

## Article

# The Effect of Pretreatment of *Tetraselmis subcordiformis* (Wille) Butcher and *Limnospira platensis* (Gomont) Ciferri et Tiboni Biomass with Solidified Carbon Dioxide on the Efficiency of Anaerobic Digestion

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## Abstract

The aim of this study was to determine the effects of low-temperature pretreatment of microalgae (*Tetraselmis subcordiformis* (Wille) Butcher) and cyanobacteria (*Limnospira platensis* (Gomont) Ciferri et Tiboni) using solidified carbon dioxide (SCO<sub>2</sub>) on the progression of methane fermentation. The experiment was carried out under batch conditions with six process variants that differed in the volumetric ratio of SCO<sub>2</sub> to the biomass tested. Changes in organic matter solubility, anaerobic digestion kinetics and overall CH<sub>4</sub> production performance were analysed. The results showed that pretreatment effectively increased the solubility of organic compounds, especially in the case of *L. platensis* biomass, where the highest increases in soluble sTOC (up to 21.6%) and sCOD (up to 14.3%) were observed. CH<sub>4</sub> yield in the most efficient variant (SCO<sub>2</sub>:biomass = 1:2.5) increased to 354 ± 16 mL CH<sub>4</sub>/gVS for *T. subcordiformis* and 403 ± 18 mL CH<sub>4</sub>/gVS for *L. platensis*, respectively. Despite the apparently less favourable physicochemical parameters of the biomass for anaerobic digestion, *L. platensis* showed a higher susceptibility to digestion and better kinetic indicators for methane fermentation. The results indicate that the efficiency of anaerobic biodegradation of biomass depends not only on the chemical composition but also on the cellular structure and physicochemical interactions during pretreatment. The use of SCO<sub>2</sub> as a disintegrant could be an effective, energy-saving method to increase the fermentation efficiency of photosynthetic microorganisms in biowaste management.

**Keywords:** *Tetraselmis subcordiformis*; *Limnospira platensis*; pretreatment; solidified CO<sub>2</sub>; microalgae; cyanobacteria; anaerobic digestion; biodegradation; energy from biomass



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## 1. Introduction

Against the backdrop of global energy and environmental challenges, microalgae and cyanobacteria are increasingly recognized as promising biomass sources for bioenergy production, particularly in anaerobic digestion and biogas generation processes [1]. Their rapid growth rates, ability to assimilate nutrients from diverse environments, and high content of organic compounds—including proteins, lipids, and carbohydrates—make them a competitive and sustainable alternative to conventional agricultural feedstocks [2]. Of particular importance is their capacity to be cultivated on marginal lands or in saline

waters without competing for arable soil or freshwater resources, aligning well with the principles of sustainable development and the circular economy [3].

Among the wide range of photosynthetic microorganisms, *Tetraselmis subcordiformis* (green microalga) and *Limnospira platensis* (cyanobacterium, commonly known as *Spirulina* sp.) exhibit particularly high biotechnological potential [4]. *T. subcordiformis* is characterized by its high lipid content and easily hydrolysable carbohydrates, whereas *L. platensis* possesses a rich protein profile and relatively flexible cellular structure [5]. Despite their favorable biochemical composition, the efficiency of methane fermentation of their biomass is often constrained by structural and physicochemical factors, including the recalcitrance of cell wall components and suboptimal C/N ratios, both of which can affect process stability and methane yield [6]. Consequently, an increasing number of studies have focused on pretreatment technologies designed to enhance substrate accessibility for anaerobic microorganisms by loosening or disrupting the cell structure [7].

In the case of microalgae and cyanobacteria, a variety of disintegration approaches have been investigated, ranging from mechanical [8] and thermal [9] to chemical [10] and enzymatic [11] methods. Although many of these techniques are effective in enhancing biodegradability, they are frequently associated with high energy demand or costly reagents, which limit their industrial feasibility and overall cost-effectiveness [12]. Considering these limitations, the use of solidified carbon dioxide (SCO<sub>2</sub>) for biomass disintegration—based on the cyclic freezing and thawing of the material—has recently attracted attention [13,14]. This process, originally developed for sewage sludge treatment, causes mechanical rupture of cell walls through ice crystal formation and rapid intracellular volume changes [15]. Previous research has demonstrated that this method can significantly increase the solubility of organic matter, accelerate biodegradation kinetics, and enhance methane production during anaerobic digestion of sewage sludge [16].

Despite numerous reports on the impact of disintegration methods on intensifying anaerobic digestion of microalgae and cyanobacterial biomass, there remains a lack of clear comparative data on technologically simple methods characterised by low energy input [17]. This is especially true for methods that effectively disrupt the cell structures of these organisms without degrading their high-energy organic components. The research gap mainly concerns the use of cryomechanical methods, such as treating biomass with solidified carbon dioxide (SCO<sub>2</sub>), which may potentially compromise both the effectiveness of cell structure disruption and the energy efficiency of the process. The mechanism of action of SCO<sub>2</sub> may be particularly effective for microalgae and cyanobacterial biomass due to their specific morphological structure, characterised primarily by a multilayered cell wall composed of polysaccharides, glycoproteins, and peptidoglycans, which form a barrier to enzymatic hydrolysis [18]. The rapid freezing and thawing cycle induces micro-cracks and increases permeability in these highly hydrated structures, allowing the release of intracellular compounds into the liquid phase [19]. In species such as *T. subcordiformis*, with a clearly defined and relatively thick cellulose-like layer, and *L. platensis*, whose cells are surrounded by a mucin sheath rich in proteins and polysaccharides, the cryomechanical action of SCO<sub>2</sub> can lead to controlled loosening of these structures without the need for aggressive chemical reagents. Importantly, the SCO<sub>2</sub> method has previously been successfully used in the disintegration of sewage sludge, where it has been shown to increase the solubility of organic matter, improve biodegradability, and enhance biogas production efficiency [20].

Considering the physicochemical similarities between the biomass of *T. subcordiformis* and *L. platensis* and sewage sludge with high moisture and organic matter content, the application of SCO<sub>2</sub> disintegration emerges as a promising research direction. However, to date, no studies have reported the use of this technology specifically for methane fer-

mentation of these microalgal and cyanobacterial biomasses. In the present study, the effects of solidified CO<sub>2</sub>-mediated disintegration on the anaerobic digestion of *T. subcordiformis* and *L. platensis* biomass were investigated for the first time. Changes in the soluble organic fraction, methane (CH<sub>4</sub>) production kinetics, and overall fermentation efficiency during batch anaerobic digestion were comprehensively analysed. Based on the results obtained, an energy balance of the process was established, enabling an assessment of the competitiveness and potential economic feasibility of the investigated technology.

## 2. Materials and Methods

### 2.1. Organisation of the Experiment

The experiment was designed and conducted in three sequential phases, divided into two research series according to the type of biomass evaluated. In the first series, the biomass of the green microalga *T. subcordiformis* was analysed, whereas in the second series the biomass of the cyanobacterium *L. platensis* was studied.

During the first phase, a comprehensive physicochemical characterisation of the organic substrates was performed, including the determination of dry matter content, organic matter, total organic carbon (TOC), total nitrogen (TN), total phosphorus (TP), C/N ratio, and the fractional composition of major biochemical constituents (proteins, lipids, and carbohydrates).

In the second phase, a low-temperature grinding method using solidified carbon dioxide (SCO<sub>2</sub>) was applied, and the effectiveness of this pretreatment was evaluated. In each series, the biomass was subjected to six variants (V0–V5), which differed in the volume ratio of SCO<sub>2</sub> to the biomass mass: V0 (control, no SCO<sub>2</sub> addition), V1 (1:10), V2 (1:5), V3 (1:3), V4 (1:2.5), and V5 (1:2). The selection of the SCO<sub>2</sub>/biomass ratio range was based on literature data and previous experimental studies [20,21]. Disintegration efficiency was assessed indirectly by measuring changes in the concentrations of soluble organic compounds, including soluble total organic carbon (sTOC) and soluble chemical oxygen demand (sCOD), in the liquid fraction after treatment.

The third phase consisted of batch methane fermentation of both raw and pretreated biomass in respirometric laboratory reactors. The efficiency and kinetics of biogas production were monitored, along with the qualitative composition of the biogas, with particular emphasis on methane (CH<sub>4</sub>) content. The six SCO<sub>2</sub>/biomass variants established in the second phase were maintained throughout this stage. The organisational workflow of the experimental study is illustrated in Figure 1.

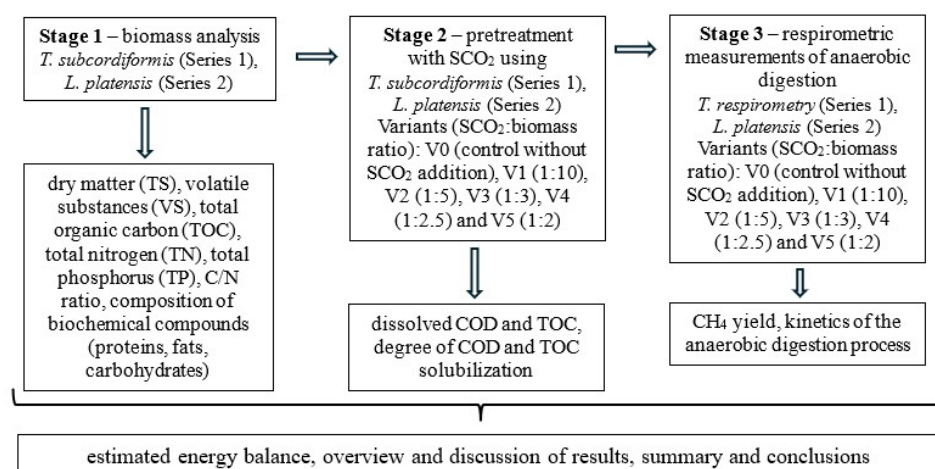


**Figure 1.** Organisation chart of the experimental work carried out.

## 2.2. *Tetraselmis Subcordiformis* and *Limnospira platensis* Biomass

The biomass used in this study was obtained from in-house cultures. The strains of *T. subcordiformis* (CCAP 161/1B) and *L. platensis* (CCAP 1440/1) were sourced from the Culture Collection of Algae and Protozoa (CCAP, Ambleside, UK). Cultivation was carried out in vertical tubular photobioreactors (V-PBR) with a working volume of 20 L under controlled conditions. For *T. subcordiformis*, F/2 medium [22] supplemented with sea salts (salinity approximately 30 PSU) was used, maintaining a temperature of  $22 \pm 1$  °C, pH of  $8.0 \pm 0.2$ , and a light intensity of  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  under a 16:8 light:dark photoperiod. For *L. platensis*, Zarrouk's medium [23] was employed, with cultivation at  $30 \pm 1$  °C, pH  $9.5 \pm 0.3$ , and identical illumination conditions. After reaching the late logarithmic growth phase, the microalgal and cyanobacterial biomass was separated from the culture medium by centrifugation at a flow rate of 35 L/h and a rotation speed of 40,000 rpm using a Z41 flow-through centrifuge (CEPA LEA Lab, Ingersheim, Germany). The resulting biomass concentrates of *T. subcordiformis* (run 1) and *L. platensis* (run 2), with a total solids content of approximately 5% TS, were collected for subsequent analyses, including physicochemical characterisation, low-temperature disintegration using solidified carbon dioxide (SCO<sub>2</sub>), and batch anaerobic digestion experiments. The main characteristics of the *T. subcordiformis* and *L. platensis* biomass used in the study are presented in Table 1.

