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Optimization of the digestion process of *Scenedesmus* sp. and *Opuntia maxima* for biogas production

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ABSTRACT

Scenedesmus biomass is not an adequate substrate for anaerobic digestion due to its low biodegradability and low biogas yield. This study aims to evaluate the anaerobic co-digestion of *Scenedesmus* microalgal biomass and *Opuntia maxima* cladodes, the latter added in order to improve the digestion process. Batch assays were conducted to evaluate possible synergistic effects in different mixtures of both substrates. Mixture with highest methane yield was digested in semi-continuous mode at different VS concentrations. Feedstock composed of 75% *O. maxima* and 25% *Scenedesmus* (VS basis) showed the highest methane yield increasing 66.4% and 63.9% that of *Scenedesmus* and *O. maxima*, respectively. In semi-continuous mode, ideal organic loading rate (OLR) with 6%VS feed concentration was 4 gVS L^{-1} d^{-1}, which yielded 292 ± 39 L_{CH4} $kgVS^{-1}$ (15 days HRT). In the case of 8%VS feed concentration ideal OLR was 5.33 gVS L^{-1} d^{-1}, which yielded 308 ± 22 L_{CH4} $kgVS^{-1}$ (15 days HRT). The co-digestion of *O. maxima* and *Scenedesmus* biomass enhanced the anaerobic digestion process and avoided inhibition caused by low C/N ratio of microalgae.

1. Introduction

Biofuels production has increased exponentially during last decade [1], as well as the different environmental and socio-economical problems that may go along with them. Microalgae, as source for biofuels, are a plausible solution for most of the problems that first generation biofuels entail. Microalgae can be mass-produced on marginal lands and wastewater without competing against terrestrial crops and tropical forests for land [2]. Moreover, microalgae show other advantages compared to other energy crops, such as high productivities [3] and high CO_2 consumption during their growth [4]. Several types of biofuels can be produced from microalgae: biodiesel, bioethanol, biohydrogen, syngas and biogas [5]. In the case of biogas production, using microalgae as feedstock, the energy coming from the sun is turned into chemical energy in the form of methane. During the digestion, organic nutrients are mineralized and they can be recycled to culture new microalgal biomass [6,7]. However, the anaerobic digestion of microalgae has typically shown two problems: low biodegradability of microalgal cells [8,9] and imbalance of nutrients due to high content of nitrogen [10,11]. The low biodegradability of cells is consequence of the composition of the cell walls of some strains (such as *Scenedesmus* and *Chlorella*). These cell walls are composed of complex carbohydrates [12] that are difficult to degrade. They protect intracellular organic molecules from bacterial attack, avoiding their degradation and conversion to biogas. Golueke et al. [13] already faced this problem when trying to digest a mix of microalgae, consisting mainly of *Scenedesmus* sp. and *Chlorella* sp. Mussgnug et al. [9] found also low cell disintegration when digesting *Scenedesmus obliquus* and *Chlorella kessleri*, but high disintegration in species without cell walls. González-Fernández et al. [14] also observed low biodegradability and biogas production when using an algae mixture composed of *S. obliquus* and *Chlorella vulgaris* as co-substrate of swine manure.

On the other hand, the low C/N ratio of the biomass is often associated with toxicity problems. Excessive ammonia nitrogen can be released inhibiting the growth of microorganisms and consequently spoiling or even stopping the digestion process. Co-digestion has been studied to overcome the low C/N ratio of microalgal biomass. High carbon content substrates were added to digesters fed with microalgal biomass, reducing ammonia concentration release inside digesters and avoiding toxicity problems. Samson and LeDuy [10] obtained good results when co-digesting *Spirulina maxima* with high carbon content residues (primary domestic sewage sludge and peat hydrolyzate). Both substrates

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produced synergistic effects on the anaerobic digestion of microalgal biomass enhancing its biogas production. Yen and Brune [11] co-digested waste paper with algal sludge. According to these authors, an adequate C/N ratio turned out in an increase of biogas and methane yield of more than twofold compared to microalgal sludge digested alone. Besides the balance C/N ratio, authors found an increase in cellulase activity during co-digestion that might be helpful in the biodegradation of algal sludge. Conversely, in other study co-digestion of microalgae with carbon-rich substrates produced no synergistic effects and a decrease in the methane yield [15].

In this work, *Opuntia maxima* is suggested as energy crop to be used in co-digestion with microalgal biomass. It is ideal for low rainfall, semiarid zones, where it grows with scarce nutrients and water inputs. It can be cultured in almost any type of soils, including marginal lands [16], where microalgae are supposed to be ideally grown to avoid competition with edible crops. It is rich in sugars, accumulates high quantities of water, has a high C/N ratio and, theoretically, it can reach high biomass productivities (up to 50 t of dry matter ha^{-1} y^{-1}) [17]. Consequently, it is an ideal energy crop for anaerobic digestion, especially, for its co-digestion with substrates of high nitrogen content, such as microalgae. *O. maxima* culture for biogas production has not being previously explored in depth. However, due to the agronomical characteristics of *O. maxima*, its use as energy crop for biogas production in Mediterranean countries is promising. In the specific case of Spain, the use of energy crops typically cultured for biogas production (e.g. maize silage in Germany) is not viable due to climatic constraints. Therefore, the study of *O. maxima* as co-substrate can contribute significantly to the development of the biogas industry.

