

Review

Fermentation of Biodegradable Organic Waste by the Family *Thermotogaceae*

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Abstract: The abundance of organic waste generated from agro-industrial processes throughout the world has become an environmental concern that requires immediate action in order to make the global economy sustainable and circular. Great attention has been paid to convert such nutrient-rich organic waste into useful materials for sustainable agricultural practices. Instead of being an environmental hazard, biodegradable organic waste represents a promising resource for the production of high value-added products such as bioenergy, biofertilizers, and biopolymers. The ability of some hyperthermophilic bacteria, e.g., the genera *Thermotoga* and *Pseudothermotoga*, to anaerobically ferment waste with the concomitant formation of bioproducts has generated great interest in the waste management sector. These biotechnologically significant bacteria possess a complementary set of thermostable enzymes to degrade complex sugars, with high production rates of biohydrogen gas and organic molecules such as acetate and lactate. Their high growth temperatures allow not only lower contamination risks but also improve substrate solubilization. This review highlights the promises and challenges related to using *Thermotoga* and *Pseudothermotoga* spp. as sustainable systems to convert a wide range of biodegradable organic waste into high value-added products.

Keywords: *Thermotoga*; *Pseudothermotoga*; thermophilic bacteria; fermentation; hydrogen; lactic acid; waste valorization; added-value products



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1. Introduction

Biodegradable organic waste from industrial processes provide versatile carbon-rich feedstocks that can be efficiently converted into high-value products and biofuels [1–3]. As described in the 2018 EU Bioeconomy Strategy report [4], the economic value of biodegradable waste is starting to be recognized by agricultural, forestry, chemical, and energy sectors. With the development of the bioeconomy, the demand for these secondary products is likely to increase, changing the economic conditions of production. More than 3300 megatonnes of residual biomass are estimated to be generated annually from barley, maize, rice, soybean, sugar cane, and wheat. In Europe only, 900 megatonnes of wastepaper, food, and plant materials are generated each year. In the fisheries sector, about 40 megatonnes of fish may be discarded each year during European commercial fishing; in the forestry sector, woody biomass residues have been estimated to be 5100 megatonnes per year globally [4].

From the perspective of constructing a circular economy based on biowaste, it is important to support the development of industrial symbiosis for feed materials, i.e., one

industry's waste becomes the starting material for another. One example is the treatment of waste and residues for energy production, including the production of biogas through anaerobic digestion of biowaste and wastewater [5–9] as well as the integrated production of chemical products and bioenergy in biorefineries [10,11]. The food processing industry is exploring the potential of recovering the energy contained in food residues on site [12–14]. A typical fermentation process consists of the controlled digestion of biodegradable materials under anaerobic conditions in closed reactors, at temperatures suitable for mesophilic or thermophilic bacteria. Fermentation products include (1) digested solids that can be used as a soil conditioner; (2) biogas that can be consumed directly or refined for higher levels of demands, such as fuels for vehicles.

In this framework, special attention has been paid to fermentation processes in which biowaste is treated for generating hydrogen gas [15,16]. Hydrogen represents a promising bioenergy fuel since it is clean, renewable, abundant, and cheap; it produces only water as the end-product when used as a fuel, without any pollutants [17–19]. Dark fermentation operated by anaerobic thermophilic bacteria is an attractive way to produce biohydrogen because of the high biogas evolution yields and the versatile feedstocks [17,18,20–24]. Among extreme thermophilic bacteria, members of the order *Thermotogales* have proven to be promising candidates due to high H₂ yields that are close to theoretical values and the ability of some members to recycle produced CO₂ into lactic acid synthesis [25–35]. These bacteria are capable of fermenting not only simple and pure sugars but also complex carbon sources with various production rates [36–41].

This review focuses on the potential of the members of the order *Thermotogales* to ferment biodegradable organic waste. Feedstock pretreatments and their effects on cultural parameters are also discussed [24,42–44]. With the development of molecular and biochemical tools applicable to extremophilic bacteria, it has been possible to demonstrate the involvement of specific enzymes and putative pathways in the uptake and degradation processes [45–47], offering new routes in the evaluation of the application of these biological systems.

The examined substrates discussed here are divided into four groups, based on their main constituents and relative origins: food waste, lignocellulosic waste, glycerol, and microalgal biomass. Their main characteristics, compositions, utility, and fermentation processes involving *Thermotogaceae* family members are discussed in subheadings, and the best results are summarized in tables. It provides a concise and precise description of the experimental results, their interpretation as well as the conclusions drawn from the studies.

2. *Thermotogaceae* Family: Features and Roles in Sugar Fermentation

2.1. General Characteristics

The phylum *Thermotogae* represents a critical node in the phylogenetic tree of bacteria. Bhandari and Gupta's classification was taken as a model because it was based on genomic data from several *Thermotogae* species, and the molecular markers were identified to estimate the relationship within the phylum [48]. Recently, with the significant advances in modern taxonomy practices, Belahbib et al. [49] have proposed some changes to Bhandari and Gupta's classification system. Nowadays, the phylum *Thermotogae*, comprising of mesophilic, thermophilic and hyperthermophilic bacteria, has more than 52 species belonging to four orders: *Thermotogales*, *Kosmotogales*, *Petrotogales* and *Mesoacidotogales* [50]. The order of *Thermotogales* includes two families, *Thermotogaceae* and *Ferroidobacteriaceae*, and their species are distinguished by the shared presence of conserved sequences [48–50]. The family *Thermotogaceae* contains two genera, *Thermotoga* and *Pseudothermotoga*, which are anaerobic, rod-shaped bacteria, surrounded by a sheath-like structure called "toga", resulting in large periplasmic spaces at the poles of each rod [28,48]. The genus *Thermotoga* retains the species *T. maritima*, *T. neapolitana*, *T. petrophila*, *T. naphthophila*, *Thermotoga* sp. EMP, *Thermotoga* sp. A7A, and *Thermotoga* sp. RQ2, while *P. lettingae*, *P. thermarum*, *P. elfii*, *P. subterranea*, and *P. hypogea* belong to the new genus *Pseudothermotoga* [28,48,49]. Members of the *Thermotogaceae* family have been isolated from geothermal environments across the

globe, including oil reservoirs, submarine hot springs, and continental solfataric springs, with their optimal growth temperature in the range of 77–80 °C [28]. They can reduce elemental sulfur and use hexoses, pentoses, disaccharides, glucans, xylans, glucomannan, galactomannan, pectin, chitin, and amorphous cellulose as main substrates during fermentation [28,48]. *Thermotoga* species generate H₂ close to the Thauer limit for anaerobic fermentation (i.e., 4 mol H₂/mol glucose), CO₂, acetate, and other minor products such as lactic acid, ethanol, and alanine [28,51]. According to the classical model of fermentation referred to as dark fermentation (DF), *Thermotoga* spp. harvest energy mainly by glycolysis via the Embden–Meyerhoff–Parnas pathway (EMP), although a simultaneous activation of 15% of the Entner–Doudoroff pathway (ED) has been described [25,52]. EMP is the most common route for oxidation of glucose (and other hexoses) and to supply energy (ATP), reducing equivalents (NADH), and pyruvate, which undergoes terminal oxidation (acetate) or is used for biosynthesis (e.g., acetyl-CoA) [25,53,54]. Moreover, some members of the *Thermotogaceae* family possess an unprecedented anaerobic mechanism, called capnophilic lactic fermentation (CLF), that represents the first example of biological non-autotrophic sequestration of CO₂ in hyperthermophilic bacteria, more advantageous than classical dark fermentation regarding the production of hydrogen through degradation of carbon substrates [32,33,55–57]. This process is activated during glucose fermentation under CO₂ sparging, and it is based on the coupling of acetate and CO₂ derived from glycolysis to produce enantiopure L-lactic acid without affecting H₂ yields [32,33,55,56,58–60]. This mechanism was extensively studied in *Thermotoga neapolitana*, and only a few members of the *Thermotoga* and *Pseudothermotoga* genera operated this CO₂ recycling mechanism [34]. Under CLF conditions, the bacteria also shift their glucose utilization through downregulation of EMP, activation of ED and/or OPP pathways, and the upregulation of some bifurcating enzymes that could supply NADH in these metabolic processes [60].

