PROTEINS: A KEY MACROMOLECULE FOR AN EFFICIENT ANAEROBIC DIGESTION OF MICROALGAE BIOMASS

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ABSTRACT

Biogas generation is the least complex technology to transform microalgae biomass into bioenergy. Since hydrolysis has been traditionally pointed out as the rate limiting stage of anaerobic digestion, the main challenge for an efficient biogas production is the optimization of cell wall disruption/hydrolysis. Among all the pretreatments tested, enzymatic treatment not only has demonstrated very effective disruption levels but also revealed the impact of microalgae macromolecular composition in the anaerobic process. Although carbohydrates have been traditionally recognized as the polymers responsible for the low microalgae digestibility, protease addition resulted in the highest organic matter solubilization and therefore also higher CH_4 production. However, the increase of protein solubilization could result in inhibition of anaerobic digestion due to the release of ammonium nitrogen. The possible solutions to overcome these negative effects are: the reduction of protein levels of biomass by culturing the microalgae in low nitrogen media and the use of ammonia tolerant anaerobic inocula.

Keywords: microalgae, anaerobic digestion, proteins, biogas, inhibition

1- INTRODUCTION

Environmental issues and energy self-sufficiency worries have led to the research of new approaches and strategies to improve traditional technologies and at the same time seek for alternatives involving renewable energies to substitute them. Anaerobic digestion is one of those traditional technologies, which has been employed for the degradation of organic residues because of many advantages such as the effectivity in the removal of biodegradable organic compounds, the applicability at any scale with a high variety of substrates and the products that are produced, which are biogas and digestate that are easy to separate and represent a way to obtain energy and fertilizers respectively [1]. Focusing on biogas, it is mainly composed by CH_4 and CO_2, but it has also other compounds such as N_2, O_2, H_2S, NH_3, water or H_2.

Among the different substrates that can be employed, microalgae are being recently studied since anaerobic digestion does not require highly concentrated biomass [2], moreover, they have also potential application in other fields such as food supplementation, or medical chemicals [3]. Microalgae biomass has a wide range of compositions depending on growth conditions and species. The main components are lipids (7-23%), carbohydrates (5-64%) and proteins (6-71%) [4]. Different compositions of microalgae produce different methane yields. This variety is related to the specie of microalgae, but also to the growth conditions (macro and micronutrients). It is especially important to highlight the composition of the cell wall because of its importance on the overall process performance. Microalgae have a chemically complex and structurally robust cell wall with low biodegradable substances that hinder the anaerobic digestion. Some of these compounds are sporopollenin, algaenan, cellulose and hemicellulose that offer a barrier to degradation [5, 6]. Cell walls are degraded by extracellular enzymes of anaerobic bacteria during anaerobic digestion. Hydrolysis is the limiting step of the process, so pretreatments are used in order to facilitate the accessibility of these extracellular enzymes, which results in an improvement of the hydrolysis. Different pretreatments have been studied such as thermal, chemical, mechanical or biological. Biological treatments are being studied lately because of their low costs and their consideration as a green technology if compared to the other pretreatments [7].

The main drawback is that it is not yet clearly stablished how enzymes and composition of the cell wall influence the whole process yield. Traditionally this importance has been awarded to carbohydrates, but, as it was pointed out, the amount of proteins out of the total composition of the microalgae could be as high as carbohydrates or even higher.

2. PRETREATMENT OF MICROALGAE TO IMPROVE BIOFUELS PRODUCTION AND BIOPRODUCTS EXTRACTION

Pretreatment has become a key step highly required to enhance biogas production from microalgae biomass [8]. Cell wall rupture or hydrolysis is needed to make available microalgae organic matter to anaerobic microorganisms [9]. Since low biodegradability is a common issue in anaerobic digestion of other substrates (such as sludge produced during wastewater treatment) a wide range of pretreatments are available to enhance the hydrolysis step [10]. Beside this, some of these techniques are regularly applied in other processes such as production of biodiesel or bioethanol [11]. In the following sections the different pretreatments reported for microalgae biomass and the performance of each of them are overviewed.

