Review

Microalgae and Cyanobacteria Biomass Pretreatment Methods: A Comparative Analysis of Chemical and Thermochemical Pretreatment Methods Aimed at Methane Production

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Abstract: Anaerobic digestion of microalgae and cyanobacteria was first proposed as a destination for algal biomass accumulated on stabilization ponds since it could not be disposed of directly in the environment. Now, the versatility of algal biomass makes them a suitable candidate to produce biofuels and other biomolecules in biorefineries. Anaerobic digestion of biomass is advantageous because it does not require the extraction of specific cellular constituents or drying of the biomass. Nevertheless, challenges remain regarding biomass concentration and their resistant cell walls, which are factors that could hamper methane yield. Many pretreatment methods, including chemical and thermochemical, have been proposed to break down the complex polymers present on the cell wall into smaller molecules. Unfortunately, the relationship between biomass solubilization and methane yield is not well defined. This article intends to review the anaerobic digestion of algal biomass and the role of chemical and thermochemical pretreatments in enhancing methane production. Several pretreatment conditions selected from the scientific literature were compared to verify which conditions actually improve methane yield. The severity of the selected pretreatments was also assessed using the combined severity factor. Results suggest that thermochemical pretreatment in less severe conditions is the most efficient, leading to a greater increase in methane yield. Only enzymatic pretreatments and some 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: anaerobic digestion; methane; algal biomass; chemical pretreatment; thermochemical pretreatment

1. Introduction

Interest in renewable energy sources based on algal biomass conversion has increased due to the versatility of such biomass for biofuel production, such as biogas/biomethane, biodiesel, biohydrogen, bioethanol, and biobutanol, depending on the chosen technology [1,2]. Biogas generation, through anaerobic digestion of the algal biomass, eliminates some of the difficulties that lead to the high cost of biofuel generation from algae, namely: drying, extraction of intracellular molecules, and conversion to energy. Anaerobic digestion is also advantageous over other conversion routes because it allows wastewater reuse, nutrient recycling, maximum biomass utilization, minimum sludge generation, low-operational costs, low-energy demand, and sustainable production of biogas [3,4]. Some authors suggest that methane production through anaerobic digestion is the most economically feasible route for an energy product generated from microalgae [5,6]. However, some technical and economic obstacles are still present for the anaerobic digestion of this kind of biomass, particularly fresh microalgae recalcitrance, low-C/N
ratio, accumulation of volatile fatty acids in the digestor, and low-methane yield [7,8]. The suitability of microalgae for biogas production has been extensively investigated to clarify various aspects and try to improve the efficiency of the process and its general sustainability [2,8–19]. Most authors agree that algal biomass is an attractive feedstock to produce biofuels (biodiesel, biogas, bioethanol) and high-value products (drugs, nutraceuticals, chemicals). However, innovative and efficient techniques are needed to make large-scale production of algal biomass possible. Nonetheless, few studies suggest solutions to fill in the gaps, especially for algal biomass pretreatment. Kannah et al. [16], for instance, propose a two-stage pretreatment, combining a cell wall-weakening step followed by the actual pretreatment to minimize the energy cost of microalgae conversion to biofuel. Other authors propose the integration between different routes of biofuel production and high-value products to increase energy generation and minimize the costs of biomass cultivation [2,19].

This article makes an extensive survey of the literature on algal biomass pretreatment methods, in particular chemical and thermochemical, aimed at increasing methane yield and making its production feasible. Chemical and thermochemical pretreatment methods were selected for further study because they are common methods for other biomasses (lignocellulosic, organic waste, waste-activated sludge) and are already used on a large scale. Such factors would be an advantage over other methods, which are still on the bench scale (ultrasound and microwave) or are more expensive (biological). The correlation between biomass solubilization and methane yield, as well as the chemical and thermochemical pretreatment conditions that effectively increase the methane yield, allowing for a positive energy balance, are also discussed in this review. The review is structured as follows: first, an overview of the anaerobic digestion process is described, with a focus on the anaerobic digestion of algal biomass, then the algal biomass pretreatment methods and the influencing factors were assessed, finishing with a comparative analysis of chemical and thermochemical pretreatment methods of algal biomass and an energy efficiency assessment of those methods. The novelty of the study is a comparative analysis of these pretreatment methods to indicate the best method and the best approach to reach higher methane yield.

2. Anaerobic Digestion

Anaerobic digestion is a complex biochemical process comprised of several sequential reactions in the absence of free oxygen. The conversion of organic matter to biogas can be understood as a four-step process: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Each of these steps is carried out by hydrolytic, acidogenic, and acetogenic bacteria and methanogenic archaea [20,21].

The typical composition of the gas mixture that makes up biogas is 50% to 75% methane, 25 to 45% carbon dioxide, and minor components such as hydrogen, nitrogen, oxygen, hydrogen sulfide, ammonia, and carbon monoxide. Methane is an energy-rich component, and its biogas content varies depending on the feedstock added to the process, the digestion system, the temperature, and the detention time [22]. Biogas containing 50% methane has a heating value of 21 MJ/Nm$^3$, and depending on the flow rate, it can be used in combustion engines, incorporated into a gas distribution grid (after proper treatment), fed to a boiler, or used to generate electricity [23].

The advantages of anaerobic digestion are that it does not require high temperature or pressure; recycling and recovery of nutrients, since the effluent can be applied as fertilizer or soil amendment; and energy recovery whenever the technical, economic, and environmental aspects are deemed adequate. The disadvantages are the specialist needs for project design, building, and operation (increasing in complexity as the scale grows); the process depends largely on temperature control; a definition of how the recovered energy is going to be used (for heat, power, or combined generation); sludge may require additional treatment (aerobic composting, humification on drying bed); treated effluent may have unattractive characteristics, thus, requiring post-treatment (physical–chemical or aerobic); the sensibility of methanogenic archaea to a number of chemicals; reactor start-
up time may be longer due to the low-growth rate of anaerobic microorganisms; sulfur compounds can have a bad odor; and small- and medium-scale anaerobic technology for solid waste treatment in low- or middle-income countries is still relatively new. The main challenges of anaerobic digestion are methane loss, relatively long treatment time, and process instability [24,25].

All kinds of biomass can be used as a substrate for biogas production, as long as their main components are carbohydrates, proteins, lipids, cellulose, and hemicellulose. Theoretical biogas yield varies depending on the distribution of the biomass constituents; each dry ton (total solids) of carbohydrates, proteins, and lipids generates 790–800, 700, and 1200–1500 nm$^3$ of biogas containing 50, 70–71, and 67–68% of methane, respectively. However, methane content higher than the theoretical is possible since CO$_2$ remains partially solubilized in the liquid phase, also known as digestate [26].

Substrates for anaerobic digestion can be solid, sludge, and liquids either concentrated or diluted. Some examples are sewage; sewage treatment sludge; animal manure; the organic fraction of municipal solid waste; food waste from households, restaurants, and hotels; industrial and commercial organic waste (food processing, beverages, meat, dairy); agricultural waste and by-products; energy crops (eucalyptus, energy-cane, sorghum, elephant grass); and residual organic material from bioethanol and biodiesel production. Currently, the most widely used substrates for biogas generation are sludge from sewage treatment, animal manure, agricultural waste, and food industry waste. Other less commonly used and explored materials for biogas production are marine biomass (algae and ascidians) and woody biomass from short rotation forests, such as willow. The agricultural sector is generally the main substrate provider for biogas production due to the large number of residues generated on farms (animal manure, straw, harvest waste) and the possibility of growing specific crops for bioenergy (e.g., energy-cane) [27].

Many biomasses studied for biofuel production present major drawbacks. Energy crops (corn, sugarcane, and plant oil) used to produce first-generation biofuels compete with food cultivation. Production of second-generation biofuels from lignocellulosic or woody biomass, agricultural waste, and municipal solid waste face many obstacles, including their low-biomass productivity, demand for large amounts of water and arable land, and lack of efficient and low-energy pretreatments. Mass cultivation of oilseeds for biodiesel production leads to extensive deforestation and changes in land use that have serious impacts on biodiversity and make it harder to reach a sustainable production of biofuels from such biomass. Third-generation biofuels are mainly connected to algal biomass, and their feedstock is the key difference between second and third-generation biofuels [28,29].

3. Anaerobic Digestion of Algal Biomass

Gouleke et al. [30] were the first authors to describe the anaerobic digestion of algal biomass when they investigated the digestion of *Chlorella vulgaris* and *Scenedesmus*. Other research followed that aimed to identify key factors that might hamper the anaerobic digestion of algal biomass [31–44].

Microalgae and cyanobacteria are promising feedstocks for technological applications due to their simple nutritional demands and high-biomass productivity. Provided they have the appropriate conditions, they grow 5 to 10 times faster than terrestrial biomass [45]. As they grow, microalgae mitigate CO$_2$ emissions by absorbing carbon and removing nutrients from wastewater [46–49]. Furthermore, microalgae cultivation does not require arable land or fresh water, making it possible to grow them in wastewater. These advantages increase the attractiveness of this biomass [50,51].

Algal biomass contains mostly lipids (2–55%), proteins (6–71%), and carbohydrates (4–64%). The proportion of these macromolecules varies in different species and growth conditions. The diversity of biochemical composition among different phyla, genera, and even between similar species may represent a challenge to the anaerobic digestion of algal
biomass [8]. Table 1 shows the typical macromolecular composition of technologically relevant microalgae species, as well as their cell wall composition.

Table 1. Approximate chemical composition and cell wall components of biotechnologically relevant microalgae.

| Species         | Chemical Composition (%) | Cell Wall Components ¹   |
|-----------------|--------------------------|--------------------------|
|                 | Carbohydrates | Proteins | Lipids | Polysaccharides sheath; lipid double-layer; peptide-glycan layer surrounded by a fibrillar layer made of cellulose and hemicellulose; cell membrane. Peptide-glycan layer; algaenan layer; fibrillar layer made of cellulose and hemicellulose; cell membrane. Algaenan layer; cellulose layer; struts; cell membrane. Algaenan layer; fibrillar layer made of cellulose and hemicellulose; cell membrane. |
| Spirulina       | 16            | 71        | 7      |
| Scenedesmus     | 17            | 56        | 14     |
| Nannochloropsis | 32            | 27        | 15     |
| Chlorella       | 17            | 58        | 22     |

¹ From the most external to the most internal layer. Adapted from [52,53].

Environmental and operational factors can significantly affect microalgae cultivation and anaerobic digestion kinetics, diminishing biomass and biogas productivity. Light intensity, nutrient availability, temperature, pH, turbulence, and salinity are key factors influencing microalgae cultivation and biomass production [54]. Biogas production during anaerobic digestion is affected by the C/N ratio, total solids, hydraulic retention time (HRT), volumetric organic load, pH, and temperature, among other factors [55].

The risk of inhibition by ammonia-nitrogen is also a limitation that should be considered in the anaerobic digestion of microalgae. This type of biomass often has a low-C/N ratio due to its high-protein content, which may be an issue since high-ammonia concentrations inhibit anaerobic microorganisms. Even when strategies to enhance microalgae digestibility are applied, the ammonia-nitrogen release may occur during the degradation of nitrogen compounds [56,57].

