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ABSTRACT

Methane production from pretreated and raw mixed microalgae biomass grown in pig manure was evaluated. Acid and basic pretreatments provided the highest volatile solids solubilisation (up to 81%) followed by alkaline-peroxide and ultrasounds (23%). Bead milling and steam explosion remarkably increased the methane production rate, although the highest yield (377 mL CH_4/g SV) was achieved by alkali pretreatment. Nevertheless, some pretreatments inhibited biogas production and resulted in lag phases of 7-9 days. Hence, experiments using only the pretreated solid phase were performed, which resulted in a decrease in the lag phase to 2-3 days for the alkali pretreatment and slightly increased biomass biodegradability of few samples. The limiting step during the BMP test (hydrolysis or microbial inhibition) for each pretreatment was elucidated using the goodness of fitting to a first order or a Gompertz model. Finally, the use of digestate as biofertilizer was evaluated applying a biorefinery concept.

Highlights

- Pretreatments solubilised volatile solids but also inhibited biogas production.
- Alkali pretreatment increased 2.3 times the methane production of the raw material.
- The removal of pretreated liquids did not improve the global methane production.
- Gompertz model fitted the results of methane production controlled by inhibition.
- Composition of digestates allows their possible valorisation as fertilizers.

Keywords: anaerobic digestion, fertilizer, inhibition, kinetic model, methane

1. Introduction

Over the past decades, the concurrent developments in society, science, and technology have resulted in a higher demand for energy. One of the principal challenges in today's society is to provide a reliable energy supply for the future, which is hindered by the increasing prices of oil and gas (Kavitha et al., 2017a). Multiple eco-friendly alternatives, such as the production of bioethanol, biodiesel or biogas from wastes, have been considered and developed to make processes more environmentally friendly and feasible. The conversion of residual biomass into biogas via anaerobic digestion is considered the simplest and most straightforward way, since it requires mild pretreatments and low-cost equipment (Kavitha et al., 2017b).

Biomass grown in wastewater treatment plants is a suitable substrate for biogas production. Among the possible biological wastewater treatment alternatives, the use of microalgae is an emerging challenge, especially for effluents such as pig manure with a high nutrient concentration. Microalgae are able to grow in these wastewaters assimilating organic matter, N and P. Although wastewater treatment coupled to the anaerobic digestion of the microalgae biomass produced is a sustainable and interesting alternative, most studies on biogas production from microalgae have focused on single species (Mussgnug et al., 2010).

The type of microalgae and the cultivation conditions are essential parameters affecting its macromolecular composition and the cell wall resistance, and hence its potential biogas production (Klassen et al., 2016). Murphy et al., (2015) reported different theoretical methane yields from each organic fractions of the biomass (1.390

L/g VS from lipids, 0.851 L/g VS from proteins, and 0.746 L/g VS from carbohydrates). Additionally, biomass grown in microalgae-based treatment plants contains resistant microalgae species and a huge number of bacteria. To evaluate the feasibility of the combined process of wastewater treatment and biomass valorisation, the study of biogas production from this type of mixed microalgae biomass is required (Jankowska et al., 2017).

The application of pretreatments to disrupt the cell wall represents a promising alternative to increase the biodegradability of mixed microalgae biomass composed of recalcitrant microalgae species. Most of the information reported in literature refers to microalgae grown in domestic wastewater. Passos et al., (2015) carried out different pretreatments such as ultrasound and hydrothermal pretreatments in a mixed microalgae biomass cultivated in domestic wastewater (*Stigeoclonium sp.* and *Monoraphidium sp.* and diatoms *Nitzschia sp.* and *Navicula sp.*). Hydrothermal pretreatment (130ºC) increased the methane yield (135 mL CH4/g VS) compared to the untreated control (106 mL CH4/g VS). However, in this case, ultrasound pretreatment (26700 J/g TS) did not significantly improve methane production. In another study, Passos et al., (2016a) studied the effect of two thermochemical pretreatments (KOH and HCl) on biogas production from microalgal biomass. They reported an increase in methane production up to 82% and 86% compared to the untreated biomass (78 mL CH4/g VS) for alkaline and acid pretreatments, respectively.

Nevertheless, Passos et al., (2016a) also observed an inhibitory effect under severe pretreatment conditions. Most of the reported degradation compounds generated by pretreatments in algae (Martín Juárez et al., 2016) or other types of biomasses were soluble and released to the liquid phase (Toquero and Bolado, 2014, Bolado-Rodríguez

et al., 2016). Therefore, the systematic comparison of biogas production using both fractions (solid and liquid fractions) or only the solid fraction of pretreated samples will provide a valuable information about the effect of the pretreatment technology on the biodegradability of biomass and generation of inhibitory compounds.

Following the valorisation as biogas of the organic matter present in microalgae, a significant load of nutrient is expected in the digestates, especially from biomass grown in wastewater with high N and P content. The use of the residual effluent from microalgae anaerobic digestion as fertilizer would lead the integral valorisation of the mixed microalgae biomass (Acién et al, 2014).

This study aimed at investigating the production of biogas by anaerobic digestion of mixed algal biomass grown in pig manure treatment plants. This work evaluated first the efficiency of different pretreatments (bead mill, alkaline, steam explosion, alkali-peroxide, ultrasound, and acid pretreatments) under two extreme operating conditions on CH_4 productivity. Furthermore, the methane productions from the whole suspension and the only solid fraction from pretreatment were compared in terms of the methane production yield to evaluate the generation of any potential inhibition induced by the pretreatments, kinetic modelling being used to identify the limiting step of the anaerobic digestion of the pretreated biomass. Finally, the composition of the digestates was analysed and their potential use as bio-fertilizers was evaluated to recover the high nutrients load of pig manure using a bio-refinery approach.