These advantageous properties of *Thermotoga* spp., i.e., valorization and transformation of biodegradable organic waste with H₂ production and, in some cases, the sequestration of CO₂ to recover energy and generate value-added products, have positioned these bacteria as promising candidates in the biotechnological field. In addition, many of their enzymes are capable of deconstructing complex biomass into basic components for fermentation [46,61]. Although biohydrogen production from hyperthermophilic bacteria is far from an industrial scale application, these studies provide common knowledge about the potential of the family *Thermotogaceae*, fueling interest in future exploration.

2.2. Fermentation of Pure Monosaccharides and Polysaccharides

Members of the *Thermotogaceae* family can ferment a wide range of mono- and polysaccharides as carbon and energy sources. In the identification processes of each new *Thermotoga* species, the authors tested a panel of pure monosaccharides to analyze strain adaptability and discovered potential alternative carbon sources for these organisms [28,36,37,62–69]. In the past a few years, independent work also evaluated the effects of monosaccharides on fermentation end-product yields, mainly in *T. maritima* and *T. neapolitana*. Glucose is the preferred substrate, and it produces the greatest amount of hydrogen, with yields higher than 3.5 mol/mol glucose [36,38,70–73]. Using other sugars as the sole carbon source, such as arabinose, fructose, mannose, galactose and ribose, resulted in similar hydrogen production rates in both species (H₂ yields around 3 mol/mol sugar) [28,36,58,73–77]. Variations in sugar concentration seem to remarkably affect the H₂ production and substrate utilization in *T. neapolitana* [72]; in *T. maritima*, lower H₂ yields have been observed (around 1.1 ± 0.1 mol H₂/mol xylose) [77].

Thermotoga spp. can also metabolize pure di- and tri-saccharides, such as sucrose, lactose, maltose and cellobiose, and polysaccharides including starch, glycogen, carboxymethyl cellulose (CMC) and cellulose [28,72,73,77–84]. The ability to hydrolyze and ferment a wide range of polysaccharides represents the basis of the great potential and versatility for biodegradable organic waste valorization by the family *Thermotogaceae*.

Cellobiose was tested with *T. maritima* at the concentration of 12.5 mmol/L, resulting in 100 mmol/L of hydrogen [77]. At the end of the fermentation, 3.6 ± 0.2 mol H₂/mol sugar was obtained, even though only 49% of the cellobiose was consumed (Table 1), suggesting that cellobiose is a difficult substrate to hydrolyze and may require a different modulation of enzyme activity. Improvements in hydrolysis of cellulosic materials and in H₂ production are possible by cocultivating *T. maritima* with *Caldicellulosiruptor saccharolyticus* [77].

Regarding sucrose, studies with *T. neapolitana* showed that the fermentation process was similar to that using glucose [72,73]. In Ngo et al., sucrose consumption rate, acetic and lactic acid production rates were comparable in batch cultures with and without pH control. A slight increase in H₂ yield was observed in sucrose-based culture (Table 1) [72]. The same trend was observed under the CLF condition in sucrose fermentation, producing 2.56 ± 0.1 mol of H₂, 25.12 ± 1.43 mM of acetate, and 16.95 ± 1.34 mM of lactic acid per mole of glucose equivalent (Table 1) [73].

Fermentation of laminarin led to H₂ yield (3.70 ± 0.17 mol per mole glucose eq) and acetic acid production (28.75 ± 0.81 mM) in *T. neapolitana* sp. *capnolactica* (*T. nea clf*) culture, similar to glucose and sucrose fermentation. However, LA level (7.60 ± 0.27 mM) was two times lower (Table 1) [73].

On CMC, a clear reduction of H₂ and organic acid production was observed in *T. nea clf* because this substrate is poorly metabolized [73,82]. Nguyen et al. described the capability of *T. neapolitana* and *T. maritima* to grow on CMC [82]. Only 95.5 ± 4.8 mL and 96.4 ± 4.8 mL H₂/g glucose eq. were produced by *T. maritima* and *T. neapolitana* growing on CMC, while 187.1 ± 9.4 and 174.5 ± 8.7 mL H₂/g glucose eq. were produced by the two strains, respectively, when growing on starch. (Table 1) [82].

The same effect was observed in *T. neapolitana* subsp. *capnolactica* growing on CMC, with a H₂ yield of 2.05 ± 0.13 mol H₂, 3.40 ± 0.30 mM of AA, and 1.18 ± 0.05 mM of LA per mol of glucose eq. In contrast to other sugars, only 10% of CMC was consumed after 72 h of fermentation, indicating that CMC should probably be pretreated to improve its accessibility to the cells [73]. No growth was observed with *P. elfii* growing on sucrose and CMC [85].

Several papers reported that *T. maritima* and *T. neapolitana* were able to degrade cellulose [17,22,86], which stimulated further research on the topic. Nguyen et al. [82] showed a drastic decrease in H₂ yields in both *T. neapolitana* and *T. maritima* growing on cellulose, with only 27.8 ± 1.3 mL H₂/g glucose eq. for *T. maritima* and 30.7 ± 1.5 mL H₂/g glucose eq. for *T. neapolitana*, suggesting that pretreatment is needed to better ferment this substrate (Table 1) [82]. In Nguyen et al. [81], pure cellulose was pretreated with three different chemical methods, acid (H₂SO₄), alkali (NaOH), and ionic liquid ([C4mim] Cl, 1-butyl-3-methylimidazolium chloride). Ionic liquid turned out to be the most effective pretreatment agent, with 18% of cellulose dissolution [81]. N₂ sparging leads to an improved H₂ production rate in *T. neapolitana* growing on cellulose, reaching 1280 ± 58.0 mL H₂/L culture and 2.20 ± 0.10 mol H₂/mol glucose eq., compared to 1.22 ± 0.067 mol H₂/mol glucose eq. without sparge; this demonstrates the feasibility of using cellulose and other complex feedstocks in *Thermotoga* fermentation [81].

Table 1. Fermentation of pure polysaccharides by *Thermotoga* spp.

Substrate	Strain	T (°C)	Start pH	Mixing Speed (rpm)	Gas Sparge	Reactor Volume (mL)	Working Volume (mL)	Substrate Consumption (mmol/L)	H ₂ yield (mol H ₂ /mol sugar)	Organic acids Production (mM)	Ref.
Sucrose	<i>T.nea cf</i>	80	7.5	250	CO ₂	3800	500	23.30 ± 0.69	2.56 ± 0.1	AA 25.12 ± 1.43 LA 16.95 ± 1.34	
Laminarin	<i>T.nea cf</i>	80	7.5	250	CO ₂	3800	500	24.73 ± 0.40	3.70 ± 0.17	AA 28.75 ± 0.81 LA 7.60 ± 0.27	[73]
CMC	<i>T.nea cf</i>	80	7.5	250	CO ₂	3800	500	2.75 ± 0.25	2.05 ± 0.13	AA 3.40 ± 0.30 LA 1.18 ± 0.05	
Sucrose	<i>T.nea</i>	75	7.5 pH control	300	N ₂	3000	1000	14.69 ± 0.06	4.95 ± 0.25	AA 25.66 LA 1.69	[72]
			7.5 w/o pH control					13.78 ± 0.70		3.52 ± 0.18	
Cellulose pretreated with [C4mim] Cl	<i>T.nea</i>	80	7.5	150	N ₂ w/o N ₂	120	40	-	2.20 ± 0.1 1.22 ± 0.067	-	[81]
Cellulose	<i>T.nea</i>	80	7.5	-	N ₂	120	50	10.18 ± 0.08	30.7 ± 1.5 *	AA 4.09	
	<i>T.mar</i>	75	6.5	-				8.82 ± 0.07		27.8 ± 1.3 *	
Starch	<i>T.nea</i>	80	7.5	-	N ₂	120	50	5.51 ± 0.09	174 ± 8.7 *	AA 22.04	[82]
	<i>T.mar</i>	75	6.5	-				6.01 ± 0.09		187 ± 9.4 *	
CMC	<i>T.nea</i>	80	7.5	-	N ₂	120	50	6.80 ± 0.08	96.4 ± 4.8 *	AA 8.97	
	<i>T.mar</i>	75	6.5	-				6.99 ± 0.08		95.5 ± 4.8 *	
Cellobiose	<i>T.mar</i>	70	7.2	90	N ₂	120	50	6.125	3.60 ± 0.2	-	[77]

All the experiments have been performed in batch, with substrate load of 5g/L. AA, acetic acid; LA, lactic acid; CMC, carboxymethyl cellulose. *T.nea cf.*, *Thermotoga neapolitana* subsp. *capnolactica*; *T.nea.*, *Thermotoga neapolitana*; *T.mar.*, *Thermotoga maritima*. H₂ yield column: * mL/g.