In addition to theoretical calculations, lab-scale and pilot-scale experiments have demonstrated the excellent potential of microalgae anaerobic digestion for methane production, obtaining specific yields higher than from other biomasses. Mussnug et al. [40] achieved methane yield from microalgae biomass 7–13% higher than from corn silage. The first study about the anaerobic digestion of microalgae biomass, conducted by Gouleke et al. [30], reached methane yields of 0.17–0.32 L CH₄/g volatile solids (VS) from Chlorella vulgaris and Scenedesmus. Studying the anaerobic digestion of two microalgae species, Phaeodactylum tricornutum and Scenedesmus obliquus, Zamalloa et al. [58] obtained methane yields of 0.36 and 0.24 L CH₄/g VS, respectively. According to Song et al. [59], the methane yields of brown, red, and green macroalgae are 0.204–0.380 L CH₄/g VS, 0.132–0.447 L CH₄/g VS, and 0.152–0.330 L CH₄/g VS, respectively, which are significantly higher than methane yields from sugar crops (0.189 ± 0.072 L CH₄/g VS) and lignocellulosic biomass (0.172 ± 0.113 L CH₄/g VS). Methane production may vary depending on algae habitat. When studying 15 species of freshwater microalgae and five species of marine microalgae, Frigon et al. [60] reported methane yields of 0.298 ± 0.083 L/g VS for marine microalgae and 0.329 ± 0.043 L/g VS for freshwater microalgae.

Table 2 shows methane yield results from the mesophilic anaerobic digestion of different microalgae species. Note that the methane yields vary greatly depending on the species, due to the chemical composition of the microalgae and its cell wall robustness. Species that lack a cell wall, e.g., Dunaliella salina, usually have higher yields. Such species have a cell membrane constituted only by a lipidic double-layer and easily degradable components [52]. Other species like Chlamydomonas reinhardtii have a glycoprotein cell wall, which is also readily degradable, thus, leading to satisfactory methane yields [61]. Nevertheless, most species have a robust cell wall, with many layers of cellulose, hemicellulose, algaenan, pectin, and peptide-glycan, among other constituents, as is the case of Chlorella sp. [15,52].
Such polymers make up resistant cell walls, hindering access to the intracellular material, therefore, hampering methane production. Algaenan, for example, is an extremely resistant aliphatic polymer that can be found in the cell wall of most microalgal species. Polymeric chain size and structure vary according to the species, ranging from 30 to as many as 120 carbons, as is the case for *Nannochloropsis* sp. and *Scenedesmus communis* [52].

It is still unknown which species from fresh or marine water are more suitable for methane production. Despite the recent developments regarding methane production from algal biomass, screening of strains is still necessary to identify which ones combine the most desirable characteristics: easy cultivation, high-biomass productivity, and easy anaerobic biodegradation. Given the results in Table 2, the selection of ideal strains is one of the most important tasks for biogas production from algal biomass, since species composition directly influences methane production.

Although the microalgal cell wall is an important feature that directly influences anaerobic digestion, the literature still lacks information about cell wall chemical composition and structure for most species. The cell wall is different from one species to another, especially its constituents, inter- and intramolecular bonds, number of layers, and thickness [62]. The chemical nature of the cell wall is an important feature when choosing the right biomass for a biotechnological process because, to access the intracellular content and convert it into biogas, the cell wall should be easily broken to minimize the costs, time, and energy required [63].
Table 2. Methane yields from mesophilic anaerobic digestion of different microalgae species.

| Algae Species                                                                 | Anaerobic Digestion Process Conditions                      | Methane Yield (mL CH₄/g VS) | Reference |
|-------------------------------------------------------------------------------|-------------------------------------------------------------|-----------------------------|-----------|
| Algae biomass: *Chlorella* sp. and *Scenedesmus* sp.                         | Batch reactor, 35 °C, 105 days                              | 260                         | [42]      |
| Algae biomass: *Scenedesmus obliquus*, *Chlorella vulgaris*                  | Batch reactor, 35 °C, 46 days                               | 117                         | [64]      |
| Algae biomass: *Stigeoclonium* sp., *Monoraphidium* sp., *Nitzschia* sp., *Amphora* sp. | Continuous reactor, 37 °C, 20 days, OLR 1.05 kg COD/m³·d  | 180                         | [65]      |
| *Arthrospira maxima*                                                          | ADMFC, 35 °C, 4 days, OLR 0.5 kg DW/m³·d                   | 173                         | [66]      |
| *Arthrospira platensis*                                                       |                                                             | 293                         |           |
| *Chlorella kessleri*                                                          |                                                             | 218                         |           |
| *Chlamydomonas reinhardtii*                                                    |                                                             | 387                         |           |
| *Dunaliella salina*                                                           | Batch reactor, 38 °C, 32 days                               | 323                         | [40]      |
| *Euglena gracilis*                                                            |                                                             | 325                         |           |
| *Scenedesmus obliquus*                                                        |                                                             | 178                         |           |
| *Botryococcus braunii*                                                        |                                                             | 343–370                     |           |
| *Chlamydomonas* sp.                                                           |                                                             | 333                         |           |
| *Isochrysis* sp.                                                              | Batch reactor, 35 °C, 34–50 days                            | 408                         | [60]      |
| *Scenedesmus dimorphus*                                                       |                                                             | 397                         |           |
| *Chlamydomonas reinhardtii*                                                    |                                                             | 263                         |           |
| *Chlorella vulgaris*                                                           | Batch reactor, 35 °C, 22 days                               | 191                         | [61]      |
| *Chlorella*                                                                   | Batch reactor, 37 °C, 45 days                               | 123                         | [67]      |
| *Chlorella minutissima*                                                        |                                                             | 166                         |           |
| *Chlorella pyrenoidosa*                                                       | Batch reactor, 36 °C, 30 days                               | 265                         | [68]      |
| *Chlorella vulgaris*                                                           |                                                             | 196                         |           |
| *Chlorella sorokiniana*                                                       | Batch reactor, 30 °C, 42 days                               | 220–280                     | [69]      |
| *Chlorella sorokiniana*                                                       | Bath reactor, 40 °C, 71–108 days                            | 212                         | [70]      |
| *Chlorella vulgaris*                                                           | Batch reactor, 35 °C, 28 days                               | 240                         | [71]      |
| *Chlorella vulgaris*                                                           |                                                             | 237                         |           |
| *Chlorella vulgaris* after lipid extraction                                   |                                                             | 219                         | [43]      |
| *Chlorella vulgaris* after chlorophyll extraction                             | Batch reactor, 35 °C, 35 days                               | 210                         |           |
| *Chlorella vulgaris* after protein extraction                                 |                                                             | 207                         |           |
| *Microcystis* spp.                                                            |                                                             | 337                         |           |
| *Nannochloropsis salina*                                                      |                                                             | 557                         | [41]      |
| *Nannochloropsis* sp.                                                         | Bath reactor, 35 °C, 30 days                                | 357                         |           |
| *Nanofrustulum* sp.                                                           |                                                             | 507                         |           |
| *Phaeodactylum tricornutum*                                                    |                                                             | 337                         |           |
Table 2. Cont.

| Algae Species                          | Anaerobic Digestion Process Conditions | Methane Yield (mL CH$_4$/g VS) | Reference |
|----------------------------------------|---------------------------------------|----------------------------------|-----------|
| *Chlorella vulgaris*                   | Batch reactor, 35 °C, 29 days          | 156                              | [72]      |
| *Chlorella vulgaris*                   | Batch reactor, 35 °C, 25 days          | 229                              | [73]      |
| *Chlorella vulgaris*                   | Batch reactor, 35 °C, 30 days          | 139                              | [74]      |
| *Hydrodictyon reticulatum* (filamentous algae) | Batch reactor, 35 °C, 25 days          | 170                              | [75]      |
| *Microcystis* spp.                     | Batch reactor, 35 °C, 30 days          | 140                              | [76]      |
| *Nannochloropsis salina*              | Batch reactor, 35 °C, 19 days          | 430                              | [77]      |
| *Phaeodactylum tricornutum*            | Batch reactor, 33 °C, 30 days          | 270                              | [58]      |
| *Scenedesmus obliquus*                 | Batch reactor, 35 °C, 33–34 days       | 130                              |          |
| *Scenedesmus obliquus*                 | Batch reactor, 35 °C, 33–34 days       | 76 $^1$                          | [58]      |
| *Scenedesmus sp.*                      | Batch reactor, 35 °C, 33–34 days       | 82 $^1$                          | [78]      |
| *Scenedesmus obtusiusculus*            | Batch reactor, 37 °C, 15 days, pH 9 (previously adapted inoculum) | 155                              | [44]      |
| *Scenedesmus sp.* and *Chlorella* spp.* | Batch reactor, 35 °C, 10 days          | 100–140                          | [24]      |

$^1$ mL CH$_4$/g COD$_{in}$. ADMFC = anaerobic digester with an integrated recirculation loop microbial fuel cell. COD = chemical oxygen demand. DW = dry weight. OLR = organic loading rate. VS = volatile solids.
4. Algal Biomass Pretreatment Methods

Rigid cell walls mean less available organic matter for the anaerobic microorganisms to convert; thus, making hydrolysis the limiting step of anaerobic digestion [79]. A growing interest in biofuels from microalgae and cyanobacteria has led to an increasing number of investigations about different pretreatment methods (Figure 1) to enhance methane yield from algal biomass [15,16,18,80].

Figure 1. The main algal biomass pretreatment methods aimed at methane production.

4.1. Mechanical Pretreatment

Mechanical pretreatment includes physical methods aimed at size reduction (milling) or cell wall disruption by provoking physical damage (ultrasound, microwave), the latter being more commonly used for microalgae biomass. Ultrasound pretreatment relies on the cavitation produced by soundwaves to break the cell wall from the inside out. Although it cannot break chemical bonds, microwaves can break hydrogen bonds of the macromolecules, altering their structures and ultimately damaging the cell wall [81,82].

In general, higher specific power or longer exposition time favors ultrasound and microwave pretreatment, leading to higher biomass solubilization and methane yield than untreated biomass (Table 3). For some species, pretreatment only promotes the rupture of the cell wall, facilitating the access of microorganisms to the intracellular material. Although the solubilization degree may seem low, enough intracellular content is released to make a significant difference between methane yields of treated and untreated biomass [62]. In other cases, biogas production seems to be linked to the specific energy applied during the pretreatment and the degree of biomass solubilization [64]. However, physical pretreatments are inefficient from the energy perspective, since the energy generated from anaerobic digestion often does not offset the energy demanded for the pretreatment step [80,83].
Table 3. Physical, thermal, and biological methods of microalgae pretreatment aimed at methane production.