Since the mono-digestion of *Scenedesmus* biomass normally did not show good results, the main goal of this work was to evaluate the anaerobic co-digestion of *Scenedesmus* sp. and *O. maxima*. A screening of the biodegradation and biogas production of several mixtures of both substrates was performed by carrying out biochemical methane potential (BMP) assays. When the most favorable mix ratio was found, semi-continuous experiments were performed to study possible inhibition processes when running a digester fed with *Scenedesmus* sp. and *O. maxima* at different feedstock concentrations.

2. Material and methods

2.1. Substrates and anaerobic biomass

Scenedesmus biomass was kindly provided by *Fundación Cajamar*. It was cultured photoautotrophically in photobioreactors located in Almería (Spain). Biomass was harvested and freeze-dried before being conserved in a place with low humidity and irradiation until its use in the anaerobic digestion assays.*O. maxima* plants grew without adding fertilizers and irrigation to simulate cultivation with low inputs. Plants were harvested in two zones of the Autonomous Community of Madrid (Spain) at different times during the experimentation. Only cladodes of one- or two-year-age were used, since old cladodes increased their proportion in lignocellulose [18], hardly biodegradable. Humidity and volatile solids content of *O. maxima* cladodes (OM) fluctuate according to local rainfall, cladode age and plant. Therefore, different cladodes were homogenized and analyzed before being used in the assays. The preparation of the mixtures included the crushing of OM and its blend with *Scenedesmus* freeze-dried biomass in the required proportion. Furthermore, in the semi-continuous assay the feedstock was adjusted to the desired VS concentration by adding tap water.

The inoculum used in the BMP assay and the first semi-continuous assay performed (6%VS concentration) was anaerobic sludge collected from a wastewater treatment plant in Madrid, working at 37 °C, mixed (50% in volume) with an inoculum with the same origin that was co-digesting OM and *Scenedesmus* in lab reactors during two weeks at low organic loading rates (OLR) (<2 gVS L^{-1} d^{-1}). The inoculum of the BMP assay showed a TS and VS concentration of 3.66% and 2.05%, respectively. Ammonia concentration was 867 mg NH_4^+ L^{-1}. pH was 8.6 and partial alkalinity (PA) was 5026 mg $CaCO_3$ L^{-1}. In the first semi-continuous assay the inoculum had a TS and VS concentration of 4.59% and 2.21%, respectively. Ammonia concentration was 992 mg NH_4^+ L^{-1}, pH was 7.57 and PA was 6184 mg $CaCO_3$ L^{-1}. In the second semi-continuous assay performed (8%VS concentration) the inoculum was obtained during the previous experimentation after more than two months working with the same substrates in semi-continuous mode. TS and VS concentration were 4.64% and 2.48%, respectively. Total ammonia concentration was 1160 mg NH_4^+ L^{-1}. pH was 7.58 and it showed a high PA (5997 mg $CaCO_3$ L^{-1}).

2.2. Biochemical methane potential (BMP) assays

BMP assays were carried out according to the guideline VDI 4630 [19]. A screening of different blends of *Scenedesmus* and OM was performed (see Table 1). These blends were placed into 1 L reactors with the corresponding amount of inoculum according to the VDI 4630 ($VS_{substrate}/VS_{inoculum}$ = 0.5). Same procedure was followed when digesting *Scenedesmus* and OM as single substrates. Every assay was performed in duplicate. Blank reactors were run only with inoculum for the determination of endogenous methane production. All reactors were tightly closed and submerged into water kept at 37 °C by a thermostatic bath. The head space of each reactor was flushed with nitrogen to ensure anaerobic conditions prior to starting the digestion tests. Content of reactors was continuously stirred by magnetic bars. The duration of the test was approximately 40 days.

Ultimate analyses of substrates were performed before blending them in order to balance C/N ratio of the mixtures. Analyses of TS, VS, total and soluble chemical oxygen demand (COD_t, COD_s), total Kjeldahl nitrogen (TKN), PA and total alkalinity (TA) and pH, were performed at the beginning and at the end of the assay. In Section 3.2 *BMP assay*, only relevant data are shown. Data of TS, VS and COD_t were used to calculate removal of each of them, having into account the influence of the inoculum. All the other parameters measured were used as control parameters.

Biogas composition and production was monitored with the Micro-Oxymax respirometer (Columbus Instrument; Columbus, OH 43204, U.S.A.). Biogas produced in the reactors was accumulated in measuring chambers, which volume was exactly calculated by the Micro-Oxymax respirometer. The fractions of CO_2 and CH_4 of the measuring chamber environments were measured periodically and their changes in level were used to compute biogas production.

Table 1
Composition of mixtures analyzed in the BMP assays and organic matter removal results.[a]

M.	*Scenedesmus* sp. (%VS)	*O. maxima* (%VS)	C/N ratio	Organic matter removal		
				COD_t (%)	TS (%)	VS (%)
1	100	0	6.0	21.3 ± 3.2	31.3 ± 9.6	48.2 ± 3.7
2	75	25	7.3	33.7 ± 14.9	48.9 ± 11.6	59.5 ± 8.2
3	50	50	9.7	72.8 ± 2.8	71.3 ± 2.7	81.5 ± 2.3
4	25	75	15.6	81.8 ± 3.2	57.2 ± 15.0	71.9 ± 11.3
5	0	100	51.3	77.6 ± 0.8	58.0 ± 2.5	80.0 ± 2.1

[a] Results exposed in table are the mean values of the duplicate reactors (±standard error). Influence of inoculum is already subtracted.

2.3. Semi-continuous assay

The assay in semi-continuous mode was performed in three continuously stirred tank reactors (CSTR) of 3 L working volume. CSTR were kept at constant temperature in the mesophilic range (37 °C) by water heated by thermostatic baths and pumped around the reactors through a double wall glass. Content of reactors was continuously stirred at 30–40 rpm.