2. Materials and methods

2.1. Microalgae biomass

Fresh mixed microalgae biomasses were cultivated in a thin-layer photobioreactor with a volume of 1200L fed with pig manure diluted at 10% at two different times of the year: February and March. The composition during February was 23.67% carbohydrates, 43.31% proteins, 16.74% lipids, 83.17% volatile solids, and 987 mg O_2/ kg of COD, all of them in a dry basis. The microalgae species were *Tetradesmus obliquus* (29%), *Tetradesmus lagerheimii* (26%), *Desmodesmus opoliensis* (16%), *Aphanothece saxicola* (11%), *Chlorella vulgaris* (5%), *Scenedesmus magnus* (4%), *Parachlorella kessleri* (3%), and others in lesser amounts. The composition during March was 38.11% carbohydrates, 24.83% proteins, 12.51% lipids, 74.5% % volatile solids and 1150 mg O_2/ kg in a dry basis. The microalgae species were *Desmosdesmus opoliensis* (47%), *Navicula reichardtiana* (27%), *Tetradesmus obliquus* (12%), *Scenedesmus sp.* (9%), and *Scenedesmus acuminatus* (5%). The biomass was supplied by the Cajamar Foundation (Almeria, Spain) and centrifuged at 78.75% (February) and 77.91% (March) of moisture and refrigerated at 4ºC prior to use.

2.2. Pretreatments

The pretreatments performed for the biomass from February were bead mill, alkaline (NaOH), steam explosion, and alkaline-peroxide (H_2O_2) pretreatments, all of them at 5% (w/w) dry weight. Two levels of bead mill pretreatments (Postma et al., 2017) were carried out: A (small beads 1.25 mm and 5 minutes) and B (big beads 2.50 mm and 60 min), using distilled water in the mill until 200 mL of total volume (Pascal Engineering Co. Ltd). The alkaline pretreatment was carried out in 1 L borosilicate bottles with NaOH 0.5M (C) and 2M (D). Adequate volumes of NaOH solutions (of the selected concentrations) were added to the known mass of microalgae to obtain 200 mL

volume, and, then, suspensions were autoclaved at 121ºC for 60 minutes (Bolado-Rodríguez et al., 2016). The steam explosion pretreatment was carried out using saturated steam at 130ºC during 5 minutes (E) and at 170ºC during 20 minutes (F) in a 5L stainless steel reactor filled with 800 mL of suspension (Alzate et al., 2012). After the selected operation time, the steam was flashed and the biomass was cooled down in another vessel (Marcos et al., 2013). For the alkaline-peroxide pretreatment, known mass of microalgae were placed in 1 L bottles and adequate volumes of H_2O_2 solutions of the selected concentrations 0.5% (G) and 7.5% (H) were added to obtain 200 mL of total volume (Martín Juárez et al., 2016). Then, the pH was adjusted to 11.5 with 2 M NaOH, a few drops of antifoam were added, and the systems were incubated in a rotatory shaker at 50ºC and 120 rpm for 60 minutes.

Ultrasound and acid (HCl) pretreatments at 5% (w/w) dry weight were performed on the biomass from March. The ultrasound pretreatment was carried to a total volume of 400 mL of microalgae biomass diluted with distilled water in Ultrasound Technology (Hielscher UIP1000hd), during 5 (I) and 21 minutes (J), (Alzate et al., 2012). Power was calculated to expend identical amount of energy (7186 J/g TS) for the two operation conditions, according to Equation (1). This consumption of energy, considered a limit value, was calculated as the difference between energy from the maximum theoretical potential of biogas production and the experimental biogas production from the raw biomass.

$$Energy = \frac{P \cdot t}{V \cdot TS} \quad \text{(Eq. 1)}$$

where P is the average ultrasonic power (Watts), t is the ultrasonic time (seconds), V is the sample volume (liters), and TS is the initial total solid concentration (g TS/L).

The acid pretreatment was carried out in borosilicate bottles with HCl 0.5 (K) and 2M (L) (Bolado-Rodríguez et al., 2016). The known mass values of microalgae were placed in 1 L bottles, adequate volumes of HCl solutions (of the selected concentrations) were added to obtain a volume of 200 mL of, and suspensions were autoclaved at 121ºC for 60 minutes. All the pretreatments were conducted in duplicate.

After the pretreatments, the resulting suspensions were centrifuged at 10000 rpm, for 10 minutes. The solid and liquid fractions were weighed. Next, the total and the volatile solids were analyzed both in the solid and liquid fractions and in the pretreated whole. Samples of whole pretreated suspensions (named 1) and only solid fractions (named 2) were stored at 4ºC for biogas production experiments. The following parameter was defined to calculate the percentage of volatile solids retained:

$$\text{Retained volatile solids} = \frac{\%SV_{solid\ fraction} \cdot g\ of\ dried\ solid\ fraction}{\%\ SV_{raw\ material} \cdot g\ of\ dried\ raw\ material} \cdot 100 \qquad \text{Eq. (2)}$$

2.3. Biogas production

Biochemical methane potential (BMP) tests were carried out to study the biodegradability of the microalgae biomass in triplicate following the protocol of Angelidaki et al., (2009). Batch mode assays were performed under mesophilic conditions in 300 mL borosilicate glass bottles with a working volume of 100mL. The effluent from a pilot scale mesophilic anaerobic digester processed mixed sludge from a municipal wastewater treatment plant, with a volatile solids (VS) concentration of 9.1 ± 0.08 g VS/kg was used as inoculum for the tests. Two series of experiments were performed to determine the influence of the pretreatment and the inhibitory effect of the compounds present in the liquid phase: (1) using the whole pretreated suspension; and (2) using only the solid fractions from pretreatments. A control test without a substrate

was also conducted which aimed to check the methanogenic activity of the inoculum (Bolado-Rodríguez et al., 2016).

NaOH or HCl were added, if necessary, to pre-neutralize the samples to pH values 8 for alkaline samples or 5.5 for acid samples. Identical mass of inoculum was used in all the BMPs tests of untreated microalgae biomass, whole suspensions, and solid fractions from pretreatments. Based on previous studies, weighed amounts of pre-neutralized algal biomass were added to obtain an identical ratio of substrate/inoculum of 0.5 g VS/g VS in all the experiments (Alzate et al., 2012). Distilled water was used to fill the 100 mL working volume, when it was required. The pH of the initial mixture was always between 6.5 and 7. Before starting the tests, the bottles were closed with rubber septa and aluminum crimps. Helium gas was circulated inside the gas chamber for 5 minutes and the test started after releasing the pressure. The bottles were placed horizontally on a rotary desk with constant mixing under mesophilic conditions in a thermostatic room (37 ± 0.5 ºC) (Bolado-Rodríguez et al., 2016).

