Influence of hydrothermal pretreatment on microalgal biomass anaerobic digestion and bioenergy production

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Abstract

Microalgal biomass grown in wastewater treatment raceway ponds may be valorized producing bioenergy through anaerobic digestion. However, pretreatment techniques seem to be necessary for enhancing microalgae methane yield. In this study, hydrothermal pretreatment was studied prior to batch and continuous reactors. The pretreatment increased organic matter solubilisation (8-13%), anaerobic digestion rate (30-90%) and final methane yield (17-39%) in batch tests. The highest increase was attained with the pretreatment at 130 ºC for 15 minutes, which was attested in a laboratory-scale continuous reactor operated at a hydraulic retention time of 20 days with an average organic loading rate of 0.7 g VS/L·day. The methane production rate increased from 0.07 to 0.12 L CH_4/L·day (58%) and the methane yield from 0.12 to 0.17 L CH_4/g VS (41%) in the pretreated digester as compared to the control. Microscopic images of microalgal biomass showed that pretreated cells had unstructured organelles and disrupted cell wall external layer, which may enchance the hydrolysis. Indeed, images of the pretreated reactor digestate showed how cells were more degraded than in the control reactor.

Keywords

Algae; Bioenergy; Biogas; Methane; Microalgae; Wastewater

1. Introduction

High rate algal ponds (HRAP) were first developed for wastewater treatment in the 1950's in California (Oswald and Golueke, 1960). This technology consists in shallow ponds with constant mixing provided by a paddle-wheel that enhances phytoplankton photosynthesis, since it allows sunlight to penetrate through the whole system. In these microalgae-based ponds, organic matter and nutrients are removed from the influent wastewater through the symbiotic relation between heterotrophic bacteria and microalgae. Thus, bacteria degrade organic carbon consuming oxygen, which is synthetized by microalgae photosynthesis. In comparison to conventional activated sludge systems, here no external aeration is needed for bacteria growth. In HRAP treating urban wastewater, biomass is composed by around 90% microalgae and 10% bacteria (García et al., 2000). Harvested microalgal biomass can be treated through anaerobic digestion, a well-known process widely used for sewage sludge treatment in conventional wastewater treatment plants (WWTP).

However, microalgal biomass has a slow anaerobic biodegradability, mainly due to its complex cell wall structure. Actually, microalgae cell wall varies greatly among species. While some species such as *Dunaliella salina* lack the cell wall, others may differ on the cell wall composition, being a protein-based cell wall for *Euglena gracilis* and a polysaccharide-based cell wall for *Scenedesmus obliquus*, conferring to the latter a more recalcitrant nature (González-Fernández et al., 2011). Moreover, predominant species in microalgal biomass grown in wastewater generally have a rigid cell wall, due to its adaptability to grow under variable ambient conditions, with predatory organisms and high organic content (Park et al., 2011).

In order to improve microalgae anaerobic digestion, pretreatment methods are currently being studied. So far it has been shown that reactors with a hydraulic retention time (HRT) of at least 20 days, preceded by some pretreatment step are required for reaching a methane yield around 0.30 L CH_4/g VS (Passos and Ferrer, 2014; González-Fernández et al., 2012). Among the investigated pretreatment techniques, thermal pretreatment has exhibited the most promising results, reaching high methane yields, while attaining positive energy balances (Passos and Ferrer, 2014;

Schwede et al., 2013). To date, temperatures from 55 to 170 °C have been applied. When thermal pretreatment is applied at temperatures higher than 100 ºC, pressure increases. In this case, thermal pretreatment is so-called hydrothermal pretreatment. Generally, it is applied at temperatures between 100-140 ºC along with pressures around 1-2 bar. As can be seen in Table 1, the methane yield may increase from 20 to 108% depending on the pretreatment conditions, and most importantly on the microalgae species used in each case.

The aim of this study was to evaluate the anaerobic digestion of microalgal biomass grown in wastewater treatment HRAP after hydrothermal pretreatment. To this end, biochemical methane potential (BMP) tests were performed with microalgae pretreated under different temperatures and exposure times. The best pretreatment condition was then studied in continuous reactors. Microscopic images where used to analyse the effect of pretreatment in microalgae cell structure and anaerobic biodegradability. Furthermore, an energy assessment was carried out in order to determine the scalability of this technology.

2. Material and Methods

2.1 Microalgal biomass

Microalgal biomass was grown in a pilot HRAP used for secondary treatment of urban wastewater. The experimental set-up was located outdoors at the laboratory of the GEMMA research group (Universitat Politècnica de Catalunya) in Barcelona (Spain). The HRAP received the primary effluent from a settling tank which had a useful volume of 7 L and a HRT of 0.9 hours. The primary effluent was pumped to the HRAP by means of a peristaltic pump with a flow rate of 60 L/d. The HRAP was built in PVC with a surface area of 1.54 m^2, a height of 0.3 m, a useful volume of 0.47 m^3 and a nominal HRT of 8 days. Average surface loading rates were ±24 g COD/m^2day and ±4 g NH_4-N/m^2day. Microalgae contact with sunlight was enhanced through continuous stirring with a bladed paddle-wheel, reaching an approximate mixed liquor flow velocity of 10 cm/s. Further information on the HRAP performance may be found elsewhere (Passos et al., 2013a).

Microalgal biomass was harvested from secondary settlers with a useful volume of 9 L and a HRT of 9 hours. Following, biomass was thickened by gravity in laboratory Imhoff cones at 4 ºC for 24 hours for reaching total solid (TS) concentration of 2.0-2.5 % (w/w). Microalgal biomass macromolecular composition was fairly stable, with 58% (± 2.5) of proteins, 19% (±1.3) of lipids and 22% (±2.7) of carbohydrates over a sampling period of four months (Passos et al., 2013a).

2.2 Hydrothermal pretreatment

Hydrothermal pretreatment was carried out in an autoclave (Autester, Selecta, Spain). For the BMP tests, pretreatment conditions were 110 ºC (1.2 bar) and 130 ºC (1.7 bar) for 15 and 30 minutes; while for the continuous reactor pretreatment conditions were 130 ºC for 15 minutes, based on previous BMP test results. Relatively low target temperatures were selected not to increase the energy demand for the thermal pretreatment and to avoid Maillard reactions which may lead to the formation of recalcitrant compounds. Exposure times (15 and 30 min) were based on literature results (Table 1). Pretreatment was performed in glass bottles of 250 mL with a useful volume of 150 mL. Bottle caps were slightly loose. During hydrothermal pretreatment biomass was placed in the autoclave and temperature was raised to the target value. In this moment, biomass was maintained under the target temperature for the whole exposure time. Then pressure was gradually released to reach atmosphere conditions. Finally, biomass was cooled to room temperature and stored at 4 ºC until use.