3. Biodegradable Organic Waste

Biodegradable organic waste represents the main end-products from agro-industrial processes and nowadays serve as popular feedstocks for anaerobic fermentation [1–3]. These biomass materials contain carbohydrates, lipids, lignocellulosic compounds and proteins, which provide a balanced supply of carbon, nitrogen, sulfur, minerals, vitamins, and other small molecules [14]. Over the years, organic biomass has acquired an increasingly important role because of their abundance and low costs. Originally considered the “waste” of industrial processes, they represent a new economic opportunity to enhance energy production [1–3,6,9,10,13].

3.1. Food Waste

Food waste is generated by the entire food system, from production to processing and to consumption, although there are considerable uncertainties about the estimated quantities related to different stages. Food waste comes from various sources including agro-industrial processes, households, and the hospitality sector. It is generally composed by carbohydrates, protein, lipids and inorganic compounds, in variable proportions depending on the source of the food waste. Their accumulation associated to population growth has become a serious problem [87]. The food processing industries are exploring the potential of recovering the energy contained in food residues on-site, through biogas production or in dedicated combined heat and power plants. Anaerobic digestion is an effective way to manage food waste, with advantages like low costs, less residual waste and production of biohydrogen [10–12,88]. Of the food waste available, only some of it will be discussed in this review based on their applications in *Thermotoga* fermentation.

3.1.1. Fruit and Vegetable Waste

Fruit and vegetable waste (FVW) is the most abundant waste obtained in wholesale markets. These substrates are mainly composed of carbohydrates, cellulose, and hemicellulose, making them good candidates to produce biohydrogen [89–91]. It is already known that these compounds are used to produce biogas and to reduce landfill maintenance costs due to their high organic content and good degradability [5,39,89–91]. Moreover, no special pretreatments are required for these substrates, simply the reduction in size with an electric blender and subsequent filtration and homogenization. This procedure guarantees the absence of extremophilic and/or halotolerant microflora that are able to produce H₂, and allows for better sugar solubilization. Saidi et al. studied fruit and vegetable waste fermentation with *T. maritima*, using a simplified medium containing natural seawater as the inorganic compound source [39]. Under this experimental condition, 3.89 ± 0.05 mol H₂/mol hexose, 1.96 mol AA/mol C₆ and 1.2 ± 0.2 mmol LA/L were obtained (Table 2) [39].

Carrot pulp is a vegetable residue obtained in carrot juice production, thus available in large quantities as a by-product. It is composed of a soluble water fraction (30%) consisting of sucrose, glucose and fructose, and a considerable amount of insoluble nonstarchy polysaccharides (NSP) (30–40% of the total dry matter) derived from cell wall hemicellulose and pectin [75,92]. Glucose was the most abundant residue in the NSP fraction, while in much lower concentrations were arabinose, galactose, mannose, rhamnose, xylose, and galacturonic acid derived from pectin [75,92]. Both the untreated material and the hydrolysate fraction were tested in *T. neapolitana* fermentation, and the importance of pretreatment was highlighted. After the enzymatic hydrolysis of the insoluble polysaccharide fraction by cellulases, the soluble sugar content in the total liquid hydrolysate increased, for example, 160 g of dry matter produced 4.0 g/L of sucrose, 39.2 g/L of glucose and 14.0 g/L of fructose. However, 30% of the initial dry matter remained insoluble [75]. *T. neapolitana* only fermented the hydrolyzated form, producing 2.4 mol H₂/mol C₆, 1.1 mol AA/mol C₆, and 0.30 mol LA/mol C₆ (Table 2). A reduction of sugar consumption rate was observed compared to pure sugar fermentation, maybe due to the insoluble residual fraction that can inhibit fermentation [75].

Potato steam peels (PSP) derive from the potato processing industry. This waste is rich in starch, and it is available in large quantities. It is generally used for animal feeding but is now regarded as a potential substrate in biohydrogen production [93]. In fact, a life cycle assessment showed that it is more beneficial to primarily use PSP to produce hydrogen and use protein-rich solids in animal feed, rather than using potato steam peels directly [94]. Mars et al. described H₂ production during *T. neapolitana* fermentation with potato steam peels as the carbon source [95]. Different pretreatment states of PSP were used as organic substrates (untreated PSP, PSP-H1 and PSP-H2). Untreated PSP is composed of 39% starch, 3.8% nitrogen, and 8.5% ash. PSP treated with alpha-amylase and then clarified, referred to as PSP-H1, contains soluble dextrans, 21 mM glucose, 7 mM acetate, and 25mM lactate. PSP-H1 further hydrolyzed with amyloglucosidase and clarified, referred to as PSP-H2, contained 407 mM glucose, 10 mM acetate, and 33 mM lactate [95]. Untreated PSP led to an H₂ yield of 3.8 mol H₂/mol glucose units with 1.80 mol AA/mol glucose units and 0.2 mol LA/mol glucose units by *T. neapolitana* (Table 2) [95]. This high hydrogen yield based on starch content in PSP could be an overestimation because other unidentified substrates in PSP may have also been consumed. Using PSP-H1 and PSP-H2, a decrease of all product yields was observed (PSP-H1: 2.6 mol H₂/mol glucose, 1.20 mol AA/mol glucose. PSP-H2: 3.3 mol H₂/mol glucose, 1.50 mol AA/mol glucose) [95]. Therefore, untreated PSP may be a suitable alternative to the use of hydrolysates.

Onion waste (OW) is the result of industrial onion (*Allium cepa* L.) cultivation, harvesting and processing. Nowadays onions are the second most important horticultural crop worldwide after tomatoes. The increase in onion demand over the years has led to an increase in onion waste production, representing an environmental concern. They are not suitable for fodder because of their aroma, and neither can they serve as an organic fertilizer because of the rapid development of phytopathogenic agents. Onion waste mainly includes undersized, malformed, diseased or damaged bulbs as well as onion skins, outer fleshy scales and roots that are generated during industrial peeling [96]. However, since onions are rich in several groups of plant compounds, such as dietary fibers (DF), fructo-oligosaccharides (FOS) and flavonoids, they have many benefits to human health [96]. An alternative solution could be their biological conversion into bioenergy and high value-added products (food and pharmacological ingredients, biogas, fertilizers, etc.) [41,97–99]. Up to 65% of the dry weight of onion waste is composed of nonstructural carbohydrates, including fructose (114 ± 1.4 mmol/L), glucose (137.5 ± 0.9 mmol/L) and sucrose (21 ± 0.7 mmol/L) [41]. They also contain sulfur, proteins, minerals, cellulose (7 ± 1.4 g/L), hemicellulose (3 ± 1.9 g/L), and essential oils. H₂ production was evaluated in *T. maritima* using onion waste alone or in combination with other FVW to provide additional nutrients for growth. Substrates were cut with an electric blender into small pieces, filtered and then homogenized [41]. Using a simplified medium containing natural seawater, cysteine-HCl and NH₄Cl as inorganic nitrogen source, *T. maritima* metabolized 60% of the carbohydrates contained in onion waste to produce 124 ± 2.5 mmol H₂/L (yield of 3.76 ± 0.5 mol H₂/mol C6), 65 ± 2.7 mmol AA/L (yield of 1.97 mol AA/mol C6), and 10 ± 1.1 mmol LA/L [41].

