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Review

A Review of Seaweed Pre-Treatment Methods for Enhanced Biofuel Production by Anaerobic Digestion or Fermentation

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Abstract: Macroalgae represent a potential biomass source for the production of bioethanol or biogas. Their use, however, is limited by several factors including, but not restricted to, their continuous supply for processing, and low biofuel yields. This review examines recent pre-treatment processes that have been used to improve the yields of either biogas or bioethanol from macroalgae. Factors that can influence hydrolysis efficiency and, consequently, biofuel yields, are highly affected by macroalgal composition, including content of salts, heavy metals, and polyphenols, structural make-up, as well as polysaccharide composition and relative content of carbohydrates. Other factors that can influence biofuel yield include the method of storage and preservation.

Keywords: macroalgae; bioethanol; biogas; hydrolysis

1. Introduction

In 2016, 29 million tonnes (wet-weight) of red, brown, and green macroalgae, also known as seaweeds, were harvested mainly for human food, animal feed, and production of hydrocolloids [1,2]. This represents a 39% increase since just 2014. It has also been accompanied by a commensurate surge in research interest in the use of both micro- and macro-algae as a source of biofuel, due to the high potential yields of these sources of biomass and growth systems that do not compete for agricultural land and fresh water [3–6]. *Saccharina japonica*, for example, was found to be 6.5 times more productive than sugarcane based on the maximum cultivated yields predicted in tonnes per hectare per year (wet-weight) [7].

Biofuel in the form of biogas from seaweed could help to reduce greenhouse gas (GHG) emissions by 42–82% compared to the use of natural gas [8,9], and contribute to achieving the EU targets by 2030: namely, total renewable energy share of 27% and reduced GHG emissions by 40% of the 1990 levels [10]. Moreover, the European Parliament has indicated that seaweed, as well as other types of waste, should contribute to at least 1.25% of energy consumption in the transportation sector by 2020 [11].

However, high water content (80–90%) of seaweed impacts negatively on the energy balance of applications that depend on dry biomass [6]. This makes seaweed undesirable for direct combustion, pyrolysis, and gasification, but suitable for processes that can produce net energy gain based on the use of wet biomass [12,13]. These methods include hydrothermal liquefaction for bio-oil production; fermentation for ethanol production, and anaerobic digestion (AD) for biogas production [14]. All three methods are still under development for the production of biofuel from seaweed [14]. Details on hydrothermal liquefaction of seaweed can be found in Milledge et al. (2014) [14] and Liu et al. (2013) [15].

This review will focus on fermentation and AD of seaweed, because seaweed has the potential to replace energy crops competing with food as a biofuel feedstock in the existing ethanol and biogas production infrastructures [16]. Gross energy yields derived from AD of *Saccharina latissimi*, for example, could reach up to 365 GJ per hectare per year [17], bearing similarities with biogas yields from maize (59–436 GJ per hectare per year), a widely used AD feedstock [18]. For fermentation and ethanol distillation, energy return on investments (EROI) for seaweed (0.44 to 1.37 in a highly efficient processing system of seaweed) can be similar to maize (1.07) [19]. In terms of their sustainability, the EnAlgae project concluded that seaweed could be cultivated with a comparable life cycle resource demand to several land plants [20]. Processes that use the entire biomass rather than just the fermentable sugars have more favourable EROIs [21] with seaweed biogas production having an EROI of 2.4, and combined production of biogas and bioethanol from seaweed having an EROI of 3.0 or greater, an EROI of 3 being the minimum value for a sustainable and viable processing system for fuel production [22]. However, the current practical yields of biogas from AD of seaweed can be as low as 79% below the theoretical maximum [23]. Low values below the theoretical maximum are also shown for ethanol yields [24].

Figure 1 provides a schematic of the biochemical processes involved in producing either biogas (Figure 1A) or bioethanol (Figure 1B). In both cases, the first stage of production requires hydrolysis of polysaccharides to sugars. In the case of biogas production, sugars from hydrolysed polysaccharides are converted to acetate, CO_2 and H_2 by natural microbial processes termed acidogenesis and acetogenesis, and thence to methane and CO_2 by methanogenesis [25]. For ethanol production, glucose and galactose from the hydrolysis of the major polymers in seaweed i.e., cellulose; starch, and ulvan (green seaweed); carrageenan and agar (red seaweed), and laminarin and fucoidan (brown seaweed), along with glucuronic acid and mannitol (brown seaweed) are converted by natural microbial processes to pyruvate by glycolysis in anaerobic respiration, thence to ethanol and CO_2 by alcoholic fermentation [25]. Unlike AD processing [26], there is a lack of natural microbial communities that can efficiently utilise fucose, rhamnose, xylose, and uronic-, and mannuronic-acid for alcohol fermentation [27–30], but metabolic engineering is increasingly facilitating the conversion of these latter sugars to pyruvate for alcoholic fermentation [11]. An in-depth review of the utilisation and conversion of these substrates to bioethanol by microorganisms can be found by Kawai and Murata (2016) [31].

Pre-treatments of biomass that modify the bioavailability of polysaccharides for their hydrolysis to sugars could have a major impact on both rate and yields of biogas or ethanol [32], enabling higher biofuel production in a given time. Methane yields, for example, have been improved by 19%–68% after the breakdown of biomass structures by mechanical, thermal, enzymatic, and chemical treatments to improve cellular access to polysaccharide-hydrolysing agents [33]. Since AD and fermentation are both dependent on the activities of microbial communities, optimisation of operating conditions to support their respective rates of microbial catalysis will also improve biofuel yields [34]. For example, by extending the solids retention time during AD, agar was hydrolysed to shorter chains and became a suitable substrate [35], possibly due to the adaptation of microorganisms [36]. The source of inoculum used can also be important where microbes can be affected by salt concentrations in seaweed [37], which may influence hydrolysis efficiency. These factors can affect methane yields when the inoculum is not acclimatised [34,38].

In the last decade, there have been continuing efforts to optimise pre-treatments to achieve better yields and lower costs. An in-depth review of the mechanisms of different pre-treatment methods, focusing on microalgae rather than seaweed, can be found in a review by Rodriguez et al. (2015) [39]. Pre-treatment options for the production of liquid biofuels have been briefly discussed by Wei, Quarterman, and Jin (2013) [16]. The process hurdles of both harvesting and post-harvest treatment of seaweed have been briefly discussed by Milledge and Harvey (2016) [6]. Jung et al. (2013) [40] have also briefly reviewed characteristics of different seaweed, highlighted microorganisms capable of hydrolysing seaweed carbohydrates, and different hydrolysis treatments developed to

produce bioethanol from seaweed. See also Michalak (2018) [41], who reviewed the experimental processing of seaweed for the production of various biofuels. However, to the authors' knowledge, there are no studies conducted to comprehensively review pre-treatment methods of seaweed for utilisation in AD or fermentation. This makes the selection of the appropriate pre-treatment method for seaweeds difficult.

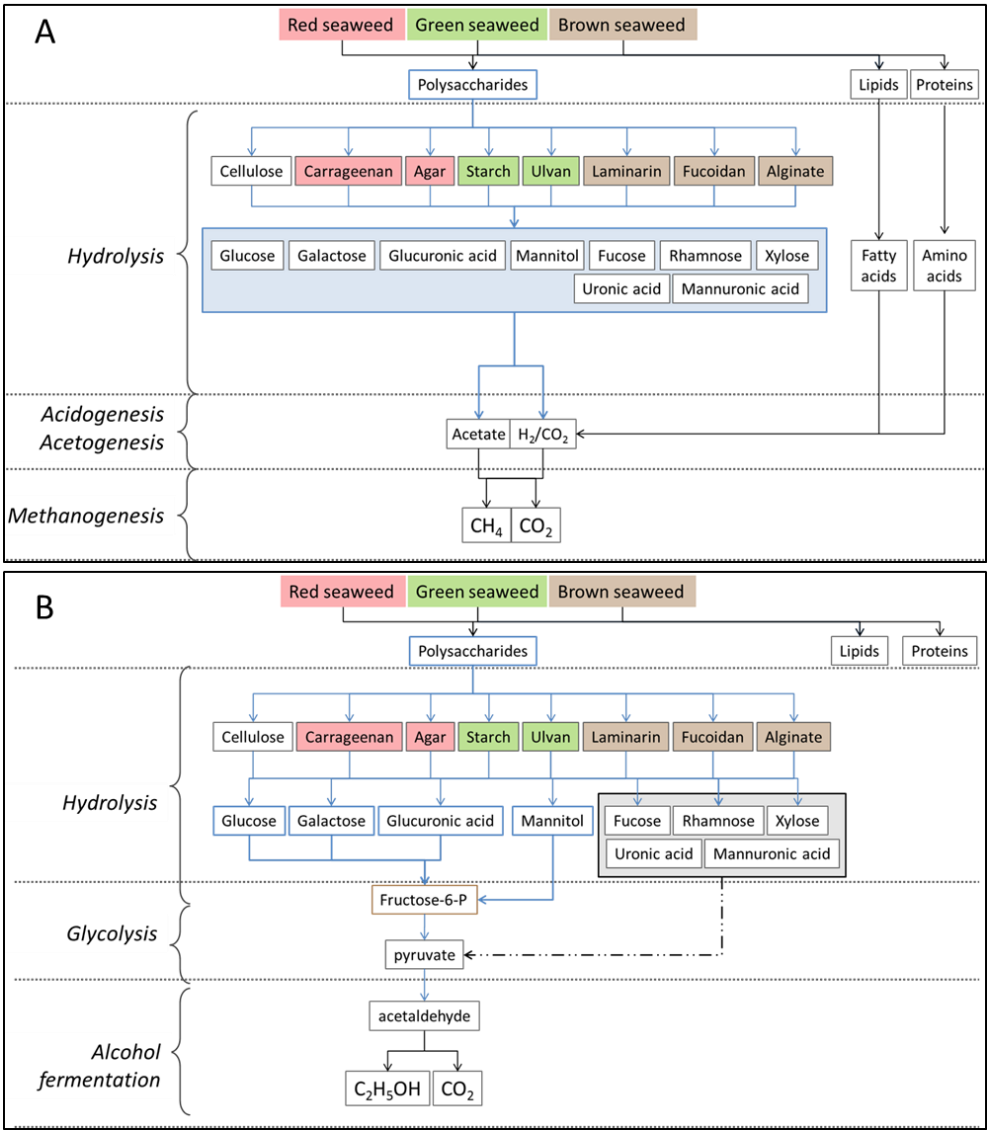

Figure 1. Major steps for biogas production (**A**), or ethanol production (**B**) from harvested seaweed. Polymeric cellulose, lipids, and proteins are found in all red, green and brown seaweeds. Additionally, the polymeric polysaccharides carrageenan and agar, colour-coded red, are typical of red seaweeds; starch and ulvan, colour-coded green, typical of green seaweeds, and laminarin, fucoidan, and alginate, colour-coded brown, typical of brown seaweeds. See also Wei, Quarterman and Jin (2013) [16].

