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Use of microalgae residues for biogas production

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HIGHLIGHTS

- *Scenedesmus* residues were successfully converted to methane.
- Extraction methods enhanced biodegradability and methane production.
- Highest methane production was obtained from amino acid-extracted biomass.
- Kinetics of the process was improved by co-digestion.

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ABSTRACT

In biorefineries, the extraction of metabolites from microalgae would produce great amount of organic residues that would need to be treated. In this work, *Scenedesmus* residues were evaluated as substrates for biogas production and compared to raw biomass (SB). Microalgae residues were generated after the extraction of amino acids (SRA) and lipids (SRL). The influence of the processes applied on physicochemical properties and anaerobic biodegradability of microalgal biomass was studied in batch digestion tests. Co-digestion of microalgae residues with carbon rich substrates was also assessed by studying synergisms and kinetics of the discontinuous process. Methane yields of SRA and SRL in mono-digestion were $272.8 \pm 7.3\ L_{CH4}\ kgVS^{-1}$ and $212.3 \pm 5.6\ L_{CH4}\ kgVS^{-1}$, respectively, increasing that of SB ($140.3 \pm 29.4\ L_{CH4}\ kgVS^{-1}$). Kinetics of the process was also improved after the extraction of amino acids and lipids. Improvements were attributed to the disruption of microalgae cell walls and the increase in the solubilization of the organic matter. The amino acid extraction process improved the digestion process in a higher extent than lipid extraction because of its higher hydrolytic effect on biomass. Co-digestion influence on methane yield depended on the co-substrates used. However, co-digestion improved kinetics of the process.

1. Introduction

Extraction on a large scale of high value compounds from microalgae would produce great amount of organic residues that would require appropriate treatment. In most microalgae species cultured without nutrient limitation protein is the main organic component [1]. These proteins can be used for human and animal nutrition. Moreover, amino acid hydrolysates can be useful for the production of bacteria and yeast in the fermentation industry, as antioxidants, as an energy source or as biofertilizer [2]. When proteins are extracted from microalgae, sugars and lipids remain in the residual biomass. Therefore, the potential for energy production from these residues is very high. Anaerobic digestion is a well-known process used for the treatment of organic residues reducing their organic load and, at the same time, producing biogas and stabilized organic matter where most of the nutrients have been mineralized. The treatment of protein-extracted microalgae residues, rich in lipids and carbohydrates, through anaerobic digestion would yield high biogas.

Microalgae are also being studied as an energy crop for biodiesel production due to their high biomass productivities and high lipid accumulation [1]. If lipids are extracted from microalgae for biodiesel production, proteins and carbohydrates would remain in the residual biomass, with the subsequent opportunity to convert these organic components into biogas by anaerobic digestion. In fact, this option has already been pointed out as crucial in order to make sustainable microalgal biodiesel [3].

However, the anaerobic digestion of microalgae has shown two main problems. Some microalgae have shown low biodegradability [4–7]. Cell walls of some microalgae species are composed of complex carbohydrates that are hardly biodegradable by bacteria [5,7]. These cell walls act as a protection of the intracellular organic macromolecules from bacterial attack, reducing biodegradability of microalgal biomass. Another drawback to the anaerobic degradation of microalgae biomass is its high nitrogen content and low

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C/N ratio, consequence of the high protein fraction. Feedstocks of low C/N ratio can produce excessive ammonia inhibiting the growth of microorganisms and consequently spoiling or even stopping the digestion process [8].

Solutions for both drawbacks have been studied. Some pretreatments are able to break microalgae cell walls. Intracellular organic molecules are released and their solubilization is increased, being available for bacterial biodegradation. Consequently, biomass biodegradability and methane yields are increased [7,9–11]. On the other hand, the low C/N ratio of microalgal biomass can be balanced by the addition of high carbon content substrates [12–15], avoiding ammonia inhibition. Results on co-digestion of microalgae show, in most cases, that it improves the digestion process through the synergistic effects produced, such as the balance of nutrients, the increase buffer capacity or the increase of enzyme activity [12–15]. Conversely, in other studies co-digestion of microalgae with carbon-rich substrates produced no synergistic effects and a decrease in the methane yield [16].

Furthermore, the extraction of intracellular metabolites, such as lipids, also increases biodegradability and methane yields, as a consequence of the disruption of the cell wall [9,10]. These extraction methods have been claimed as similar to other types of pretreatments applied to microalgae that seek to enhance the biodegradability and the methane yield [11]. Therefore, either by applying pretreatments or by the extraction of metabolites the biodegradability of intracellular organic molecules of microalgae is increased leading to a higher accumulation of ammonia from degraded proteins inside digesters. In fact, Schwede et al. [11] observed inhibition caused by ammonia and salts accumulation during the anaerobic degradation of thermally pretreated *Nannochloropsis salina* in semi-continuously fed reactors. The combination of pretreatments or metabolite-extraction processes and co-digestion could be the solution to increase the anaerobic biodegradability of microalgal biomass avoiding at the same time ammonia toxicity.

The main goal of this study was to assess *Scenedesmus* residues as substrates for anaerobic digestion and to compare the digestion process of these residues and the digestion process of raw *Scenedesmus* biomass. Moreover, co-digestion assays were performed to study the effect of adding carbon-rich substrates to microalgae residues. Microalgae residues were generated after two different processes of amino acid and lipid extraction. Lipid-extracted microalgae residues have been assessed as substrate for biogas production several times. However, to the authors' knowledge, this is the first time that amino acid-extracted microalgae residues are evaluated for biogas production.

2. Materials and methods

2.1. Substrates and anaerobic biomass

Scenedesmus biomass (SB) and residues were kindly provided by *Fundación Cajamar* and University of Almería (Spain).

In order to extract amino acids, *Scenedesmus* biomass underwent an enzymatic hydrolysis process described in detail in Romero García et al. [2]. This process included a pretreatment to break microalgae cell walls by mechanical means (horizontal-bed ball-mill). After this pretreatment the enzyme Viscozyme® was added in order to reduce the viscosity of the solution to increase the yield of the enzymatic hydrolysis process. Viscozyme® is an enzyme with activity betaglucanase-cellulase-xylanase thus breaking carbohydrates and reducing their influence in the enhancement of the viscosity of the medium. After 30 min, the enzyme Alcalase® 2.5 L was added at pH 8.0 (kept constant by the addition of NaOH 1 M during 120 min). Then, pH was decreased by the addition of H_2SO_4 1 M and the enzyme Flavourzyme® 1000 L was added (pH was kept constant at 7.0 by H_2SO_4 1 M during 60 min). All these three steps were performed at 50 °C. Overall, biomass was subjected to 50 °C during 3.5 h (0.5 h during the viscosity reduction process and 3 h during the enzymatic hydrolysis). Finally, in order to deactivate enzymes, biomass was heated up to 75 °C during 15 min. After the separation of amino acid hydrolysate, residual biomass (from now on referred as SRA) was freeze-dried to make easier its transport, manipulation and conservation.