To enhance H₂ production, several experiments were carried out by combining different amounts of onion waste (0–200 mL) and 100 mL of other fruit and vegetable waste (FVW). The increase in onion waste levels significantly improved substrate consumption (69.8% without OW and 79% with 200 mL of OW), H₂ yield (3.24 ± 0.5 mol H₂/mol C6 without OW and 3.75 ± 0.8 mol H₂/mol C6 with 200 mL of OW), and acetate yield (1.67 mol AA/mol C6 without OW and 1.99 mol AA/mol C6 with 200 mL of OW) [41]. An economical and efficient H₂ production process was finally obtained by the removal of inorganic nitrogen sources and a surplus of onion waste (400 mL) (Table 2) [41].

3.1.2. Fish Waste

Supplemented of fish waste (FW) can be used to overcome the low nitrogen content in fruit and vegetable waste to sustain *T. maritima* cultures [40]. The fish waste from sardines

represents a highly biodegradable product. It is available in large quantities and rich in nitrogen, making it a good candidate to balance the C/N ratios in growth media. The reduction of C/N ratio by increasing fish waste counterparts (range 0–250 mL) significantly enhanced substrate consumption (from 69.85% at 47 C/N ratio to 96% at 12 C/N ratio), H₂ yield (from 3.24 ± 0.1 at 47 C/N ratio to 3.87 ± 0.1 at 12 C/N ratio), and organic acids production (AA: 56 ± 1.5 mmol/L at 47 C/N ratio to 99.5 ± 2.6 mmol/L at 12 C/N ratio; LA: 10.1 ± 1.1 mmol/L at 47 C/N ratio to 33.4 ± 2.9 mmol/L at 47 C/N ratio) [40]. In this example, a net increase of H₂ production was observed, resulting in 285 ± 2.9 mmol/L of H₂ (yield of 3.86 mol H₂/mol hexose) with 148 ± 3.5 mmol/L of AA (yield of 1.94 mol AA/mol C6) and 49 ± 1.3 mmol/L of LA (Table 2) [40].

3.1.3. Rice straw

Rice straw is produced as a by-product of rice production, and represents one of the major lignocellulosic industrial residues in the world [100]. It is the vegetative part of the rice plants (*Oryza sativa* L.), cut at grain harvest or after. It may be burned, ploughed down as a soil improver, used as a feed for livestock or to produce biofuels such as bioethanol [101]. It is composed of 41.4% cellulose, 19.6% hemicellulose, 22.8% total lignin (3% acid-soluble lignin and 19.8% acid-insoluble lignin), and 10.9% ash [102]. Over the years, different chemical pretreatments (e.g., thermal NH₃, thermal dilute H₂SO₄, combined pretreatments) were investigated to improve the conversion of residues to fermentable compounds, thus improving their utility in anaerobic digestion [103–105]. Korean rice straw has been used as a growth substrate for *T. neapolitana* [102]. To reduce the percentage of lignin in the matrix and to release the more accessible sugars contained in the cellulose and hemicellulose, a combined protocol consisting of two steps was proposed [102,106,107]. Rice straw particles and 10% ammonium hydroxide solution were thoroughly mixed and autoclaved at 121 °C for 60 min. Then the water-washed solid fractions were hydrolyzed with 1.0% sulfuric acid under autoclaving condition at 121 °C for 50 min, and the hydrolysate mixture was finally neutralized by 5 N of NaOH solution before its use as a carbon source [102,107]. After hydrolysis, the solid fraction consisted of 62.6% glucose, 3.04% xylose, and 5.29% lignin, whereas the liquid fraction was composed of 3.93% glucose and 16.16% xylose. Moreover, 78% of the lignin was removed, and an effective hemi-cellulose hydrolysis of 81.6% was observed. The liquid fraction was then used for the fermentation: 85.4% of the rice straw was consumed, including 95.7% of the xylose conversion and 73.0% of the glucose conversion. H₂ production was 112.38 ± 7.66 mL/L, with a yield of 2.7 mmol H₂/g straw (Table 2) [102]. Compared with the untreated form (not shown) and other chemical pretreatments, hydrogen production was noticeably increased, demonstrating that the combination of an efficient pretreatment and the capability of *T. neapolitana* to completely metabolize glucose and xylose can offer many advantages in rice straw valorization. Inhibitory effects on the growth rate due to chemical reagents used in the hydrolytic treatment should be overcome for industrial exploitation of this process.

Table 2. Fermentation of food waste by *Thermotoga* spp.

Substrate	Matrix Components	Sugar Components	Pretreatment Type	Pretreatment Method	Substrate Load (g/L)	Strain	T (°C)	Start pH	Mixing Speed (rpm)	Volume tot. (mL)	Working Volume (mL)	H ₂ yield (mol/mol sugar)	Organic Acids Yield (mol/mol sugar)	Organic Acids (g/L)	Ref.
Carrot pulp	Glucose, fructose, sucrose, polysaccharides	Glucose, fructose	Enzymatic	Enzymes	10	<i>T.nea</i>	72	6.8/7	350	2000	1000	2.7	AA 1.3 LA 0.17	AA 7.20 LA 1.34	[75]
												2.4	AA 1.1 LA 0.30	AA 10.79 LA 4.08	
Rice straw	Cellulose, hemicellulose, lignin	Glucose, xylose	Untreated	-	10	<i>T.nea</i>	75	7.5	150	120	40	2.27 ± 0.01	-	-	[102]
			Chemical	NH ₃								2.68 ± 0.02			
				H ₂ SO ₄								2.61 ± 0.01			
				Combined NH ₃ /H ₂ SO ₄								2.70 ± 0.01			
Potato steam peels	Starch	Glucose	Enzymatic	Enzymes	10	<i>T.nea</i>	75	6.9	350	2000	1000	3.8	AA 1.8 LA 0.20	-	[95]
Molasses	Glucose, fructose, sucrose	Glucose, fructose, sucrose	-	-	20	<i>T.nea</i>	77	8.5	100	116	40	2.6 ± 0.1	AA 1.5	-	[38]
Cheese whey	Lactose, proteins, lipids	Lactose	-	-	12.5	<i>T.nea</i>	77	8.5	100	116	40	2.4 ± 0.1	AA 1.0	-	
Fruit and vegetable waste	Cellulose, hemicellulose	Glucose	Mechanical	Shredding	8.1	<i>T.mar</i>	80	7	150	2200	1100	3.89	AA 1.96	AA 5.39	[39]
					20 (plus FW)					2500		3.86	AA 1.94	AA 12.28 LA 5.49	[40]
Onion waste	Glucose, fructose, sucrose, cellulose, hemicellulose	Glucose, fructose, sucrose	Mechanical	Shredding	200 * OW	<i>T.mar</i>	80	7	150	2500	1100	3.76 ± 0.5	AA 1.97	AA 5.33 LA 1.12	[41]
					400 * OW 100 * FVW							3.67 ± 0.8	AA 1.85	AA 9.27 LA 1.96	

All the experiments have been performed in batch cultures. H₂ yields, mol H₂/mol sugars; *T.nea*, *Thermotoga neapolitana*; *T.mar.*, *Thermotoga maritima*; FW, Fish waste; OW, Onion waste; FVW, Fish vegetable waste. Substrate load column: * mL.