Organic matter solubilisation was determined to evaluate the effectiveness of the pretreatment prior to BMP tests. The solubilisation degree (%) was calculated according to Eq. 1, where VS corresponds to total volatile solids, VS_s corresponds to soluble volatile solids and the sub-indexes refer to pretreated (p) and control (o) biomass.

$$S\,(\%) = \frac{(VS_s)_p - (VS_s)_o}{VS - (VS_s)_o}\,100$$

(Eq. 1)

2.3 Biochemical methane potential tests

BMP tests were used to compare the anaerobic biodegradability of pretreated and non-pretreated microalgal biomass. To this end, microalgal biomass (1.5 L) was harvested once for all trials. Digestate from a full-scale anaerobic reactor treating sewage sludge in a WWTP near Barcelona (Spain) was used as inoculum. The selected substrate to inoculum ratio was 0.5 g VS_s/g VS_i (Passos et al., 2013b), corresponding to 28 g of microalgae (substrate) and 32 g of sludge (inoculum) per bottle. Serum bottles (160 mL) were filled with distilled water up to 100 mL, flushed with Helium gas, sealed with butyl rubber stoppers and incubated at 35 ºC until biogas production ceased. A blank treatment with only inoculum was used to quantify the amount of methane produced by endogenous respiration. Each pretreatment was performed in duplicate, whereas the control (non-pretreated biomass) and blank (inoculum) were performed in triplicate. Biogas production was calculated by subtracting the blank results to each trial. The methane content in biogas was analyzed twice a week by gas chromatography (GC).

2.4 Continuous reactors

The influence of pretreatment on microalgae anaerobic digestion performance was monitored using two lab-scale reactors (2 L), with a useful volume of 1.5 L. In this manner, control and pretreated biomass were simultaneously investigated. Reactors were operated under mesophilic conditions (37 ± 1 ºC) by implementing an electric heating cover (Selecta, Spain). Constant mixing was provided by a magnetic stirrer (Thermo Scientific). Biogas production was measured by water displacement and the methane content was analysed twice a week by GC. The same volume (75 mL) was purged from and added to the digesters using plastic syringes on a daily basis. Reactors were operated at a HRT of 20 days and were considered to be under steady-state after three complete HRT. Afterwards, anaerobic digestion performance was monitored during 2-3 complete HRT (8 weeks). Thus, the reactors were operated over a period of 104 days, in which the pretreated reactor was fed with microalgal biomass after hydrothermal pretreatment and the control reactor was fed with non-pretreated biomass. Microalgal biomass was harvested once a week and stored at 4 ºC until use.

2.5 Analytical methods

All analyses were carried out in triplicated and results are given as mean values. Microalgal biomass was characterised by the concentration of TS, VS, chemical oxygen demand (COD), total Kjeldhal nitrogen (TKN) and ammonia nitrogen ($N\text{-}NH_4^+$) according to Standard Methods (APHA-AWWA-WPCF, 1999). Soluble samples for VS and $N\text{-}NH_4$ analysis were obtained by centrifugation (UNICEN20, 4200 rpm, 8 min, 20 ºC) and filtration (glass fiber filter 47 mm and pore size 1 μm). pH was analysed with a Crison Portable 506 pH-meter. Regarding the continuous reactors, TS, VS and pH were determined twice a week, while COD, TKN, $N\text{-}NH_4^+$ and volatile fatty acids (VFA) were determined once a week.

VFA were analysed in soluble phase by gas chromatography (GC) (Agilent Technologies 7820A), according to the procedure described by Passos et al. (2013b). Similarly, the methane content in biogas was measured with a GC (Trace GC Thermo Finnigan) equipped with a Thermal Conductivity Detector, according to the procedure detailed previously (Passos et al., 2013b).

2.6 Microscopic images

Microscopic images were used to provide qualitative information on the effect of hydrothermal pretreatment on the cell structure and anaerobic biodegradability. Samples were taken once the continuous reactors were stable.

Microalgae species identification and cell wall integrity images were taken with an optical microscope (Aixoplan Zeiss, Germany), equipped with a camara MRc5, using the software Axioplan LE. Basic microalgae diversity morphotypes were identified from classical specific literature (Palmer, 1962; Bourelly, 1966). For transmission electron microscopy (TEM) images, biomass was centrifuged at 2000 rpm for 5 min and fixed in a mixture of 2% paraformaldehyde and 2,5% glutaraldehyde, as described in our previous study (Passos et al., 2014a). Samples were examined using a JEOL 1010 TEM at 100 kV accelerating voltage.

2.7 Statistical analysis

In BMP tests, anaerobic digestion kinetics were fit by the least square method. The effect of hydrothermal pretreatment on the methane production rate and yield was determined by the ANOVA test using R Commander Statistical Software. $\rho = 0.05$ was set as the level of statistical significance.

2.8 Energy assessment

An energy assessment of microalgal biomass anaerobic digestion with and without pretreatment step was carried out for evaluating its scalability. To do so, parameters for full-scale reactors were estimated from experimental data, considering a flow rate of 100 m^3/d and a useful volume of 2,000 m^3 corresponded to 20 days HRT. Energy input was divided in to electricity and heat demands. Parameters used are summarised in Table 2.

For the anaerobic digestion of non-pretreated microalgal biomass, input heat was calculated as the energy required to heat influent biomass from ambient temperature (T_a) to digestion temperature (T_d), according to Eq. 2. The density (ρ) and specific heat (γ) of microalgal biomass were assumed to be the same as those of water, 1,000 kg/m^3 and 4.18 kJ/kg·ºC, respectively. Heat losses through the reactor wall were considered, the heat transfer coefficient (k) was assumed to be 1 W/m^2·d (Metcalf and Eddy, 2003). The reactor wall surface area (A) was calculated from the reactor useful volume, considering a 2:1 diameter to height ratio; while the reactor bottom and top were not accounted for (Metcalf and Eddy, 2003).

$$E_{i,heat} = \rho Q \gamma \, (T_d - T_a) + kA \, (T_d - T_a) 86.4 \qquad \text{(Eq. 2)}$$

where: $E_{i,heat}$: input heat (kJ/d); ρ: density (kg/m^3); Q: flow rate (m^3/d); γ: specific heat (kJ/kg·ºC); T_d: anaerobic digestion temperature (37 ºC); T_a: ambient temperature (20 ºC); k: heat transfer coefficient (W/m^2·ºC); A: surface area of the reactor wall (m^2).