The extraction of lipids from *Scenedesmus* biomass was performed using hexane as solvent in a standard Soxhlet apparatus. Lipid-extracted residual biomass (from now on referred as SRL) was also freeze-dried.

SRA was co-digested in batch mode with paper sludge (PS) and *Opuntia maxima* cladodes (OM) in order to increase the C/N. PS is an abundant residue in the recycling paper industry with costs associated to its treatment or disposal. Due to its high carbon content, in principle it is a suitable co-substrate for anaerobic co-digestion of high nitrogen content feedstocks. PS was composed of sludge of the deinking process and of biological sludge from a wastewater treatment plant. Both came from the same factory in Madrid. Sludge produced in the deinking process was mainly composed of cellulose, ink and $CaCO_3$ whereas biological sludge, produced in the wastewater treatment plant of the factory, was around 6% in weight of the final sludge. PS was dried and finely milled to produce particles as small as possible in order to increase the surface contact with anaerobic biomass. *O. maxima* is rich in carbon and yield high biogas. It has been proposed in Mediterranean countries as an energy crop [17], since it can grow with high biomass yields with low water and fertilizer inputs [18]. OM was harvested in Madrid, where it was growing without fertilizers nor irrigation. Young cladodes (one or two years) were the only part of the plant harvested, since old cladodes increased their fraction in lignocellulosic material [19], hardly biodegradable. The cladodes were grinded and homogenized before their use; however the sample still contained some lumps because of its high mucilage content.

SRL was co-digested with residual glycerin (GLY) obtained during a biodiesel production process from waste cooking oil. GLY is rich in carbon and has been proved to be beneficial to the digestion process when added in low amounts to lipid-extracted microalgae residues [14,20]. Glycerol residue used in this study was not liquid and had to be homogenized by an intensive blending. However, samples still contained lumps and showed color gradients.

Different inoculums were used in the batch assays performed. In the first BMP assay (SRA–PS), the anaerobic biomass was anaerobic sludge adapted to the co-digestion of *Scenedesmus* biomass and *O. maxima* in laboratory reactors. Total and volatiles solids (TS and VS) concentration were 39.4 g L^{-1} and 23.3 g L^{-1}, respectively. Total Kjeldahl nitrogen (TKN) concentration was 2.2 g L^{-1}. Total and soluble chemical oxygen demand (COD_t and COD_s) were 33,333 and 2149 mgO_2 L^{-1}, respectively, and it showed a high partial alkalinity (PA) (3868 mg$CaCO_3$ L^{-1}) and a low intermediate alkalinity (IA) (950 mg$CaCO_3$ L^{-1}), consequently a low IA/PA ratio (0.25). pH was 7.8.

In the second BMP assay (SRA–OM) the anaerobic biomass was obtained during the digestion process of SRA in continuous mode in lab reactors. TS and VS concentration was 47.7 gTS L^{-1} and 27.8 gVS L^{-1}. TKN concentration was 4.0 g L^{-1}. COD_t and COD_s were 51,830 and 7877 mgO_2 L^{-1}, respectively, and it showed a high PA (6634 mg$CaCO_3$ L^{-1}) and a low IA (1398 mg$CaCO_3$ L^{-1}), with a consequent low IA/PA ratio (0.21) pH was 7.9.

In the third BMP assay (SRL–GLY) the anaerobic biomass was obtained during the digestion process in continuous mode of diluted SRA. TS and VS concentration were 58.3 g L^{-1} and 32.4 g L^{-1}, respectively. TKN concentration was 4.8 g L^{-1}. COD_t and COD_s were 61,867 mgO_2 L^{-1} and 8003 mgO_2 L^{-1}, respectively,

with an IA/PA ratio under 0.3 (0.22), corresponding to a PA of 8878 $mgCaCO_3\ L^{-1}$ and an IA of 1952 $mgCaCO_3\ L^{-1}$. pH was 8.2. All inoculums came originally from a wastewater treatment plant located in Madrid (Spain), working at 37 °C treating the primary sludge produced in the plant.

2.2. Biochemical methane potential (BMP) assays

The BMP assays were carried out according to the guideline VDI 4630 [21]. SB, SRA and SRL were used as substrates in mono-digestion. Also, screenings of different blends of SRA–PS, SRA–OM and SRL–GLY were performed in different BMP assays in order to study the influence of the co-substrates on the anaerobic digestion process. PS and OM were also mono-digested to study their methane potential. Feedstocks were placed into 1L reactors with the corresponding amount of inoculum according to the VDI 4630 ($VS_{substrate}/VS_{inoculum}$ = 0.5). Moreover, TS concentration inside reactors did not exceed 10% to ensure an appropriate mass transfer.

Every batch was performed in duplicate for SB, SRA, PS and the mixtures of SRA–PS. In the case of the mixtures of SRA–OM and SRL–GLY, due to the heterogeneity of OM and GLY samples, the batches were performed in triplicate. Blank reactors were run in duplicate with the inoculum for the determination of endogenous methane production. Reactors were tightly closed and submerged into water kept at 37 °C with a thermostatic bath. The head space of each reactor was flushed with nitrogen gas to ensure anaerobic conditions before starting tests. The content of reactors was continuously stirred by magnetic bars. Duration of the different tests ranged between 32 and 40 days.

Before doing the mixtures elemental analyses of the substrates were performed in order to balance the *C*/*N* ratio. Analyses of TS, VS, COD_t, COD_s, TKN, PA, TA and pH were performed at the beginning and at the end of each assay. Volatile acids (VA) and total ammonia nitrogen (TAN) were measured at the beginning and at the end of the assay for mixtures composed of SRA and OM and SRL and GLY.

Biogas composition and production was monitored with the Micro-Oxymax respirometer (Columbus Instrument; Columbus, OH 43204 USA). The biogas produced in the reactors was accumulated in the measuring chambers. The volume of the measuring chambers was exactly calculated by the Micro-Oxymax respirometer. The percentage gas levels of CO_2 and CH_4 of the measuring chambers were measured periodically and their changes in level were used to compute CO_2 and CH_4 production rates. Between the measures of gas composition of each chamber, the sensors were purged in order to avoid cross contamination. This purge was made using atmospheric air; therefore, check valves were installed between the measuring chambers and the reactors to prevent the entrance of oxygen.

2.3. Kinetics of degradation

The kinetic of the discontinuous process was analyzed for the different batch assays performed. The following kinetics equation previously proposed in various studies [22–24] was used:

$$G = G_m[1 - exp(-k_0 * t)]$$

where G is the volume of methane accumulated (NmL) after a time t (d), G_m is the maximum volume of methane (NmL) accumulated at an infinite digestion time and k_0 is the observed specific rate constant of the overall process (day^{-1}). This model is a first order kinetic model and describes the methane production in a batch assay as an exponential curve. Methane is the main metabolite produced during the anaerobic digestion and therefore it can be used to study the kinetic of the process. This model gives useful information of the kinetics such as the initial methane production rate and the total methane yield, which can be used for process design [24].

