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Crude Microalgae Extract for Increased CO_2 Capture and Higher Biomass Production in Algal Cultivation Systems

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Abstract

Efficient inorganic carbon supply is a common limitation in microalgal cultivation, particularly in waste-derived media such as anaerobic digestate. Carbonic anhydrase (CA) accelerates the interconversion of CO_2 and bicarbonate and may therefore enhance carbon utilisation under conditions where inorganic carbon is abundant but not readily available. In this study, crude CA-containing extracts (aCA) were prepared from *Scenedesmus*-dominated algal biomass, and CA activity was quantified using an esterase assay (EAA). Although EAA activities varied depending on biomass pretreatment (0.15–0.47 U g^{-1} DW), the physiological response to extract addition was consistent. In batch cultures of *Chlorella sorokiniana* grown in diluted digestate, aCA supplementation increased the specific growth rate (SGR) by 21–82%. In contrast, stimulation in a mineral medium was minimal, indicating that the benefit of aCA addition is most apparent under reduced inorganic carbon availability. In semi-continuous cultivation, repeated extract addition sustained a higher biomass productivity over time (rather than a specific growth rate). These results demonstrate that crude microalgal extracts containing CA can improve growth performance in digestate-based cultures and may offer a simple, low-cost approach to enhancing inorganic carbon utilisation in waste-integrated algal production systems.

Keywords: carbonic anhydrase; microalgae; anaerobic digestate; carbon dioxide

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1. Introduction

In full-scale algae biomass production systems, CO_2 availability is often a limiting factor for growth because such systems are typically designed for optimal light or nutrient utilisation rather than for ensuring a sufficient carbon supply. Consequently, many cultivation facilities rely on the injection of pure CO_2 gas, which represents one of the major operational costs [1]. CO_2 injection has been reported to account for up to 27% of total algal production costs [2], and for 60–70% of the expenses associated with supplying nutrients for algal growth [3]. Despite this investment, a large fraction of the injected CO_2 escapes to the atmosphere: in raceway ponds, only approximately 13–20% of supplied CO_2 is actually absorbed [3,4].

CO_2 availability to the photosynthetic system is governed by a series of bidirectional processes driven by concentration gradients. Enhancing the rate of any of these steps can improve the photosynthetic CO_2 availability. Common strategies, such as increasing the surface area, bubbling, reducing bubble size, or extending bubble paths, focus on optimising gas–liquid mass transfer. Alternative cultivation modes, including biofilm or attached-growth systems, further improve CO_2 contact and shorten diffusion pathways, leading to enhanced CO_2 fixation rates [5,6]. Mixotrophic cultivation or algal–bacterial consortia, in which CO_2 is generated intracellularly or through microbial respiration, represent additional biological routes for increasing CO_2 availability [7,8].

A complementary strategy targets inorganic carbon utilisation kinetics within the culture medium. Carbonic anhydrase (CA) catalyses the reversible interconversion between CO_2 and bicarbonate and thereby influences the distribution and availability of inorganic carbon for photosynthesis. While algae and cyanobacteria naturally express CA as part of their carbon-concentrating mechanisms, external CA supplementation represents a biochemical approach that operates independently of physical mass transfer optimisation and can act in parallel with other CO_2 utilisation strategies.

Building on this concept, the absorption of CO_2 can be enhanced by adding CA directly into the culture medium [9,10]. CA is a zinc-containing metalloenzyme that catalyses the rapid, reversible hydration of CO_2 to bicarbonate and protons, thereby regulating the inorganic carbon balance and facilitating key biological processes such as photosynthesis, respiration, and CO_2 transport. Carbonic anhydrases are among the fastest known enzymes, with catalytic rates ranging from approximately 10^4 to 10^6 reactions per second, depending on substrate diffusion [11]. Cytosolic carbonic anhydrase II (CA II), also found in green microalgae, can reach a specific turnover rate of 1.4×10^6 s^{-1} [12].

In algal cultures, CA enhances the availability of inorganic carbon by accelerating the interconversion between CO_2, bicarbonate, and carbonate species [13]:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+$$

By catalysing CO_2 hydration, CA increases carbon availability for photosynthesis and can substantially improve algal growth and biomass productivity, particularly in dense cultures or under CO_2-limited conditions [10,14]. For example, the addition of CA from bovine erythrocytes increased the biomass productivity of *Nannochloropsis* sp. from 22.7 ± 0.5 mg L^{-1} d^{-1} (control) to 37 ± 3 mg L^{-1} d^{-1} (free CA) and to 40 ± 1 mg L^{-1} d^{-1} when immobilised in buoyant alginate beads [10].

Although CA supplementation offers an energetically favourable alternative to continuous CO_2 injection, commercial CA is expensive. Furthermore, conventional isolation from animal tissues (e.g., bovine erythrocytes) requires cell disruption, extraction with detergents or organic solvents, and purification using affinity chromatography on sulfonamide-derivatized or CNBr-activated matrices. These procedures involve reagents such as Triton X-100, chloroform–ethanol mixtures, and p-aminomethylbenzenesulfonamide–Sepharose, and typically require repeated dialysis and concentration steps [15–18]. Such multi-step protocols are chemically intensive, energy-demanding, and poorly suited for large-scale or sustainable CO_2 capture applications. Similar purification strategies in protein bioprocessing have been shown to dominate both cost and environmental burden, with downstream processing accounting for up to ~80% of total production costs, primarily due to chromatography steps and extensive buffer consumption [19]. Process mass intensity (PMI) analyses further illustrate this burden, reporting several thousand kilograms of material input per kilogram of purified product, more than 90% of which is associated with water and buffer use [20]. Life cycle assessments further indicate E-factors (including water) exceeding

10^4 g waste per g product, underscoring the intrinsic resource intensity of conventional multi-step purification routes [21].

In contrast, microalgae represent a renewable, non-toxic source of CA that can be extracted using simple mechanical disruption in water or mild green solvents [12,22,23]. Here, "non-toxic" refers to the biological origin of the enzyme from microalgal biomass and to the use of mild extraction procedures that avoid the toxic organic solvents and hazardous purification reagents commonly employed in conventional protocols.

In microalgae, CA is the core component of the carbon concentrating mechanism (CCM), enabling efficient CO_2 uptake and fixation [24,25]. Multiple CA isoforms have been identified in distinct cellular locations, including the chloroplasts, cytosol, plasma membrane, cell wall, and periplasmic space [13,26,27], and belong to several genetic classes (α, β, γ, δ, ε, η, and θ). Their expression and localization vary across species and environmental CO_2 conditions [26].