In the case of microalgae pretreatment, input heat was calculated as the energy required to heat influent biomass from T_a to pretreatment temperature (T_p), i.e. 130 ºC, subtracted by the heat recovered when cooling down biomass from T_p to T_d (Eq. 3). Heat would be recovered by means of a heat exchanger, with an efficiency ϕ of 85% (Lu et al., 2008). Heat losses through the reactor walls were also accounted for.

$$E_{i,heat} = \rho Q \gamma \left(T_p - T_a\right) - \rho Q \gamma \left(T_p - T_d\right) \emptyset + kA \left(T_d - T_a\right) 86.4$$

(Eq. 3)

where: $E_{i,heat}$: input heat (kJ/d); ρ: density (kg/m^3); Q: flow rate (m^3/d); γ: specific heat (kJ/kg·ºC); T_d: anaerobic digestion temperature (37 ºC); T_a: ambient temperature (20 ºC); T_p: pretreatment temperature (130 ºC); □: heat recovery from pretreated biomass; k: heat transfer coefficient (W/m^2·ºC); A: surface area of the reactor wall (m^2).

Input electricity (Eq. 4) for both control and pretreated digesters, was estimated from the energy required for biomass pumping and reactor mixing, assumed to be 1,800 kJ/m^3 and 300 kJ/m$^3_{reactor}$·d, respectively (Lu et al., 2008).

$$E_{i,elecricity} = Q\theta + V\omega$$

(Eq. 4)

where: $E_{i,electricity}$: input electricity (kJ/d); Q: flow rate (m^3/d); θ: electricity consumption for pumping (kJ/m^3); V: useful volume (m^3); ω: electricity consumption for mixing (kJ/m$^3_{reactor}$·d).

The energy output from the anaerobic digestion was calculated from the methane yield, according to Eq. 5 and 6. The lower heating value of methane (ξ) was assumed to be 35,800 kJ/m^3 CH_4 (Metcalf and Eddy, 2003). An efficiency of 90% on energy conversion was considered

$$E_o = P_{CH4}\ \xi\ OLR\ V\ \eta$$

(Eq. 5)

where: E_o: output energy (kJ/d); P_{CH4}: methane yield (m^3CH_4/kg VS); ξ: lower heating value of methane (kJ/m^3CH_4); OLR: organic loading rate (kg VS/m^3·d); V: useful volume (m^3); η: energy conversion efficiency (%).

Finally, results were expressed as energy balance (ΔE) and energy ratio (E_o/E_i) for both control and pretreated reactors. The energy balance was calculated as the difference between the energy output and energy input (heat and electricity) (Eq. 6), while the energy ratio was calculated from the energy output over the energy input (heat and electricity) (Eq. 7).

$$\Delta E = E_o - (E_{i,heat} + E_{i,electricity})$$

(Eq. 6)

$$E_o/E_i = \frac{E_o}{(E_{i,heat} + E_{i,electricity})}$$

(Eq. 7)

3. Results and Discussion

3.1 Effect of hydrothermal pretreatment on biomass solubilisation and anaerobic biodegradability in BMP tests

Microalgal biomass solubilisation, anaerobic digestion rate and methane yield were improved after hydrothermal pretreatment under all conditions assayed (Table 3). Soluble VS increased by 8-9% after pretreatment at 110 ºC and by 13-15% after pretreatment at 130 ºC. Temperature rather than exposure time seemed more important for biomass solubilization; since only small differences were noticed between 15 and 30 min (Table 3). This is in accordance with our previous study on thermal pretreatment at temperatures below 100 ºC (Passos et al., 2013). However, results attained were lower than expected. For instance, hydrothermal pretreatment of *Chlorella* sp. and *Scenedesmus* sp. biomass at 120 ºC attained a solubilisation of 30% (Cho et al., 2013). Furthermore, COD solubilisation of *Acutodesmus obliquus* and *Oocystis sp.* biomass and *Microspora* sp. biomass was increased by 37% and 40% after pretreatment at 140 ºC for 15 min, respectively; while *Scenedesmus* sp., *Clamydomonas* sp. and *Nannocloropsis* sp. biomass reached a solubilisation of 16% under the same conditions (Alzate et al., 2012). The latter results are more similar to those found in our study. This is probably due to the different microalgae species used in each case. Indeed, microalgal biomass grown in wastewater is commonly formed by species with resistant cell walls forming flocs in order to adapt to the diverse conditions, e.g. seasonality and predators. These characteristics may hamper biomass solubilisation and anaerobic biodegradability. It has been shown that microalgae pretreatment may not disrupt the cell wall, however by damaging the cell structure, it seems to assist the anaerobic digestion process (Passos et al., 2014a).

BMP tests showed that hydrothermal pretreatment was effective at enhancing microalgae anaerobic biodegradability. Increased anaerobic digestion rate (30-90%) and final methane yield (17-39%) were observed when compared to the control (Table 3; Fig. 1). These results are in accordance with previous BMP tests of mixed microalgae cultures. For instance, the methane yield of *Scenedesmus* sp., *Clamydomonas* sp. and *Nannocloropsis* sp. biomass increased by 19 and 33% after pretreatment at 110 and 140 ºC for 15 min; while for *Acutodesmus obliquus* and *Oocystis* sp. biomass the methane yield increased by 11 and 33% under the same pretreatment conditions (Alzate et al., 2012). However, much higher values were found for *Chlorella* sp. and *Scenedesmus* sp. biomass and *Microspora* sp. biomass, which reached from 50 to 120% higher methane yield as compared to non-pretreated samples (Alzate et al., 2012; Cho et al., 2013). Indeed, it has been shown that microalgae anaerobic biodegradability is species-specific and depends mainly on the cell wall structure (Mussgnug et al., 2011). In our case, the methane yield was improved by 24 and 39% after pretreatment at 110 and 130 ºC for 15 min, respectively. The best results in terms of anaerobic digestion rate and methane yield were attained when pretreatment was performed at 130 ºC for 15 min (0.36 d^{-1}; 0.17 L CH_4/g VS).