| Code | Method                  | Species/Concentration                              | Pretreatment Conditions | Solubilization Increase (%) | Methane Yield (NmL CH\textsubscript{4}/g VS) | Reference |
|------|-------------------------|---------------------------------------------------|-------------------------|------------------------------|---------------------------------------------|-----------|
| U1   | Ultrasound              | *Chlorella sorokiniana*, 13.8 g/L COD\textsubscript{T} | 220 W, 30 min           | 28.5\textsuperscript{b}     | 458.43                                      | 44.3      |
| U2   | Ultrasound              |                                                    | 400 W, 20 min           | 33.3\textsuperscript{b}     | 414.12                                      | 30.4      |
| U3   | Ultrasound              |                                                    | 400 W, 30 min           | 43.2\textsuperscript{b}     | 424.68                                      | 33.7      |
| U4   | Ultrasound              |                                                    | 400 W, 40 min           | 56.8\textsuperscript{b}     | 421.87                                      | 32.8      |
| U5   | Ultrasound              | Mixed biomass (*Monoraphidium* sp., *Stigeoclonium* sp., *Nitzschia* sp., *Amphora* sp.) | 80 W, 30 min, 128.9 MJ/kg TS | 8.0 \* | 81.80 \textsuperscript{i} | 153.50 \textsuperscript{j} | 87.7 \textsuperscript{i} | [84] |
| U6   | Ultrasound              | *Stigeoclonium* sp., *Nitzschia* sp., *Amphora* sp.) | 70 W, 30 min           | 43.2\textsuperscript{b}     | 147.70                                      | 20.0      |
| U7   | Ultrasound              | *Scenedesmus* sp., 4.48 g/L TS                    | 300 W, 9 min, 64,400 kJ/kg TS | 7.5 \textsuperscript{d} | 167.24                                      | 60.1      |
| U8   | Ultrasound              | Mixed biomass (*Monoraphidium* sp., *Stigeoclonium* sp., *Nitzschia* sp., *Amphora* sp.) | 900 W, 3 min, 64,400 kJ/kg TS | 7.4 \textsuperscript{d} | 117.63                                      | 71.6      |
| T1   | Thermal                 | *Chlorella sorokiniana*, 13.8 g/L COD\textsubscript{T} | 80 °C, 20 min           | 6.6 \textsuperscript{b}     | 374.81                                      | 18.0      |
| T2   | Thermal                 | Microalgae mixed biomass from high-rate ponds, 17.9 g/L COD\textsubscript{T} | 55 °C, 10 h            | 402.0 \textsuperscript{e} | 154.57                                      | 40.0      |
| T3   | Thermal                 | Microalgae mixed biomass from high-rate ponds, 17.9 g/L COD\textsubscript{T} | 75 °C, 10 h            | 1058.0 \textsuperscript{e} | 169.88                                      | 53.0      |
| T4   | Thermal                 | *Scenedesmus* sp., 4.48 g/L TS                    | 95 °C, 10 h             | 1184 \textsuperscript{e}    | 128.7 \textsuperscript{j} | 57.0      |
| T5   | Thermal                 | Microalgae mixed biomass from high-rate ponds, 17.9 g/L COD\textsubscript{T} | 70 °C, 25 min           | 90.0 \textsuperscript{a}   | 89.3 \textsuperscript{j} | 9.0       |
| T6   | Thermal                 | *Scenedesmus* sp., 4.48 g/L TS                    | 80 °C, 25 min           | 130.0 \textsuperscript{a}   | 128.7 \textsuperscript{j} | 57.0      |
| T7   | Thermal                 | *Scenedesmus* sp., 14.0 g/L COD                   | 70 °C, 180 min          | na                           | 85.0 \textsuperscript{j} | 11.8      |
| T8   | Thermal                 | *Scenedesmus* sp., 14.0 g/L COD                   | 90 °C, 180 min          | na                           | 123.7 \textsuperscript{j} | 123.7     |
| T9   | Thermal                 | *Chlorella* sp., 27.9 g/L COD                     | 65 °C, 4 h              | 41.0                         | 297.00                                      | 41.0      |
| T10  | Thermal                 | Microalgae mixed biomass from high-rate ponds, 2.5% TS | 110 °C, 1, 2 bar, 15 min | 8.0 \textsuperscript{c} | 150.0                                       | 25.0      |
| T11  | Hydrothermal            | *Chlorella vulgaris*, 32.1 g/L COD\textsubscript{T} | 110 °C, 1, 2 bar, 30 min | 8.8 \textsuperscript{c} | 140.00                                      | 17.0      |
| T12  | Hydrothermal            |                                                    | 130 °C, 1, 7 bar, 15 min | 15.0 \textsuperscript{c}    | 170.00                                      | 42.0      |
| T13  | Hydrothermal            |                                                    | 130 °C, 1, 7 bar, 30 min | 13.3 \textsuperscript{c}    | 160.00                                      | 33.0      |
| T14  | Hydrothermal            |                                                    | 140 °C, 3 bar, 10 min, 350 rpm | 42.0 \textsuperscript{f} | 219.80 \textsuperscript{j} | 40.0      |
| T15  | Hydrothermal            |                                                    | 160 °C, 6 bar, 10 min, 350 rpm | 54.0 \textsuperscript{f} | 256.30 \textsuperscript{j} | 64.0      |
| T16  | Hydrothermal            |                                                    | 180 °C, 10 bar, 10 min, 350 rpm | 69.0 \textsuperscript{f} | 226.50 \textsuperscript{j} | 45.0      |
### Table 3. Cont.

| Code | Method     | Species/Concentration | Pretreatment Conditions | Solubilization Increase (%) | Methane Yield (NmL CH₄/g VS) Without PT | Methane Yield (NmL CH₄/g VS) With PT | Variation (%) | Reference |
|------|------------|------------------------|-------------------------|-----------------------------|----------------------------------------|--------------------------------------|---------------|-----------|
| T17  | Hydrothermal | *Scenedesmus obliquus*, 20 g/L TS | 165 °C, 7 bar, 30 min | 40.0¹ | 159.00 | 383.60 | 141.3 | [88] |
| T18  | Hydrothermal (solar powered) | *Chlorella pyrenoidosa*, 0.961 g TS/g DW | 155 °C, 30 min, flow rate 40 L/h (semi-continuous) | na | 221.70 | 348.00 | 57.0 | [89] |
| E1   | Enzymatic | *Scenedesmus obliquus*, 25 mg sCOD/g TS | Cellulase + endogalactouronase mix, 17.5 U/g TS, 50 °C, 24 h | organic matter solubilization between 35.0% and 45.0%¹ | 159.00 | 897.75 | 464.6 | [88] |
| E2   | Enzymatic | *Scenedesmus obliquus*, 25 mg sCOD/g TS | Estearase + protease mix, 12.5 U/g TS, 50 °C, 24 h | 159.00 | 617.70 | 288.5 | 214.6 | [88] |
| E3   | Enzymatic | *Chlorella sorokiniana*, 95 mg sCOD/g TS | Cellulase + endogalactouronase mix, 17.5 U/g TS, 50 °C, 24 h | 169.30 | 532.68 | 214.6 | 187.1 |                     |
| E4   | Enzymatic | *Chlorella sorokiniana*, 95 mg sCOD/g TS | Estearase + protease mix, 7.5 U/g TS, 50 °C, 24 h | 169.30 | 486.08 | 187.1 | 187.1 |                     |
| E5   | Enzymatic | *Arthrospira maxima*, 300 mg sCOD/g TS | Cellulase + endogalactouronase mix, 12.5 U/g TS, 50 °C, 24 h | 111.00 | 879.60 | 214.6 | 187.1 |                     |
| E6   | Enzymatic | *Arthrospira maxima*, 300 mg sCOD/g TS | Estearase + protease mix, 7.5 U/g TS, 50 °C, 24 h | 111.00 | 927.00 | 214.6 | 187.1 |                     |
| E7   | Enzymatic | *Porphyridium cruentum*, 3.4 g COD₄/L | Protease 0.5 mL/g dry biomass, pH 8.0–8.5, 55 °C, 9 h | 32.2¹ | 130.00 | 230.00 | 77.0 | [90] |
| E8   | Enzymatic |   | Enzyme commercial cocktail, 1% E/S, pH 4.8, 24 h | 42.0¹ | 430.80 | 35.6 |                     |                     |
| E9   | Enzymatic |   | Enzyme commercial cocktail, 1% E/S, pH 4.8, 24 h | 48.0¹ | 317.66 | 38.7 |                     | [91] |
| E10  | Enzymatic | *Chlorella sorokiniana* | Commercial cellulase, 1% E/S, pH 4.8, 24 h | 36.0¹ | 552.66 | 74.0 |                     |                     |
| E11  | Enzymatic |   | Commercial cellulase, 1% E/S, pH 7.0, 24 h | 34.0¹ | 545.68 | 71.0 |                     |                     |
| E12  | Enzymatic |   | Enzyme commercial cocktail, 1% E/S, pH 7.0, 24 h | 50.0¹ | 570.84 | 70.0 |                     |                     |
Table 3. Cont.

| Code | Method | Species/Concentration | Pretreatment Conditions | Solubilization Increase (%) | Methane Yield (NmL CH₄/g VS) | Reference |
|------|--------|-----------------------|-------------------------|------------------------------|-------------------------------|------------|
|      |        |                       |                         | Without PT | With PT | Variation (%) |
| E13  | Biological | Mixed culture of bacteria and microalgae, composed mainly by *Oocystis* sp., 31.3 g/L CODₜ | 100 U/L laccase-rich broth from *Trametes versicolor*, 100 rpm / 20 min | na | 83.0 | 144.00 | 20.0 | [92] |
| E14  | 100 U/L commercial laccase, 20 min, 100 rpm, 25 °C | 100.00 | 74.0 |
| E15  | Biological | *Scenedesmus* sp., 60.9 g/L CODₜ | pretreatment in the 1st stage, fermentation reactor: 40 d, SRT = 7 d, HRT = 7 d. | na | na | 214.00 | na | [93] |

*a S (%) = (sCODᵣ × 100)/(COD₀) [sCOD= soluble COD, 0 = before pretreatment, r = after pretreatment]. *b S (%) = (VSSᵣ × 100)/(VSS₀) [VSS = volatile suspended solids, 0 = before pretreatment, r = after pretreatment]. *c S (%) = (sVSᵣ – sVS₀)/(VSᵣ – sVS₀) × 100 [sVS = soluble volatile solids, VS = total volatile solids, 0 = before pretreatment, r = after pretreatment]. *d S (%) = (sVSᵣ)/VS [sVS = soluble volatile solids, VS = total volatile solids, r = after pretreatment]. *e S (%) = [(sVSSᵣ/VS) – (sVSS₀/VS)]/(sVSS₀/VS) × 100 [sVSS = soluble volatile suspended solids, 0 = before pretreatment, r = after pretreatment]. *f considering soluble carbohydrates. *g considering sCOD/g TS ratio. *h considering sCOD/g dry biomass ratio. *i S (%) = [(sVSSᵣ – VSS₀)/VSS₀] × 100 [sVSS = soluble volatile suspended solids, 0 = before pretreatment, r = after pretreatment]. *j NmL CH₄/g COD. na = not available, E/S = enzyme/substrate, TSAD = two-stage anaerobic digestion.
4.2. Thermal Pretreatment

In thermal pretreatment, biomass can be heated at temperatures ≤ 100 °C. When higher temperatures followed by a pressure increase are applied, it is considered a hydrothermal pretreatment [82]. Both time and temperature influence biomass solubilization and, in some cases, the temperature has more influence on thermal pretreatment, resulting in a positive correlation between biomass solubilization and methane yield (Table 3). On the one hand, high temperatures solubilize organic matter and partially hydrolyze macromolecules such as carbohydrates, lipids, and proteins. On the other hand, they may also favor the formation of inhibitory chemicals affecting the metabolism of anaerobic microorganisms, especially ammonia [94]. Hence, many authors noticed a decrease in biomass digestibility when the microalgae were treated with harsh temperature and pressure in comparison to those treated with milder conditions [72]. Many studies have demonstrated that to increase methane production from microalgae biomass, pretreatment temperatures should range between 55 and 170 °C [83,95].

