A novel pre-treatment for the methane production from microalgae by using N-methylmorpholine-N-oxide (NMMO)

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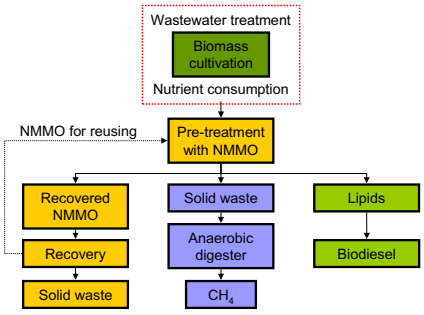
HIGHLIGHTS

- The NMMO damages the *N. oculata* cells, increasing the biodegradability.
- The lipid fraction remains inside the microalgae cells after the pre-treatment.
- The drying of *C. vulgaris* may have reduced the effectiveness of the pre-treatment.
- The NMMO could be fully recycled since does not decompose during the pre-treatment.

GRAPHICAL ABSTRACT



ABSTRACT

The aim of this work was to study the effect of the solvent N-methylmorpholine-N-oxide (NMMO) to pre-treat *Nannochloropsis oculata* before the anaerobic digestion process. The results indicated that the pre-treatment affects the characteristics of the cell wall, which consequently becomes more susceptible to the microorganisms attack during anaerobic digestion. The methane production was increased by 43% after the pre-treatment, from 238 ± 6 mL_{CH4}/g_{VS} until 339 ± 4 mL_{CH4}/g_{VS}. On the contrary, the methane production from *Chlorella vulgaris* decreased after the pre-treatment from 251 ± 4 mL_{CH4}/g_{VS} to 231 ± 3 mL_{CH4}/g_{VS}. The failure on the pre-treatment was attributed to the particular characteristics of the substrate in consequence of a previous drying step.

1. Introduction

Microalgae have the ability accumulate high amount of lipids, being promising feedstocks for biofuels (Rawat et al., 2013). Over the past years, researchers turned their attention to produce biodiesel from microalgae. Unfortunately, some challenges still required still need to be faced to scale up the biodiesel production, mainly derived from the high water content in microalgae cultures (Rawat et al., 2013). The anaerobic digestion (AD) is an alternative to produce energy from microalgae, in this case, biogas. The AD does not require the biomass drying, thus reducing the cost associated with harvesting (Kwietniewska and Tys, 2014). Moreover, AD results favourable when the biomass has low lipid content for the biodiesel production (Pragya et al., 2013). Some microalgae species such as *Chlorella* sp. and *Nannochloropsis* sp. demonstrated their suitability to grow in different wastewaters consuming nutrients, reducing the demand for fertilizer during their cultivation (He et al., 2013; Caporgno et al., 2015b). This allows coupling the cultivation and the AD of microalgae in a wastewater treatment plant (WWTP), with environmental and economic benefits.

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The organic solvent N-methylmorpholine-N-oxide (NMMO) has facilitated the AD of carbohydrates in lignocellulosic material, which is characterised by high resistance to degradation (Teghammar et al., 2014; Kabir et al., 2014). Since the NMMO is usually used as an aqueous solution, is does not require the biomass drying. However, the amount of water affects the process (Jeihanipour et al., 2010a). This solvent is commercially used in the Lyocell fibre-production process due to the feasibility to act as solvent for cellulose at mild conditions (90–130 °C and ambient pressure) (Zheng et al., 2014). The NMMO shows low toxicity, high thermal stability, and the possibility of being recovered and reused, amongst other advantages (Jeihanipour et al., 2010a). The NMMO affects hydrogen bonds and weaken van der Waals forces between the cellulose chain molecules, thus changing the cellulose structure (Zheng et al., 2014). Some of these changes, for example a decreased crystallinity and an increased porosity make cellulose more susceptible to AD (Jeihanipour et al., 2010a).

This paper is the first attempt to pre-treat microalgae, *Nannochloropsis oculata* and *Chlorella vulgaris*, using NMMO in order to improve the methane production.

2. Methods

2.1. Materials

N. oculata and *C. vulgaris* species were provided by AlgoSource (Alpha Biotech, Asserac, France). They were cultivated in a raceway unit placed in a greenhouse with thermal control, and harvested via centrifugation. *N. oculata* microalgae was received as frozen slurry with 28% solids and stored at −15 °C. In order to facilitate the pre-treatment, the biomass was dried before the experiment. *C. vulgaris* was received dried, and the solid was stored in a dessicator.

NMMO 50% w/w in aqueous solution was concentrated to 85% as described by Jeihanipour et al. (2010a).