OM shows different water content depending on the season in which it is harvested, whereas microalgae biomass concentration depends on the harvesting methods. The mixture of both substrates which yielded highest methane at the end of the BMP assays was fed at two different constant concentrations in assay 1 and assay 2 (6%VS and 8%VS, respectively). These concentrations are expected if harvesting of *Scenedesmus* is made by flocculation (with possible centrifugation later) and OM has high or middle content of water (90–94%). Lower VS concentrations were not studied since very low feedstock concentrations are normally considered anti-economic due to large volumes of digesters required. A higher VS concentration would require a very energy intensive drying method of microalgae or very low water content in OM; therefore also discarded.

Different OLR were tried for each of the assays (see Table 2). OLR levels were increased when constant biogas production was achieved, as suggested in VDI 4630. Feed and discharge of reactors were done manually every day.

Reactors were monitored by means of chemical and physical analysis of the digestate and biogas production and composition. pH was measured daily. TS, VS, COD_t and COD_s, PA and TA, volatile acids (VA), TKN and total ammonia nitrogen (TAN) were measured once a week.

Volume and composition of biogas was monitored continuously using *Milligascounters*® (Dr. Ing. Ritter Apparatebau GMBH & Co. KG; Bochum, Germany). Biogas produced was accumulated in 5 L bags and its composition was measured each time bags were full by means of an *Awite® serie 6 gas analyzer* (Awite Bioenergie GmbH, Langenbach, Germany).

2.4. Analytical methods

Ultimate analysis (C, H, N, S) were carried out by combustion using a LECO TruSpec CHNS macroanalyzer. pH analyses were performed using a Crison pH sensor submerged directly into the reactors. The pH of SB was analyzed after dilution with water at a ratio 1:2.5 (substrate:water) and agitation during two hours. TS and VS were analyzed according to APHA. PA and TA were determined by titration to pH 5.75 and 4.3, respectively [20]. COD_t and COD_s were analyzed by an adaptation of the 410.4 method of U.S. EPA. SB was leached for two hours at a ratio substrate: water 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. Free ammonia (FA), as unionized ammonia (NH_3), was calculated using TAN concentration considering the equilibrium between NH_4^+ and NH_3 inside reactors, which depends on temperature and pH [21]. VA were analyzed by steam distillation and titration of the distillate to pH 8.6. Analyses were performed in triplicates.

Gas analysis during the semi-continuous assay was performed by different sensors that are part of the *Awite® serie 6 gas analyzer*. Infrared sensors were used to measure methane (0–100%) and carbon dioxide (0–100%) and electrochemical sensors were used to measure hydrogen sulfide (0–5000 ppm), hydrogen (0–5000 ppm) and oxygen (0–25%).

Table 2
Experimental design of the semi-continuous assay.

Assay	VS concentration (%)	T (°C)	HRT (days)	OLR (gVS L^{-1} d^{-1})
1	6	37	30	2
		37	15	4
		37	10	6
2	8	37	40	2
		37	20	4
		37	15	5.33
		37	12	6.67

2.5. Kinetics of degradation

The kinetic of the discontinuous process was analyzed for the different batch assays performed. The following kinetics equation previously proposed in several studies [22,23] 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 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 [23].

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 v11.5*. k_0 is highly dependent on operational factors, such as temperature, stirring and the initial substrate concentration [23]. 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.

2.6. Statistical analysis

ANOVA statistical analyses were performed to determine significant differences of biogas yield, methane yield and digester efficiency among the OLR's tried in the semi-continuous assays. These parameters are helpful to determine optimal operation conditions.*ANOVA* analysis can be run only if populations are normally distributed and their variances are equal. These two requirements are fulfilled with biogas and methane yield. If there were significant differences ($p < 0.05$), HSD Tukey *post hoc* analysis was used to determine which groups were significantly different ($p < 0.05$). Digester efficiency was normally distributed but with heterogeneous variances. Therefore, the Welch statistic was used. If there were significant differences ($p < 0.05$), Games-Howell *post hoc* analysis was used to determine which groups were significantly different ($p < 0.05$).

Linear correlations between some parameters analyzed during the two semi-continuous assays were studied using the Pearson correlation coefficient (r). Correlations were considered significant with a confidence interval of at least 95% ($p < 0.05$). Correlation studies were performed individually for each assay.

All statistical analyses were performed using the *SPSSv.11.5.1* software.

3. Results and discussion

3.1. Characterization of substrates

Composition of *Scenedesmus* and *O. maxima* cladodes (OM) is shown in Table 3. Both substrates had a high fraction of volatile solids, indicating a high percentage of organic matter. *Scenedesmus* biomass showed a low C/N ratio, as it was expected due to the high protein content of microalgae [3], whereas OM used in both batch and semi-continuous assays showed a high C/N ratio. C/N ratio of OM used in batch assay was higher than that used in semi-continuous assays. However, after mixing it with *Scenedesmus* biomass the resulting C/N ratios of the feedstocks were similar.

The pH of *Scenedesmus* biomass was close to neutral, whereas pH of OM was acid (around 4.6) and it might affect the digestion process. Fraction of soluble COD in *Scenedesmus* biomass was low (9.0%) indicating a low solubility of organic matter. The low solubility of *Scenedesmus* biomass has also been observed by González-Fernández et al. [24]. On the other hand, COD_s fraction was very high in OM (28.4%). The high solubilization of organic matter suggested that it was highly available for microorganisms.