2.1. Energy demanding pretreatments: thermal, thermo-chemical and mechanical pretreatments

Pretreatments are classified in four groups: thermal, mechanical (ultrasound and microwave), chemical (acidic, alkaline, and ozonation) and thermo-chemical (combination of acidic or alkaline with a high temperatures) and biological (enzymatic). Many studies have been done in recent years to improve biogas production using those pretreatments (Table 1). Most of them have been only assessed in Biochemical Methane Potential (BMP) assays while there is little information of the effect of pretreatments when the digestion is conducted in semi–continuous operated reactors (Figure 1) [12].

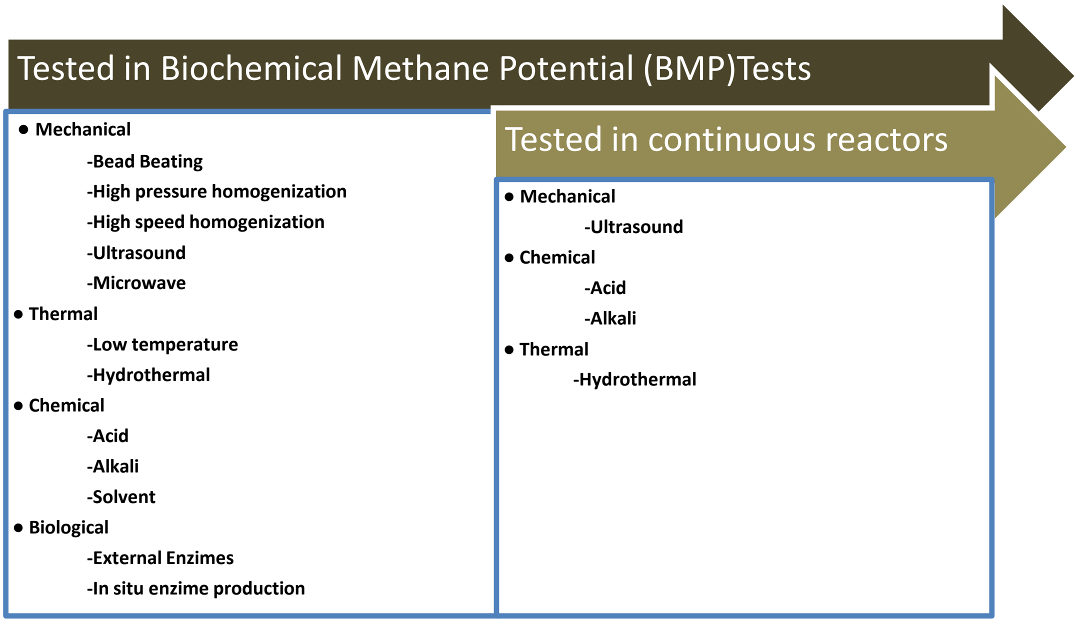

Figure 1. Research concerning the different types of pretreatment

Given that thermal energy is available in biogas production installations, the most used pretreatment is thermal application.

Thermal pretreatments involve biomass heat up in a wide range of temperatures (50-270°C) and time (from minutes to hours). An example of this variety are the experiments carried out by Passos and Ferrer (2014) and Passos and Ferrer (2015) [13, 14] where they applied 75°C-95°C for 10 hours, and 130 °C for 15 minutes, respectively, to test the influence of thermal pretreatments on energy production. Low thermal pretreatment (80°C) for 15 min applied to *Scenedesmus* increased 1.6-fold its methane production (128.7 mL CH_4/g COD_{in}) compared to untreated biomass (81.8 mL CH_4/g COD_{in}) [15]. Similar temperatures were tested in *Chlorella* biomass (70 and 90°C) for 0.5 h resulting in a methane yield of 37 % and 48% in compared to basal values (322 mL CH_4/ g VS_{add}) [16]. Higher temperatures (130°C for 15-30 min) were also tested, resulting in 28% methane yield increase if compared to the control (105.6 mL CH_4/ g VS_{add})[17].