In this review, pre-treatments will refer to the downstream processing steps of seaweed after harvesting and before AD or fermentation, which are suitable for improving biofuel yields at industrial scales. To set the scene, an overview of the chemical and structural composition of different seaweeds precedes methods for their pre-treatment, including its storage and preservation. Different methods recently utilised to break down seaweed into less complex substrates for improved hydrolysis of their polysaccharides are emphasised. Intrinsic inhibitors or those formed after pre-treatments that can limit biofuel yields and its commercialisation are also discussed.

2. Structural and Chemical Composition

2.1. Moisture and Salt Content

Seaweeds have a higher water content than many terrestrial crops [6]. The higher heating value (HHV) of seaweeds is lower than terrestrial energy crops due to the high ash content of the former [42]; higher calorific values have been achieved in seaweeds that have been demineralised [43]. Seaweed also has a higher salt (sodium chloride) content than terrestrial plants, with salt being 15% dry weight (DW) of unwashed *Sargassum muticum* [44].

2.2. Structural Composition

Structural differences exist between the red, green, and brown seaweed. A review by Kloareg and Quatrano (1988) thoroughly underlines these differences [45]. Briefly, the primary skeletal cell wall component in brown seaweed is cellulose, while xylan, mannan, and cellulose are found in green and red seaweed [45,46]. Notably, green and red seaweeds have been found solely with crystalline xylan and mannan forming the skeletal backbone, which can also change with the growth of the seaweed [45]. These polysaccharides form microfibrils which have different structural configurations, where cellulose and mannans are characterised by flat ribbons, while xylans are in a helix configuration [45]. These microfibrils have variable orientations depending on the species, either having an organised structure or being randomly distributed within each layer [47].

Furthermore, these microfibrils are associated with matrix polysaccharides which include different sulphated or carboxylic polysaccharides depending on the species (Table 1) [48]. For example, sulphated fucans were suggested to play a role in 'interlocking' the cellulosic backbone [49]. In brown seaweed, proteins were also found associated with sulphated fucans and phenols [49]. The attachment between phenols and alginates are likely to play an important role in the rigidity of cell wall structures [49]. Additionally, phenols can also be inhibitory to microorganisms involved in biofuel production, as will be discussed in later sections. The structural overview of cell walls in green and brown seaweed are shown in Figure 2.

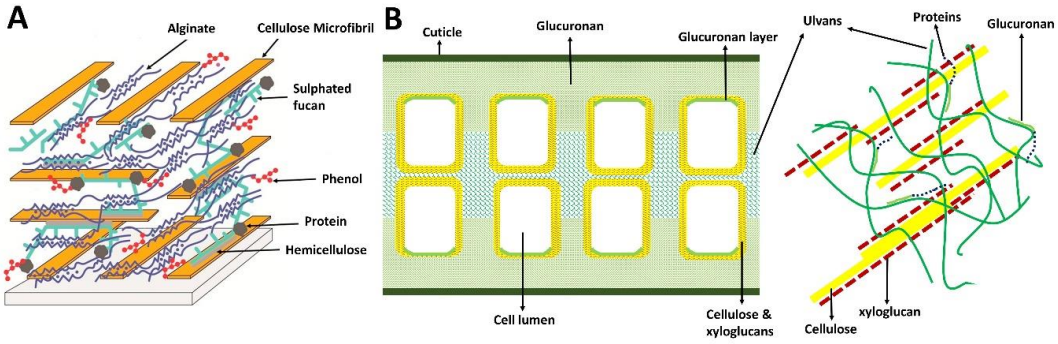

Figure 2. (**A**) Cell wall model of brown seaweed (Fucales) [50], modified for simplification (obtained permission for re-use). (**B**) Cell wall polysaccharide distribution of green seaweed (*Ulva* spp.); far right figure shows closer interactions between polysaccharides, adapted from Lahaye and Robic (2007) [51]. Reprinted with the permission of the American Chemical Society.

Ulvans found in green seaweeds, comprised of xylose, rhamnose, uronic acid, and galactose, are relatively resistant to degradation. They may, therefore, act to limit access to, and biodegradation of, other polysaccharides, especially cellulose and starch [52]. Bobin-Dubigeon et al. (1997) [52] suggested similar roles of alginates in brown seaweeds and carrageenans in red. Thus, the use of alginate lyase to break down brown seaweed was found to release only small amounts of sulphated fucans [49], whilst effects of cellulase and alginate lyase treatment of *Laminaria digitata* were insignificant on biogas yields [53]. Cell wall architecture in seaweeds may, therefore, be similar to that of the plant cell wall in land-plants: chains of β-1,4-linked glucose molecules in crystalline cellulose microfibrils provide

structural support but are protected from hydrolysis to glucose by most natural microorganisms, in the case of seaweeds, by a matrix of sulphated fucans and alginates in brown seaweeds, carrageenans and agar in red, and ulvans in green seaweeds [52], and in land-plants, by polymeric lignin.

Sargassum spp., a brown seaweed, is more recalcitrant to digestion compared to *Gracilaria* spp., a red seaweed, and *Ulva* spp., a green seaweed [35]. The insoluble fibre content in brown seaweed has been found to vary greatly in different seasons, with total fibre content ranging from 36–54% in *Hizikia Fusiformis* [54]. A general overview of fibre content in two different studies suggests that brown seaweed may generally have a higher fibre content (10%–75%) compared to red (10%–59%) or green (29%–67%) seaweed [55,56]. Consequently, pre-treatment methods may need to be tailored depending on the seaweed type and structural composition.

2.3. Polysaccharides

The suitability of different types of pre-treatment for processing seaweed is likely to vary with differences in seaweed chemical composition [57,58]. Chemical profiling of 107 seaweed types showed many similarities between different seaweed groups [59], with red and green seaweed having more similar characteristics in their water-soluble and insoluble components compared to brown seaweed, which held more unique characteristics [59]. However, clear distinctions between them are found in their sugar and amino acid compositions [60].

The polysaccharides and sugars in different types of seaweeds and their unique characteristics are shown in Table 1. The structures of these polysaccharides were highlighted by Wei, Quarterman, and Jin (2013) [16]. Seaweeds generally have high sulphur content due to the presence of sulphated polysaccharides with different amounts of sulphate groups in different polysaccharides, which also varies in different phyla and genera [61,62], where brown seaweed contain sulphated fucans [63]; red seaweed contains sulphated galactans (agar and carrageenan); and green seaweed contains sulphated xyloarabinogalactans or other sulphated heteropolysaccharides, depending on the species [62]. It should be noted that this is highly simplified and more detailed work can be found by Kloareg and Quatrano (1988) [45] and Synytsya et al. (2015) [48].

Table 1. Sugars and polysaccharides in red, green, and brown seaweed.

Seaweed Type	Polysaccharides	Sugars	Ref.
Red	Agar [1], carrageenan [1], agaropectin, cellulose, xylans, mannans	D-galactose, D-fructose, 3,6-anhydro-D-galactose, glucose	[45,60,64]
Green	Ulvan [1], starch, xylopyranose, glucopyranose, xyloglucan, glucuronan, cellulose, hemicellulose	Glucose, xylose, uronic acids, rhamnose, galactose	[52,60]
Brown	Fucoidan [1], laminaran, alginates [1], cellulose	Mannitol, glucose, guluronate, mannuronate, glucuronate, sulphated fucose	[60,65,66]

[1] Main matrix polysaccharides associated with microfibrils. Other matrix polysaccharides of green seaweeds are also available but not mentioned here [48].

The biochemical composition of seaweeds' polysaccharides influences their structural configurations and properties [67], with alginates described as egg-box like (Figure 2), carrageenans in a double helix, and ulvans in a bead-like configuration [45,68]. These differences are also likely to influence the effects of pre-treatments and biofuel yields. Alginic acid and its sodium salt were found to be recalcitrant to AD with average methane yields equivalent to only 23%–28% of their theoretical methane potential, considerably below that of cellulose [69].

2.4. Chemical Composition Variability

Seaweed composition varies between species, seasons, and geographical location due to differences in sea currents, light intensities, and temperatures [65]. The amino acid content in *S. latissima* in August

was almost double that in June and ash and mineral contents also increased [70]. Polysaccharides, such as fucoidan, also show different degrees of branching, sulphation, and chain length at different times of the year [65]. Seaweeds growing in the presence of higher heavy metal contents also produced more cell wall polysaccharides [71]. Furthermore, phenolic compounds that are potential inhibitors of AD and fermentation (Section 4.5.2) also vary spatio-temporally in seaweeds [72].