**Table 1.** Characteristics of the *T. subcordiformis* and *L. platensis* biomass used in the experiments.

| Parameter                  | Unit     | Species-Series                       |                                 |
|----------------------------|----------|--------------------------------------|---------------------------------|
|                            |          | <i>T. subcordiformis</i><br>Series 1 | <i>L. platensis</i><br>Series 2 |
| Total solids (TS)          | [%FM *]  | $5.1 \pm 0.3$                        | $5.2 \pm 0.4$                   |
| Volatile solids (VS)       | [%TS]    | $84.5 \pm 1.5$                       | $89.6 \pm 1.2$                  |
| Mineral solids (MS)        | [%TS]    | $15.5 \pm 1.5$                       | $10.4 \pm 1.2$                  |
| Total carbon (TC)          | [mg/gTS] | $475 \pm 28$                         | $442 \pm 35$                    |
| Total organic carbon (TOC) | [mg/gTS] | $437 \pm 21$                         | $418 \pm 26$                    |
| Total nitrogen (TN)        | [mg/gTS] | $53.6 \pm 2.7$                       | $63.4 \pm 3.1$                  |
| C/N ratio                  | –        | $8.9 \pm 0.3$                        | $7.0 \pm 0.2$                   |
| Total phosphorus (TP)      | [mg/gTS] | $12.6 \pm 1.1$                       | $19.8 \pm 1.4$                  |
| pH                         | –        | $7.85 \pm 0.06$                      | $9.42 \pm 0.08$                 |
| Proteins                   | [%TS]    | $33.5 \pm 1.9$                       | $46.2 \pm 2.5$                  |
| Lipids                     | [%TS]    | $18.4 \pm 0.9$                       | $8.5 \pm 0.7$                   |
| Saccharides                | [%TS]    | $26.1 \pm 1.8$                       | $18.9 \pm 1.5$                  |

\* FM—fresh mass.

## 2.3. Solid Carbon Dioxide—SCO<sub>2</sub>

Commercially available solid carbon dioxide (SCO<sub>2</sub>), supplied by a certified manufacturer (Sopel Sp. z o.o., Białystok, Poland), was used in this study. The material was provided in the form of homogeneous granules with a diameter of  $3.0 \pm 1.0$  mm and was a high-purity product approved for direct contact with food (food grade) [24]. SCO<sub>2</sub> complied with all safety requirements for use in laboratory and technological settings. It is non-toxic, non-flammable, odourless, and tasteless.

Owing to its physicochemical properties, particularly its high heat absorption during sublimation, solid CO<sub>2</sub> has been employed as a low-temperature agent for the disruption of microalgal and cyanobacterial biomass. This characteristic induces rapid temperature fluctuations within the biological matrix, which, in combination with ice crystal formation

inside the cellular structure, can result in substantial damage to cell walls and membranes. SCO<sub>2</sub> was stored and dispensed strictly in accordance with the manufacturer's instructions and applicable safety regulations [25].

#### 2.4. Anaerobic Sludge Inoculum

In the methane fermentation experiments of *T. subcordiformis* biomass (series 1) and *L. platensis* biomass (series 2), anaerobic sludge from a full-scale digester at the municipal wastewater treatment plant in Białystok, Poland, was used as a source of active methanogenic microflora. This digester operates under stable organic loading rates (OLR) of 1.9 to 2.2 gVS/L·d and a hydraulic retention time (HRT) of 20 to 22 days, functioning in mesophilic conditions at temperatures between 35 and 37 °C. The inoculum was collected directly from the operating digester and filtered through a 2 mm mesh sieve to remove coarse solids prior to use. It was then acclimatised under laboratory conditions for 48 h without the addition of fresh substrate to stabilise microbial activity and minimise the effects of endogenous degradation. This prepared inoculum was characterised by an active methanogenic population and stable physicochemical parameters, ensuring reproducibility and reliability of the subsequent experimental results.

The physicochemical properties of the anaerobic sludge used as inoculum reflected the characteristics of an active methanogenic microflora. The total solids (TS) content was  $3.8 \pm 0.2\%$  FM, with organic matter (VS) representing  $59.4 \pm 3.9\%$  TS and the remaining mineral fraction (MS) accounting for  $40.6 \pm 4.1\%$  TS. The total carbon content (TC) reached  $361 \pm 38$  mg/g TS, of which the organic carbon (TOC) comprised  $315 \pm 19$  mg/g TS. Total nitrogen (TN) was  $32.1 \pm 2.8$  mg/g TS, corresponding to a C/N ratio of  $9.8 \pm 0.3$ . Total phosphorus (TP) was  $2.2 \pm 0.3$  mg/g TS, and the pH was slightly alkaline at  $7.19 \pm 0.13$ . Among the main organic fractions in the sludge, proteins predominated at  $21.1 \pm 1.7\%$  TS, while lipids accounted for  $2.1 \pm 0.2\%$  TS, and simple sugars comprised  $2.9 \pm 0.9\%$  TS.

#### 2.5. Test Stations

##### 2.5.1. Biomass Pretreatment with SCO<sub>2</sub>

The pretreatment of microalgal and cyanobacterial biomass was conducted in open laboratory reactors (JLT 6, VELP Scientifica, Milan, Italy) equipped with mechanical stirrers and a temperature control system. Separate experimental treatments were performed for each species, namely *T. subcordiformis* (series 1) and *L. platensis* (series 2).

In each experiment, 200 mL of fresh biomass was mixed with the designated amount of solidified carbon dioxide (SCO<sub>2</sub>) at an initial temperature of  $20 \pm 1$  °C, in accordance with the previously established process variants. Mixing was carried out at 50 rpm in an open reactor system until complete sublimation of the SCO<sub>2</sub> occurred. After sublimation, once the sample temperature had returned to approximately the initial value ( $\sim 20$  °C), the treated suspension was immediately introduced into the anaerobic digestion systems. This procedure was standardised for both species to ensure comparability of the results in subsequent phases of the study.

##### 2.5.2. Respirometric Batch Fermentation Bioreactors

The methanogenic potential of the biomass of the microalgae *T. subcordiformis* (series 1) and the cyanobacteria *L. platensis* (series 2) was evaluated by the amount of methane (CH<sub>4</sub>) produced, which was measured using an automated AMPTS II system (Automatic Methane Potential Test System, BPC Instruments AB, Lund, Sweden). Anaerobic digestion was carried out under controlled temperature conditions of  $38 \pm 1$  °C in glass bioreactors with a total capacity of 1000 mL. Each reactor was equipped with an independently controlled, vertical, bladeless stirrer operating at 100 rpm for 30 s every 5 min to ensure adequate homogenisation of the system.

At the beginning of the experiment, 500 mL of anaerobic sludge inoculum and the corresponding amount of biomass of the test substrate were added to each vessel. The initial organic load (OLR) was set at  $5.0 \pm 0.3$  gVS/L. Anaerobic conditions in the reactors were achieved by flushing the mixture with nitrogen gas at a flow rate of 100 L/h for 3 min. CH<sub>4</sub> production was automatically monitored using the AMPTS II system software, which recorded the CH<sub>4</sub> volume and converted it to standard conditions (101.3 kPa, 0 °C, dry gases). Measurements were continued until five consecutive results differed by less than 1%, indicating completion of organic matter degradation. CH<sub>4</sub> production results were expressed as volume per gramme of organic matter fed into the reactor (mL/gVS). To eliminate the influence of CH<sub>4</sub> from endogenous microbial activity in the inoculum, a correction was made for the CH<sub>4</sub> produced by the anaerobic sludge itself. The carbon dioxide (CO<sub>2</sub>) produced during fermentation was selectively captured in a 100 mL sorption module with 3 M NaOH solution, which guaranteed an absorption efficiency of over 98%. The experimental setup for the investigation of anaerobic fermentation in respirometric reactors is shown in Figure 2.