As early as the 1980s, the addition of crude algal extract to *Dunaliella salina* raceway ponds has been shown to increase biomass yield from 0.25 g L^{-1} to 0.375 g L^{-1}, demonstrating the potential of algae-derived CA for enhancing productivity [9]. Li et al. [12] demonstrated the aqueous extraction and partial purification of cytosolic CA II from *Chlorella vulgaris*, showing that crude extracts retained CA activity and exhibited kinetic properties comparable to mammalian CAs. Ores et al. [23] later confirmed that crude extracts from both marine and freshwater microalgae can yield measurable CA activity without harsh purification.

More recently, extraction using deep eutectic solvents (DESs) has been shown to preserve CA activity while simultaneously enhancing CO_2 solubility. Craveiro et al. [22] tested DESs composed of ChCl:Urea, ChCl:PEG, and PEG:Urea as solvents for CA extraction from *Tisochrysis lutea*, *Chlorella vulgaris*, and *Spirulina* sp., reporting specific CA activities up to 0.70 mU mg^{-1} and CO_2 solubilities up to ~4 g CO_2 g^{-1} DES. PEG-based DESs exhibited the best performance. These findings suggest that crude, unpurified algal extracts may be sufficient to promote CO_2 hydration and stimulate algal growth, offering a low-cost and environmentally benign alternative to purified animal enzymes for biological CO_2 capture.

The integration of this approach might also be attractive for microalgal cultivation in waste-derived media such as wastewater and anaerobic digestate. These substrates supply substantial macro- and micronutrient loads, enabling a simultaneous biomass production and removal of nitrogen, phosphorus, and organic matter [28–33], while supporting circular resource flows and reducing reliance on mineral media. However, optimisation remains challenging because the conditions that maximise nutrient removal do not always support optimal algal growth. Several studies have shown that biomass productivity in digestate-based cultures can be significantly enhanced through CO_2 supplementation [33], indicating that inorganic carbon availability may influence growth performance in such systems. Under these conditions, carbonic anhydrase may support algal growth by improving the utilisation of inorganic carbon already present in the medium.

Digestate-based cultivation could be improved by carbonic anhydrase due to the distinct inorganic carbon speciation and buffering characteristics of digestate compared to mineral media. Anaerobic digestates typically exhibit an elevated alkalinity and high concentrations of bicarbonate (HCO_3^-), resulting from the degradation of organic matter and ammonium accumulation, and are commonly maintained at circumneutral to alkaline pH. Under such conditions, the dominant inorganic carbon form is bicarbonate rather than dissolved CO_2, which can limit the direct CO_2 availability for microalgal carbon fixation. Carbonic anhydrase can enhance the interconversion between bicarbonate and CO_2, thereby increasing the bioavailable CO_2 fraction at the cell surface and supporting photosynthetic carbon assimilation [34].

The aim of this study was therefore to determine whether crude algal extracts containing carbonic anhydrase can stimulate microalgal growth in laboratory cultures, and to evaluate their potential as a low-cost tool for enhancing CO_2 utilisation in waste-derived cultivation systems.

2. Materials and Methods

2.1. Preparation of Crude Algal Extract Containing Carbonic Anhydrase (aCA)

A crude algal extract containing carbonic anhydrase (aCA) was prepared from fresh, frozen, or dry biomass, originally harvested from open raceway ponds (Ljubljana, Slovenia). Cultures were concentrated using a sedimentor and a vibro-filter system (VibroLab-3500, Sani Membranes, Farum, Denmark).

Several cell disruption methods were preliminary compared: high-pressure homogenisation (600 bar, 3 cycles of 10 min), high-speed homogenisation (75% power; 2 × 1 min with a 1 min pause), ultrasonication (Labsonic 2000, B. Braun, Melsungen, Germany; 4 min at max power, 50 W), freezing (−20 °C; 24 h), drying (35–37 °C; 24 h), and manual grinding (2 min). Different cell disruption methods may influence enzyme accessibility and the composition of crude extracts. Mechanical, thermal, and solvent-based treatments can differentially affect cell wall integrity, protein release, and the co-extraction of pigments, metabolites, or cell debris, potentially altering measured enzyme activity and biological effects. In preliminary exploratory tests, no obvious differences in the growth response of *C. sorokiniana* were observed between extracts prepared using different disruption approaches. In the present study, drying followed by manual grinding was therefore selected as a pragmatic compromise between extraction efficiency, operational simplicity, and suitability for on-site application. Consequently, the resulting extracts are not intended to represent maximal CA recovery, but rather a reproducible and scalable preparation reflecting conditions relevant for applied algal cultivation systems.

For aCA preparation, one gram of dry algae biomass (dried 24 h at 35–37 °C) was mechanically disrupted using a manual ceramic grinder prior to extraction in 50 mL of solvent. The solvent used depended on the experiment and included Tris buffer, DES, or deionized water. After 1 h of extraction at room temperature, in daylight and without stirring, samples were centrifuged at 11,000× *g* for 20 min, and the supernatant was used as the aCA solution for growth experiments. In one experiment, the disrupted algae suspension was used without centrifugation to evaluate the contribution of cell debris.

The deep eutectic solvent (DES) was prepared from urea and polyethylene glycol (PEG) in a 1:2 molar ratio, following Craveiro et al. [22]. An 80% (*w*/*w*) DES solution was used to lower the viscosity [22].

Commercial CA (cCA) from bovine erythrocytes (C3934, Sigma-Aldrich, Merck, Darmstadt, Germany) was prepared in Tris-SO_4^{2-} at concentrations of 1, 0.1, and 0.01 g L^{-1} and was used for the positive control.

2.2. Carbonic Anhydrase Activity Assay

Carbonic anhydrase activity was quantified using the p-nitrophenyl acetate (pNPA) esterase activity assay (EAA) following Ores et al. [23]. This assay exploits the fact that the same active site responsible for CO_2 hydration also catalyses pNPA hydrolysis.

A stock solution of pNPA (Sigma-Aldrich) was prepared in methanol according to the manufacturer's instructions (63 mg in 10 mL), stored at 8 °C for no longer than a week, and diluted 1:100 immediately before use.