3. Storage and Preservation

The combined effects of the seasonal growth of seaweeds and the fluctuations in levels of fermentable sugars present in seaweeds harvested in different seasons suggest there is only a small window for harvesting during the year to obtain the optimal biomass composition for biofuel production [73]. Therefore, in many geographical locations, there is a need for effective preservation methods if seaweed is to be used for biofuel production throughout the year.

3.1. Drying for Storage

Drying of seaweeds to a moisture content <22% is recommended before storage [73]. This not only improves shelf life but can also reduce transportation costs [6]. Drying of seaweed can involve drying on platforms directly under the sun, in greenhouse drying facilities, or by using solar thermal drying systems that may also utilise electricity [73,74]. Methods that rely on solar energy would be less suitable and more energy consuming in temperate climates. Alternatively, to reduce energy consumption, Milledge and Harvey (2016) [6] have suggested mechanical dewatering to reduce moisture content before drying. However, screw-pressing brown seaweed has been found to be ineffective unless the seaweed is pre-treated with hydrochloric acid [75].

3.2. Ensilage for Storage and Preservation

Ensiling is a practice used in the production of forages from terrestrial crops to preserve wet biomass. Ensiling involves the biochemical conversion of water-soluble carbohydrates into mainly lactic acid and other organic acids by anaerobic microorganisms [6], creating a pH drop that prevents the growth of spoilage microorganisms [76]. For forages, the ideal conditions have been indicated as the following: at least 25% DW; high concentrations of water-soluble carbohydrates for lactic acid bacteria to initiate and sustain fermentation; at least 10^6 colony forming units of lactic acid bacteria per gram of fresh biomass; and low buffering capacity to rapidly reduce the pH [58,76]. A rapid drop in pH is required to prevent a cascade of pH-increasing effects caused by the growth of clostridia, where clostridia utilise lactic acid and water-soluble carbohydrates, and produce butyric acid and CO_2 [58].

However, seaweeds have high buffering capacity due to their high anionic acid content, with additional inoculation of lactic acid bacteria having mixed successes in lowering the pH for different seaweed species [58]. A pH of lower than 4.7 is required to prevent the growth of clostridia in *Saccorhiza polyschides* [76], while below pH 4.48–4.10 is sufficient for *S. latissima* [58]. Sandbakken et al. (2018) [77] found that no sugar loss was detected during 6 months of storage when *S. latissima* was stored anaerobically at a pH < 4 by the addition of sulphuric and formic acid.

Success in ensiling seaweed is also highly affected by the season of harvest and the seaweed species, which influences the biochemical composition of the biomass [58,76,78]. For example, high polyphenol content in *Ascophyllum nodosum* may contribute to low acid production by inhibiting fermentative microorganisms, thereby preventing a successful pH drop [76]. Pre-processing methods can also have significant effects on ensiling success. In washing treatments used prior to ensiling, different results for the HHV were found between the ensiled and non-ensiled seaweed: Redden et al. (2016) [78] found an increase (seawater washed); Herrmann et al. (2015) [76] found an increase (cold water washed); Cabrita et al. (2017) [58] found an increase (cold freshwater washed); and Milledge and Harvey (2016a) [44] found an insignificant difference in HHV (unwashed seaweed). The differences in washing could also play a role in the extent of inorganic and organic material loss during ensiling [78,79]. Additionally, maceration of biomass prior to ensilage, using a macerator, was shown to increase the fermentation rate and lactic acid

concentration [80,81], as well as reduce ethanol production during ensilage [82]. By contrast, chopping of seaweed before ensilaging, which may form larger particle sizes and reduce the rate of hydrolysis compared to maceration, reduced overall leachate loss, loss of total solids (TS), and volatile solids (VS) [44], allowing more of the biomass to be available for further downstream processing.

Unsuccessful ensilaging of *Gracilaria vermiculophylla* has been partly attributed to its high water content [58], which could be reduced by dewatering before ensilaging. Dewatering techniques include screw-pressing, using chemical additives, saline solutions or salting [75,83]. Screw-pressing itself has been found to increase ethanol yields of *Laminaria digitata* [83]. However, these methods will also need to be selectively chosen for different types of seaweeds as it was found that red and brown seaweeds required different dewatering methods [57].

From literature searches, the production of ethanol from ensiled seaweed has received little attention. AD has thus far delivered mixed results: use of ensiled *A. nodosum* showed enhanced methane production compared to untreated *A. nodosum*, regardless of VS losses during storage and provided the leachate was also co-digested, but ensiling *S. latissima* had no significant effect on methane production and a negative effect for *S. polyschides* [76]. Milledge et al. (2018) [79] also found no significant difference between methane production from ensiled and fresh *S. muticum*. Further research is required to establish the conditions optimal for successful preservation of different seaweed types for non-seasonal use in biofuel production.

4. Seaweed Hydrolysis Methods

4.1. Mechanical Treatment

Mechanical pre-treatment to improve access by hydrolysing agents to polysaccharides mainly affects the physical structure of seaweeds. A summary of mechanical pre-treatments performed on seaweed before ethanol or methane production is shown in Tables 2 and 3. The range of mechanical pre-treatments includes size reduction, beating, washing, and sonication of seaweed. Direct comparison between each treatment and between different seaweeds by direct comparisons of percentage change in yields is difficult as various authors treat the biomass in diverse manners. This includes differences in enzymes used for saccharification, yeasts used for fermentation, inoculum to substrate (I/S) ratios, and the biochemical methane potential (BMP) measurement methods which can affect the final gas volume [84]. Methane yields are presented rather than biogas yields where possible due to variable methane composition (<40% to ~70%) depending on the feedstock and BMP test period [85,86]; with typical values around 60% CH_4, 40% CO_2, and other trace gases [87].

The biodegradability index (BI) is used to indicate the efficiency of the particular pre-treatment approach relative to the theoretical yield [88], and is used to compare the effectiveness of the pre-treatments more easily. It is calculated by the methane or ethanol yield after pre-treatment divided by the authors' provided theoretical yield and presented as a percentage. The change in the biodegradability index (BI), similar to the % change, is also used to indicate the effectiveness of the pre-treatment relative to the untreated control seaweed. Different methods were used to calculate theoretical yields: some authors have estimated the theoretical ethanol yield according to glucan content (0.57 g ethanol g^{-1} glucan) [89], while others have used glucose which includes hydrolysis gain (0.51 g ethanol g^{-1} glucose) [90]. Since glucose is often not the primary fermentable substrate, others have used laminarin and mannitol concentrations [91]. Ethanol yields have been converted to the same units where possible. Where authors had tested more than one variable, tables show the variables with the highest ethanol or methane yields. The tables cannot give a complete picture of the most effective pre-treatment method, but demonstrate the effectiveness of each pre-treatment method for the particular species harvested at a particular time of year, using a specific approach.

Table 2. Ethanol yield following mechanical pre-treatment of seaweed.

Algae (Harvest Time)	Pre-treatment	Fermentation	Ethanol Yield (mg g^{-1} DW)	% Change	BI (%)	ΔBI * (%)	Ref.
		Size reduction					
Gelidium sesquipedale (unknown time)	Freshwater washed and air-dried. *Test*: Cutting milled then centrifugally milled, 12,000 rpm. *Control*: Cutting milled: <2 mm.	Simultaneous saccharification and fermentation (SSF): 5% seaweed loading fermented with Haliatase enzyme (β-glucanase, carragenase, agarase), and *S. cerevisiae*, stirred (37 °C, 72 h (h))	351	+80	69	+79.8 [1]	[92]
Ulva lactuca (unknown time)			527	−4.4	64	−4.3 [1]	
Chaetomorpha linum (September 2010)	Freshwater washed, dried (40 °C, 48 h) *Test*: 25 g ball milled (25 balls), 18 h, 180 rpm to <2 mm size. *Control*: untreated biomass	SSF: 10% pre-treated seaweed pre-hydrolysed, inoculated: cellulase enzymes, and *S. cerevisiae*, 32 °C, 200 h	180	+63.6	77	+41.9 [2]	[89]
		Washing					
L. digitata (July 2009)	*Test*: Freshwater washed (W) Oven dried (OD) (70 °C, 72 h); frozen (−20 °C) and OD (FOD). *Control*: Unwashed, OD or FOD. All milled to <1 mm.	SSF: 5% seaweed with laminarinase and yeast *Pichia angophorae* stirred at 24 °C, 88 h	*W + OD*: 12.3 µL g^{-1} DW	−9.6	19	+49.2 [3]	[91]
			W + FOD: 11.1 µL g^{-1} DW	−26	15	−8.1 [3]	
		Sonication					
U. rigida (unknown time)	Dried (70 °C, 48 h), ground: ≤1 mm particle size. SSF: 4% (*w*/*v*) seaweed, amyloglucosidase, α-amylase, cellulase enzymes, buffer, and *S. cerevisiae*. *Test*: Incubated in sonicator bath, 40 kHz, 120W, 37 °C, 3 h. *Control*: conventional incubator, 150 rpm, 37 °C, 48 h		64.7 [4]	+58.8 [4]	65	−2.9 [5]	[90]

* BI based on calculation from theoretical yield presented in literature based on: [1] unknown; [2] glucans; [3] laminarin, mannitol, and glucose; [5] glucoses; [4] Calculated from yields in literature.

Table 3. Methane yields following mechanical pre-treatment of seaweed.