Although thermal pretreatments normally present positive results in terms of methane yield, these methods involved some drawbacks such as the formation of recalcitrant compounds that could potentially decrease the performance of the process [18, 19].

Mechanical pretreatments are commonly employed to disrupt different kind of organic substrates in industrial processes. Ultrasound treatment has been applied to disrupt microalgae cell wall in different bioprocess devoted to biofuel production, such as ethanol production with *Chlorella* [20] and biodiesel generation with *Spirulina* [21]. In case of anaerobic digestion, ultrasound pretreatment has shown positive results in terms of methane yield enhancement. Ultrasound (128.9 kJ/g at 80°C and 30 min) has been applied in *Scenedesmus* biomass resulting in an increase of 19% methane production compared to basal values (128 mL CH_4/ g COD_{in}) [22], whereas Gonzalez-Fernandez et al. (2012) applied 128.9 MJ/Kg to enhance methane yield from 81.8 mL CH_4/ g COD_{in} to 153.5 mL CH_4/ g COD_{in} [23]. Ultrasound pretreatment (70 W for 30 min) was also applied for *Monoraphidium sp* and *Stigeoclonium sp* biomass to enhance methane yield from 105.6 mL CH_4/ g COD_{in} to 196 mL CH_4/ g COD_{in} [17]. Alzate et al. (2012) found increases of 6-24 % in CH_4 yield of a mixture of microalgae biomass after the pretreatment at different energy inputs (10; 27; 40; 57 MJ/kgTS) [18]. After testing these energy levels, no significant increases in methane production were found above 10 MJ per kg TS.

The main limitation of ultrasound pretreatment is the high energy input required when compared to thermal, chemical or biological methods [13]. In addition, in contrast to thermal pretreatments, the energy is required as electricity; therefore it is more difficult to use self-produced energy during the pretreatment in a biogas production plant.

Chemical methods have been less employed than thermal and mechanical and they are often combined with of heat pretreatment.

Cell wall disruption with alkali and acid pretreatments has been tested with positive results in different processes of bioenergy generation with microalgae biomass (ethanol, buthanol and biomethane) [24, 25]. Studies of solubilisation of the microalgae biomass before anaerobic digestion have been reported using thermo-alkaline methods. Different doses of CaO (4 and 10%) and different temperatures (25, 55 and 72 °C) resulted in a solubilisation of proteins and carbohydrates of 32.4% and 31.4% respectively, and methane yield was enhanced by 25% from basal values of 260 mL CH_4/ g VS_{add} [26]. Another experiment, which improved the solubilisation of the raw biomass was carried out in *Chlorella* and *Scenedesmus* using NaOH (0.5, 2 and 5% v/v) although increase of methane yield was not observed by Mahdy et al. (2014a) [27]. Besides, acidic pretreatment was tested in *Chlorella* where different concentrations of hydrochloric acid (0.5-10% w/w) at 121°C for 20 min enhanced the solubilisation of carbohydrates (92%) [28]. Recently, the application of ozone as pretreatment has shown variable increases between 6 and 66 % on the CH_4 yield and the disruptive effect of this compound on the cell wall was evidenced by electron microscopy [29]. One of the main limitations of this pretreatment is the need to readjust the pH previously to the anaerobic digestion. In this manner, chemical costs make this type of pretreatments limited. Additionally, some the chemicals need to be removed previous the anaerobic digestion since they can be toxic for anaerobes [30]

2.2. Low Energy Demanding Pretreatments: Biological Pretreatments applied to microalgae

Compared to the previous pretreatments, **biological pretreatments** involve reduced energy demands. These pretreatments include the use of enzymes or microorganisms to hydrolyze the microalgae cell wall. Given the scarce information related to the cell wall composition, a wide range of biocatalysts have been tested. In principle, given the similarities between higher plants and microalgae, the most studied catalysts are cellulas, hemicellulose, amylase and pectinase [11, 31, 32]. Moreover, another enzymes are used, such as lysozyme, which was used for the enhancement of fermentable sugars for bioethanol production in *Microcystis aeruginosa* [33].

