CHAPTER 1

PRETREATMENT METHODS OF MICROALGAE BIOMASS FOR BIOGAS PRODUCTION

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ABSTRACT: Interest in the production of biofuels from microalgae biomass is growing. The benefits of microalgae and cyanobacteria over terrestrial plants are that they help reduce CO_2 emissions, can grow in nutrient-rich wastewater, have simple nutritional requirements, and have high productivity. However, low cell concentration in cultivation and the recalcitrance of raw algae biomass hinder methane yields. Most species of microalgae and cyanobacteria have a rigid cell wall made of layers of cellulose, hemicellulose, pectin, peptidoglycan, sporopollenin, and other resistant polymers. Many pretreatment methods have been proposed to break down complex cell wall polymers into smaller, soluble, and biodegradable molecules. However, the relationship between biomass solubilization and methane yield is not well defined. Furthermore, most studies do not provide an assessment of the energy balance of working conditions, with energy demand being a key challenge for biomass pretreatments. Mechanical, thermal, chemical, and thermochemical are all energy-intensive methods, either due to the energy required for heating biomass or the energetic cost of producing chemicals, or both. An in-depth analysis of the effects of chemical and thermochemical pretreatments of algal biomass for methane production suggests that less severe conditions should be preferred for microalgae biomass. Only enzymatic pretreatment and a few thermal pretreatments result in a positive energy balance. The large-scale implementation of pretreatment methods requires technological innovations to reduce energy consumption and its integration with other processes in wastewater treatment plants.

KEYWORDS: biogas, biomethane, microalgae, pretreatment.

1. INTRODUCTION

Climate change caused by increased greenhouse gas emissions, especially CO_2, has driven the search for alternative energy sources that could replace fossil fuels (Singh *et al.*, 2016). According to the IPCC (Intergovernmental Panel on Climate Change), global warming needs to be limited to 1.5 °C by 2030, or well below 2 °C by 2050, so that the impacts on Earth's ecosystems and human life are as minimal as possible (IPCC, 2018). Due to its dependence on fossil fuels, the energy sector is one of the biggest contributors to the release of CO_2 into the atmosphere. The Global Carbon Budget report revealed that in 2018, 36.6 Gton of CO_2 was emitted because of the use of fossil fuels (Friedlingstein *et al.*, 2019; Peters *et al.*, 2020). Thus, a transition to a low-carbon economy must happen in the next few years, which requires increasing the share of renewable energy sources in the global energy mix (Jackson *et al.*, 2019).

Anaerobic digestion is a complex biochemical process carried out by a mixed microbial population of anaerobic methanogenic archaea and bacteria that degrade organic matter. This consolidated technology for the biological treatment of waste and wastewater is applied to a wide range of organic materials (Khanal, 2008).

The anaerobic digestion process does not require high temperature or pressure. Its advantages include i) the ability to process a wide range of organic materials, solids, and liquids (concentrated or diluted); ii) the possibility of recycling or recovering nutrients from digestate; and iii) the possibility of recovering energy in the form of methane after biogas purification. The disadvantages include i) the requirement of skilled professionals to design, construct, and operate the reactor; ii) the need to control the temperature, pH, and other operational parameters; and iii) the sensitivity of methanogenic archaea to chemical compounds present in the raw material and imbalances in the reactor.

Anaerobic digestion has also gained prominence as a bioenergy production route because the biogas generated in the process could be used as a source of thermal and/or electrical energy. Thus, many biomasses, including algae biomass, have become as candidates for anaerobic digestion. For energy conversion, the main components of the biomass must be carbohydrates, proteins, fats, cellulose, and hemicellulose. Angelidaki and Sanders (2004) estimated that the methane potential from anaerobic digestion of lipids, proteins, and carbohydrates corresponds to 1,040 mL CH_4/g VS (volatile solids), 496 mL CH_4/g VS, and 415 mL CH_4/g VS, respectively.

Biogas is a colorless and, depending on its composition, odorless gas. Its typical composition is 50 to 75% methane (CH_4) and 25 to 45% carbon dioxide (CO_2), with smaller amounts of hydrogen (H_2), nitrogen (N_2), hydrogen sulfide (H_2S), oxygen (O_2), ammonia (NH_3), and carbon monoxide (CO). The energy component of biogas is methane, which, after biogas purification, can be used as fuel for industries, households, or transportation. The percentage of methane in biogas varies according to the chemical composition of the

digested material. Operating parameters and imbalances in the anaerobic reactor also affect methane production.

To increase the methane yield from the anaerobic digestion of different biomasses, several authors have proposed pretreatment methods to solubilize recalcitrant molecules present in the biomass (Rodriguez *et al.*, 2015). Thermochemical pretreatment, using acidic or alkaline reagents, is a widely used method done prior to the energy conversion of raw materials such as lignocellulosic biomass (Amin *et al.*, 2017), algae biomass (Bohutskyi; Betenbaugh; Bouwer, 2014; Candia-Lomeli *et al.*, 2022), and waste activated sludge (Kim; Yu; Lee, 2013; Torres; Lloréns, 2008). Despite aiding biomass degradation and enhancing the availability of organic matter to the digesting microorganisms, there is no correlation between the increase in biomass solubilization and the increase in methane yield resulting from pretreatment. Therefore, a deeper investigation is needed of traditional and novel biomass pretreatments that benefit the anaerobic digestion, not only solubilization. The aim of this chapter is to present the most employed pretreatment methods and their effect on microalgae and cyanobacteria biomass (here called algae biomass, excluding macroalgae).

2. BIOMASS PRETREATMENTS

Although anaerobic digestion is a mature biotechnology to convert organic matter into biogas, most biomasses explored as energy sources are resistant to degradation. This results in less methane yield than the theoretical methane potential presented by those biomasses. To tackle this issue, many pretreatment methods have been investigated to enhance biomass biodegradability by microorganisms in anaerobic digestion (Córdova; Passos; Chamy, 2019; de Carvalho e*t al.*, 2020; Kannah *et al.*, 2021).

The main goal of the pretreatment is to disorganize the rigid structure of the cell wall responsible for the biomass recalcitrance. Microalgae have a complex cell wall often comprised of highly polymerized polysaccharides. Thus, most pretreatment methods seek to solubilize hemicellulose, depolymerize cellulose into smaller sugars, decrease crystallinity, and/or modify cellulose microfibrils by increasing its surface area. Other organic constituents like proteins can also be affected by the pretreatment, releasing smaller, easily degradable molecules.