3.1.4. Molasses

Molasses is one of the main products of the sugar cane or sugar beet industry, commonly used to produce alcohol and food flavoring. It is mainly composed of glucose, fructose and sucrose, and high amounts of organic nitrogen, vitamins and salts [108–112]. It can be employed without any pretreatments, avoiding additional nitrogen sources like yeast extract and peptone in hydrogen production by fermentative bacteria [111]. *T. neapolitana*, *T. maritima*, *T. naphthophila* and *T. petrophila* were able to produce H₂ from molasses with both suspended and immobilized cells, and in particular *T. neapolitana* showed comparable yields to pure glucose under the same conditions [38]. The fermentation process in complete medium leads to efficient H₂ production of 2.6 ± 0.1 mol H₂/mol C6 and acetic acid production of 1.5 mol AA/mol C6 (Table 2) [38]. The removal of vitamins, micronutrients, tryptic soy broth, yeast extract, MgCl₂, and CaCl₂ from the growth medium of *T. neapolitana* achieved a 70% reduction of medium cost, without significant loss of performance in molasses fermentation (2.95 ± 0.09 mol H₂/mol C6 and 1.0 mol AA/mol C6) [38]. These findings were confirmed by Frascari et al., who developed a kinetic model of biohydrogen production by molasses fermentation in *T. neapolitana*, in which several parameters were considered as fundamental to further optimize the fermentation process, such as the effects of H₂, O₂ and substrate inhibition [113].

3.1.5. Cheese Whey

Cheese whey is the wastewater originating from the precipitation and removal of milk casein during cheese-making. It represents a renewable resource in the food industry for its high lactose content. The milk type used in the cheese production (cow, goat, sheep, buffalo, and other mammals) influences the characteristics of the produced cheese whey. For example, bovine whey contains 70–80% lactose, 9% proteins, 8–20% minerals and other minor components, such as some hydrolyzed peptides of k-casein, lipids and bacteria [114]. Due to its high organic content, it cannot be directly discharged into water bodies and is not easily treatable in municipal/consortium purification plants, thus becoming an environmental problem for the dairy industry [115]. Cheese whey is commonly used as direct animal feed, or to be produced as protein and lactose powders for human food and livestock feed. Biological treatment involving the microbial conversion of the lactose contained in cheese whey represents one of the best approaches to obtain value-added products, such as organic acids, bioalcohols, gases (e.g., hydrogen, methane) and bioplastics [116]. Cheese whey was evaluated as the substrate for selected *Thermotoga* spp. (*T. neapolitana*, *T. maritima*, *T. naphthophila* and *T. petrophila*) to better resist high H₂ concentrations [38]. In terms of H₂ production rate, *T. neapolitana* was markedly superior to the other three strains, obtaining 2.4 ± 0.1 mol H₂/mol glucose eq. in complete medium (Table 2). The use of minimal medium supported neither growth nor H₂ production of *T. neapolitana*, probably because the protein content of the cheese whey was not readily usable [38]. Kinetic studies in Frascari et al. showed that immobilized and suspended cells performed similarly in cheese whey-based reactors [113].

3.2. Lignocellulosic Waste

Lignocellulosic waste arises from agricultural and wood industries, and represent the largest renewable feedstock for industrial fermentation [117–124]. Lignocellulosic materials are composed of heterogeneous polysaccharides derived from the photosynthesis process representing a potentially inexpensive carbon source of hexose and pentose sugars. More than 90% of plant dry weight is composed of cellulose (30–60%) and hemicelluloses (20–40%). Cellulose is a complex carbohydrate consisting of monomeric glucose units, and hemicelluloses are polysaccharides consisting of different pentose and hexose sugars units (mainly xylose, arabinose, glucose, and galactose). Cellulose and hemicelluloses form the structural components of plant cell walls, providing mechanical resistance and protection against pathogens. They tightly bound to lignin (10–25%), which is a class of cross-linked polymers rich in aromatic subunits, relatively hydrophobic and heterogeneous, with differ-

ent degrees of polymerization [120,125,126]. The major hurdle in the industrial exploitation of lignocellulosic waste as an energy feedstock comes from the need to first hydrolyze them and then remove the lignin from the cellulose and hemicellulose by economical and efficient processes [122,123,126,127]. Due to its hydrophobic and heterogeneous nature, lignin is resistant to acid and base hydrolysis, representing an obstacle for accessing the fermentable polysaccharides for biogas production. Moreover, lignin contains certain oligosaccharides and phenolic compounds that can act as growth inhibitors, representing another significant obstacle during the degradation of the cell walls [10,126,128].

Several chemical, physical, biochemical, and biological pretreatments have been proposed over the years to increase the biodegradation of lignocellulosic compounds and release their fermentable parts [10,42,44,81,118,126,129–131]. Hyperthermophilic bacteria are known to ferment the lignocellulosic biomass because they contain many relevant thermostable glycoside hydrolases [10,61,86,130]. The high growth temperatures also promote partial detachment of lignin from the hemicellulose–cellulose assembly and the degradation of growth inhibitors [81,129,130,132].

3.2.1. Miscanthus Waste

Miscanthus is a woody rhizomatous C4 perennial grass which represents an advantageous lignocellulosic energy crop adapted to various bioenergy processes, replacing fossil fuel resources. It combines high biomass production per hectare in various climates, suitable biomass composition for various thermochemical or biochemical conversions, and a positive environmental footprint (lowest water requirement, lowest N, P, and K fertilization, low greenhouse gas emissions, low invasiveness, etc.) [133]. About 62.5% of the total dry matter consists of cellulose and hemicellulose, whose main components are glucans and xylans. The total lignin content of the grass is around 25.0%, consisting mainly of acid-insoluble compounds, which makes a pretreatment step mandatory [129,130].

There have been several studies comparing different pretreatments to make *Miscanthus* biomass fermentable by *Thermotoga* spp. In early studies on *P. elfii*, the best *Miscanthus* hydrolysate was obtained involving a combination of mechanical extrusion and incubation with sodium hydroxide. The pretreatment caused a substantial delignification of the biomass and significantly improved C5 and C6 sugars, reaching a final monosaccharide concentration around 32 g/L in the hydrolysate [129]. *P. elfii* could grow on *Miscanthus* hydrolysates, consuming glucose and xylose simultaneously, and reaching high hydrogen (82.2 mM) and acetic acid (42.4 mM) production, even slightly higher than growing on glucose [129]. The *Miscanthus* fermentation was also demonstrated in *T. neapolitana* cultures [130]. Based on the previous work, different alkali pretreatments were investigated to reduce lignin insoluble fractions in the hydrolysates. The NaOH incubation at 85 °C for 16 h was found to be the optimal condition. Afterwards, enzymatic hydrolysis with cellulases was performed at 50 °C for 24 h to facilitate the release of arabinose, glucose, and xylose. *T. neapolitana* grown on 14 g/L of hydrolysate gave 3.2 mol H₂/mol C₆, 1.4 mol AA/mol C₆, and 11.2 mmol LA/L (Table 3), demonstrating the efficiency of these pretreatment steps in *Thermotoga* spp. fermentation [130]. When hydrolysate concentrations exceeding 28 g/L, the H₂ and acetate yields substantially dropped to 2.0 mol H₂/mol C₆ and 1.1 mol AA/mol C₆, since the fermentability of the substrate was reduced [130].

3.2.2. Garden Waste

Garden and park waste are generated from the maintenance of private gardens and public parks, and represent an economic substrate to produce biohythane (a mixture of hydrogen and methane, usually with 10 to 25% hydrogen in volume) and hydrogen via anaerobic dark fermentation [77,134,135]. In general, we can identify three major components: an organic fraction from garden grass, small bushes containing an undefined organic content and inorganic elements (on a dry matter basis, 0.6% N, 0.1% P, and 1.0% K), and ash, whose content is related to the amount of soil present. On an annual base, wet

garden waste contains 40% water, 30% organic matter, 30% ash, and a low content of trace elements (Cd, Cr, Cu, Hg, Ni, Pb, and Zn) [136].

Abreu et al. [77] estimated biohydrogen production by *T. maritima* from garden waste with a glucans/xylans ratio of 3:1 and a lignin content higher than 30% [77]. To develop a sustainable process, the biomass was homogenized, and no harsh chemical pretreatments were performed. H₂ production from garden waste by *T. maritima* reached 45.1 ± 4.6 L of H₂ per Kg of organic matter with 3.8 ± 0.2 mmol/L of AA (Table 3) [77]. These results were not very encouraging in comparison to data obtained using pure sugars, suggesting the inability of *T. maritima* to ferment the more recalcitrant fraction of the garden waste. Therefore, efficient pretreatments are needed to make the waste more accessible to fermentation.