The specific rate constant (k_0) was calculated analytically from experimental data, assuming G_m as the maximum volume of methane produced at the end of each assay. A linear least square regression procedure was used to determine k_0 with a confidence interval of 95% using *SPSS v*11.5. k_0 is highly dependent on operational factors, such as temperature, stirring and the initial substrate concentration [24]. In this study conditions were kept constant and therefore the comparison of k_0 values can be useful to determine the influence of the addition of co-substrates on the kinetics of the process.

2.4. Analytical methods

Ultimate analysis (C, H, N, S) were carried out by combustion using a LECO TruSpec CHNS macroanalyzer. Determination of major and trace elements (Al, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, P, Pb, Sr, Ti, V and Zn) was done by inductively coupled plasma atomic emission spectroscopy (ICP-AES) with a VARIAN 735-ES plasma optical emission spectrometer. K and Na were analyzed by flame atomic emission spectrometry with a Perkin Elmer (2280) emission spectrometer. Mercury (Hg) was analyzed using a Milestone DMA-80 Direct Mercury Analyzer. Protein, carbohydrate and lipid fractions of microalgae and OM were theoretically estimated by the use of the *R*-value concept and constituents' calculation method [14,25]. pH of solid substrates (SB, SRA, SRL and PS) was analyzed after dilution with water at a ratio 1:2.5 (substrate:water) and agitation during two hours. pH of OM and reactors was measured submerging directly into them a pH sensor. TS and VS were analyzed according to APHA [26]. PA and TA were determined according to Ripley et al. [27] by titration to 5.75 and 4.3 pH, respectively. COD_t and COD_s of reactors and substrates were analyzed according to the 410.4 method of US EPA. For the measurement of COD_s, solid substrates were leached for two hours at a ratio substrate: water of 1:10, centrifuged at 5100 rpm during five minutes and the supernatant was filtered for the determination of COD_s. TKN was determined by the Kjeldahl method and TAN was analyzed with the same method but without the destruction step. VA were analyzed by steam distillation and titration of the distillate to pH 8.6. Analyses of each sample were performed in triplicates.

3. Results and discussion

3.1. Composition of substrates and effects of the extraction methods on microalgal biomass

Composition of substrates is shown in Table 1. SB showed a low *C*/*N* ratio (5.9) due to its high nitrogen content (6.8%). The high nitrogen content is consequence of the high fraction of proteins in the biomass (42.5%). Carbohydrate and lipid fractions accounted for 12.3% and 16.9% of the dry matter, respectively. COD_t was 1109.5 g O_2 kg^{-1} and COD_s was 100.3 g O_2 kg^{-1}. Only 9.0% of the COD was in soluble form. Therefore, only a low fraction of organic matter was readily available for bacterial degradation. pH of microalgal biomass was neutral and therefore adequate for anaerobic digestion.

The *C*/*N* ratio of SRA was 7.2, higher than that of raw biomass. Part of the nitrogen content of microalgae was extracted from the biomass in the form of amino acids, however, it was still below the optimal range for microorganisms, considered to be between 10 and 30 [24]. Therefore, inhibition due to ammonia accumulation is possible, justifying the search for co-substrates that increase the *C*/*N* ratio of the feedstock. pH of SRA is acid, but close to neutral

Table 1
Composition of substrates (elemental analysis expressed in% of dry matter).

	SB	SRA	SRL	PS	OM
TS (%)	93.6	96.2	90.0	99.9	6.4
VS (%)	67.1	59.4	64.3	32.7	4.9
COD_t ($gO_2\ kg^{-1}$)	1109.5	1011.1	961.5	715.6	172.1
COD_s ($gO_2\ kg^{-1}$)	100.3	254.5	169.4	16.8	NA
Ashes (%d.m.)	28.3	38.3	28.5	67.3	23.4
Carbohydrates (%d.m.)	12.3	10.9	29.3	NA	58.5
Proteins (%d.m.)	42.5	31.3	35.6	NA	6.9
Lipids (%d.m.)	16.9	19.6	6.5	NA	11.2
pH	7.25	6.70	6.76	7.14	4.63
C (%)	39.9	35.8	34.8	24.2	37.4
H (%)	5.5	4.8	5.4	2.5	5.1
N (%)	6.8	5.0	5.7	0.3	1.1
S (%)	0.5	0.5	0.5	0.1	ND
C/*N*	5.9	7.2	6.1	80.7	34.3
P (%)	2.0	3.0	4.4	0.03	0.17
Ca (%)	8.21	13.0	5.0	21.0	3.05
K (%)	0.9	0.5	1.4	0.1	3.80
Mg (%)	0.5	0.6	1.2	0.74	1.12
Al (%)	0.004	0.02	0.02	3.0	0.02
Na (ppm)	3000	6400	7000	1600	97
Ba (ppm)	33	44	27	110	70
Be (ppm)	<6	<6	<6	<6	<6
Bi (ppm)	<6	<6	<6	<6	<6
Cd (ppm)	<6	<6	<6	<6	<6
Co (ppm)	<6	<6	<6	<6	<6
Cr (ppm)	<6	<6	<6	20	<6
Cu (ppm)	35	40	27	188	11
Fe (ppm)	801	760	4000	1600	82
Mn (ppm)	308	320	620	36	281
Mo (ppm)	<6	<6	<6	<6	<6
Ni (ppm)	<6	<6	<6	18.0	<6
Pb (ppm)	<6	<6	<6	38.0	12
Sr (ppm)	554	700	193	320	155
Ti (ppm)	<6	<6	9	960	<6
V (ppm)	<6	<6	14	<6	<6
Zn (ppm)	78	100	84	62	47
Hg (ppb)	NA	9.5	9.5	70.9	85

ND = non-detected; NA = no analyzed.

(6.7). Protein fraction of SRA (31.3%) was lower than that of SB. COD_t and COD_s were 1011.1 and 254.5 $gO_2\ kg^{-1}$, respectively. COD_s was 2.5-fold higher than in SB, whereas the relative fraction of soluble COD was increased up to 25.17%. The increase was attributed to different facts. First, the rupture of microalgae cell walls which releases intracellular organic components previously contained within the cell wall. Secondly, hydrolyzed organic matter that remained in the residual biomass such as hydrolyzed proteins that could not be separated, carbohydrates that were hydrolyzed because of the action of Viscozyme® and lipids that were probably also hydrolyzed due to basic and slightly acid environments and mild temperatures that were kept at different phases during the amino acid extraction process. Actually, basic and acid thermochemical pretreatments have shown to induce carbohydrate and protein hydrolysis and subsequent solubilization, increasing biodegradability of microalgal biomass [28]. Content of calcium and sodium in SRA was the highest compared to the other microalgal biomasses used (13% and 6400 ppm, respectively). However, by diluting the substrate with the inoculum concentrations decreased to 0.22% of calcium and 110 ppm of sodium. This concentration of calcium is lower than the moderately inhibitory concentration described by McCarty [29], whereas the concentration of Na is inside the range described as stimulatory by the same author.