4.3. Biological Pretreatment

Biological pretreatment can be performed by adding enzymes or hydrolytic microorganisms (e.g., bacteria and fungi) to the biomass under controlled temperature and pH conditions (Table 3). Cellulases are often used for pretreating species whose cell wall is composed of cellulose and hemicellulose, e.g., *Chlorella* sp. and *Chlorococcum* sp. [81]. Biological pretreatment does not release chemicals that are toxic or inhibitory for the anaerobic digestion microorganisms, which is an advantage over other pretreatment methods, such as thermal or chemical. However, it requires a long time, the enzymes are specific to a substrate, and the cost of enzyme production is still high [96]. The choice of an enzyme must consider the chemical composition of the microalgae and of its cell wall, requiring an investigation about which enzyme or a mix of enzymes is most suitable for a given microalgae species [7,96].

Biological pretreatment using enzymes generated locally, e.g., using a fungal broth, can be more efficient in improving methane yield than using single commercial enzymes. This is due to a synergistic effect between multiple enzymes secreted by the fungi and enzymes secreted by the hydrolytic bacteria that participate in anaerobic digestion [91,92].

Some authors consider the first stage (acidogenic phase) of the two-stage anaerobic digestion as a form of biological pretreatment since it is dominated by fermentative bacteria digesting the organic matter [97]. The fermentation step can be aided by adding external enzymes or hydrolytic microorganisms [93].

4.4. Chemical Pretreatment

Chemical pretreatment can be performed by adding an alkali, acid, or oxidizing agent (Table 4) over the biomass. The solubilization of cell wall components and intracellular organic matter (carbohydrates, lipids, and proteins) by chemical pretreatment has been extensively investigated, and the main factors affecting this pretreatment method are chemical concentration, biomass concentration, temperature, and time [52,98]. The relationship between those factors and biomass solubilization is discussed in Section 4.4.3.

Overall, the advantages of chemical pretreatment include a high-solubilization degree and low-energy demand. Disadvantages include the cost of chemicals, pH imbalance, inhibition of the methanogenic microorganisms due to the buildup of toxic ions and molecules released during pretreatment, and the corrosion of the reactors [82,99].

4.4.1. Acid Pretreatment

Acid pretreatment or acid hydrolysis is widely used for lignocellulosic biomass due to its efficiency in depolymerization of cellulose and hemicellulose, converting them into lower molecular weight sugars [97]. When pretreatment is performed using concentrated acid and/or high temperatures, the acid can react with solubilized sugars producing furfural
and 5-hidroxymethylfurfural $^{[96,100]}$. According to Ghasimi et al. $^{[101]}$, microbial growth and methanogenic activity during anaerobic digestion (mesophilic and thermophilic) are inhibited starting at 2 g/L of furfural or 5-hidroximetilfurfural. Acid hydrolysis can use either concentrated or diluted acids. Concentrated acid leads to more efficient carbohydrate solubilization, formation of toxic by-products, and equipment corrosion, thus, demanding more resistant construction materials $^{[82]}$.

4.4.2. Alkaline Pretreatment

Solubilization of organic matter by alkali addition (alkaline hydrolysis) occurs due to many reactions between the alkaline reagent and biomass components. In this process, saponification of uronic acids and esters occurs, as well as reactions involving carboxylic groups and the neutralization of acid intermediaries $^{[102]}$. Alkalis increase the biomass superficial area by swelling after saponification reactions $^{[42,103]}$, favoring the attack of the digesting microorganisms $^{[61]}$ and protein solubilization $^{[52,74]}$. Biomass solubilization achieves better results when monovalent alkalis are used instead of divalent ones since divalent alkalis ionize only partially in aqueous media $^{[102]}$.
Table 4. Chemical and thermochemical pretreatments of algal biomass for methane production.

| Code | Species/Concentration | Pretreatment Conditions | Solubilization $^a$ | Methane Yield (NmL CH$_4$/g VS) | References |
|------|-----------------------|-------------------------|---------------------|-------------------------------|------------|
|      |                       |                         | $S_f$ (%) | Variation (%) | Without PT | With PT | Variation (%) |                     |
| A1   | Scenedesmus sp., 6 g/L COD$_T$ | H$_2$SO$_4$ 0.1% v/v, 150 $^\circ$C, 1 h | 37.5 | 35.5 | 253.10 | 93.4 | [104] |
| A2   | Scenedesmus sp., 6 g/L COD$_T$ | H$_2$SO$_4$ 0.2% v/v, 150 $^\circ$C, 1 h | 36.9 | 34.6 | 130.90 | na | na |               |
| A3   | Scenedesmus sp., 6 g/L COD$_T$ | Bubbling CO$_2$ up to pH 2, 150 $^\circ$C, 1 h | 41.6 | 39.6 | na | na |               |
| A4   | Scenedesmus sp., 6 g/L COD$_T$ | HCl, pH 1, 50 $^\circ$C, 1 h | 53.9 | 87.5 | 0.0 | −100.0 |               |
| A5   | Scenedesmus sp., 6 g/L COD$_T$ | HCl, pH 1, 100 $^\circ$C, 1 h | 57.4 | 100.0 | 60.0 | −68.4 |               |
| A6   | Scenedesmus sp., 6 g/L COD$_T$ | HCl, pH 1, 150 $^\circ$C, 1 h | 68.2 | 137.5 | 20.0 | −89.5 |               |
| A7   | Scenedesmus sp., 6 g/L COD$_T$ | HCl, pH 3, 50 $^\circ$C, 1 h | 39.5 | 37.5 | 190.00 |               | [105] |
| A8   | Scenedesmus sp., 6 g/L COD$_T$ | HCl, pH 3, 100 $^\circ$C, 1 h | 44.9 | 56.3 | 140.0 | −26.3 |               |
| A9   | Scenedesmus sp., 6 g/L COD$_T$ | HCl, pH 3, 150 $^\circ$C, 1 h | 64.6 | 125.0 | 160.0 | −15.8 |               |
| A10  | Scenedesmus sp., 6 g/L COD$_T$ | HCl, pH 5, room temp. | 4.5 | 15.4 | 123.0 $^c$ | 18.3 |               |
| A11  | Microalgae biomass after lipid extraction, 10 g/L COD$_T$ | HCl, pH 4, room temp. | 7.2 | 84.6 | 107.0 $^c$ | 2.9 |               |
| A12  | Microalgae biomass after lipid extraction, 10 g/L COD$_T$ | HCl, pH 3, room temp. | 10.8 | 176.9 | 124.0 $^c$ | 19.2 | [106] |
| A13  | Microalgae biomass after lipid extraction, 10 g/L COD$_T$ | HCl, pH 2, room temp. | 18.2 | 366.7 | 174.0 $^c$ | 67.3 |               |
| A14  | Microalgae biomass after lipid extraction, 10 g/L COD$_T$ | HCl, pH 1, room temp. | 24.3 | 523.1 | 217.0 $^c$ | 108.7 |               |
| A15  | Mixed biomass of Chlorella sp. and Monoraphidium sp., 14.45 g/L TS | HCl 0.05% m/m, pH 1, 80 $^\circ$C, 2 h | na | 964.0 $^d$ | 142.50 | 82.4 |               |
| A16  | Mixed biomass of Chlorella sp. and Monoraphidium sp., 14.45 g/L TS | HCl 1.25% m/m, pH 0.6, 80 $^\circ$C, 2 h | na | 1008.0 $^d$ | 78.13 | 94.59 | 21.1 | [99] |
| A17  | Mixed biomass of Chlorella sp. and Monoraphidium sp., 14.45 g/L TS | HCl 2.0% m/m, pH 0.4, 80 $^\circ$C, 2 h | na | 965.8 $^d$ | 89.05 | 14.0 |               |
| A18  | Isochrysis galbana, 4.5 g/L COD$_T$ | H$_2$SO$_4$ 0.2% v/v, 40 $^\circ$C, 16 h | 8.4 | 27.1 | 15.90 | 71.5 |               |
| A19  | Chlorella vulgaris, 26.2 g/L COD$_T$ | H$_2$SO$_4$ 4M, pH 2, room temp. | na | na | 113.10 | −18.6 |               |
| A20  | Chlorella vulgaris, 26.2 g/L COD$_T$ | H$_2$SO$_4$ 4M, pH 2, 120 $^\circ$C, 20 min | na | 500 $^e$ | 221.80 | 59.7 |               | [74] |
| A21  | Chlorella vulgaris, 26.2 g/L COD$_T$ | H$_2$SO$_4$ 4M, pH 2, 120 $^\circ$C, 40 min | na | 700 $^e$ | 228.80 | 64.7 |               |
| A22  | Mixed biomass of Desmodesmus opoliensis (47%), Scenedesmus obtusiusculus (32.2 g/L COD$_T$ | HCl, pH 2, room temp., 1 h | na | na | 46.00 | 228.6 |               |
| A23  | Mixed biomass of Desmodesmus opoliensis (47%), Scenedesmus obtusiusculus (32.2 g/L COD$_T$ | HCl 3% m/m, 105 $^\circ$C, 1.7 h | 60.0 | 821.4 | 234.00 | 296.00 | 26.5 | [109] |
| A24  | Mixed biomass of Desmodesmus opoliensis (47%), Scenedesmus obtusiusculus (32.2 g/L COD$_T$ | H$_2$SO$_4$ 4M, pH 2; room temp. | 11.0 | 143.0 | 191.00 | 210.00 | 9.9 | [110] |
| A25  | Navicula reichardtiana (27%), Tetraselmis obliquus (12%), Scenedesmus sp. (9%), and Scenedesmus acuminatus (5%), 74.5% VS | HCl 0.5 M, pH 0.3, 121 $^\circ$C, 1 h | 40.0 | 135.3 | 216.00 | 205.20 | −5.0 | [111] |
| Code | Species/Concentration | Pretreatment Conditions | Solubilization $^a$ | Methane Yield (NmL CH$_4$/g VS) | References |
|------|------------------------|-------------------------|---------------------|---------------------------------|------------|
|      |                        |                         | $S_T$ (%)          | Without PT                      |            |
|      |                        |                         | Variation (%)      | With PT                          |            |
|      |                        |                         |                   | Variation (%)                    |            |
| B4   | *Spirulina maxima*, 55.7 g/L COD$_T$ | NaOH, pH 11, 50 °C, 1 h | 50.0               | 210.0                           | [105]      |
| B5   |                        | NaOH, pH 11, 100 °C, 1 h | 60.0               | 220.0                           |            |
| B6   |                        | NaOH, pH 11, 150 °C, 1 h | 60.0               | 240.0                           |            |
| B7   |                        | NaOH, pH 13, 50 °C, 1 h | 55.0               | 90.0                            |            |
| B8   |                        | NaOH, pH 13, 100 °C, 1 h | 70.0               | 140.0                           |            |
| B9   |                        | NaOH, pH 13, 150 °C, 1 h | 78.0               | 80.0                            |            |
| B10  | Mixed biomass,         | NaOH 5 g/L, pH 10, room temp. | 63.0               | 195.0 $^c$                      | [102]      |
| B11  | 56.2 g/L COD$_T$       | NaOH 20 g/L, pH 12, room temp. | 75.4               | 19.50 $^c$                      |            |
| B12  |                        | NaOH 20 g/L, pH 10, 140 °C, 30 min | 80.0               | 66.30 $^c$                      |            |
| B13  |                        | NaOH 20 g/L, pH 10, 140 °C, 30 min | 85.1               | 226.20 $^c$                     |            |
| B14  | *Chlorella*, 45.6 g/L COD$_T$ | NaOH 13.7 g/L, pH 13, room temp. | 20.0               | 12.48 $^c$                      |            |
| B15  | *Nannochloropsis*, 51 g/L COD$_T$ | NaOH 10.8 g/L, pH 14, room temp. | 30.0               | 10.0 $^{f}$.                    |            |
| B16  | *Thalassiosira weissflogii*, 46.1 g/L COD$_T$ | NaOH 20 g/L, pH 13, room temp. | 60.0               | 20.0 $^{f}$.                    |            |
| B17  | *Tetraselmis*, 42.2 g/L COD$_T$ | NaOH 7.8 g/L, pH 13, room temp. | 60.0               | 10.0 $^{f}$.                    |            |
| B18  | *Tetraselmis*, 42.2 g/L COD$_T$ | NaOH 20 g/L, pH 14, room temp. | 70.0               | 5.0 $^{f}$.                     |            |
| B19  | *Pavlova cf.*, 56.1 g/L COD$_T$ | NaOH 13.7 g/L, pH 12.5, room temp. | 70.0               | na $^f$                         | [7]        |
| B20  | *Chlorella*, 45.6 g/L COD$_T$ | NaOH 20 g/L, pH 13, 120 °C, 30 min | 80.0               | 84.8                            |            |
| B21  | *Nannochloropsis*, 51 g/L COD$_T$ | NaOH 20 g/L, pH 14, 120 °C, 30 min | 95.0               | 363.00                          | [112]      |
| B22  | *Thalassiosira weissflogii*, 46 g/L COD$_T$ | NaOH 20 g/L, pH 12, 120 °C, 30 min | 60.0               | 363.00                          |            |
| B23  | *Tetraselmis*, 42.2 g/L COD$_T$ | NaOH 6 g/L, pH 13, 120 °C, 30 min | 90.0               | 5.0 $^{f}$.                     |            |
| B24  | *Pavlova cf.*, 56.1 g/L COD$_T$ | NaOH 20 g/L, pH 13, 120 °C, 30 min | 90.0               | 363.00                          |            |
| B25  | *Chlorella*, (30%) and *Scenedesmus*, (70%) | NaOH, pH 11, room temp. | 5.4                | 336.00                          |            |
| B26  |                        | NaOH, pH 11, room temp. | 5.0                | 336.00                          | [112]      |
| B27  | 12.3 g/L COD$_T$       | NaOH, pH 13, room temp. | 2.0                | 324.5                           |            |
| B28  | *Nannochloropsis salina*, 97% TS | NaOH, pH 11, room temp. | na $^f$            | 213.00                          | [113]      |
| B29  |                        | NaOH 0.8 g/L, room temp., 12 h | 55.0               | 289.90                          |            |
| B30  | *Spirulina platensis*, 60.4 g/L COD$_T$ | NaOH 1.6 g/L, room temp., 12 h | 57.0               | 278.80                          | [114]      |
| B31  |                        | NaOH 4.0 g/L, room temp., 12 h | 20.0               | 297.50                          |            |
| B32  |                        | CaO 4% m/m, pH 12, room temp., 4 d | 10.0               | 294.90                          |            |
| B33  |                        | CaO 4% m/m, pH 12; 55 °C, 24 h | 10.0               | 282.00                          |            |
| B34  | Mixed biomass of *Chlorella* and *Scenedesmus*, 23.5 g/L COD$_T$ | CaO 4% m/m, pH 12, 72 °C, 24 h | 15.0               | 255.00                          | [42]       |
| B35  |                        | CaO 10% m/m, pH 12, room temp., 4 d | 13.0               | 260.00                          |            |
| B36  |                        | CaO 10% m/m, pH 12, 55 °C, 24 h | 20.0               | 292.00                          |            |
| B37  |                        | CaO 10% m/m, pH 12, 72 °C, 24 h | 25.0               | 325.00                          |            |
### Table 4. Cont.