3.2 Effect of hydrothermal pretreatment on the anaerobic digestion performance in continuous reactors

The optimal pretreatment condition (130 ºC; 15 min) was thereafter tested in laboratory-scale continuous reactors. During the whole experimental period, both control and pretreated reactors were operated with an organic loading rate around 0.7 g VS/L·day and a HRT of 20 days (Table 4). Weekly average methane yield from each reactor is shown in Fig. 2; hydrothermal pretreatment clearly enhanced anaerobic digestion performance. The methane production rate and methane yield of non-pretreated microalgal biomass were 0.07 L CH_4/L·day and 0.12 L CH_4/g VS, respectively, with a VS removal around 30%. After the pretreatment step, the methane production rate increased to 0.12 L CH_4/L·day (58% increase) and the methane yield to 0.17 L CH_4/g VS (41% increase), with a VS removal around 40%. In fact, the methane production rate and yield were significantly higher for the pretreated reactor in comparison with the control (Table 5). As can be seen in Fig. 2, especially for the control reactor, the methane yield reached very low values of 0.06 L CH_4/g VS. Microalgae biodegradability and pretreatment effectiveness are species-specific and therefore, higher methane yields may be reached when biomass is composed by species with less complex cell wall structure than those typically found in HRAP treating wastewater (e.g. diatoms). Indeed, in our previous studies, microalgal biomass harvested from the same pilot system reached average methane yields of 0.17 L CH_4/g VS (Passos et al., 2014a) and 0.18 L CH_4/g VS (Passos and Ferrer, 2014). In these cases, biomass was mainly composed by *Monoraphidium* sp. and *Stigeoclonium* sp. Changes in methane yield in the long term are normal, since the composition of microalgal biomass varies over time in open ponds treating wastewater (Park et al., 2011; Passos et al., 2014b). This occurs due to many factors, such as environmental conditions (e.g. solar radiation, temperature and precipitation), influent wastewater composition (e.g. toxic compounds) or external contamination (e.g. plants, microfauna and bacteria). In fact, both reactors showed a decreasing trend in the average methane yield, although it was consistently higher in the pretreated one (Fig. 2).

Concerning the stability of digesters, pH values were stable during the whole period, ranging from 7.0 to 7.6 (Table 4). Regarding ammonium concentration, the reactor effluent exhibited between 300 and 350 mg N-NH_4/L, which is below toxic concentrations of 1.7 g/L (Schwede et al., 2013). VFA were not detected before and after pretreatment, and only very low concentrations of 45 mg COD/L were found in both effluents (Table 4).

Nitrogen mineralisation was calculated as the difference in concentration of organic nitrogen before and after anaerobic digestion of pretreated and non-pretreated biomass. For this, organic nitrogen was calculated as the difference between the total Kjeldhal nitrogen (TKN) and ammonium concentration (Table 4). According to the results, hydrothermal pretreatment increased organic nitrogen removal. For the control reactor, nitrogen mineralisation was in average 24%, while after hydrothermal pretreatment, it was 34%.

So far, the sole study dealing with microalgae hydrothermal pretreatment prior to anaerobic digestion in continuous reactors was the one by Schwede et al. (2013), in which the methane yield of *Nannochloropsis salina* was increased from 0.13 to 0.27 L CH_4/g VS (108%). In regards to thermal pretreatment at lower temperatures (< 100 ºC), the methane yield of microalgal biomass grown in wastewater treatment HRAP increased by 33% after pretreatment at 100 ºC for 8 hours (Chen and Oswald, 1998) and around 70% after pretreatment at 75 and 95 ºC for 10 hours (Passos and Ferrer, 2014). As previously mentioned, the variation in the results obtained may be attributed to the characteristics of the microalgae species investigated in each case. Our biomass was not a pure microalgae culture; on the contrary, it was formed by a mixed culture of microalgae and bacteria growing in HRAP for wastewater treatment. Biomass biodegradability depends on characteristics such as microalgae species, content of bacteria and microfauna, biofilm, growing conditions, macromolecular composition, among others. In microalgal biomass grown in open ponds treating wastewater a spontaneous ecosystem is formed. In our previous study, we observed that during periods where microalgae species with resistant cell wall are present, hydrolysis step in anaerobic digestion is hampered leading to low methane yields (Passos et al., 2014b).

3.3 Microscopic analysis of microalgae cells after preteratment and anaerobic digestion

Optical microscope images of non-pretreated and pretreated microalgal biomass before and after anaerobic digestion are shown in Figure 3. Towards the end of the experiment, microalgal biomass was mainly composed by *Oocystis* sp. Non-pretreated microalgae are shown in Fig. 3a and 3b before and after anaerobic digestion, respectively. In the digestate (Fig. 3b), most *Oocystis* sp. cells were not disrupted, suggesting that methane was produced by anaerobic biodegradation of other microalgae, flocs containing extracellular polymeric substances and/or other organisms, such as bacteria. This was already found for *Scenedesmus* biomass anaerobic digestion after thermal pretreatment at 70 ºC (González-Fernández et al., 2012).

Pretreated microalgae are shown in Fig. 3c and 3d before and after anaerobic digestion, respectively. After hydrothermal pretreatment, *Oocystis* sp. cells were affected and damaged (Fig. 3c). Although the cell wall was still present, organelles were unstructured, pigmentation was lower and there were many granules. Note that chloroplasts, which were clearly detected in fresh biomass (Fig. 3a), were completely disrupted in pretreated biomass (Fig. 3c). In the digestate, almost no cells were found (Fig. 3d). This suggests that the increase in methane yield after pretreatment was due to microalgae which could not be digested without pretreatment.

These observations were confirmed by TEM images of non-pretreated (Fig. 4a-b) and pretreated (Fig. 4c-d) *Oocystis* sp. cells. Damaged intracellular structure can be observed in Fig. 4c. The space between the cell wall and cytoplasm indicates that the pretreatment disrupted organelles. Furthermore, the external layer of the cell wall of *Oocystis* sp. was disrupted (Fig. 4d). In fact, *Oocystis* sp. has distinct cell wall layers. A detailed microscopic investigation on *Oocystis apiculata* by Fujino and Itoh (1994) showed that the cell wall was formed by three different layers; an outer and inner layer composed by amorphous material and a middle layer composed by microfibril structures. According to our TEM images, *Oocystis* sp. showed at least two different cell wall layers, and an outer structure affected by the pretreatment step. The disruption of microalgae cell wall surely enhanced microalgae anaerobic biodegradability.