Biogas production in the headspace of each bottle was measured periodically by a manual pressure transmitter (PN5007, range 0–1 bar, IFM Electronics) over a period of 30-45 days. The biogas composition was determined by gas chromatography. Specific methane yields are expressed as the volume of methane under standard conditions, i.e. 0ºC and 1 atm for gases, as defined by the International Union of Pure Applied Chemistry (IUPAC), per gram of VS in the substrate fed into the assay (N mL CH_4/g VS). Theoretical methane yields, calculated from the ratio of COD/VS performed for every substrate, were 415 mL and 540 mL CH_4/g VS for February and March, respectively.

After the anaerobic digestion, the possible use of selected digestates as fertiliser was evaluated, analysing TS, VS, elements (C, H, N, S, P), heavy metals (Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Zn, Hg), and pathogens (*Salmonella spp.* and *E. Coli*).

2.4. Kinetic models

First order model (Eq. (3)) and the modified Gompertz equation (Eq. (4)) were applied to fit the cumulative methane production data from the experiments (Lay et al., 1996). The first order model fits successfully results of anaerobic biodegradability tests when the hydrolysis reaction is the rate-limiting step. The modified Gompertz model fits better the cumulative methane production in batch assays when occurs inhibition, assuming that the methane production is function of bacterial growth (Bolado-Rodríguez et al., 2016). Moreover, the model parameters were calculated by minimizing the least square difference between observed and predicted values.

$$B = B_0 \cdot [1 - \exp(-k_H \cdot t)] \quad \text{(Eq. 3)}$$

$$B = B_0 \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{B_0}(\lambda - t) + 1\right]\right\} \quad \text{(Eq. 4)}$$

In these equations, B represents the cumulative methane production (mL CH_4/g VS) and t is the time of the assay (d). These models estimate the methane production potential B_0 (mL CH_4/ g VS, related to the substrate biodegradability), the hydrolysis coefficient k_H (d^{-1}), the maximum biogas production rate Rm (mL CH_4/g VS d), and the lag time λ (d).

2.5. Analytical methods

The identification, quantification, and biometry measurements of microalgae were carried out by microscopic examination (OLYMPUS IX70) of microalgae samples

(fixed with lugol acid at 5% and stored at 4°C prior to analysis) according to Sournia, (1978). The COD concentration was determined according to APHA Standard Methods (2005). The total and volatile solids were measured following the NREL (Van Wychen and Laurens, 2015a). The carbohydrate content was determined by acid hydrolysis and HPLC-RI using an NREL procedure (Van Wychen and Laurens, 2015b). The protein content in the raw materials was correlated with the Total Nitrogen Kjeldahl, multiplied by a factor of 5.95, and the lipid content was determined by the Kochert method (González Lopez et al., 2010). The determination of the carbon, nitrogen, and hydrogen content of the biomass was performed using a LECO CHNS-932 analyzer, while phosphorus, sulphur, and all the heavy metals analyses were carried out spectrophotometrically after acid digestion in a microwave according to the internal protocol of the Laboratory of Instrumental Analysis of The University of Valladolid.

The CO_2, H_2S, CH_4, O_2, and N_2 concentrations in the gas phase of biogas samples were determined using a Varian CP-3800 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 mm × 15 μm) and a CP-Pora BOND Q (25 m × 0.53 mm × 15 μm) columns (Posadas et al., 2015). The analysis of *Salmonella spp.* and *Escherichia Coli* were measured following the UNE-EN ISO 6579:2003/A1:2007 and UNE-EN ISO 9308-2:2014, respectively.

3. Results and discussion

3.1. The pretreatments effect in terms of volatile solids solubilisation

Mixed biomasses were used in this study with different macromolecular compositions as shown in Section 2.1. These differences, mainly in carbohydrate and protein compositions, had an influence on the biogas production and kinetic. So, the

comparison between pretreatments applied to the different biomasses was only studied in terms of general results. Molinuevo-Salces et al., (2016), who treated swine slurry at different temperatures, illumination periods, and NH_4^+ concentrations, also observed the influence of operational conditions in the biomass composition. Carbohydrate content increased from 35%-40% under non-favorable conditions and up to 50%-60% in the summer experiments.

Mass balances were made for all the experiments considering retained volatile solids in the solid fraction and released volatile solids in the liquid fraction. Additionally, the total mass of the both fraction from pretreatment were considered. The differences found between the initial VS and the total VS after pretreatment were always lower than ±10%. All the performed pretreatments solubilised volatile solids, but in different amounts, as shown in Figure 1 as the percentage of volatile solids retained. The alkaline and acid methods involved a high solubilisation of volatile solids while the bead milling or ultrasound methods solubilised only a small fraction of these solids. Contrary to what was expected, the retained volatile solid yield of alkaline-peroxide pretreatment was high, much like the results of the mechanical ultrasound method. This high solid recovery compared to the results of the basic pretreatment could be related to the low concentration of NaOH in these experiments. The most intense condition only increased remarkably volatile solids solubilisation for acid pretreatment with yields of retained volatile solids decreasing from 40% to 19%. A light increase was found for alkaline-peroxide (from 81% to 73%) and ultrasound pretreatments (from 86 to 76%). As previously reported for alkaline-peroxide pretreatment of mixed microalgae biomass composed mainly by *Scenedesmus* (Martín

Juárez et al., 2016), no clear effect of severity in the studied range was observed for other pretreatments apart from the acid one.

Passos et al., (2016a) applied KOH and HCl at different concentrations (0.5, 1.25, and 2% w/w) at 80ºC for 2 hours to the biogas production from microalgal biomass grown in urban wastewater treatment. They reported around 50% of TOC solubilisation for the acid pretreatment and up to 200% for the alkaline pretreatment with respect to the thermal pretreatment (80ºC, 2 hours) as their control.