SRL showed a low *C*/*N* ratio (6.1) and a lower percentage of carbon compared to SB and SRA due to the extraction of part of its lipids. In fact, the lipid fraction was 6.5%, much lower than in SB and SRA, evidencing the effectiveness of the lipid extraction method applied. The total and soluble COD were 961.5 and 169.4 $gO_2\ kg^{-1}$, respectively. COD_s increased 1.69 times compared to SB as a consequence of the lipid extraction method. The relative fraction of soluble COD increased up to 17.6% in SRL. The increase in the COD soluble fraction was lower than in the case of SRA. The lower fraction of COD_s in SRL was attributed to the lack of the enzymatic hydrolysis during the lipid extraction process.

PS had a high carbon and low nitrogen content; therefore it can balance the low *C*/*N* ratio of SRA. VS content was low (32.7% of TS), indicating that PS had a high content of inert material. Moreover, it showed a high content of calcium, part of $CaCO_3$, which would increase reactors alkalinity, providing reactors with a high buffer capacity. Calcium has been described as inhibitory by some authors. McCarty [29] already described calcium concentrations to be moderately inhibitory above 0.25–0.35%, whereas it was strongly inhibitory with concentrations above 0.8%. Although calcium concentration in the dried substrate was 21%, by mixing it with the inoculum it was diluted. The highest calculated calcium concentration was found in the batch containing PS as sole substrate, with a concentration of 0.7%, therefore being moderately inhibitory according to McCarty [29]. Highest concentrations of heavy metals in PS were found for iron (1600 ppm) and titanium (960 ppm). Concentrations are high when referring to the dried substrate, however, after dilution with the inoculum concentrations were 53.5 ppm and 32.1 ppm for iron and titanium, respectively. Heavy metals can affect the digestion process by inhibition. Inhibitory concentration thresholds depend on a large number of factors such as physic-chemical form, differences in substrate, bacteria genre, and environmental factors [30]; therefore it is not easy to determine whether certain heavy metal would act as inhibitory. In these cases, low concentrations of iron and titanium were not probably affecting the digestion process.

OM showed high water content (93.6%) and high VS content (76.1% of TS). The *C*/*N* ratio of OM was high due to its high carbon content (37.4%), consequence of the high carbohydrate fraction (58.5%). It showed a low pH (4.63) that in a continuous-mode digestion may have to be neutralized to avoid the acidification of reactors. However, in batch assays, where there is a high alkalinity due to the high inoculum to substrate ratio, it was not necessary. The content of light and heavy metals is not remarkable in OM.

GLY was obtained after the transesterification of waste cooking oil for biodiesel production in lab scale. GLY was not purified since a purification method would suppose an increase in the costs associated to its use in the anaerobic digestion process. Therefore, it contained crude fatty acids that did not react during the transesterification process, water and salts. TS and VS of GLY were 81% and 72.6%, respectively. An estimation of the glycerol content was performed by the method used by Ooi et al. [31] that separate the unreacted free fatty acids and the salts from a solution of concentrated glycerin. The concentrated glycerin solution obtained was 39.8 ± 3.6% (in weight basis) of the residual glycerin (GLY) used in batch assays as co-substrate.

3.2. Methane yield of microalgae

In Fig. 1, biogas and methane yield are shown, as well as the methane content of the biogas and the fraction of COD_s (%) of the different kind of microalgal biomasses employed in the batch assays.

SB showed low organic matter degradation and consequently low biogas and methane yields (177.4 ± 34.2 $L_{biogas}\ kgVS^{-1}$, 140.3 ± 29.4 $L_{CH4}\ kgVS^{-1}$, 21.3 ± 3.2%COD_t removal and 48.2 ± 3.7%VS removal). The cell wall of *Scenedesmus* is composed of complex carbohydrates [32], hardly biodegradable that impede the degradation of intracellular organic molecules by microorganisms. Other authors have already pointed out the low biodegradation of *Scenedesmus* and other microalgae genre such as *Chlorella*, which

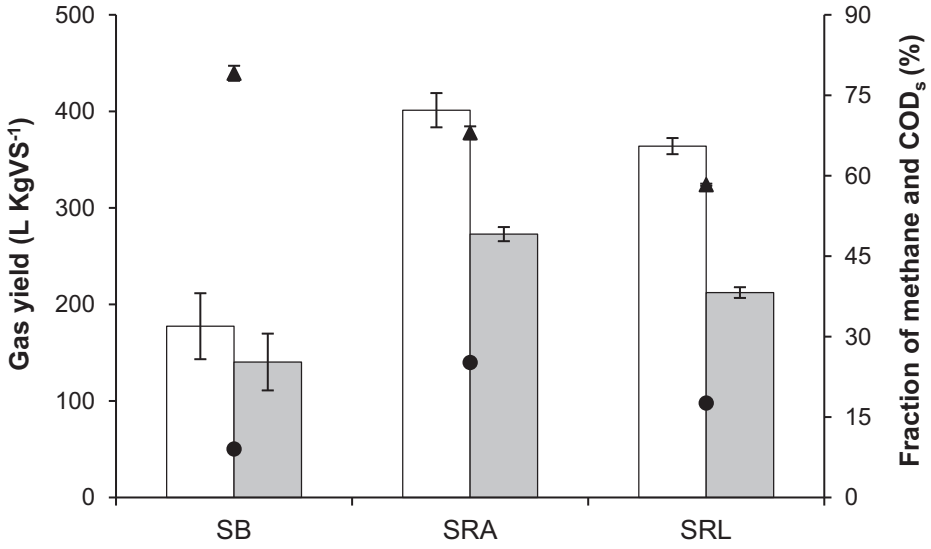

Fig. 1. Biogas yield (white bars), methane yield (gray bars), methane content of biogas (▲) and COD_s fraction (●) of the three types of microalgal biomass tested. Error bars indicate standard error of the mean value.

have cell walls that impede the action of the microorganisms responsible for the anaerobic degradation [5]. The low fraction of COD_s of SB suggests that organic compounds were not available for bacteria, probably contained within the cell.

