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Effect of Cultivation Parameters on Fermentation and Hydrogen Production in the Phylum *Thermotogae*

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**Abstract:** The phylum *Thermotogae* is composed of a single class (*Thermotogae*), 4 orders (*Thermotogales, Kosmotogales, Petrotogales, Mesoaciditogales*), 5 families (*Thermotogaceae, Fervidobacteriaceae, Kosmotogaceae, Petrotogaceae, Mesoaciditogaceae*), and 13 genera. They have been isolated from extremely hot environments whose characteristics are reflected in the metabolic and phenotypic properties of the *Thermotogae* species. The metabolic versatility of *Thermotogae* members leads to a pool of high value-added products with application potentials in many industry fields. The low risk of contamination associated with their extreme culture conditions has made most species of the phylum attractive candidates in biotechnological processes. Almost all members of the phylum, especially those in the order *Thermotogales*, can produce bio-hydrogen from a variety of simple and complex sugars with yields close to the theoretical Thauer limit of 4 mol H₂/mol consumed glucose. Acetate, lactate, and L-alanine are the major organic end products. Thermotogae fermentation processes are influenced by various factors, such as hydrogen partial pressure, agitation, gas sparging, culture/headspace ratio, inoculum, pH, temperature, nitrogen sources, sulfur sources, inorganic compounds, metal ions, etc. Optimization of these parameters will help to fully unleash the biotechnological potentials of *Thermotogae* and promote their applications in industry. This article gives an overview of how these operational parameters could impact *Thermotogae* fermentation in terms of sugar consumption, hydrogen yields, and organic acids production.

**Keywords:** anaerobic bacteria; hydrogen yields; fermentation rate; organic acids; nitrogen; carbon dioxide

1. **Introduction**

The phylum *Thermotogae* is comprised of thermophilic, hyperthermophilic, mesophilic, and thermo-acidophilic anaerobic bacteria that originated from geothermally heated environments (Table 1) [1,2]. Recent phylogenetic analyses based on gene markers/core genome inferences, comparative genomics, and whole-genome relatedness have led to a taxonomic revision of the phylum, with a single class (*Thermotogae*), 4 orders (*Thermotogales, Kosmotogales, Petrotogales, Mesoaciditogales*), 5 families (*Thermotogaceae, Fervidobacteriaceae, Kosmotogaceae, Petrotogaceae, Mesoaciditogaceae*), and 13 genera, i.e., *Thermotogae* (T.) [3], *Pseudothermotoga* (Pseudot.) [2,4], *Fervidobacterium* (F.) [5], *Thermospho* (Ts.) [6], *Kosmotoga* (K.) [7], *Mesotoga* (Ms.) [8], *Defluviitoga* (D.) [9], *Geologa* (G.) and *Petrotoga* (P.) [10], *Marinitoga* (Mn.) [11], *Oceanotoga* (O.) [12], *Mesoaciditoga* (M.) [13], and *Athalassatoga* (A.) (Table 1) [2,4,14]. *Thermotogae* are able to grow under mesophilic (*Kosmotogales, Mesoaciditogales, Petrotogales*) and thermophilic conditions (*Thermotogales*), but most species have optimal growth temperatures in the range of 45–80 °C (Table 1). They are Gram-negative
bacteria, except for *D. tunisiensis*, which shows a positive result in Gram staining [9]. Apart from *K. shengliensis*, whose cells are in a coccoid form, *Thermotogae* cells are rod-shaped and encapsulated by a unique outer membrane, named “toga” [1,8,15]. Usually, the cells grow singly or in pairs, but it is also possible to observe chains surrounded by a unique toga [1,2]. Cell length is typically less than 20 µm, except for *F. gondwanense* and some members of the *Petrotoga* genus, whose cells can reach to 50 µm long (Table 1) [2,10]. Almost all species grow at neutral pH, and NaCl tolerances are high among *Geotoga*, *Oceanotoga*, and *Petrotoga* species (Table 1). Numerous studies have reported that members of the phylum can grow on both simple (e.g., glucose, galactose, fructose, lactose, maltose, mannose, sucrose) and complex carbohydrates (e.g., starch, glycogen, cellulose, keratin) (Table 1). Genes, transcriptional factors, and regulatory mechanisms driving the carbohydrates utilization have been identified for multiple members of the phylum [16–18]. ABC transporters for the uptake of a broad list of sugars have also been characterized [19–23].

All species of the phylum, except for *Mesotoga* spp., have tremendous potentials in biotechnological production of H₂, especially the order *Thermotogales*, as their hydrogen yields are close to the theoretical maximum value (Thauer limit) of 4 mol H₂/mol glucose [1,4,24]. Acetate, lactate, and L-alanine are the major organic products of the sugar fermentation [1]. *Ms. prima* and *Ms. infera* produce mainly/only acetate from sugar utilization without H₂ formation [8,25–27]. Lactate is produced by *T. maritima*, *T. neapolitana*, and *Mn. camini* in variable quantities depending on growth conditions [11,28–31]. Other significant products include ethanol (has been measured in *Geotoga*, *Petrotoga*, *Kosmotoga*, and *Oceanotoga* spp.); isovalerate, isobutyrate, and/or propionate (have been measured in *Mn. camini* and *K. olearia*); L-glutamate, alpha-aminobutyrate, hydroxyphenyl-acetate, or phenylacetate (have been measured in *F. pennavorans*) [1,32] (Table 1). Among these fermentation end-products, lactic acid has been widely used in various industries such as food, cosmetic, pharmaceutical, and chemical industries, although its primary application is serving as the building block for the production of biodegradable polyactic acid (PLA) [33]. Ethanol is an important industrial commodity; it is used as a food additive and a renewable biofuel; it is also contained in many cosmetics, households, and sanitizer products [34]. Moreover, a plethora of thermostable enzymes, harbored by most of these bacteria, are valuable components for many industrial and biotechnological applications [17,35–44].

Hydrogen (H₂) is considered a green and sustainable alternative to traditional fossil fuels and is capable of mitigating greenhouse gas emissions. Using hydrogen in fuel cells or combustion engines produces heat and electricity with water as the only waste. As the current abiotic hydrogen production method is energy-consuming and still causes pollution, emphasis must be given to biological production of the energy from renewable sources [45,46]. Biological synthesis of H₂ can use a wide range of organic substrates as feedstocks, including agro-industrial wastes and algal biomass, and may operate under various environmental conditions [1,46–54]. In addition, high temperatures help to improve the solubilization of substrates, reduce fermentation time, and lower contamination risks [55]. Although hydrogen production by *Thermotoga* species is considered one of the most challenging biological systems, no application using pure *Thermotoga* cultures has been reported at the industrial scale.

Releasing hydrogen is an efficient way to dissipate excessive reductants generated during the fermentative conversion of organic substrates. The process is generally referred to as dark fermentation (DF) and is typically influenced by environmental conditions such as pH, cell growth rate, and hydrogen partial pressure [24,56,57].