3.2. BMP assay

Main results obtained from the BMP assays regarding organic matter removal are summarized in Table 1. Biodegradability (in terms of COD_t removed), biogas and methane production of *Scenedesmus* sp. (M.1) were very low, 21.3 ± 3.2%, 177.4 ± 34.2 L_{biogas} $kgVS^{-1}$ and 140.3 ± 29.4 L_{CH4} $kgVS^{-1}$, respectively. These results showed that *Scenedesmus* biomass is hardly biodegradable, as it has been pointed out in other studies [9,14,24]. The low biodegradability is consequence of its cell wall, which is composed by complex carbohydrates [12], containing even sporopollenin like biopolymer, very difficult to degrade by microorganisms.

Table 3
Physical and chemical characteristics of substrates. Ultimate analyses expressed in % of dry matter.

	Scenedesmus sp.	*Opuntia maxima*[a]	*Opuntia maxima*[b]
TS (%)	93.6	14.1	10.9 (5.8)
VS (%TS)	71.7	81.7	79.8 (3.2)
COD_t (gO_2 kg^{-1})	1109.5	208.9	136.7 (51.2)
COD_s (gO_2 kg^{-1})	100.3	NA	38.8 (4.7)
pH	7.25	4.66	4.63
C (%)	41.6	36.9	37.35 (0.21)
N (%)	6.98	0.72	1.0 (0.3)
C/N ratio	5.95	51.3	36.3 (11.8)

NA: not analyzed; ND: non-detectable; (±standard deviation).
[a] *O. maxima* cladodes used in batch assays;
[b] *O. maxima* cladodes used in semi-continuous assays.

OM was highly biodegradable as sole substrate (M.5). Removal of COD_t was 77.8 ± 0.8% and biogas production was high (302.3 ± 9.7 L_{biogas} $kgVS^{-1}$). However, biogas methane content was poor (47.1%). This was probably consequence of the composition of young cladodes, very rich in soluble carbohydrates (up to 63.9% according to Sánchez [17]). Carbohydrates degrade faster than other organic molecules; nevertheless they yield biogas poor in methane [25]. A high proportion of soluble carbohydrates could also cause a destabilization at the beginning of the anaerobic reaction. Acidogenic and acetogenic bacteria worked faster than methanogenic bacteria and consequently, CO_2 could be produced at high rates at the beginning of the process.

The co-digestion of microalgae and OM improved organic matter removal of the mixtures compared to the mono-digestion of microalgal biomass. This was probably consequence of two reasons. First, the decrease of *Scenedesmus* in the mixture, that has shown to be poorly biodegradable; and secondly, the increase of the C/N ratio that improved microorganisms activity and therefore biodegradability. For M.4 methane yield was 233.6 ± 16.4 L $kgVS^{-1}$ and increased by 66.4% and 63.9% compared to *Scenedesmus* biomass and OM digested alone (see Fig. 1). The methane yield obtained experimentally from this mixture indicated synergistic effects, since the mixture yielded more methane than that calculated from the methane potential of each individual substrate [23].

The use of OM as co-substrate also improved the kinetics of the process. The specific rate constant (k_0) of *Scenedesmus* in mono-digestion was 0.0902 ± 0.0025 d^{-1}. The addition of OM increased k_0 of all mixtures. Raw *C. vulgaris* showed a slightly higher k_0 than raw *Scenedesmus* in this study (0.10 d^{-1} vs. 0.0902 d^{-1}, respectively). Thermal pretreatment at 120 °C during 20 and 40 min applied to *C. vulgaris* increased k_0 up to 0.23 d^{-1} and 0.17 d^{-1}, respectively. Thermal pretreatments caused the disruption of microalgae cell walls and the solubilization of the organic matter, making easier its degradation [26]. Specific rate constants of M.2, M.3 and M.4 were 0.0915 ± 0.0051 d^{-1}, 0.1122 ± 0.052 d^{-1} and 0.098 ± 0.0049 d^{-1}, respectively. On the other hand, the specific rate constant of OM in mono-digestion was the highest (0.1262 ± 0.0122 d^{-1}). OM is mainly composed of carbohydrates that are easily biodegradable, and therefore, the digestion process proceeded faster in mixtures containing OM. A faster digestion process could result in a decrease of the costs by reducing digester volumes and HRT.

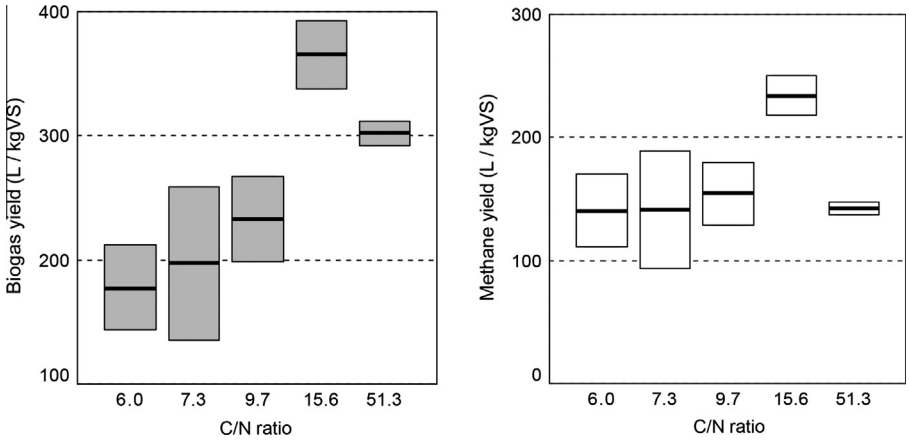

Fig. 1. Mean biogas and methane yield of the different mixtures. Blank influence on each reactor is subtracted. The edges of rectangles indicate value of duplicates.

