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Anaerobic Digestion And Co-Digestion of Oleaginous Microalgae Residues for Biogas Production

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Microalgae have been suggested as one of the most promising feedstock for the production of bioenergy and biofuels, including biodiesel and aviation fuels, because of the high oil content of selected species. In the context of biofuel production from microalgae, anaerobic digestion of microalgal biomass residues after oil extraction has the potential to make the process more sustainable and increase the energy efficiency. The main goal of this study was to assess microalgae residues as substrates for anaerobic digestion and investigate their potential for biomethane production. Biochemical Methane Potential (BMP) tests were carried out on a microalgal species, *Nannochloropsis gaditana*, selected for its high oil content and fast growth rate. The methane potentials observed for the microalgae residue after lipid extraction were higher than those recorded for the raw microalgae samples. Co-digestion with cellulose did not clearly enhance the anaerobic digestion performances.

1. Introduction

Owing to concerns being raised about the use of arable land and food crops for bioenergy generation, research into biofuel production using alternative biomass sources has been receiving increasing attention in the last years. Microalgae have been suggested as one of the most promising feedstock for the production of bioenergy and biofuels, due to high growth rates, high photosynthetic efficiencies, CO_2 fixation capability, large accumulation of oil compared to oleaginous plants, ability to thrive in harsh environments such as seawater, alkaline lakes, non-potable industrial wastewater, arid and barren land areas, avoiding competition for fresh water and arable land (Suali and Sarbatly, 2012). The potential for the use of microalgae as a feedstock for bioenergy production has been discussed in the literature, with an increasing focus on the production of advanced biofuels, including biodiesel and aviation fuels, because of the high oil content of selected species (Pragya et al., 2013). However, biofuel production from microalgal feedstock has several challenges to overcome before it can become a mainstream industry capable of producing the quantity of biofuel required at a competitive price. Challenges faced by the industry include demand for fertilizer due to microalgae's significant utilization of nutrients, as well as high energy inputs required for harvesting and dewatering biomass and for the lipid extraction and conversion processes. Anaerobic digestion of microalgal biomass residues after oil extraction can offer a pathway to eliminate some of the overheads of the production cycle by producing biogas for utilization in electricity or thermal energy production (Ward et al., 2014). Methane produced by the anaerobic digestion of the lipid-spent microalgae can contribute to the energy requirements of the microalgae biomass production and fuel processing stages. In addition, the recovery of valuable nutrients from biomass via anaerobic digestion which can be used for microalgae culture is essential for the sustainability of the algae biofuel industry (Ward et al., 2014). Thus, in the context of biofuel production from microalgae, the anaerobic digestion has the potential to make the process more sustainable and increase the energy efficiency. In this framework, it is important to make representative assessments of the methane yields available from a selected feedstock. This will provide useful preliminary information for the design of energy recovery systems using the lipid-extracted microalgae residues. Information on the methane

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yields could then be used in process modelling and cost analysis to evaluate the feasibility of such an integrated biofuel production platform from microalgae feedstock. In this context, the biochemical methane potential assay constitutes a useful tool to determine the methane conversion yield of a specific substrate.
The main goal of this study was to assess microalgae residues as substrates for anaerobic digestion and investigate their potential for biomethane production. A microalgal species, *Nannochloropsis gaditana*, selected for its high oil content and fast growth rate, was tested for biogas production. Biochemical Methane Potential (BMP) tests were carried out to evaluate biogas production capacity from raw microalgae and microalgae residue after lipid extraction. The digestion tests were performed in batch reactors in mesophilic conditions. Moreover, in order to overcome problematically low carbon/nitrogen ratio associated with microalgae biomass, co-digestion assays were performed to study the effect of adding carbon-rich substrates to microalgae residues. Co-digestion mixtures of microalgae residue and cellulose with different carbon/nitrogen ratios were assessed in BMP tests.