For activity measurement, 0.2 mL of enzyme solution was added to a cuvette containing 1.8 mL of 50 mM Tris-SO_4 buffer (pH 7.4). After the addition of 1 mL of pNPA working solution, the absorbance at 405 nm was recorded for 210 s. The slope of absorbance increase

($\Delta A/\Delta t$) was used to calculate enzymatic activity according to Ores et al. [23], with a molar extinction coefficient of ε_{405} = 18,000 M^{-1} cm^{-1} for p-nitrophenol. One enzyme unit (U) corresponded to the release of 1 µmol p-nitrophenol per minute under assay conditions. Specific activity was expressed as U g^{-1} of extract.

Preliminary activity measurements were also conducted using the Wilbur–Anderson (WA) assay [35,36] for comparative screening.

2.3. Microalgae Strains and Cultivation Conditions

2.3.1. *Chlorella sorokiniana* Laboratory Cultures

Chlorella sorokiniana (CCAP 211/8K; adapted to anaerobic digestate) (Figure 1) was cultivated under controlled laboratory conditions: 60–70 µmol m^{-2} s^{-1} PAR (fluorescent white light), with 14 h light/10 h dark photoperiod, and temperature maintained at 24–26 °C. The strain has been continuously maintained at Algen since 2015 in a diluted liquid fertiliser (Plantfert U, Marcoser, Galati, Romania), referred to here as the mineral medium (MIN). The composition of MIN (pH 7.2) was: 333 mg L^{-1} total N, 333 mg L^{-1} P_2O_5, 300 mg L^{-1} K_2O, 1 mg L^{-1} B, 0.03 mg L^{-1} Co, 1 mg L^{-1} Cu, 2 mg L^{-1} Fe, 1 mg L^{-1} Mg, 1 mg L^{-1} Mn, 0.03 mg L^{-1} Mo, 3 mg L^{-1} S, and 1 mg L^{-1} Zn.

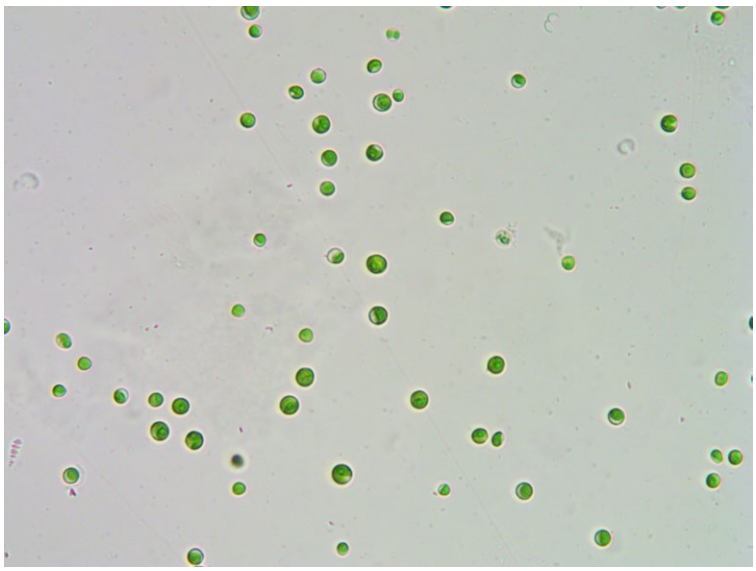
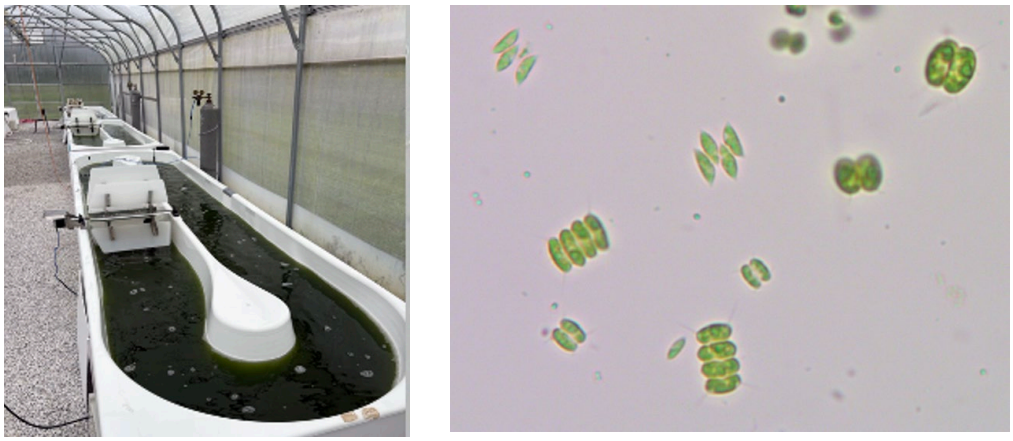


Figure 1. *Chlorella sorokiniana* (CCAP 211/8K) under a light microscope (T690C-PL-5M, AmScope, Irvine, CA, USA; 400× magnification).

2.3.2. Pond Culture Used for aCA Extraction

Algae biomass used for preparing aCA originated from a 7.5 m^2 open raceway pond located in a greenhouse in Ljubljana, Slovenia. The pond has been operated with anaerobic digestate from a food waste biogas plant (KOTO, Ljubljana, Slovenia) as the nutrient source since 2015 [32]. The culture was maintained in semi-continuous mode: 25% of the culture was harvested twice a week into sedimentors, where the algae sedimented overnight. Sedimented algae were dried as described above. Harvested culture was replaced with fresh water and digestate. Culture was maintained at an OD_{680} around 1. The average biomass yield in summer, when the algae were harvested and dried, was 11.1–13.1 g m^{-2} day^{-1} [32]. The culture was dominated by *Scenedesmus dimorphus* (Turpin) Kützing 1834 and *S. quadricauda* (Turpin) Brébisson 1835 (Figure 2), both naturally inoculated in 2015 and present in the culture ever since [32].



(**a**) (**b**)

Figure 2. (**a**) Algae ponds fed with anaerobic digestate; (**b**) pond culture with predominant species *Scenedesmus dimorphus* and *S. quadricauda* (under light microscope AmScope T690C-PL-5M, 400× magnification).

2.4. Growth Experiments

To evaluate the effect of aCA on algal growth, *C. sorokiniana* was cultivated in 250 mL Erlenmeyer flasks filled with 100 mL of medium. Cultures were inoculated at the initial OD_{680} of 0.3 ± 0.1 (in some tests 0.5 ± 0.1).

Two media were used:

- MIN: mineral medium;
- DIG: diluted anaerobic digestate medium.

Digestate medium contained 5% digestate (pH 7.1) in batch tests and 30% digestate (pH 7.5) in semi-continuous tests.