3.2. Biogas production

3.2.1. Test 1: BMP of untreated raw materials and of pretreated whole suspensions

The anaerobic digestion of whole suspensions after the pretreatments was carried out to harness volatile solids released in the liquid phase and to avoid a separation step. Figures 2a and 3a present the cumulative methane production curves from Test 1 in terms of methane production (the volume of methane gas produced per gram of volatile solid in the substrate). This test worked with untreated and pretreated whole suspensions from the microalgae biomass from February. Figure 4a presents the results of the microalgae biomass from March. Other terms such as biodegradability – defined as the percentage of the theoretical methane yield determined for raw substrates – and normalized production of methane (NP) – defined as the ratio between the production of methane per gram of VS from treated and untreated microalgae biomass – are used in this discussion.

For both biomasses, the biodegradability of the untreated microalgae was 39% with respect to the theoretical methane yield (415 mL CH_4/g VS for February and 540 mL CH_4/g VS for March algae). These values of biogas production from untreated

biomass are comparable to a range of 106 mL to 146 mL CH_4 g/COD as reported by Molinuevo-Salces et al., (2016) who worked with different microalgae biomasses grown in pig manure. Contrary to our experiment, Passos et al., (2016b) reported lower methane yields in the biomass from March than in the biomass from February, with values of 72 mL and 128 mL CH_4/g COD, respectively.

The highest methane production of all the assays was achieved by alkaline pretreatment at the high NaOH concentration (D1) after overcoming an initial delay, with 377 mL CH_4/g VS; 91% of biodegradability and an NP value of 2.34. Although C pretreatment reported a slightly higher volatile solids release than D, the biogas production was remarkably lower and very similar to the untreated biomass (C1: 173 mLCH_4/g VS; 42% biodegradability and NP 1.08) and also contained a considerable lag phase. Passos et al., (2016a) reported increases on methane production of 82% with respect to the untreated biomass for alkaline pretreatment at low NaOH concentrations (0.5%, 80ºC, 2 hours), but the methane production from the untreated biomass was very low in this study (78 mL CH_4/g VS).

The second-best result was achieved by the alkaline-peroxide pretreatment, but working with a low peroxide concentration (G1: 279 mL CH_4/g VS; 67% of biodegradability and NP 1.73). In this case, the increase in the severity of the condition caused methane production to be slightly lower than the methane production of the untreated material (H1: 148 mL CH_4/g VS; 36% of biodegradability and NP 0.92), probably due to an inhibition that could not be coped with.

Despite the low effect on biodegradability, some pretreatments such as bead milling and steam explosion had an advance of methane production. Biomass pretreated with both pretreatments achieved 90% of its total methane production at day 4. This

advance was also reported by Gruber-brunhumer et al., (2015) but they reached an increase of 51% (289 mL CH_4/g VS) using milling (100 g of biomass mixed with 40 g of glass beads for 20 minutes, cooling to 20ºC) with respect to the untreated biomass (191 mL CH_4/g VS). No enhancement of methane production was observed at severe conditions of both pretreatments, reporting NP values of 1.00 and 0.91 for B1 and F1, respectively. For the mildest conditions, methane production increased slightly, reaching NP values of 1.06 and 1.11 for A1 and E1, respectively. Passos et al., (2015) reported a significant increase of 28% on the methane yield by hydrothermal pretreatment at 130ºC for 15 minutes (135 mL CH_4/g VS) with respect to the untreated mixed microalgae biomass from urban wastewater treatment.

The other pretreatment assays recorded no improvement with respect to the untreated biomass in terms of methane production and biodegradability. Acid pretreatments provided even lower methane production than untreated material with an NP of 0.95 for K1 and 0.90 for L1. However, Passos et al., (2016a) reported an increase of methane production of 86% with respect to the untreated biomass for acid pretreatment at 0.5%, 80ºC for 2 hours. However, as mentioned previously, the methane production in this study was very low.

Surprisingly, the biogas production was remarkably reduced by ultrasound pretreatment and further for the higher time conditions (J1: 137 mL CH_4/g VS; 25% of biodegradability and NP 0.66). The lag phase detected in biogas production from ultrasound pretreated biomass confirmed the possible inhibitory effect of this method. The decrease on biogas production with pretreatment time, even expending identical energy amount, could be related with the higher impact of time in inhibition. Similar behavior was observed by Passos et al., (2015) with no increase in methane production

by ultrasound pretreatment. Gruber-brunhumer et al., (2015) reported an increase of 52% (292 mL/g VS) with respect to the untreated biomass by ultrasound pretreatment but they expended 20000 J/g TS, working with pure microalgae (*Acutodesmus obliquus*).

3.2.2. Test 2: BMP of solid fraction from pretreatments

Cumulative methane production curves from Test 2 are presented in Figures 2b and 3b (for February) and in Figure 4b (for March). These figures show the results from the solid fractions after the pretreatments and the results from the untreated microalgae biomasses.

In this test, the solid fractions from alkaline pretreatment again provided the highest increase in methane production. Material pretreated with NaOH 2M (D2) achieved methane production values of 296 mL/g VS, 71% of biodegradability and 1.84 of NP. Despite the fact that these values were the highest for Test 2, they were lower than the results achieved from whole suspension, demonstrating that the VS of liquid fractions were more biodegradable than the VS of solids. However, this behavior was not detected with the solid fraction of NaOH 0.5M which reached a higher methane production (232mL CH_4/g of VS) than whole fraction, with 56% biodegradability and an NP 1.44. In this case, the inhibition was reduced or avoided by removing the liquid phase since most of the possible inhibitory compounds were soluble. This low inhibition was confirmed with the shortening of lag phase with respect to experiments with whole suspensions.

Apart from the alkaline pretreatment, only acid pretreatment with HCl 2M increased the methane production (L2: 250 mL CH_4/g of VS; 46% biodegradability and

NP 1.20) with respect to the untreated biomass and to the whole suspension. The inhibition played a key role in this pretreatment and decreased when the liquid fraction was removed.