2.2. Microalgae pre-treatment

The pre-treatment process is described in Fig. 1. The microalgae sample, 6 g of dried biomass, was added into a round-bottom flask containing 94 g of 85% NMMO solution and placed in an oil bath at 120 °C for 3 h in atmospheric conditions. Agitation was provided by a magnetic stirrer. After the 3 h heating, 150 mL of boiling deionised water was added to stop the process. The pre-treated microalgae were recovered by centrifugation as a solid fraction and washed with deionised water to eliminate the NMMO prior to the anaerobic digestion. The supernatant, which is the recovered NMMO, and liquid fraction from washing were collected together for further analysis. The pre-treatment was evaluated in three different opportunities.

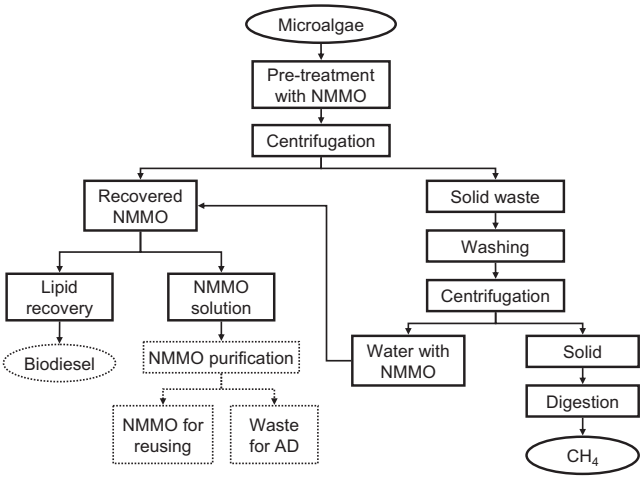

Fig. 1. Scheme of the pre-treatment process and the uses of the different products.

Microalgae samples, before and after the pre-treatment, were observed under a light microscope to evaluate their integrity The samples were also analysed by Fourier Transform Infrared (FTIR) spectroscopy to observe the changes in the protein, carbohydrate and lipid content in the biomass. Protein, carbohydrate and lipid content in raw and pre-treated samples were quantified as described by Caporgno et al. (2015a). In *C. vulgaris*, the carbohydrate content was calculated by the difference between total organic matter content and the contents of proteins and lipids.

2.3. Lipid extraction

The lipid content was analysed in the mixture containing the NMMO solutions collected after the microalgae pre-treatment and after washing, as indicated in Fig. 1. A sample of the mixture containing the NMMO was mixed with 10 ml of hexane; the hexane phase containing lipids was separated by centrifugation (3500 rpm, 10 min). This step was repeated three times. Hexane was dried under anhydrous sodium sulphate and evaporated in a rotary evaporator. For the lipid analysis in the pre-treated microalgae, a sample of microalgae was re-suspended in deionised water and acidified until pH 2 prior to the hexane addition. The results were expressed as gram of extractable lipids per gram of volatile solids in the dry microalgae used for the experiments, g/g_{VS}.

A thin-layer chromatography (TLC) was performed in order to evaluate the composition of the extracted-lipids. The lipids were dissolved in hexane and spotted on a TLC plate which was then developed in a solvent system of hexane/diethyl ether/acetic acid (60:40:1, v/v/v). Separated compounds were visualised under iodine vapour and identified by using authentic standards. The fatty acids in the extracted lipids were identified and quantified as described by Olkiewicz et al. (2014).

2.4. Anaerobic digestion

The AD experiments were performed in batch reactors at 33 °C in triplicate. The solid samples were re-suspended in deionised water for better handling. The inoculum consisted of digested sludge described in Caporgno et al. (2015a). The total solids (TS) and the volatile solids (VS) in all the substrates and in the inoculum were analysed according to standard methods as described by Caporgno et al. (2015a), and the substrate to inoculum ratio was adjusted to 1:2 $VS_{Substrate}$:$VS_{Inoculum}$ in all reactors.

The methodology for quantifying the biogas production and composition, the volatile fatty acid concentration (VFA), the ammonia concentration and the substrates biodegradability is fully described in Caporgno et al. (2015a).

3. Results and discussion

3.1. Lipid recovery after pre-treatment

Since the lipids are not solubilised in the NMMO solution due to the differences in the polarity of lipids and the NMMO solution, the released lipids can be recovered by hexane addition. The results indicated that 3.0 ± 0.2 and 0.9 ± 0.3 g/g_{VS} were recovered from the NMMO solution after *N. oculata* and *C. vulgaris* pre-treatment respectively. These were minor fractions considering the initial lipid content in both microalgae species (Table 1). The results indicated that the lipids were not released during the pre-treatment

and remained in the pre-treated microalgae instead. Although the NMMO is usually considered as ionic liquid (IL) (Zheng et al., 2014), completely opposite results have been reported by Olkiewicz et al. (2015) using the same microalgae but different IL; the authors indicated almost complete lysis and high lipid-recovery from IL.