Each medium included three treatments:

(1) aCA addition: 5% (*v*/*v*) of crude extract,
(2) Positive control: cCA with matching activity (based on EAA measurements' value U),
(3) Negative control: no CA added.

Optical density at 680 nm was measured 2–3 times per week (UV-6100S UV/VIS Spectrophotometer) as an indicator of algal biomass concentration.

The specific growth rate (SGR) during the exponential phase was calculated as follows:

$$\mu = (ln(X_2) - ln(X_1))/(t_2 - t_1)$$

where X_1 and X_2 are the OD_{680} values at times t_1 and t_2.

2.5. Digestate Composition

The liquid digestate used in this study was sourced from a pilot-scale anaerobic digestion plant at the Lavrion Technological and Cultural Park (LTCP), Attica, Greece. The facility is part of the LIFE CIRCforBIO integrated biorefinery operated by the National Technical University of Athens (NTUA), processing diverse biowastes, including source-separated food waste, brewery spent grains, spent coffee grounds, potato residues, and citrus by-products.

Digestate was stored under controlled conditions to minimise compositional changes. Its main characteristics are summarised in Table 1.

Table 1. Characteristics of liquid digestate used as algae cultivation media.

Parameter	Value
pH	8.18 ± 0.32
Alkalinity (mg $CaCO_3$ L^{-1})	3450 ± 353
Conductivity (mS cm^{-1})	3.71 ± 0.83
NH_4-N (mg L^{-1})	294.28 ± 4.28
TN (mg L^{-1})	470.63 ± 79.43
PO_4-P (mg L^{-1})	30.08 ± 10.96
TP (mg L^{-1})	38.01 ± 5.22
TC (mg L^{-1})	691.15 ± 95.25
IC (mg L^{-1})	567.73 ± 84.70
TOC (mg L^{-1})	157.13 ± 8.52
COD (mg L^{-1})	438.67 ± 31.72
K (mg L^{-1})	717.42 ± 287.48
Na (mg L^{-1})	87.07 ± 10.84
Ca (mg L^{-1})	43.78 ± 23.25
Mg (mg L^{-1})	28.47 ± 7.95
Fe (mg L^{-1})	n.d.
Cr (mg L^{-1})	n.d.
Cu (mg L^{-1})	n.d.
Mn (mg L^{-1})	n.d.
Ni (mg L^{-1})	n.d.
Cd (mg L^{-1})	n.d.
Pb (mg L^{-1})	n.d.

n.d.—not detected.

2.6. Statistical Analysis

Statistical analysis was performed in R (version 4.4.2; Posit Software, PBC, Boston, MA, USA). Microalgal growth was analysed using repeated optical density measurements at 680 nm (OD_{680}) collected over time for each treatment and experiment. For each experiment, growth trajectories were analysed using linear mixed-effects models, with time (Day) included as a continuous fixed effect and treatment-related factors (medium, CA treatment, and DES presence, where applicable) included as categorical predictors. Random intercepts were included to account for repeated measurements within individual cultures. Where experimental designs differed among experiments, model structures were adjusted accordingly to include only estimable terms. Model assumptions were evaluated using residual diagnostics. Post hoc comparisons focused on differences in growth rates between treatments, assessed as differences in OD_{680} slopes over time with adjustment for multiple testing.

3. Results

3.1. Carbonic Anhydrase Activity of Crude Algal Extracts

The crude algal extracts (aCA) displayed a measurable carbonic anhydrase activity, and the activity varied depending on the biomass processing method and extraction solvent. DES-based extracts were challenging to quantify using the esterase activity assay (EAA) due to their high viscosity, which interfered with spectrophotometric measurements. Similarly, the Wilbur–Anderson (WA) assay was difficult to apply to DES extracts due to excessive bubbling during CO_2 injection. Therefore, WA results were used only for preliminary screening.

Preliminary WA tests indicated that DES extracts prepared from dried, ground algae exhibited the highest apparent CA activity, followed closely by extracts from fresh or frozen biomass. Extracts from high-pressure-homogenised algae showed slightly a lower activity,

although the differences were small. The observed WA activity range was comparable to that of 0.01 g L^{-1} commercial CA (cCA).

Overall, the extracts prepared from fresh biomass exhibited the highest EAA-measured activity, followed by frozen and dried biomass (Tables 2 and 3). For frozen and fresh biomass, the aCA activity corresponded to approximately 0.1–1 g L^{-1} of cCA activity. The extracts prepared from dried biomass showed an activity similar to 0.1 g L^{-1} cCA. Dry algal extracts in DES could not be quantified reliably due to the intense yellow-green coloration that overlapped with the 405 nm detection wavelength.

Table 2. Carbonic anhydrase activity (U) of crude algal extracts and commercial CA measured using the p-nitrophenyl acetate esterase activity assay (EAA).

Extracts and Controls	Supernatant (U)	Suspension (U)
Frozen algae aCA—water	0.0017 ± 0.0010	0.0014 ± 0.0002
Dry algae aCA—water	0.0006 ± 0.00003	0.0003
Frozen algae aCA—DES	0.0015 ± 0.0003	0.0028
Dry algae aCA—DES	n.d.	n.d.
Fresh algae aCA—Tris	0.0024	/
cCA 1 g/L	0.0058 ± 0.0021	/
cCA 0.1 g/L	0.0011 ± 0.0003	/
cCA 0.01 g/L	$0.0002 \pm 4.2 \times 10^{-5}$	/
dH_2O	$0.0001 \pm 1.5 \times 10^{-5}$	/

Note: SD is reported only for samples with replicate measurements; single measurements are shown without SD. n.d.—not detected; "/"—no measurement.

Table 3. Specific carbonic anhydrase activity (U g^{-1}) of crude algal extracts prepared under different extraction conditions.

	Supernatant (U g^{-1})	Suspension (U g^{-1})
Frozen algae aCA—water	0.28 ± 0.16	0.23 ± 0.03
Dry algae aCA—water	0.15 ± 0.01	0.08
Frozen algae aCA—DES	0.24 ± 0.04	0.47
Fresh algae aCA—Tris	0.40	

Note: SD is reported only for samples with replicate measurements; single measurements are shown without SD.