Even though researchers have intensively studied some methods and implemented them at an industrial scale, like chemical and hydrothermal pretreatments, a universal pretreatment method for biomass does not exist. Cell wall composition varies greatly between the different microalgae species (Table 1). Some microalgae and cyanobacteria lack a rigid cell wall, having only a cell membrane, while others have many external layers protecting the cell (de Carvalho *et al.*, 2020; D'Hondt *et al.*, 2017; Kahnnah *et al.*, 2021). Additionally, most pretreatment methods are unspecific, which can lead to the formation of undesirable products that hamper methane production.

Table 1 – Cell wall components of biotechnologically relevant microalgae.

Microalgae Species	Cell Wall Components	References
Scenedesmus	Peptide-glycan layer; sporopollenin layer; fibrillar layer made of cellulose and hemicellulose; cell membrane.	de Carvalho *et al.*, 2019 Oliveira; Bassin; Cammarota, 2022 D'Hondt *et al.*, 2017 Spain; Funk, 2022 Weber *et al.*, 2022
Nannochloropsis	Sporopollenin layer; cellulose layer; fibrillar layer; cell membrane.	
Chlorella	Sporopollenin layer; fibrillar layer (cellulose and hemicellulose); cell membrane.	

Some research attempting to determine microalgae cell wall composition relies on sequential pretreatments to depolymerize polysaccharides and polypeptides, in addition to releasing lipidic layers protecting the cell wall. Although cellulose and hemicellulose are regarded as part of the cell wall of *Chlorella vulgaris*, little is known about the chemical structure of its other components. Aside from cellulose, Weber *et al.* (2022) found the presence of chitin- and pectin-like polysaccharides in the cell wall of *C. vulgaris*. These polyssacharides release monosaccharides like galactose, mannose, rhamnose, glucosamine, and uronic acids, identified in *C. vulgaris* hydrolysates (Weber *et al.*, 2022). Glucose, rhamnose, and mannose monomers were released from the cell wall of *Scenedesmus* sp. after enzymatic pretreatment (Spain; Funk, 2022). The cell wall of microalgae can be comprised of proteins, lipids, glycoproteins, and inorganic material (Ye *et al.*, 2023). In addition, the arrangement of those complex macromolecules within the cell wall layers may increase the complexity and recalcitrance of microalgae biomass.

No pretreatment method and/or condition is suitable for all biomasses. Furthermore, a pretreatment is unlikely to promote simultaneously all the desired results because it exerts different effects on the cell wall components (da Silva *et al.*, 2013). Thus, choosing a pretreatment is still a challenging step for the energetic conversion of biomass. This chapter presents and describes some of the most common pretreatments applied to algae biomass aimed at improving methane yields, with special attention given to chemical, thermochemical, and enzymatic pretreatments. Figure 1 is an overview of the classification of biomass pretreatment methods.

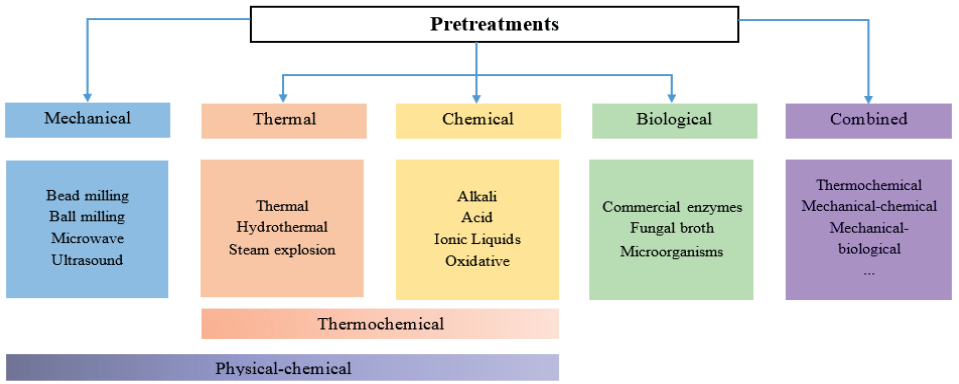

Figure 1 – Biomass pretreatment methods

2.1 MECHANICAL PRETREATMENTS

Mechanical pretreatment methods can rupture the cell wall structure by physical damage mainly. Bead milling is a simple mechanical pretreatment for microalgae and cyanobacteria cell wall disintegration. In this method, an algae suspension is added to a grinding chamber filled with ceramic beads (Schwenzfeier; Wierenga; Gruppen, 2011). Cell wall rupture by bead milling can release intracellular content, enhancing the availability of organic matter for anaerobic digestion.

Ball milling is conducted on either dry or wet biomass in a rotary drum filled with balls of different sizes and materials. This method reduces the particle size and significantly decreases the crystallinity of cellulose (da Silva *et al.*, 2013). Amorphous cellulose is more susceptible to degradation than the crystalline form; thus, the biomass becomes easier to digest after ball milling treatment.

Ultrasound breaks the cell wall from the inside out due to the cavitation produced by magnetic waves that penetrate the cells (Jankowska; Sahu; Oleskowicz-Popiel, 2017; Rodriguez *et al.*, 2015). Microwave pretreatment promotes an increase in the kinetic energy of water dipoles, causing the rupture of hydrogen bonds of the macromolecules, altering their structures and ultimately damaging the cell wall (Rodriguez *et al.*, 2015).

2.1.1 Advantages and disadvantages of mechanical pretreatments

Mechanical methods often produce a rise in temperature sufficient to break intermolecular bonds between polysaccharides. Thus, in addition to mechanical forces, the locally increased temperature contributes to cell wall weakening, resulting in increased biomass degradability. In the case of ball or bead milling, the temperature rises as a side result of the friction between the spheres and the biomass. In microwave pretreatment, the heating from the electromagnetic waves is enough to break hydrogen bonds.