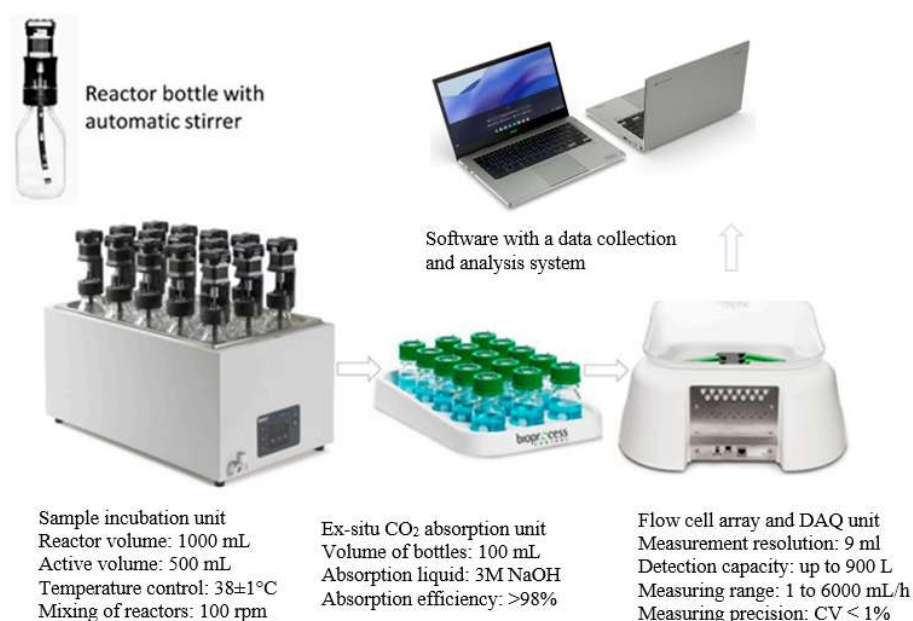


**Figure 2.** Research stand for respirometric measurements of anaerobic digestion.

## 2.6. Analytical Methods

The physicochemical analysis of the soluble fraction of the fermentation medium included the determination of parameters such as chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP). These parameters were determined spectrophotometrically with a Hach DR 6000 photometer (Hach, Düsseldorf, Germany) after sample digestion in a HT200S heating block (Hach, Germany) using High-Speed Digestion (HSD) technology. Total carbon (TC), total organic carbon (TOC), inorganic carbon (IC) and total nitrogen (TN) were measured by catalytic oxidation and chemiluminescence using a TOC-L CPH/CPN analyser with a TNM-L module (Shimadzu, Kyoto, Japan). The protein content was calculated indirectly using the conversion factor  $\text{TN} \times 6.25$  and the content of reducing sugars was determined colorimetrically with anthrone reagent at a wavelength of 600 nm using a DR 2800 spectrophotometer (Hach-Lange GmbH, Düsseldorf, Germany). Lipid extraction was performed using the Soxhlet method with a B-811 lipid extractor (Büchi AG, Flawil, Switzerland). To determine the degree of dissolution of the organic matter and to evaluate its solubilisation efficiency, the samples for the TOC and COD analyses were centrifuged for 3 min at 9000 rpm (ROTINA 380 centrifuge, Hettich, Tuttlingen, Germany).

and then filtered through 1.2- $\mu\text{m}$  membranes. For the determination of TOC, TC and TN in the solid phase (dry weight), the samples were dried at 105 °C and analysed with a Flash 2000 element (Thermo Scientific, Delft, the Netherlands). The pH value was determined using a laboratory pH metre (Model 1000 L, VWR International, Radnor, PA, USA). The total solids (TS), the organic fraction (VS) and the mineral fraction (MS) were determined gravimetrically, taking into account the successive drying and calcination of the samples in a muffle furnace.

## 2.7. Computational and Statistical Methods

The degree of solubilisation was assessed by the change in the proportion of soluble fractions of chemical oxygen demand (sCOD) and total organic carbon (sTOC) after biomass pretreatment at low temperature with solid  $\text{CO}_2$  ( $\text{SCO}_2$ ). Process efficiency was measured by the relative change in sCOD and sTOC content expressed in relation to the total concentration of a given parameter before disintegration. Calculations were performed according to Equations (1) and (2):

$$\text{Degree of solubilization (COD)} = [(s\text{COD}_{S1} - s\text{COD}_{S0}) / (\text{COD}_{T0} - s\text{COD}_{S0})] \cdot 100 \quad (1)$$

$$\text{Degree of solubilization (TOC)} = [(s\text{TOC}_{S1} - s\text{TOC}_{S0}) / (\text{TOC}_{T0} - s\text{TOC}_{S0})] \cdot 100 \quad (2)$$

where

- $s\text{COD}_{S0}$  and  $s\text{TOC}_{S0}$  are the initial concentrations of the soluble fractions of COD and TOC [mg/L], respectively, before pretreatment;
- $s\text{COD}_{S1}$  and  $s\text{TOC}_{S1}$  are the concentrations of these fractions after the application of  $\text{SCO}_2$ ;
- $\text{COD}_{T0}$  and  $\text{TOC}_{T0}$  represent the total content of the analysed indicators in the biomass of microalgae and cyanobacteria before pre-treatment.

This approach allows the quantitative evaluation of the efficiency of cell disruption and the transfer of organic compounds into the soluble fraction, which is an important parameter related to the increase of the bioavailability of substrates in methane fermentation processes.

A modified Gompertz model was used to quantitatively characterise the dynamics of the methane fermentation process and to evaluate the potential and progression of methane production. This model is often used to describe the cumulative gas production in batch systems, especially for microbiological processes with a pronounced lag phase and a logarithmic phase. The mathematical relationship of the model is shown in Equation (3):

$$V(t) = V_{\max} \cdot \exp \left\{ -\exp \left[ \frac{R_{\max} \cdot e}{V_{\max}} \cdot (\lambda - t) + 1 \right] \right\} \quad (3)$$

- $V(t)$ —cumulative  $\text{CH}_4$  quantity at time  $t$ , [mL/gVS];
- $V_{\max}$ —maximum asymptotic  $\text{CH}_4$  yield [mL/gVS];
- $R_{\max}$ —maximum  $\text{CH}_4$  production rate [mL/gVS/d];
- $\lambda$ —duration of the lag phase [d];
- $t$ —fermentation time [d];
- $e$ —Euler's constant ( $\approx 2.71828$ ).

The characteristic sigmoidal shape of the model allows a clear distinction between three phases of the process: the initial adaptation of the fermentation microflora ( $\lambda$ ), the phase of intensive gas production (defined by  $R_{\max}$ ) and the saturation phase ( $V_{\max}$ ). The model parameters were estimated by non-linear regression of the empirical data on cumulative methane production using the least squares method. Curve fitting was performed

in MATLAB R2023a (function `nlinfit`), Python 3.9.0 (library SciPy, function `curve_fit`) or R 4.3.0 (function `nls`), depending on the available software. In order to minimise the effects of experimental disturbances on the accuracy of the estimates, smoothing procedures were applied to the raw data before analysis. The quality of the fit was assessed using the coefficient of determination ( $R^2$ ) and root mean square error (RMSE). The models were fitted independently for the curves of methane production from the biomass of *T. subcordiformis* and *L. platensis*, which allowed a comparative kinetic analysis of the two seasonal biomass variants. The modified Gompertz model was used because of its strong ability to describe the kinetics of biogas production in batch processes, incorporating key kinetic data on CH<sub>4</sub> production. This model provides a good fit to empirical data for microalgae and organic sludge fermentation, as confirmed by numerous studies in the literature [26,27].

The energy consumption ( $E_s$ ) [Wh] was estimated from Equation (4) using the power ( $P$ ), mass ( $M$ ) and efficiency ( $Y$ ) data of the dry ice generator (SCO<sub>2</sub>) adopted for the P3000 Pelletiser model (Cold Jet, Loveland, CO, USA):

$$E_s = (P_{SCO_2} \cdot M_{SCO_2}) / Y_{SCO_2} \quad (4)$$

where

- $P_{SCO_2}$ —appliance power (W);
- $M_{SCO_2}$ —mass of the SCO<sub>2</sub> produced (kg);
- $Y_{SCO_2}$ —efficiency of the appliance (kg/h).

The amount of output energy ( $E_{out}$ ) resulting from methane production was determined according to Equation (5):

$$E_{out} = Y_{CH_4} \cdot CV_{CH_4} \cdot \text{vs. (FM)}_{biomass} \quad (5)$$

where

- $Y_{CH_4}$ —methane yield (L/kgVS, L/kgFM);
- $CV_{CH_4}$ —calorific value of methane (Wh/L);
- $VS_{biomass}$ —VS of the *T. subcordiformis* / *L. platensis* biomass used (kgVS);
- $FM_{biomass}$ —FM of the *T. subcordiformis* / *L. platensis* biomass used (kgFM).

The net energy yield ( $E_{nout}$ ) was calculated as the difference between the energy obtained in the tested variant and the reference energy (variant V1—control) according to Equation (6):

$$E_{nout} = E_{out}(V_x) - E_{out}(V_1) \quad (6)$$

The final net energy balance ( $E_{net}$ ) was calculated according to Equation (7) as the difference between the net energy output and the energy consumption:

$$E_{net} = E_{nout} - E_s \quad (7)$$

All experiments were performed in triplicate, and results are presented as means  $\pm$  standard deviation. This approach ensured the reliability of the data and enabled assessment of the reproducibility of the results in terms of statistical analysis. Statistical analysis of the results obtained was performed using Statistica 13.3 PL software (StatSoft, Inc., Tulsa, OK, USA) [28]. Before carrying out comparisons between the groups, the data distribution was checked for normality using the Shapiro–Wilk test. The homogeneity of variance was tested simultaneously using the Levene test. A one-way analysis of variance (ANOVA) was performed to assess the significance of the differences between the mean values. If statistically significant differences were found ( $p \leq 0.05$ ), post hoc tests were performed

using the Tukey HSD procedure to determine differences between individual group pairs. The level of statistical significance was set at  $\alpha = 0.05$  for all analyses [28].

### 3. Results and Discussion

#### 3.1. Characterisation of the Biomass of *T. subcordiformis* and *L. platensis*

The comparative analysis of basic chemical and physicochemical properties of the microalgae biomass *T. subcordiformis* (series 1) and the cyanobacterium *L. platensis* (series 2) shows significant differences in the composition of both species (Table 1). The total solids (TS) content of both biomasses was comparable, at  $5.1 \pm 0.3\%$  of fresh weight for *T. subcordiformis* and  $5.2 \pm 0.4\%$  for *L. platensis*, indicating a similar level of material dehydration. However, significant differences ( $p \leq 0.05$ ) were observed in volatile solids (VS) content, which amounted to  $84.5 \pm 1.5\%$  of TS for *T. subcordiformis* and  $89.6 \pm 1.2\%$  of TS for *L. platensis*. The higher proportion of the volatile fraction in *L. platensis* biomass may indicate greater fermentation potential, consistent with numerous studies reporting a positive correlation between vs. content and substrate methane yield [29].

