1: Range of main physical-chemical parameters of vinegar wastewaters available in literature

BOD ($\text{g} \cdot \text{L}^{-1}$)	COD ($\text{g} \cdot \text{L}^{-1}$)	TSS ($\text{mg} \cdot \text{L}^{-1}$)	TN ($\text{mg} \cdot \text{L}^{-1}$)	TP ($\text{mg} \cdot \text{L}^{-1}$)	Electrical conductivity ($\text{mS} \cdot \text{cm}^{-1}$)	pH	Reference
4.80	10.00	150.19	-	-	2.00	4.00	Yilmaz et al., 2018
-	0.74	-	20.50	7.40		5.60	Huo et al., 2020
-	33.16	650.00	-	-	1.80	3.03	Kargi et al., 2013

Note: BOD = biological oxygen demand, COD = chemical oxygen demand, TSS = total suspended solids, TN = total nitrogen, TP = total phosphorus.

3.2 Effect of vinegar supplementation under mixotrophic conditions

To overcome these limitations, a two-stage cultivation strategy was adopted. During the first week cultures were grown under autotrophic conditions in JM, whereas during the second week vinegar was added to induce mixotrophic growth. Vinegar supplementation significantly enhanced biomass accumulation and growth kinetics compared with the control medium (Table 2). The addition of 0.5 mL vinegar increased the $X_{\max}$ to $3.52 \pm 0.31 \text{ g} \cdot \text{L}^{-1}$, with a μ of $0.14 \pm 0.01 \text{ day}^{-1}$ and a Q_x of $400 \pm 50 \text{ mg} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$.

Increasing the supplementation to 1 mL vinegar further increased biomass productivity ($Q_x = 440 \pm 100 \text{ mg} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$) and biomass concentration ($X_{\max} = 4.28 \pm 0.8 \text{ g} \cdot \text{L}^{-1}$), although the growth rate slightly decreased ($\mu = 0.11 \pm 0.02 \text{ day}^{-1}$) and doubling time increased ($t_d = 6.82 \pm 1 \text{ days}$).

The highest biomass concentration was obtained with 2 mL vinegar, reaching $6.3 \pm 1.28 \text{ g} \cdot \text{L}^{-1}$, although growth kinetics were slightly lower ($\mu = 0.10 \pm 0.01 \text{ day}^{-1}$) compared with the control medium. These results suggest that increasing the availability of organic carbon enhances biomass accumulation under mixotrophic conditions. However, the relationship between organic carbon availability and growth performance was not linear. While higher vinegar supplementation increased final biomass concentration, it did not lead to a proportional improvement in growth kinetics. This suggests that, beyond a certain threshold, the addition of organic substrates may induce metabolic constraints or shifts in cellular energy allocation, potentially affecting the

efficiency of biomass production. Therefore, maximizing organic input does not necessarily correspond to optimal process performance.

Table 2: Growth kinetics of *Limnospira platensis* under mixotrophic cultivation with vinegar supplementation and varying salinity.

Growth medium	μ (day ⁻¹)	t_d (day ⁻¹)	X_{max} (g · L ⁻¹)	Q_x (mg · L ⁻¹ · day ⁻¹)
JM	0.18 ± 0.03	4.00 ± 0.70	2.42 ± 0.48	260 ± 6.2
V	0.02 ± 0.02	-	0.56 ± 0.04	10 ± 3
VM	0.04 ± 0.06	-	0.46 ± 0.06	20 ± 4
JM+V0.5	0.14 ± 0.01	5.11 ± 0.42	3.52 ± 0.31	400 ± 50
JM+V1	0.11 ± 0.02	6.82 ± 1.00	4.28 ± 0.80	440 ± 100
JM+V2	0.10 ± 0.01	6.87 ± 0.60	6.30 ± 1.28	390 ± 70

Note: JM = Jourdan medium as control, V = vinegar, VM = vinegar + must, JM + V0.5 = control + 0.5 mL vinegar, JM + V1 = control + 1 mL vinegar, JM + V2 = control + 2 mL vinegar.

The improved growth performance can be attributed to the simultaneous assimilation of organic carbon derived from acetic acid together with inorganic carbon sources present in the medium (bicarbonate and CO₂). Similar improvements in biomass production under mixotrophic conditions have been reported for *Limnospira* sp. and other microalgae cultivated using organic substrates such as acetate or molasses (Andrade et al., 2007; Setyoningrum et al., 2015). Nevertheless, unlike approaches that primarily focus on the use of organic substrates as simple growth enhancers, the present results indicate that the effectiveness of mixotrophic cultivation strongly depends on the way organic carbon is introduced into the system. The controlled supplementation strategy adopted in this study allowed the decoupling of biomass growth and metabolic stimulation, highlighting the importance of process control in achieving stable and reproducible outcomes.

Since the increase in biomass between 1 and 2 mL vinegar supplementation did not correspond to a significant improvement in growth kinetics, 1 mL vinegar was selected as the optimal condition for the subsequent salinity experiments.