The main disadvantage of mechanical pretreatments is the energy demand. To become economically feasible, the methane yield must compensate for the energetic and economic cost of the pretreatment. A balance between the energy demand and the recovered energy is hard to achieve for most pretreatments because mechanical equipment consumes extensive amounts of electricity. Since recalcitrant algae species usually need pretreatments with higher power input, those methods become economically unattractive to implement at large scales. Research has indicated that ultrasound is not recommended for protein-rich microalgae species because it may cause aggregation of proteins. Therefore, the overall recalcitrance of biomass is not eliminated (Oleszek; Krzemińska, 2021).

2.2 THERMAL PRETREATMENTS

Thermal pretreatments usually occur in temperatures ≤ 100 °C (thermal pretreatment) or very high temperatures with increased pressure (hydrothermal pretreatment) at different times (Rodriguez *et al.*, 2015). High temperatures solubilize organic matter and partially hydrolyze macromolecules, including carbohydrates, lipids, and proteins. Most studies on thermal pretreatment of algae biomass have been conducted with a temperature range from 55 to 170 °C (Kendir Çakmak; Ugurlu, 2020; Passos; García; Ferrer, 2013).

Wilson and Novak (2009) studied the effect of temperature on the degradation of carbohydrates, lipids, and proteins by applying thermal pretreatment to starch, cellulose, fatty acids, and albumin. The authors found that above 150 °C the degradation of proteins is accompanied by the generation of ammonia; unsaturated fats are converted to volatile fatty acids, such as acetic and propionic acid; and saturated fats form long-chain fatty acids (Wilson; Novak, 2009). These observations prove that high temperatures produce inhibitory compounds for the metabolism of anaerobic microorganisms. This reduces the digestibility of biomass treated under harsher temperatures compared to milder conditions (Mendez *et al.*, 2014).

Hydrothermal pretreatments are usually done at temperatures ≥ 160 °C. In these conditions, hemicellulose autohydrolysis takes place. Water at a high temperature disrupts the chemical bonds between the hemicellulose polymeric chain and its ramifications. Thus, acetic acid is formed locally and contributes to further hemicellulose depolymerization. Moreover, hydrothermal pretreatment can be catalyzed by adding chemicals like HCl, H_2SO_4, SO_2, and CO_2. Acid catalysts promote the hydrolysis of the hemicellulose fraction, increasing the yield of soluble sugars. Nevertheless, in addition to corrosion problems, acid catalysts can react with solubilized monomeric sugars to form inhibitors. More drawbacks related to adding chemicals will be discussed in the section on chemical pretreatments.

The chemical reactions that take place in steam explosions are very similar to hydrothermal pretreatment. Temperatures usually range between 160 and 220 °C, and biomass is hydrolyzed in the presence of steam. Steam explosion also relies on physical

mechanisms to disrupt biomass constituents. As the temperature rises, there is also an increment in pressure. At the end of residence time, the reactor is then suddenly depressurized so that the quick pressure drop destroys the cell wall fibers and enhances biomass porosity.

The main factors affecting hydrothermal and steam explosion pretreatments are temperature, residence time, and the addition of catalysts. In hydrothermal pretreatment, the reactor heating and cooling steps should be as fast as possible to guarantee that most chemical reactions occur at the desired temperature. Duration of thermal pretreatment usually compensates for temperature, as higher temperatures demand shorter residence times and vice versa (Oliveira; Bassin; Cammarota, 2022). Biomass thermal hydrolysis can be improved by the addition of chemical catalysts, which also reduce the duration of pretreatment. Thermochemical pretreatments will be discussed in another section.

2.2.1 Advantages and disadvantages of thermal pretreatments

The main advantages of thermal pretreatments include high biomass solubilization and efficient release of monomeric sugars; previous biomass size reduction is not mandatory; the addition of chemicals is optional; and it is easy to implement at industrial scales.

At high temperatures, the degradation of lipids and proteins can lead to the accumulation of long-chain fatty acids and ammonia, respectively, both of which inhibit the metabolism of methanogenic microorganisms (Wilson; Novak, 2009). High temperatures also favor Maillard reactions between amino acids and reducing sugars, forming new complex molecules and decreasing protein solubility. Thus, the formation of inhibitory and recalcitrant compounds accounts for the lower digestibility of biomass pretreated at high temperatures, indicating the need to prioritize milder temperatures for thermal pretreatments (Solé-Bundó *et al.*, 2017). Other drawbacks include the economic costs derived from the intense consumption of heat and/or electricity for reactor heating.

Table 2 shows the increase in methane yield after the mechanical and thermal pretreatments of algae biomass.

Table 2 – Methane yields after mechanical and thermal pretreatments of microalgal biomass

Microalgae species	Pretreatment method	Pretreatment conditions	Methane yields (NmL CH_4/g VS)			Reference
			Without PT	With PT	Variation (%)	
Scenedesmus obliquus	Thermal	155 °C, 30 min	159	233	47	Ometto *et al.*, 2014
		165 °C, 30 min		259	63	
	Hydrothermal	155 °C, 5 bar, 30 min		242	52	
		165 °C, 7 bar, 30 min		384	141	
	Ultrasound	100 W, 10 MJ/kg TS, 8 min		243	53	
Chlorella sorokiniana	Thermal	145 °C, 30 min	169	206	22	
		165 °C, 30 min		173	2	
	Hydrothermal	120 °C, 2 bar, 30 min		173	2	
		165 °C, 7 bar, 30 min		318	88	
	Ultrasound	100 W, 20 MJ/ kg TS, 10 min		164	-3	
		100 W, 35 MJ/ kg TS, 20 min		244	44	
Scenedesmus sp.	Ultrasound	80 W, 129 MJ/ kg TS, 30 min	82	154	88	González-Fernandes *et al.*, 2012
	Thermal	80 °C, 25 min		129	57	
Chlorella sorokiniana	Ultrasound	220 W, 30 min	318	458	44	Córdova, Passos, Chamy, 2018
	Thermal	80 °C, 20 min		375	18	
Mixed microalgae biomass	Microwave	900 W, 64.4 MJ/kg TS, 3 min	118	210	79	Passos *et al.*, 2013
Mixed microalgae biomass	Microwave	900 W, 3 min, 70 MJ/kg VS	170	270	58.8	Passos *et al.*, 2014
Chlorella sp.	Thermal	65 °C, 4 h	211	297	41	Scarcelli *et al.*, 2020
Chlorella vulgaris	Hydrothermal	160 °C, 6 bar, 10 min		256	64	
Acutodesmus obliquus	Ultrasound	20 MJ/kg TS	156	174[a]	50	Gruber-Brunhumer *et al.*, 2015
	Bead milling	20 min		198[a]	72	
	Thermal	100 °C, 60 min		149[a]	29	

[a] Nm^3 CH_4/g COD (chemical oxygen demand)

2.3 CHEMICAL AND THERMOCHEMICAL PRETREATMENTS

Chemical methods have already been implemented on a large scale to pretreat waste activated sludge and organic wastes prior to anaerobic digestion. Thus, many researchers have been trying to adapt those methods for algae biomass.