Information on microalgal biomass characteristics using microscopic images is crucial to understand the effect of pretreatments on the cell structure and, consequently, on the anaerobic digestion performance. As can be seen in Fig. 2, the methane yield of both digesters had a decreasing trend over the experimental period. This decrease was more evident in the control reactor, which varied from 0.16 to 0.08 L CH_4/g VS. This variation was probably due to changes in microalgal biomass characteristics and/or species. In fact, it has been already reported that microalgae anaerobic digestion performance is species-specific (González-Fernández et al., 2011; Passos and Ferrer, 2014). In the same way, pretreatment efficiency also depends on the microalgae species. This means that changes in biomass over time may have had a higher impact on the methane yield of non-pretreated microalgae, which decreased from 0.16 to 0.08 L CH_4/g VS, as compared to pretreated biomass, which decreased from 0.20 to 0.15 L CH_4/g VS.

Since microalgal biomass from wastewater treatment systems changes over time, further research should couple microalgae digestion in continuous reactors with periodic biomass characterization to elucidate the effect of microalgae species on the methane yield of the reactor.

3.4 Energy assessment

The energy assessment of microalgae anaerobic digestion with and without hydrothermal pretreatment was based on experimental results in continuous reactors (Table 6). Since global energy balances were calculated by subtracting the energy input (heat and electricity) to the energy output (methane production), positive values indicate net energy production in the system. As can be observed, neither the control reactor nor the pretreated reactor attained a positive energy balance, i.e. -2.24 and -5.94 GJ/d, respectively. After pretreatment, the energy output increased from 5.41 to 7.67 GJ/d; however the energy input for heating influent biomass was also higher: 12.83 GJ/d as compared to 6.87 GJ/d for the control reactor.

One of the main issues concerning the high energy input for the pretreatment step is the low solids content in microalgal biomass. Indeed, Schwede et al. (2013) incorporated biomass dewatering to reach a solids concentration of 25 %, and by doing so only 7% of the heat generated from biogas (317 kWh) was consumed in the thermal pretreatment (23 kWh).

In our case, the energy balance was recalculated including a centrifugation step to determine the minimum solids concentration for reaching a neutral energy balance. This corresponds to a biomass concentration increase from 2.3 to 7.4% TS (3.2 times higher biomass concentration). Consequently, the energy input was recalculated according to a new flow rate of 31.25 m^3/d, reactor volume of 625 m^3, and reactor wall surface area of 214 m^2; instead of 100 m^3/d, 2000 m^3 and 465 m^2, respectively without thickening step (Table 2). The energy input for the centrifuge was estimated considering an electricity consumption υ of 0.04 kWh/kg TS (Suh and Rosseaux, 2002), according to Eq. 7. In this hypothetic scenario, the energy output after centrifugation was assumed to be the same as for the non-thickened biomass.

$$E_{i,centrifuge} = Q \times \upsilon \times TS \times 3600 / 100 \quad (7)$$

where: $E_{i,centrifuge}$: input electricity for the centrifuge (kJ/d); Q: flow rate (100 m^3/d); υ: electricity consumption (0.04 kWh/kg TS); TS: influent total solids concentration (23 kg TS/m^3) and 3600 is the conversion from kWh to kJ .

According to the results, both the pretreatment and thickening steps were crucial for reaching a positive energy balance (Table 6). In this scenario, the control digester still had a negative energy balance of -0.40 GJ/d, while the pretreated reactor had a neutral energy balance (E_o=E_i). Alternatively, lower temperature pretreatment (75 ºC) could be used even without a thickening step, leading to a net energy production of 3 GJ/d (Passos and Ferrer, 2014).

It is worth taking into consideration that after biomass thickening the OLR would increase from 0.7 to 2.2 g VS/L·d. This may affect microalgae methane yield and, consequently, the energy output. A previous study using batch tests showed that the methane yield of thermally pretreated *Chlorella vulgaris* and *Scenedesmus* sp. biomass did not decrease after increasing the solids concentration from 16 to 130 g TS/L (Mendez et al., 2014). However, in continuous reactors, *Scenedesmus* biomass methane yield decreased from 0.21 to 0.14 L CH_4/g VS when the OLR was increased from 1.3 to 2.2 g VS/L·d due to ammonia inhibition (Alzate, 2014). Conversely, the same microalgae species pretreated at 90 ºC had a similar methane yield when digested at an OLR of 1 kg COD/m^3·day (97 mL CH_4/g COD) and 2.5 kg COD/m^3·day (111 mL CH_4/g COD); with no ammonia toxicity detected (González-Fernández et al., 2013). Thus, literature results on the effect of the OLR on microalgae anaerobic digestion in the range needed to reach a neutral energy balance (2.2 g VS/L·d) are not conclusive. Furthermore, biomass concentration and consequently the OLR needed for reaching a neutral energy balance would decrease if more biodegradable biomass was digested, leading to higher methane yield and energy output Indeed, the average methane yield observed during this period was the lowest found so far in our pilot plant and could be regarded as the worst case scenario (Passos and Ferrer, 2014; Passos et al., 2014a; Passos et al., 2014b).

4. Conclusions

Hydrothermal pretreatment was evaluated for improving the anaerobic digestion of microalgal biomass grown in high rate algal ponds for wastewater treatment. The pretreatment increased VS solubilisation (8-13%), anaerobic digestion rate (30-90%) and final methane yield (17-40%) in BMP tests. The best pretreatment condition (130 ºC and 15 min) was further evaluated in continuous reactors, obtaining a methane production rate of 0.12 L CH_4/L·d and a methane yield of 0.17 L CH_4/g VS, 58% and 41% increase in comparison with the control, respectively. Moreover, microscopic images taken towards the end of the experiment showed how *Oocystis* sp. cells were damaged after the pretreatment. Indeed, pretreated cells had unstructured organelles and disrupted external cell wall layer, which possibly enhanced subsequent anaerobic digestion.

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Table 1. Hydrothermal pretreatment for improving microalgae biogas production.