3.2.3. Paper Sludge

Paper sludge is a solid industrial waste, arising from the paper industry which can be partially used in cement as a Supplementary Material [137,138]. Landfilling and incineration are also common handling options. However, companies from different sectors are looking for new solutions to reduce costs and their impact on the environment. Wastepaper sludge is becoming an economical and profitable material that can be used, after hydrolysis, in green technologies; for example, as a substrate for microbial fermentation to obtain hydrogen. This is due to its chemical and mineralogical composition: besides its low sulfate content, it is rich in minerals (calcium, silicon and aluminum etc.), proteins (22–52%), lignin (20–58%), carbohydrates (0–23%), lipids (2–10%), and cellulose (2–8%) [139]. The first example of paper sludge fermentation was reported for *P. elfii* [137]. The hydrolysate was obtained after digestion for 48 h using a chemical-enzymatic approach involving H₂SO₄, resulting in 12.8 g/L of glucose and 2.4 g/L of xylose [137]. To search for the medium components for optimal hydrogen production, the bacterium was cultivated in different conditions with defined and complex media. Data demonstrated that *P. elfii* grew on paper sludge hydrolysate. In complex medium, hydrogen production, sugar consumption and acetate production rates were similar to glucose fermentation (approximately 30 mM of H₂, 15mM of glucose consumption, 15mM of AA and less than 5mM of LA on paper sludge). Only a slight reduction in hydrogen production was observed without salts (less than 20 mM of H₂), while a net decrease in both hydrogen and acetate production, together with the glucose consumption rate (approximately 11 mM of H₂ and 7 mM of AA with 6 mM of glucose consumed) was observed without yeast extract, indicating that this component was essential for optimal results [137]. The hydrogen production rate on paper sludge hydrolysate was around 48% of the theoretical hydrogen yield of 4 mol of hydrogen/mol of C₆ sugar, suggesting that there were still possibilities to improve the biomass pretreatments and cultural conditions to utilize this waste (Table 3) [137].

Table 3. Fermentation of lignocellulosic waste and microalgal biomass by *Thermotogaceae*.

Substrate	Matrix Components	Sugar Components	Pretreatment type	Pretreatment Method	Substrate Load (g/L)	Strain	T (°C)	Start pH	Mixing Speed (rpm)	Volume tot. (mL)	Working Volume (mL)	H ₂ Yield (mol/mol sugars)	Organic Acid(g/L)	Ref.
<i>Miscanthus</i>	Cellulose, hemicellulose, lignin	Glucose, xylose	Mechanical, chemical	Extrusion NaOH	14	<i>T.nea</i>	80	7	350	2000	1000	3.2	AA 10.29 LA 1.25	[130]
			Chemical, enzymatic	NaOH enzymes	10	<i>P.elfii</i>	65	8	-	100	30	60.36 *	AA 3.52	[129]
Garden waste	Glucans, Xylans, lignin	Glucans, xylans	Mechanical	Shredding	5	<i>T.mar</i>	70	7.2	90	120	50	41.5 **	AA 0.31	[77]
Paper sludge	Proteins, lignin, carbohydrates, lipids, cellulose	Glucose, xylose	Chemical, enzymatic	H ₂ SO ₄ -enzymes	11	<i>P.elfii</i>	65	7.2	100	30	-	-	-	[137]
<i>Chlamydomonas reinhardtii</i>	Starch	Glucose	Enzymatic	Enzymes	5	<i>T.nea</i>	75	7/7.4	150	120	40	2.5 ± 0.3	-	[140]
<i>Thalassiosira weissflogii</i>	Protein, chrysolaminarins	Chrysolaminarins	Chemical	MeOH	2	<i>T.nea</i>	80	7.5/8	250	3800	500	1.9 ± 0.1	AA 1.57 LA 0.112	[141]

All the experiments have been performed in batch cultures. Pretreatment protocols are described in the appropriate sections. H₂ yields, mol H₂/mol sugars; *T.nea*, *Thermotoga neapolitana*; *T.mar*, *Thermotoga maritima*; *P.elfii*, *Pseudothermotoga elfii*. H₂ yield column: * mL/L; ** L/Kg.

3.3. Glycerol

Crude glycerol is the major by-product of the biodiesel industry, generated by base-catalyzed transesterification during the biodiesel production processes [142]. It represents a green, biodegradable and abundant feedstock that can be widely used in pharmaceuticals, cosmetics, soaps, toothpastes, paints, and other commercial products [71,143]. Since around 1 kg of glycerol waste is generated for every 10 kg of biodiesel produced, its abundance has increased due to the dramatic growth of the biodiesel industry, although its economic value has decreased in the last few years [142]. Developing advanced sustainable systems is essential to a wider range of applications of crude glycerol without increasing the refining costs [144]. The classical refining processes, such as filtration, chemical additions, and fractional vacuum distillation are sometimes too expensive for small and middle-sized producers [145,146]. From this perspective, economic and alternative ways of using crude glycerol have been studied, like fatty acid production, animal feed, biological conversion [144,146–150]. Among these options, anaerobic digestion to biogas (e.g., methane and hydrogen) production from fermentative microorganisms represents a promising approach, which produces high levels of biogas in small reactors and enjoys several advantages, such as low nutrient requirements, energy savings, and generation of a stabilized digestate [142–144,151]. The chemical compositions of crude glycerol are not well defined and are dependent on the parent feedstock and biodiesel production processes, e.g., the type of catalyst used, the transesterification efficiency, recovery efficiency of the biodiesel and other impurities [144,146]. Generally, every feedstock contains around 50–60% (wt) of glycerol, 12 to 16% of alkalis, especially in the form of alkali soaps and hydroxides, 15 to 18% of methyl esters, 8 to 12% of methanol, and 2 to 3% of water [146]. In addition to methanol and soaps, crude glycerol also contains Ca, Mg, P, or S [146], K, Na, C, N, and proteins (0.05 to 0.44%), etc. [142]. The impurities present in the raw substrate, such as spent catalysts, salts after neutralization, residual methanol, methyl esters, oil/fat, soap and free fatty acids, have to be removed to make the substrate suitable for further applications [146,152]. As a carbon source, glycerol was tested for biohydrogen production via anaerobic fermentation by thermophilic bacteria. Since glycerol is a more reduced compound compared to other substrates like glucose or xylose, it has the potential to generate more NADH and H₂ during catabolism [153].

Some controversies exist concerning the ability of *Thermotogaceae* family members to utilize glycerol. Early studies reported that *T. maritima* contained the coding sequences for a complete pathway for glycerol uptake, although glycerol had to enter into the cell by diffusion in other strains (e.g., *T. neapolitana*), [154–156]. Therefore, a putative degradation pathway based on the *T. maritima* genome was proposed, i.e., glycerol enters the cell either by diffusion or facilitated transportation and enters glycolysis via glycerol-3-phosphate. The involvement of a glycerol kinase and an uncharacterized NAD⁺ or FAD-dependent multimeric glycerol-3-phosphate dehydrogenase has been hypothesized [154].

Two research groups experimented with the possibility of fermenting glycerol in *T. maritima*, *T. neapolitana*, and *P. elfii*, obtaining conflicting results. Eriksen et al. observed growth only if glycerol was supplemented simultaneously with one or more sugars; none of the three species grew if glycerol was the sole carbon source (data not shown) [36]. The surplus of NADH generated during glycerol conversion may influence the activity of the bifurcating hydrogenases present in these bacteria. In fact, 2 mol of NADH and 2 mol of reduced ferredoxin were produced in glycerol conversion, changing the conventional stoichiometric ratio for hydrogenase activity from 1:2 to 1:1 [153,157,158].