2. Materials and Methods

2.1 Materials

Nannochloropsis gaditana was used as a microalgal model in the present study. This microalgae was supplied as dried powder by AlgaSpring B.V. (The Netherlands).
Lipid-spent microalgae residue was obtained from raw microalga following the procedure reported in Section 2.2. All solvents used for lipid extraction were of high purity grade (>99%) and were purchased from Sigma-Aldrich (Italy). Both the raw, non-lipid exhausted microalgae (ANX) and the microalgae residue after lipid extraction (A) were used in anaerobic digestion tests. α-Cellulose (C) provided by Sigma-Aldrich (Italy) was employed in co-digestion experiments with microalgae residues at two different A:C ratios (AC1, AC2).

2.2 Lipid extraction

Different lipid extraction methods from *Nannochloropsis gaditana* were tested: the Bligh and Dyer method, a modified Bligh and Dyer method, and the Soxhlet method with different solvent systems.
The first extraction method was following the Bligh and Dyer method (Bligh and Dyer, 1959). 1 g of algal biomass was blended with 8 mL of distilled water in a 100 mL conical flask, vortexed for 5 min and then sonicated using an ultrasonic bath for 10 min at 25 °C. Then 30 mL of solvent mixture (chloroform – methanol 1:2 v/v) was added to form a single phase solution. The mixture was homogenized with a magnetic stirrer at 25 °C for 20 min, and then sonicated with an ultrasonic bath for 10 min. 10 mL of chloroform were added, and the mixture was vortexed for 5 min. Then 10 mL of distilled water were added to form a two phase system, and the mixture was vortexed for 5 min. The phases were separated by centrifugation for 10 min at 2000 rpm. The upper layer was transferred to a flask. The lower layer (chloroform phase) was filtered through filter paper, and the filtrate was collected. A second extraction of the solid residue with chloroform was performed, and the chloroform phase was added to the first extract. Solvents were evaporated, and the residues were further dried in an oven at 60 °C for 12 h. Lipids were determined gravimetrically.
The second extraction method tested was a modification of the Bligh and Dyer procedure in which methanol and chloroform were replaced by propan-2-ol and cyclohexane, respectively (Schlechtriem et al., 2003). 1 g of algal biomass was weighed in a 100 mL conical flask, and mixed with 20 mL of propan-2-ol and 25 mL of cyclohexane. The mixture was vortexed for 5 min and then placed in an ultrasonic bath at room temperature for 10 min. 27.5 mL of water were then added to obtain a mixture of water : propan-2-ol : cyclohexane 11:8:10. The mixture was mixed again for 5 min. The different phases were separated by centrifugation at 2000 rpm for 10 min and the organic phase (upper layer) transferred to an evaporation flask. A second extraction with 25 mL of cyclohexane containing 13% w/w propan-2-ol was done. After centrifugation, the cyclohexane phase was added to the first extract. The solvents were evaporated to dryness, and the extract and the residues were further dried in an oven at 60 °C for 12 h, after which the samples were weighed.
A standard Soxhlet extraction apparatus was used for the extraction of lipids with the Soxhlet method. In a typical experiment, 1 g of microalgae sample was weighed accurately into a Soxhlet cellulose extraction thimble and transferred to the extraction chamber in the Soxhlet apparatus. A 100 mL aliquot of the extraction solvent was transferred into the solvent cup. The following solvents were used for extraction: n-hexane, ethanol, n-hexane – ethanol 3:1, n-hexane – ethanol 1:1, chloroform – ethanol 1:1. The extraction was allowed to proceed for 3 h. Further experiments were carried out by varying the extraction time (1.5 – 6 h). At the end of the extraction process, the solvent was removed using a rotary evaporator. Both the lipid extract and the residue in the extraction thimble were placed in an oven at 60 °C for 12 h, and weighed.
For all the extraction methods employed, the initial microalgae sample, the lipid extract and the residue were characterized by thermogravimetric analysis. The ratio of the mass of the extract (on a dry basis) to the initial algal biomass (dry basis) determined the extraction yield. All analyses were performed at least in duplicate.

For the production of the lipid-spent microalgae residue employed in anaerobic digestion tests, the Soxhlet method with n-hexane – ethanol 3:1 as solvent was selected. In a typical experiment, 10 g of microalgae sample, 500 mL of solvent, and an extraction time of 6 h were used. Several replicates were performed, and the recovered residues were finally joined, characterized, and used in anaerobic digestion experiments.