| Code | Species/Concentration | Pretreatment Conditions | Solubilization $^a$ | Methane Yield (NmL CH$_4$/g VS) References |
|------|----------------------|-------------------------|---------------------|------------------------------------------------|
|      |                      |                         | $S_f$ (%) | Variation (%) | Without PT | With PT | Variation (%) | |
| B38  | **Chlorella sp.**    | NaOH 0.05%, 50 °C, 24 h | 15.8     | 139.4        | 110.00 $^c$ | 101.20 $^c$ | 0.80 $^c$ |
| B39  |                     | NaOH 2.0%, 50 °C, 24 h  | 16.2     | 145.5        | 125.00 $^c$ | 115.00 $^c$ | 8.90 $^c$ |
| B40  |                     | NaOH 5.0%, 50 °C, 24 h  | 18.2     | 175.8        | 155.00 $^c$ | 150.00 $^c$ | 5.00 $^c$ |
| B41  |                     | NaOH 0.05%, 50 °C, 48 h | 15.8     | 139.4        | 130.00 $^c$ | 115.00 $^c$ | 5.00 $^c$ |
| B42  |                     | NaOH 2.0%, 50 °C, 48 h  | 17.6     | 166.6        | 160.00 $^c$ | 145.00 $^c$ | 8.00 $^c$ |
| B43  |                     | NaOH 5.0%, 50 °C, 48 h  | 19.8     | 200.0        | 155.00 $^c$ | 135.00 $^c$ | 16.00 $^c$ |
| B44  |                     | NaOH 0.05%, 50 °C, 24 h | 4.1      | 115.8        | 150.00 $^c$ | 135.00 $^c$ | 11.00 $^c$ |
| B45  |                     | NaOH 2.0%, 50 °C, 24 h  | 5.1      | 168.4        | 165.00 $^c$ | 150.00 $^c$ | 22.00 $^c$ |
| B46  |                     | NaOH 5.0%, 50 °C, 24 h  | 16.8     | 784.2        | 135.00 $^c$ | 120.00 $^c$ | 20.00 $^c$ |
| B47  | **Scenedesmus sp.**  | NaOH 0.05%, 50 °C, 48 h | 4.7      | 147.4        | 146.00 $^c$ | 131.00 $^c$ | 8.50 $^c$ |
| B48  |                     | NaOH 2.0%, 50 °C, 48 h  | 7.8      | 310.5        | 140.00 $^c$ | 125.00 $^c$ | 9.00 $^c$ |
| B49  |                     | NaOH 5.0%, 50 °C, 48 h  | 17.7     | 669.6        | 138.00 $^c$ | 123.00 $^c$ | 2.20 $^c$ |
| B50  | Mixed biomass of **Chlorella sp.** and **Monoraphidium sp.**, 14.45 g/L COD, 2% w/w biomass/alkaline H$_2$O$_2$, 94.29 g TS/L | KOH 0.5%; pH 12.6, 80 °C, 2 h | na | 1943.5 $^d$ | 145.10 $^d$ | 86.00 $^d$ |
| B51  |                     | KOH 1.25%; pH 12.6, 80 °C, 2 h | na | 2043.5 $^d$ | 140.14 $^d$ | 80.78 $^d$ | 28.00 $^d$ |
| B52  |                     | KOH 2.0%; pH 12.6, 80 °C, 2 h | na | 2184.7 $^d$ | 138.00 $^c$ | 123.00 $^c$ | 2.20 $^c$ |
| B53  | **Scenedesmus obtusiusculus**, 14.45 g/L COD, 2% w/w biomass/alkaline H$_2$O$_2$, 94.29 g TS/L | 2% w/w biomass/alkaline H$_2$O$_2$, 50 °C, 1.5 h solution (1.25% v/v), 50 °C, 1.5 h | 15.4 | 100.0 $^a$ | 208.40 $^a$ | 34.50 $^a$ |
| B54  |                     | NaOH 4.0 M, pH 10, 120 °C, 20 min | 27.0 | 251.0 $^a$ | 227.10 $^a$ | 46.50 $^a$ |

$^a$ calculated for this study: $S_f$ = solubilization degree before and after pretreatment. $S_f$ (%) = (sCOD$_0$/(COD$_T$) × 100; $S_f$ (%) = (sCOD$_f$/(COD$_T$f) × 100; COD$_T$ = total COD (g/L); sCOD$_0$ = soluble COD (g/L) before pretreatment; sCOD$_f$ = soluble COD (g/L) after pretreatment; Solubilization increase (%) = [(S$_0$/S$_f$) − 1] × 100. $^b$ Methane yield increase (%) = [(Y$_{CH_4}$-PT/Y$_{CH_4}$-B) − 1] × 100. $^c$ NmL CH$_4$/g COD. $^d$ considering total organic carbon (TOC) increase (%) after pretreatment. $^e$ considering soluble carbohydrates. $^f$ as informed by the original paper. na = not available.
4.4.3. Factors Influencing Algal Biomass Solubilization in Chemical and Thermochemical Pretreatments

- **Temperature**

Both acid and alkaline pretreatment have been less explored at room temperature, with most studies using temperatures ranging from around 50 to 100 °C and greater (Table 4). The combination of the temperature plus an acid or alkali allows pretreatments to occur under mild conditions, demanding less time and energy, which potentially lowers pretreatment costs. Such a combination leads to biomass solubilization greater than the ones achieved by chemical pretreatment at room temperature or even by thermal pretreatment alone [7,42,72,102].

Figure 2 shows the increase in biomass solubilization after chemical or thermochemical pretreatment using NaOH, compared to the solubilization of untreated biomass. The dot and whisker represent the mean and standard deviation of the solubilization increase obtained in several studies conducted with the species highlighted in the legend. To construct the figure, results from references [7,102,112,114] and [7,61,102,105], shown in Table 4, were used for chemical and thermochemical pretreatments with NaOH. The colors in the two columns represent the contribution share of the different microalgae species in calculating the mean and standard deviation. Thus, in the 14 chemical pretreatment studies, the most studied biomasses were *Spirulina platensis* (21.4% of the total) and a mixture of *Chlorella* sp. and *Scenedesmus* sp. (21.4%), followed by the biomasses of *Tetraselmis* sp. (14.3%) and unspecified mixed biomass (14.3%). While in the 24 thermochemical pretreatment studies, *Chlorella* sp. (29.2%), *Scenedesmus* sp. (25%), and *Spirulina maxima* (25%) were the most studied biomasses.

![](image)

**Figure 2.** Mean and standard deviation for solubilization increase in different microalgae species after chemical and thermochemical pretreatment with NaOH. The colors in the two columns represent the contribution share of the different microalgae species in calculating the mean and standard deviation. Data from references [7,61,102,105,112,114].

The increase in solubilization in the chemical pretreatment ranged from −1.2% (*Thalassiosira weissflogii*) to 325% (unspecified mixed biomass), with an average of 91.3 ± 105.4%. At the same time, the increase in solubilization in the thermochemical pretreatment ranged from 53.5% (*Pavlovia cf* sp.) to 784.2% (*Scenedesmus* sp.), with an average of 226.9 ± 183.3%. The
wide ranges of values are due to the different characteristics of the studied biomasses. The lowest values occur when the biomass contains species without a cell wall or a simple cell wall. In these cases, the alkaline pretreatment at room temperature becomes irrelevant to biomass solubilization. In contrast, the highest values are from biomasses containing species with a more rigid cell wall and a more significant alkaline pretreatment contribution to solubilization. The mean solubilization increase achieved at higher temperatures (thermochemical pretreatment) is 2.5-fold greater than the mean increase obtained by chemical pretreatment at room temperature. The difference between the averages is statistically significant (p-value of 0.006 in Student’s t-test with a 95% confidence interval).