3.2. Effects of Crude Algal Extracts on Chlorella sorokiniana Growth

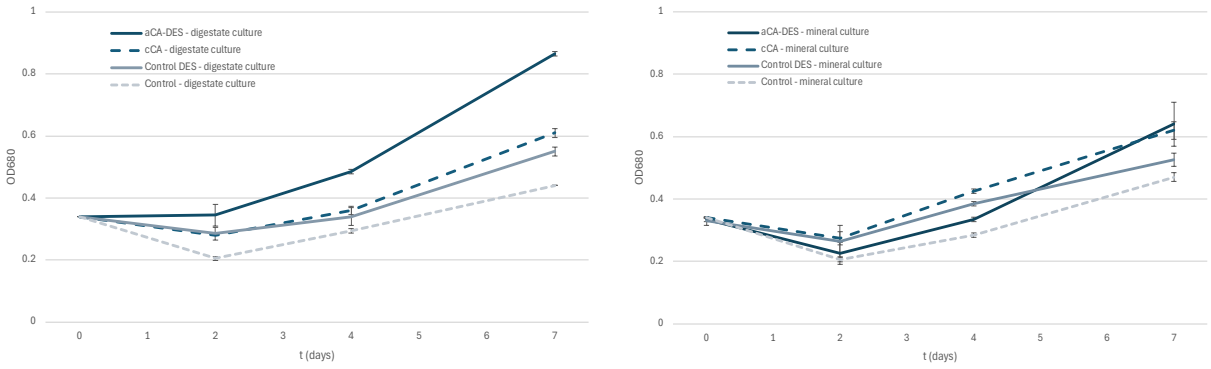
3.2.1. Batch Cultivation with aCA Extracted in DES

The crude extracts prepared in DES (aCA–DES) stimulated the growth of *C. sorokiniana* in both digestate (DIG) and mineral (MIN) media (Figure 3). In digestate, cultures supplemented with aCA–DES separated early from the controls, while cultures treated with commercial CA (cCA) closely tracked the DES-only control. In the mineral medium, aCA–DES also promoted growth relative to the negative control after the first days of cultivation; however, DES alone stimulated early growth, resulting in a less pronounced visual separation among treatments than in digestate.

Consistent with these growth trajectories, the specific growth rates (SGRs) in digestate were highest for aCA–DES cultures (0.080 ± 0.010 d^{-1}), exceeding those of the DES control (0.057 ± 0.004 d^{-1}), the control without DES (0.066 ± 0.003 d^{-1}), and the cCA treatment (0.068 ± 0.002 d^{-1}). In the mineral medium, aCA–DES also showed the highest SGR (0.091 ± 0.023 d^{-1}) compared with the DES control (0.060 ± 0.020 d^{-1}), the control without DES (0.072 ± 0.000 d^{-1}), and the cCA treatment (0.071 ± 0.003 d^{-1}).

Model-based comparisons of growth-rate slopes (OD_{680} vs. Day) supported a treatment effect only under specific conditions. In the digestate medium with DES present, the growth-rate slope of aCA–DES cultures was higher than that of the corresponding control

(t = −3.309, p = 0.002). No other slope contrasts in this experiment were supported after adjustment for multiple testing.



(**a**) (**b**)

Figure 3. Growth of *C. sorokiniana* in (**a**) digestate or (**b**) mineral media with added CA.

3.2.2. Batch Cultivation with aCA Extracted in Water

Because DES alone stimulated algal growth, additional experiments were conducted using water-extracted aCA (aCA–water) to disentangle the effects of the extract from those of the solvent. The visual inspection of growth curves showed that aCA–water cultures increased OD_{680} relative to the control from approximately day 2 onward in both mineral and digestate media (Figure 4). In digestate, growth was slower during the early phase, but aCA–water cultures exceeded the corresponding controls by day 4.

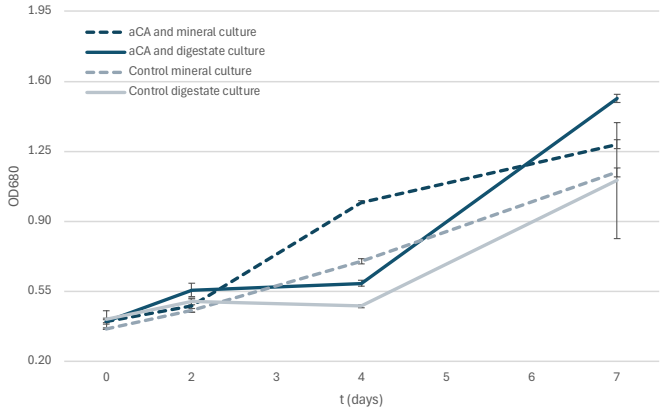
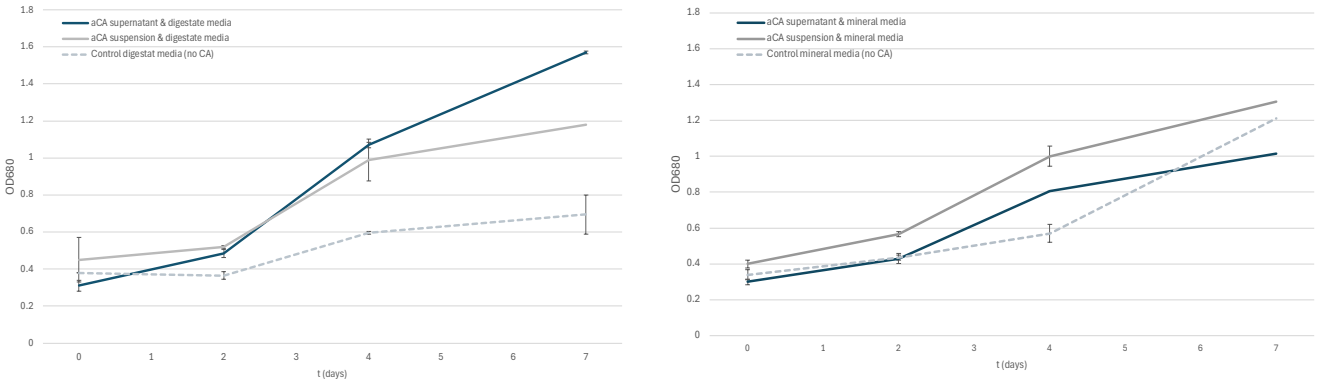


Figure 4. Growth of *C. sorokiniana* in digestate and mineral media supplemented by aCA.