The capability of *T. neapolitana* to ferment glycerol waste was also demonstrated by Ngo et al. [151]. Before use, crude glycerol waste was pretreated to avoid inhibition of bacterial growth by removing the solvents present (e.g., methanol and/or ethanol) by rotary evaporation at 45 °C, and the solid fraction was precipitated by centrifugation at 15,000 rpm for 15 min [151]. H₂ yield was around 1.97 ± 0.09 mol H₂/mol glycerol, obtained without any other modification. Several cultural parameters were also important to enhance glycerol fermentation, including pH, N₂ sparging, sodium chloride concentration and yeast

extract. Under specific conditions (i.e., N₂ sparging and buffering agent), H₂ could reach 2.7 ± 0.1 mol H₂/mol glycerol; considerable production of acetic acid was also observed (22.35 ± 1.05 mmol/L) (Table 4) [151]. Another study [71] confirmed this ability with pure and waste glycerol, obtaining 1.3 ± 0.06 mol H₂/mol of glycerol waste consumed and a percentage of acetic and lactic acid comparable to pure glycerol results (data not shown) [71]. During fermentation, more acetic acid than lactic acid was produced, implying that the H₂-acetate pathway predominated over the lactate one [70].

Again, in the study of Maru et al. [153], both *T. neapolitana* and *T. maritima* metabolized pure glycerol and produced H₂ at 2.65 mol H₂/mol glycerol for *T. neapolitana* and 2.75 mol H₂/mol glycerol for *T. maritima* (Table 4) [153]. In order to improve glycerol fermentation, cultural conditions were optimized in *T. maritima* by testing the glycerol content, yeast extract concentration, and pH control. Maximum H₂ yields were 2.86 mol H₂/mol glycerol for *T. neapolitana* and 2.84 mol H₂/mol glycerol for *T. maritima* in the optimized conditions [154].

3.4. Microalgal Biomass

Microalgae are photosynthetic unicellular organisms living individually, in chains or groups in a wide range of aquatic habitats; they can tolerate different light intensities, temperature, salinity and pH values [159]. They can be cultured in large scale by different methods and conditions, and represent a potential feedstock for the coproduction of different forms of energy. Several species were recently investigated as a fuel source since they contain large quantities of lipids useful for biodiesel production [159,160]. For example, marine diatoms contain up to 50% of lipids per biomass dry weight [161]. Moreover, to valorize all microalgal biomass components, the soluble polysaccharides of photosynthetic biomass could play an important role for biohydrogen production through DF [140].

T. neapolitana can metabolize different microalgal biomass. Nguyen et al. [140] and Dipasquale et al. [141] studied *T. neapolitana* fermentation on the biomasses of *Chlamydomonas reinhardtii* and *Thalassiosira weissflogi* respectively [140,141]. In the former case, algal biomass was pretreated in two different ways (heat-HCl and Termamyl enzyme) to disrupt the algal cell walls and release starch for fermentation [140]. Termamyl enzyme pretreatment, performed by a thermostable α -amylase from *Bacillus licheniformis* at 90°C for 30 min, was the most effective process to optimize the hydrolysis [140]. This pretreatment maximized H₂ yield (2.5 ± 0.3 mol H₂/mol glucose eq) when compared to that obtained with other pretreatment methods (<2.2 mol H₂/mol glucose eq) or with pure starch fermentation (1.5 ± 0.1 mol H₂/mol glucose eq) (Table 3) [140].

In the latter study [141], chemical extraction with MeOH was performed on the *Thalassiosira weissflogi* biomass to separate the water-soluble fraction from the lipid fraction [141]. The aqueous diatom extracts mainly contained 0.4 g/L protein and 2.3 g/L sugar eqs (chrysolaminarins). Although 81.8% of sugars in microalgal extract were consumed in 48 h of fermentation by *T. neapolitana*, H₂ yields (1.9 ± 0.1 mol H₂/mol glucose eq) (Table 3) were lower in comparison to those obtained from complex and simple sugars (around 2.7 mol H₂/mol glucose eq.) [141]. The co-occurring decrease of lactate and acetate production suggested a minor availability of pyruvate in cultures of *T. neapolitana* on diatom extracts (Table 3) [141].

Depending on the origin of microalgal biomass, targeted strategies could be adopted to optimize the fermentation medium or to increase the carbohydrate content.

Table 4. Fermentation of glycerol by *Thermotoga* spp.

Substrate	Pretreatment Type	Pretreatment Method	Substrate Load (g/L)	Strain	T (°C)	Start pH	Mixing Speed (rpm)	Reactor Volume (mL)	Working Volume (mL)	H ₂ Yield (mol H ₂ /mol Sugar)	Organic Acids (g/L)	Ref.
Pure glycerol	-	-	5	<i>T.nea</i> <i>T.mar</i>	80	7.5	200	120	25	$\frac{2.65}{2.75}$	-	[153]
Biodiesel waste (1% glycerol)	Mechanical	Evaporation, centrifugation	5	<i>T.nea</i>	80	7.5	-	120	40	2.70 ± 0.10	AA 1.85	[151]
Pure glycerol	-	-	2.5	<i>T.nea</i> <i>T.mar</i>	80	8	200	120	25	$\frac{2.86}{2.84}$	AA 2.21 LA 1.74	[154]
Biodiesel waste (1% glycerol)	Mechanical	Evaporation, centrifugation	3	<i>T.nea</i>	75	7.5	-	120	40	1.3 ± 0.06	-	[71]

All the experiments have been performed in batch cultures. AA, acetic acid; LA, lactic acid.

4. Molecular Basis of Sugar Catabolism and Hydrolytic Enzymes in the Family *Thermotogaceae*

In recent years, several bacterial genomes of genus *Thermotoga* were sequenced (e.g., *T. maritima*, *T. neapolitana*, *T. thermarum*, RQ7), revealing their versatility in utilizing various organic carbon sources [162–166]. Many members of the family *Thermotogaceae* possess all the genes needed for glucose catabolism by EMP, ED and OPP pathways (Supplemental Table S1), as also supported by the presence of key enzymes, such as phosphofructokinase (PFK, E.C. 2.7.1.11), 2-dehydro-3-deoxyphosphogluconate aldolase (KDPG aldolase, E.C. 4.1.2.14), and 6-phosphogluconate dehydrogenase (6PDGH, E.C. 1.1.1.44) [54]. Interestingly, these pathways showed an environmental-dependent activation mechanism in *T. neapolitana*, because the insufflation of CO₂ instead of N₂ induced the upregulation of the genes involved in ED and OPP [60]. Another peculiarity of some *Thermotogales* members (e.g., *T. maritima*) is the presence of an unconventional triosephosphate isomerase (TIM, E.C. 5.3.1.1) linked to phosphoglycerate kinase (PGK, E.C. 2.7.2.3). This anomalous association leads to a bifunctional tetrameric protein, which showed an increased stability and catalytic activity at high temperatures [167].

On the other hand, *Thermotogaceae* showed the presence of genes involved in monosaccharides conversion to glucose inducing an alternative flux to EMP and ED, enabling *Thermotogaceae* to use alternative sugar substrate sources [168–170]. Examples of these are uronate isomerase (E.C. 5.3.1.12), xylose isomerase (E.C. 5.3.1.5), mannose-1-phosphate guanylyltransferase (E.C. 2.7.7.22), phosphomannomutase (E.C. 5.4.2.8), mannose-6-phosphate isomerase (E.C. 5.3.1.8) and others. These enzymes could operate in the conversion of monosaccharides to glucose and/or glycolysis intermediates. *Thermotogaceae* also possess enzymes related to glucuronic and galacturonic acid metabolism, which provide an additional and specific feed into the ED pathway.

Different species of *Thermotogaceae* prefer different monosaccharides. Experiments performed using mixtures of glucose, fructose, arabinose, and xylose displayed similar behaviors in *T. neapolitana* and *T. maritima* which clearly catabolized glucose and xylose instead of arabinose, while *T. RQ2* quickly consumed fructose [76]. In this context, it is not surprising to find in *Thermotogaceae* genomes the entire regulons for monosaccharide metabolism and the ABC transporters for the import/export of simple and complex sugars [76]. The fine regulation of these mechanisms leads to efficient catabolism of the sugar substrates [60,163,171].