Although pretreatments at higher temperatures lead to high solubilization, the hydrolysis of lipids and proteins in such conditions may form long-chain fatty acids (LCFA) and ammonia, both of which are inhibitors of methanogenic metabolism [94]. Higher temperatures also favor the formation of Maillard reactions between proteins, amino acids, and reducing sugars, thereby generating new complex molecules in the soluble phase. The presence of inhibitory and recalcitrant substances explains the lower digestibility of biomass treated under high temperatures, signaling the need to prioritize thermal pretreatments at mild temperatures [42].

• **Time reaction**

  High temperatures are beneficial for organic matter solubilization and reduce pretreatment time compared to pretreatment time at room temperature. Typically, temperatures greater than 100 °C led to satisfactory solubilization degrees within 20 min to 2 h (Table 4). Different microalgae species reached solubilizations ranging from 60 to 95% after 30 min of hydrolysis with NaOH (6–20 g/L) at 120 °C [7]. At mild temperatures, the pretreatment time seems to have little influence on the solubilization results. For instance, the mixed biomass composed of *Chlorella* and *Scenedesmus* demanded 4 days of pretreatment with 10% w/w CaO to achieve a solubilization degree of 13%, while 25% was achieved after 24 h of pretreatment at 72°C [42]. Pretreatment of *Chlorella* and *Scenedesmus* by applying 5% w/w NaOH at 50 °C led to similar solubilization results of 18.2 and 16.2%, respectively, after 24 h, and of 19.8 and 17.7% after 48 h, respectively [61].

• **Chemicals concentration**

  Regardless of the acid or alkali used in the pretreatment, some authors noticed that biomass solubilization increases only to a certain level, at which it either remains the same or decreases even when more concentrated chemicals are used. Penaud et al. [102] studied the alkaline pretreatment of mixed algal biomass at room temperature with different NaOH concentrations. When the alkali concentration was increased from 4.6 to 26.1 g/L, the biomass solubilization increased from 63 to 75.4%. Pretreatment of *Spirulina platensis* using 0.8 and 1.6 g/L NaOH performed by Du et al. [114] reached a final solubilization degree of 61% in both cases, corresponding to a 56% increase over untreated biomass. With 4.0 g/L NaOH, however, the degree of biomass solubilization remained identical to that of untreated biomass.

  Similar solubilization results were achieved by acid pretreatment of *Scenedesmus* using 0.1 and 0.2% v/v H₂SO₄ [104]. Acid pretreatments of mixed biomass containing *Chlorella* and *Monoraphidium* sp. with HCl concentrations ranging from 0.5 to 2.0% w/w resulted in increasing concentration of soluble sugars (glucose, xylose, arabinose) with the increase in HCl concentration [99]. Juárez et al. [111] investigated the thermochemical pretreatment of a mixed microalgal biomass (47% *Desmodesmus opoliensis*, 27% *Navicula reichardtiana*, 12% *Tetradesmus obliquus*, 9% *Scenedesmus* sp., and 5% *Scenedesmus acuminatus*) at 121°C with HCl for one hour. By increasing HCl concentration from 0.5 to 2.0 mol/L, the volatile solids concentration in the solid fraction of the hydrolyzed biomass decreased from 40 to 19%.

• **pH**

  Using solutions with different NaOH concentrations but the same pH (12), Penaud et al. [102] obtained very similar solubilization results, meaning that pH plays an important
role in alkaline pretreatment. Moreover, pH seems to be beneficial to biomass solubilization as it approaches high levels of acidity or alkalinity. Cho et al. [112] assessed the pretreatment of mixed algal biomass (Chlorella sp. and Scenedesmus sp.) using NaOH at room temperature. The solubilization degree reached around 5.0% after pretreatments at pH 9 and 11; whereas, at pH 13, a final solubilization of 25% was obtained. Passos et al. [99] investigated the pretreatment of mixed biomass of Chlorella and Monoraphidium sp. at 80 °C for 2 h, with HCl at three different pHs (1, 0.6, and 0.4). Their results showed that pH 0.6 led to the highest biomass solubilization.

- Cell wall composition

Bohutsky et al. [7] evaluated the chemical and thermochemical pretreatment of five microalgae species: Chlorella sp. and Nannochloropsis sp., both species have rigid cell walls; Thalassiosira weissflogii, with a silica cell wall; Tetraselmis sp., with a cell wall composed of glycoproteins; and Pavlova cf sp., which does not have a cell wall. All five species were treated at room temperature with NaOH concentrations up to 20 g/L. Biomass solubilization increased by 10 to 20% for Chlorella and Nannochloropsis sp. when 10.8 and 13.7 g/L NaOH was used. Such a low-solubilization increase was probably due to the complex cell walls of such species, making it harder for the alkali alone to depolymerize its constituents. Solubilization of Tetraselmis sp. doubled after pretreatment at room temperature using 20 g/L NaOH, while for the other species solubilization remained close to that of untreated biomass. In the case of species that lack cell walls (e.g., Pavlova cf) or have a simple cell wall (e.g., Thalassiosira weissflogii), organic matter is readily available, making alkali pretreatment at room temperature irrelevant to biomass solubilization. Thermochemical pretreatment, in turn, enhanced the solubilization of four of the five species. Chlorella sp. and Nannochloropsis sp. reached solubilization degrees of 80 and 95%, respectively, after pretreatment at 121 °C for 30 min with 20 g/L NaOH.

- Algal biomass concentration

There is a consensus that a concentration step before the anaerobic digestion of algal biomass (thus, before pretreatment as well) is crucial for the good performance of the anaerobic reactor. Overall, microalgae concentration in culture media is low, regardless of the cultivation method (open ponds or closed photobioreactors), meaning that only a small portion of the influent is actually organic matter available to be digested [115]. Furthermore, depending on the operational conditions of the anaerobic digester, processing a large volume of liquid may wash out the anaerobic microorganisms [116]. Most lab-scale experiments reported in the scientific literature use centrifugation for the harvesting and concentration of microalgal biomass. Nevertheless, in pilot-scale and large-scale, less energy-intensive and more economic methods must be chosen [115]. Nevertheless, only a few studies have investigated the synergistic effect between algal biomass concentration methods and pretreatment prior to anaerobic digestion.

Although concentrated biomass is better for anaerobic digestion, different works show different trends for algal biomass concentration and solubilization. Alzate et al. [117] studied the anaerobic digestion of three different microalgae mixtures (A, B, and C) using three different concentrations of each. According to them, when the biomass concentration decreased from 10 to 3 g TS/kg, methane yields decreased for all three mixtures. On the other hand, increasing biomass concentration from 10 to 20 g TS/kg decreased the methane yield for mixture A but had the opposite effect for microalgae mixtures B and C. In the work of Santos et al. [107], biomass concentration was the most significant factor \( p = 0.05 \) influencing solubilization. Thus, higher methane production was obtained by the anaerobic digestion of I. galbana at the maximum concentration (4500 mg COD/L) after thermochemical pretreatment with dilute acid. The statistical model presented by Rincón-Pérez et al. [109] showed that biomass concentration would have a negative effect on solubilization. However, the highest methane yield (296 mL CH\(_4\)/g VS\(_{in}\)) was achieved when the maximum microalgae concentration was used for anaerobic digestion (30 g TS/L).
4.4.4. Influence of Chemical and Thermochemical Pretreatments on Anaerobic Digestion of Algal Biomass

- Solubilization

Overall, methane yield increases as the organic matter available for anaerobic digestion increases. Table 4 presents some examples of improved methane yields after anaerobic digestion of chemically and thermochemically treated algal biomass [99,104,106,107]. Following this trend, higher solubilizations should lead to higher methane yields. However, the relationship between algal biomass solubilization and methane production is not well defined. Results reported in the literature show that for both chemical pretreatment at room temperature and thermochemical, methane yields seldom follow biomass solubilization trends, especially under harsh pretreatment conditions [7,105].

The less soluble the organic matter is, the lower the expected methane conversion is. However, after severe pretreatment (e.g., high-chemical concentration), hydrolysis is not the limiting step anymore. Thus, low-methane yields from treated biomass can be ascribed to the formation of complex molecules in the soluble phase via Maillard reactions. High temperatures and extreme pH contribute to Maillard reactions, forming recalcitrant substances [42,102] that decrease overall biodegradability and methane yields, even though more organic matter is available after pretreatment. Inhibition due to sodium buildup was ruled out as the cause of low-methane yields in the experiments of Penaud et al. [102], because the lack of correlation between solubilization and methane yield was also observed for biomass pretreated with KOH, Mg(OH)\(_2\), and Ca(OH)\(_2\). In conclusion, often the pretreatment conditions that result in higher solubilizations are not the same that lead to the highest methane yield [61,99,112].

- Ammonia accumulation

Microalgae and cyanobacteria are known for their high-protein content. In the first stage of anaerobic digestion, hydrolytic bacteria secrete enzymes that hydrolyze proteins into amino acids, generating free ammonia and favoring a low-C/N ratio [7,118]. The same happens with chemical pretreatment due to the reactions between proteins and the chemical reagent, especially with alkalis promoting protein degradation [52].

Ammonia may inhibit anaerobic digestion for various reasons. For instance, it permeates easily through the cell membrane, changing the intracellular pH. Free ammonia buildup can also inhibit specific enzymes involved in methane production [118,119]. Traditionally, N-NH\(_3\) concentrations are considered inhibitory between 1500 and 3000 mg/L to methanogenic microorganisms at pH values higher than neutral, while concentrations over 3000 mg N-NH\(_3\)/L are toxic to methanogenic microorganisms regardless of pH [120]. However, recent studies have reported inhibition occurring at N-NH\(_3\) concentrations ranging from 1500 to 10,000 mg/L for mesophilic and thermophilic anaerobic digestion of a variety of substrates [121,122].

- Fatty acid accumulation

During anaerobic digestion, lipids are first hydrolyzed into long-chain fatty acids (LCFAs) by fermentative hydrolytic bacteria. Then, the acidogenic bacteria convert them into volatile fatty acids (VFAs), such as acetic, propanoic, and butanoic acids. In turn, VFAs are consumed by the acetogenic bacteria. Although they are not toxic to the methanogenic metabolism, accumulation of VFAs is a signal of imbalance and may acidify the reactor, lowering methane production. Long-chain fatty acids adsorb into the cell wall or cell membrane of Gram-negative bacteria, causing transport issues and slowing down bacterial activity [123]. At high concentrations, LCFAs inhibit both their degradation by acetogenic bacteria and the degradation of VFAs by methanogenic archaea. The higher the concentration of LCFAs in the reactor, the longer the lag phase before methane production starts and the lower the biogas production [113,124].

LCFA accumulation was observed in the anaerobic digestion of *Nannochloropsis salina* after alkaline pretreatment at pH 11, resulting in low concentrations of VFAs and a biogas without any methane. According to the authors, under alkaline conditions, there are
insufficient protons for the β-oxidation reaction performed by acetogenic organisms during the degradation of fatty acids. In addition, the high pH also strongly inhibits the growth of acetogenic and methanogenic organisms, suppressing the subsequent steps of anaerobic digestion [113].

- Salt accumulation

Inorganic salts are essential for microbial growth and cell metabolism. However, high- or even moderate-salt concentrations can be toxic or inhibitory [99,125]. Alkaline reagents used in chemical and thermochemical pretreatments or as pH neutralizers after acid pretreatments favor the accumulation of Na\(^+\) or K\(^+\) ions in the biomass. In addition, because salts adhere to the biomass surface, marine and halotolerant species often present higher salinity [116].