Algae (Harvest Time)	Pre-treatment	I/S Ratio; Source	BMP Method	CH_4 Yield (mL g^{-1} VS)	% Change	BI (%)	ΔBI (%)	Ref.
			Size reduction					
G. vermiculophylla (March 2012)	*Test*: Unwashed and macerated (UM); washed and macerated (WM); washed, dried (37 °C) and macerated (WDM). *Control*: without maceration.	4:1; brewery wastewater treatment plant (WWT)	Glass vials with rubber stopper, aluminium crimp, 37 °C, 28 days.	*UM*: 338	+14.6	-	-	[93]
				WM: 481	+11.9	-	-	
				WDM: 349	+7.7	-	-	
G. vermiculophylla	Frozen at harvest (unknown time of harvest). *Test*: Fresh water washed, macerated (M). *Control*: Fresh water washed and chopped (2 × 2 cm)	6:1 in 500 mL; lab reactor using cattle manure	Bottles with rubber stoppers and aluminium crimp, 53 °C, 34 days	147	+11.4	-	-	[94]
C. linum				195	+17.5	-	-	
U. lactuca				255	+67.7	-	-	
S. latissima				333	−2.1	-	-	
Laminaria spp. (November 2013)	*Test*: Ball milled (20 balls) unwashed seaweed, dried at 80 °C for 24 h Particle size: 1–2 mm. *Control*: cut, unwashed.	1:1.33 in 400 mL; WWT	Bottle sealed with adaptor attached to gas measuring device (GMD), 38 °C, 25 days, manually shaken	*1 mm*: 241	−26.5	-	-	[95]
				2mm: 260	−20.7	-	-	
F. vesiculosus (Autumn 2014)	Frozen until treatment *Test*: Washing (W) and chopped (C) (<5 mm); unwashed (UW) and chopped; washed and not chopped (NC). *Control*: not washed or chopped	5:1 in 60 mL; WWT	Serum bottles, gas measured with syringe, 37 °C, 30 days.	*W + C*: 81.1	+574.3	25 [1]	-	[23]
				UW + C: 67.3	+493.6	21 [1]	-	
				W + NC: 73.1	+527.5	23 [1]	-	

Table 3. *Cont.*

Algae (Harvest Time)	Pre-treatment	I/S Ratio; Source	BMP Method	CH_4 Yield (mL g^{-1} VS)	% Change	BI (%)	ΔBI (%)	Ref.
			Beating					
Laminaria spp. (November 2013)	*Test*: Cut without washing and beaten (Hollander beater), 76 µm gap, 10 min (min). *Control*: only cut, unwashed.	1:1.33 in 400 mL; WWT	Bottle with adaptor attached to GMD, 38 °C, 25 days, shaken manually	335	+2.1	-	-	[95]
Laminaria spp. (May 2014) *A. nodosum* (August 2014)	*Test*: Cut without washing and beaten (Hollander beater), 76 µm gap, 15 min. *Control*: only cut, unwashed.	1.2:1 (*Laminaria* spp.), 3:1 (*A. nodosum*) in 400 mL; WWT	Bottle attached to GMD, 38 °C, 14 days, shaken manually	240 169	+8.6 +30	- -	- -	[96]
			Washing					
U. lactuca (June 2011)	*Test*: Washed and dried (room temperature) (24 h). *Control*: Unwashed Both frozen (−20 °C), grinded: 10–15 mm.	3:1 in 400 mL; reactor using grass, dairy slurry and seaweed.	Bioprocess AMPTS II system, 37 °C, 30 days.	221	+33.9	55 [1]	+53.2 [1]	[97]
U. lactuca (April)	*Test*: Washed 2% (*w*/*v*) seaweed in water, 24 h, chopped (2 × 2 cm) (C) or macerated (M). *Control*: Unwashed (C or M)	8:1 in 500 mL; reactor using cattle manure.	Bottles with rubber stoppers, aluminium crimp, 52 °C, 42 days.	*W* + *C*: 171 *W* + *M*: 200	−1.7 −26.2	- -	- -	[98]
L. digitata (July 2009)	Milled to <1 mm particle size. *Test*: Freshwater washed (W), oven dried (70 °C, 72 h) (OD) or frozen (−20 °C) and OD (FOD). *Control*: Unwashed, OD or FOD.	6:1 in 500 mL; Unknown	Bottle with rubber stoppers, aluminium caps, shaken, 35 °C, 35 days.	*W* + *OD*: 202.9 *W* + *FOD*: 248.1	−13.8 +29.4	- -	- -	[91]
S. muticum (June 2017)	*Test*: Freshwater washed *Control*: unwashed Both frozen (−20 °C), then blended.	9:1 in 400 mL; paper WWT	Automated CJC system, 37 °C, 28 days.	177	−21.3	48 [1,2]	−21.3 [1,2]	[79]
L. digitata (March (M) and September (S))	*Test*: Washed in cold water (CO) (15 °C); hot water (H) (40 °C), 3 min, cut (4 cm) *Control*: unwashed, cut to 4 cm.	2:1 in 400 mL; reactor using grass, dairy slurry and seaweed.	Bioprocess AMPTS II system, 37 °C, 30 days.	*M*,*CO*: 258 *M*,*H*: 283 *S*,*CO*: 303 *S*,*H*: 326	+5.3 +15.5 +8.2 +16.4	59 60 67 76	+13.4 +15.4 +8.0 +22.6	[88]
			Sonication					
U. rigida (July–September 2013)	*Test*: 30 mL blended seaweed (80% (*w*/*v*) in water), sonicated (5 min, 40 kHz, 120 W) Control: 80% *w*/*v*, (assumed) blended	1:1 in 500 mL; Unknown	Bottles with rubber stoppers, gas measured by syringe plunger, 37 °C 48 days.	–	+10.2	57 [3]	+6.6 [3]	[99]

[1] based on Buswell equation; [2] calculated based on elemental analysis provided (using Buswell equation [100,101]); [3] presumably based on theoretical methane yields per gram of chemical oxygen demand (COD).

4.1.1. Size Reduction

Chopping or milling of the biomass is commonly used to increase the surface area to volume ratio, in order to improve the hydrolysis of complex carbohydrates to sugars for fermentation or AD (Figure 1A,B) [16,102]. However, the same milling techniques used for lignocellulosic terrestrial plants may not elicit the expected increase in surface area for seaweed [83]: milling of *L. digitata*, which has flat blades, did not significantly increase its surface area [103].

Mechanical wet milling of *L. digitata* using cutting discs did not enhance glucose release [103]. Similarly, Amamou et al. (2018) [92] found that neither vibro-ball milling nor centrifugal milling of *Ulva lactuca* affected its sugar release. Centrifugal milling of *U. lactuca* showed a 4.4% reduction in ethanol yield. However, the same treatment on *Gelidium sesquipedale* showed up to a 129% increase in sugars released, increasing the ethanol yield by 80% compared to only cutting milled seaweed (Table 2). Regardless, *U. lactuca* still showed higher ethanol yields than *G. sesquipedale*, indicating that *U. lactuca* required less processing as it only required a cutting mill. Ball milling of *Chaetomorha linum* also enhanced bioethanol production by 63.6% compared to non-milled biomass (Table 2) [89].

The differences in the cell wall ultrastructure of seaweed can determine the beneficial value of mechanical treatment, where those with more fibrous cell walls would benefit from size reduction [94]. Experimental results by Nielsen and Heiske (2011) [94] showed that chopped or macerated samples of different seaweed species could have different effects on methane production (Table 3). Oliveira, Alves and Costa (2014) [93] found an increase of 8–16% in specific methane production after maceration of *G. vermiculophylla*, whilst Tedesco, Mac Lochlainn, and Olabi (2014) [104] also found an increase in methane yields with decreased particle size of *Laminaria* spp. On the other hand, Montingelli et al. (2016) [95] found reduced methane yields after milling of *Laminaria* spp., which they attributed to the inhibitory effect of volatile fatty acid (VFA) accumulation and the subsequent pH drop in the acidogenesis phase (Figure 1A) due to higher hydrolysis rates aided by higher surface areas [95,104]. It has been proposed that a mixture of smaller and larger particle sizes would be beneficial for increasing methane yields during AD [104].

4.1.2. Beating

Apart from size reduction by the cutting action, beating also involves pounding the seaweed against a plate, enabling the production of seaweed pulp at different consistencies depending on the machine setting [95]. A Hollander beater has been investigated by a number of researchers [86,95,96,104,105]. A comparison between beating, milling and microwave pre-treatments of *Laminaria* spp. found that beating was the most effective pre-treatment to enhance methane production from seaweed [95], and more effective than drying before ball milling in terms of net energy gain.

Montingelli et al. (2016) and Montingelli et al. (2017) [95,96] reported only marginal increases in methane yields from beaten seaweed compared to those that were only cut (Table 3), but rates of degradation were improved. Furthermore, although higher methane yields were obtained from the beating of *Laminaria* spp. collected in November compared to collection in May (Table 3), similar conclusions on faster hydrolysis rates were drawn [95]. On the other hand, beating of *A. nodosum* at low VS concentrations also enhanced methane yields, but an increase in hydrolysis rate was not observed [96].

4.1.3. Washing

Washing in freshwater is a pre-treatment step often used in a wide variety of seaweed biofuel research studies (See also Section 3.2 above) [30,34,42,106–112]. Washing has been used to remove inert impurities, such as gravel and sand, limiting their build-up in reactors [98]. Washing also removes salts which can be inhibitory at high concentrations to both methane production and enzymatic hydrolysis for bioethanol production [89,113]. Chisti (2013) [114], in a review of the constraints to the

commercialisation of algal fuels, suggested that seaweed should be washed in fresh water to reduce the salt content.

Adams, Schmidt, and Gallagher (2015) [91] found that higher ethanol yields were obtained from unwashed samples compared to washed samples of *L. digitata* (Table 2), attributed to the loss of water-soluble carbohydrates such as fermentable laminarin and glucose [91].