Consistent with these trends, the specific growth rate (SGR) in digestate was higher for aCA–water cultures (0.092 ± 0.002 d^{-1}) than for the control (0.061 ± 0.013 d^{-1}). In the mineral medium, SGRs were similar between treatments (aCA–water: 0.110 ± 0.005 d^{-1}; control: 0.110 ± 0.001 d^{-1}). Model-based comparisons of growth-rate slopes (OD_{680} vs. Day) supported a treatment effect in digestate (Control vs. aCA: t = −2.399, p = 0.025), whereas the corresponding contrast in the mineral medium was not supported after adjustment for multiple testing.

The comparison of the water-extracted aCA supernatant and suspension revealed a medium-dependent growth response (Figure 5). In the digestate medium, the supernatant produced the most consistent growth stimulation and the highest specific growth rate (SGR; 0.102 ± 0.004 d^{-1}), exceeding that of the suspension (0.070 ± 0.031 d^{-1}) and the control (0.056 ± 0.008 d^{-1}). In the mineral medium, SGRs were similar for the supernatant

(0.074 ± 0.010 d^{-1}) and suspension (0.073 ± 0.003 d^{-1}) and were slightly lower than for the control (0.089 ± 0.012 d^{-1}).



(**a**) (**b**)

Figure 5. Comparison of growth of *C. sorokiniana* in (**a**) digestate and (**b**) mineral culture with addition of aCA–water solution before (suspension) and after (supernatant) centrifugation.

Model-based comparisons of growth-rate slopes (OD_{680} vs. Day) detected no supported treatment differences in the mineral medium. In digestate, however, both aCA preparations increased the growth-rate slope relative to the control (aCA–supernatant vs. control: t = 7.392, $p < 0.001$; aCA–suspension vs. control: t = 3.370, $p = 0.005$), and the supernatant also showed a higher growth-rate slope than the suspension (t = 4.022, $p < 0.001$). In the control treatment, growth-rate slopes were higher in the mineral than in the digestate medium (t = 3.890, $p < 0.001$), consistent with the generally faster growth in the mineral medium.

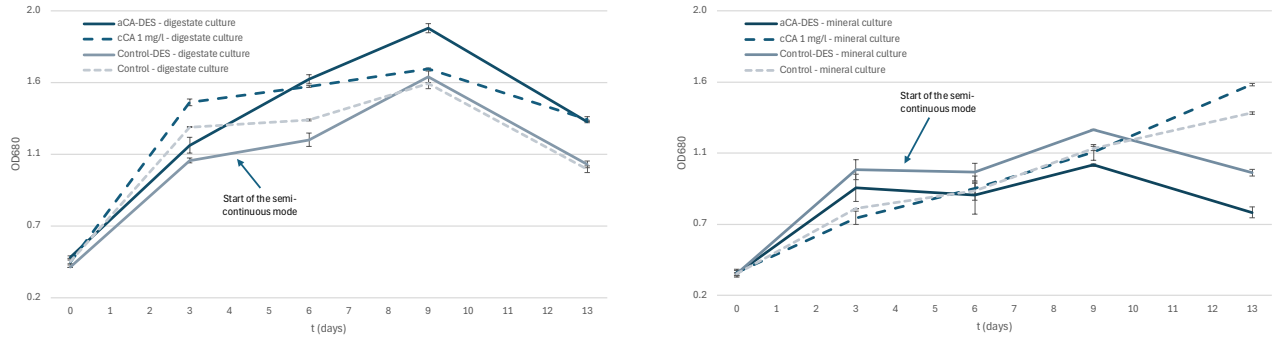
Suspension treatments exhibited slightly higher OD680 values at day 0, reflecting the optical contributions from disrupted biomass.

3.3. Semi-Continuous Growth with aCA Addition

Semi-continuous cultivation was applied to simulate operational conditions relevant for large-scale systems, with cultures diluted twice per week (30% medium replacement) and freshly prepared aCA or cCA re-supplied at each dilution (Figures 6 and 7). In the experiment using DES-extracted aCA (Figure 6), the growth trajectories indicated a higher biomass accumulation in digestate than in the mineral medium; however, the model-based analysis did not detect statistically supported differences in the growth-rate slopes between media. Similarly, no statistically supported effects of enzyme addition (aCA or cCA) on growth-rate slopes were detected. Consistent with these results, Tukey-adjusted post hoc comparisons under DES-present conditions did not reveal statistically supported differences among the treatments within either the mineral or the digestate medium.

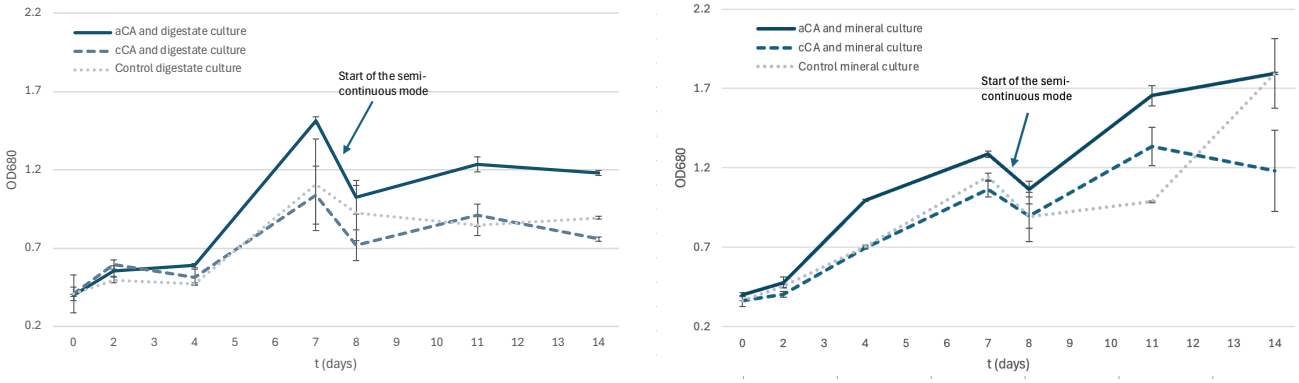
Nevertheless, an inspection of the time series shows that aCA-supplemented cultures in digestate repeatedly reached higher OD_{680} values than the corresponding controls across successive dilution cycles.

In the water-extracted aCA experiment, cultures showed a slow initial growth across treatments; therefore, the semi-continuous regime was initiated after one week of cultivation. Following this transition, OD_{680} increased after each medium replacement (Figure 7). Because the first dilution at day 7 removed 30% of the biomass and resulted in a transient decrease in OD_{680}, growth-rate comparisons were restricted to the post-dilution phase (days 8–13) to focus on the steady semi-continuous segment.