Regarding total carbon (TC) and total organic carbon (TOC), *T. subcordiformis* exhibited slightly higher values ( $475 \pm 28$  mg/g TS and  $437 \pm 21$  mg/g TS, respectively) than *L. platensis* ( $442 \pm 35$  mg/g TS and  $418 \pm 26$  mg/g TS, respectively); these differences were not statistically significant ( $p \leq 0.05$ ). In a study [30], lower organic carbon content was reported for *T. subcordiformis* biomass ( $351.8$  mg/g TS), highlighting the considerable influence of culture conditions and strain on this parameter. Literature data for *L. platensis* report TOC values of  $434.3 \pm 12.7$  mg/g TS [31], which align with the results of the present study.

Total nitrogen (TN) content was significantly higher in *L. platensis* biomass ( $63.4 \pm 3.1$  mg/g TS) than in *T. subcordiformis* ( $53.6 \pm 2.7$  mg/g TS), resulting in a significantly lower C/N ratio for the cyanobacterial biomass ( $7.0 \pm 0.2$ ) compared to the microalgal biomass ( $8.9 \pm 0.3$ ) ( $p \leq 0.05$ ). A low C/N ratio may promote intensive growth of anaerobic microorganisms but also carries the risk of excessive ammonia formation and potential inhibition of methanogenesis [32]. The total phosphorus (TP) content was significantly higher in *L. platensis* ( $19.8 \pm 1.4$  mg/g TS) than in *T. subcordiformis* ( $12.6 \pm 1.1$  mg/g TS) ( $p \leq 0.05$ ).

Analysis of the main biopolymer fractions revealed fundamental metabolic differences between the species. *L. platensis* contained significantly more protein ( $46.2 \pm 2.5\%$  TS) than *T. subcordiformis* ( $33.5 \pm 1.9\%$  TS) ( $p \leq 0.05$ ), consistent with its naturally nitrogen-rich profile [33,34]. These findings are corroborated in [35], reporting protein content of  $37.4 \pm 0.5\%$  TS in *L. platensis*. Conversely, *T. subcordiformis* was characterised by significantly higher lipid and carbohydrate contents,  $18.4 \pm 0.9\%$  TS and  $26.1 \pm 1.8\%$  TS, respectively, compared to  $8.5 \pm 0.7\%$  TS and  $18.9 \pm 1.5\%$  TS in *L. platensis* ( $p \leq 0.05$ ). Literature data [36,37] further demonstrate that culture conditions can markedly influence the qualitative composition of biomass, resulting in variability in protein, lipid, and carbohydrate contents across studies.

The presence of lipids can enhance the energy value of the substrate but necessitates careful adjustment of process parameters to prevent accumulation of long-chain fatty acids (LCFA), which may be toxic to methanogenic microorganisms [38]. Differences were also observed in the pH of the fresh biomass: *T. subcordiformis* exhibited an almost neutral pH ( $7.85 \pm 0.06$ ), whereas *L. platensis* was distinctly alkaline ( $9.42 \pm 0.08$ ).

#### 3.2. Indirect Indicators of Pretreatment Efficiency

Pretreatment with solid carbon dioxide (SCO<sub>2</sub>) significantly affected the solubility of organic matter in the tested biomass samples of *T. subcordiformis* (series 1) and *L. platensis*

(series 2). Table 2 presents changes in the concentrations of dissolved TOC and COD, as well as the calculated degree of solubilization.

**Table 2.** Changes in the concentrations of indicators characterizing the dissolved organic matter content of biomass pretreated with  $\text{SCO}_2$ .

| Seria                                           | Variant | $\text{SCO}_2$ :Biomass | COD Dissolved<br>[mg $\text{O}_2$ /L] | TOC Dissolved<br>[mg/L] | Degree of<br>Solubilisation<br>COD [%] | Degree of<br>Solubilisation<br>TOC [%] |
|-------------------------------------------------|---------|-------------------------|---------------------------------------|-------------------------|----------------------------------------|----------------------------------------|
| <i>Tetraselmis<br/>subcordiformis</i> —series 1 | V0      | –                       | $384 \pm 28$                          | $142 \pm 17$            | –                                      | –                                      |
|                                                 | V1      | 1:10                    | $2634 \pm 186$                        | $1040 \pm 83$           | $5.4 \pm 0.4$                          | $2.9 \pm 0.5$                          |
|                                                 | V2      | 1:5                     | $4817 \pm 256$                        | $3273 \pm 179$          | $10.6 \pm 0.6$                         | $13.8 \pm 1.0$                         |
|                                                 | V3      | 1:3                     | $5955 \pm 447$                        | $3996 \pm 241$          | $13.2 \pm 0.9$                         | $16.7 \pm 0.7$                         |
|                                                 | V4      | 1:2.5                   | $5998 \pm 401$                        | $4327 \pm 288$          | $13.4 \pm 0.7$                         | $18.3 \pm 1.4$                         |
|                                                 | V5      | 1:2                     | $6041 \pm 379$                        | $4375 \pm 312$          | $13.5 \pm 0.6$                         | $18.5 \pm 1.2$                         |
| <i>Limnospira platensis</i> —series 2           | V0      | –                       | $392 \pm 34$                          | $153 \pm 19$            | –                                      | –                                      |
|                                                 | V1      | 1:10                    | $2953 \pm 197$                        | $1209 \pm 98$           | $6.0 \pm 0.3$                          | $3.2 \pm 0.6$                          |
|                                                 | V2      | 1:5                     | $5412 \pm 303$                        | $3904 \pm 204$          | $11.7 \pm 0.8$                         | $16.3 \pm 1.2$                         |
|                                                 | V3      | 1:3                     | $6487 \pm 549$                        | $4612 \pm 277$          | $14.2 \pm 0.9$                         | $19.7 \pm 0.8$                         |
|                                                 | V4      | 1:2.5                   | $6504 \pm 492$                        | $4980 \pm 360$          | $14.3 \pm 0.8$                         | $21.5 \pm 1.7$                         |
|                                                 | V5      | 1:2                     | $6511 \pm 438$                        | $5010 \pm 392$          | $14.3 \pm 0.7$                         | $21.6 \pm 1.6$                         |

In the control samples (V0), in which no disintegration treatment was applied, the soluble chemical oxygen demand (sCOD) and soluble total organic carbon (sTOC) values were relatively low, ranging from  $384 \pm 28 \text{ mgO}_2/\text{L}$  to  $142 \pm 17 \text{ mg/L}$  for *T. subcordiformis* and from  $392 \pm 34 \text{ mgO}_2/\text{L}$  to  $153 \pm 19 \text{ mg/L}$  for *L. platensis* (Table 2). The differences between the two series in this variant were not statistically significant ( $p \leq 0.05$ ), confirming a comparable level of natural solubility of organic substances in both biomass types.

A systematic increase in the concentrations of soluble organic matter was observed with increasing  $\text{SCO}_2$  dose, clearly demonstrating the effectiveness of the disintegration treatment. Even at the lowest  $\text{SCO}_2$ :biomass ratio tested (variant V1, 1:10), sCOD and sTOC increased significantly relative to the control samples ( $p \leq 0.05$ ). For *T. subcordiformis*, these values were  $2634 \pm 186 \text{ mgO}_2/\text{L}$  (sCOD) and  $1040 \pm 83 \text{ mg/L}$  (sTOC), while for *L. platensis* they were  $2953 \pm 197 \text{ mgO}_2/\text{L}$  and  $1209 \pm 98 \text{ mg/L}$ , respectively. This corresponded to solubilisation levels of  $5.4 \pm 0.4\%$  for COD and  $2.9 \pm 0.5\%$  for TOC in *T. subcordiformis*, and  $6.0 \pm 0.3\%$  (COD) and  $3.2 \pm 0.6\%$  (TOC) in *L. platensis* (Table 2). Differences between the species were statistically significant for sTOC ( $p \leq 0.05$ ).

In the subsequent variants (V2–V5), in which progressively higher  $\text{SCO}_2$  doses were applied, further increases in organic matter solubilisation were observed. The highest sCOD and sTOC values were measured in V5 (1:2), reaching  $6041 \pm 379 \text{ mgO}_2/\text{L}$  and  $4375 \pm 312 \text{ mg/L}$  for *T. subcordiformis*, and  $6511 \pm 438 \text{ mgO}_2/\text{L}$  and  $5010 \pm 392 \text{ mg/L}$  for *L. platensis*, respectively. This corresponded to solubilisation degrees of  $13.5 \pm 0.6\%$  (COD) and  $18.5 \pm 1.2\%$  (TOC) for *T. subcordiformis*, and  $14.3 \pm 0.7\%$  (COD) and  $21.6 \pm 1.6\%$  (TOC) for *L. platensis* (Table 2). In both species, a plateau in the disintegration effect was observed between variants V3 and V5, with no statistically significant differences in sCOD and sTOC ( $p > 0.05$ ), suggesting that maximum efficiency in cellular structure disruption was achieved under these conditions. Similarly, studies on sewage sludge pretreatment using  $\text{SCO}_2$  reported systematic increases in sCOD with successive dry ice doses, reaching  $480 \text{ mg/L}$  at a 1:2 ratio,  $600 \text{ mg/L}$  at 1:1.3, and  $889 \text{ mg/L}$  at a 1:1 ratio [39].

Across all SCO<sub>2</sub>-treated variants (V1–V5), *L. platensis* exhibited significantly higher sCOD and sTOC values ( $p \leq 0.05$ ), which may be attributed to distinct morphological and biochemical characteristics [40]. Its higher initial pH (9.42 vs. 7.85 in *T. subcordiformis*) and lower lipid content (8.5% TS vs. 18.4% TS) likely facilitated the release of hydrophilic organic compounds during rapid expansion and thawing of the cellular structure [41]. Additionally, the high protein content in *L. platensis* (A. *platensis*) (46.2% TS) may have contributed to the elevated sTOC. Conversely, the substantial lipid content in *T. subcordiformis* may have limited the release of soluble fractions under low-temperature disintegration conditions [42].