When it comes to the addition of other microorganisms, cellulase-secreting bacteria was added to *Chlorella vulgaris*. The results showed an increase of 18% organic matter solubilization against the control which proved the biomass hydrolysis [34]. Some other enzymatic cocktails for microalgae cell wall hydrolysis include proteases and lacases. In this sense, commercial proteases cocktails (Alcalase) were employed in *Chlamydomonas reinhardtii* and *Chlorella vulgaris* displaying solubilisation of carbohydrates and proteins of 86-96% and an [35]. As it is observed in Table 1, almost all tested pretreatments improve methane production yield although it seems there is not a direct linkage between solubilisation and methane enhancement. Biological approaches and specifically, enzymatic pretreatments are being used recently to identify which is the most recalcitrant microalgae macromolecule in the context of biogas production [35].

3. BIOLOGICAL APPROACH TO ENHANCE BIOGAS PRODUCTION: ENZYMATIC PRETREATMENT

As it was pointed out, these methods are energetically competitive since most of the time their temperature requirement is low and only need smooth shaking. Despite of the high economic cost of the enzymatic cocktails [36], the use of biocatalysts can provide crucial information to identify the macromolecule hampering anaerobic digestion of microalgae biomass. Moreover, the costs could be reduced either by producing enzymes in situ [34] or by sludge bioagumentation [35, 36, 37].

Opposite to other pretreatments, biological reactions show a high selectivity and absence of inhibitory compounds, therefore, biocatalysts do not only disrupt the cell wall, but they also hydrolyze the macromolecules during biological pretreatment. Different parameters must be taken into account such as pH, temperature, enzyme dose, and exposure time. Likewise given the different macromolecular composition, structural features and cell wall composition among microalgae strains, a wide range of biocatalysts can be found in literature.

3.1. Carbohydrases

Carbohydrases are in charge hydrolysing carbohydrates polymers into simple sugars. Cellulases, amylases and amyloglucosidases have been tested in microalgae biomass to enhance its methane yield. Studies have been carried out in order to assay the influence of this fraction in the process since it is believed that it is the responsible of the toughness of the cell wall. *C. vulgaris* and *Scenedesmus* were treated applying Viscozyme, Celluclast, and Pectinase reaching 84 and 36% of carbohydrates solubilisation respectively and enhancing the methane yield 1.2-fold [40]. Amilolytic enzymes were produced by submerged fermentation and solid state fermentation and purified to hydrolyze de polysaccharides in *Spirulina* producing yields of 332% and 205% if compared to the crudes [41] . Combination of different enzymes were also studied when cellulases from *Trichoderma reseei* were mixed with metal oxides to treat *Chlorella* biomass resulting in a glucose yield of 91% of theoretical maximum [42]. Enzymatic hydrolysis was also combined with acid hydrolysis in *Chlorella sorokiniana* and *Nannochloropsis gaditana* improving the sugar release [43]. Carbohydrases were used to facilitate lipid extraction using enzymes (exoglucanase, endoglucanase, xylanase and laccase) produced by different biomass-degrading bacteria improving it up to 40% [44].

3.2. Lipases

Lipids could be very useful for anaerobic digestion due to the high potential yield of this fraction if compared to other macromolecular constituents, 1.014 compared to 0.496 and 0.415 $LCH_4\ g^{-1}$, in lipids, proteins and carbohydrates, respectively [45]. However, long chain fatty acids are formed when lipids are hydrolyzed, which can easily make the system unstable [46]. Because of this, studies are mainly focused on the optimum concentration of lipids that makes possible to carry out the process without inhibition. In this way, experiments were carried out at different lipid concentration observing inhibition in methane production when this fraction summed up 31% of the total substrate [47], and research was also conducted in order to develop strategies to avoid this inhibition [48]. It is worth to notice that lipids accumulation in microalgae biomass has been in deep studied to produce liquid fuels [49, 50].