Alkalis and acids are traditional reagents for chemical and thermochemical pretreatments; therefore, the solubilization of organic matter from multiple biomass sources by those methods has been extensively studied. Oxidizing agents and ionic liquids have gained attention as an alternative to traditional chemicals. The most influential factors in the chemical and thermochemical pretreatment of algae biomass are the concentration of the reagent, the concentration of the biomass, the temperature, and the contact time between the biomass and the chemicals (Oliveira; Bassin; Cammarota, 2022).

Table 3 shows the increase in methane yield after chemical and thermochemical pretreatments of algae biomass. For acid and alkaline pretreatments, temperatures above 100 °C usually allow good solubilization for 20 min to 2 h. At mild temperatures, the contact time seems to have little influence on the solubilization result (Solé-Bundó *et al.*, 2017; Mahdy *et al.*, 2014a).

Regardless of the type of alkali or acid chosen for the pretreatment, some authors have observed that the solubilization of biomass tends to stabilize or even decrease after a certain concentration of reagent (Du *et al.*, 2020; Penaud; Delgenès; Moletta, 1999). The solubilization increases as the pH moves toward higher values of either acidity or alkalinity. Species with a rigid cell wall, like *Chlorella* and *Nannochloropsis*, usually demand harsher pretreatment conditions. In this case, thermochemical pretreatment is preferable over chemical pretreatment at room temperature. On the other hand, chemical pretreatment seems to be indifferent for the anaerobic digestion of microalgae species with no cell wall because they are already easy to digest (Bohutskyi; Betenbaugh; Bouwer, 2014).

Table 3 – Methane yields after chemical and thermochemical pretreatments of microalgal biomass

Microalgae specie	Pretreatment method	Pretreatment conditions	Methane Yields (mL CH_4/g VS)			Reference
			Without PT	With PT	Variation (%)	
Oscillatoria tenuis	acid	H_2SO_4 4M, pH 2; room temp.	191	210	10	Cheng *et al.,* 2018
Microalgae biomass after lipid extraction	acid	HCl, pH 1, room temp.	104 [c]	217 [c]	109	Sposob *et* al., 2020
Mixed biomass of *Chlorella* sp. and *Monoraphidium* sp.	acid - thermochemical	HCl 0.05% m/m, pH 1, 80 °C, 2 h	78	142	82	Passos *et al.*, 2016
Isochrysis galbana	acid - thermochemical	H_2SO_4 0.2% v/v, 40 °C, 16 h	9	16	72	Santos *et al.*, 2014
Scenedesmus obtusiusculus	acid - thermochemical	HCl 3% m/m, 105 °C, 1.7 h	234	296	26	Rincón-Pérez *et al.*, 2020
Scenedesmus sp.	acid - thermochemical	H_2SO_4 0.1% v/v, 150 °C, 1 h	131	253	93	Marques *et al.*, 2018
Mixed microalgae biomass of *Desmodemus opoliensis* (47%) and others	acid - thermochemical	HCl 2 M, pH 0.3, 121 °C, 1 h	216	250	20	Juárez *et al.,* 2018
	alkali	NaOH 0.8 g/L, room temp., 12h	279	290	4	Du *et al.*, 2020
Spirulina platensis	alkali	NaOH 1.6 g/L, room temp., 12h		298	7	
	alkali	NaOH 4.0 g/L, room temp., 12h		295	6	
Thalassiosira weissflogii	alkali	NaOH 20 g/L, pH 13, room temp.	unavailable		20	Bohutskyi; Betenbaugh; Bouwer, 2014
Mixed microalgae biomass	alkali	530 mg N-NH_3/L, pH 9.5, 22°C, agitation (300 rpm)	136	162	19	Wang; Park, 2015
Mixed biomass of *Chlorella* and *Scenedesmus*	alkali - thermochemical	CaO 4% m/m, pH 12, 72 °C, 24 h	260	287	10	Solé-Bundó *et al.*, 2017
Scenedesmus obtusiusculus	alkali - thermochemical	NaOH 4 M, pH 10, 120 °C, 20 min	155	227	46	Candia-Lomeli *et al.,* 2022

[a] NmL CH_4/g COD.

a) Acid pretreatment

Acid pretreatment is a common pretreatment method for lignocellulosic biomass, and many researchers have adapted this type of pretreatment to microalgal biomass (Ariunbaatar *et al.*, 2014). Hemicellulose is an amorphous polymer susceptible to the attack of hydrogen protons during acid hydrolysis. The detailed reaction mechanism between the acid reagent and the hemicellulose polymer has been studied and can be found elsewhere (Miguez *et al.*, 2023).

The most common reagents for acid pretreatment are hydrochloric acid (HCl) or sulfuric acid (H_2SO_4). Especially in the case of anaerobic digestion, accumulation of Cl^- and SO_4^{-2} may be harmful to the methanogenic microorganisms, and concentrated acid should be avoided. The conditions of acid pretreatment should be carefully chosen and monitored to minimize the formation of undesired degradation products. Severe pretreatment conditions may lead to a reaction between the acid and the released sugars. This decreases the number of available sugars after pretreatment and results in the formation of inhibitory compounds, especially when concentrated acid is applied.

The main inhibitory products formed during acid hydrolysis are furfural (FF), which originates from xylose degradation, and 5-hidroximetilfurfural (HMF) due to glucose degradation (Amin *et al.*, 2017; Bohutskyi; Betenbaugh; Bouwer, 2014). Inhibition of microbial growth and methanogenic activity has been reported at 2 g/L of FF or HMF in mesophilic or thermophilic anaerobic digestion (Ghasimi *et al.*, 2016). Acid pretreatment of mixed algae biomass of *Chlorella* and *Monoraphidium* sp. with HCl 1.25% at 80 °C for 2 h led to the formation of 3.8 mg/L of FF. When HCl concentration increased to 2.0%, FF concentration rose to 6.9 mg/L, and 1.3 mg/L HMF was also present (Passos *et al.*, 2016a). In this case, the degradation products at these concentrations did not impair anaerobic digestion. However, higher acid concentrations led to more FF and HMF formation.