After the pre-treatment, cells with different appearances were distinguished (Fig. S1 in the Supplementary material); however, it was impossible to identify if the lipid fraction remained inside the microalgae cells with the naked eye. Due to the strong alkaline pH of the NMMO solution (Navard, 2012), one of the possibilities was that the lipids remains in the solid as calcium and magnesium salts, product of the reaction between the free fatty acids (FFA) and the metal ions present in the solution. Since this reaction can be reversed by acidification (Olkiewicz et al., 2014), this hypothesis was verified by the analysis of the lipid in the pre-treated microalgae after acidifying the sample until pH 2. The results indicated that 14.4 ± 0.5 and 3.0 ± 0.1 g/g_{VS} were recovered from *N. oculata* and *C. vulgaris* respectively after acidification of the pre-treated solid. These lipid yields represent 83% and 77% of the total lipid recovered with hexane from *N. oculata* and *C. vulgaris* respectively, indicating that the major part of the lipid fraction remained in the solid fraction.

The TLC analysis revealed that the different lipid fractions had similar compositions. The fractions were not only FFA, but also contained monoglycerides, diglycerides and triglycerides. This refused the hypothesis about the lipids remaining as calcium and magnesium salts, and suggested that the pre-treatment modified the microalgae cell walls and facilitated the lipid extraction from the microalgae after acidification. On the other hand, the similarity in the lipid fractions revealed that the lipids recovered from the NMMO solution came from part of the biomass which was completely damaged during the pre-treatment.

The lipid fractions were converted into biodiesel. The fatty acid profile agreed with the composition reported for both microalgae species using different extraction methods (Olkiewicz et al., 2015). The *N. oculata* profile was dominated by cis-5,8,11,14,17-E icosapentaenoic (20:5), cis-9 Palmitoleic (C16:1) and Palmitic (C16:0), whereas the *C. vulgaris* profile, by Linolenic (C18:3), Palmitic (C16:0), cis-10-heptadecenoic (C17:1), Linoleic (C18:2), Oleic (C18:1) and cis-9 Palmitoleic (C16:1).

3.2. Methane production from the pre-treated microalgae

As can be observed in Fig. 2, the methane production from the raw microalgae species resulted similar for *N. oculata* and *C. vulgaris*. Regarding the pre-treatment, it can be observed that the NMMO pre-treatment has positively affected the methane production from *N. oculata*, which increased from 238 ± 6 to 339 ± 4 mL_{CH4}/g_{VS} after pre-treatment, representing a 43% increase. On the contrary, *C. vulgaris* showed an 8% decrease after the pre-treatment, from 251 ± 4 to 231 ± 3 mL_{CH4}/g_{VS}.

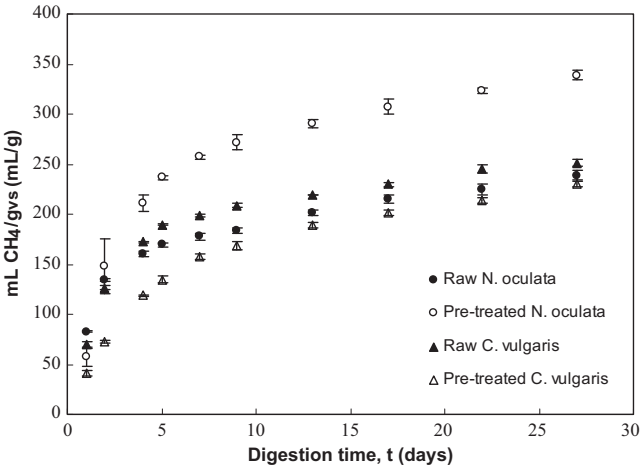

Fig. 2. The methane production curves from raw and pre-treated *N. oculata* and *C. vulgaris*. Symbols: ● raw *N. oculata*, ○ pre-treated *N. oculata*, ▲ raw *C. vulgaris* and ◆ pre-treated *C. vulgaris*. Batch reactors at 33 °C.

The theoretical methane production based on the substrate compositions (Table 1) resulted in 596 and 576 mL_{CH4}/g_{VS} for raw and pre-treated *N. oculata*, and 496 and 494 mL_{CH4}/g_{VS} for raw and pre-treated *C. vulgaris* respectively. In terms of theoretical methane production, lipids almost double proteins and carbohydrates (Caporgno et al., 2015b); thus higher values resulted for *N. oculata*. Based on these values, the biodegradability increased from 40% to 59% for *N. oculata* after the pre-treatment, and slightly decreased from 51% to 47% for *C. vulgaris*.