The mixture with the overall best performance in the BMP assay was M.4; therefore it was used in the semi-continuous assays.

3.3. Co-digestion in semi-continuous mode

Main results obtained during the semi-continuous assays are shown in Table 4 (6%VS feed concentration, first assay) and Table 5 (8%VS feed concentration, second assay).

3.3.1. Organic matter degradation

COD_t and VS removals showed a decreasing tendency when HRT decreased, since short HRT prevented organic matter to be completely degraded.

In the first assay the highest VS removal was 63.0 ± 3.6% for the lowest OLR. VS removal decreased when OLR increased reaching a minimum of 51.5 ± 2.9%. COD_t removal suffered the highest variation when the OLR was increased up to 6 gVS L^{-1} d^{-1}. For the first two OLR levels, COD_t removal was acceptable, being higher than 50%. When the OLR was increased again, the COD_t removal decreased by 46.8% compared to the highest removal.

In the second assay, organic matter degradation was in the same range as in the first assay. The highest VS removal was 64.1 ± 1.8% for the lowest OLR, and it decreased with the increasing OLR. At an OLR level of 6.67 gVS L^{-1} d^{-1} (12 days HRT) VS removal was 52.0 ± 1.1%. COD_t removal was high during the whole assay. For the lowest OLR, COD_t removal was 66.8 ± 4.4%. As OLR increased, removal of COD_t also decreased, but remained around 50%.

These results showed that the mixture of OM and *Scenedesmus* was highly biodegradable even at short HRT and high OLR. VS removal was above 50% even for a HRT of 10 days. Other studies have shown a low organic matter removal of *Scenedesmus* biomass as single substrate, even when pretreatments were applied to ease its biodegradation. González-Fernández et al. [24] used raw *Scenedesmus* and thermally pretreated *Scenedesmus* at low OLR and 15 days HRT observing around 30–44%COD_t removal for pretreated biomass.

The mix of OM and microalgal biomass enhanced the digestion process by increasing the degradation of organic matter and consequently increasing biogas production. In anaerobic digestion processes it is very important to reduce the necessity of pretreatments or large digesters in order to make the process environmentally friendly and economically viable. The high degradation of this feedstock even at high OLR and short HRT contribute to this goal. However, due to the low biodegradation of *Scenedesmus* biomass observed in other studies in semi-continuous digestion [24], it was important to determine whether both substrates were used in the same extent by anaerobic microorganisms. Nitrogen mineralization was analyzed to determine the degree of degradation of microalgae inside the digesters.

3.3.2. Nitrogen mineralization

The main contributor of nitrogen in the system studied were the proteins of microalgal biomass. Nitrogen mineralization is an indicator of the degree of degradation of microalgae since organic nitrogen is converted to ammonia nitrogen during the anaerobic digestion process. On the other hand, large nitrogen mineralization and the subsequent ammonia accumulation inside digesters could lead to the inhibition of microorganisms.

Nitrogen mineralization is indicated by TAN concentration (mg NH_4^+ L^{-1}) inside digesters. In the first assay TAN decreased at the first OLR level to almost half of it at second and third OLR levels. The evolution of TAN concentration during the digestion process of *Scenedesmus* and OM at 6%VS concentration is shown in Fig. 2a. TAN and FA concentrations showed a negative correlation with the OLR ($p < 0.01$).

In the second assay, TAN concentration was 1080.0 ± 92.1 mg NH_4^+ L^{-1} at 2 gVS L^{-1} d^{-1} (40 days HRT). The evolution of TAN concentration during the digestion of *Scenedesmus* and OM at 8%VS concentration is shown in Fig. 3a. As in the first assay, TAN and FA were negatively correlated with the OLR ($p < 0.01$).

The fraction of ammonia nitrogen (N–NH_4^+) in the feedstock was 8.2% of the total nitrogen. At 40 days HRT, 31.4% of total nitrogen was in the form of N–NH_4^+ in the digestate. When HRT decreased to 20, 15 and 12 days, the fraction of N–NH_4^+ decreased to 13.5%, 14.9% and 11.0% respectively. These low fractions of N–NH_4^+ suggested that the degree of degradation of microalgae was very low. As it was indicated already, *Scenedesmus* cells have hardly biodegradable cell walls that prevent microorganisms to degrade their cells content. In fact, the low mineralization of nitrogen observed during the whole assay suggests that a long HRT is needed to allow microorganisms to degrade *Scenedesmus*. Mussgnug et al. [9] observed intact *S. obliquus* cells after more than 6 months in batch

Table 4
Conditions inside digesters and biogas composition at the different OLR with a feed concentration of 6% VS (mean values of triplicate reactors ± standard deviation).

OLR (gVS $L_{digester}^{-1}$ d^{-1})	2	4	6
HRT (days)	30	15	10
TS removal (%)	45.3 ± 6.6	42.5 ± 2.0	38.2 ± 2.4
VS removal (%)	63.0 ± 3.6	55.6 ± 2.0	51.5 ± 2.9
COD_t removal (%)	55.3 ± 5.4	53.0 ± 4.9	29.4 ± 5.1
COD_s (mgO_2 L^{-1})	2010 ± 205	3272 ± 747	11,524 ± 4225
VA ($gHAc_{eq}$ L^{-1})[a]	0.83 ± 0.42	1.02 ± 0.26	1.85 ± 0.85
PA ($mgCaCO_3$ L^{-1})	5011 ± 102	4336 ± 619	4452 ± 320
IA/PA ratio	0.16 ± 0.02	0.24 ± 0.03	0.58 ± 0.22
TAN (mg NH_4^+ L^{-1})	916.6 ± 97.7	515.8 ± 152.6	550.0 ± 87.5
CH_4 (%)	48.7 ± 1.2	49.8 ± 1.9	45.5 ± 2.5

[a] $gHAc_{eq}$ = g acetic acid equivalents.