Apart from the formation of inhibitors, acid pretreatment requires high-quality construction materials for the reactors to avoid corrosion, increasing the economic costs (Rodriguez *et al.*, 2015). Another drawback of this method is the need for pH adjustment before anaerobic digestion, because the anaerobic community is very sensitive to environmental changes and operates within a strict pH range, usually between 6.8 and 7.2.

b) Alkaline pretreatment

Alkaline pretreatment is a conventional method for solubilizing waste activated sludge prior to anaerobic digestion. Therefore, many studies rely on this method to propose a pretreatment for microalgae biomass (Oliveira; Bassin; Cammarota, 2022). Alkaline pretreatment causes saponification of uronic acids and esters from hemicellulose, resulting in the swelling of the fibers and surface area increase (Penaud; Delgenès; Moletta, 1999; Singh; Sharma, 2022). It also decreases the cellulose crystallinity, making it easier to break by hydrolytic enzymes from the microorganisms. Proteins can also be solubilized by

alkaline pretreatment (D'Hondt *et al.*, 2017; Mendez *et al.*, 2013). These structural changes in biomass favor the access of the digesting microorganisms to organic matter and enhance methane production (Mahdy *et al.*, 2014a).

NaOH, KOH, CaO, and $Ca(OH)_2$ are the most commonly employed reagents for alkaline pretreatment. However, according to some authors, monovalent alkalis should be preferred over divalent alkalis, because the latter partially ionize in aqueous media (Oliveira; Bassin; Cammarota, 2022). In a novel approach, Wang *et al.* (2019) achieved positive results, improving methane yield using an ammonia-based alkaline pretreatment.

c) Ionic liquids

Ionic liquids (ILs) are organic salts that are liquid at temperatures < 100 °C. Those liquids are composed of an inorganic anion and an organic cationic counterpart. The thermodynamic and physical-chemical properties (like melting point, viscosity, density, and solubility) of ILs are very particular and vary greatly as a function of their components. Replacing the anionic, cationic, or both ions is enough to change the characteristics of ILs (Holm; Lassi, 2011). This allows for new ILs to be prepared for specific uses.

Since they do not release toxic by-products or dangerous gases, those chemicals have been studied as green solvents for many applications. Furthermore, the ability of cellulose solubilization by some ILs allows biomass pretreatment with the goal of cellulose recovery and further conversion into bioenergy (Hou *et al.*, 2017).

Solubilization of cellulose by ILs is related to the breakage of intra and intermolecular chemical bonds of the cellulose fibrils (da Silva *et al.*, 2013). ILs with chloride or formate anions are suitable for cellulose solubilization, especially because of the hydrogen bonds formed between the chloride anion and the hydroxyl group of cellulose. However, chloride-based ILs require temperatures > 70 °C for biomass pretreatment, while formate-based ILs have low stability (Holm; Lassi, 2011). More stable options are acetate-based or phosphate-based ILs. Regarding the cationic component, Holm and Lassi (2011) found that ILs with small cations are more efficient at dissolving cellulose.

Cellulose solubility in ILs depends on cellulose crystallinity, temperature, pretreatment duration, and initial cellulose concentration (Holm; Lassi, 2011). Moreover, the presence of water in the system may lead to the formation of inhibitory compounds, especially at high temperatures. The chemical structure of cellulose can be altered by chemical reactions, resulting in undesired derivatization. Thus, for biomass pretreatment, the ILs must be water-free and without any impurities (Holm; Lassi, 2011). After pretreatment, the cellulose fraction can be recovered by adding a counter solvent, such as water (da Silva *et al.*, 2013; Holm; Lassi, 2011).

Despite the potential for fractionation of microalgae biomass by ILs, they are mainly used for lipid recovery and high-value products. Since microorganisms in anaerobic digestion are not restricted to a class of molecules, the recovery of a specific biomass fraction may not be economically attractive for methane production.

d) Thermochemical

Both acid and alkaline pretreatments of microalgae biomass have been minimally explored at room temperature, with most studies conducted at temperatures around 50 °C and above, including > 100 °C. The combination of an acid or alkaline reagent and temperature makes it possible to disintegrate biomass in less time than chemical pretreatments at room temperature or thermal treatment alone (Bohutskyi; Betenbaugh; Bouwer, 2014; Mendez *et al.*, 2014; Penaud; Delgenès; Moletta, 1999; Solé-Bundó *et al.*, 2017).

Since hydrothermal pretreatment leads to autohydrolysis of hemicellulose, it is sometimes referred to as a thermochemical pretreatment, especially when a chemical catalyst is added. Autohydrolysis was discussed in the Thermal pretreatments section.

2.3.1 Advantages and disadvantages of chemical and thermochemical pretreatments

In general, chemical and thermochemical pretreatments lead to a high degree of biomass solubilization. However, their disadvantages include the cost of reagents, changes in the pH of the digester, accumulation of ions or degradation intermediates that inhibit methanogenic activity, corrosion of equipment, and high energy demand (Oliveira; Bassin; Cammarota, 2022). The disadvantages of ammonia-based pretreatments are mainly related to ammonia recuperation. However, ammonia recovery and recycling are feasible, and this step is crucial for anaerobic digestion because free ammonia may inhibit methanogenic activity.

After pretreatment, the greater organic matter available is expected to increase the methane yield compared to untreated biomass. Anaerobic digestion of different microalgae species after chemical and thermochemical pretreatment resulted in significant increases in methane yield compared to raw biomass (Marques *et al.*, 2018; Santos *et al.*, 2014). However, the scientific literature includes many examples in which increased solubilization of organic matter did not lead to a higher methane yield, especially when harsh conditions were chosen for chemical and thermochemical pretreatments (Bohutskyi; Betenbaugh; Bouwer, 2014; Cho *et al.*, 2013; Passos *et al.*, 2016a).