The pre-treatment damaged the *N. oculata* cells, remaining the lipid fraction in the cells. The lipids became then available for microorganisms, increasing the methane production drastically. The carbohydrate fraction may have also contributed to increase the methane production. The cell wall of *Nannochloropsis* sp. is characterised by a thick and multilayered wall composed of polysaccharides (Bohutskyi et al., 2014). The addition of water to stop the pre-treatment produced the carbohydrates precipitation, which constitute part of the solid fraction recovered by filtration. The NMMO has demonstrated its feasibility to enhance the digestibility of low-digestible carbohydrates (Zheng et al., 2014); however, based on the low carbohydrate content in *N. oculata*, the methane production enhancement can be mainly attributed to the lipid fraction. In spite of *C. vulgaris* and *N. oculata* show similar cell walls composition (Bohutskyi et al., 2014), the cell aggregates originated during *C. vulgaris* drying remained partially unaffected after the pre-treatment (Fig. S1d–f). These aggregates hampered the action of the NMMO, thus the pre-treatment failed at increasing the digestibility of the low-digestible carbohydrates. The intra- and intermolecular hydrogen bonds were unaffected by the pre-treatment (Fig. S2b). Considering the higher

Table 1
Characteristics of the inoculum and the substrates used in the experiments.

Parameter	Inoculum	*Nannochloropsis oculata*		*Chlorella vulgaris*	
		Raw[a]	Pre-treated[b]	Raw[a]	Pre-treated[b]
TS (g/L)	17.9 ± 0.4	63.8 ± 0.4	18.3 ± 0.2	9.5 ± 0.6	36.6 ± 2.9
VS/TS	0.62	0.89	0.83	0.78	0.63
Lipids ($g/100g_{VS}$)	–	21.9 ± 0.4	18.9[c]	6.5 ± 0.6	5.6[c]
Proteins ($g/100g_{VS}$)	–	60.9 ± 3.4	56.2 ± 4.6	48.7 ± 4.0	53.1 ± 0.4
Carbohydrates ($g/100g_{VS}$)	–	16.7 ± 1.5	23.2 ± 0.9	41.2 ± 0.7[d]	37.5[d]

[a] Suspension in deionised water.
[b] Suspension in deionised water after washing.
[c] Calculated considering the lipid recovery from the NMMO solution.
[d] Calculated by difference between difference between total organic matter content and the contents of proteins and lipids.

carbohydrate fraction in *C. vulgaris* compared with *N. oculata*, the digestion of the carbohydrate fraction can contributes considerably to the final methane production. On the contrary, the lipid fraction is low in *C. vulgaris*, thus does not increase the methane production. Moreover, the lipid fraction still remained inaccessible for the microorganisms after the pre-treatment due to the microalgae aggregates. The lower methane production in pre-treated samples can derive from the minor changes in the biomass composition.

The pH ranged 7.41–7.53, the concentration of ammonium nitrogen 736–825 mg/L and VFA were not detected at the end of the experiments. These parameters corroborated that all digesters operated at optimum conditions. The biogas composition, analysed several times during the experiments, resulted in 71.3 ± 1.9% and 71.2 ± 2.2% CH_4 for *N. oculata* and *C. vulgaris* respectively; the pre-treatment did not influence the biogas composition.

3.3. Other considerations for economic feasibility

The pre-treatment with NMMO is high-energy consuming since the solvent is heated at 120 °C. However, all the pre-treatments reported in the literature are usually carried out at 90 °C or higher (Teghammar et al., 2014; Jeihanipour et al., 2010a, 2010b; Kabir et al., 2014); the reason is that the NMMO 85% in water is a solid at room temperature. The pre-treatment here proposed could be optimised either reducing the temperature and the duration of the pre-treatment or reducing the energy demand by heat-integration.

Unlike the chemical pre-treatments usually reported, this method allows recovering the NMMO for its reutilisation, being essential to make similar processes economically feasible (Jeihanipour et al., 2010b). It has been proved that the NMMO can be reused for the pre-treatment, but the characteristics of the biomass strongly affects recycling (Jeihanipour et al., 2010b; Kabir et al., 2014). The NMMO has not been reused in the present experiments; however, the ^{1}H NMR spectroscopy analyses demonstrated the solvent stability after the pre-treatment of *N. oculata* at 120 °C for 3 h (Fig. S3).

4. Conclusions

The digestibility of *N. oculata* was successfully increased after the pre-treatment with NMMO. On the contrary, *C. vulgaris* digestibility was negatively affected; the drying step in the biomass production process influenced the digestion process more than the pre-treatment itself.

The NMMO is currently used in industrial processes and presents several advantages over some other solvents; however, the pre-treatment method requires optimisation in order to design an economically viable process. The NMMO does not decompose during the pre-treatment and could be fully recycled.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.11.069.

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