According to the classical model of dark fermentation, theoretically up to 4 mol of hydrogen may be produced from each mole of glucose, which is converted to acetate and CO₂ (Thauer limit Figure 1) [24]. When hydrogen accumulates, pyruvate is diverted away from acetate production. In this case, excessive NADH from glycolysis is not used in the energetically favorable manner to synthesize acetate and H₂ but dissipated via synthesizing other metabolic products such as lactic acid, L-alanine, ethanol, butyrate, and valerate (Figure 1) [24]. Synthesis of hydrogen in *Thermotogae* species is performed...
by the heterotrimeric [FeFe]-hydrogenase, an electron-bifurcating enzyme that couples the endergonic reduction of $H^+$ to hydrogen by NADH to the exergonic reduction of $H^+$ to hydrogen by reduced ferredoxin (Figure 1) [58]. Because the hydrogenase uses both NADH and reduced ferredoxin as electron donors, hydrogen yield is influenced by factors that affect both reductants.

The value of these bacteria in biotechnological processes is rising sharply since the discovery of the bifurcating hydrogenase and will probably be enhanced with a full elucidation of the molecular and biochemical properties of the processes. Despite decades of efforts in the development of genetic tools to engineer these species, only a few of thermostable selectable markers and genetic modifications with low stability are reported, which makes it still difficult to perform genetic modifications of these organisms [59–61]. However, these difficulties could be offset by their well-known susceptibility to mutations under environmental pressures [62,63].

In recent years, many researchers have been focusing on the optimization of fermentation performance towards the production of hydrogen and other target end-products [30,43,64–71].