Mixed effects of washing seaweed on methane production have been found for different species (Table 3). Washed and macerated *U. lactuca* had around 26% lower methane yields than the unwashed and macerated [98], but 34% higher methane yields when washed and wilted (dried at room temperature) compared to unwashed and wilted [97], with an increase in BI of 53%. A 42% increase in methane production was found for *G. vermiculophylla* when washed and macerated compared to the unwashed and macerated [93]. Washed and cut *L. digitata* was also found to have higher methane yields compared to unwashed and cut [88]. Furthermore, even though Table 3 indicates a negative impact on methane yields after washing of *S. muticum*, statistical analysis showed that washing did not affect yields [79]. Loss of readily digested substrates or removal of hydrolytic bacteria from seaweed surfaces were suggested to slow down the initial rate of methane production [79], indicating that initial stages of AD could be affected (Figure 1A).

Hot water washing (40 °C) of *L. digitata* increased VS content by up to 31%, due to the removal of ash and nitrogenous compounds, compared to cold water washing (15 °C) and unwashed [88]. The lower ash to VS ratio for hot water washing, proposed to contribute to higher methane yields [88], do not seem to correlate with the changes in methane content for other washing experiments. Low ash to VS ratio was observed in *A. nodosum*, but the lower methane content was attributed to polyphenols [38]. Furthermore, washing may also reduce vital trace elements such as cobalt and selenium that have a considerable effect on AD [115]. Hence, the change in the mineral content of the seaweed after washing may also be a contributory factor in the lack of correlation between methane production and lower ash to VS ratio following washing.

In summary, if washing is found to have insignificant roles in biofuel yields, the removal of a washing process could greatly benefit industrial downstream seaweed processes for biofuel production, saving large quantities of water. However, salt accumulation in continuously-stirred digesters can cause further problems to the microbial community [38] and, therefore, industrial-scale digesters may need to be salt-acclimated if unwashed seaweed is to be used (see also Section 4.5.1).

4.2. Thermal Treatment

Thermal pre-treatment is able to release sugars and extract polysaccharides from seaweed. The treatment of the brown seaweed, *Nizimuddinia zanardini*, at 121 °C released up to 84% of components such as hemicelluloses and mannitol [116], and resulted in a 22% higher methane production compared to untreated seaweed (Table 4) [116]. Autoclave treatment of the red and green seaweed, *Gelidium amansii* and *C. linum*, also increased bioethanol yields when compared to non-heat treated biomass (Table 5) [89,117]. The increase in bioethanol yields was correlated with an increase in exposed fibres and eroded surfaces of the seaweed, enabling higher enzymatic degradation.

However, certain structures of the seaweed such as the cortex of the seaweed were not affected by autoclave treatment, indicating the importance of the structural make-up of seaweed and the appropriate pre-treatment method required to hydrolyse these components effectively. The following section discusses different thermal pre-treatments of seaweed.

Table 4. Methane yields following thermal pre-treatment of seaweed.

Algae (Harvest Time)	Treatment	I/S Ratio; Source	BMP Method	CH_4 Yield (mL g^{-1} VS)	% Change	Ref.
Ulva spp. (Korea, Spring 2014)	Freshwater rinsed, blended into slurry *Thermal*: no chemical, 90 °C *HCl*: 0.1 M, 90 °C *NaOH*: 0.1 M, 90 °C All magnetically stirred (10 mins), oven (6 h), shaken 1 min every half hour. *Control*: untreated slurry	unknown (35 mL slurry and 70 mL inoculum); sewage sludge digester	Bottles with rubber stopper and aluminium cap, 35 °C, 30 days. Shaken manually intermittently.	*Thermal*: 293.0 *0.1 M HCl*: 284.8 *0.1 M NaOH*: 251.3	+15.8 +12.7 −0.7	[118]
S. latissima (August 2010)	Defrosted, shredded into slurry. *Test*: steam exploded 130 °C or 160 °C, 10 mins. *Control*: untreated slurry	7:1 in 700 g; sewage treatment plant.	Bottles with rubber stopper, aluminium screw caps, shaker (90 rpm, 37 °C). Re-fed day 67, biogas shown: day 119.	*130 °C*: 268 *160 °C*: 260	+20.2 +16.6	[119]
N. zanardini (July)	Washed, dried (40 °C, 24 h); hammer milled to <1 mm. *Test*: 5% seaweed, 121 °C, 0.5 h. *Control*: untreated.	unknown; WWT	Bottles closed with rubber stopper, aluminium caps, 37 °C, 40 days.	143	+22	[116]
Laminaria spp. (November 2013)	*Test*: Cut seaweed, Freshwater immersed, microwaved (560 W) till water boiled, held for 30 s. *Control*: cut unwashed seaweed.	1:1.33 in 400 mL; WWT	Bottles sealed with adaptor attached to GMD, 38 °C, 25 days, shaken daily.	244	−25.6	[95]
F. vesiculosus (October 2014)	Cut and grounded (mortar and pestle) *Test*: microwaved (700 W), 3 mins *Control*: not microwaved	1:3; WWT	Bottles with rubber stopper and metal cap, 37 °C, 22 days. Shaken daily.	146.9	+92.3	[120]

Table 5. Ethanol yields following thermal pre-treatment methods.

Algae (Harvest Time)	Treatment	Fermentation	Ethanol Yield (mg g^{-1} DW)	% change	BI [1] (%)	ΔBI [1] (%)	Ref.
C. linum (July 2009)	Washed, dried (40 °C) and milled. *Thermal*: 4% (*w*/*v*) seaweed, autoclaved (200 °C), 10 mins, 1 bar. *Wet oxidation (WO)*: same as thermal, 12 bars O_2 *Steam explosion*: 1.2 kg (35% DW, 1.9 MPa), 200 °C, 5 mins. *Plasma assisted*: 2.5 g, 1% O_3, 1 h. *Control*: untreated biomass	SSF: 10% pre-treated seaweed pre-hydrolysed, inoculated with cellulase enzymes and *S. cerevisiae* (32 °C 200 h).	*Thermal*: 150 *WO*: 170 *Steam*: 130 *Plasma*: 150	+36.4 +54.5 +18.2 +36.4	68.4 77.2 66.7 71.9	+25.7 +41.9 +22.6 +32.2	[89]

[1] BI based on calculation from theoretical yield presented in literature (glucan theoretical yield).

4.2.1. Microwave

Microwave pre-treatment appears suitable for seaweeds due to its high moisture content which facilitates a quick rise in temperature and pressure inside the cells, allowing cell wall rupturing [121,122], and enabling an increase in surface area for subsequent bioethanol or biogas production processes (Figure 1A,B). The mechanisms and the use of microwave pre-treatment of biomass for bioenergy production were reviewed by Kostas, Beneroso, and Robinson (2017) [123].

Microwave pre-treatment has mostly been used in the extraction of high-value products and polysaccharides, such as agar, carrageenan, and fucoidan from seaweed [121,124–127]. When microwave thermal treatment and a more conventional thermal treatment were compared using *Undaria pinnatifida*, the microwave treatment was not only more effective at extracting the polysaccharide, fucoidan, but it also degraded it to lower molecular weight compounds [128]. Subsequently, higher bioethanol or methane yields could be achieved, provided suitable microorganisms capable of utilising the compounds are available.

Another advantage is its rapid heating time that could stabilise and minimise sugar degradation at high temperatures, resulting in lower concentrations of inhibitory product, such as furfural compounds (5-hydroxymethyl furfural and furfural), formic acid, and levulinic acid, often formed during conventional inductive heating [116,129–131]. Microwave treatment of *A. nodosum* (150 °C, 0.4 M sulphuric acid (H_2SO_4), 1 min) showed no furfural production, and 5-hydroxymethyl furfural production was as low as 0.01 g L^{-1} [132]. Likewise, microwave-assisted acid hydrolysis of a red seaweed, *Eucheuma denticulatum*, also produced only 0.24 g L^{-1} 5-hydroxymethyl furfural at 150 °C, 0.1 M H_2SO_4 [133].

Furfural compounds lower ethanol yields by inhibiting cell growth of yeasts and enzymes during glycolysis (Figure 1B) [134,135]. Ethanol yields were enhanced during microwave treatment of *A. nodosum*, with ethanol production up to 60.7% of the theoretical yield [132]. Microwave pre-treatment of *F. vesiculosus* also increased methane production by 92% compared to the untreated seaweed [120]. This is a 10% enhancement in BI compared to washing and chopping of *F. vesiculosus*, which had similar harvesting time and BMP measurement method. However, microwave pre-treatment was unsuitable for *Laminaria* spp. where it was held at boiling point for 30 s [95]. This was found to lower methane yields by 27% compared to untreated seaweed (only cut). Its current small-scale application (20–50 mL in volume) and low ethanol concentration obtained (0.7% (*v*/*v*)) also calls for further investigations and optimisations [132,136].

4.2.2. Steam Explosion

Steam explosion, widely used as a lignocellulosic pre-treatment process, has not been highly investigated as a thermal pre-treatment method for seaweed, possibly because the latter is considered to be much less recalcitrant. Steam explosion involves both thermal and mechanical means to hydrolyse the seaweed. Steam explosion of *S. latissima* for 10 min at 130 °C and 160 °C both showed an increase in methane production compared to untreated seaweed (Table 4) [119].