2.3 Anaerobic digestion tests

Biochemical methane potential (BMP) was determined according to the methodology described by Ragaglini et al. (2014), summarized as follows. Anaerobic digestion was carried out in 2 L batch reactors and the assays were conducted in triplicates on the different samples. Figure 1 shows a scheme and a photograph of the experimental units used. Each reactor received 270 g of inoculum that was suspended in a basal mineral medium, prepared according to the ISO 11734 standard (ISO, 1995), up to a final filled volume of 1 L. Three blank experiments were also performed with 270 g of inoculum, demineralised water and minerals only. The inoculum was originated from the methanogenic stage of a mesophilic anaerobic digester, fed mainly with energy crops (maize silage), agroindustrial residues and poultry manure. The anaerobic sludge was sieved through a 1 mm mesh, then its total solids and volatile solids content were determined (57.4 g kg^{-1} and 36.9 g kg^{-1}, respectively). Before the beginning of the assay, in order to adapt the bacterial culture to the degradation of the microalgal biomass, untreated microalgae and cellulose were added to the sludge, then the inoculum was pre-digested for 21 days at 37 °C.

The substrates were added to the reactors according to a ratio between the inoculum and the substrate (I:S) equal to 2:1 on the basis of their volatile solids content (VS). Once the reactors were loaded with the different substrates, the batches were sealed and flushed with nitrogen, in order to obtain anaerobic conditions. Subsequently, the vessels were incubated at 37 ± 1 °C until biogas production became negligible (45 days). Biogas pressure in each reactor was continuously measured by pressure piezo-resistive transducers and continuously recorded by a dedicated Programmable Logic Controller (PLC) connected to a PC. The cumulative volume of biogas produced in each reactor at each time was calculated according to the ideal gas law and the molar volume of ideal gases at standard temperature and pressure conditions (1 bar, 273.15 K). Methane concentration was measured at discrete time intervals by gas chromatography (micro-GC Agilent 3000). Both the pressure reduction due to biogas removal at each sampling interval and the biomethane content of the sampled gas were considered in estimating the cumulative biogas production of each batch. Finally, in order to obtain the Biochemical Methane Potential (BMP) of each substrate, expressed in NL of CH_4 per kg of volatile solids (VS), the residual (or intrinsic) methane potential of the inoculum obtained in blank experiments was removed.

2.4 Analytical methods

Total solids (TS) and volatile solids (VS) were determined on fresh samples according to standard methods (APHA, 2005). Moisture, volatile matter, fixed carbon and ash content of samples were determined by thermogravimetric analysis. Thermogravimetric analysis was performed using a TA Instruments Q-500 thermobalance. Ultimate analysis was carried out with a LECO TruSpec CHN Elemental Analyzer.

2.5 Kinetic and statistical analyses

The kinetics of anaerobic digestion was analysed by regressing on time the cumulated methane measured in each reactor and by calculating the time at which the inflection point (I) of the accumulation curve was reached. Curve fitting according to a Modified Gompertz model (Ragaglini et al., 2014) was performed using the MATLAB® software. The anaerobic digestion parameters of the different substrates were analysed by one-way ANOVA and mean separation was carried out by LSD test ($p<0.05$ level) when significant differences were recorded.

Figure 1: Scheme (left) and photo (right) of the experimental units used for anaerobic digestion tests. Each batch reactor has a gas inlet for flushing at the beginning of the assay (1), a compressed air filter (2), a T-pipe connector (3), a pressure transducer (4) and an opening controlled by an electrovalve (5).