SRA was highly biodegradable. 61.8 ± 1.6% of COD_t and 58.1 ± 4.3% of VS were removed during the assay. Biogas and methane yields were also very high (401.2 ± 17.7 L_{biogas} $kgVS^{-1}$ and 272.8 ± 7.3 L_{CH4} $kgVS^{-1}$), increasing that of SB by 2.26 and 1.94 times, respectively. The amino acid extraction process enhanced to a great extent the biodegradability of *Scenedesmus* biomass. The main reason is the mechanical rupture of the cell wall of microalgae and the subsequent hydrolysis and solubilization of the organic matter. Additionally, after the extraction of amino acids the residual biomass increased its *C*/*N* ratio compared to the raw biomass. The increase in *C*/*N* was also supposed to benefit the development of the digestion process by favoring bacterial activity. Such high biogas and methane yields showed that the anaerobic digestion of residual biomass could improve the profitability of the process of amino acid extraction by reducing the energy costs. Finally, the amino acid extraction process can be considered as a pretreatment that enhances the biodegradability of microalgal biomass by disrupting the cell wall and increasing the solubilization of the organic matter by the hydrolysis of organic macromolecules, consequently, increasing the bioavailability of the intracellular organic components of microalgae.

SRL biodegradation was satisfactory. COD_t and VS removal were higher than 60% after 40 days (67.2 ± 0.9% and 63.8 ± 1.5%, respectively). Biogas and methane yield were 364.0 ± 8.4L $kgVS^{-1}$ and 212.3 ± 5.6 L $kgVS^{-1}$, respectively. That means an increase of 2.06-fold the biogas yield and 1.51-fold the methane yield of SB, but a decrease of 10.2% of the biogas yield and 28.5% of the methane yield compared to SRA. Lipids are the organic compound which yield most methane (1.014 L_{CH4} $kgVS^{-1}$), followed by proteins (0.851 L_{CH4} $kgVS^{-1}$) and carbohydrates (0.415 L_{CH4} $kgVS^{-1}$) [3]. As SRL suffered a process of extraction of lipids biogas and methane yields were slightly lower in SRL than in SRA. The lipid extraction method did not include a pretreatment to break microalgal cell walls. Therefore, the increase in methane production despite lipid extraction should be consequence of a disruption of the cell walls caused by the forced extraction of intracellular lipids. Other authors have also observed an increase in the methane yield after lipid extraction from microalgae, attributing this increase to the disruption of the cell wall caused by the solvent-based oil extraction [11]. Methane content in biogas was higher for SB (79.1%) than for SRA (68.0%) and SRL (58.3%). The high carbohydrate fraction of SRL could cause the decrease in the methane content of the biogas compared to the other two types of biomasses.

The specific rate constants (k_0) of SB, SRA and SRL were 0.0902 ± 0.0025 d^{-1}, 0.108 ± 0.007 d^{-1} and 0.101 ± 0.005 d^{-1}, respectively. SRA and SRL showed a higher k_0 compared to SB. Both extraction methods applied to microalgal biomass increased the soluble fraction of the organic matter, increasing its availability for microorganisms and the rate of methane production. SRL showed a slightly lower k_0 than SRA. The enzymatic hydrolysis process increased the soluble fraction of the organic material as it has been already described, providing a more readily available organic matter for microorganisms and therefore increasing the rate of degradation. Similar k_0 values, 0.08–0.14 d^{-1}, were obtained by Mendez et al. [28] in batch digestion of thermochemically pretreated *Chlorella vulgaris*.

Several authors have studied the influence of different kind of pretreatments on the biodegradability of *Scenedesmus* biomass. González-Fernández et al. [33] assessed ultrasonic and thermal pretreatments on *Scenedesmus* biomass by performing batch tests. They obtained higher methane yields than with untreated biomass. Methane yields varied between 69.5 and 153.5 L_{CH4} $kgCOD^{-1}$ depending on the energy applied during the pretreatments. Using the TS/COD ratio (0.56) indicated by these authors, methane yield based on TS can be calculated and it oscillated between 124.1 and 274.1 L_{CH4} $kgTS^{-1}$. Methane yields obtained in this study from *Scenedesmus* residues were inside this range, being 168.3 L_{CH4} $kgTS^{-1}$ for SRA and 151.7 L_{CH4} $kgTS^{-1}$ for SRL. Since some of the organic material was extracted prior digestion, it was logical to obtain methane yields close to the lower value of the range obtained by González-Fernández et al. [33] with pretreated biomass. Keymer et al. [9] compared the influence of a lipid extraction (LE) method on the biodegradability of *Scenedesmus* biomass. Methane yield was comparable to the result obtained in this work for SRL, being 240 L_{CH4} $kgVS^{-1}$ the methane yield obtained by Keymer et al. [9] and 212.3 L_{CH4} $kgVS^{-1}$ the methane yield from SRL. Results suggest that after the extraction of high-value metabolites from microalgae the methane yield of the residual biomass is increased in a similar way to which it is done by the application of thermal or ultrasound pretreatments. In studies performed in

continuous mode with thermally pretreated *N. salina* inhibition caused by high ammonia release and salt content was observed [11]. Although bacteria adapted to such conditions, the biogas productivity was lowered. The high ammonia release was a consequence of the increase in the biodegradability caused by the thermal pretreatment applied to biomass. Therefore, it is reasonable to expect ammonia inhibition when digesting microalgae residues in continuous mode. Consequently, it is necessary to seek for co-substrates that balance the *C*/*N* ratio. This option is further discussed in the next section.

The sale of amino acids or lipids should be the main income of a microalgae industry devoted to the commercialization of these compounds. The anaerobic digestion of microalgae residues could be a factor that would increase the economic viability of these processes by reducing their energy needs that are normally high due to the necessity of dewatering or drying microalgal biomass [3]. As a first approach, batch results obtained in this work show that it deserves to keep on researching on the coupling of microalgae industries and anaerobic digestion. However, assays in continuous mode have to be performed to optimize the process and to evaluate possible constraints and/or advantages that are not observable in batch digestion, such as inhibition due to accumulation of toxic compounds or the possibilities of nutrient recovery by recycling the digestate as medium for growing microalgae, which would suppose additional savings in fertilizers. Thus, continuous assays can give enough information to determine the viability of the anaerobic digestion as a final step to treat microalgae residues.

3.3. Co-digestion assays

3.3.1. Co-digestion of SRA and PS

In Table 2, the experimental design of the co-digestion assay of SRA and PS is shown. Results obtained showed that the biodegradation of PS as a sole substrate was difficult. COD_t and TS removal was not detected. Some reduction of VS could be observed (26.0 ± 1.9%). As a consequence, biogas and methane yields were low (78.6 ± 45.9 L_{biogas} $kgVS^{-1}$, 50.6 ± 28.2 L_{CH4} $kgVS^{-1}$). The low biodegradability suggests that the cellulose contained in PS is recalcitrant or not bioavailable. In fact, the soluble fraction of COD was only 2.4% of the total COD. In Fig. 2 the biogas yield, the methane yield and the methane percentage of biogas are shown for the different mixtures of SRA and PS analyzed, as well as for each individual substrate. The biogas composition was similar for all the mixtures analyzed; however, the increase of the *C*/*N* ratio, corresponding with an increase in the fraction of PS in the mixture, caused a decrease in biogas and methane yield, as a consequence of the low biodegradability of PS. El-Mashad [16] observed a similar behavior when *Spirulina platensis* was co-digested with switchgrass. The decrease in biogas and methane yield with increasing proportion of switchgrass in the mixtures suggested, according to the author, that the high proportion of carbohydrates and lignin of switchgrass affected negatively the final methane yield. In the case of PS, the high content of calcium could have also caused a negative impact in the digestion process. A high proportion of PS in the mixture caused a high content of calcium in the reactor, which could act as inhibitor with concentrations above 0.25% [29]. The concentration of calcium inside reactors was calculated to be 0.7%, 0.58%, 0.51% and 0.38% for M1.5, M1.4, M1.3 and M1.2, respectively; therefore there could have been some inhibition inside reactors containing PS.