Anaerobic fermentation in *Thermotogae* depends on many cultivation parameters such as hydrogen partial pressure, agitation, gas sparging, culture/headsapce ratio, inoculum, pH, temperature, nitrogen sources, sulfur sources, inorganic compounds, and metal ions. The effect of each factor on $H_2$ yield, sugar consumption rate, and formation of biotechnologically interesting end-products are discussed here. Main data are also summarized in extensive tables, citing the most important studies, with the information on their cultivation systems (e.g., reactor type, incubation periods, batch vs. continuous modality).
| Genus               | Species                     | Isolation               | Temp. Range/ Optimal (°C) | pH Range/ Optimal | Cell Dimension (Long by Wide) (µm) | Growth Substrates                                      | NaCl Range/ Optimal (%) | Electron Acceptor | End Products | Ref. |
|---------------------|-----------------------------|-------------------------|---------------------------|-------------------|------------------------------------|--------------------------------------------------------|--------------------------|-------------------|--------------|------|
| Thermotoga         | Thermotoga petrophila       | Oil reservoir, Japan    | 47–88/80                  | 5.2–9.0/7.0       | 2.0–7.0 by 0.7–1.0                 | YE, peptone, glucose, fructose, ribose, arabinose, sucrose, lactose, maltose, starch, cellulose | 0.1–5.5/1.0              | S0; Thio       | AA, LA, C02, H2 | [72] |
|                     | Thermotoga naphthophila    | Oil reservoir, Japan    | 48–86/80                  | 5.4–9.0/7.0       | 2.0–7.0 by 0.8–1.2                 | YE, peptone, glucose, galactose, fructose, mannitol, ribose, arabinose, sucrose, lactose, maltose, starch | 0.1–6.0/1.0              | S0; Thio       | AA, LA, C02, H2 | [72] |
| Thermotoga         | Thermotoga maritina         | Golden, vent, Japan    | 55–90/80                  | 5.5–9.0/6.5       | 1.5–11.0 by 0.6                    | ribose, xylose, glucose, sucrose, maltose, lactose, galactose, starch, glycogen | 0.2–3.8/2.7              | Fe (III)       | S0, Thio    | [3]  |
|                     | Thermotoga profunda        | Hot spring, Japan       | 50–72/60                  | 6.0–8.6/7.4       | 0.8–2.1 by 0.4                     | glucose, trehalose, cellobiose, arabinose, xylose, ribose, pyruvate | n. d                    | S0; Thio       | n. d        | [73] |
|                     | Thermotoga caldofonis       | Hot spring, Japan       | 55–85/70                  | 6.0–8.6/7.4       | 1.2–3.5 by 0.5                     | glucose, maltose, trehalose, cellobiose, arabinose, xylose, ribose, pyruvate, starch | n. d                    | Thio            | n. d        | [73] |
|                     | Thermotoga neapolitana     | Submarine, thermal vent | 55–95/77                  | 6.0–9.0/7.5       | 1.5–11.0 by 0.6                    | fructose, fucose, galactose, mannose, rhamnose, pyruvate, glucosamine, lactulose, turanose, glycerol, dextrin, ribose, xylose, glucose, sucrose, maltose, lactose, starch, glycogen | 0.2–6.0/2.0              | S0            | AA, ALA, C02, H2 | [74] |
| Pseudothermotoga    | Pseudothermotoga lettingae | Thermophilic bioreactor | 50–75/65                  | 6.0–8.5/7.0       | 2.0–3.0 by 0.5–1.0                 | glucose, EIOH, acetate, formate                          | 0.0–2.8/1.0              | S0; Thio, AQDS; Fe(III) | AA, ALA, C02, H2, EIOH, AA, BA, C02, H2 | [75] |
|                     | Pseudothermotoga elfii     | Oil reservoir, Japan    | 50–72/66                  | 5.5–7.5/7.5       | 2.0–3.0 by 0.5–1.0                 | glucose, arabinose, fructose, lactose, maltose, mannose, ribose, sucrose, xylose | 0.0–2.8/1.0              | Thio           | AA, C02, H2   | [76] |
|                     | Pseudothermotoga hypoga    | Oil reservoir, Africa   | 56–90/70                  | 6.1–9.1/7.3–7.4   | 2.0–3.0 by 0.5–1.0                 | fructose, galactose, glucose, lactose, maltose, mannose, sucrose, xylose, xylan | 0.0–1.5/0.2              | Thio           | AA, ALA, C02, H2, EIOH | [77] |
| Pseudothermotoga    | Pseudothermotoga subterranea | Oil reservoir, Paris   | 50–75/70                  | 6.0–8.5/7.0       | 3.0–10.0 by 0.5                    | YE, peptone, tryptone, casein                           | 0.0–2.4/1.2              | Cys, Thio     | n. d        | [78] |
|                     | Pseudothermotoga thermarum | Hot spring, Africa      | 55–84/70                  | 6.0–9.0/7.0       | 1.5–11.0 by 0.6                    | starch, glucose, maltose                               | 0.2–0.5/0.35             | S0            | n. d        | [6]  |
| Genus            | Species                | Isolation                      | Temp. Range/ Optimal ('C) | pH Range/ Optimal | Cell Dimension (Long by Wide) (µm) | Growth Substrates                                                                 | NaCl Range/ Optimal (%) | Electron Acceptor | End Products | Ref.  |
|------------------|------------------------|--------------------------------|---------------------------|------------------|-----------------------------------|-----------------------------------------------------------------------------------|--------------------------|-------------------|--------------|-------|
| *Fervidobacterium* | *Fervidobacterium nodosum* | Hot spring, New Zealand       | 40–80/ 65–70             | 6.0–8.0/ 7.0     | 1.0–2.5 by 0.5–0.55               | glucose, sucrose, starch and lactose                                              | n.d./<1.0                | 5°               | AA, LA, CO₂, H₂, ETOH, But, Val | [5]  |
|                  | *Fervidobacterium pennavorans* | Hot spring, Portugal          | 50–80/ 70               | 5.5–8.0/ 6.5     | 2.0–20.0 by 0.5                   | cellobiose, starch, glycogen, pullulan, glucose, fructose, maltose, xylose, native feathers | 0.0–4.0/ 0.4             | 5°, Thio         | AA, CO₂, ALA, Glu, ETOH, But, H₂, BuOH | [79] |
|                  | *Fervidobacterium islandicum* | Hot spring, Iceland           | 50–80/ 65               | 6.0–8.0/ 7.2     | 1.0–4.0 by 0.6                    | pyruvate, ribose, glucose, maltose, raffinose, starch, cellulose                   | 0.0–1.0/ 0.2             | 5°, Thio         | LA, AA, CO₂, ETOH, CO₂, iBut, iVal | [80] |
|                  | *Fervidobacterium riparium* | Hot spring, Russia            | 46–80/ 65               | 5.7–7.9/ 7.8     | 1.0–3.0 by 0.4–0.5                | peptone, YE, pyruvate, glucose, xylose, fructose, maltose, succrose, cellobiose, starch, xylan, CMC, cellulose, filter paper | 0.0–1.0/ 0.0             | 5°               | H₂, AA, CO₂, PPA, iBut, But | [81] |
|                  | *Fervidobacterium gondwanense* | Hot spring, Australia         | 45–80/ 65–68            | 5.5–8.5/ 7.0     | 4.0–4.0 by 0.5–0.6                | cellobiose, amylopectin, maltose, starch, dextrin, xylose, glucose, pyruvate, lactose, fructose, mannose, CMC, galactose | 0.0–0.6/ 0.1             | 5°               | ETOH, AA, LA, CO₂ H₂ | [82] |
|                  | *Fervidobacterium thailandese* | Hot spring, Thailand          | 60–88/ 78–80            | 6.5–8.5/ 7.5     | 1.1–2.5 by 0.5–0.6                | glucose, maltose, sucrose, fructose, cellobiose, CMC, cellulose, starch            | <0.5/0.5                  | 5°               | n.d.         | [83]  |
|                  | *Fervidobacterium changbaicum* | Hot spring, China             | 55–90/ 75–80            | 6.3–8.5/ 7.5     | 1.0–8.0 by 0.5–0.6                | glucose, lactose, fructose, sucrose, maltose, starch, sorbitol, cellobiose, trehalose, galactose, mellibiose, pyruvate, glycercin | 0.0–1.0/ 0.0             | 5°               | n.d.         | [84]  |
| *Thermosipho*    | *Thermosipho africanus* | Hot spring, Africa            | 53–77/ 75               | 6.0–8.0/ 7.2     | 3.0–4.0 by 0.5                    | glucose, ribose, maltose, starch, galactose, fructose, sucrose                     | 0.11–3.6                  | 5°, Thio         | AA, H₂, CO₂, ETOH, LA | [85]  |
|                  | *Thermosipho japonicus* | Hydrothermal vent, Japan      | 45–80/ 72               | 5.3–9.3/ 7.2–7.6 | 3.0–4.0 by 0.5                    | YE, peptone, and tryptone, maltose, glucose, galactose, starch, sarcharose, ribose, casein | 0.7–7.9/ 4.0             | 5°, Thio         | n.d.         | [86]  |
|                  | *Thermosipho geolei*  | Oil reservoir, Russia         | 45–75/ 70               | 6.0–9.4/ 7.5     | 2.0–3.0 by 0.4–0.6                | Glucose, peptone, beef extract, YE                                               | 0.5–7.0/ 2.0–3.0          | 5°               | H₂, AA, ALA, CO₂, iVal | [87]  |