(**a**) (**b**)

Figure 6. Semi-continuous cultivation of *C. sorokiniana* with aCA-DES in (**a**) digestate and (**b**) mineral medium.



(**a**) (**b**)

Figure 7. Semi-continuous cultivation of *C. sorokiniana* with aCA–water in (**a**) digestate and (**b**) mineral medium.

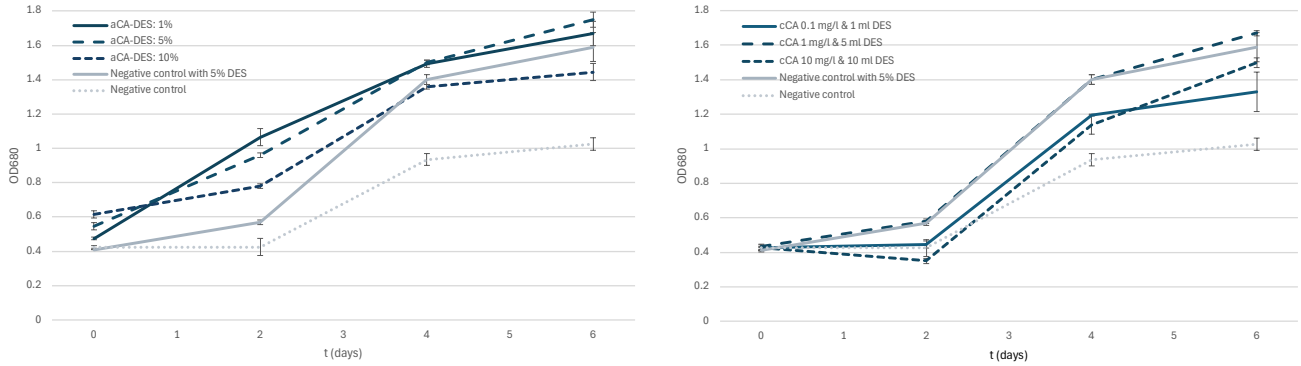
Within this time window, model-based comparisons indicated that, in the mineral medium, the growth-rate slope of cCA-treated cultures was lower than that of the control (control vs. cCA: t = 2.728, $p = 0.032$), whereas no supported difference between aCA and the control was detected. In the digestate medium, no treatment-related differences in growth-rate slopes were supported. Comparisons between media within each treatment showed that growth-rate slopes were higher in the mineral than in the digestate medium for both the control (t = 4.588, $p < 0.001$) and aCA treatments (t = 3.008, $p = 0.006$) during the post-dilution phase.

Although slope-based comparisons did not detect a statistically supported treatment effect for aCA under semi-continuous operation, the time series consistently showed post-dilution recovery, with aCA-supplemented cultures repeatedly returning to higher OD_{680} values than the corresponding controls after medium replacement.

To enable direct comparisons between aCA and cCA under DES-containing conditions, DES was added to cCA treatments in proportional volumes (Figure 8). Growth trajectories and slope-based analyses indicated that the DES concentration influenced early growth responses. Treatments receiving intermediate DES volumes (1–5%) showed higher early OD_{680} values than those receiving higher DES volumes, while both aCA–DES and cCA–DES treatments followed similar patterns corresponding to the amount of DES supplied.

Consistent with these trends, slope-based comparisons showed that the DES-only control (5% DES) had a higher growth-rate slope than the control without DES (t = −4.47, $p = 0.001$). Relative to this control, aCA–DES treatments at 1% and 5% also showed higher

growth-rate slopes (t = −3.58, $p = 0.017$; t = −3.85, $p = 0.008$), whereas the 10% aCA–DES treatment did not show a supported change after adjustment. For cCA, DES-supplemented treatments at 5% and 10% increased the growth-rate slopes relative to the control (t = −4.72, $p < 0.001$; t = −3.52, $p = 0.021$), while the lowest DES level did not show a supported effect.



(**a**) (**b**)

Figure 8. Growth of *C. sorokiniana* in mineral medium with addition of (**a**) aCA-DES or (**b**) cCA-DES.

When treatments were compared directly with the DES-only control, no additional supported changes in growth-rate slopes were detected for either aCA or cCA (all $p \geq 0.12$), indicating that under these conditions the observed differences were primarily associated with the DES fraction rather than with CA addition.

4. Discussion

This study demonstrates that crude carbonic anhydrase extracted directly from microalgae can enhance biomass productivity in algae cultures. Previous studies have primarily focused on purified mammalian carbonic anhydrase or crude extracts applied in standard mineral media [9,10,12,22]. Here, we show that minimally processed extracts obtained from *Scenedesmus*-dominated pond biomass can stimulate growth also in an industrially relevant waste-derived substrate, highlighting a practical and low-cost strategy for improving carbon utilisation in circular bioeconomy systems.

The crude algal extracts (aCA) prepared in this study exhibited a measurable carbonic anhydrase activity, although the values varied depending on the biomass processing method and extraction solvent. Extracts obtained from fresh and frozen biomass showed a higher EAA-measured activity than those from dried biomass, although the preliminary WA assay tests showed the opposite. The specific EAA activities measured (0.08–0.47 U g^{-1} DW) were comparable to microalgae-dialysed extracts in DESs (urea:PEG) of *Chlorella vulgaris* (0.70 U g^{-1} DW (±18%)) and *Spirulina* sp. (0.18 U g^{-1} DW (±53%)) in Craveiro et al.'s study [22]. Measuring CA activity in DES-extracted samples proved challenging, largely due to the high viscosity, background extract coloration, and non-enzymatic pNPA hydrolysis. These factors complicate spectrophotometric detection, an issue already identified by Craveiro et al. [22], who showed that DES alone can exhibit esterase-like activity in EAA, and thus measured CA-specific activity also in the phosphate buffer extracts, obtaining higher values than in DES (1.57 U g^{-1} (±7%) for *Chlorella vulgaris*). Ores et al. [23] measured specific activity in the 10 mM Tris-HCl buffer extracts and also obtained higher values (17.0 ± 1.3 U g^{-1} for *C. vulgaris* and 10.9 ± 0.9 U g^{-1} for *Scenedesmus obliquus*). Direct comparison of specific activity values across studies should be interpreted with caution. The reported activities depend strongly on the assay type (e.g., esterase versus Wilbur–Anderson assays), assay conditions (pH, temperature, buffer composition), and how activity is normalised (per unit dry weight, protein, or extract).