3.3. Proteases

Protein fraction is degraded by proteases. These enzymes hydrolyze peptides into amino acids. The use of proteases is receiving particular interest in last years, especially in combination with other pretreatments or enzymes contained in commercial cocktails [11, 51]. Hydrolysis of proteins was studied by combining sonication and enzymatic pretreatment enhancing the solubilisation of proteins by 56% [52]. Likewise, recent research has conferred the proteins special importance in the anaerobic digestion process since microalgae biomass exhibits a high content (until 75 %). In this way, different approaches have been researched. *C. vulgaris* enhanced 2.6-fold its methane production when pretreated with Alcalase [53]. In the same way, *C. vulgaris* biomass was treated with proteases in a prevailing carbohydrates biomass, reaching a higher methane yield (5-6.3-fold) than the biomass pretreated with carbohydrases [40] and a high enhancement of methane yield in *C.vulgaris* (1.72-fold) and *Scenedesmus* (1.53-fold) pretreated with proteases was also achieved [54]. These results suggest that proteins are the molecules that hindered anaerobic digestion instead of carbohydrates. For this reason, different strategies are arising to assay this fraction, which is lately considered as a key macromolecule.

4. Biomass Proteins in anaerobic digestion of microalgae

Microalgae have been reported as a high heterogeneous substrate, which holds a sturdy cell wall that makes the hydrolysis step more difficult [35]. After applying a pretreatment, solubilisation of organic matter increases, and the main fractions to produce biogas, which are proteins, lipids and carbohydrates, are more accessible.

Anaerobic digestion is divided in four different phases named as hydrolysis, acidogenesis, acetogenesis and methanogenesis (**Figure 2**).

4.1. The relevance of microalgae proteins in the hydrolysis stage of anaerobic digestion

The first biological process involved in anaerobic digestion is hydrolysis, which is the limiting step and its effectiveness is crucial for the overall process as some authors already pointed out [2, 40]. Focusing on proteins, they are hydrolyzed into amino acids by extracellular enzymes secreted by different bacteria such as *Clostridium, Vibrio, Peptococcus, Bacillus, Proteus, Bacteroides* [35]. Moreover, research showed higher methane production when proteases were applied if compared to raw biomass and biomass treated with carbohydrases, methane production was enhanced by 51% showing the benefits of having proteins in the soluble phase [40]. Similar results were reported in *C. sorokiniana* biomass using proteases and esterases, increasing the hydrolysis (35-45%) resulting in a higher methane yield (3-fold) when compared to raw biomass [2]. In addition, enhancement of methane yield (37%) was also attributed to protease activity when biomass (blue algae) was stored [55].

During the second step, acidogenesis, a facultative consortia of bacteria ferment amino acids transforming them into volatile fatty acids (VFA) such as acetic, propionic and butyric acids [56]. Acetogenesis is the third step of the process and it consists in the degradation of VFA producing acetate, CO_2 and H_2 by some species such as *Syntrophobacter or Syntromonas* [23]. Eventually, these products are further transformed into methane by two different pathways. The first one is called “acetoclastic methanogenesis”, where acetate produces methane. The second is called “hydrogenotrophic methanogenesis”, where methane is produced by CO_2 and H_2.