Synergism of various substrates can be identified when methane yield of their co-digestion is higher than the sum of the methane yield of each individual substrate [24,34]. On the other hand, if the methane yield of the mixture is lower than the sum of the methane yield of each individual substrate, antagonism takes place. The methane yield obtained during the mono-digestion of each substrate can be considered its theoretical methane potential. Therefore, synergistic or antagonistic effects can be evaluated by adding proportionally methane yield of each individual substrate of the mixture and comparing it to the methane yields obtained experimentally. Theoretical methane potentials of individual substrates are 272.8 ± 7.3 L_{CH4} $kgVS^{-1}$ for SRA and 50.6 ± 28.23 L_{CH4} - $kgVS^{-1}$ for PS. The co-digestion assays with PS revealed that only in M1.2 (*C*/*N* = 10) the methane yield was higher than the expected one (Table 3); therefore, synergistic effects occurred in this mixture. In the other two mixtures analyzed (M1.3 and M1.4) methane yields did not differ from expected yields, and therefore, no synergistic or antagonistic effects occurred.

The specific rate constants (k_0) of M1.2, M1.3 and M1.4 were similar: 0.120 ± 0.006 d^{-1}, 0.113 ± 0.005 d^{-1}, 0.123 ± 0.006 d^{-1}, respectively. k_0 was improved in all co-digestion trials compared to the mono-digestion of individual substrates. k_0 of the mono-digestion process of SRA and PS were 0.108 ± 0.007 d^{-1} and 0.094 ± 0.008 d^{-1}, respectively. Therefore, the kinetics of the process was improved when SRA and PS are co-digested, however, the *C*/*N* variations caused no significant differences in the kinetics once that both substrates have been mixed.

3.3.2. Co-digestion of SRA and OM

In Table 4, the composition of the different mixtures analyzed in this BMP assays are shown. OM was used as co-substrate in order to enhance the biogas production. However, its addition caused a decrease in biogas and methane yields, as well as in methane content of biogas (Fig. 3). M2.3 (*C*/*N* ratio of 15) showed a slightly better behavior than the other mixtures, however, no significant differences were observed in the biogas yields amongst the different *C*/*N* ratios analyzed. Although the biogas and methane yields decreased when OM was added, the organic matter removal increased. The decrease in the methane yield with the increase in the organic matter removal was consequence of the biochemical composition of OM. Carbohydrate, protein and lipid fractions of the young cladodes used in this study were estimated to be 58.5%, 6.9% and 11.2% of the dry matter, respectively. Carbohydrates are highly biodegradable; however, their methane potential is lower than that of other organic molecules such as protein and lipids. Moreover, biogas produced from carbohydrates is typically rich in CO_2, consequently, methane content of biogas in the mixtures that contained OM decreased. On the other hand, SRA

Table 2
Composition of mixtures of SRA–PS and organic matter degradation during batch assay.

BMP assay	Mixtures	%SRA	%PS	*C*/*N*	Organic matter rem. (%)		
		(VS basis)			COD_t	TS	VS
SRA + PS	1.1	100	0	7.2	61.8 ± 1.6	41.5 ± 4.8	58.1 ± 4.3
	1.2	74	26	10.0	50.4 ± 2.2	26.8 ± 0.9	52.0 ± 1.3
	1.3	49	51	15.0	52.7 ± 6.6	10.5 ± 1.8	34.2 ± 1.7
	1.4	35	65	20.0	30.0 ± 4.4	12.6 ± 2.3	33.8 ± 2.3
	1.5	0	100	80.7	ND	ND	26.04 ± 1.9

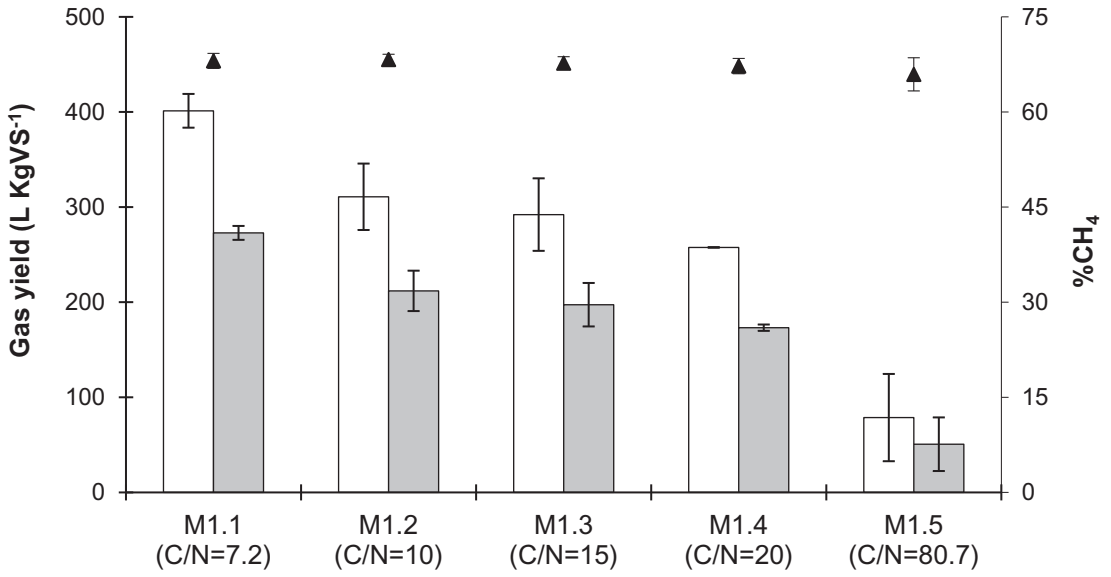

Fig. 2. Biogas yield (white bars), methane yield (gray bars) and methane content of biogas (▲) of the co-digestion of SRA and PS. Error bars indicate standard error of the mean value.

Table 3
Analysis of synergistic/antagonistic effects during the co-digestion assays.