| *Thermosipho*    | *Thermosipho auctus*   | Hydrothermal vent, Atlantic Ocean | 37–75/ 70              | 5.6–8.2/ 6.6     | 1.2–6.0 by 0.4–0.9                | YE, beef extract, glucose, maltose, sucrose, starch, dextrin, CMC, cellulose      | 1.0–5.5/ 2.0             | 5°               | AA, H₂, CO₂, ETOH | [88]  |
|                  | *Thermosipho globiformans* | Hydrothermal vent             | 40–75/ 68               | 5.0–8.2/ 6.8     | 2.0–4.0 by 0.5                    | YE, tryptone, starch                                                              | 0.2–5.2/ 2.5             | 5°               | Fe₂O₃        | n.d.  |
| Genus             | Species            | Isolation                  | Temp. Range/ Optimal (°C) | pH Range/ Optimal | Cell Dimension (Long by Wide) (µm) | Growth Substrates                                                                 | NaCl Range/ Optimal (%) | Electron Acceptor | End Products         | Ref. |
|-------------------|--------------------|----------------------------|---------------------------|-------------------|----------------------------------|--------------------------------------------------------------------------------|--------------------------|-------------------|---------------------|------|
| Thermosipho      | melanesiensis      | Hydrothermal vent, Pacific Ocean | 50–75/ 70                | 4.5–8.5/ 6.5–7.5   | 1.0–3.5 by 0.4–0.6                | BHI, malt extract, tryptone, sucrose, starch, glucose, maltose, lactose, cellulose, galactose | 1.0–6.0/ 3.0            | S⁰               | H₂, AA, ALA, CO₂   | [90] |
| Thermosipho      | activus            | Riftia sheath, Guaymas Basin | 44–75/ 65                 | 5.5–8.0/ 6.0       | 1.5–10.0 by 0.3–0.8               | glucose, maltose, cellulose, filter paper, chitin, xylan, pectin, xanthan gum, YE, beef extract, tryptone, casein, keratin, arabinose, xylose, gelatin | 0.3–6.0/ 2.5            | S⁰, Fe (III)    | AA, H₂, CO₂        | [91] |
| Thermosipho      | atlanticus         | Hydrothermal vent, Atlantic Ocean | 45–80/ 65                | 5.0–9.0/ 6.0      | 1.0–2.6 by 0.2–0.6               | cellubiose, xylose, starch, LA, maltose, mannose, trehalose, lactose, arabinose, galactose, mannitol, peptone, casamino acids, gelatin, BHI, YE, glucose | 1.5–4.6/ 2.3            | S⁰, Thio, Cys     | AA, iVal, H₂, Gly, ALA, Pro | [92] |
| Geotoga           | subterranea        | Oilfields, USA              | 30–60/ 45                 | 5.5–9.0/ 6.5       | 4.0–7.5 by 0.5                    | mannose, starch, maltodextrins, glucose, lactose, sucrose, galactose, maltose | 0.5–10/ 4.0             | S⁰               | H₂, CO₂, AA, EtOH  | [10] |
| Geotoga           | petraea             | Oilfields, USA              | 30–55/ 50                 | 5.5–9.0/ 6.5       | 3.0–20.0 by 0.6                   | mannose, starch, maltodextrins, glucose, lactose, sucrose, galactose, maltose | 0.5–10/ 3.0             | S⁰               | H₂, CO₂, AA, EtOH  | [10] |
| Petrotoga         | miiotherma          | Oilfields, USA              | 35–65/ 55                 | 5.5–9.0/ 6.5       | 2.0–7.5 by 0.6                    | mannose, starch, maltodextrins, glucose, lactose, sucrose, galactose, maltose, maltodextrins, xylose | 0.5–10/ 2.0             | S⁰               | H₂, CO₂, AA, EtOH  | [10] |
| Petrotoga         | olearia              | Oil reservoir, Russia       | 37–60/ 55                 | 6.5–8.5/ 7.5       | 0.9–2.5 by 0.3–0.6               | arabinose, xylose, cellobiose, dextrin, sucrose, glucose, fructose, maltose, ribose, trehalose, xylan, pyruvate, peptone, starch | 0.5–8.0/ 2.0            | S⁰               | H₂, AA, LA, ALA, EtOH | [93] |
| Petrotoga         | sibirica             | Oil reservoir, Russia       | 37–55/ 55                 | 6.5–9.4/ 8.0       | 0.9–2.5 by 0.3–0.6               | sucrose, glucose, fructose, maltose, ribose, trehalose, xylan, pyruvate, peptone, galactose | 0.5–7.0/ 1.0            | S⁰               | H₂, AA, LA, ALA, EtOH | [93] |
| Petrotoga         | mobilis             | Oilfield, North Sea         | 40–65/ 58–60              | 5.5–8.5/ 6.5–7.0   | 1.0–50.0 by 0.3–1.5               | starch, xylan, maltodextrin, maltose, cellobiose, sucrose, lactose, glucose, galactose, fructose, arabinose, xylose, rhamnose | 0.5–9.0/ 3.0–4.0        | S⁰, Thio          | H₂, CO₂, AA, EtOH  | [94] |
| Petrotoga         | halophilica         | Offshore oil, Africa        | 45–65/ 60                 | 5.6–7.8/ 6.7–7.2   | 2.0–45.0 by 0.5–0.7               | arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, rhamnose, ribose, starch, sucrose, xylose, xylan, pyruvate | 0.5–9.0/ 4.0–6.0        | S⁰               | AA, LA, ALA, H₂, CO₂ | [95] |
| Genus       | Species            | Isolation                  | Temp. Range/ Optimal (°C) | pH Range/ Optimal | Cell Dimension (Long by Wide) (µm) | Growth Substrates                                      | NaCl Range/ Optimal (%) | Electron Acceptor | End Products | Ref.       |
|-------------|--------------------|-----------------------------|---------------------------|-------------------|-----------------------------------|--------------------------------------------------------|------------------------|------------------|--------------|------------|
| Petrotoga   | *mexicana*         | Offshore oil, Africa        | 25–65/55                  | 5.8–8.5/6.6       | 1.0–30.0 by 0.5–0.7              | arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, rhamnose, ribose, starch, sucrose, xylose, xylan, pyruvate. | 1.0–20.0/3.0           | S0, Thio, Sulfite | AA, LA, H2, CO2, ALA | [96]       |
| Petrotoga   | *japonica*         | Oil reservoir, Japan        | 40–65/60                  | 6.0–9.0/7.5       | 2.5–7.0 by 0.25–0.75             | starch, xylan, maltose, cellobiose, sucrose, lactose, glucose, galactose, fructose, casamino acids, mannose, arabinose, xylose, ribose. | 0.5–9.0/0.5–1.0         | S0, Thio     | AA, H2, CO2, ALA | [97]       |
| Marinitaoga | *piezophila*       | Hydrothermal chimney, Pacific Ocean | 45–70/65                | 5.0–8.0/6.0       | 1.0–1.5 by 0.5                   | starch, fructose, glucose, galactose, maltose, cellobiose, ribose, acetate | 1.0–5.0/3.0            | S0, Thio, Cys | n.d.         | [98]       |
| Marinitaoga | *litoralis*        | Hot spring, Indian Ocean    | 45–70/65                  | 5.5–7.5/6.0       | 1.0–7.0 by 0.8–1.0               | cellobiose, galactose, glucose, glycogen, lactose, maltose, ribose, starch, BHI, casamino acids, casein, peptone, pyruvate, tryptone, YE | 0.8–4.6/2.6            | n.d.          |             | [99]       |
| Marinitaoga | *okinawensis*      | Hydrothermal chimney, Okinawa | 30–70/55–60              | 5.5–7.4/5.5–5.8   | 1.5–5.0 by 0.5–0.8               | YE, tryptone, peptone, starch, glucose, glycine, pyruvate, 3-IAA, LA, CO2, H2, HPA, PA | 1.0–5.5/3.0–3.5        | S0, Thio, Cys | n.d.         | [100]      |
| Marinitaoga | *hydrogentolens*   | Hydrothermal chimney, Atlantic Ocean | 35–65/60                | 4.5–8.5/6.0       | 1.5–5.0 by 0.5–0.8               | glucose, starch, glycogen, chitin, YE, BHI, peptone, casein, pyruvate, maltose | 1.0–6.5/3.0–4.0        | S0, Thio, Cys | AA, EtOH, Fe, H2, CO2 | [101]      |
| Marinitaoga | *artic*            | Hydrothermal chimney, Norwegian | 45–70/65               | 5.0–7.5/5.5       | 1.0–5.0 by 0.5–0.8               | glucose, trehalose, maltose, sucrose, maltodextrin, starch, pectin, meat extract, tryptone, YE, pyruvate, fructose, mannose, cellobiose, cellulose, peptone | 1.5–5.5/2.5            | S0, Cys      | n.d.         | [102]      |
| Marinitaoga | *camini*           | Hydrothermal chimney, Atlantic Ridge | 25–65/55                | 5.0–9.0/7.0       | 2.0–3.0 by 0.5–1.0               | BHI, gluten, peptone, tryptone, pyruvate, glucose, fructose, maltose, cellobiose, sucrose, starch, cellulose, CMC, pectin, chitin | 1.0–4.5/2.0            | S0, Cys      | AA, iBut, iVal, H2, 3-IAA, LA, CO2, HPA, PA | [11]       |
| Oceanotoga  | *teriensis*        | Offshore oil, India         | 25–70/55–58              | 5.5–9.0/7.5       | 1.5–1.7 by 0.5–0.7               | glucose, fructose, cellobiose, arabinose, raffinose, rhamnose, sucrose, xylose, ribose, starch, ETOH, formate, acetate, BHI, YE, bio–tryptinase | 0.0–12/4.3             | S0, Thio     | AA, H2, CO2, EtOH | [12]       |
| Defluviitoga| *tunisiensis*      | Mesothermic digester        | 37–65/55                 | 6.7–7.9/6.9       | 3.0–30.0 by 1.0                  | arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, cellulose, xylan | 0.2–3.0/0.5            | S0, Thio     | AA, H2, CO2, | [9]        |