Sodium concentrations greater than 3 g/L are highly toxic to methanogenic microorganisms, while K\(^+\) concentrations between 2.5 and 4.5 g/L cause moderate inhibition and strong inhibition above 12 g/L [99]. Toxicity from salt accumulation could hamper biogas productivity when high-alkali concentrations are used to achieve high-biomass solubilization. Passos et al. [99], for instance, achieved a better methane yield treating biomass with less alkali (0.5% w/w KOH, 80 °C, 2 h) than with the biomass treated using 2% w/w KOH at the same temperature. The concentration of potassium ions was 2.9 and 12.8 g/L for 0.5% w/w KOH and 2% w/w, respectively, indicating the inhibitory effect on methanogenic microorganisms associated with K\(^+\) accumulation.

In the work of Santos et al. [107], pretreatment with HCl resulted in 34% decreased methane production from anaerobic digestion of the marine species *I. galbana*, compared to the untreated microalgae. The poor performance of anaerobic digestion after pretreatment was ascribed to Na\(^+\) buildup on the biomass since the sodium concentration was 3.6 g/L. This high concentration was due to the use of NaOH to neutralize pH after acid pretreatment. By neutralizing the pH with Ca(OH)\(_2\) and adding a washing step after pretreatment, the sodium concentration in the biomass dropped to 65 mg/L and the methane yield increased by 71.5% compared to the digestion of untreated biomass.

5. Comparative Analysis of Chemical and Thermochemical Pretreatments Methods for Algal Biomass Aimed at Methane Production

To determine which conditions actually benefit methane production from the anaerobic digestion of algal biomass, a comparative analysis between chemical and thermochemical pretreatment methods was performed using the data from Table 4. Of all 78 methods listed, the ones using NaOH and HCl were preselected considering they are widely employed in the literature. To assess the impact of pretreatment conditions on anaerobic digestion, the percentual variation in biomass solubilization degree (S\(_p\)) and methane yield (Y\(_p\)) were calculated based on the results for treated and untreated biomass. Then, a screening was performed adopting the following criteria: (i) solubilization increase \(\geq 50\%\) and (ii) a positive variation in methane yield. The screening resulted in 28 pretreatment conditions, half of them being thermochemical using sodium hydroxide. Finally, the correlation between the pretreatment conditions (chemical concentration, temperature, and time) and the variables S\(_p\) and Y\(_p\) was analyzed within four pretreatment categories: (a) chemical using NaOH; (b) thermochemical using NaOH; (c) chemical using HCl; and (d) thermochemical using HCl.

From all chemical pretreatments using NaOH presented in Table 4, only five (B10, B18, B25, B29, and B30) showed a positive correlation between biomass solubilization increase and increased methane yield. Nevertheless, apart from B10, the increase in methane yield was lower than 10%, meaning that alkaline pretreatment at room temperature has little influence on the final yield of anaerobic digestion. This is probably due to the high-alkali concentrations needed to raise pH, leading to a Na\(^+\) buildup and inhibition of methanogenic microorganisms [102,125]. Indeed, a negative effect of chemical concentration on methane production is observed. It is common to use NaOH concentrations up to 20 g/L, especially
to reach $pH \geq 12$, rendering NaOH pretreatments that are unfavorable for algal biomass conversion into biogas.

Almost all thermochemical pretreatments using NaOH listed in Table 4 allowed for a rise in biomass solubilization $\geq 50\%$. Nonetheless, only eight of them (B4–B6, B12, B20, B21, B45, B46) allowed for a positive variation in methane productivity, but no correlation was found between increased solubilization and improved methane production. As discussed earlier, biomass pretreatment plays an important role in organic matter solubilization; but, apart from biological pretreatment, most methods are not selective. Both temperature and reagent can break down a large number of molecules that make up the cell wall and other biomolecules; however, there is no way to control or predict which substances will be formed. Although they are in the soluble phase, recalcitrant components will not be used by the microorganisms, so there is no guarantee that every solubilized organic molecule will be converted to methane. Still, for thermochemical pretreatment with NaOH, no correlation was found between alkali concentration and methane yield. However, temperature provides a positive effect on methane yield. Temperature can break hydrogen links between molecules, denature proteins, and depolymerize carbohydrates. Compared to other methods, thermal pretreatment alone has proven to be very efficient for anaerobic digestion, leading to methane yields similar or even superior to those obtained by physical or chemical pretreatments [112]. Time, unlike temperature, has a negative effect on methane yield, probably due to the formation of recalcitrant components during lengthy pretreatments.

Data about the acid pretreatment of algal biomass at room temperature for methane production are still scarce. Sposob et al. [106] attained a direct correlation between biomass solubilization and methane production when testing chemical pretreatment using HCl. Lower pH led to higher solubilization and higher yields. Nevertheless, the correlation found in this study cannot be generalized for other algal species or pretreatment conditions different from those investigated by the authors.

The majority of thermochemical pretreatments using HCl listed in Table 4 attained solubilizations over 50%, but only five of them (A15–A17, A23, and A27) raise the methane yield above 20%. There is no correlation between solubilization increase and the gain in methane yield from pretreated biomass. The reagent concentration has a strong negative influence on methane production. Carbohydrates broken down by a concentrated acid may generate 5-hidroximetilfurfural and furfural; both inhibit the anaerobic microorganisms and impair methane production [100,101]. Regarding pretreatment time and temperature, mild temperatures and a longer duration seem to favor anaerobic digestion. The strategy chosen by Passos et al. [99] is a good example of the best conditions for thermochemical pretreatment with HCl: diluted reagent (0.5% w/w), a long time (2 h), and mild temperature (80 °C), resulting in a methane yield 82% higher than untreated biomass.

The dataset analyzed herein confirms the trends found in the scientific literature regarding the lack of correlation between biomass solubilization and methane production from algal biomass. Biomass degradability is clearly governed by multiple factors, beyond those mostly investigated so far. In the case of chemical and thermochemical pretreatments, this indicates the need to continue exploring alternative reagents and experimental conditions. For instance, alkali pretreatment is a traditional choice for many biomasses, but it has little effect on algal biomass. Assessing pretreatment conditions based on their effects on methane yields prioritizes the best anaerobic digestion results, aiding in the choice of methods that actually benefit energy production rather than focusing on maximizing biomass solubilization. Moreover, the diversity of algal biomass chemical composition makes some pretreatment conditions effective for a particular species and indifferent to another. Therefore, identifying and choosing species that are more susceptible to pretreatment can also be a way to optimize methane production.

Table 5 shows the combined severity factors for thermochemical pretreatments selected for the comparative analysis. The severity factor is a concept introduced by Overend and Chornet [126] and was originally associated with steam explosion pretreatment of
lignocellulosic biomass. According to them, the hemicellulose conversion into a xylose reaction follows first-order kinetics in which the kinetic rate constant \((k)\) follows the Arrhenius Law. \(R_0\) is a factor known as the reaction order and combines the effects of time and temperature of the steam explosion pretreatment. The severity factor is given by \(\log R_0\). Since the steam explosion can be aided by an acid catalyst such as HCl or H\(_2\)SO\(_4\), a combined severity factor (CS) was proposed by Chum et al. [127] to account for the effect of the catalyst.

### Table 5. Thermochemical pretreatment conditions and their associated combined severity factors.

| Pretreatment | Temperature (°C) | Time (min) | pH | \(R_0\) | \(\log R_0\) | \(\log CS\) |
|--------------|------------------|------------|----|--------|-------------|------------|
| A1           | 150              | 60         | 2  | 1779.66| 3.25        | 1.25       |
| A15          | 80               | 2          | 1  | 30.92  | 1.49        | 0.49       |
| A16          | 80               | 2          | 0.6| 30.92  | 1.49        | 0.89       |
| A17          | 80               | 2          | 0.4| 30.92  | 1.49        | 1.09       |
| A18          | 40               | 960        | 1.1| 16.43  | 1.22        | 0.12       |
| A23          | 105              | 102        | 1.1| 143.16 | 2.16        | 1.06       |
| A27          | 121              | 60         | 0.3| 249.15 | 2.4         | 2.1        |
| B4           | 50               | 60         | 3  | 2.02   | 0.31        | −2.69      |
| B5           | 100              | 60         | 3  | 60     | 1.78        | −1.22      |
| B6           | 150              | 60         | 3  | 1779.66| 3.25        | 0.25       |
| B12          | 140              | 30         | 4  | 451.72 | 2.65        | −1.35      |
| B20          | 120              | 30         | 1  | 116.41 | 2.07        | 1.07       |
| B21          | 120              | 30         | 0  | 116.41 | 2.07        | 2.07       |
| B24          | 120              | 30         | 1  | 116.41 | 2.07        | 1.07       |

\[ R_0 = t \times \exp\left(\frac{T_h - T_{ref}}{14.75}\right) \] [126]. where \(t\) = pretreatment time (min), \(T_{ref}\) = reference temperature (100 °C), \(T_h\) = pretreatment temperature (°C), 14.75 = constant factor associated with the hydrolysis activation energy. CS—combined severity: \(\log CS = \log R_0 - pH\) [127]. For alkaline pretreatment, pH is replaced by pOH.

Negative CS (Table 5) was ascribed to non-severe pretreatments whenever low temperatures, pH, or duration time were used. Figure 3 shows the relationship between combined severity and the variation in biomass solubilization (\(S_p\)) and methane yield (\(Y_p\)). For acid pretreatment, higher solubilizations are associated with CS between 0.5 and 1.5, while the highest methane improvement happens at lower severities (Figure 3a). For alkaline pretreatment, except for B24, severe conditions are better for improving both solubilization and methane yield (Figure 3b). In B24, pretreatment achieved no difference for the anaerobic digestion of Pavlova_c_f since this species lacks a rigid cell wall and only has a cell membrane [7]. Severe pretreatments (i.e., higher log CS) more efficiently break down polysaccharides, increasing the availability of organic matter for anaerobic digestion. However, extreme conditions can lead to the formation of inhibitory compounds, making more severe pretreatment ineffective for methane production [128].
Figure 3. Relationship between the combined severity factor of thermochemical pretreatments with HCl and NaOH, solubilization (S_p), and methane yield (Y_R). (a) Thermochemical pretreatment with acid (HCl). (b) Thermochemical pretreatment with alkali (NaOH).

6. Energy Assessment

Data from Tables 3 and 4 were used to assess the energy efficiency of algal biomass pretreatment methods. When an energy balance was not provided by the original work, the energy required for biomass pretreatment was calculated from the available information, according to Equations (1) [129] and (2) [112]:

\[
E_{in} = \eta \Delta P_P \frac{V C S}{10^3}, \quad (3)
\]

where \(E_{in}\) (kJ/g VS or kJ/g COD) is the energy input required for raising the biomass temperature from room temperature \((T_0 = 25 ^\circ C)\) to pretreatment temperature \((T_p, ^\circ C)\), \(\rho\) is the density of the microalgae suspension (considered the same of pure water, 1.0 kg/L), \(V\) is biomass volume (1 L), \(\gamma\) is the specific heat of microalgae suspension (considered the same as pure water, 4.18 kJ/kg.°C), \(m_{VS}\) is the weight of biomass submitted to pretreatment (g VS or g COD), \(k\) is the heat transfer coefficient (1.0 W/m².°C) [88,129,130], \(t\) is the pretreatment duration time (min), and \(A\) is the surface area (0.061 m²) of a vertical cylindrical reactor with a total useful volume of 1.5 L and height/diameter ratio equals 2 [131].

\[
E_{in} = \eta \Delta P_P \frac{V R P_e}{C_S V}, \quad (2)
\]

where \(E_{in}\) (kJ/g VS or kJ/g COD) represents the energy cost of manufacturing the reagents for chemical and thermochemical pretreatments. \(V_R\) is the volume (L) or mass (g) of reagents used in pretreatment; \(Y_R\) is the cost of chemicals (0.16 USD/mL HCl or 0.11 USD/g NaOH); \(P_e\) converts the monetary cost into energy cost and is equivalent to the inverse of the energy price for the industrial sector (27,481 kJ/USD); \(C_S\) is the concentration of biomass suspension (g VS/L or g COD/L); and \(V\) (L) is the volume of biomass submitted to pretreatment. The price of reagents was based on a commercial supplier’s website in July 2022, and energy prices (0.131 USD/kWh) were based on the average global electricity cost in 2021 [132].