3. Results and Discussion

Preliminary experiments were carried out to evaluate the efficacy of different methods for the extraction of lipids from *Nannochloropsis gaditana*, and identify the most suitable one for the production of the lipid-spent microalgae residue to be used in the anaerobic digestion tests. The use of solvents for the extraction of lipids from algal biomass has been widely reported because of its simplicity in operation, and potential for upscaling to industrial plant level. The Bligh and Dyer method, a modified Bligh and Dyer method, and the Soxhlet method, methods widely used for lipid extraction from microalgal biomass (Li et al., 2014), were tested with respect to lipid extraction efficiency. Similar extraction yields were observed for the Bligh and Dyer method (11.8 %), the modified Bligh and Dyer method (11.0 %), and the Soxhlet method. With respect to the Soxhlet method, different solvents with varying polarities were used in the extraction of algal biomass; the experiments trials involved the use of single solvents, and selected binary solvent mixtures (Ramluckan et al., 2014). Hexane proved to have the lowest efficiency for the extraction of lipids (8.9 %), while higher extraction yields were observed with binary mixtures of different solvents (hexane – ethanol 3:1, 11.7 %; hexane – ethanol 1:1, 12.3 %); it may be inferred that a range of lipids varying from polar to non-polar were present in the algal biomass. Even if high extraction yields (17.6 %) were observed using a chloroform – ethanol mixture, the use of chloroform as solvent was discarded in the present work, since a severe inhibitory effect of the residual chloroform on methane production was observed during anaerobic digestion tests by Yun et al. (2014). Evidence of strong inhibition of methanogenic activity during tests within microalgal residues after oil extraction with a chloroform/methanol mixture was reported also by Ramos Tercero et al. (2014). On the other hand, the experimental results reported in the study of Yun et al. (2015) showed that the inhibition of n-hexane on methane yield was negligible up to 2 g COD/L. A n-hexane: ethanol 3:1 mixture was thus selected as extraction solvent. The results of experiments carried out by increasing the extraction time revealed a slight increase of extraction efficiency (11.7 % and 14.1 % extraction yield after 3 h and 6 h, respectively, while a low yield of 5.7 % was obtained after 1.5 h extraction time). For the production of the lipid-spent microalgae residue employed in anaerobic digestion tests, the Soxhlet method with n-hexane – ethanol 3:1 as solvent was thus employed, following the experimental procedure reported in Section 2.2. As a result, an average lipid extraction yield of 12.4 % was obtained. The TS and VS content of the obtained microalgae residue, as well as its elemental composition, are reported in Table 1, and compared with the composition of the microalgae. The high N content of the microalgae is even increased in the residue, as the latter was enriched of proteins.
Biochemical Methane Potential (BMP) tests were carried out to evaluate the biogas production capacity from raw microalgae and microalgae residue after lipid extraction. Moreover, co-digestion assays were performed to evaluate the effects of adding cellulose to the digestion of algae residue. Different co-digestion mixtures were assessed in BMP tests (Table 2). Several researchers investigated co-digestion, in order to achieve higher methane yields from substrates that are poorly exploited by mono-digestion, owing to their unbalanced characteristics. In fact, problematically low carbon/nitrogen ratio associated with microalgae biomass has been acknowledged as a limiting factor in the anaerobic digestion of microalgae (Montingelli et al., 2015). Therefore, microalgae can be co-digested with carbon-rich substrates (e.g. other waste streams or biomass) to increase the overall C/N ratio and reduce the chance of ammonia toxicity. Algal biomass has been co-digested with various substrates, e.g. corn silage (Schwede et al., 2013), waste paper (Yen and Brune, 2007), organic fraction of municipal solid waste and sewage sludge (Da Ros et al., 2015).

Table 1: Total Solids (TS), Volatile Solids (VS), ash content, and elemental composition of substrates used in digestion experiments (ar: as received; daf: dry ash free)

Substrate	TS (%, ar)	VS (%, ar)	Ash (%, dry)	C (%, daf)	H (%, daf)	N (%, daf)
Microalgae	95.6	87.2	8.8	56.3	7.9	8.1
Microalgae residue	96.6	87.6	9.4	54.9	7.3	9.2
Cellulose	91.8	91.7	0.1	43.8	6.2	0.0

Table 2: Carbon/Nitrogen ratio of substrates used in digestion and co-digestion tests (mixture composition expressed on the basis of the Volatile Solids content)

Substrate	Acronym	Composition	C/N
Microalgae	ANX	Microalgae 100%	7.0
Microalgae residue	A	Microalgae residue 100%	6.0
Co-digestion mixture 1	AC1	Microalgae residue – Cellulose 50:50	10.8
Co-digestion mixture 2	AC2	Microalgae residue – Cellulose 25:75	20.3
Cellulose	C	Cellulose 100%	∞