Table 5
Conditions inside digesters and biogas composition at the different OLR with a feed concentration of 8%VS (mean values of triplicate reactors ± standard deviation).

LR (gVS $L_{digester}^{-1}$ d^{-1})	2	4	5.33	6.67
HRT (days)	40	20	15	12
TS removal (%)	48.9 ± 2.7	46.1 ± 3.6	40.8 ± 3.4	38.8 ± 0.4
VS removal (%)	64.1 ± 1.8	57.0 ± 8.5	54.1 ± 2.6	52.0 ± 1.1
COD_t removal (%)	66.8 ± 4.4	48.4 ± 2.1	53.3 ± 2.2	49.0 ± 4.0
COD_s (mgO_2 L^{-1})	3604 ± 186	5531 ± 788	5559 ± 1865	6917 ± 2771
VA ($gHAc_{eq}$ L^{-1})[a]	NA	1.21 ± 0.08	1.72 ± 0.07	1.99 ± 0.12
PA ($mgCaCO_3$ L^{-1})	6923 ± 206	6460 ± 98	6847 ± 213	6240 ± 5
IA/PA ratio	0.25 ± 0.02	0.30 ± 0.01	0.34 ± 0.02	0.41 ± 0.00
TAN (mg NH_4^+ L^{-1})	1080.1 ± 92.1	612.5 ± 41.1	695.8 ± 13.0	567.9 ± 25.3
CH_4 (%)	51.3 ± 1.0	50.8 ± 1.5	52.1 ± 1.0	50.8 ± 1.4

NA = not analyzed.
[a] $gHAc_{eq}$ = g acetic acid equivalents.

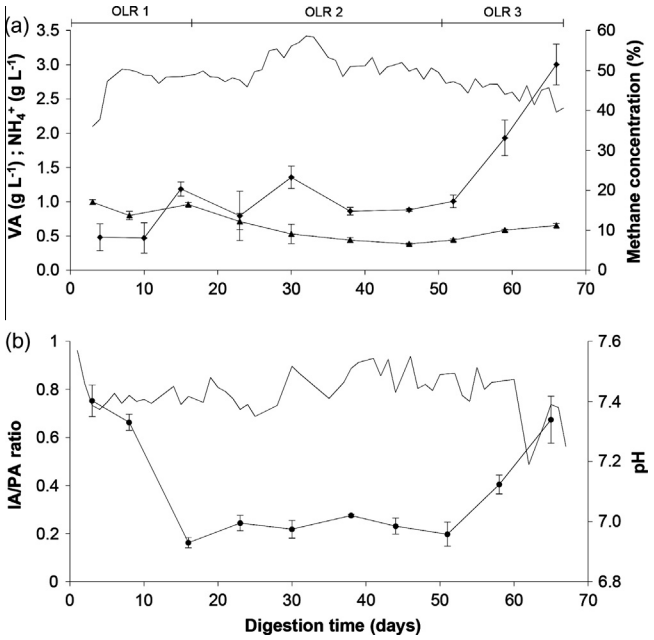

Fig. 2. (a) VA (♦), TAN (▲), and CH_4 concentration (-); (b) IA/PA ratio (●) and pH (-) during the co-digestion of *Scenedesmus* and OM at 6%VS concentration. Error bars indicate standard deviation.

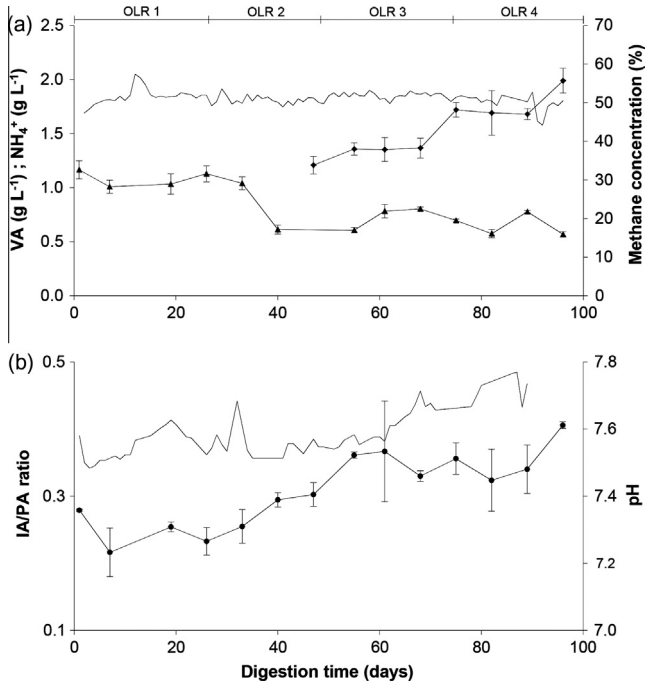

Fig. 3. (a) VA (♦), TAN (▲), and CH_4 concentration (-); (b) IA/PA ratio (●) and pH (-) during the co-digestion of *Scenedesmus* and OM at 8%VS concentration. Error bars indicate standard deviation.

digestion. Therefore, it seems likely that the potential biogas yield of the mixture of OM and *Scenedesmus* was not being fully obtained. It would be worth evaluating the extra energy yield reachable by applying pretreatments that break microalgae cell walls to make intracellular organic molecules accessible for microorganisms. There are several works dealing with pretreatment of microalgal biomass. Different techniques have shown promising results, such as sonication [27], high temperature [8,26,27] or thermal hydrolysis [28]. However, in some studies pretreated microalgae have shown inhibition problems when digested in continuous mode due to ammonia accumulation [29]. The combination of pretreatments and co-digestion could be a solution to increase biogas production without showing toxicity problems due to ammonia accumulation. In fact, this option has already been proved by co-digesting thermally pretreated *Nannochloropsis salina* and corn silage [30].