Equation (1) accounts for the energy necessary to elevate the microalgae temperature from room temperature to pretreatment temperature, and Equation (2) accounts for the energetic costs of chemicals. Thus, the energy demand for thermal pretreatment is given by Equation (1), and is calculated for thermochemical pretreatment by adding Equations (1) and (2). Energy generated by anaerobic digestion of treated biomass, \(E_{out}\), is given by Equation (3) [112,129]:

\[
E_{out} = \frac{\eta \Delta P_P^2}{10^3}, \quad (3)
\]
The most efficient condition for methane production (80 °C, 30 min) reached a net energy ratio of 2.7. Other physical methods (ultrasound, hydrothermal, and steam explosion) were explored in the same study [86] and, although they reached better methane yields, the energy demand was too high. Pretreatment of *Chlorella sp.* at 65 °C for 4 h [86] has the second best net energy ratio among thermal pretreatments in Table 3. Ometto et al. [88] also attained a positive energy balance for *Scenedesmus obliquus* hydrothermal pretreatment at 165 °C and 30 min. However, Passos et al. [85] demonstrated that even pretreatments at temperatures below 100°C do not achieve a positive energy balance if the biomass concentration is less than 2% TS, highlighting the importance of a biomass concentration step prior to pretreatment. Recently, to overcome the energy demand of thermal pretreatment, some authors investigated systems that use solar energy to heat biomass. Xiao et al. [89] proposed a solar-based system for hydrothermal pretreatment (155 °C, 30 min) of *Chlorella pyrenoidosa.*

*Figure 4.* Net energy ratio of different algal biomass pretreatment methods and methane yield increment ($Y_p$).

Positive energy balance was achieved by thermal pretreatment of *Chlorella sorokiniana.* The most efficient condition for methane production (80 °C, 30 min) reached a net energy ratio of 2.7. Other physical methods (ultrasound, hydrothermal, and steam explosion) were explored in the same study [80] and, although they reached better methane yields, the energy demand was too high. Pretreatment of *Chlorella sp.* at 65 °C for 4 h [86] has the second best net energy ratio among thermal pretreatments in Table 3. Ometto et al. [88] also attained a positive energy balance for *Scenedesmus obliquus* hydrothermal pretreatment at 165 °C and 30 min. However, Passos et al. [85] demonstrated that even pretreatments at temperatures below 100°C do not achieve a positive energy balance if the biomass concentration is less than 2% TS, highlighting the importance of a biomass concentration step prior to pretreatment. Recently, to overcome the energy demand of thermal pretreatment, some authors investigated systems that use solar energy to heat biomass. Xiao et al. [89] proposed a solar-based system for hydrothermal pretreatment (155 °C, 30 min) of *Chlorella pyrenoidosa.*

where $\eta$ is the methane recovery efficiency (0.9), $\Delta P$ is the increase in methane yield after pretreatment (NmL CH$_4$/g VS$_n$ or NmL CH$_4$/g COD$_{in}$) and $\xi$ is the lower heating value of methane (35.8 kJ/L CH$_4$) [133].

To be economically feasible, the net energy ratio ($NER = E_{out}/E_{in}$) must be higher than 1.0, meaning that the energy produced is enough to offset the energy demand. If $NER < 1.0$, energy needs to be supplemented by other sources (preferably renewable). However, this implies an extra investment in the process, making it less attractive from an economic point of view. Figure 4 shows the net energy ratio for many algal biomass pretreatment methods considering the increase in methane yield ($Y_p$) achieved by pretreatment. When log $NER > 0$, the energy balance is positive because more energy is produced than required; log $NER = 0$ means that all produced energy is consumed in the process, and when log $NER < 0$, the energy produced is not enough to offset the demand, leading to a negative energy balance.

Only enzymatic pretreatment and four thermal pretreatment conditions achieve log $NER > 0$. Biological pretreatment is frequently cited as less energy-intensive, as it does not require high temperatures or equipment with high-electricity consumption. In the works of Ometto et al. [88] (Table 3, E1 to E6) and Çakmak and Ugurlu [90] (Table 3, E7), this method showed a favorable energy balance due to significant increases in methane yield after enzymatic pretreatment. However, the high cost of commercial enzymes still prohibits the implementation of this method on a large scale [131]. An economic assessment performed by Çakmak and Ugurlu [90] revealed that the price of enzymes should range between 0.18 and 0.28 €/L to make the process economically feasible under the investigated conditions.
Energy generated from the anaerobic digestion of treated biomass was 3.64 times higher than the energy demand for pretreatment alone, and the net energy ratio for the whole process (pretreatment + anaerobic digestion) was 1.45. Similarly, Vassalle et al. [134] obtained a net energy ratio of 2.0 for the co-digestion of primary sludge and thermally pretreated algal biomass by using a solar-based system to heat the biomass.

Mechanical pretreatments are less attractive due to their high-energy consumption. In the work of Passos et al. [83], a neutral energy balance was achieved for ultrasound pretreatment only when biomass was concentrated in a previous step.

Many studies address the effects of chemical and thermochemical pretreatments in the anaerobic digestion of algal biomass, but only a few investigate the energy efficiency of such methods. Candia-Lomeli et al. [44] studied four methods for alkaline pretreatment of *Scenedesmus obtusiusculus*. Although the best solubilization occurred when using 15% CaO and H$_2$O$_2$ 2.5% v/v, anaerobic digestion was performed using biomass treated with less severe conditions to decrease the energy demand and overall process costs. Among the mild conditions tested, pretreatment with NaOH 4 M led to a 47% increase in methane yield compared to the untreated biomass, and when H$_2$O$_2$ 1.5% was used as alkali, a 34% increase was achieved. Moreover, the extra energy produced with treated biomass allowed for a 14.3% cost reduction. However, in their study, anaerobic digestion was performed in alkaline pH using an anaerobic sludge previously adapted to such conditions. That means that no pH adjustment was needed after pretreatment, reducing the demand for chemicals. In addition, an already adapted sludge may be more resilient to imbalances that otherwise could inhibit methanogenic microorganisms, as discussed in Section 4 (e.g., salt and free ammonia buildup).

Among all the pretreatment conditions selected for the comparative analysis, only eight of them contained enough information to determine the energy balance. None of the assessed chemical and thermochemical pretreatments achieved NER > 1.0. Even though chemical pretreatment is performed at room temperature, severe pH conditions are often required to maximize biomass solubilization [112,113], implying concentrated reagents that pose a risk of toxicity or inhibition for the anaerobic microorganisms. Thus, the increase in methane yield after alkaline pretreatment is often very low, and the energy produced is not enough to offset the energy demand. Thermochemical pretreatments assessed in this work were more efficient in enhancing methane production than chemical ones. Nonetheless, neither of them achieved a positive energy balance. These conclusions are in accordance with the work of Marques et al. [131], after assessing 24 pretreatment methods, they indicated that alkali pretreatment is the least efficient for algal biomass anaerobic digestion, and thermochemical pretreatment is not efficient from an energy perspective.

Anaerobic digestion of algal biomass is not proposed as a stand-alone system. Rather, it should be implemented as an integrated process within wastewater treatment plants and biorefineries. Within this context, pretreatment might become feasible by optimizing the other existing processes. Ometto et al. [135] investigated six different scenarios for a wastewater treatment plant using microalgae for nitrogen and phosphorus removal in a tertiary treatment. The plant consists of an activated sludge unit, a stabilization pond, a flotation unit (to separate microalgae biomass from the treated effluent), a hydrothermal pretreatment unit for microalgae biomass, and an anaerobic reactor. When the conventional flotation unit was replaced by a more efficient one, the energy produced by co-digestion of treated algal biomass with activated sludge corresponded to 76% of the energy demand of the entire process. In this case, the energy almost doubled compared to the scenario in which no pretreatment was applied, and it was 1.33 times greater than using a diluted air flotation unit [135]. Passos et al. [136] assessed the energy balance for a 1500 m$^3$/d wastewater treatment plant (WWTP) operating for a year. In a previous study, the same authors had determined that thermal pretreatment at 75 $^\circ$C for 1 h was the most efficient, resulting in a 44% increase in methane yield from algal biomass co-digestion with activated sludge. Thus, this condition was selected for the WWTP assessment. When all biogas produced was used to generate heat and electricity in a combined heat and power unit (CHP), pretreatment
was essential to guarantee the self-sufficiency of the WWTP throughout the year. Without algal biomass pretreatment, thermal energy from external sources should be supplied to the plant during the winter months [136].

Anaerobic digestion can be integrated into other biological processes, such as dark fermentation and photo-fermentation, to increase the energy recovery from microalgae. Phanduang et al. [137] employed the Chlorella sp. biomass as a substrate to produce hydrogen and methane through integrated dark fermentation (DF), photo-fermentation (PF), and anaerobic digestion (AD) processes. The total energy yield increased from 0.51 kJ/g VS (with DF alone) to 1.86 kJ/g VS (with integrated DF-PF), and 5.98 kJ/g VS (with integrated DF-AD). However, the integrated processes were still not energetically feasible due to the high energy demand of the acid-hydrothermal pre-treatment employed for biomass hydrolysis. Policastro et al. [138] also recommend the use of mixed cultures containing dark- and photo-fermentative bacteria, enriched with photosynthetic non-sulfur bacteria (PNSB), such as Rhodopseudomonas sp., to reduce costs and enhance the energy performance of integrated processes. According to the authors, dark-fermentative bacteria convert complex substrates to simple compounds, which can be assimilated by PNSB without high-cost pretreatments or two separate steps of dark- and photo-fermentation.

7. Conclusions

Although pretreatment with NaOH is widely used, its efficiency for algal biomass is debatable since most cases analyzed led to an increase in methane yield of less than 20%. In contrast, acid pretreatment at room temperature has only been minimally explored, and more studies are needed to evaluate its effects on the anaerobic digestion of algal biomass. No correlation was found between biomass solubilization and methane increment for chemical and thermochemical pretreatments using alkali or acid. The analyses suggest that thermochemical pretreatment with dilute acid, temperatures below 100 °C, and long duration should be preferred, resulting in less severe conditions. Only enzymatic pretreatment and, in some cases, thermal pretreatment result in a positive energy balance.

In summary, there is still room for improvement in algal biomass pretreatment methods aimed at anaerobic digestion. Features including the suitability of different microalgae species considering their chemical composition; novel reagents; combined pretreatment methods; less energy-intensive technologies, such as solar-powered devices; and process integration should be further explored.

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