BMP assay	M.	*C*/*N* ratio	Expected[a] (L_{CH4} $kgVS^{-1}$)	Obtained (L_{CH4} $kgVS^{-1}$)
SRA + PS	1.2	10	128.4–149.3	173.1 ± 3.3
	1.3	15	159.5–177.5	197.3 ± 22.9
	1.4	20	215.0–227.8	211.9 ± 21.2
SRA + OM	2.2	10	220.7–227.2	166.7 ± 7.2
	2.3	15	181.6–187.5	177.1 ± 10.0
	2.4	20.1	163.4–168.9	156.7 ± 12.9
	2.5	23.7	155.5–161.0	165.7 ± 14.9

[a] Calculated from theoretical values of each individual substrate. Low figure of the interval corresponds to the calculation using mean values of each substrate and higher figure of the interval corresponds to the calculation using maximum values (average + standard error) of each substrate.

Table 4
Composition of mixtures of SRA–OM and organic matter removal during batch assay.

BMP assay	Mixtures	%SRA	%OM	*C*/*N*	Organic matter rem. (%)		
		(VS basis)			COD_t	TS	VS
SRA + OM	2.1	100	0	7.2	61.8 ± 1.6	41.5 ± 4.8	58.1 ± 4.3
	2.2	60	40	10.0	76.6 ± 2.2	39.6 ± 5.4	62.3 ± 3.7
	2.3	30	70	15.0	66.7 ± 11.0	49.1 ± 1.8	75.1 ± 1.2
	2.4	16	84	20.1	79.5 ± 9.0	61.6 ± 5.4	82.3 ± 4.8
	2.5	10	90	23.7	78.9 ± 5.2	50.2 ± 4.4	77.9 ± 0.3
	2.6	0	100	34.3	77.6 ± 0.8	58.0 ± 2.5	80.0 ± 2.1

were rich in proteins (31.3%) and lipids (19.6%) yielding biogas with a higher methane content when digested as a sole substrate.

In other studies microalgae were co-digested and improvements in the digestion process were observed. Yen and Brune [13] observed in digesters operated with waste paper and algal sludge (composed of *Chlorella* and *Scenedesmus*) a twofold higher (292.5 L_{CH4} $kgVS^{-1}$) methane yield than that observed in algae digestion alone, and the improvement was ascribed to the nutrient balance. Zhong et al. [15] also observed an improvement in the digestion process of Taihu blue algae when the *C*/*N* ratio of the feedstock was increased by the addition of corn straw. The improvement of the methane yield was 61.7% and it was attributed to the greater number of nutrients and the increased buffer capacity of the system. The comparison of the results obtained by these authors and the results obtained in this study point out the need for studying co-digestion individually for each combination of substrates, without the possibility of extrapolating results for biomasses of similar origin.

In the co-digestion of SRA and OM no synergistic effects were observed for any of the mixtures analyzed (Table 3). Theoretical methane potentials of individual substrates were 272.8 ± 7.3 L $kgVS^{-1}$ for SRA and 142.5 ± 5.33 L $kgVS^{-1}$ for OM. For M2.3, M2.4 and M2.5 (with a *C*/*N* ratio of 15, 20.1 and 23.7, respectively) the obtained methane yields were very close to the expected ones. In the case of M2.2 (with a *C*/*N* ratio of 10), the obtained methane yield was clearly lower than the expected yield. Antagonistic effects have been described in other studies when substrates of different nature are co-digested in defined proportions [34]. This could be the case of this specific mixture.

It is important to point out that the balance of the *C*/*N* ratio is not the only factor to take into account for the digestion of high nitrogenous substrates. It can be observed that the synergistic or

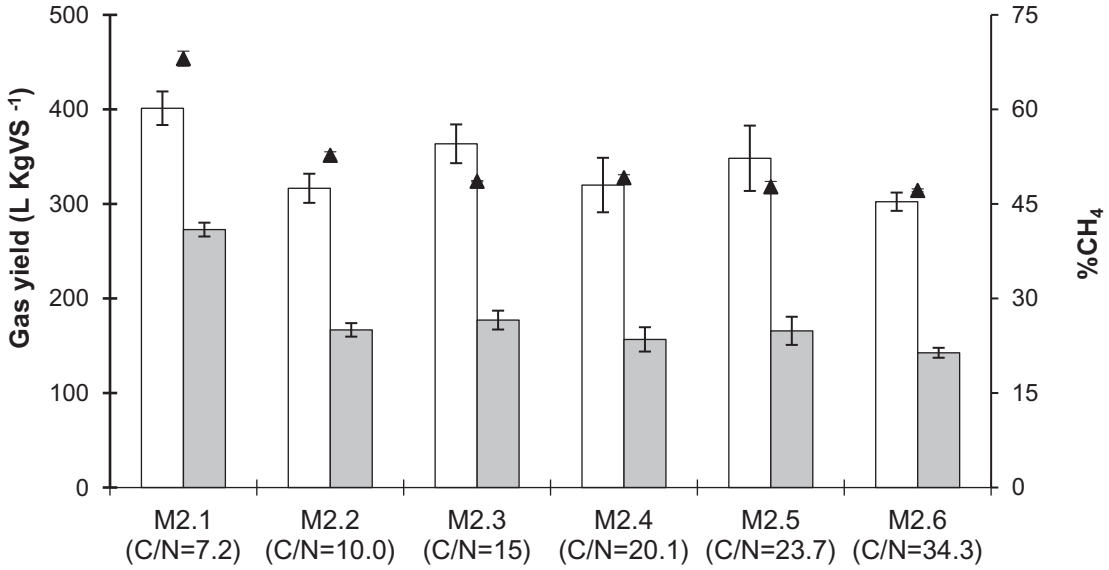

Fig. 3. Biogas yield (white bars), methane yield (gray bars) and methane content of biogas (▲) of the co-digestion of SRA and OM. Error bars indicate standard error of the mean value.

antagonistic effects were not dependent on the *C*/*N* ratio. The co-digestion of SRA and PS showed synergistic effects at a *C*/*N* of 10, whereas co-digestion of SRA and OM showed antagonistic effects at the same *C*/*N*.

There were important differences among the specific rate constant of the different mixtures of SRA–OM and the individual substrates. An increase in the *C*/*N* as a consequence of a higher content of OM in the mixture caused an increase in k_0 up to a *C*/*N* of 20.1 ($0.154 \pm 0.011\ d^{-1}$, $0.168 \pm 0.008\ d^{-1}$ and $0.2495 \pm 0.0205\ d^{-1}$ for *C*/*N* ratios of 10, 15 and 20.1, respectively). *C*/*N* ratios higher than 20.1 caused a decrease in k_0 ($0.181 \pm 0.011\ d^{-1}$ at *C*/*N* ratio of 23.7), suggesting an optimum *C*/*N* around 20.1 for the kinetics of the process. Co-digestion of SRA and OM caused an improvement in the specific rate constant compared to the mono-digestion of individual substrates ($0.108 \pm 0.007\ d^{-1}$ for SRA and 0.132 ± 0.013 for OM). Moreover, the specific rate constant of mixtures containing OM were higher than these containing PS. OM is mainly composed of carbohydrates that are easily biodegradable, and therefore, the digestion process proceeded faster in mixtures containing OM as co-substrate than in mixtures containing PS.