In another experiment comparing five different pre-treatment methods for *C. linum* (Table 5), steam-exploded seaweed at 200 °C for 5 min produced around 18% higher ethanol yields than untreated seaweed [89]. However, the yield of ethanol was lower than other pre-treatments tested (wet oxidation, autoclave, and plasma-assisted), which was attributed to the loss of glucans from biomass solids [89]. The fermentation was carried out on the solid fraction of the pre-treated biomass as the slurry was pressed to remove half of the liquid, with loss of 31% DW. It would, therefore, be of interest to understand bioethanol yields from the liquid and solid fraction to determine the effectiveness of steam explosion of seaweed on bioethanol production. Its high energy costs will also need to be compensated by significantly higher biofuel yields and, therefore, may not be an appropriate technology for seaweed.

4.2.3. Other Thermal Pre-Treatment Methods

Other thermal pre-treatment methods include wet oxidation and plasma-assisted pre-treatment. Only one author had used these methods for the pre-treatment of seaweed (Table 5). Plasma-assisted pre-treatment involves the generation of ozone in the reactor which is thought to react and degrade unsaturated organic compounds within the biomass [137]. Ozone has also been found to reduce polyphenol concentrations [138]. Unlike steam explosion, there was no loss of dry matter after pre-treatment, a small loss in glucan, xylan, and arabinan, and no production of 5-hydroxymethyl furfural or furfural [89]. Even though the combined levels of glucan, xylan, and arabinan in the pre-treated biomass was lower than untreated biomass, the pre-treatment may have allowed higher enzymatic hydrolysis of the remaining biomass and, subsequently, higher ethanol production than untreated seaweed.

Wet oxidation was also an effective method in increasing the glucan content of the pre-treated biomass compared to the untreated seaweed [89]. Interestingly, wet oxidation resulted in the high production of formic acid and acetic acid, but its ethanol yield was 54% more than untreated biomass, and only 0.01 g g^{-1} DW lower than the ball milled pre-treated sample. However, the direct comparison between the two would not be completely accurate as the seaweeds were harvested in different seasons.

4.3. Chemical Treatment

Enhancements in hydrolysis and solubilisation of seaweed during acid and alkali pre-treatments make them highly investigated methods prior to fermentation or AD [139]. Other chemicals, such as ionic liquids, 'organosolv' (a solvent-based pre-treatment), and sodium chlorite, have also been investigated as pre-treatment methods for seaweed [140,141]. Chemical properties play an important role in the hydrolysis of seaweed's polysaccharides. Jmel et al. (2018) [140] highlighted that the viscosity of 1-ethyl-3-methylimidazolium acetate (an ionic liquid) limited its hydrolysis potential as its access into the internal matrix was prevented by the inability to pass through the carbohydrate cell wall matrix. An in-depth investigation of the degradation of *U. rigida* by ionic liquids has been achieved by Pezoa-Conte et al. (2015) [142]. High solubilisation of seaweed and the subsequent high concentrations of reducing sugars have been achieved as a result of using different chemicals and thermochemical methods [99,139,143].

4.3.1. Alkali or Acidic Treatment

The addition of alkalis, often sodium hydroxide (NaOH), was proposed to cause swelling of fibres and increase pore sizes, enabling the release of sugars from within cell walls, which facilitates efficient subsequent enzymatic hydrolysis or subsequent fermentation [144]. For acidic pre-treatment, H_2SO_4, hydrochloric acid (HCl), and flue gas condensates with low pH values have been investigated (Table 6). Acids are thought to hydrolyse cellulose, hemicellulose, and other storage carbohydrates such as laminarin [139,145]. More concentrated acid solutions are more effective at hydrolysing seaweed's cell walls to release its cell contents [145]. However, using acids has been criticised: as a hazard risk (especially concentrated acid), for incurring high acid recycling costs, and for acid-resistant process equipment requirements [145].

The benefits of increased solubility of seaweed on biogas yields appeared quite limited after pre-treatment with NaOH or HCl (Table 6). Pre-treatment of *F. vesiculosus* with 0.2 M HCl (80 °C, 12 h) enhanced methane yield by almost 2.5 times compared to untreated seaweed and was 1.6 times higher than hydrothermal treatment (80 °C, 24 h) [146]. Comparatively, a similar treatment of *Ulva* spp. with 0.1 M or 0.2 M HCl or NaOH at 60 °C, 75 °C, and 90 °C did not increase methane yields compared to only thermal pre-treatment (Table 4) [118]. However, both experiments used different methods for BMP determination, making the direct comparison between the two experiments challenging. Notably, concentrations of furfural compounds present after thermo-chemical treatment of lignocellulosic biomass were not inhibitory to AD of cellulose [134], suggesting that thermo-chemical hydrolysis of seaweed could be useful in increasing biogas yields if the appropriate seaweed species is chosen.

Table 6. Methane yields following thermochemical pre-treatment of seaweed.

Algae (Harvest Time)	Pre-treatment	I/S Ratio; Source	BMP Method	CH_4 Yield (mL g^{-1} VS)	% Change	Ref.
G. vermiculophylla (March 2012)	Algae washed, macerated *Test*: 0.1, 0.3, 0.5 g NaOH g^{-1} seaweed (20 °C, 55 °C, 90 °C, 1 bar, 3.5 bar, 6 bar, 60 and 90 mins) *Control*: Untreated	4:1; brewery WWT	Glass vials with rubber stopper and aluminium crimp, 37 °C, 24 days	353–380	−21 to −26.6	[93]
P. palmata (March 2010)	Dried (40 °C), chopped (2 × 2 cm) *Test*: 0.04 g NaOH g^{-1} TS (50 g L^{-1}): 20–70 °C, 24 h; 160 °C, 0.5 h. 0.02 g HCl g^{-1} TS, 160 °C, 0.5 h. *Control*: untreated	2:1 in 400 mL; sugar WWT	Glass vials with rubber stopper and aluminium crimp, 35 °C, 60 days.	*20–70 °C*: 362–365 *160 °C NaOH*: 282 *160 °C HCl*: 268	+17.5 to 18.5 −8.4 −13	[147]
Seaweed mixture: *Spermothamnion* family (80-90%), *Chaetophorales* family (5–15%), eelgrass (2–5%) (August, 2011)	Dried (54 °C), shredded *Test*: Only H_2O; flue gas condensate (FGC) (pH 1.2); 0.05 M HCl (80 °C, 2 h); 0.2 M HCl (80 °C, 1.5 h) *Control*: Untreated	2:1 in 2 L; Mixture: WWT, maize silage and cattle manure, seaweed adapted sludge.	Fermenter tanks with CO_2 absorbing unit, gas drying unit, and gas volume sensor, 37 °C, 22 days.	*H_2O*: 80 *FGC*: 108 *0.05 M HCl*: 66 *0.2 M HCl*: 121	−8.0 +24.1 −24.1 +39.1	[148]
Ulva spp. (March 2015)	Washed, sun dried (1–2 weeks) *Test*: 0.04 g NaOH g^{-1} TS (20 °C, 24 h); 0.04 g HCl g^{-1} TS (150 °C, 0.5 h) *Control*: untreated	2:1 in 400 mL; sugar wastewater industry	Glass vials with rubber stopper, aluminium crimp, 35 °C until no gas production.	*NaOH*: 148 *HCl*: 77	+12.1 −41.7	[144]
Ulva spp. (Spring 2014)	Fresh water rinsed, blended to slurry. *Test*: 500 mL slurry, no chemical; 0.01 M HCl; 0.1 M NaOH. All 90 °C, 6 h, manual shaking every 0.5 h, 1 min. *Control*: untreated	Sewage sludge digester	Bottles with rubber stopper, aluminium cap, 35 °C, 30 days, shaken manually intermittently.	*Only thermal*: 293.0 *0.1M HCl*: 284.8 *0.1 M NaOH*: 251.3	+15.8 +12.7 −0.7	[118]
L. digitata (Unknown time)	Fresh Water rinsed, dried (75 °C, 24 h), milled. *Test*: 20% solids loading, 2.5% citric acid (CA); 6% citric acid; 1% lactic acid (LA), autoclaved (120 °C, 1 h, 1 atm) *Control*: untreated	2:1 in 30 mL; bovine slurry adapted to seaweed.	Serum bottles (pH 7.3–7.5) with rubber stopper and aluminium crimp, 35 °C, 32 days.	*2.5% CA*: 237 *6% CA*: 69 *LA*: 161	+3.9 −69.7 −29.4	[53]
F. vesiculosus (Unknown time)	Dried, crushed, homogenised *Test*: 0.2 M HCl (80 °C, 12 h); FGC (pH 2.2, 0.13 M, 80 °C, 24 h) *Control*: untreated	2:1 in 2 L; mixture: WWT, corn silage, seaweed adapted sludge.	Fermenter tanks, CO_2 absorbing unit and gas volume sensor, 37 °C, 20 days.	*HCl*: 116 *FGC*: 65	+147 +38.3	[146]

Production of furfural compounds is, however, a major issue when using acid pre-treatment for bioethanol production [149]. When 20 seaweed species collected from the same geographical location were subject to the same pre-treatment (0.2 M H_2SO_4, 121 °C, 15 min), different ethanol yields and different concentrations of 5-hydroxymethyl furfural were obtained [149]. High concentrations of 5-hydroxymethyl furfural appears to be related to low ethanol yields even though high levels of galactose for fermentation are seen (Figure 3). The removal of 5-hydroxymethyl furfural by activated charcoal can successfully increase ethanol yields per gram of galactose present [129]. Interestingly, 5-hydroxymethyl furfural concentrations decreased during fermentation of *G. amansii*, suggesting the metabolism of these compounds by the yeasts [129]. Use of these compounds as a carbon source by aerobic bacteria has also been discussed by Monlau et al. (2014) [134].

Some successes have been achieved for bioethanol production using thermo-chemical pre-treatment methods [150]. The species-dependent effect of thermochemical pre-treatment, with differential effects on solubilisation of seaweed and subsequent biofuel yields [145], appear to be associated with differences in the biochemical composition of the seaweed. For example, fucoidan and alginate were depolymerised at different temperatures [151,152] and, therefore, the proportion of these components in each macroalgal type could affect the extent of seaweed hydrolysis under the same conditions.