Free ammonia (FA) is the most toxic form of ammonia for microorganisms. FA is fully permeable through bacterial membrane, therefore it may cause severe toxicity at very low concentrations (>100 mg L^{-1}) [31]. During the first and second assay, FA concentration was always below 36 mg NH_3 L^{-1} and 60 mg NH_3 L^{-1}, respectively. Therefore, results suggested that co-digestion of microalgae with OM avoided TAN and FA concentrations to be over the levels reported as toxic for anaerobic microorganisms.

3.3.3. Biogas production

In Fig. 4 biogas yield and methane yield are shown for the two assays. Significant differences among OLR are indicated by different lower-case letters next to each point on the graph.

In the first assay biogas and methane yields reached their maximum at 4 gVS L^{-1} d^{-1} with significant differences compared to the others OLR (see Fig. 4a). At 2 gVS L^{-1} d^{-1} biogas and methane yields should theoretically be at least the same as at 4 gVS L^{-1} d^{-1}. Organic matter stayed more time inside the digesters; therefore, it should have been converted in a greater extent to biogas. However, biogas and methane yields at 2 gVS L^{-1} d^{-1} were lower. The reason of the lower yields at 2 gVS L^{-1} d^{-1} was probably an insufficient

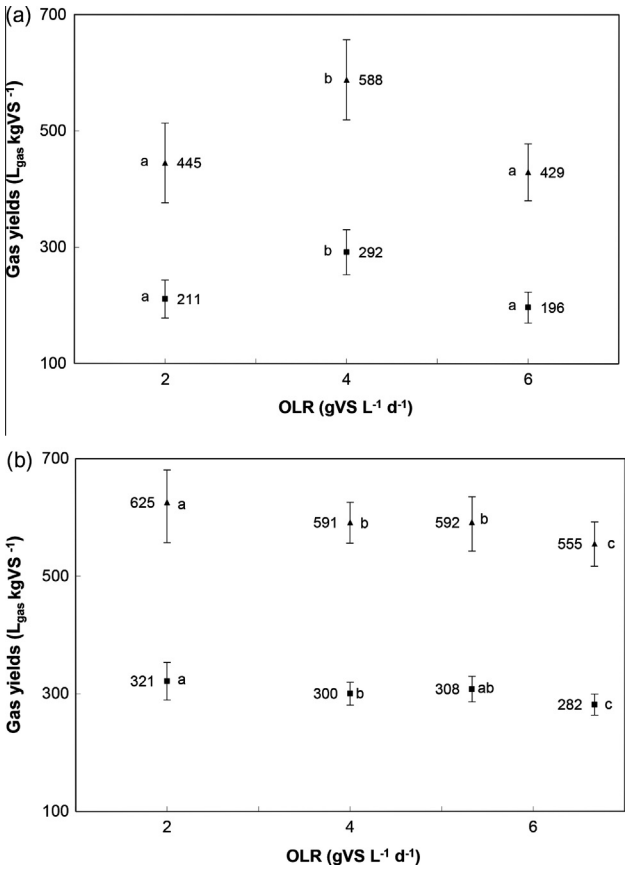

Fig. 4. Biogas (triangles) and methane (squares) yields at different OLR: (a) 6%VS feedstock concentration; and (b) 8%VS feedstock concentration. Error bars indicate standard deviation.

preadaptation of the inoculum to the co-digestion of microalgae and *O. maxima* at this initial phase of operation. On the other hand, at 6 gVS L^{-1} d^{-1}, biogas and methane yields decreased substantially before the collapse of the system. Control parameters suggested that the collapse of the system was probably consequence of the short HRT applied (10 days) and the washout of anaerobic microorganisms. Although IA/PA ratio increased over 0.3 (upper limit for a stable process) [20], there was no sign of overloading of the system besides the decrease of the biogas yield: the pH did not decrease below 7.2 and VA accumulation did not increase over 3 $gHAc_{eq}$ L^{-1} (see Fig. 2). Moreover, VA were not correlated with methane yield. In CSTR a short HRT produce the washout of microorganisms, since they are removed faster than they can reproduce [31].

In the second assay biogas and methane yields were higher than in the previous assay (see Fig. 4b). Highest biogas and methane yields were obtained for the lowest OLR and longest HRT. Gas yield remained constant, around 600 L_{biogas} $kgVS^{-1}$ and over 300 L_{CH4} $kgVS^{-1}$ at the second and third OLR tried, with no significant differences between them. At the last OLR tried, biogas yield decreased to 555 ± 38 L_{biogas} $kgVS^{-1}$ and methane yield to 282 ± 18 L_{CH4} $kgVS^{-1}$. VA concentration showed a negative correlation with biogas and methane yield ($p < 0.05$), indicating a possible inhibition of methanogens due to VA accumulation. However, concentration of VA was below 2 $gHAc_{eq}$ L^{-1} (see Fig. 3a). Higher concentrations of VA can be tolerated by methanogens as long as pH is inside the optimal range [31]. The pH remained inside adequate limits during the whole process although at the end of the experimentation IA/PA ratio increased over 0.3 (see Fig. 3b).