Table 1. Cont.
### Table 1. Cont.

| Genus     | Species            | Isolation          | Temp. Range/ Optimal (°C) | pH Range/ Optimal | Cell Dimension (Long by Wide) (µm) | Growth Substrates                                                                 | NaCl Range/ Optimal (%) | Electron Acceptor | End Products              | Ref. |
|-----------|--------------------|--------------------|---------------------------|-------------------|-------------------------------------|----------------------------------------------------------------------------------|------------------------|-------------------|------------------------|------|
| Mesotoga  | *Mesotoga infera*  | Deep aquifer, France | 30–50/ 45                | 6.2–7.9/ 7.4      | 2.0–4.0 by 1.0–2.0                  | arabinose, cellobiose, fructose, galactose, glucose, lactose, LA, mannose, maltose, raffinose, ribose, sucrose, xylose | 0.0–1.5/ 0.2          | $S^0$             | AA, CO$_2$              | [26] |
|           | *Mesotoga prima*   | Sediment, USA      | 20–50/ 57                | 6.5–8.0/ 7.5      | 1.0 by 0.2                          | xylose, fructose, ribose, sucrose, mannose, galactose, lactose, maltose, peptone, tryptone, casamino acids, glucose, arabinose, cellobiose, casein, pyruvate | 2.0–6.0/ 4.0          | $S^0$, Thio, Sulfite | AA, But, iBut, iVal, 2–MeBu | [8]  |
| Kosmotoga | *Kosmotoga arenicorallina* | Hot spring, Japan | 50–65/ 60                | 6.2–8.0/ 7.1      | 1.1–2.7 by 1.1–1.9                  | xylose, maltose, glycerol                                                        | 1.0–6.0/ 3.0           | $S^0$, Cys           | n.d.                   | [103]|
|           | *Kosmotoga pacifica* | Hydrothermal field, Pacific Ocean | 33–78/ 70                | 6.2–8.0/ 7.1      | 1.0 by 0.6                          | maltose, YE, peptone, BHI, glycerol, tryptone, xylose, glucose, fructose, cellobiose, trehalose, LA, propionate, glutamate | 0.5–6.0/ n.d.         | $S^0$, Cys           | n.d.                   | [104]|
|           | *Kosmotoga olearia* | Fluid, North Sea   | 20–80/ 65                | 5.5–8.0/ 6.8      | 0.8–1.2 by 0.4–0.7                  | maltose, ribose, sucrose, starch, casamino acids, tryptone, pyruvate              | 1.0–6.0/ 2.5–3.0       | Thio               | H$_2$, CO$_2$, AA, EIOH, PPA | [7]  |
|           | *Kosmotoga shengiensis* | Oilfield, China   | 45–75/ 65                | 6.0–8.0/ 7.0      | 0.7–0.9                             | glucose, acetate, mEtOH, galactose, fructose, xylose, sucrose, maltose, sorbitol, lactose, xylan, arabinose, formate, rhamnose, glycerol, pyruvate, starch, LA | 0.0–4.0/ 1.5           | $S^0$, Thio, Sulfate | AA, LA, LA, CO$_2$, H$_2$ | [15]|
| Athalassatoga | *Athalassatoga saccharophila* | Hot spring, Japan | 30–60/ 55                | 4.5–7.5/ 5.5–6.0  | 0.8–2.0 by 0.7–0.8                  | arabinose, fructose, glucose, lactose, maltose, mannose, ribose, sucrose, xylose, starch, glycogen, peptone, YE | <1/0.0                | Fe (III), Thio, Cys | AA, iBut, iVal       | [14] |
| Mesoaciditoga | *Mesoaciditoga laevis* | Hydrothermal vent, Pacific Ocean | 45–65/ 57–60             | 4.1–6.0/ 5.5–5.7  | 0.8–1.0 by 0.4                      | YE, peptone, maltose, sucrose, glucose, xylose, ribose, starch, tryptone          | 0.5–6.0/ 3.0           | $S^0$, Thio, Cys           | n.d.                   | [13] |
2. Operating Conditions

2.1. H₂ Partial Pressure (P_H₂)

Since Thermotogae members are hydrogen producers, tolerance to hydrogen produced by the bacteria on its own gaseous production, known as the “hydrogen partial pressure (P_H₂)” effect, is one of the primary parameters being extensively investigated [51,70,105]. The highest hydrogen tolerance has been observed in the genus Marinitoga. Mn. camini and Mn. piezophila were able to grow with H₂ concentrations up to 40% and 60%, respectively. Mn. hydrogenitolerans and Mn. okinawensis can grow under 100% H₂ atmosphere with only minor inhibition on growth and fermentation [100,101]. Their remarkable resistance to high H₂ levels is probably related to the typical habitats in which Marinotoga species thrive [100]. However, the growth of Thermotogae species is often inhibited by H₂ accumulation, and the metabolism of these organisms undergoes a series of rearrangements to suit P_H₂ levels in the bioreactor headspace. The majority of literature data refers to H₂ percentages in gaseous phase, although some studies have been reporting values of P_H₂. Partial pressure around 607 mbar led to decreased levels of biomass production, glucose consumption rate, and H₂ production in both T. neapolitana and T. maritima [106,107]. Boileau et al. [107] highlighted a shift of T. maritima glucose catabolism from acetic acid towards lactic acid when P_H₂ increased from 7 to 607 mbar (Table 2) [106,107]. In contrast, low P_H₂ (less than 80 mbar) promoted acetic acid accumulation. Biomass production and glucose consumption rate are unaffected when P_H₂ is maintained within the range of 7.1–178.5 mbar (Table 2) [105,106]. In fact, P_H₂ lower than 200 mbar is required for optimal growth in reactors, and P_H₂ around 2900 mbar completely inhibits growth in T. maritima [1,45,49,108,109].

Hydrogen evolution is driven by a bifurcating hydrogenase (H₂ase) that couples the oxidation of reduced ferredoxin (Fd) and NADH with the reduction of protons to H₂ (Figure 1) [58]. In dark fermentation, pyruvate is converted to acetate and ATP, which thermodynamically drives the H₂-acetate pathway. Under high H₂ partial pressure, hydrogenase activity is inhibited, NADH consumption stops, pyruvate is diverted away from acetic acid production, and lactic acid synthesis becomes the only mechanism for recycling reduced electron carriers (Figure 1) [28–30,57,64,106,110]. Synthesis of lactic acid by the lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate with the concomitant conversion of NADH to NAD⁺ (Figure 1). The depletion of the pyruvate pool, as occurs with the synthesis of lactic acid, negatively affects hydrogen yield, preventing it from reaching the theoretical maximal value (Figure 1) [24]. This problem can be overcome by enhancing the liquid-to-gas mass transfer and keeping H₂ concentrations low in experimental conditions (See Section 2.2) or by using mixed cultures with microbial species that are able to oxidize H₂ [27,111].
Figure 1. Schematic representation of *Thermotogae* metabolic fermentation. Dark fermentation (black arrows) of glucose leads to the production of $\text{H}_2$ and acetate. An increase in $\text{CO}_2$ concentration in the reactor headspace induces the recycling of Ac-CoA and $\text{CO}_2$ into lactate without impairing the synthesis of biogas (blue arrows). This process is named “Capnophilic lactic fermentation (CLF)” [30,31,56,70]. The main end-products of *Thermotogae* fermentation are $\text{H}_2$, lactate, and acetate. Other fermentation products are reported in red. Fe-Fe $\text{H}_2$ase = [Fe-Fe] hydrogenase; PFOR = Pyruvate ferredoxin oxidoreductase; LDH = Lactate dehydrogenase; Fd = Ferredoxin.