Therefore, the solubilization of organic matter does not clearly translate into the availability of readily digestible substrates for the anaerobic microorganisms. Most authors attribute this to the occurrence of Maillard reactions between the solubilized molecules, such as amino acids and reducing sugars (Penaud; Delgenès; Moletta, 1999; Solé-Bundó *et al.*, 2017). These reactions are favored by the high temperature and extreme pH of the pretreatments and result in the formation of recalcitrant molecules, leading to decreased biodegradability and low methane production (Penaud; Delgenès; Moletta, 1999).

Because they release the inner moisture of the originally solid biomass, chemical, thermal, hydrothermal, and thermochemical pretreatments result in a biomass slurry. This

mixture of liquid and solid fractions does not need further separation for methane production. However, this biomass slurry is a complex mixture of soluble degradable and recalcitrant molecules. After pretreatment, the degree of biomass degradation is usually measured by the rise in soluble COD (chemical oxygen demand), which is chemically unspecific. Some works have assessed the final concentration of soluble sugars released by pretreatment (Passos *et al*., 2016). Other than that, little is known about the chemical composition of biomass slurry. This information could provide new perspectives on the relation between biomass solubilization and methane yields.

2.4 ENZYMATIC PRETREATMENT

Enzymatic pretreatment is an environmentally friendly process due to its low energy costs, high yield of fermentable sugars released from biomass under mild operating conditions, absence of corrosive problems, and few by-products (Yun *et al*., 2014). Since the enzymes target specific substrates, no inhibitory by-products are formed, thus preserving the anaerobic digestion microorganisms. However, it requires a longer residence time, in addition to the high cost of commercial enzymes (Bohutskyi; Bouwer, 2012).

When selecting the enzyme or enzyme mixture to be used, it is necessary to consider i) the chemical composition of the biomass, especially its cell wall; ii) the optimum operating conditions such as pH, temperature, exposure time, and enzyme/substrate ratio; and iii) the costs associated with using the enzymes (Córdova *et al*., 2018). Operating conditions must also be carefully chosen, as unsuitable conditions, especially pH and temperature, can deactivate enzymes due to denaturation. The enzyme/microalgae ratio also directly influences the efficiency of enzymatic activity.

To determine the relative importance of the parameters used for enzymatic hydrolysis, Shokrkar *et al*. (2017) evaluated the effects of microalgae biomass concentration, temperature, pH, and hydrolysis time on the enzymatic hydrolysis of a mixed culture of microalgae using α-amylase and cellulases. They determined that pH was the parameter with the greatest influence on the enzymatic hydrolysis process, followed by substrate concentration, temperature, and hydrolysis time.

The efficiency of enzymatic pretreatment is directly related to the enzymes used and the operating conditions. In the enzymatic hydrolysis of *C. sorokiniana* with cellulase (150 U/mL at 50 °C), Yin *et al*. (2010) reduced the total insoluble solids by about 30% after three hours of pretreatment.

The combined use of enzymes can improve the cell wall degradation of microalgae. In both cases, a cellulase mix can target cellulose and hemicellulose separately. Moreover, the cell wall of many microalgae species has outer layers comprised of biopolymers other than carbohydrates. Thus, a mix of cellulases, proteases, and other types of enzymes can benefit biomass degradation.

The pretreatment of *Chlorella vulgaris* with an enzyme mixture of lysozyme and sulfatase achieved a 98.6% permeability, while the permeability obtained using each enzyme individually was 7.6% for lysozyme; 1.2% for sulfatase (Gerken; Donohoe; Knoshaug, 2013).

Ometto *et al*. (2014) also reported the use of enzymes for the cell wall degradation of *Scenedesmus obliquus* and *Arthrospira maxima.* They found an increase in soluble COD depending on the enzymes used in the process. The use of α-amylase with *S. obliquus* showed no significant differences compared to biomass without pretreatment, while esterase and pectinase increased soluble COD by more than 30 mg/g total solids. The highest release of soluble COD was obtained with enzyme mixture preparations, which indicates that it is preferable to use enzyme cocktails to achieve greater solubilization of the insoluble compounds.

Table 4 shows the increase in methane yield after enzymatic pretreatment of different algae biomasses. Pretreatment of mixed biomass of *Chlorella* and *Scenedesmus* sp. by Avila *et al*. (2020) resulted in greater solubilization with an enzyme dose of 2%; however, anaerobic digestibility was lower than with pretreatments at a lower dose (1%). On the other hand, Mahdy *et al*. (2014c) found that decreasing enzyme dosages in *Chlorella* pretreatment concomitantly decreased hydrolysis efficiency and methane production.

Regarding biofuel production, Ciudad *et al*. (2014) observed an increase of approximately 100 mL CH_4/g VS after *Botryococcus braunii* pretreatment for 24 h with an enzyme extract. Hom-Diaz *et al*. (HOM-DIAZ *et al*., 2016) evaluated the increase in biogas production by enzymatic pretreatment of a mixed microalgal biomass (predominantly *Oocystis* sp.). The authors evaluated the performance of commercial laccase and an enzymatic extract produced by *Trametes versicolor* fungi, both at a dose of 100 U/L. Results showed that when using commercial enzymes, methane yield increased by 20%, while pretreatment with fungal broth increased methane yield by 74% compared to non-pretreated biomass. The difference in results may be associated with the presence of other enzymes, radicals, and other mediators produced by *T. versicolor* in culture, achieving greater solubilization of the cells.

In addition to increasing the availability of soluble matter, enzyme mixtures can provide higher methane yields. Gruber-Brunhumer *et al*. (2015) obtained a 30.3% increase in methane yield after pretreating *Acutodesmus obliquus* with an enzyme mixture. Ehimen *et al*. (2013) found that an enzyme mixture of lipase, xylanase, α-amylase, cellulase, and protease, in the pretreatment of *Rhizoclonium*, resulted in the highest methane yield, with an increase of 31%. Passos *et al*. (2016a) achieved an 8–15% increase in methane yield from microalgae biomass after pretreatment with an enzyme mixture including cellulase, glucohydrolase, and xylanase.