Common components of fruit and vegetable waste are galactose and rhamnose [76,163]. As showed in Supplemental Table S1, the entire set of enzymes, related to Leloir pathway, were encoded by *Thermotogaceae* genomes. This pathway is specifically involved in the galactose metabolism. These enzymes include aldose 1-epimerase (E.C. 5.1.3.3), galactokinase (E.C. 2.7.1.6), galactose 1-phosphate uridylyl-transferase (E.C. 2.7.7.12), phosphoglucomutase (E.C. 5.4.2.2) and UDP-glucose 4-epimerase (E.C. 5.1.3.2), converting galactose to glucose 6-phosphate. The ability of *Thermotogales* in rhamnose metabolism was defined by the presence of rhamnose isomerase (E.C. 5.3.1.14), rhamulose kinase (E.C. 2.7.15), and rhamulose 1-phosphate aldolase, resulting in the biosynthesis of dihydroxyacetone phosphate and lactaldehyde [172]. Interestingly, this metabolic pathway is connected to both glycolysis and lactate dehydrogenase metabolism. The rhamnose metabolism pathway is totally absent in the genus *Pseudothermotoga* [76].

Complex sugars from different sources such as plant and algal biomass were also efficiently metabolized by *Thermotogaceae* [73,95,129,130,140]. Plant storage polysaccharides as well as starch and sucrose could be easily used as the carbon source by using enzymes such as α -amylase, α -glucosidase, pullulanase, and others (Supplemental Table S2). Starch is composed by α -glucose residues mainly linked by α -1,4/1,6 glycosidic bonds. The two main high molecular weight components of starch are the linear polymer amylose and the branched polymer amylopectin [173]. *Thermotogaceae* genomes reported the complex set of depolymerizing enzymes able to catalyze the catabolism of both linear and branched starch polymers (Supplemental Table S2). Non-reducing ends are attacked by

enzymes such as hydrolases, β -amylase producing small oligosaccharides, while enzymes capable of hydrolyzing α -1,6 glycosidic bonds in pullulan are defined pullulanases [173]. These enzymes ensure degradation and linearization of complex polysaccharides into a monosaccharide unit. Intriguingly, although starch is a rare carbon source in deep marine environments, especially in a hot thermal vent, extremophiles repeatedly showed starch-hydrolyzing genes in their genomes, suggesting starch as an important carbon source for their metabolism [173]. Homologous starch catabolic enzymes have been identified and characterized in a number of hyperthermophilic genera, namely *Pyrococcus*, *Thermococcus*, *Sulfolobus*, *Pyrodictium* [173].

Lignocellulosic biomass represents a recalcitrant source of organic compound that requires a number of enzymatic processes to depolymerize [174]. The ability of *Thermotogaceae* to metabolize cellulose and hemicellulose is related to the presence of a number of cellulolytic and hemicellulolytic enzymes. Examples of these are β -glucosidase, α -arabinofuranosidase, endo-1,3- β -xylanase, endo-1,4- β -xylanase, endo-1,4- β -mannanase etc., (Supplemental Table S2). In particular, the lignocellulosic biomass showed the presence of mannans which represent a specific form of storage and cell wall polysaccharide [175]. *Thermotogaceae* showed the presence of a number of genes involved in mannans catabolism, namely mannonate dehydratase, D-mannonate oxidoreductase, alpha- and beta-mannosidases. Microarray analyses revealed a dramatic reorganization of *Thermotogaceae* transcriptomes when bacterial growth on a polysaccharide mix was compared to the growth on glucose. These data connected the ability of *Thermotogaceae* to ferment individual carbohydrates to the versatile set of ABC transporters [76]. The hemicellulolytic enzymes from *T. neapolitana* were tested to solubilize lignocellulosic products from barley straw and corn bran, which improved the yield of fermentable sugars up to 65% compared to traditional systems [86]. *T. maritima* cellulase has also been overexpressed in tobacco and *Arabidopsis* chloroplasts to maximize the production of this cellulolytic enzyme [175]. The biomass of brown algae and diatoms, particularly polysaccharides such as sucrose and laminarin, were easily fermented by *Thermotogaceae* [73]. It is worth pointing out that genes coding for laminarinase, endoglucanase (β 1 \rightarrow 3 and β 1 \rightarrow 4), glucosidase (alpha and beta), and similar enzymes are frequently noticed in *Thermotogaceae* genomes. Interesting differences were reported between *Thermotoga* and *Pseudothermotoga* genomes, regarding polysaccharide catabolic enzymes (Supplemental Table S2). The ability of *Thermotogales* to use microalgal biomass as an organic source could be confirmed by the presence of genes related to lipid catabolism, such as lipase (CTN_RS06200), glycoside hydrolase 4 related to glycolipids and sphingolipids (CTN_RS09115), and alpha-galactosidase related to glycolipids (CTN_RS06915).

5. Conclusions and Future Perspective

Biodegradable organic waste is a promising carbon source to be exploited in a more circular and sustainable worldwide economy. Their abundance and heterogeneity in terms of compositional and structural features, associated to their origins, allow them to be widely used for biogas production, mainly biohydrogen, biofuels such as bioethanol, and value-added products (acetic acid, lactic acid, etc.). In the last few years, microbial anaerobic fermentation has become a promising way to obtain high production yields of bioenergy and green chemicals, and hyperthermophilic bacteria capable of metabolizing complex sugars via a dark fermentation process represent the new frontier of biotechnological development. The hyperthermophilic family *Thermotogaceae*, including the *Thermotoga* and *Pseudothermotoga* genera, are recognized for their ability to produce H₂ from many complex substrates.

This review demonstrates that *Thermotoga* and *Pseudothermotoga* spp. have an enormous biotechnological potential in fermenting organic waste originated from food, glycerol, lignocellulosic, and microalgal biomasses. In particular, *T. maritima*, *T. neapolitana* and *P. elfii* have been recognized as the best candidates in this scientific landscape. Their ability to degrade complex substrates is due to their unique metabolic and genomic features.

Many studies have been performed to find the best employment strategies and to identify putative transporters, enzymes, pathways, limiting factors, and pretreatment methods. The extreme growth temperatures of these bacteria not only reduce contamination by environmental bacteria but also make complex substrates easier to solubilize, avoiding, in some cases, the pretreatment step, which helps to preserve major components of the substrates and increase their availability.

Several studies were carried out to investigate the effect of different mechanical, thermal, chemical, and biological pretreatment methods on biodegradable organic waste to develop more sustainable processes. They can also include the combined use of different substrates to balance nutritional requests. The synergistic activities of two strains may also be exploited to metabolize complex substrates. For example, *C. saccharolyticus* can provide thermostable cellulolytic and xylanolytic enzymes, allowing the growth on complex lignocellulosic carbon sources and the co-metabolization of a wide range of monosaccharides including both pentose and hexose sugars. On the other hand, *T. maritima* and *T. neapolitana* can grow either on various C5 and C6 sugars, starch, glycogen, or complex organic substrates with hydrogen yields close to the maximum theoretical values. Co-cultivating *C. saccharolyticus* and *Thermotoga* can maximize the utilization of cellulosic substrates while ensuring optimal H₂ yield.

The collective knowledge we gained so far will allow us to experiment with several waste fermentation strategies with members of the *Thermotogaceae* family. The existence of unprecedented pathways, like the capnophilic lactic fermentation pathway discovered in *T. neapolitana*, which pairs CO₂ and acetate to produce lactic acid at high yields and at the same time detoxifies the environment from CO₂, further illustrates the great potentials of the *Thermotoga* and *Pseudothermotoga* genera in establishing a sustainable economy based on waste elimination and exploitation.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/resources10040034/s1>, Table S1: List of *Thermotogales* genes related to glycolytic pathways. Data have been exported from Ensembl Bacteria database; Table S2: List of *Thermotogales* genes related to organic catabolism. Data have been exported from Ensembl Bacteria database.

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