Biogas and methane yields obtained in this work were in the same range as results obtained in other study that evaluated the co-digestion of *Scenedesmus* and *Chlorella* [11]. Several assays of co-digestion with paper residues indicated an optimum C/N ratio of 22.6 with a maximum methane yield of 321 L $kgVS^{-1}$ at an HRT of 10 days and an OLR of 5 gVS L^{-1} d^{-1}. In this study the same maximum yield was obtained, although here longer HRT were needed. Alternatively, González-Fernández et al. [24] digested in semi-continuous mode thermally pretreated *Scenedesmus* as single substrate obtaining approximately 215 L_{CH4} $kgVS^{-1}$ at low OLR (1.3 gVS L^{-1} d^{-1}) and 15 days HRT (calculated according to the VS/COD ratio (0.5175) obtained from the description of the biomass).

Digester efficiency is a very important parameter to determine the feasibility of a biogas plant. Fig. 5 shows digester efficiency (in terms of L_{CH4} $m^{-3}_{digester}$ d^{-1})). Significant differences among OLR of the same assay are indicated by different lower-case letters next to each point on the graph.

In the first assay, at an OLR of 2 gVS L^{-1} d^{-1}, digester efficiency was very low, being below 500 L_{CH4} m^{-3} d^{-1} (Fig. 5a). When the OLR was increased, digester efficiency increased up to 1166 ± 154 L_{CH4} m^{-3} d^{-1}. However, when OLR was increased again, digester efficiency did not differ significantly compared to previous OLR and remained at the same level before biogas production stopped. Digester efficiency showed a positive correlation with OLR ($p < 0.01$). Considering biogas yield, methane yield and digester efficiency, it can be concluded that 4 gVS L^{-1} d^{-1} and 15 days HRT were the optimum operation parameters with 6%VS feed concentration.

In the second assay digester efficiency was slightly enhanced (see Fig. 5b) compared to the first assay. Significant differences were observed among all OLR tried, being the lowest digester efficiency 643 ± 64 L_{CH4} m^{-3} d^{-1} for the lowest OLR. Highest digester efficiency was 1877 ± 121 L_{CH4} m^{-3} d^{-1} for the highest OLR. Again, digester efficiency showed a positive correlation with OLR level ($p < 0.01$). Considering digester efficiency and methane yield, results suggested that the optimal operational parameters were

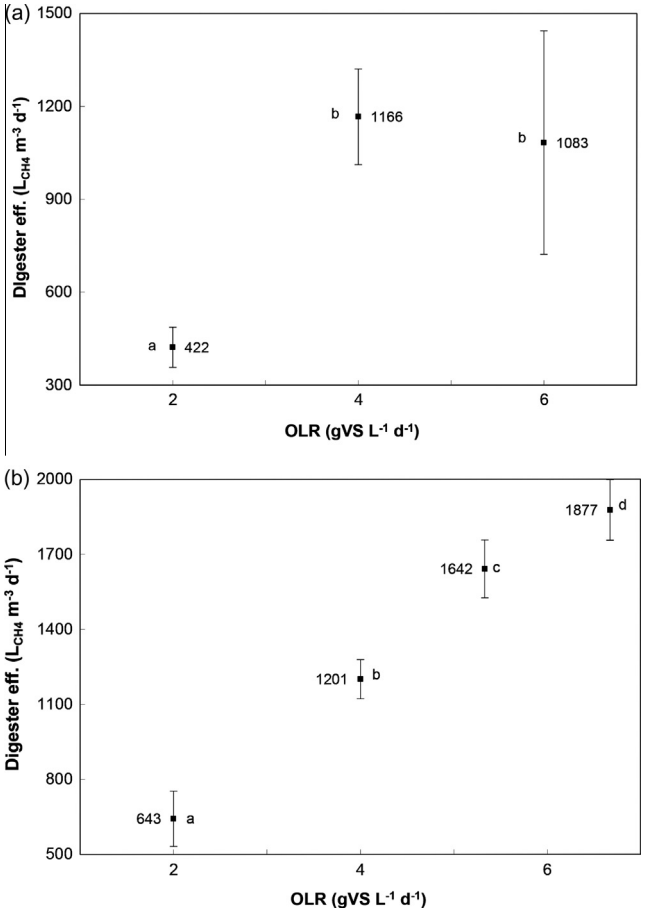

Fig. 5. Digester efficiency at different OLR: (a) 6%VS feedstock concentration; and (b) 8%VS feedstock concentration. Error bars indicate standard deviation.

5.33 gVS L^{-1} d^{-1} and 15 days HRT, yielding 592 ± 43 L_{biogas} $kgVS^{-1}$ and 308 ± 22 L_{CH4} $kgVS^{-1}$, with a digester efficiency of 1642 ± 115 L_{CH4} m^{-3} d^{-1}.

4. Conclusions

The co-digestion of *O. maxima* and *Scenedesmus* improved methane yield and kinetics of the discontinuous process compared to the mono-digestion of both substrates. The best mix ratio turned out to be 75% *O. maxima*-25% *Scenedesmus* (VS basis). In semi-continuous assay, optimum OLR with 6%VS feedstock concentration was 4 gVS L^{-1} d^{-1} (15 days HRT), yielding 292 ± 39 L_{CH4} $kgVS^{-1}$. With 8%VS concentration, optimum OLR was 5.33 gVS L^{-1} d^{-1} (15 days HRT), yielding 308 ± 22 L_{CH4} $kgVS^{-1}$. Co-digestion of *O. maxima* and *Scenedesmus* ran stable at high OLR with high methane yield and no ammonia inhibition.

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