These results clearly confirm the effectiveness of SCO<sub>2</sub> pretreatment in enhancing the bioavailability of organic matter in microalgal and cyanobacterial biomass. A SCO<sub>2</sub>:biomass volume ratio exceeding 1:3 achieved maximum solubilisation, with further increases in the SCO<sub>2</sub> dose yielding no significant improvement. *L. platensis* demonstrated higher susceptibility to disintegration, suggesting it is a potentially more effective substrate for methane fermentation following low-temperature cell disruption. Other studies exploring microalgal biomass pretreatment have reported comparable results. For example, a biochemical approach using bacterial activity and nickel nanoparticles for disintegration of *Chlorella vulgaris* increased sCOD from 0.64 gO<sub>2</sub>/L in untreated biomass to 4.8–7.2 gO<sub>2</sub>/L after pretreatment, corresponding to solubilisation degrees of 3.2% to 36% depending on the variant [43]. Hydrothermal depolymerisation at 150 °C for 60 min increased sCOD by 23.2%, nearly tenfold relative to the control [44]. These findings indicate that SCO<sub>2</sub>-based pretreatment represents a promising and competitive alternative, combining low operating costs with relatively simple technological requirements.

### 3.3. Anaerobic Respirometry Measurements

The results concerning the efficiency of CH<sub>4</sub> production and the kinetic parameters of anaerobic digestion of *T. subcordiformis* (series 1) and *L. platensis* (series 2) biomass clearly demonstrate a positive effect of SCO<sub>2</sub> pretreatment on process performance. Both the specific biogas yield, the reaction rate constant (k), and the CH<sub>4</sub> production rate (r) exhibited an increasing trend from the control (V0, untreated biomass) to variant V4, in which the volumetric SCO<sub>2</sub>:biomass ratio was 1:2.5. Further increases in the SCO<sub>2</sub> dose in variant V5 did not produce significant ( $p \leq 0.05$ ) improvements, indicating that a plateau in pretreatment efficiency had been reached. A summary of the parameters characterising the efficiency and progress of the anaerobic digestion process for the tested organic substrates is presented in Table 3.

**Table 3.** Efficiency and kinetic indicators of CH<sub>4</sub> production as a function of the substrate used and the SCO<sub>2</sub> pretreatment variant.

| Parameter       | Unit   | <i>T. subcordiformis</i> Biomass—Series 1 |             |             |             |             |             |
|-----------------|--------|-------------------------------------------|-------------|-------------|-------------|-------------|-------------|
|                 |        | V0                                        | V1          | V2          | V3          | V4          | V5          |
| CH <sub>4</sub> | mL/gVS | 280 ± 11                                  | 298 ± 11    | 321 ± 12    | 337 ± 15    | 354 ± 16    | 377 ± 12    |
| k               | 1/day  | 0.16 ± 0.02                               | 0.14 ± 0.01 | 0.18 ± 0.02 | 0.18 ± 0.02 | 0.18 ± 0.03 | 0.18 ± 0.01 |
| r               | mL/day | 44.8 ± 2.1                                | 41.7 ± 1.9  | 57.8 ± 1.3  | 60.7 ± 1.4  | 63.7 ± 1.4  | 67.9 ± 1.1  |
| Parameter       | Unit   | <i>L. platensis</i> Biomass—Series 2      |             |             |             |             |             |
|                 |        | V0                                        | V1          | V2          | V3          | V4          | V5          |
| CH <sub>4</sub> | mL/gVS | 301 ± 10                                  | 322 ± 14    | 342 ± 14    | 362 ± 16    | 403 ± 18    | 420 ± 12    |
| k               | 1/day  | 0.19 ± 0.02                               | 0.19 ± 0.02 | 0.22 ± 0.03 | 0.22 ± 0.02 | 0.22 ± 0.01 | 0.22 ± 0.01 |
| r               | mL/day | 57.2 ± 1.2                                | 61.2 ± 1.4  | 75.2 ± 0.9  | 79.6 ± 1.0  | 88.7 ± 0.8  | 92.4 ± 0.9  |

When *T. subcordiformis* biomass was used, the total CH<sub>4</sub> production efficiency increased from  $280 \pm 11$  mL CH<sub>4</sub>/gVS (V0) to  $354 \pm 16$  mL CH<sub>4</sub>/gVS (V4), corresponding to a statistically significant ( $p \leq 0.05$ ) increase of approximately 26% (Table 3, Figure 3). A further increase to  $377 \pm 12$  mL CH<sub>4</sub>/gVS was observed in variant V5, although the difference relative to V4 was no longer statistically significant ( $p \leq 0.05$ ). In a study [45], the application of sonication to *Tetraselmis* sp. biomass resulted in a substantial increase in biogas yield from  $195.3 \pm 3.6$  to  $425.3 \pm 5.5$  mL/g VS, with CH<sub>4</sub> production ranging from  $114.4 \pm 2.1$  to  $289.4 \pm 7.4$  mL CH<sub>4</sub>/gVS [45]. Although the absolute CH<sub>4</sub> yields reported in that study were higher than those obtained with SCO<sub>2</sub> pretreatment, the use of dry ice remains a competitive solution when process economics are considered. This is particularly relevant given the potential for recovering SCO<sub>2</sub> during biogas upgrading and biomethane enrichment, which can enhance both the economic and environmental viability of the process and enable closure of the CO<sub>2</sub> cycle in biogas plants [46].

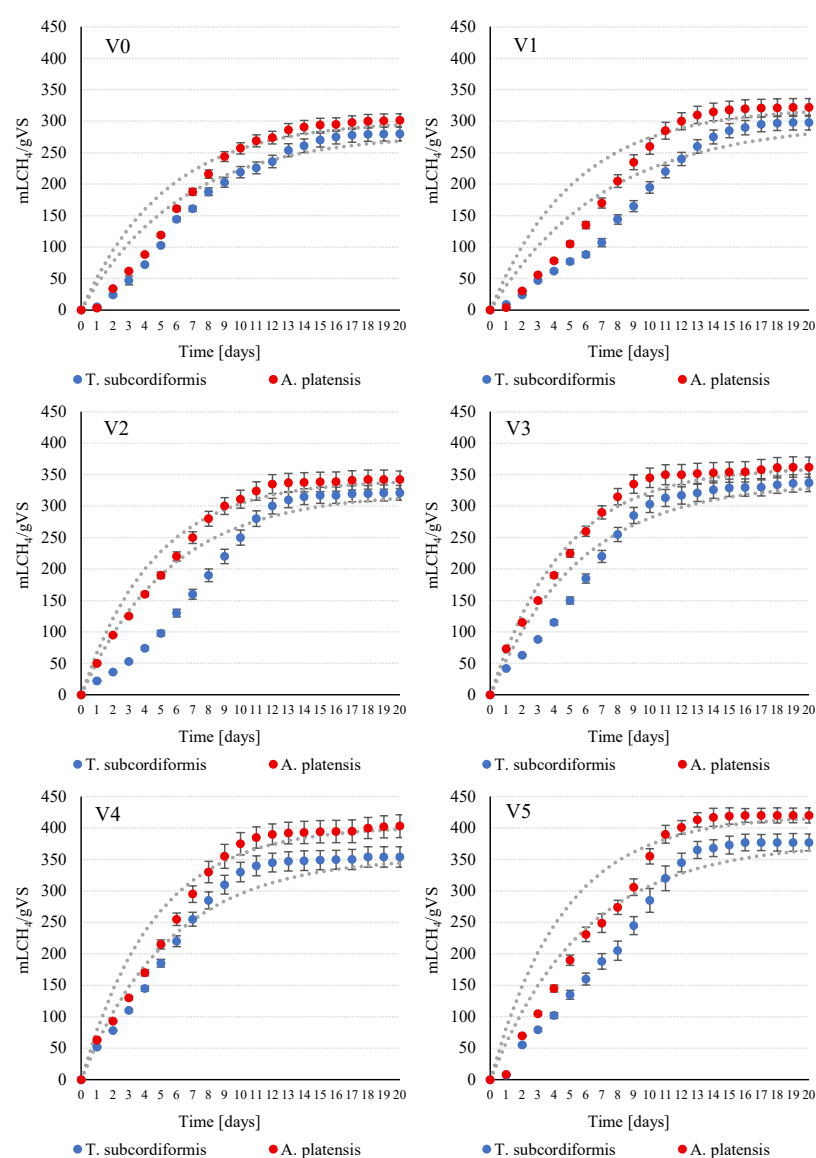


**Figure 3.** The course of CH<sub>4</sub> production in the anaerobic digestion process of the tested substrates in the following experimental variants.

A similar trend was observed for the CH<sub>4</sub> production rate ( $r$ ), which increased from  $44.8 \pm 2.1$  mL CH<sub>4</sub>/d (V0) to  $63.7 \pm 1.4$  mL CH<sub>4</sub>/d (V4) and subsequently to  $67.9 \pm 1.1$  mL CH<sub>4</sub>/d in V5, with no significant difference relative to V4 ( $p \leq 0.05$ ) (Table 3). The reaction

rate constant ( $k$ ) values varied within a narrower range (0.14–0.18 1/d) and did not differ significantly among the variants, suggesting that the observed improvement in  $\text{CH}_4$  production efficiency was primarily associated with intensified methanogenesis rather than acceleration of the hydrolysis or acidogenesis phases.

In the case of *L. platensis*, all analysed parameters were higher than those observed for *T. subcordiformis*, independent of the experimental variant. The initial  $\text{CH}_4$  production efficiency in V0 was  $301 \pm 10$  mL  $\text{CH}_4$ /gVS and systematically increased to  $403 \pm 18$  mL  $\text{CH}_4$ /gVS in V4 (Table 3, Figure 3), representing a 34% increase. In variant V5,  $\text{CH}_4$  production reached  $420 \pm 12$  mL  $\text{CH}_4$ /gVS, although, similar to *T. subcordiformis*, the increase relative to V4 was not statistically significant ( $p \leq 0.05$ ). Previous studies [47] demonstrated that mechanical pretreatment of *A. platensis* (*L. platensis*) biomass can also enhance methanogenesis, increasing  $\text{CH}_4$  production by 47%, from 113 to 166 mL  $\text{CH}_4$ /gVS. The efficiency gain observed with  $\text{SCO}_2$  pretreatment in this study (~40%) is therefore comparable, while offering potential economic advantages. For green algae of the genus *Scenedesmus* sp., a more pronounced  $\text{CH}_4$  yield increase (~80%) from 54 to 97 mL  $\text{CH}_4$ /gVS has been reported [47]. These findings highlight that both the pretreatment method and the species' intrinsic characteristics are critical factors in achieving enhanced  $\text{CH}_4$  production.