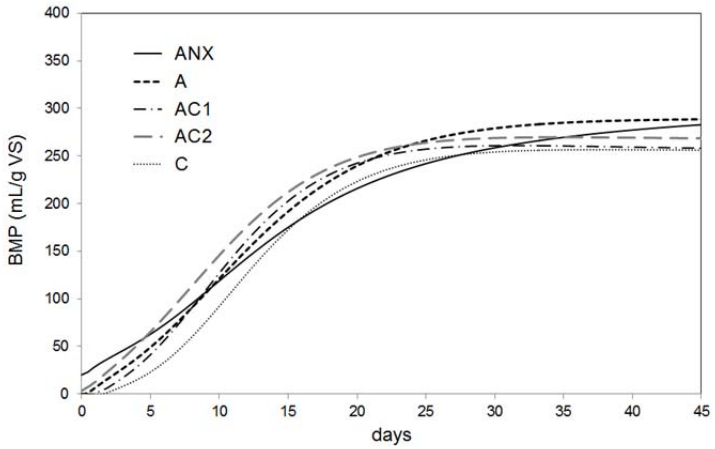

Figure 2: Cumulative methane production for the substrates listed in Table 2.

Moreover, several studies focused on pre-treatments on microalgae biomass, since their availability for anaerobic degradation has often been found to be hampered by cell wall structures (Passos et al., 2014). Some authors found that lipid extraction could break down cell walls, as well as proteins and carbohydrates into monomers, thus increasing their availability for anaerobic bacteria and increasing CH_4 production. At opposite, unbroken cells may hinder access to lipids, proteins and carbohydrates, resulting in lower methane yields despite the higher lipid content (Hernandez et al., 2014).
The results obtained in this work showed a complex outcome (Figure 2, Table 3), in partial agreement with the cited literature. The methane yield of microalgae residues (A) was slightly higher than that of non-extracted biomass (ANX). Therefore, a positive effect of lipid extraction might be inferred, in line with previous findings. The methane potentials observed in both extracted and non-extracted *Nannochloropsis gaditana* samples were also similar or higher than values reported in the literature (Frigon et al., 2013).
The large variability of methane yields shown by co-digestion scenarios, and particularly by AC2, affected the results, making the co-digestion scenarios not clearly favourable. The microalgal digestion scenarios (A, ANX, AC1, AC2) did not show statistically significant differences, thus implying that co-digestion with cellulose did not clearly enhance the anaerobic digestion performances. A large variability in cellulose degradation could be hypothesized when cellulose was digested with microalgal biomass, ranging from successful co-digestion and increased BMPs, owing to an optimal C/N ratio (Rincòn et al., 2010), to a poor overall performance. The digestion rate seemed to be slightly improved by cellulose supplement, while the microalgae residue (A) was degraded more quickly than the non-extracted biomass (ANX). As expected, cellulose mono-digestion showed the lower digestion rate, as the process reached the inflection point in a longer time span (about 11 days). The methane content of the biogas reflected the nitrogen content of the substrates, from the richer to the poorer (A>ANX>AC1>AC2), in line with other studies (Rincòn et al., 2010).

Table 3: Biochemical Methane Potential (BMP), inflection point of the methane accumulation curve (I) and methane content of the biogas produced by the considered substrates. Standard errors are reported within brackets. Different letters indicate significant differences at p<0.05

Substrate	BMP (mL CH_4 g VS^{-1})			I (days)			Methane content (%)		
ANX	287.0	(47.2)	a	9.86	(1.59)	ab	72.21	(0.92)	a
A	288.9	(46.7)	a	9.53	(1.10)	ab	78.93	(11.25)	a
AC1	257.4	(15.6)	a	8.85	(2.28)	b	59.87	(0.40)	b
AC2	268.0	(74.0)	a	8.45	(2.91)	b	58.93	(1.40)	b
C	255.6	(32.5)	a	10.92	(0.23)	a	61.34	(4.55)	b

4. Conclusions

Biochemical Methane Potential tests were carried out to evaluate biomethane production capacity of a microalgae residue obtained after lipid extraction from *Nannochloropsis gaditana*. The methane yields observed for the microalgae residue were higher than those recorded for the raw microalgae sample. Co-digestion with cellulose did not clearly enhance the anaerobic digestion performances.

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