Microalgae species	**Pretreatment conditions**	**Reactor**	**Methane yield (increase)**	**References**
Clamydomonas sp., *Scenedesmus* sp. and *Nannocloropsis* sp.	110, 140 ^{o}C 15 min	BMP	0.32 and 0.36 L CH_4/g VS (19 and 33%)	Alzate et al., 2012
Acutodesmus obliquus and *Oocystis* sp.	110, 140 ^{o}C 15 min	BMP	0.22 and 0.26 L CH_4/g VS (11 and 31%)	Alzate et al., 2012
Microspora sp.	110, 140 ^{o}C 15 min	BMP	0.41 and 0.38 L CH_4/g VS (62 and 50%)	Alzate et al., 2012
				Bohutski et al., 2014
Chlorella sp. and *Scenedesmus* sp.	120 ^{o}C 30 min	BMP	0.40 L CH_4/g VS (20%)	Cho et al., 2013
Nannocloropsis salina	100-120 ^{o}C 2 h	CSTR	0.57 L CH_4/g VS (108%)	Schwede et al., 2013

Note: BMP stands for biochemical methane potential tests and CSTR stands for continuous stirred tank reactors.

Table 2. Energy assessment parameters.

Parameter	Unit	Value	Reference
Density of water (ρ)	kg/m^3	1,000	Metcalf and Eddy, 2003
Specific heat of water (γ)	kJ/kg ºC	4.18	Metcalf and Eddy, 2003
Ambient temperature (T_a)	ºC	20	Assumed
Anaerobic digestion temperature (T_d)	ºC	37	This study
Pretreatment temperature (T_p)	ºC	130	This study
Flow rate (Q)	m^3/d	100	Assumed
Heat transfer coefficient (k)	W/m^2·ºC	1	Metcalf and Eddy, 2003
Heat recovery by heat exchanger (ϕ)	%	85	Lu et al., 2008
Useful volume (V)	m^3	2,000	Calculated
Surface area of the reactor wall (A)	m^2	465	Calculated
Energy consumption for pumping (θ)	kJ/m^3	1,800	Lu et al., 2008
Energy consumption rate for mixing (ω)	kJ/m^3·d	300	Lu et al., 2008
Lower heating value of methane (ξ)	kJ/m^3	35,800	Metcalf and Eddy, 2003
Organic loading rate (OLR)	Kg VS/m^3·d	0.70	This study (Table 4)
Methane yield (P_{CH4})	m$^3_{CH4}$/kg VS	0.12; 0.17	This study (Table 5)
Energy conversion efficiency (η)	%	90	Assumed

Table 3. BMP test of microalgae under different hydrothermal pretreatment conditions.

Temperature (ºC)	**Time (min)**	**Solubilisation (%)**	**Anaerobic digestion rate (d^{-1})**	**Methane yield (L CH_4/g VS)**
-	-	-	0.19	0.12
110	15	8.0 (0.62)	0.26	0.15
110	30	8.8 (0.61)	0.25	0.14
130	15	15.0 (1.04)	0.36	0.17
130	30	13.3 (0.93)	0.31	0.16

Table 4. Influent and digested microalgal biomass characteristics with and without hydrothermal pretreatment over the steady state period. Mean values (standard deviation).

Parameter	**Control reactor**	**Pretreated reactor**
Operating conditions		
HRT (days)	20	20
OLR (g VS/L·day)	0.70 (0.12)	0.71 (0.10)
OLR (g COD/L·day)	2.30 (1.8)	2.54 (2.3)
Influent composition		
pH	7.8 (0.5)	7.8 (0.4)
TS [% (w/w)]	2.25 (0.44)	2.44 (0.55)
VS [% (w/w)]	1.33 (0.30)	1.46 (0.68)
VS/TS (%)	61 (3.1)	63 (4.1)
COD (g/L)	20.0 (4.4)	22.6 (5.5)
TKN (g/L)	1.3 (0.3)	1.3 (0.4)
N-NH_4 (mg/L)	14.9 (4.4)	25.0 (6.4)
VFA (mg COD/L)	0	0
Effluent composition		
pH	7.1 (0.2)	7.2 (0.3)
TS [% (w/w)]	1.67 (0.13)	1.34 (0.27)
VS [% (w/w)]	0.96 (0.10)	0.79 (0.13)
VS/TS (%)	58 (1.7)	59 (4.7)
COD (g/L)	14.3 (1.0)	11.4 (1.8)
TKN (g/L)	1.0 (0.1)	1.0 (0.1)
N-NH_4 (mg/L)	311.5 (25.3)	351.5 (16.2)
VFA (mg COD/L)	43.5 (13.3)	46.5 (8.2)
Removal efficiency		
VS removal [% (w/w)]	28 (3.5)	40 (4.5)
COD removal [% (w/w)]	29 (2.8)	38 (5.0)

Table 5. Biogas production from microalgal biomass with and without hydrothermal pretreatment over the steady state period. Mean values (standard deviation).

Parameter	Control reactor	Pretreated reactor
Methane production rate (L CH_4/L·d)	0.07 (0.01)	0.12 (0.02) [a]
Methane yield (L CH_4/g VS)	0.12 (0.04)	0.17 (0.02) [a]
Methane yield (L CH_4/g COD)	0.08 (0.02)	0.11 (0.02) [a]
Methane content in biogas (% CH_4)	68 (3)	68 (5)

[a] Stand for significantly higher values between paired columns ($\rho = 0.01$)

Table 6. Energy assessment of microalgal biomass anaerobic digestion with and without hydrothermal pretreatment.

Parameter	**Without thickening step**		**With thickening step (7.4% TS)**	
	Control	**Pretreatment**	**Control**	**Pretreatment**
$E_{i,heat}$ (GJ/d)	6.87	12.83	1.80	3.29
$E_{i,electricity}$ (GJ/d)	0.78	0.78	0.20	0.20
$E_{i,centrifuge}$ (GJ/d)	-	-	3.31	3.31
E_o (GJ/d)	5.41	7.67	5.41	7.67
ΔE (GJ/d)	-2.24	-5.94	-0.38	0.01
E_o/E_i	0.71	0.56	0.93	1.00