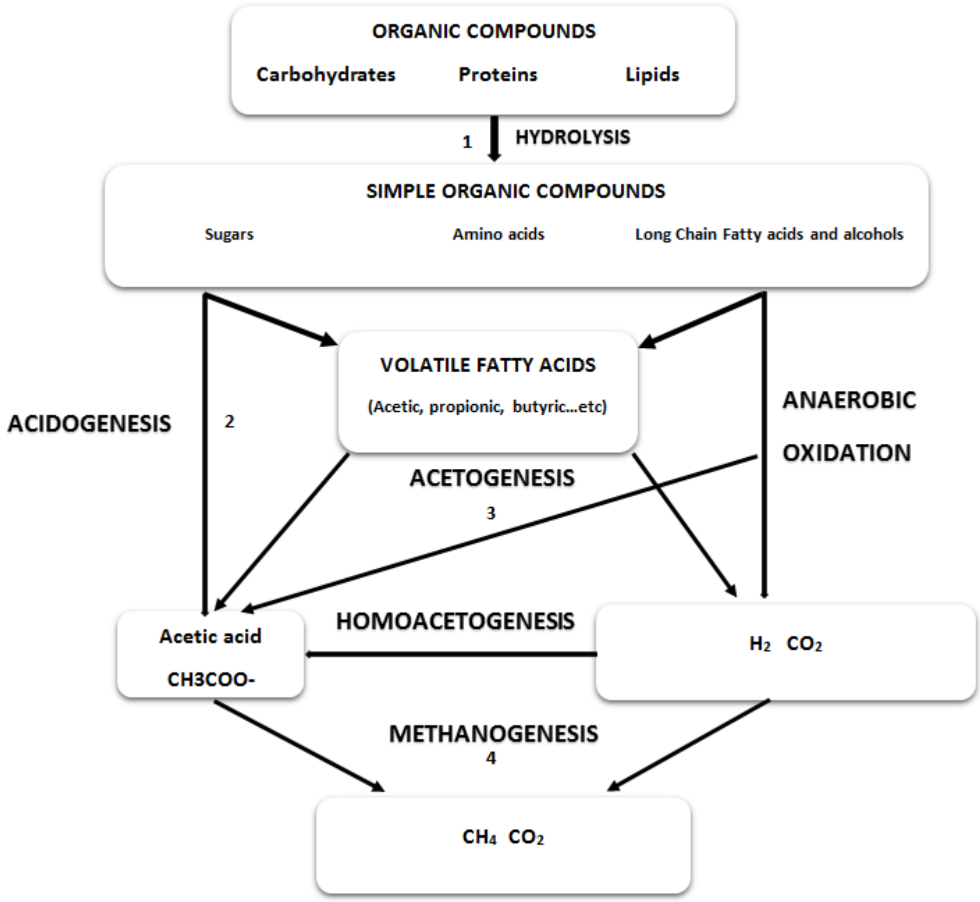

Figure 2. Anaerobic digestion steps

As it was indicated, microalgae are a very heterogeneous substrate. Because of this variety, composition of the cell wall differs among the different species, but there are also intra-specie variations based on the growth conditions, which eventually produce different methane production yields [57]. Different strategies have been developed in order to modify the composition of microalgae to improve biomass productivity. These methodologies encompass the modification of the growth media through nitrogen starvation, phosphate limitation, or high Fe^{+3} concentrations [36]. In fact, modifications were tested when *Arthrospira platensis* grew in phosphorous limitation resulting in an enrichment of the carbohydrates fraction [58]. Likewise, production of hydrogen was induced in *C. reinhardtii* when sulfur starvation was applied [59].

4.2. The relevance of microalgae proteins in the methanogenesis stage of anaerobic digestion

Out of the subsequent stages involved in anaerobic digestion, during methanogenesis hydrogen and acetic acid is converted to methane gas and carbon dioxide. This last stage is performed by archaea. When compared to anaerobic bacteria involved in anaerobic digestion, archaea are more sensitive to toxic compounds and also exhibited lower growth rates. According to Henze *et al*., acidifiers present ten to twentyfold higher growth rates and fivefold conversion rates than methanogens [1, 60]. With regard to their sensibility toward toxic compounds, methanogens present lower tolerance against ammonium nitrogen. Ammonia diffuses freely through the permeable membrane of methanogens cells causing changes in intracellular pH and resulting in potassium deficiency and/or proton imbalance. Beside this, ammonium could inhibit enzymes that are involved in methane production [61]. As a result, the high concentration of total ammonia (ammonia/ammonium) can lead to volatile fatty acids accumulation. This last process involves acidification which in turns inhibits the methanogen activity. Therefore, the main drawback of digesting the proteins fraction is the high amount of nitrogen released in form of ammonium that can inhibit methane formation.