3.3 Influence of salinity on growth kinetics

Using the optimal vinegar supplementation (JM+V1), the influence of salinity was investigated by modifying the NaCl concentration in the culture medium (0.5, 1, and 2 g · L⁻¹). The highest growth performance was observed at 1 g · L⁻¹ NaCl, corresponding to the standard salinity of the JM (Table 2).

Under these conditions, cultures exhibited the highest specific growth rate ($\mu = 0.24 \pm 0.02$ day⁻¹) and the shortest doubling time ($t_d = 2.94 \pm 0.17$ days), with a Q_x of 560 ± 144 mg · L⁻¹ · day⁻¹. At 0.5 g · L⁻¹ NaCl, the growth rate decreased ($\mu = 0.086 \pm 0.01$ day⁻¹) and doubling time increased (8.63 ± 0.98 days), although biomass accumulation remained relatively high (5.90 ± 0.07 g · L⁻¹).

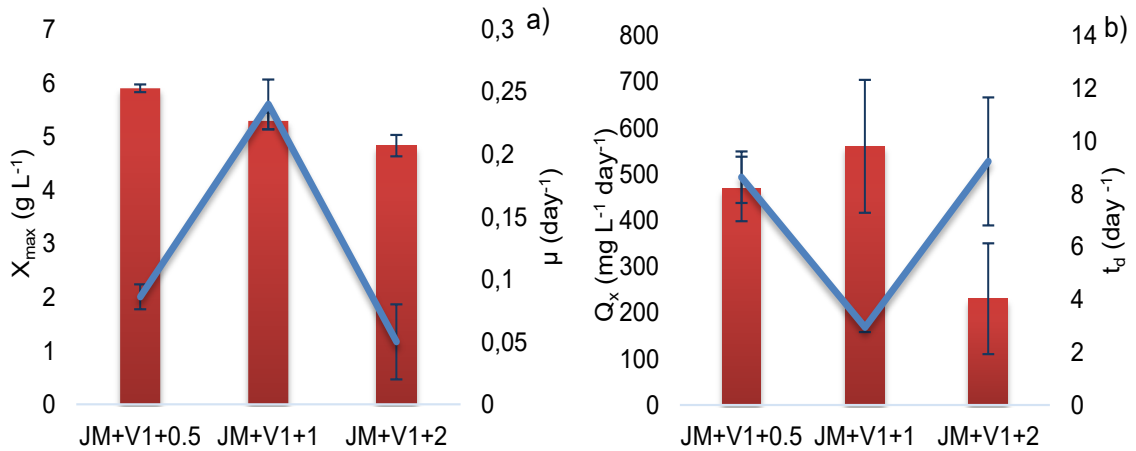


Figure 1: Relationship between growth parameters of *Limnospira platensis* under mixotrophic conditions with vinegar supplementation and varying NaCl concentrations (0.5, 1 and 2 g · L⁻¹). (a) Biomass concentration (X_{max}) vs specific growth rate (μ), (b) Biomass productivity (Q_x) vs t_d (doubling time)

Conversely, the highest salinity tested ($2 \text{ g} \cdot \text{L}^{-1} \text{ NaCl}$) resulted in a substantial reduction in growth performance, with a growth rate of $0.05 \pm 0.03 \text{ day}^{-1}$, a doubling time of 9.23 ± 2.43 days, and Q_x of $230 \pm 120 \text{ mg} \cdot \text{L}^{-1} \text{ day}^{-1}$. These results indicate that both hypo-saline and hyper-saline conditions negatively affect growth kinetics, likely due to osmotic stress that disrupts cellular ion homeostasis and metabolic activity. Similar responses to salinity stress have been reported in *Limnospira* sp. (Russo et al., 2024).

Overall, the results demonstrate that the successful integration of agro-industrial WWs into microalgal cultivation systems relies on a multi-factor optimization approach, in which organic carbon supplementation and environmental conditions must be jointly controlled. The identification of an operational window, where biomass productivity is enhanced without compromising culture stability, represents a key outcome of this work and provides a basis for the development of more robust and scalable cultivation processes.

4. Conclusions

Vinegar-derived effluents alone cannot sustain efficient growth of *Limnospira platensis* due to their low nutrient content and acidic pH. However, when used as an organic supplement under mixotrophic conditions, vinegar significantly enhanced biomass production. Among the tested conditions, the highest biomass concentration ($6.30 \text{ g} \cdot \text{L}^{-1}$) was obtained with 2 mL vinegar supplementation, while the best growth performance in terms of growth kinetics was observed with 1 mL vinegar combined with $1 \text{ g} \cdot \text{L}^{-1} \text{ NaCl}$, resulting in the highest μ (0.24 day^{-1}) and Q_x ($560 \text{ mg} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$). These findings demonstrate the potential of vinegar-derived waste streams as supplementary carbon sources for *L. platensis* cultivation and highlight their possible role in the valorization of agro-industrial by-products within sustainable microalgae-based bioprocesses. This study provides a process-oriented framework for integrating agro-industrial waste streams into microalgal cultivation systems through controlled supplementation strategies, moving beyond conventional approaches based on direct WW utilization.

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