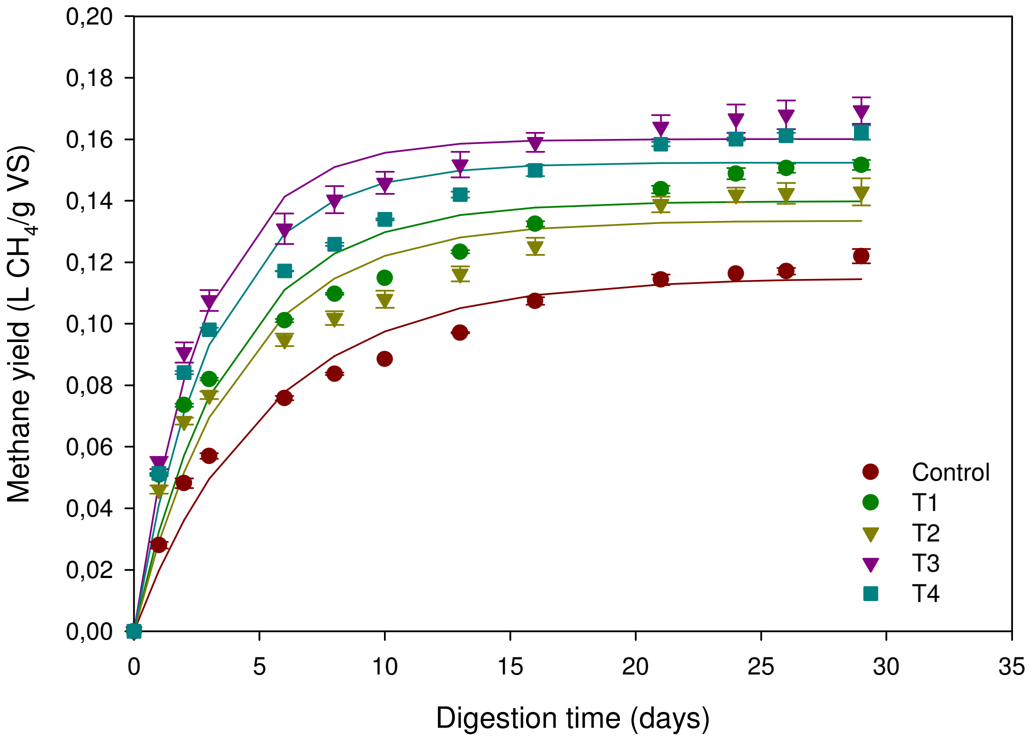

Figure 1. Accumulated methane yield of microalgal biomass after hydrothermal pretreatment. Note: Error bars stand for standard deviation of BMP replicates.

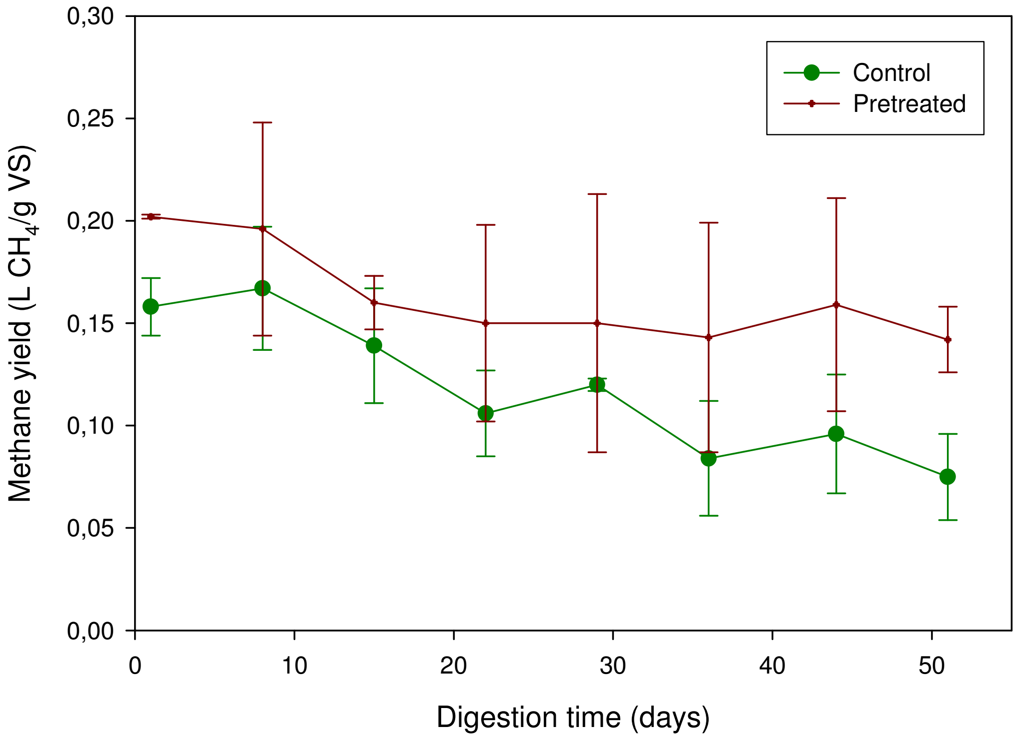

Figure 2. Average methane yield (weekly values) of non-pretreated (control) and pretreated microalgal biomass anaerobic digestion. Note: Error bars stand for standard deviation of weekly averages.