Some studies have indicated that proteins play a crucial role in algae biogas production, since proteins account for 40–60% of the dry weight of algae. The addition of protease increased the solubilization of the substrate and had an incremental effect on the methane

yield (Magdalena; Ballesteros; González-Fernández, 2018). The enzymatic pretreatment of *Chlorella vulgaris* and *Scenedesmus* sp. with protease increased the methane yield by 71% and 62%, respectively, compared to the pretreatment using a carbohydrase mixture (Mahdy; Ballesteros; González-Fernández, 2016). Similarly, pretreatment with *Porphyridium cruentum* protease increased solubilization (32.3%) more than the mixture of cellulase and carbohydrase, with a maximum methane yield of 230 mL CH_4/g VS, which showed an improvement of 77% (Kendir Çakmak; Ugurlu, 2020). A total biogas yield of 42.73 mL/g VS was obtained from the pretreatment of *Spirulina subsalsa* BGLR6 with a multi-enzyme mix containing protease, cellulase, and hemicellulase at a dosage of 10% for 24 hours (Dar; Phutela, 2020).

Apart from the high economic cost of commercial enzymes, the main challenge of this method is the diversity of microalgae cell walls. Some species of industrial importance are well known, e.g., Chlorella genus. However, little data is available about the chemical composition and ultrastructure of other species that have a potential for energetic uses and biorefinery platforms. Therefore, investigation is needed on various enzymes and their suitability for different types of microalgae (Bohutskyi; Betenbaugh; Bouwer, 2014; Bohutskyi; Bouwer, 2012).

Table 4 – Methane yields after enzymatic pretreatment of algae biomass

Microalgae Species	Pretreatment Conditions	Methane Yield (NmL CH_4/g VS)			References
		Without PT	With PT	Variation (%)	
Chlorella sp. and *Scenedesmus*	Mix of commercial enzymes, 1% E/S, 0.5 h, 37 °C, 100 rpm	124.2	447.5	260	Avila *et al*., 2021
	Mix of commercial enzymes, 1% E/S, 0.5 h, 37 °C, 100rpm	124.2	640.9	416	
	Poligalacturonase, 1% E/S, 0.5 h, 37 °C, 100 rpm	124.2	652	425	
Chlorella vulgaris	Protease, 0.585 U/g TS, 3 h, 80 °C, 130 rpm	160.4	255.6	59	Mahdy *et al.,* 2014c
Acutodesmus obliquus	Enzymatic cocktail (cellulase, protease, β-glucanase, and xylanase), 10% E/S, 24 h, 37 °C	191.2	218	14	Gruber-Brunhumer *et al.,* 2015
Arthrospira máxima	Lipomod™ 957 (esterase, protease), 24 h, 50 °C	200[a]	1545[a]	672	Ometto *et al*., 2014
Oocystis sp.	Cellulase, 1%, 37 °C, 6 h	188.6	203	7.6	Passos *et al*., 2016
Oocystis sp.	Enzymatic cocktail (cellulase, glucohydrolase, and xylanase), 1%, 37 °C, 6 h	188.6	217.3	15.2	Passos *et al*., 2016
Oocystis sp.	Commercial laccase, 100 U/L, 25 °C, 25 min, 100 rpm	83	100	20	Hom-Diaz *et a*l., 2016
Oocystis sp.	Laccase from *Trametes versicolor,* 100 U/L, 25 °C, 25 min, 100 rpm	83	144	74	
Scenedesmus obliquus	Depol™ 40 L (cellulase, endogalactouronase), 24 h, 50 °C	283.3[a]	1425[a]	403	Ometto *et al*., 2014
Botryococcus braunii	Enzymatic extract of *Anthracophyllum discolor* 1000 U/L, room temperature, 24 h, 200 rpm	325.6	521	60	Ciudad *et al*., 2014

[a] biogas yield, VS = volatile solids, TS = total solids, E/S = enzyme/substrate ratio.

2.4.1 Advantages and disadvantages of enzymatic pretreatments

Enzymatic pretreatment is a very promising method for cell wall disruption before anaerobic digestion. Some results show an increase of more than 100% in methane production from enzymatically treated microalgae. Moreover, since enzymes are substrate-specific, the formation of inhibitory molecules or undesired by-products, such as complex recalcitrant glycopeptides, is much less likely to occur. However, this same feature of substrate-specificity requires a tailored choice of enzymes or enzyme mix for a given microalgae species. For example, a single enzyme type may be insufficient for microalgae with lipid capsules or pectin layers. Due to the scarce information about the cell wall composition of microalgae, enzyme selection may take several tests to achieve the ideal choice and operation conditions. Thus, more research is needed to better understand this pretreatment.

Additionally, commercial enzymes are expensive, which hinders their application at demonstrative and industrial scales. Alternative methods should be developed to decrease the enzyme production costs. Residual agricultural biomass can be used for enzyme preparation through fermentative biotechnologies.

3. TECHNICAL AND ECONOMIC OBSTACLES TO ANAEROBIC DIGESTION OF MICROALGAE

Interest in the production of third-generation biofuels from microalgae and cyanobacteria biomass is growing. Microalgae biomass is very versatile for the production of high-value-added biochemicals and biofuels. Biogas/biomethane, biodiesel, bioethanol, biohydrogen, and biobutanol can be obtained from microalgae biomass depending on the route employed (Cavinato *et al.*, 2017; Wang *et al.*, 2022).

Microalgae and cyanobacteria have additional benefits over terrestrial plants. As photosynthetic microorganisms, they help reduce CO_2 emissions. Their ability to grow in nutrient-rich wastewater allows them to be cultivated without clean water or fertilizers (Rusten; Sahu, 2011; SALAMA *et al.*, 2017). Other advantages of algae biomass include their simple nutritional requirements and high productivity, since under suitable conditions, they grow 5 to 10 times faster than terrestrial biomasses (Kröger; Müller-Langer, 2012).

The first study of anaerobic digestion of microalgae was carried out by Golueke et al. (1957) who achieved a methane yield of 0.17–0.32 L CH_4/g VS from the microalgae *Chorella vulgaris* and *Scenedesmus*. Bench and pilot experiments, as well as theoretical calculations, have demonstrated the enormous potential of anaerobic digestion of microalgae for methane production, with a higher specific yield than other biomass sources (Mussgnug *et al.*, 2010; Zamalloa; Boon; Verstraete, 2012).