The biodegradability of VS on the solid fraction from the alkaline-peroxide pretreatment at mild conditions was very low (G2: 95 mL CH_4/g of VS; 23% biodegradability and NP 0.59), showing a drastic reduction with respect to whole suspension but also to the untreated material. The VS retained in the solid fraction was high in this experiment (81%), and the possible high biodegradability of VS solubilized into the liquid fraction cannot justify this huge difference.

In the same way, bead milling pretreatment did not advance the anaerobic digestion of the solid fraction. However, a slight increase in methane production was only observed for B2 (180 mL CH_4/g of VS; 41% biodegradability and NP 1.12). Results of the other applied pretreatments were similar to those obtained from the whole suspension experiments.

In order to calculate the global methane production balance, the losses of volatile solids solubilized to the liquid phase during the pretreatment and removed in these experiments must be considered (Figure 1). Referring the methane production from the pretreated solid to the initial VS in the raw biomass before the pretreatment, only the bead mill pretreatment for 60 minutes (B2) slightly enhanced the methane production with respect to the untreated biomass, with an NP of 1.08. For the other pretreatments, the increase in methane production by gram of volatile solid did not counteract the volatile solids' losses in the removed liquid fraction. If VS removal is considered, even the pretreatments with the highest biodegradability provided global NP values lower than 1, such as 0.38 (C2) and 0.56 (D2) for alkaline pretreatment or 0.39

(K2) and 0.23 (L2) for acid pretreatment.

3.3. Kinetics

Two different models were tested to fit the experimental results of cumulative methane production and to calculate the kinetic parameters. The first order model considers the hydrolysis reaction as the limiting step while the modified Gompertz equation considers bacterial growth and, hence, the inhibition of the process as the limiting step. Table 1 shows the model kinetic parameters that provided the best fit of methane production for each pretreatment and operational condition, working with the whole suspension and with only the solid fraction.

In the case of the biomass from February, methane production from untreated and bead mill pretreatment (A and B) were fit using the first order kinetic. Bead mill pretreatment is a mild method, which gently opens the cell wall, generating scarce amounts of degradation compounds. Thus, the hydrolysis reaction was the limiting step in these cases. The methane potentials obtained for all the bead mill experiments were similar to that of the untreated microalgae biomass. The rapid increase of methane production previously mentioned for experiments with whole suspensions was reflected in the hydrolysis coefficient, which remarkably increased even more at the mildest conditions (A1).

Gompertz model was required for fitting the whole suspensions and solid fractions from alkaline conditions. This pretreatment was the most effective, increasing the methane potential up to 234% for NaOH 2M when working with the whole suspension. As expected, the lag period (inhibition) was longer for experiments with whole suspensions due to the presence of degradation compounds in the liquid fraction.

However, the inhibition effect decreased with the NaOH concentration while also increasing the maximum biogas production rate. Pretreatment with NaOH 0.5 M caused a high lag phase but the mild conditions did not open the structure and enhance the methane production potential. The lag phase using only solid fractions was shorter, and pretreatment increased the methane production potential by nearly 150% for NaOH 0.5M and 200% for NaOH 2M. Nevertheless, they did not achieve the results that were obtained by using the whole fractions at a high NaOH concentration. Moreover, the high mass losses by solubilisation in these experiments should be still considered. Passos et al., (2016a) also used the Gompertz model to fit the methane production from microalgae grown in urban wastewater and pretreated with KOH, even while working with lower concentrations. They reported lag phases that increased with the alkaline concentration from 1.20 days with KOH 0.5% up to 6 days with KOH 2.0%.

The results of steam explosion pretreatment were fit with first order model as the untreated biomass, with hydrolysis as the limiting step. The pretreatment increased the kinetic coefficients of whole suspensions three times (E1 and F1), but the methane production only increased 11% for E1. The results of methane production were similar to the untreated material. As detected in the bead milling pretreatment, the steam explosion pretreatment reduced the reaction time when working with whole suspensions, but maintained or slightly increased the biogas production.

Regarding alkaline-peroxide pretreatment, all the conditions were fit with the first order model except for H2 which required the use of the Gompertz model. This behaviour was the opposite of that noticed in other chemical pretreatments because the inhibition appeared using only the solid fractions. Nevertheless, the methane production potential of H2 achieved the values of the untreated material with a lag period of 3 days

while B_0 decreased remarkably for G2 (with milder conditions and no apparent inhibition). Regarding the whole suspensions, G1 practically doubled the methane production potential but decreased the kinetic. This effect was exactly the opposite when increasing the pretreatment severity.

The untreated biomass from March was fit with a first order model with higher methane production potential but a lower kinetic coefficient than the untreated biomass from February. The experimental results from all the assayed pretreatments were fit using the Gompertz model with a long lag phase from 6.2 to 10.7 days, showing a remarkable inhibitory effect. The only pretreatment providing a certain increase of methane production potential (20%) was the acid pretreatment at severe conditions (L2), when using only the solid fraction but with the longest lag phase (10.7). Passos et al., (2016a), working with HCl, reported lag phases that increased with the acid concentration (0.43 days for 0.5%, 3 days for 1.25%, and 5 days for 2%), but all the experiments required the Gompertz model to fit the results.

Additional research is necessary in order to identify the inhibitory compounds generated by some of the pretreatments, which were unexpectedly retained in the solid phase. Further continuous anaerobic digestion tests would provide relevant information about acclimation of microorganisms to the pretreated substrates, which would enhance both methane production yields and microbial kinetic.