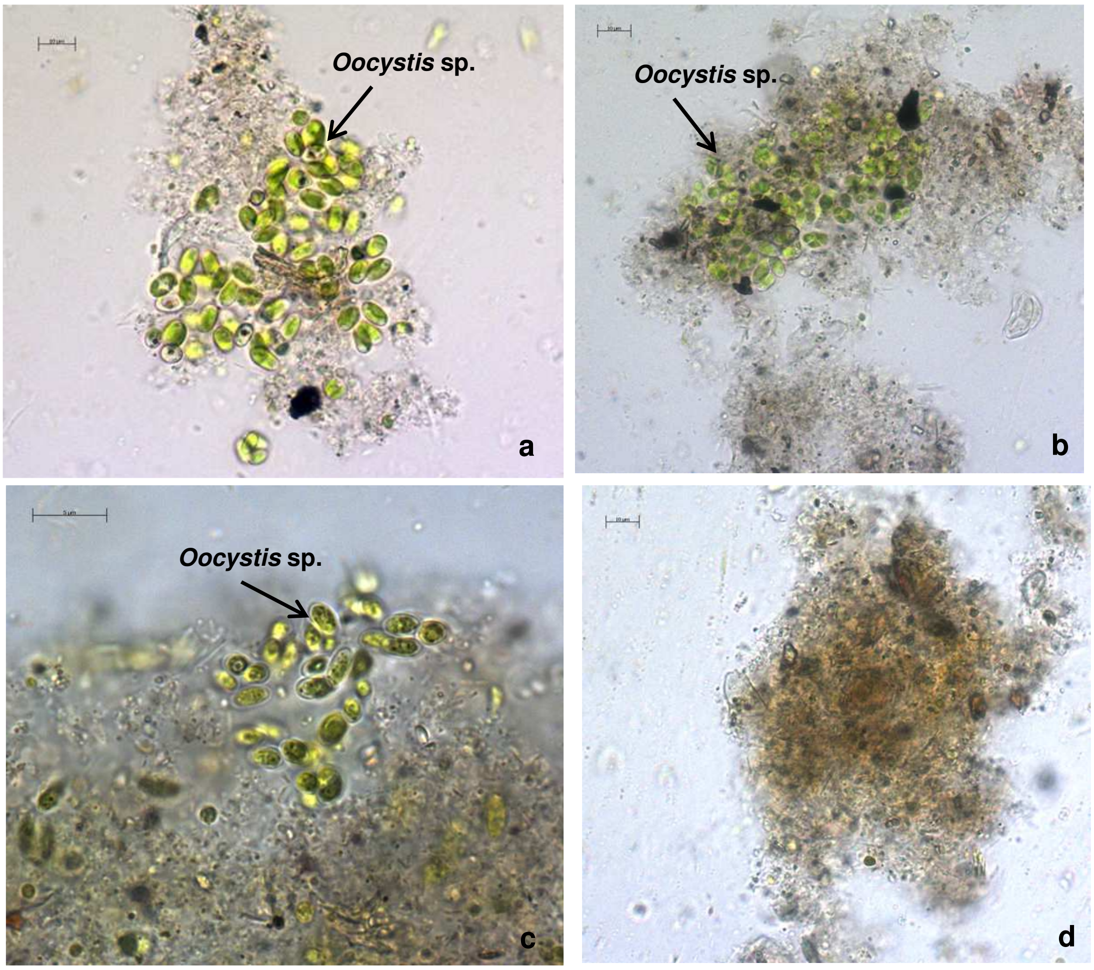

Figure 3. Optical microscope images of *Oocystis* sp. before (a, c) and after (b,d) anaerobic digestion; the first row shows non-pretreated (a, b) and the second row pretreated microalgal biomass (c, d). Note: scale bar in Fig. 3c is 20 µm and not 5 µm**.**

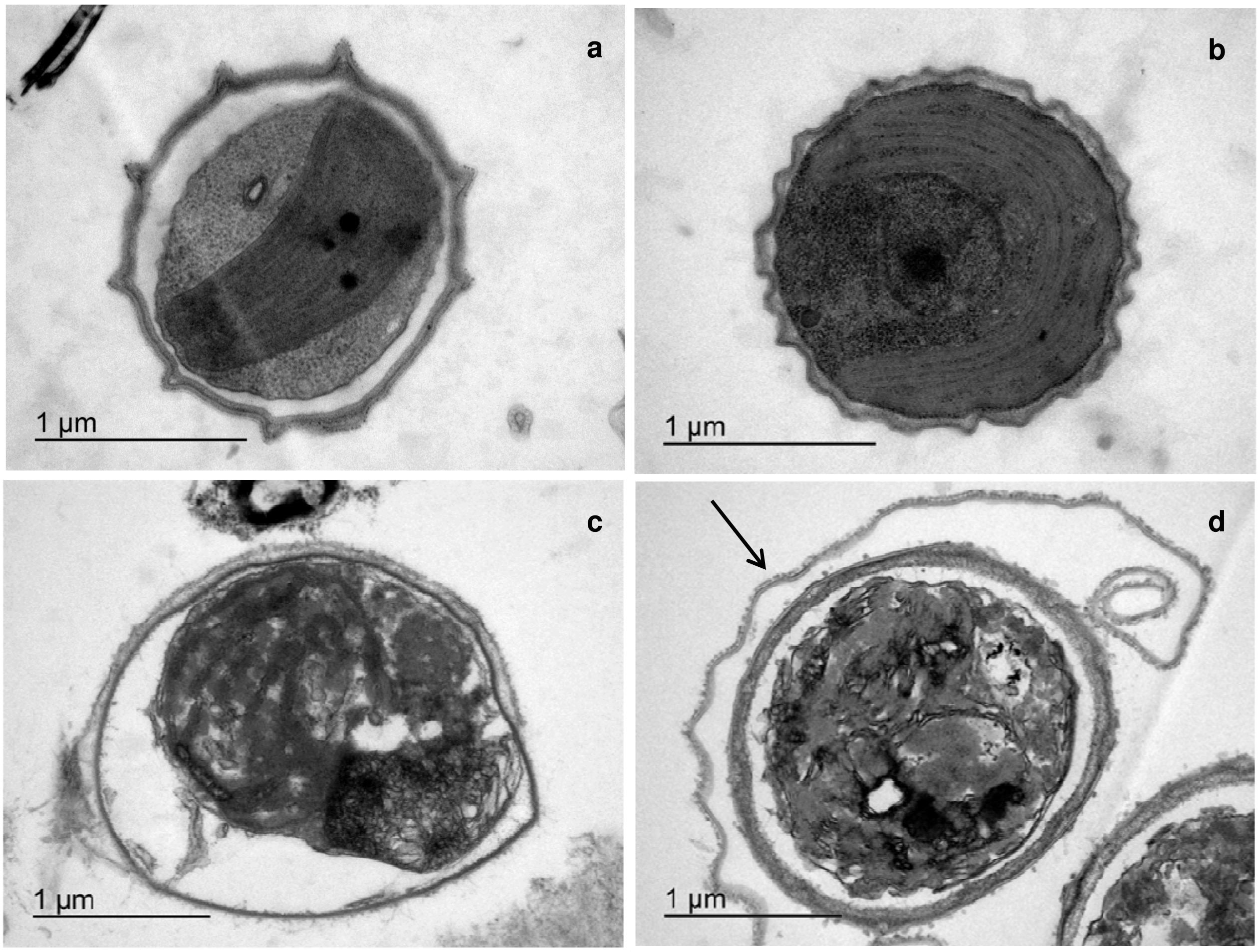

Figure 4. TEM images of non-pretreated (a, b) and pretreated (c, d) *Oocystis* sp. The pretreatment disrupted cell organelles (c) and the external layer of microalgae cell wall (d).