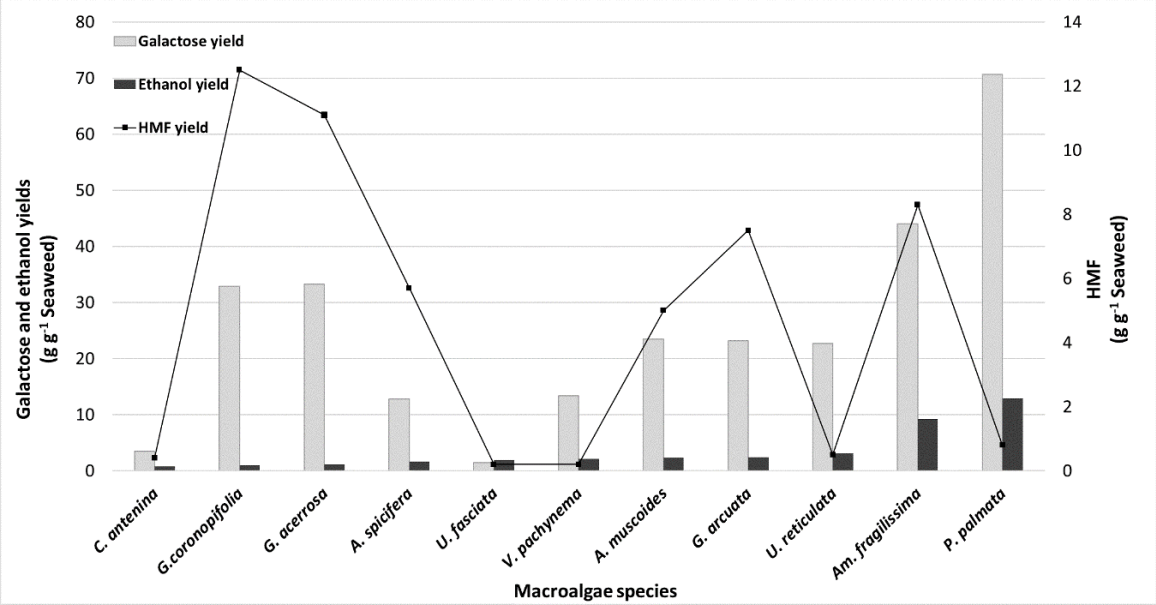

Figure 3. Galactose, 5-hydroxymethyl furfural (HMF), and ethanol yields from different seaweed species using data from Mutripah et al. (2014) [135].

High salt content in seaweeds, including calcium, sodium, magnesium, and potassium salts, can also negatively impact ethanol yields by increasing salinity levels during fermentation [145]. Salinity can also be enhanced by the use of acid and alkali treatments of seaweed [118,145] and, therefore, this pre-treatment method may not be appropriate for seaweeds with high salt contents if high biofuel yields were to be achieved (see also Section 4.5.1).

Additionally, seaweeds adsorbing different concentrations of heavy metals could be differentially solubilised by acid hydrolysis, where the presence of aluminium and iron ions in microalgae affected optimal concentrations of sulphuric acid required for optimal reducing sugar yields [153]. Based on response surface methodology (RMS), for the same seaweed substrate, different experimental conditions (reaction temperature, acid concentration, and reaction time) affects the amounts of 5-hydroxymethyl furfural, glucose, and galactose formed [154].

The difficulty in identifying the appropriate pre-treatment to yield the least inhibitory by-product and high biofuel yields suggest that an approach carried out by Dandikas et al. (2014) [155] and

Dandikas et al. (2018) [156] may be useful for seaweed. This involves performing a primary component analysis of numerous different species of seaweed harvested during different times of year, and locations, and identifying the correlation of different components to biofuel yields. Not only would this help to identify the appropriate pre-treatment method for different seaweed, but also identify the characteristics of suitable seaweeds that can be mass-cultivated for biofuel production.

4.3.2. Peroxide Treatment

This pre-treatment method uses thermal treatment to enhance the disruption of seaweed crystalline structures and hydrogen bonds by hydroxyl radicals [157]. A higher cellulose to glucose conversion rate of 88.1% has been achieved using this treatment compared to alkali and acid pre-treatments of seaweed [32,157], after hot water pre-treatment (100 °C, 30 min) followed by a hydroxyl radical reaction step (0.018% H_2O_2 and 11.9mM $FeSO_4$) [32]. Additionally, hydroxyl radical pre-treatment of 4 different species of seaweed all showed an increase in glucose yield per gram dry matter compared to untreated seaweed, indicating an increase in enzymatic digestibility [157]. The risk of producing inhibitory furfurals is also reduced with peroxide treatment and polysaccharides, such as laminarin and alginates, remained intact with this pre-treatment [157,158].

4.4. Biological Treatment

Biological pre-treatment involves the use of enzymes or the direct use of microorganisms for the degradation of seaweed. Recently, Perez et al. (2018) [159] studied the capabilities of laminarinase and cellulase in saccharifying *Sargassum* spp. Commercial enzymes such as Celluclast 1.5L, Novozym, and other cellulase preparations, or a mix of enzymes for enzymatic saccharification after acid hydrolysis, have also been used [30,150,160,161].

For AD purposes, direct addition of suitable microorganisms able to degrade the complex polysaccharides within seaweeds is more effective than use of enzymes for improving biogas yields (Table 7) [99,162]. Decomposed seaweed collected from the beach also produced higher biogas yields compared to fresh seaweed [163]. Unwashed *Ulva* spp. digested using seaweed-adapted slurry containing seawater, mud, and sand showed higher methane yields and a higher biodegradability of 36% compared to washed seaweed digested with seaweed-adapted slurry, initially fed food waste (Table 7). Treatment of *Laminaria japonica* using *Vibrio harveyi* and *Vibrio alginolyticus*, capable of producing alginate lyase to break down alginates, also yielded higher amounts of VFAs available for biogas production compared to alkali pre-treatment [164].

V. harveryi can also grow on cellobiose [164], which may have enabled the increase in VFA compared to untreated seaweed during the pre-treatment of red and green seaweed by *V. harveryi* and *V. alginolyticus* [165]. Multiple mechanisms of biological pre-treatment have also been discovered using *Aspergillus fumigatus* to degrade *Ulva* spp. via solid-state fermentation [144]. Methane yield enhancements were also observed when treating a mixture of seaweed from the Mexican Caribbean using *Trametes hirsuta*, known to enzymatically degrade lignocellulosics [166].

There are also continuous isolations of new enzymes and microorganisms capable of increasing hydrolysis of seaweed's complex polysaccharides [167,168]. Microbes isolated from sheep fed with seaweed could degrade the seaweed's polysaccharides, but methane yields were higher from individual polysaccharides than the total biomass even though similar levels of acetate (a component of VFA) were produced [169]. This could be due to polyphenols present within seaweed (see Section 4.5.2) [170,171].

Table 7. Methane yield following biological pre-treatment of seaweed.

Algae (Harvest Time)	Pre-treatment	Inoculum	BMP Method	CH_4 Yield (ml g^{-1} VS)	% Change	BI (%)	ΔBI (%)	Ref.
Ulva rigida (July–September 2013)	*Test*: 7.5 mL *A. niger* filtrate to 50 mL blended seaweed (80% (*w*/*v*) in water), 50 °C, 100 rpm, 2 h. Repeated with β-glucosidase. *Control*: untreated seaweed.	I/S ratio: 1:1 in 500 mL Source: (unknown) treatment plant.	Bottles with rubber stoppers, 37 °C. Gas measured using syringe plunger.	-	*A. niger*: +33 [1]	63 [2]	+17.1 [2]	[99]
				-	*β-glu.*: +28 [1]	58 [2]	+7.8 [2]	
Ulva spp. (March 2015)	*Test*: washed, sun dried (1–2 weeks), grounded. 35% seaweed in Mandels' salt solution, autoclaved (120 °C, 20 mins); inoculated: *A. fumigatus* SL1 conidia suspension; incubated (50 °C, eight days). *Control*: untreated.	I/S ratio: 2:1 in 400 mL with buffer and nutrients Source: sugar wastewater industry.	Vials with rubber stopper and aluminium crimp, 35 °C till gas production halts.	153	+15.9	57 [3]	+16.3 [3]	[144]
Ulva spp. (June 2013)	*Test*: 100 g washed or unwashed seaweed (macerated <5 mm) in 100 g freshwater or thalassic hydrolytic inoculum; 3 days, 37 °C. *Control*: untreated with hydrolytic inoculum	Source: Freshwater: washed seaweed-adapted, original slurry: food waste. Thalassic: Unwashed seaweed-adapted, original slurry: seawater, mud, sand	100 g of respective methanogenic inoculum, 37 °C, 6 days.	77.7	*Freshwater*: +42.8	27	+42.4 [3]	[172]
				180.9	*Thalassic*: +72	63	+71.8 [3]	
L. digitata (unknown time)	Freshwater rinsed, dried (75 °C, 24 h), milled. 20% (*w*/*v*) seaweed in water with: *Cellulase (C)*: 37 °C; *Alginate lyase (AL)*: 37 °C; *or Celluclast® 1.5L (C1.5)*: 40 °C. All incubated: 300 rpm, 24 h *Control*: water (room temp., 24 h)	I/S ratio: 2:1 in 30 mL (pH 7.3–7.5) Source: bovine slurry (seaweed-adapted).	Bottles with rubber stopper and aluminium crimp, 35 °C, 32 days.	*C*: 232	+1.8	-	-	[53]
				AL: 225	−1.3	-	-	
				C1.5: 72	−68.4	-	-	

[1] BI calculated from theoretical methane yields based on per gram COD; [2] based on biogas yields; [3] BI calculated from theoretical yields based on crude protein, lipids and carbohydrates.