The  $\text{CH}_4$  production rate ( $r$ ) increased in series 2 from  $57.2 \pm 1.2$  mL  $\text{CH}_4$ /d (V0) to  $88.7 \pm 0.8$  mL  $\text{CH}_4$ /d (V4), and further to  $92.4 \pm 0.9$  mL  $\text{CH}_4$ /d in V5, with no statistically significant difference relative to V4 ( $p \leq 0.05$ ) (Table 3). The reaction rate constant ( $k$ ) increased systematically from  $0.19 \pm 0.02$  1/d (V0) to  $0.22 \pm 0.01$  1/d (V4–V5), in contrast to *T. subcordiformis*, suggesting that  $\text{SCO}_2$  pretreatment improved substrate availability not only during methanogenesis but also during the hydrolysis and acidogenesis phases.

Comparison between the two biomass types indicates that *L. platensis* achieved significantly ( $p \leq 0.05$ ) higher  $\text{CH}_4$  production and kinetic indices, which can be attributed to its thinner cell wall that is more susceptible to cryogenic damage induced by  $\text{SCO}_2$  [48]. The systematic increase in  $k$  further suggests a faster initial phase of anaerobic digestion for this biomass. From an applied perspective, the plateau in V5 indicates an optimal  $\text{SCO}_2$  dose at V4, above which further increases in pretreatment intensity do not significantly improve fermentation efficiency. This plateau may reflect the limited structural potential of the biomass for additional damage or negative effects such as partial denaturation of cellular compounds or supersaturation of the  $\text{CO}_2$  system, potentially impairing microbial activity [49].

Respirometric studies confirmed higher anaerobic digestion efficiency of *L. platensis*, despite its apparently less favourable physicochemical parameters compared to *T. subcordiformis* (Table 1). This demonstrates that process effectiveness, particularly following  $\text{SCO}_2$  pretreatment, depends not only on the chemical composition but also on cell structure, surface properties, and physicochemical interactions induced during pretreatment [20]. Despite lower lipid and sugar contents in *L. platensis* ( $8.5 \pm 0.7\%$  TS and  $18.9 \pm 1.5\%$  TS, respectively) compared to *T. subcordiformis* ( $18.4 \pm 0.9\%$  TS and  $26.1 \pm 1.8\%$  TS), the cyanobacterial biomass exhibited significantly higher protein content ( $46.2 \pm 2.5\%$  TS vs.  $33.5 \pm 1.9\%$  TS). In previous studies comparing biomethane production between microalgae and cyanobacteria, *A. platensis* (*L. platensis*) displayed higher methanogenic potential than *Scenedesmus* sp., producing 110 mL  $\text{CH}_4$ /gVS compared to 60 mL  $\text{CH}_4$ /gVS [47].

The higher efficiency of *L. platensis* is likely attributable to the combined effects of structural, biochemical, and physicochemical factors: its greater susceptibility to disintegration, higher protein and nutrient content, and alkaline pH ( $9.42 \pm 0.08$  vs.  $7.85 \pm 0.06$  for *T. subcordiformis*) which promotes  $\text{CO}_2$  dissolution and bicarbonate formation, enhancing buffering capacity and facilitating substrate availability [50–52]. The significantly lower ash content ( $10.4 \pm 1.2\%$ ) also reduces dilution of the organic fraction and limits potential ionic interferences that could negatively affect enzymatic activity [53]. As a filamentous

cyanobacterium with a thin, cellulose-free cell wall, *L. platensis* is more susceptible to mechanical disruption by sublimating SCO<sub>2</sub> compared to the more resistant cell wall of *T. subcordiformis* [54,55].

From a biochemical perspective, *L. platensis* also contained higher total nitrogen ( $63.4 \pm 3.1$  mg/g VS) and phosphorus ( $19.8 \pm 1.4$  mg/g VS) contents than *T. subcordiformis*, which may support the growth of methanogenic consortia by supplying essential nutrients [56]. Although excessive concentrations of these elements can be toxic, under controlled anaerobic conditions combined with SCO<sub>2</sub> pretreatment, they likely enhance microbial metabolism. Overall, the superior CH<sub>4</sub> production efficiency of *L. platensis* following SCO<sub>2</sub> pretreatment appears to result from a synergy of structural, biochemical, and physicochemical factors, emphasizing that evaluation of biomass fermentation potential should consider not only compositional parameters but also physical properties, processability, and dynamic changes induced by pretreatment [57].

Biomass pretreatment with SCO<sub>2</sub> significantly alters substrate availability for anaerobic microorganisms, affecting both the kinetics of hydrolysis and the progression of subsequent stages of methane fermentation [58]. The action mechanism of SCO<sub>2</sub> involves the physicochemical disruption of cell walls, resulting in the release of intracellular organic compounds. Simultaneously, CO<sub>2</sub> dissolved in water lowers the local pH, which can modulate the activity of hydrolytic enzymes and selectively inhibit the activity of sensitive methanogen groups [59].

The microbiological consequences of this pretreatment can be multifaceted. Increased monomer availability during the initial phase of hydrolysis accelerates the production rate of volatile fatty acids (VFAs), primarily acetate, propionate, and butyrate [60]. With a moderate SCO<sub>2</sub> dose and a well-adapted methanogenic inoculum, the increased VFA concentration is rapidly converted to CH<sub>4</sub>, resulting in higher fermentation rates and efficiency. However, excessive SCO<sub>2</sub> doses can cause VFA accumulation, especially propionate and butyrate, which indicate potential disruptions in syntrophy between acetogenic bacteria and methanogens [61]. This effect may result from a local decrease in pH and increased free ammonia concentration following protein solubilisation, which consequently limits methanogen activity and alters their community composition, shifting the balance between acetoclastic and hydrogenotrophic methanogens [62].

Furthermore, biomass composition significantly influences the microbial response to SCO<sub>2</sub> pretreatment. Cyanobacteria with high protein content release larger amounts of nitrogen after treatment, increasing the risk of ammonia inhibition, while green algae with a higher proportion of polysaccharides in their cell wall structure exhibit a more moderate increase in sVFA at the same SCO<sub>2</sub> to biomass ratio. Therefore, optimising the SCO<sub>2</sub> dose and biomass/SCO<sub>2</sub> ratio is crucial for maintaining microbial balance and maximising methane yield [63].

The use of SCO<sub>2</sub> as a pretreatment method for microalgal and cyanobacterial biomass can have a dual effect. Appropriately selected doses stimulate hydrolysis and accelerate the conversion of VFA to CH<sub>4</sub>, while excessive doses can destabilise the microbiome and lead to fatty acid accumulation [64]. In designing methane fermentation processes for microalgal and cyanobacterial biomass, SCO<sub>2</sub> pretreatment should be regarded as a tool for modulating substrate availability. Its effectiveness depends on precise dosage adjustment and control of parameters such as pH, free NH<sub>3</sub> concentration, and C/N ratio in the process system. Monitoring VFA profiles, sCOD, and microbiological parameters, including methanogen community composition, is essential for assessing the effectiveness of this approach and its impact on process stability. Table 4 compares the efficiency of anaerobic digestion of microalgal and cyanobacterial biomass depending on the pretreatment method used.

**Table 4.** Efficiency of anaerobic digestion of microalgae and cyanobacteria biomass according to the pretreatment method used.

| Species                                                                                                                               | Method       | Pretreatment (PT) Conditions                                                                                                            | Methane Yield<br>(mL CH <sub>4</sub> /g VS) |               | Reference |
|---------------------------------------------------------------------------------------------------------------------------------------|--------------|-----------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------|---------------|-----------|
|                                                                                                                                       |              |                                                                                                                                         | Without PT                                  | With PT       |           |
| <i>Scenedesmus</i> sp.,<br>4.48 g/L TS                                                                                                | Ultrasound   | 80 W, 30 min, 128.9 MJ/kg TS                                                                                                            | 81.80                                       | 153.5         | [65]      |
|                                                                                                                                       | Thermal      | 70 °C, 25 min<br>80 °C, 25 min                                                                                                          |                                             | 89.3<br>128.7 |           |
| <i>Chlorella sorokiniana</i> , 13.8 g/L<br>COD <sub>T</sub>                                                                           | Ultrasound   | 220 W, 30 min                                                                                                                           | 317.66                                      | 458.43        | [66]      |
|                                                                                                                                       |              | 400 W, 20 min                                                                                                                           |                                             | 414.12        |           |
|                                                                                                                                       |              | 400 W, 30 min                                                                                                                           |                                             | 424.68        |           |
|                                                                                                                                       |              | 400 W, 40 min                                                                                                                           |                                             | 421.87        |           |
|                                                                                                                                       | Thermal      | 80 °C, 20 min                                                                                                                           |                                             | 374.81        |           |
| Mixed biomass ( <i>Nitzschia</i> sp.,<br><i>Stigeoclonium</i> sp., <i>Navicula</i> sp.,<br><i>Monoraphidium</i> sp.),<br>31.49 g/L TS | Ultrasound   | 70 W, 30 min, 20 kHz, 27 MJ/kg TS                                                                                                       | 105.6                                       | 113.7         | [67]      |
|                                                                                                                                       | Microwave    | 900 W, 3 min, 34.3 MJ/kg TS                                                                                                             |                                             | 127.7         |           |
| Microalgae mixed biomass from<br>high-rate ponds,<br>16.7 g/L COD <sub>T</sub>                                                        | Microwave    | 300 W, 9 min, 64,400 kJ/kg TS                                                                                                           | 117.63                                      | 167.24        | [68]      |
|                                                                                                                                       |              | 600 W, 4.5 min, 64,400 kJ/kg TS                                                                                                         |                                             | 188.34        |           |
|                                                                                                                                       |              | 900 W, 3 min, 64,400 kJ/kg TS                                                                                                           |                                             | 210.06        |           |
| <i>Chlorella</i> sp.,<br>27.9 g/L COD                                                                                                 | Thermal      | 65 °C, 4 h                                                                                                                              | 211                                         | 297           | [69]      |
| <i>Scenedesmus obliquus</i> , 20 g/L TS                                                                                               | Hydrothermal | 165 °C, 7 bar, 30 min                                                                                                                   | 159                                         | 383.6         | [70]      |
| <i>Porphyridium cruentum</i> ,<br>3.4 g COD <sub>T</sub> /L                                                                           | Enzymatic    | Protease 0.5 mL/g dry biomass,<br>pH 8.0–8.5, 55 °C, 9 h                                                                                | 130                                         | 230           | [71]      |
| <i>Scenedesmus</i> sp.,<br>60.9 g/L COD <sub>T</sub>                                                                                  | Biological   | TSAD, rumen m-orgs as<br>pretreatment in the 1st stage,<br>fermentation reactor: 40 d,<br>SRT = 7 d, HRT = 7 d.                         | na                                          | 214           | [72]      |
| Mixed culture of bacteria and<br>microalgae, composed mainly by<br><i>Oocystis</i> sp.,<br>31.3 g/L COD <sub>T</sub>                  | Biological   | 100 U/L laccase-rich broth from<br><i>Trametes versicolor</i> , 100 rpm/20 min<br>100 U/L commercial laccase,<br>20 min, 100 rpm, 25 °C | 83                                          | 144<br>100    | [73]      |