2.2. Shaking Speed, Culture/Headspace Volume Ratio, Gas Sparging, and Inoculum

Growth and metabolism of thermophilic bacteria are reported to be strongly affected by an increase in the hydrogen level, which makes the metabolic reactions thermodynamically unfavorable [112]. Many effective strategies have been developed to overcome the $\text{H}_2$ feedback inhibition, such as gas sparging, vigorous stirring, or simply increasing the gas/liquid volume ratio in the reactor. $\text{H}_2$ saturation is dependent on the partial pressure of hydrogen in the culture medium and its mass transfer from liquid to gas phase. As a matter of fact, the mass transfer of $\text{H}_2$ from liquid to gas can be improved by applying vigorous agitation in bioreactors [69,106]. Increased $\text{H}_2$ production rate, glucose consumption rate, and lactic acid synthesis have been observed in *T. neapolitana* cultures with agitation at 200 rpm, compared to static cultures, although the final $\text{H}_2$ yields were similar [106]. Comparable hydrogen yields were also observed when the agitation speed was 300 and 500 rpm, e.g.,
3.0 ± 0.0 mol H$_2$/mol glucose at 300 rpm vs. 3.2 ± 0.1 mol H$_2$/mol glucose at 500 rpm, with a mild improvement in fermentation rate (Table 2) [69]. In xylose fermentation, the highest hydrogen and organic acid yields have been reported at 400 rpm when tested in the range of 300–600 rpm [113].

To improve hydrogen liquid-gas mass transfer, Dreschke et al. [69] designed a new method that recirculated the H$_2$-rich biogas (GaR) into the T. neapolitana subsp. capnolactica broth with agitation (300, 500 rpm). This combination accelerated the H$_2$ evolution rate and glucose consumption rate during glucose fermentation, compared to the treatments including agitation but excluding GaR. Nonetheless, levels of the end-products, except for H$_2$ yield, were not significantly altered by the combined parameters (Table 2) [69].

Since $P_{\text{H}_2}$ depends on the culture/headsphere volume ratio in the bioreactors, its impacts on the performance of fermentation have also been investigated, mainly in batch reactors. Nguyen et al. [64] have experimented various culture/headsphere volume ratio from 8.3% (10 mL/120 mL) up to 50% (60 mL/120 mL) in T. neapolitana and T. maritima cultures [64]. At 8.3%, the H$_2$ production is the highest for both species (890 mL H$_2$/L medium in T. neapolitana and 883 mL H$_2$/L medium in T. maritima). H$_2$ production gradually diminished, and lactic acid production was promoted with increasing culture volumes [30,64,110]. d’Ippolito et al. [30] found 1:3 culture/headsphere volume was the most suitable ratio for high hydrogen yields [30]. When these conditions were optimized, T. neapolitana resulted in H$_2$ yields between 3.46–3.85 mol H$_2$/mol glucose [30,114].

Gas sparging, mainly with N$_2$, is the most common method to reduce hydrogen partial pressure by removing H$_2$ and CO$_2$ produced from sugar fermentation in closed bioreactors [56,108,115,116]. Under nitrogen sparging conditions, the overall yield of H$_2$ in T. neapolitana fermentation was about two-fold of the non-sparged cultures, e.g., 1.82 vs. 3.24 mol H$_2$/mol glucose or 1.14 vs. 2.20 mol H$_2$/mol xylose (Table 2). The levels of acetic acid and butyrate also increased [110]. Moreover, the fermentation performance was remarkably improved when N$_2$-sparging was coupled with pH control in T. neapolitana using pure glycerol as the sole carbon source (Table 2) [116]. Keeping pH close to neutral improved the glucose utilization and H$_2$-acetate production rates. In contrast, lactic acid production was lowered under these conditions (0.255 mmol/L with pH control and sparging vs. 0.36 mmol/L with pH control but no sparging) (Table 2) [116].

The use of a CO$_2$-enriched atmosphere significantly increased both glucose consumption rate and hydrogen production rate, even though the molar yield was comparable to that of N$_2$-sparging (Table 2) [31]. Surprisingly, supplementation of CO$_2$ to T. neapolitana cultures induced an unexpected metabolic shift from acetate to lactic fermentation without any significant change in hydrogen production (3.6 mol/L/mol glucose) (Table 2) [31]. Experiments with labeled precursors revealed that part of the exogenous CO$_2$ was biologically coupled with acetyl-CoA to give lactic acid when the cultures were sparged with CO$_2$ gas or enriched in sodium bicarbonate (Figure 1) [117]. This process, named Capnophilic Lactic Fermentation (CLF), has the surprising feature to produce more lactic acid than expected from the classical dark fermentation model where H$_2$ production is impaired by the onset of by-passing pathways (Figure 1) [31,56,117–119]. In dark fermentation, hydrogen and lactic acid levels competed for a common pool of reducing power. Whereas, in CLF, the H$_2$ level remained high, probably due to additional sources of reductants to sustain NADH-dependent pathways (Figure 1) [118–120]. Recently, an additional increase in lactic acid production occurred in a T. neapolitana mutant that was isolated from a culture adapted to continuous exposure to CO$_2$ [62]. Sparging with CO$_2$ was also performed on the culture of other Thermotogales species, whose metabolic response was qualitatively and quantitatively diverse (Table 2) [70]. CO$_2$-enriched conditions promoted glucose consumption rate and lowered biogas production in almost all tested species [70]. T. caldifontis, Pseudot. elfii, Pseudot. thermarum, Pseudot. lettingiae, and Pseudot. subterranea did not show substantial variations in the levels of the fermentation products compared to cultures in an N$_2$-enriched atmosphere [70]. T. neapolitana, T. maritima, T. profunda, and Pseudot. hypogea species responded to CO$_2$ by reducing the fermentation rate. T. neapolitana subsp. capno-
lactica was the only species to increase lactic acid and H\textsubscript{2} yield moving from N\textsubscript{2}-sparging to CO\textsubscript{2}-sparging \[70\]. Generally speaking, the supplementation of external gas (N\textsubscript{2} or CO\textsubscript{2}) successfully improves the fermentation performance in most species and lowers the inhibitory effect of H\textsubscript{2} accumulation, but it inevitably causes an undesired dilution of hydrogen in evolved gases. In this context, the recirculation of the H\textsubscript{2}-rich biogas method prevents hydrogen saturation in the bioreactor without negatively affecting the content of the produced biogas \[69\].

The initial biomass concentration (size of inoculum) also has an unexpected impact on the fermentation of thermophilic bacteria. Using various initial biomass concentrations of \textit{T. neapolitana subs. capnolactica} (in the range of 0.46–1.74 g CDW/L) under CO\textsubscript{2} atmosphere, hydrogen yield and the distribution of end-products were unaffected (Table 2) \[68\]. However, increasing inoculum size from 0.46 to 1.74 g/L reduced the fermentation time from 7 h to 3 h \[68\]. Moreover, the hydrogen production rate, glucose consumption rate, and biomass growth rate were increased \[49,50,68\]. It is worth pointing out that Ngo et al. \[116\] reported a reverse correlation between hydrogen production rate and inoculum size, stating that high initial biomass corresponded to a mild reduction of hydrogen production rate \[116\].