In a scenario of bioenergy production in form of biogas produced from anaerobic fermentation of microalgae, two strategies to avoid inhibition by ammonium can be applied. The first one is to modify the growth conditions by providing the microalgae with a poor nitrogen medium. Biogas productivity was modified using this method in different studies [61, 62, 63, 64, 65]. This strategy can be easily applied by using urban wastewater as culture media, which normally presents considerable lower nitrogen concentrations than synthetic salt mediums (≈ 60 *vs.* 300-600 mg N/L^{-1}). *C.vulgaris* was grown in wastewater media, which resulted in a high accumulation of carbohydrates, which eventually enhanced methane production [40]. In addition to reducing the amount of proteins in the biomass, by using wastewater as culture media the biogas production can be coupled to treatment of the effluents by oxygenation mediated by microalgae.

The second approach is through bioaugmentation of the sludge, which consists in introducing anaerobic microorganisms for one specific goal. As a matter of fact, this strategy was successful in order to enhance methane yield (18-38%) after adding *Clostridium thermocellum* at various inoculum ratios to degrade microalgae cellulose [37]. Thus, anaerobic microorganisms that are tolerant to high NH_4^+ concentrations should be provided to accomplish this goal. The use ammonia tolerant inocula has been recently demonstrated as efficient option for digestion of mixtures of *C. vulgaris* and cattle manure [66]. In this study the effectiveness of adapted methanogens resulted in an increase of 33 % in the potential conversion of biomass to methane. Although it is generally believed that ammonia levels above 3 g/L have toxic effect on the methanogens, the resistance of methanogens can be increased by exposing the microorganisms to high nitrogen concentrations (67).

5. CONCLUSIONS

Anaerobic digestion of microalgae has been presented as a promising alternative for generation of bioenergy. The implementation of this process requires a disruption of the rigid cell wall in order to release to organic matter for methanogens. Enzymatic pretreatment with proteases shows the best performance in terms of organic matter solubilization and methane production. This fact shows that protein embedded in microalgae cell wall is causing the low biodegradability. However, solving this problem with protease addition could result in methanogens inhibition mediated by high ammonia concentrations. Two solutions are proposed: the reduction of nitrogen levels of microalgae biomass using a low nitrogen concentration culture media and the use of ammonium highly tolerant anaerobic inocula.

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Table 1. Summary of pretreatments applied before anaerobic digestion of microalgae biomass

Pretreatment used	**Microalgae species used**	**Conditions**	**Methane yield increased (%)**	**References**
Thermal	*Chlorella* sp.	70°C for 0.5 h	37	(Wang et al. 2017)
		90°C for 0.5 h	48	
		121°C for 0.3 h	108	
Thermal	*Scenedesmus*	80°C; 1.6-fold	Not appreciated	(Gonzalez-Fernandez et al. 2012)
Thermal	*Monoraphidium* sp	95°C for 10 h	72	(Passos et al. 2015)
	Stigeoclonium sp	130°C for 0.25 h	28	(Passos et al. 2015)
Thermo-alkaline	*Scenedesmus sp.*	96h at 25 °C and 24 h at 55 and 72 °C	25	(Solé-Bundó et al. 2017)
	Chlorella sp.	CaO concentrations (0, 4 and 10%)		
Thermo-alkaline	*Scenedesmus* sp.	0.5, 2 and 5% w/w NaOH dosages	Not appreciated	(Mahdy et al. 2014a)
Ultrasound	*Scenedesmus*		19,27	(Luo et al. 2014)
Ultrasound	*Monoraphidium* sp	70 W; 30 min; 26.7 MJ/kg TS	Not appreciated	(Passos et al. 2015)
	Stigeoclonium sp			
Ultrasound	*Scenedesmus*		Not appreciated	(Gonzalez-Fernandez et al. 2012)
	Stigeoclonium sp			
Microwave irradiation	*Monoraphidium* sp	900 W; 3 min; 34.3 MJ/kg TS	21	(Passos et al. 2015)
Biological	*Chlorella vulgaris*	Cellulase-secreted bacteria	Not appreciated	(Kavitha et al. 2017)
Biological	*Chlamydomonas reinhardtii*	Viscozyme L	1.17-fold	(Mahdy et al. 2014b)