Table 4. Cont.

| Species                                           | Method         | Pretreatment (PT) Conditions                                 | Methane Yield<br>(mL CH <sub>4</sub> /g VS) |         | Reference            |
|---------------------------------------------------|----------------|--------------------------------------------------------------|---------------------------------------------|---------|----------------------|
|                                                   |                |                                                              | Without PT                                  | With PT |                      |
| <i>Scenedesmus</i> sp.,<br>6 g/L COD <sub>T</sub> | Thermochemical | H <sub>2</sub> SO <sub>4</sub> 0.1% <i>v/v</i> , 150 °C, 1 h | 130.9                                       | 253.1   | <a href="#">[44]</a> |
| <i>Chlorella</i> sp.                              | Thermochemical | NaOH 0.05%, 50 °C, 24 h                                      | 137.17                                      | 110.00  | <a href="#">[74]</a> |
|                                                   |                | NaOH 2.0%, 50 °C, 24 h                                       |                                             | 125.00  |                      |
|                                                   |                | NaOH 5.0%, 50 °C, 24 h                                       |                                             | 155.00  |                      |
|                                                   |                | NaOH 0.05%, 50 °C, 48 h                                      |                                             | 130.00  |                      |
|                                                   |                | NaOH 2.0%, 50 °C, 48 h                                       |                                             | 160.00  |                      |
|                                                   |                | NaOH 5.0%, 50 °C, 48 h                                       |                                             | 135.00  |                      |
| <i>Oscillatoria tenuis</i>                        | Chemical       | H <sub>2</sub> SO <sub>4</sub> 4M, pH 2; room temp.          | 191                                         | 210     | <a href="#">[75]</a> |

na = not available.

### 3.4. Energy Balance

Based on the experimental results, the energy efficiency of the applied pretreatment process was evaluated for the biomass of the microalga *T. subcordiformis* and the cyanobacterium *L. platensis*. The analysis relied on data from respirometric measurements, allowing the calculation of key energy parameters, including methane yield ( $Y_{CH_4}$ ), energy output ( $E_{out}$ ), net energy ( $E_{nout}$ ), net energy gain ( $E_{net}$ ), the calorific value of methane ( $CV_{CH_4}$ ), and the energy input associated with the pretreatment technology ( $E_s$ ).

In the first phase, the effects of progressively increasing the volumetric ratio of  $SCO_2$  to biomass ( $SCO_2/C_v$ , 0.1–0.5) were assessed. For *T. subcordiformis*,  $CH_4$  yield increased from 280 mL  $CH_4$ /gVS in the control sample to 377 mL  $CH_4$ /gVS in V5, representing a 34.6% improvement. This clearly demonstrates the effectiveness of  $SCO_2$ -induced cell disruption in enhancing substrate bioavailability for methanogenic microorganisms. In comparison, the use of  $SCO_2$  in sewage sludge pretreatment resulted in a 14% increase in  $CH_4$  production relative to the control [76]. The results for *T. subcordiformis* are thus highly promising and exceed the effectiveness of other disintegration methods. For instance, sonication of *Tetraselmis* sp. (MUR 233) biomass yielded 252 mL  $CH_4$ /gVS versus 248 mL  $CH_4$ /gVS in the control, while hydrothermal depolymerisation increased  $CH_4$  production from 104–163 mL  $CH_4$ /gVS to 204–316 mL  $CH_4$ /gVS, corresponding to an average increase of ~95% [44]. Although hydrothermal methods achieve higher absolute yields, they are considerably more energy- and cost-intensive.

Correspondingly, the energy output ( $E_{out}$ ) for *T. subcordiformis* increased from 110.7 Wh to 149.0 Wh, while the net energy ( $E_{net}$ ), defined as the difference between energy output and energy input ( $E_{nout} - E_s$ ), reached 38.3 Wh in V5, indicating a positive energy balance for the pretreatment process (Figure 4, Table 5).

A similar trend was observed for *L. platensis*, albeit with significantly ( $p \leq 0.05$ ) higher magnitudes.  $CH_4$  yield increased from 301 mL  $CH_4$ /gVS (control) to 420 mL  $CH_4$ /gVS at the highest  $SCO_2$  dose, corresponding to a 39.5% increase. The energy output reached 179.4 Wh, and the net energy gain was 47.6 Wh, with a comparable energy input ( $E_s$ ) of 3.22 Wh (Figure 4, Table 4). These results indicate that *L. platensis* is more susceptible to  $SCO_2$  pretreatment, likely due to morphological and structural differences such as thinner cell walls and a higher proportion of easily biodegradable compounds (e.g., proteins and soluble sugars) that become more accessible to fermentative microorganisms [46,77].

These findings align with previous reports showing that mechanical disruption of *A. platensis* (*L. platensis*) cells increased methane production by 47% [46].  $SCO_2$  pretreatment efficacy is further supported by studies on sewage sludge, where the highest  $CH_4$  production efficiency of  $380 \pm 13$  mL  $CH_4$ /gVS was achieved with a  $SCO_2$ :granular sludge ratio of 1:3, representing nearly a fourfold increase relative to untreated material [78]. Conversely, some studies indicate that pretreatment may not always enhance methane yields. For example, hydrothermal depolymerisation of *Desertifilum tharense* reduced  $CH_4$  yield from 261.8 mL  $CH_4$ /gVS (control) to 201.5–235 mL  $CH_4$ /gVS [79], whereas enzymatic treatment of *Spirulina subsalsa* BGLR6 increased  $CH_4$  production by 65% to 768.92 mL  $CH_4$ /gVS [80].

A comparative analysis clearly demonstrates the superior energy efficiency of *L. platensis* under identical pretreatment conditions. Variants subjected to  $SCO_2$  pretreatment exhibited significantly higher  $Y_{CH_4}$  and  $E_{out}$  values than *T. subcordiformis*, with a net energy gain difference ( $E_{net}$ ) of up to 12.5 Wh in V5. Such differences have important implications for industrial-scale biogas production from microalgal biomass. By comparison,  $SCO_2$  pretreatment of granulated sewage sludge achieved a maximum net energy gain of  $1047.85 \pm 20$  kWh/Mg TS at a  $SCO_2$ /AGS volume ratio of 0.3 [20].

In other studies, the use of ultrasonic disintegration (UD) of *Pinnularia* sp. biomass did not result in a positive net energy balance in any of the tested variants. In the control

sample, a net energy gain of  $1.394 \pm 0.19$  Wh/gVS was obtained. After UD application, significantly lower values were observed, ranging from  $0.943 \pm 0.22$  Wh/gVS to  $0.453 \pm 0.21$  Wh/gVS [81]. A strong negative correlation ( $R^2 = 0.8870$ ) was found between the duration of UD exposure and the net energy gain. The studies showed that a positive energy balance was achieved during the pretreatment of *Scenedesmus* sp. biomass. In the most effective variants, these values were  $0.231 \pm 0.02$  Wh and  $0.086 \pm 0.07$  Wh. In the remaining variants, the difference in net energy gain was negative [81].