2.3. pH

As the fermentation of sugars leads to the production and accumulation of organic acids, the pH is decreasing during the process, which may inhibit bacterial growth before the substrates are completely consumed \[30,106,113\]. Two factors impose a strong inhibition on bacterial growth and H\textsubscript{2} production: rapid decrease in pH due to the accumulation of byproducts and feedback inhibition caused by H\textsubscript{2} accumulated in the headspace \[65,105–108,113,121\].

Thus, pH is a critical factor to control sugar consumption and direct end-products formation \[65,67,117,119,122\]. Gradual pH drop causes enzyme activity loss \[123\]. To overcome pH-induced limitations on \textit{Thermotoga} fermentation, several studies were performed with pH adjustments \[51,67,121\]. In pH-controlled cultures (~6.5–7.0), H\textsubscript{2} and acetic acid production predominated over lactic acid and peaked around 20 h \[113\]. In contrast, lactic acid production only started when pH declined to around 5.0 \[113\].

The addition of NaOH at regular intervals and the use of buffering reagents have been regarded as the best-performing methods with serum bottles \[56,66,67,113\]. The optimum pH for growth and hydrogen production is 6.5–7.0 in \textit{T. maritima} and 6.5–7.5 in \textit{T. neapolitana} depending on substrates and growth conditions \[64,113,122\]. Moreover, pH 7.0 provides the most promising results in terms of H\textsubscript{2} and organic acids production in \textit{T. neapolitana} \[113,122\]. A pH shift from 5.5 to 7.0 improved H\textsubscript{2} yield from 125 to 198 mL H\textsubscript{2}/L medium in \textit{T. neapolitana} \[61\]. With \textit{T. neapolitana} cells immobilized on ceramic surfaces using glucose as the carbon source, the highest hydrogen production was observed in the pH range of 7.7–8.5 \[51\]. Further increase in the range of pH to 8.0–9.0 led to a dramatic decrease in the biogas evolution \[64\].

Different organic and inorganic buffers have been examined for their effect on anaerobic fermentation under various growth conditions and buffer concentrations \[51\]. According to Cappelletti et al. \[51\], 0.1 M HEPES resulted in the best performance, compared to MOPS, PIPES, HPO\textsubscript{4}^{-}/H\textsubscript{2}PO\textsubscript{4}^{-}, or Tris-HCl buffer in \textit{T. neapolitana} batch cultures growing on glucose under N\textsubscript{2} atmosphere \[51\]. The good buffering properties of HEPES, whose pK (7.55) is near the optimal pH of \textit{T. neapolitana}, was also demonstrated for \textit{T. neapolitana} cultures growing on different complex carbon sources (cheese whey, molasses, or waste glycerol) \[51,122\]. In another study, 0.05 M HEPES was found to be sufficient under N\textsubscript{2} sparging atmosphere (Table 2) \[113\]. Under CLF conditions, 0.01 M MOPS, TRIS, or HEPES buffers provided satisfactory results for both H\textsubscript{2} and lactic acid synthesis in \textit{T. neapolitana subs. capnolactica} (Table 2) \[67\]. More specifically, H\textsubscript{2} synthesis was found to be the highest in MOPS, while TRIS promoted acetic acid formation (Table 2) \[67\]. The highest value of
lactic acid synthesis was 14.9 ± 0.3 mM in phosphate buffer compared to 11.3 ± 0.6 mM in the standard condition (Table 2) [67].

The buffering capacity of HCO$_3^-$ is sufficient to maintain near to optimal pH for growth (~6.5), facilitating the complete substrate degradation and desired by-product formation (Table 2) [31,56,67].

In other studies, itaconic acid was successfully used as a physiological buffer to enhance hydrogen production in *T. neapolitana* growing on glucose or glycerol [121,122]. During the cultivation with 1.5 g/L itaconic acid, the pH slowly dropped from 7.5 to 6.8 over 99 h, while the same pH change was reached within 48 h in cultures not buffered [122]. Although itaconic acid is only poorly catabolized, it affected the overall metabolism of *T. neapolitana* because H$_2$ and acetic acid production were almost 1.4-fold higher than the control, while lactic acid production was reduced by nearly 100% compared to the control (Table 2) [122]. In addition, Ngo and Sim [122] found that the performance of *T. neapolitana* fermentation growing on waste glycerol was improved by almost 40% by adding itaconic acid into the culture medium [122].

### 2.4. Temperature

Due to their origin from hot habitats, bacterial species of the phylum *Thermotogae* can live and grow at temperatures in the range of 40–90 °C (Table 1). Some species such as *K. olearia*, *O. teriensis*, *Ms. prima*, and *P. mexicana* can thrive at mesophilic temperatures (Table 1) [7,8,96,100], and other species such as *F. changbaicum*, *F. thailandese*, *T. maritima*, *Pseudot. hypogea*, and *T. neapolitana* share the ability of growing at temperatures close to 90 °C (Table 1) [3,74,77,83,94]. For a long time, researchers have selected an operating temperature of 70 °C [104,117] or 80 °C [105] to cultivate *T. neapolitana* and *T. maritima* without careful investigation of the impacts on fermentation. Nguyen et al. [64] explored changes of H$_2$ production with temperatures ranging from 55 to 90 °C for *T. neapolitana* and *T. maritima*. Both cultures showed approximately 100 mL H$_2$/L medium at 55 °C and a maximum of 200 mL H$_2$/L medium at 75–80 °C, with a decrease to 150 H$_2$/L medium at 90 °C [64]. In *T. neapolitana*, high temperatures (77–85 °C) enhanced glucose uptake (2.2 mmol/L at 60 °C and 11.0 mmol/L at 77–85 °C) and boosted hydrogen yields (2.04 mol H$_2$/mol consumed glucose at 60 °C and 3.85 mol H$_2$/mol at 77 °C) [65]. This positive effect was also found for acetic acid (2.0 mmol/L at 60 °C and 18.0 mmol/L at 85 °C) and lactic acid production (no production at 60 °C and 1.25 mmol/L at 85 °C) (Table 2) [65]. Studies conducted on *T. maritima* hydrogenase demonstrated that this enzyme is unstable at the ambient temperature and its activity increased considerably with rising temperature (an activity of 25 units/mg at 20 °C and 110 units/mg at 90 °C [123].