However, technical and economic obstacles to the anaerobic digestion of this type of biomass remain. Low cell concentration in cultivation and the recalcitrance of raw algae

biomass hinder methane yields (Bohutskyi; Betenbaugh; Bouwer, 2014; Zabed *et al.*, 2020). Inhibition of anaerobic digestion may occur due to the accumulation of volatile fatty acids from lipid degradation or free ammonia from protein degradation.

In general, the concentration of cells in the cultures is low, less than 1 g/L in open ponds and between 2 and 6 g/L in closed photobioreactors (Roselet *et al.*, 2019). In practice, the low cellular concentration in culture media means that only a small part of the raw material is organic matter available to be converted to methane. This demands the adoption of harvesting technology to recover the cells, since they do not stick together due to electrostatic repulsion (Chatsungnoen; Chisti, 2019; Vandamme; Foubert; Muylaert, 2013).

The ions present in the culture medium surround the cells, forming a stable suspension. Therefore, separation strategies based on sedimentation of the biomass result in low efficiency. However, processes based on centrifugation, filtration, and membrane separation are generally more efficient but costly (Chatsungnoen; Chisti, 2019; Milledge; Heaven, 2013). According to some calculations, biomass harvesting can represent 30% or more of the total biomass production cost, making the process economically unviable (Sharma; Stal, 2013). There is an imperative need for cheap and easily scalable microalgae harvesting technology to upscale methane production from this biomass.

The methane yield from the mesophilic anaerobic digestion of various species of microalgae and cyanobacteria varies considerably from species to species and is affected by the chemical composition of the biomass and the rigidity of the cell wall. Some species have no cell wall and show high yields, for example, *Dunaliella salina* (325 mL CH_4/g VS, Mussgnug *et al.*, 2010). Others, such as *Chlamydomonas reinhardtii*, have a cell wall composed mostly of glycoproteins, which is easily degradable and allows for good methane yields (263 mL CH_4/g VS, Mahdy *et al.*, 2014b).

However, most species have a rigid cell wall made of layers of cellulose, hemicellulose, pectin, peptidoglycan, and sporopollenin, among other resistant polymers (D'Hondt *et al.*, 2017; de Carvalho *et al.*, 2020). These polymers make it difficult for anaerobic microorganisms to degrade the cells, consequently reducing methane yield, such as from *Scenedesmus obliquus* (130 mL CH_4/g VS, Zamalloa *et al.*, 2012). Although microalgae cell wall composition is a key feature to choose the best operational conditions for pretreatments, chemical characterization of the cell wall is a complex task, especially given the number of microalgae species suitable for energy conversion. Thus, information in the literature is very scarce, but some efforts have been made with converging results.

Finally, a key challenge for biomass pretreatments is the energy demand. Most studies do not provide an energy balance assessment of the working conditions. Authors have collected data available in scientific literature to compare different methods (Oliveira; Bassin; Cammarota, 2022; Marques; Araújo; Cammarota, 2019). Mechanical, thermal, chemical, and thermochemical are all energy-intensive methods, either due to the energy required for biomass heating or the energetic cost of chemical production, or both.

Table 5 offers a comparison of the pretreatments discussed in this chapter. Oliveira *et al.* (2022) conducted an in-depth analysis of the effects of chemical and thermochemical pretreatments of algal biomass for methane production. The results suggest that less severe conditions are preferable for microalgae biomass. Considering the energetic cost of the assessed pretreatments, only enzymatic pretreatment and a few thermal pretreatments result in a positive energy balance (Oliveira; Bassin; Cammarota, 2022).

Table 5 – Characteristics of different pretreatment methods for methane production

Pretreatment	Changes biomass composition	Substrate specific	Formation of inhibitors	Energy-intensive	Corrosive	Post-treatment adjustments*
Acid	+	-	+	-	+	+
Alkaline	+	-	+	-	-	+
Ionic Liquids	+	+	-	-	-	+
Ball Milling	-	-	-	+	-	-
Bead Milling	-	-	-	+	-	-
Microwave	+	-	-	+	-	-
Ultrasound	+	-	-	+	-	-
Thermal	+	-	+	+	-	-
Hydrothermal	+	-	+	+	-	-
Steam Explosion	+	-	-	+	-	-
Thermochemical	+	-	+	+	-	+
Enzymatic	+	+	-	-	-	-

*pH adjustment, biomass washing, chemicals recovery ...

+ = yes; - = no

CONCLUSIONS

Mechanical pretreatments are inefficient from the energy perspective because the energy generated from anaerobic digestion often does not offset the energy required for the pretreatment step. Thermal pretreatments are more efficient but energy-intensive and can lead to the formation of inhibitory and recalcitrant compounds with lower digestibility, indicating that milder temperatures are more suitable for thermal pretreatments.

Chemical and thermochemical pretreatments lead to a high degree of biomass solubilization but have high energy demand and result in the formation of inhibitors of methanogenic activity, in addition to the cost of reagents and corrosion of equipment. Although chemical pretreatments with NaOH are widely used, the efficiency of this method for algae biomass is questionable because most of the studies analyzed obtained an increase in methane yield of less than 20%. In contrast, there is a lack of data on acid

pretreatment at room temperature.

In both chemical pretreatments at room temperature and thermochemical pretreatments, an increase in the solubilization of organic matter does not necessarily lead to a higher biogas yield, especially if the conditions applied in the pretreatment are more severe. The pretreatment conditions in which greater solubilization is achieved may be different from those that contribute to a higher methane yield. In other words, the relationship between increased solubilization and methane yield has not been well defined.

Enzymatic pretreatment is a very efficient method for cell wall disruption. However, the selection of enzymes depends on the cell wall composition of microalgae, requiring several tests to achieve the ideal enzyme blend and operation conditions. Furthermore, enzymatic pretreatment requires different types of enzymes and the production of enzyme complexes at a lower cost than commercially available enzymes.

Although algae biomass is a promising raw material for methane production, most species are resistant to degradation. Thus, some pretreatment methods have been intensively studied to provide a solution to the recalcitrance of biomass. Although some chemical and hydrothermal pretreatments have been implemented at an industrial scale, there is no universal biomass pretreatment method. It is impossible to name a pretreatment method and/or condition suitable for all biomasses. Thus, the pretreatment choice is still a challenging step for the energetic conversion of microalgae biomass, and more studies are needed to establish a viable pretreatment condition that uses algae biomass to obtain biogas/methane.

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