3.4. Fertilisers analysis

Table 2 shows the composition of some residues after anaerobic digestion in order to evaluate their possible application as fertilizers. Digestates from tests that achieved higher methane production than untreated biomass were selected (alkaline,

alkaline-peroxide, and acid pretreatments). The content of nitrogen was clearly reduced in the samples from alkali media due to the effect of basic pH on protein release and ammonia stripping. The NPK content of digestate from pretreated samples was always lower than from untreated biomasses, but higher than the minimum legal threshold value of 7% (w/w). This excess was very low for samples from the biomass from March. The ratio C/N increased in basic pretreatments, because of N removal, but remained lower than the maximum allowed value of 15. The content of As was much lower than the maximum limit of 50 mg/kg. The minimum legal content of the other analysed elements depends of the fertilizers use: extensive and grazing cultivation, fertirrigation or horticultural use, and foliar; but Cu and Mn supplementation would likely be necessary (Reglamento CE 2003/2003, 2003).

Regarding microbiology, the digestate from the untreated biomass from February did not contain pathogens and the results did not provide information about a possible sterilization effect of these pretreatments. However, a clear sterilizing effect of acid pretreatment was observed, remarkably reducing the *E.coli* content of the final digestate.

In summary, the digestates from anaerobic digestion of algal biomass grown in pig manure have a potential application as fertilizers. The initial microalgae biomass composition should be considered, mainly for the variability of nitrogen content throughout the year and the cultivation conditions.

4. Conclusions

Acid and alkaline pretreatments solubilised high percentage of VS but induced a remarkable inhibition. The highest methane production enhancement was achieved with

whole broth of alkaline (234%) and alkaline-peroxide (173%) pretreatments, while bead mill and steam explosion increased the methane production rate by a factor of 5 and 3, respectively. The methane yield was not improved by removing the liquid phase. The fitting to kinetic models revealed the impact of each pretreatment in terms of hydrolysis or inhibition. Finally, the composition of the digestates, with NPK higher than 7% (w/w) and C/N lower than 15, allows their use as fertilizers.

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Figure Captions

Figure 1. Percentage of volatile solids retained in the solid fractions with respect to the initial content of volatile solids.

Figure 2. Experimental results and fitting curves of cumulative methane production: (a) untreated and whole pretreated fraction of microalgae biomass (Test 1) and (b) untreated and solid pretreated fraction of microalgae biomass (Test 2). A: bead mill 5 minutes; B: bead mill 60 minutes; C: NaOH 0.5M; D: NaOH 2M.

Figure 3. Experimental results and fitting curves of cumulative methane production: (a) untreated and whole pretreated fraction of microalgae biomass (Test 1) and (b) untreated and solid pretreated fraction of microalgae biomass (Test 2). E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%.

Figure 4. Experimental results and fitting curves of cumulative methane production: (a) untreated and whole pretreated fraction of microalgae biomass (Test 1) and (b) untreated and solid pretreated fraction of microalgae biomass (Test 2). I: ultrasound 5 minutes; J: ultrasound 21 minutes; K: HCl 0.5M; L: HCl 2M.

Tables

Table 1. Kinetic model and parameters of fitting equations of cumulative methane production from untreated and pretreated microalgae biomass using whole suspension and solid fractions from pretreatment.

Sample[a]	Kinetic Model[b]	B_0[c]	k_H[d]	λ[e]	R_m[f]	R^2[g]
Untreated_February	First order	154	0.167			0.9914
A1	First order	161	0.852			0.9805
A2	First order	158	0.168			0.9821
B1	First order	154	0.711			0.9951
B2	First order	172	0.166			0.9933
C1	Gompertz Model	168		9.34	18.18	0.9943
C2	Gompertz Model	226		1.89	15.90	0.9921
D1	Gompertz Model	362		7.80	27.09	0.9710
D2	Gompertz Model	295		2.63	19.97	0.9960
E1	First order	172	0.487			0.9890
E2	First order	153	0.246			0.9912
F1	First order	135	0.528			0.9868
F2	First order	150	0.147			0.9865
G1	First order	297	0.100			0.9788
G2	First order	100	0.112			0.9775
H1	First order	141	0.491			0.9913
H2	Gompertz Model	153		3.04	11.10	0.9957
Untreated_March	First order	214	0.055			0.9901
I1	Gompertz Model	167		7.39	19.11	0.9870
I2	Gompertz Model	158		7.91	16.10	0.9957
J1	Gompertz Model	133		7.79	15.34	0.9954
J2	Gompertz Model	99		8.60	14.00	0.9918
K1	Gompertz Model	197		6.18	26.63	0.9733
K2	Gompertz Model	200		9.25	24.35	0.9994
L1	Gompertz Model	185		8.44	18.36	0.9965
L2	Gompertz Model	238		10.67	37.96	0.9930

[a] Codes: Pretreatment: A: bead mill 5min; B: bead mill 60 min; C: NaOH 0.5M; D: NaOH 2M; E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%; I: ultrasound 5 min; J: ultrasound 21 min; K: HCl 0.5M; L: HCl 2M. Fractions used: 1, whole slurry and 2, solid fraction.

[b] B_0: methane production potential (mL CH_4·g VS^{-1}). (Equations 3 and 4).

[c] k_H: hydrolysis coefficient in the first order kinetic model (d^{-1}). (Equation 3).

[d] λ: lag time (d). (Equation 4).

[e] R_m: maximum biogas production rate in the Gompertz model (mL CH_4·g VS^{-1}·d^{-1}). (Equation 4).

[f] R^2: coefficient of determination.

Table 2. Main parameters analyzed for the characterization as a fertilizer of anaerobic digestate of untreated and selected pretreated algal biomasses