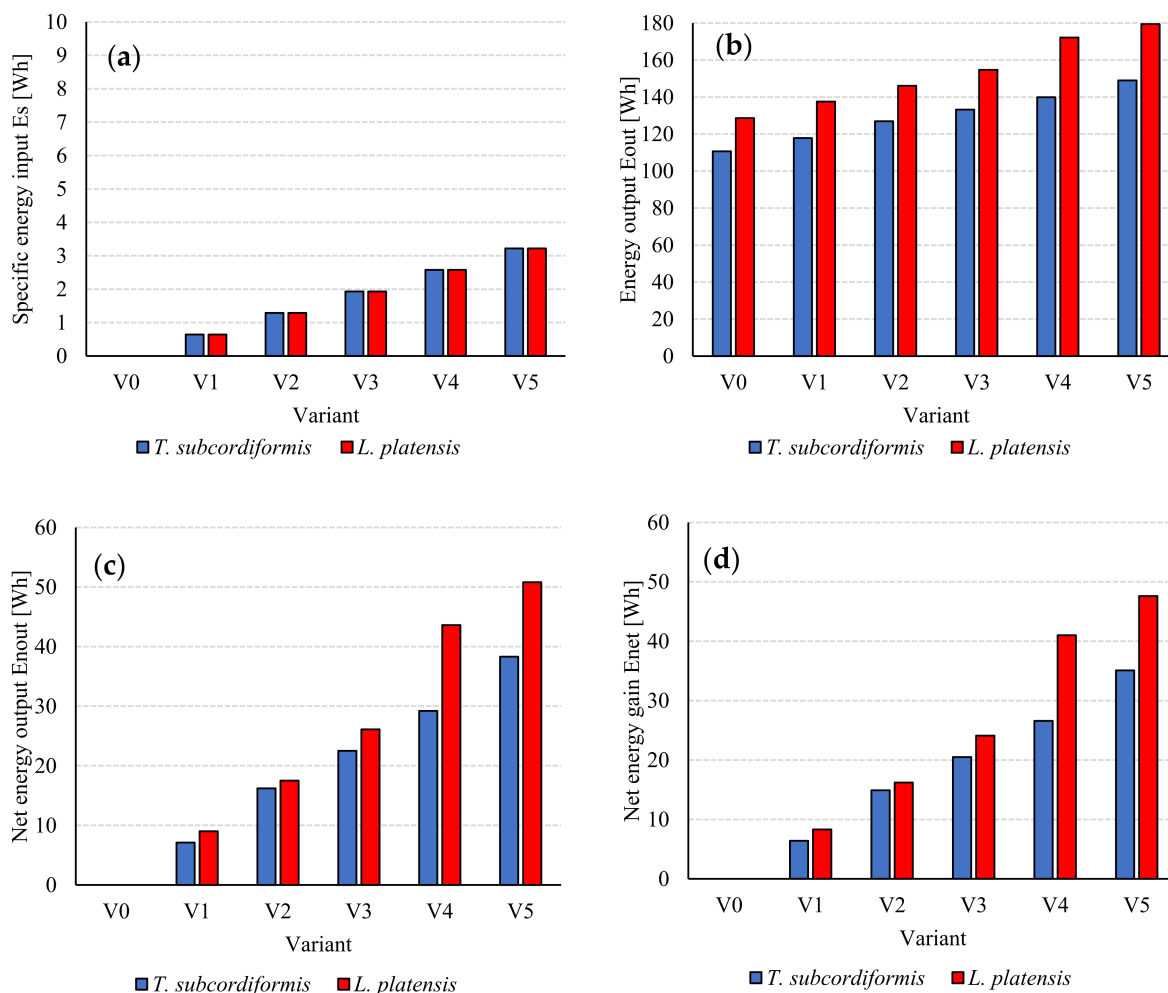


**Figure 4.** Representation of the components of the energy balance depending on the type of biomass used and the experimental variant tested, including specific energy input— $E_s$  (a); energy output— $E_{out}$  (b); net energy output— $E_{net}$  (c); net energy gain— $E_{net}$  (d).

Regarding the evaluation of the effectiveness of the applied  $\text{SCO}_2$  pretreatment, it should be emphasised that the increase in energy input associated with the production of the agent ( $E_s$ ) was proportional to the amount of  $\text{SCO}_2$  applied (ranging from 0 to 3.22 Wh). Importantly, the corresponding increases in energy output and net energy consistently exceeded these inputs, demonstrating a favourable energy balance. This observation holds even when considering energy losses related to the production and handling of dry ice, which are often cited as a key limitation of this method. From a practical perspective, the results of this study confirm that  $\text{SCO}_2$  pretreatment constitutes an effective and relatively low-energy approach for processing microalgal and cyanobacterial biomass. In particular, the results obtained for *L. platensis* are highly promising, highlighting its potential as a cost-efficient and high-yield substrate for biogas production, offering a viable alternative to conventional organic wastes.

**Table 5.** Energy balance of the experimental variants of biomass pretreatment of *T. subcordiformis* and *L. platensis* with SCO<sub>2</sub> resulting from the respirometric measurements of anaerobic digestion.

| <i>Tetraselmis subcordiformis</i> Biomass—Series 1 |                      |             |          |           |                           |                        |                         |                        |                           |          |                             |                              |                            |            |             |            |
|----------------------------------------------------|----------------------|-------------|----------|-----------|---------------------------|------------------------|-------------------------|------------------------|---------------------------|----------|-----------------------------|------------------------------|----------------------------|------------|-------------|------------|
| Variant                                            | SCO <sub>2</sub> /Cv | ρCv<br>kg/L | VCv<br>L | MCv<br>kg | ρSCO <sub>2</sub><br>kg/L | VSCO <sub>2</sub><br>L | MSCO <sub>2</sub><br>kg | PSCO <sub>2</sub><br>W | WSCO <sub>2</sub><br>kg/h | Es<br>Wh | Y CH <sub>4</sub><br>L/kgVS | Y CH <sub>4</sub><br>L/kg FM | CV CH <sub>4</sub><br>Wh/L | Eout<br>Wh | Enout<br>Wh | Enet<br>Wh |
| 0                                                  | 0                    |             |          |           |                           |                        |                         |                        |                           |          | 280                         | 12.1                         |                            | 110.7      | 0           | 0          |
| 1                                                  | 0.1                  |             |          |           |                           | 0.1                    | 0.156                   |                        |                           | 0.644    | 298                         | 12.8                         |                            | 117.8      | 7.1         | 6.4        |
| 2                                                  | 0.2                  |             |          |           |                           | 0.2                    | 0.312                   |                        |                           | 1.288    | 321                         | 13.8                         |                            | 126.9      | 16.2        | 14.9       |
| 3                                                  | 0.3                  | 1.03        | 1        | 1.04      | 1.56                      | 0.3                    | 0.468                   | 4500                   | 1090                      | 1.932    | 337                         | 14.5                         | 9.17                       | 133.2      | 22.5        | 20.5       |
| 4                                                  | 0.4                  |             |          |           |                           | 0.4                    | 0.624                   |                        |                           | 2.576    | 354                         | 15.3                         |                            | 139.9      | 29.2        | 26.6       |
| 5                                                  | 0.5                  |             |          |           |                           | 0.5                    | 0.78                    |                        |                           | 3.22     | 377                         | 16.2                         |                            | 149.0      | 38.3        | 35.1       |
| <i>Limnospira platensis</i> Biomass—Series 2       |                      |             |          |           |                           |                        |                         |                        |                           |          |                             |                              |                            |            |             |            |
| Variant                                            | SCO <sub>2</sub> /Cv | ρCv<br>kg/L | VCv<br>L | MCv<br>kg | ρCO <sub>2</sub><br>kg/L  | VSCO <sub>2</sub><br>L | MSCO <sub>2</sub><br>kg | PSCO <sub>2</sub><br>W | WSCO <sub>2</sub><br>kg/h | Es<br>Wh | Y CH <sub>4</sub><br>L/kgVS | Y CH <sub>4</sub><br>L/kg FM | CV CH <sub>4</sub><br>Wh/L | Eout<br>Wh | Enout<br>Wh | Enet<br>Wh |
| 0                                                  | 0                    |             |          |           |                           |                        |                         |                        |                           |          | 301                         | 14.0                         |                            | 128.6      | 0           | 0          |
| 1                                                  | 0.1                  |             |          |           |                           | 0.1                    | 0.156                   |                        |                           | 0.644    | 322                         | 15.0                         |                            | 137.6      | 9.0         | 8.3        |
| 2                                                  | 0.2                  |             |          |           |                           | 0.2                    | 0.312                   |                        |                           | 1.288    | 342                         | 15.9                         |                            | 146.1      | 17.5        | 16.2       |
| 3                                                  | 0.3                  | 1.03        | 1        | 1.04      | 1.56                      | 0.3                    | 0.468                   | 4500                   | 1090                      | 1.932    | 362                         | 16.9                         | 9.17                       | 154.7      | 26.1        | 24.1       |
| 4                                                  | 0.4                  |             |          |           |                           | 0.4                    | 0.624                   |                        |                           | 2.576    | 403                         | 18.8                         |                            | 172.2      | 43.6        | 41.0       |
| 5                                                  | 0.5                  |             |          |           |                           | 0.5                    | 0.78                    |                        |                           | 3.22     | 420                         | 19.6                         |                            | 179.4      | 50.8        | 47.6       |

Explanation of symbols Table 5: SCO<sub>2</sub>/Cv—volume ratio of SCO<sub>2</sub> to raw biomass; ρCv—specific density of raw biomass; VCv—volume of raw biomass; MCv—mass of raw biomass; ρSCO<sub>2</sub>—density of SCO<sub>2</sub>; VSCO<sub>2</sub>—volume of SCO<sub>2</sub>; MSCO<sub>2</sub>—mass of SCO<sub>2</sub>; PSCO<sub>2</sub>—generator power SCO<sub>2</sub>; WSCO<sub>2</sub>—efficiency of SCO<sub>2</sub> generator; Es—specific energy input; Y CH<sub>4</sub>—methane yield; CV CH<sub>4</sub>—methane calorific value; Eout—energy output; Enout—net energy output; Enet—net energy gain.

## 4. Conclusions

The application of solid carbon dioxide (SCO<sub>2</sub>) as a disintegration pretreatment effectively enhanced the solubilization of organic matter in the biomass of *L. platensis* and *T. subcordiformis*. The soluble total organic carbon (sTOC) concentration increased to approximately 5.0 g/L for *L. platensis* and 4.4 g/L for *T. subcordiformis*, indicating efficient cellular disruption and improved substrate bioavailability.

This improvement translated into a substantial enhancement of methane fermentation performance. The methane yield reached about 420 mL CH<sub>4</sub>/g VS for *L. platensis* and 370 mL CH<sub>4</sub>/g VS for *T. subcordiformis*, corresponding to increases of approximately 40% and 35%, respectively, compared with the untreated controls. The optimal SCO<sub>2</sub>-to-biomass volume ratio of 1:2.5 ensured maximal disintegration efficiency; higher doses produced no statistically significant improvement, indicating a plateau in process effectiveness.

Although *T. subcordiformis* contained a higher lipid fraction, *L. platensis* exhibited superior methane yield and faster fermentation kinetics. This can be attributed to its favorable structural and biochemical characteristics, such as a thinner cell wall, higher protein content, and more suitable intracellular pH, which collectively promote microbial accessibility and enzymatic hydrolysis. Overall, SCO<sub>2</sub>-assisted disintegration represents a sustainable, low-energy, and scalable pretreatment method that significantly enhances both methane yield and process kinetics, confirming its potential for efficient bioenergy recovery from microalgal biomass.

In light of the results obtained, it is important to highlight certain limitations of this study, which may indicate directions for further work. The experiments were conducted on a laboratory scale under batch fermentation conditions, limiting the possibility of directly transferring the results to continuous industrial systems. Future research is recommended to characterise the microbial community of the process and to assess the energy and economic efficiency of the technology on a pilot or semi-technical scale, which would allow a more comprehensive assessment of its application potential.

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