4.5. Inhibitor Removal

As discussed in previous sections, there is a range of inhibitors, such as phenolics and salts, implicated in limiting production yields of both ethanol during fermentation and methane during AD. The removal of these inhibitors as part of the pre-treatment process before fermentation and AD may not only improve biofuel yields, but also obtain potential high-value by-products. The following sections further discusses the role of these inhibitors and the possible removal methods of these compounds.

4.5.1. Salts

Although low salt concentrations can stimulate microbial growth, high salt concentrations ($\geq$10 g L^{-1}) inhibit the methanogenesis phase of AD (Figure 1A) through an increase of osmotic pressure or dehydration of methanogenic microorganisms [37,173]. The sodium cation predominantly determines the toxicity of salt but other light metal ions, such as potassium, are also toxic to methanogens at high levels [174]. An optimal sodium concentration for mesophilic methanogens in waste treatment processes of 230 mg Na L^{-1} has been recommended [175]. However, the approximate level of sodium found in seawater is 14 g Na L^{-1}, and at this level, mesophilic methanogenic activity is halved [175–177].

Anaerobic digesters can be acclimatised to higher salt levels if they are continuously exposed to gradually increasing salt concentration by adaptation of methanogens rather than salt shock [173,178]. After acclimation, the sodium concentration to halve methanogenic activity can increase to 37.4 g Na L^{-1} [175]. Thalassic conditions are capable of producing higher methane yields when digesting unwashed seaweed compared to freshwater conditions digesting washed seaweed (Table 7). It, therefore, appears possible to produce biogas from seaweed without fresh water washing, which increases production costs. Otherwise, the high salt concentration could also be mitigated by mixing the seaweed biomass with other types of biomass to 'dilute' the salt [13].

4.5.2. Phenolics

Phenols are a diverse group of polymerisation products of phloroglucinol (1,3,5-trihydroxybenzene), widely distributed in plants and algae with >8000 phenolic compounds being separated from terrestrial and marine organisms [179,180]. High levels of phenolics are found in many seaweeds, with brown seaweed containing up to 14% DW [181]. The phenolic compounds in cell wall structures are generally considered to be used as a chemical defence mechanism against grazers, bacteria, fungi, and other epiphytes [72,182].

Low molecular weight phlorotannins, ranging from 126 kDa to 650 kDa [183], damage microbial cells by altering membrane permeability, including those of gram-negative bacteria [184], causing leakage of intracellular components and inactivating essential enzymatic systems; with lower molecular weight phenolics being more toxic to microorganisms than high molecular weight compounds [134]. Phenolic extracts from *S. muticum* showed antimicrobial activity against some aerobic bacteria [185]. Hierholtzer et al. (2013) [186] highlighted the role of phlorotannins in bactericidal effects on anaerobic microorganisms found in sludge from wastewater treatment plant. Resistive microbes to the bactericidal effects were also detected, suggesting that biogas production is still possible from seaweed containing phlorotannins. Polyphenols extracted from brown seaweed also inhibited α-amylase, α-glucosidase, and lipase [187,188], implying possible enzymatic inhibition by seaweed during the hydrolysis stages of fermentation and AD (Figure 1A,B).

The mode of action of phlorotannins, found in brown seaweed, on anaerobic microorganisms remains obscure, and there is little information available regarding their influence on mixed microbial cultures found in anaerobic digesters [186]. Concentrations of up to 7% gallic acid, epicatechin, and phloroglucinol did not inhibit the breakdown of simple and readily digestible compound, glycerol, but there were statistically significant interactions on methane yield between high levels of phenolics and complex substrates [69]. Tabassum et al. (2016) [189] found an association between the high phenolic content in *A. nodosum* and reduced methane yields. Moen et al. (1997) [170] found that

the methanogenesis phase of AD was inhibited by polyphenols (Figure 1A), and biogas production was improved during AD of *A. nodosum* when polyphenols were 'fixed' by formaldehyde. Moreover, alginate degradation was suggested to be limited by the binding of polyphenols to alginates which limited the degradation by alginate lyases during AD [171]. It may, therefore, be beneficial to understand the concentrations of phlorotannins in a reactor during AD of seaweed to elucidate the magnitude of its inhibitory role.

The removal of polyphenols using formaldehyde would be too costly on a large-scale. Alternatively, there is a growing body of research showing the potential uses of polyphenols and seaweed extracts, including pharmacology and food industry use [187,190,191]. Thus, the removal of phlorotannins before AD or fermentation may form the basis of a useful biorefinery, yielding potentially bioactive substances at the same time as improving biofuel yield. These phenolic compounds could be extracted as part of the pre-treatment method. For example, microwave-assisted extraction has been achieved [192], and subsequent fermentation and AD could be carried out on remaining residues [193].

4.5.3. Heavy Metals

Seaweeds can accumulate heavy metals on cell wall polysaccharides and are able to bind and chelate to heavy metals [194]. These heavy metals include Fe, Pb, Cd, Mn, and Cu which have been measured in seaweed in the Bulgarian Black Sea [195], as well as Zn, Cr, and Hg in seaweed from the Aegean Sea [196]. Heavy metals are indicated as inhibitors of AD, provided the freely available ions exceed specific inhibitory threshold values of the particular heavy metal [197]. The potential additive inhibitory effect of different heavy metals may also lower these thresholds [197]. However, the removal of heavy metals using iminodiacetic acid (IDA) cryogel adsorbents reduced methane yields during the two-stage AD of seaweed compared to those without heavy metal removal [198]. This was suggested to be due to higher sulphate content as a consequence of heavy metal removal, resulting in an increase in H_2S and sulphates as less are precipitated off [198]. Furthermore, the concentrations of heavy metals initially present may not have exceeded threshold values to cause inhibitory effects.

Nevertheless, heavy metal removal will be required if seaweeds are to be used in a biorefinery, with digestate being used as soil conditioners or fertilisers [199]. For example, cadmium concentrations found in the digestate of a digester digesting seaweed harvested in Germany was above legal limits for fertilisers [148]. Nkemka and Murto (2012) [199] have suggested using sulphide precipitation followed by IDA-cryogel carriers to remove and possibly recover these metal ions, while others have suggested carbonate or hydroxide precipitation [197].

4.5.4. Other

Inhibitors forming as a result of thermal or thermo-chemical pre-treatment, including 5-hydroxymethyl furfural and furfural, have been discussed by Shobana et al. (2017) [200]. Briefly, removal of these compounds can be achieved by methods such as 'overliming' (providing calcium ions and a high pH, often using calcium hydroxide [201]), use of ethyl acetate, or using activated carbon [200]. Otherwise, different hydrolysis reactor configurations have been found to reduce the formation of these inhibitory compounds [130]. However, the cost-effectiveness of these methods will need to be analysed for the subsequent increase in biofuel yields after detoxification.

5. Conclusions

Seaweed biomass has the potential to serve as a feedstock for biofuel production but is currently harvested primarily for high-value natural products and food only. A biorefinery approach to processing seaweed would open up opportunities to sustainably process the biomass into a spectrum of food and feed ingredients, bio-based chemicals, and materials, as well as biofuel, without affecting current industries. However, to fully realise the value of seaweed biomass in terms of its potential to produce biofuel, appropriate seaweed pre-treatments will be required. Green, brown, and red seaweeds differ in terms of their polysaccharide composition and contents, and the arrangement of

these polymers in cell wall architectures. In order to maximise yields of biofuel, be it biogas from AD processing, or bioethanol from alcoholic fermentation, or both, these polysaccharides need to be hydrolysed at appropriate rates to their component sugars if they are to then serve successfully as feedstock for processing to biofuels by the corresponding microbial communities. Biofuel yields from either AD or fermentation of seaweed are also greatly dependent on seasonality of harvested seaweed and their location at the time of harvest, since these factors influence salt content, heavy metal content, polyphenols, structural make-up, and the relative content of the different polymeric carbohydrates.

This review has shown that there is a range of different methods available that will variously break down seaweed cell wall architecture, improve access to seaweed polymers for their initial hydrolysis, and remove/prevent formation of compounds, such as furanic acids and phenolics, that might be inhibitory to subsequent microbial metabolic processes. These include reducing particle size, beating, thermal and thermo-chemical methods, and biological methods, such as the addition of specific microbial inocula for AD processes or addition of cellulolytic enzymes for fermentation. Acid or alkali pre-treatments, whilst effective in swelling fibres and hydrolysing polymers, unfortunately increase the risk of producing inhibitory compounds and consequently amongst chemical treatments, appear less attractive than for example, the use of peroxides. Washing seaweed in freshwater is a common practice to remove inhibitory salt, but since results to date have been mixed in terms of the effects on biofuel yield, the value of this pre-treatment warrants further research to reduce impacts on sustainability. On the other hand, a major area of research need lies in devising practices that could reduce the heavy metal content in seaweeds: seaweeds accumulate these on negatively-charged polysaccharide components, and their presence not only reduces biofuel yields but requires monitoring to ensure safe practice in the management of subsequent digestate. Finally, note should be made of pre-treatments based on ensiling seaweed. Although its effect on subsequent biofuel yields is not well-developed, this practice could serve to ensure continuity in biomass supply throughout the year, as well as overcome the influence of seasonality on seaweed properties, and thereby significantly improve processing sustainability in the longer term.

In summary, with a sound understanding of the history and source of seaweed and its biomass composition and properties, it becomes possible to design suitable seaweed pre-treatments that will maximise sustainability and the EROI for biofuel production, at lowest cost.

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