	Untreated_February	C2	D1	D2	G1	Untreated_March	K1	K2	L1	L2
ST	1.090	1.232	1.453	2.504	1.423	1.824	1.931	1.843	2.048	2.366
SV[a]	51.656	46.541	36.619	23.695	45.566	40.185	34.147	33.900	35.800	23.732
C[a]	34.260	21.270	17.670	10.180	19.260	21.100	21.890	17.220	19.100	14.720
N[a]	7.500	2.640	1.870	1.360	2.470	2.420	2.120	1.800	1.710	1.520
P[a]	4.105	4.091	3.765	2.155	3.470	2.177	1.749	2.167	1.669	1.772
S[a]	1.705	1.383	1.348	0.801	1.284	1.073	0.958	1.060	0.935	0.841
Hg[a]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Al[a]	1.041	0.817	0.762	0.375	0.691	0.547	0.512	0.623	0.519	0.517
As[a]	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Ca[a]	5.018	5.252	6.036	2.229	4.830	10.079	9.754	9.677	9.457	9.854
Cr[a]	0.003	0.003	0.003	0.001	0.002	0.002	0.002	0.002	0.002	0.002
Cu[a]	0.020	0.024	0.032	0.014	0.020	0.013	0.017	0.013	0.011	0.010
Fe[a]	2.399	1.783	1.626	0.824	1.532	1.219	1.057	1.280	1.065	1.090
K[a]	3.013	2.308	1.643	1.387	2.489	1.267	0.912	1.139	0.836	0.936
Mg[a]	1.156	0.987	0.939	0.433	0.923	0.461	0.367	0.642	0.384	0.495
Mn[a]	0.023	0.049	0.064	0.022	0.032	0.000	0.000	0.000	0.000	0.000
Ni[a]	0.006	0.005	0.005	0.002	0.004	0.003	0.003	0.003	0.003	0.003
Pb[a]	0.005	0.004	0.004	0.002	0.003	0.003	0.003	0.003	0.003	0.003
Zn[a]	0.248	0.246	0.362	0.144	0.237	0.169	0.104	0.179	0.118	0.141
Salmonella[b]	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence
E.coli[c]	<1	<1	<1	<1	<1	1.10E+05	1.00E+05	9.10E+04	<1	1.30E+03

[a]: percentage in dry weight (g*100/g dried)
[b]: 25g. Limit: absence
[c]: NMP/g. Limit: <1.0E3

Figure 1

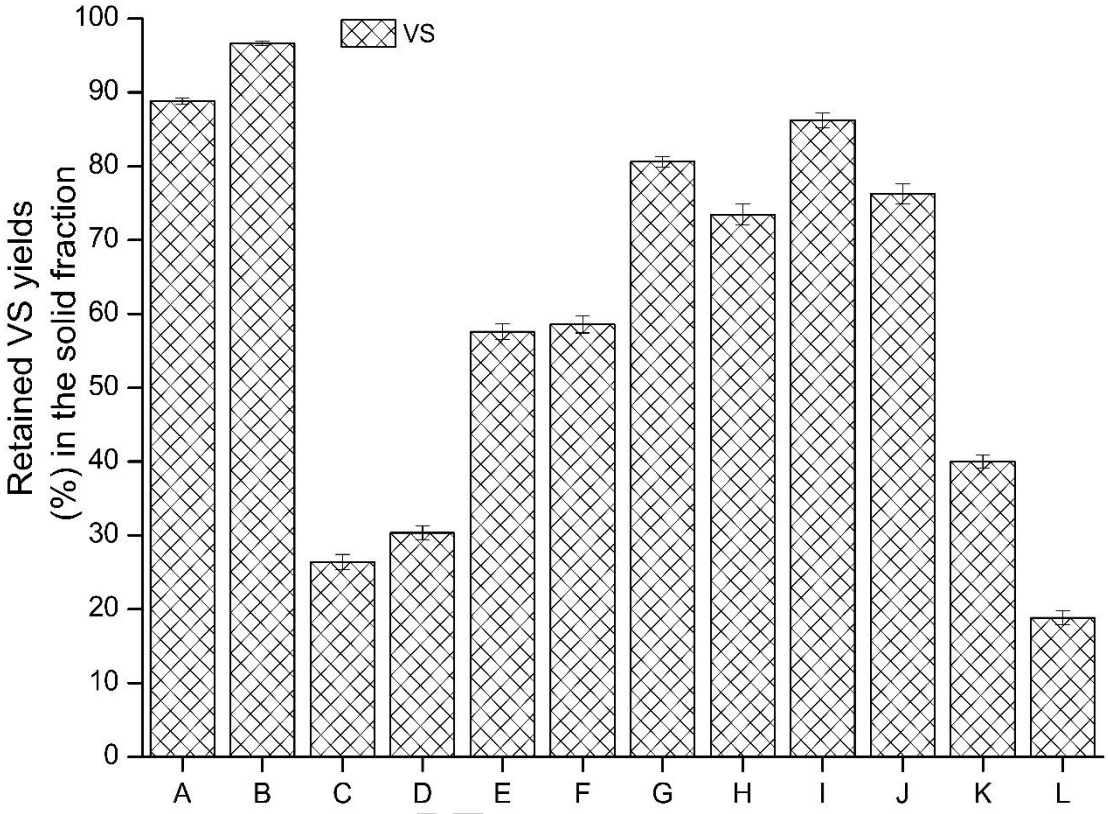

Figure 2

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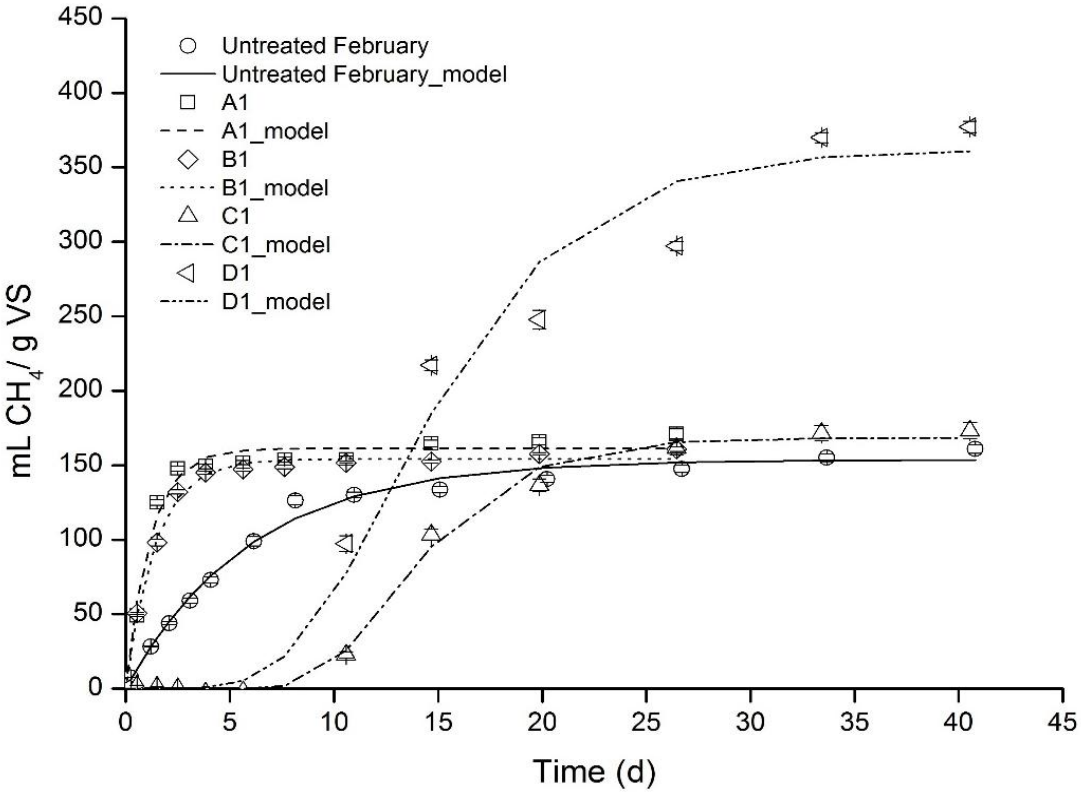