3.3.3. Co-digestion of SRL and GLY

In Table 5, mixtures analyzed in the BMP assays are described. The addition of GLY aimed for an increase in methane production. GLY can balance the *C*/*N* ratio of the feedstock introduced into the digester and it gives a source of organic matter able to be readily converted into biogas. However, due to its low alkalinity and its fast degradation, it can destabilize reactors causing a high accumulation of VFA that can reduce digesters pH [35]. Regarding the composition of the mixtures, M3.2 is done based on the assumption of using the exact quantity of glycerol obtained during the transesterification process of the lipids extracted from microalgae, assuming an extraction method that is able to extract the total lipid content [14]. The other mixtures were prepared in order to study the effect of higher *C*/*N* ratios and higher content of GLY, assuming that it is possible to have larger amounts of glycerin available.

The addition of GLY to SRL had different effects on the methane yield (Fig. 4). The addition of 2.3% GLY (VS basis) to SRL (M3.2) caused a slight decrease in methane yield ($185.7 \pm 25.7\ L_{CH4}$ - $kgVS^{-1}$) compared to SRL. However, the high variation of the results obtained from M3.2 did not allow authors to conclude that there was a difference between the addition of glycerin in this amount and the mono-digestion of SRL. Mixtures containing 11.1% and 19.8%VS of GLY showed a higher methane yield than SRL as sole substrate. This increase was higher in M3.3 than in M3.4, being 20% and 7.4% compared to SRL, respectively. The increase in the methane yield was attributed to the higher *C*/*N* ratio that was supposed to favor bacterial activity and to the increased availability of organic matter, since glycerol is easily biodegradable. On the other hand, VS and COD removal increased slightly in most of the co-digestion batches. COD and VS removal during the mono-digestion of SRL was higher than 60%, whereas the removal of VS when SRL was co-digested with GLY was also higher than 60% in all samples. COD removal was lower than 50% for M3.2, whereas M3.3 and M3.4 showed a COD removal slightly higher to that obtained for SRL in mono-digestion.

The benefit of using glycerol as co-substrate of lipid-extracted microalgae has been showed in other works. Ehimen et al. [14] obtained an increase in the methane yield of 4–7% depending on the lipid extraction method applied to microalgae. Residual glycerin has also been used as co-substrate of pig manure, increasing the

Table 5
Composition of the mixtures of SRL and GLY analyzed.

BMP assay	Mixtures	%SRL	%GLY	*C*/*N*	Organic matter rem. (%)		
		(VS basis)			COD_t	TS	VS
SRL + GLY	3.1	100	0	6.1	67.2 ± 0.9	44.8 ± 1.6	63.8 ± 1.5
	3.2	97.7	2.3	6.2	46.0 ± 7.5	41.3 ± 5.3	63.1 ± 6.1
	3.3	88.9	11.1	6.8	70.9 ± 6.8	48.1 ± 6.2	68.0 ± 2.6
	3.4	80.2	19.8	7.5	73.5 ± 3.5	51.2 ± 3.2	64.7 ± 4.9

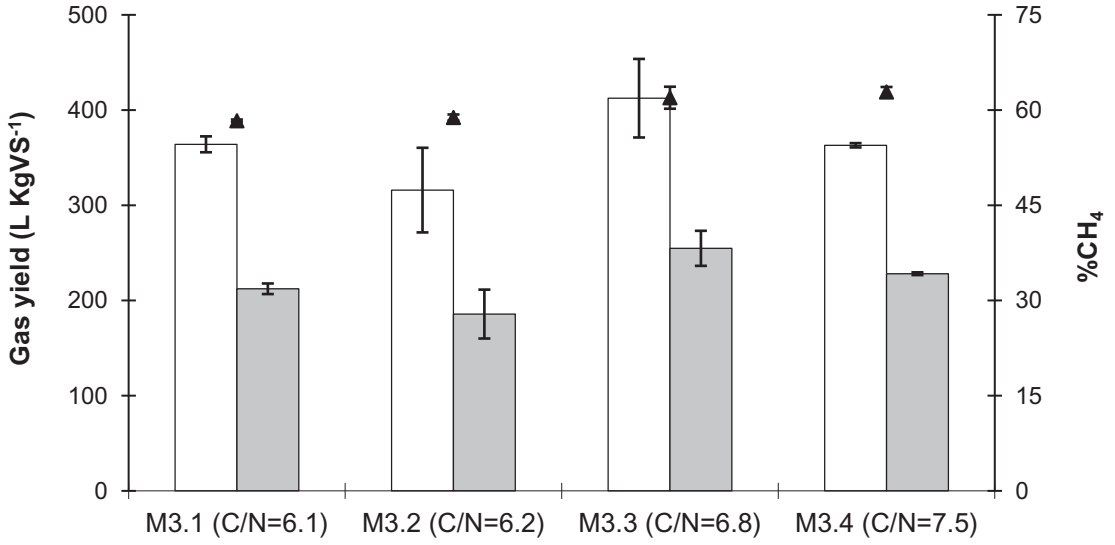

Fig. 4. Biogas yield (white bars), methane yield (gray bars) and methane content of biogas (▲) of SRL–GLY co-digestion assay. Error bars indicate standard error of the mean value.

methane yield in mixtures composed of up to 60% of residual glycerin (fresh matter basis) [35]. Mixtures of higher GLY content were not tested in this study; however, having into account the growing glycerin oversupply due to the increase in the biodiesel industry, it could be worth to study the effect of adding larger quantities of GLY.

The specific rate constant (k_0) in the mono-digestion of SRL was $0.101 \pm 0.005\ d^{-1}$. Higher fractions of GLY in the mixtures caused an increase in k_0 (0.105 ± 0.006 for M3.2; 0.106 ± 0.004 for M3.3 and 0.189 ± 0.014 for M3.4). The increase in k_0 was attributed to the rapid degradation of GLY with the subsequent higher volume of methane produced in the first days of the digestion process.

4. Conclusions

Methane yield of *Scenedesmus* residues generated after amino acid or lipid extraction increased substantially compared to raw biomass. Also, the rate of methane production was improved. The improvements were attributed to the disruption of the cell wall and the organic matter solubilization. Co-digestion with carbon-rich substrates improved the kinetics of the process. However, the effect of co-digestion on the methane yield was not the expected at the beginning of the experiments. Generally, codigestion caused a decrease in methane yields. The high methane yields obtained in the monodigestion of *Scenedesmus* residues show that the coupling of microalgae industry and anaerobic digestion looks promising.

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