In addition, crude extracts may contain pigments, metabolites, or solvents that interfere with spectrophotometric measurements or contribute to background reactions, particularly in DES-based systems. These methodological differences likely account for much of the variability in reported specific activities and do not necessarily reflect intrinsic differences in carbonic anhydrase content or catalytic potential.

The physiological results of aCA addition were substantially clearer than the biochemical measurements. In general, crude algal extracts, whether prepared in water or DES, stimulated the growth of *C. sorokiniana*. The strongest and most consistent effects occurred in digestate-based media, where cultures supplemented with aCA showed SGR increases of 21–82%, depending on the extraction solvent and preparation. In mineral media, the stimulative effect was weaker or absent, indicating that aCA benefits are most pronounced under carbon-limited conditions.

The markedly stronger effect of carbonic anhydrase (CA) observed in digestate-based cultures compared with the mineral medium can be attributed to the chemical characteristics of the digestate. The liquid digestate exhibited a higher pH (8.18 $\pm$ 0.32) and substantial alkalinity (3450 $\pm$ 353 mg $CaCO_3$ L^{-1}), together with elevated inorganic carbon (IC: 567.73 $\pm$ 84.70 mg L^{-1}), creating a large bicarbonate reservoir. At such an elevated pH and alkalinity, inorganic carbon is predominantly present as bicarbonate, while freely dissolved CO_2 represents only a small fraction of the total carbon pool. Under these conditions, carbon acquisition may become kinetically limited by the conversion of bicarbonate to CO_2 rather than by total inorganic carbon availability.

Although microalgae possess carbon-concentrating mechanisms and intracellular carbonic anhydrase that enable bicarbonate uptake and utilisation, these pathways depend on energy-requiring transport and enzymatic conversion steps that occur only after inorganic carbon has entered the cell [34]. Consequently, carbon assimilation can still be constrained by processes outside the cell, particularly when external CO_2 concentrations are low. Under such conditions, additional carbonic anhydrase activity at or near the cell surface can partially relieve these kinetic constraints by accelerating bicarbonate-to-CO_2 conversion in the surrounding medium, thereby maintaining a local CO_2 supply for diffusion into the cell. Such an effect has already been demonstrated in bicarbonate-rich systems and CA-amended cultures [37,38].

In addition, the digestate also showed a high conductivity (3.71 $\pm$ 0.83 mS/cm) and considerable total and organic carbon (TC: 691.15 $\pm$ 95.25 mg L^{-1}; TOC: 157.13 $\pm$ 8.52 mg L^{-1}), reflecting a complex matrix in which ongoing microbial mineralisation continually produces CO_2 and bicarbonate. CA may help recapture part of this internally generated carbon that would otherwise degas from the culture. Furthermore, the nutrient-rich composition of digestate (e.g., NH_4-N: 294.28 $\pm$ 4.28 mg L^{-1}; PO_4-P: 30.08 $\pm$ 10.96 mg L^{-1}) supports rapid algal growth and may promote the upregulation of carbon-concentrating mechanisms, particularly under conditions where CO_2 becomes limiting during cultivation [33]. The importance of digestate composition and tailoring for optimal algal performance has also been highlighted in controlled digestate-based cultures [31].

The growth-promoting effect of crude algal extract is consistent with previous studies that demonstrated an enhanced biomass productivity in cultures supplemented with commercial CA or crude enzyme extracts. For instance, the addition of bovine CA increased the productivity of *Nannochloropsis salina* by 63–76% [10], and Bloch et al. [9] reported a 50% biomass increase in *Dunaliella salina* raceway ponds supplemented with crude algal lysates. In Brettfeld et al. [39], CO_2-loaded DESs stimulated microalgal growth, increasing the optical density by 12–113% and biomass by 23–53% depending on the DES formulation and cultivation time.

Several experiments demonstrated that DES alone stimulated microalgal growth, indicating that the solvent is not an inert carrier. Accordingly, the growth responses observed in aCA–DES treatments reflect the combined influence of solvent-related effects and enzyme activity. In this context, DES is best interpreted as providing a baseline growth stimulation that is independent of carbonic anhydrase, while any additional enhancement attributable to aCA becomes apparent only under specific cultivation conditions, particularly in digestate-based media. This separation avoids the over-attribution of growth effects to enzyme activity and is consistent with the experimental observations across media and cultivation modes. Previous studies have shown that PEG-based DESs can influence CO_2 solubility and inorganic carbon availability in aqueous systems [22], which may contribute to this baseline stimulatory effect.

While CA activity is a plausible driver of growth stimulation, the EAA cannot definitively attribute the observed effects solely to CA. The crude extracts likely contain additional components, peptides, pigments, osmolytes, amino acids, or micronutrients, that may influence growth. Li et al. [12] provided strong indirect evidence that CA is present in crude extracts from *C. vulgaris*, based on CO_2-hydration kinetics, inhibition by acetazolamide, metal-ion sensitivity, pH activity profiles, and the presence of a ~29 kDa protein band consistent with CA II. Our comparison of supernatant versus suspension extracts suggests that water-insoluble cell debris does not substantially change the stimulatory effect on growth, although DES suspensions showed a higher measured activity.

Semi-continuous cultivation, which better mimics the operational conditions of large-scale algal systems, demonstrated that repeated aCA addition can sustain elevated biomass productivity in digestate-based cultures. This observation is particularly relevant, as digestate-fed systems often face a trade-off between wastewater treatment efficiency and biomass productivity. Improving inorganic carbon utilisation using crude extracts presents a low-cost, scalable strategy to mitigate carbon limitation without increasing CO_2 injection.

However, these findings, while robust under controlled laboratory conditions, may underestimate the complexity of carbon dynamics, pH fluctuations, microbial interactions, and gas-transfer limitations in full-scale raceway ponds; therefore, pilot-scale testing will be essential to validate the operational relevance of aCA addition in real cultivation systems.

Overall, the results support the hypothesis that crude algal extracts containing CA can improve algal growth, particularly in waste-derived media such as anaerobic digestate. This approach offers a potentially economical and environmentally friendly alternative to CO_2 injection or purified enzyme supplementation, with strong applicability for circular bioeconomy systems aiming to recover nutrients and carbon from waste streams.

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Abbreviations

The following abbreviations are used in this manuscript:

CA	Carbonic Anhydrase
DES	Deep Eutectic Solvent
DIG	Digestate-Based Media
MIN	Mineral-Based Media
SGR	Specific Growth Rate
WA	Wilbur–Anderson Assay
EAA	Esterase Activity Assay

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