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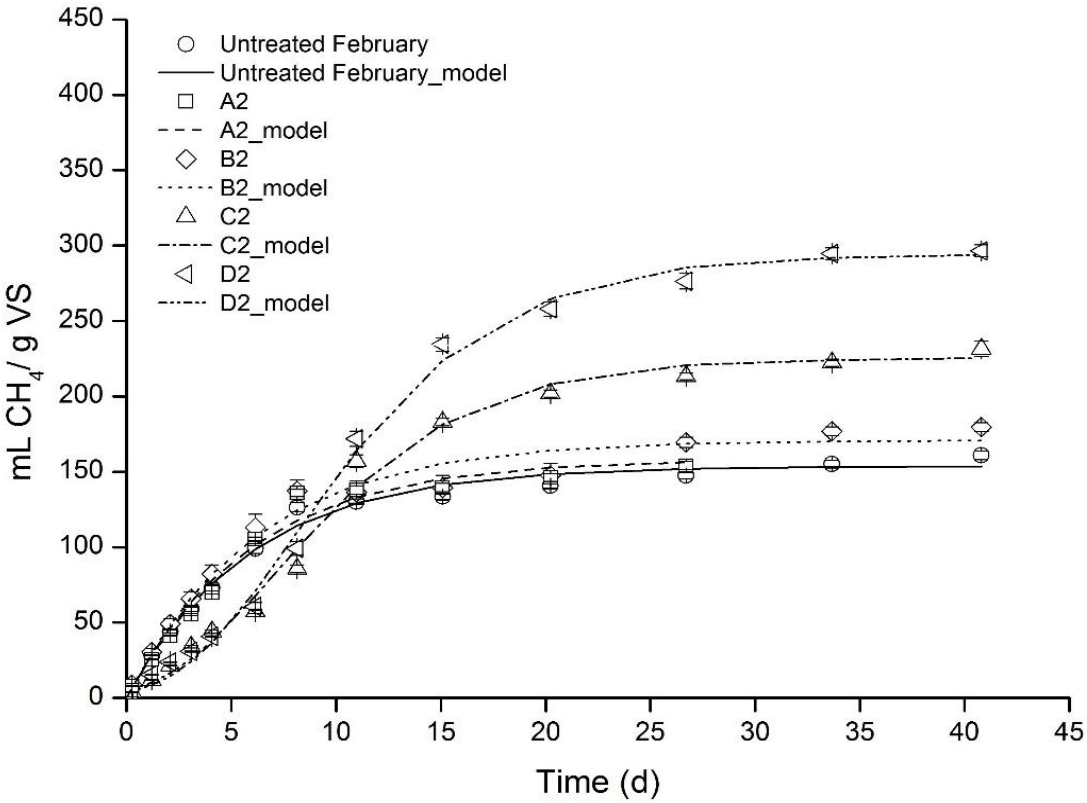
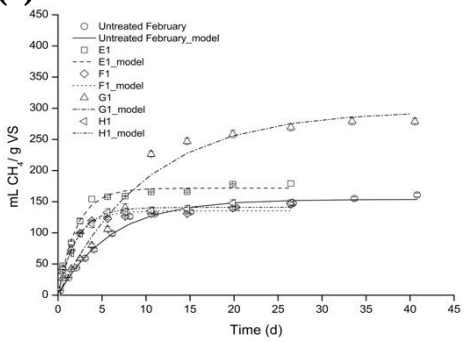


Figure 3

(a)



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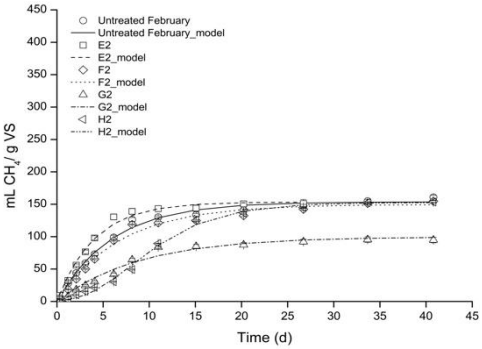
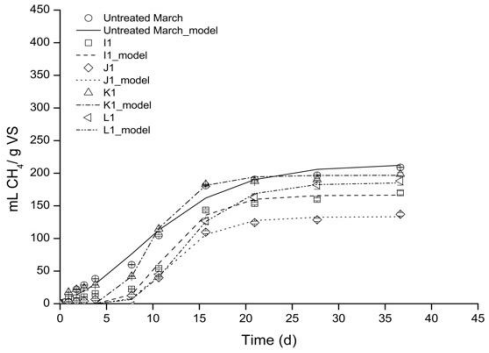


Figure 4

(a)



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