### 2.5. Oxygen (O$_2$)

*Thermotogae* members occur in various hot ecosystems, including hot springs, deepsea, and shallow hydrothermal vents, and may also be exposed to O$_2$ in these ecological niches [1254]. Indeed, despite their anaerobic nature, O$_2$ tolerance is variable in the phylum; for example, *Thermotoga*, *Fervidobacterium*, and *Geotoga* genera can grow only under strictly anaerobic conditions, while *K. olearia* can survive in up to 15% O$_2$ [10]. With elemental sulfur, *Ts. atlanticus* can grow with up to 8% O$_2$ in the headspace [92]. Geochemical and microbial analyses demonstrated the wide distribution of *Thermotogae* species in ecosystems that are not only anaerobic but also partially oxygenated [124]. For this reason, the question of O$_2$ tolerance and microaerophilic metabolism of *Thermotogae* has been addressed by several studies [65,105,106,125–129]. Some researchers have demonstrated that low concentrations of O$_2$ are tolerated by *T. neapolitana* and *T. maritima* [127,128]. An O$_2$ insensitive hydrogenase has been described in *T. neapolitana*, explaining why microaerobic H$_2$ production and O$_2$ tolerance could take place in this bacterium [130]. Additionally, *Pseudot. hypogea* and *T. maritima* contain an NADH oxidase that may serve as an O$_2$ detoxification system [131,132]. Lakhal et al. [129] demonstrated O$_2$ consumption over 12 h during the stationary phase of *T. maritima* in a batch reactor without reducing agent [129]. O$_2$ presence
reduced glucose fermentation rate and significantly shifted metabolism towards lactic acid production in *T. maritima* (Table 2). This change can probably be explained by O$_2$ sensitivity of the hydrogenase [129]. Furthermore, *T. maritima* overproduced enzymes involved in reactive oxygen species (ROS) detoxification, iron-sulfur cluster synthesis/repair, cysteine biosynthesis, and a flavoprotein homologous to the rubredoxin of *Desulfovibrio* species that exhibited an oxygen reductase activity [127].

Van Ooteghem et al. [121] reported that O$_2$ concentration decreased during the growth of *F. pennavorans*, *P. miotherma*, *Ts. africanus*, *Pseudot. elfii*, and *T. neapolitana*. In these experiments, the H$_2$ yield greatly exceeded the theoretical limit of 4 mol H$_2$/mol glucose in *F. pennavorans*, *Pseudot. elfii*, and *T. neapolitana* fermentation [121]. These surprisingly high H$_2$ yield have led to the hypothesis of an unidentified aerobic pathway using O$_2$ as a terminal electron acceptor in these bacteria which may not be obligate anaerobes [121]. However, aerobic metabolism is not supported by the genomic sequence of *T. maritima*, although the enzymes involved in the pentose phosphate pathway and an NADPH-reducing hydrogenase have been identified in the genome [16]. To explain the increased yield of H$_2$ by *T. neapolitana* in microaerobic conditions and the existence of a catabolic process requiring O$_2$, van Ooteghem et al. [121] used malonic acid as an inhibitor of succinate dehydrogenase and thus the O$_2$-dependent metabolism. Even if the coding sequence for succinate dehydrogenase has not been identified in the *T. maritima* genome, hydrogen generation was completely inhibited for >40 h in the presence of malonate, postulating that malonate in the medium was no longer available to block catabolism [121]. Then, Eriksen et al. [106] demonstrated that malonic acid was not metabolized by *T. neapolitana* cultures but the exposure to malonic acid clearly affected the metabolism as reduced production of lactic acid and increased H$_2$ yield were observed [106]. Against these findings, other researchers reported a reduction of H$_2$ rate and production in *T. neapolitana* cultures after the injection of 6% O$_2$ [65,106]. The reduction of O$_2$ consumes reducing equivalents that are then unavailable to produce H$_2$. The total duration of *T. maritima* fermentation in the batch reactor was delayed about 67 h under O$_2$-induced stress [129]. In addition, the consumption rate of glucose was drastically reduced and the metabolism of *T. maritima* shifted towards lactic acid production due to inhibition of the O$_2$-sensitive hydrogenase [129].

From a technical point of view, several strategies were adopted to remove dissolved O$_2$ in the bioreactor: [I] sparging the culture with N$_2$, CO$_2$ or a mixture of both gases; [II] heating the medium; [III] adding a reducing agent such as sodium sulfide or cysteine-HCl in the medium; [IV] maintaining a positive pressure in the bioreactor headspace [31,56,62,67,70,105,106,113,121].
Table 2. Effects of operating conditions on *Thermotoga* fermentation. MOPS: Morpholinopropane-1-sulfonic acid; HEPES: 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid; TRIS: tris(idrossimetil)amminometil cloridrato; CDW: Cellular dry weight; AA: Acetic acid; LA: Lactic acid; ALA: Alanine; But: Butyrate; IA: Itaconic acid; GaR: recirculation of H$_2$-rich biogas. Experiments were performed in different bioreactor configurations: B = Batch; CSTR = Continuous-flow Stirred-Tank Reactor; CSABR: Continuously Stirred Anaerobic Bioreactor; SB = Serum bottles. H$_2$ column: $^a$ H$_2$ yield = mol H$_2$/mol consumed substrate; $^b$ mL/L culture. * Values extrapolated from the graphical representation of data.

| Parameter | Organism | T (°C) | Culture Type | Mixing Speed (rpm) | Reactor/Working Volume (L) | Substrate Loaded (mmol/L) | Operational Parameter | Substrate Consumed (mmol/L) | Products |
|-----------|----------|--------|--------------|--------------------|----------------------------|---------------------------|----------------------|--------------------------|----------|
| $P_{H2}$ (mbar) | *T. maritima* | 80 | B | 350 | 1.4/0.1 Glucose (28) | | | $P_{H2}$ = 7.1 ± 0.4 | 19.8 ± 1.1 | 2.34 | 25.0 ± 1.4 | 10.5 ± 0.5 | **[107]** |
| | | | | | | | | $P_{H2}$ = 71.4 ± 2.1 | 19.7 ± 1.4 | 2.44 | 24.6 ± 2.4 | 11.0 ± 0.6 | |
| | | | | | | | | $P_{H2}$ = 178.5 ± 3.5 | 17.2 ± 0.9 | 2.32 | 20.1 ± 1.0 | 9.4 ± 0.5 | |
| | | | | | | | | $P_{H2}$ = 606.9 ± 18.7 | 13.4 ± 0.7 | n. d. | 13.0 ± 0.7 | 11.0 ± 0.6 | |
| Stirring Speed (rpm) | *T. neapolitana* | 75 | CSABR | 300 | 3.0/1.0 Xylose (33.3) | | | | |
| | | | | | | | | 400 | 32.56 | 2.94 ± 0.15 | 41.8 ± 2.16 | 4.8 ± 0.22 | **[113]** |
| | | | | | | | | 500 | 32.03 | 2.31 ± 0.12 | 44.62 ± 2.16 | 4.84 ± 0.22 | |
| | | | | | | | | 600 | 31.87 | 2.24 ± 0.11 | 41.12 ± 2.0 | 1.89 ± 0.11 | |
| | | 80 | CSTR | 300 | 3.0/2.0 Glucose (28) | | | | |
| | | | | | | | | 500 | 24.7 ± 1.2 | 3.5 ± 0.2 | 39.2 ± 1.2 | 4.4 ± 0.1 | **[69]** |
| | | | | | | | | 300 | 24.9 ± 0.2 | 3.3 ± 0.1 | 38.7 ± 2.2 | 5.1 ± 0.5 | 0.8 ± 0.0 | |
| Gas sparging | *T. neapolitana* subsp. capnolactica | 80 | SB | 250 | 3.8/1.0 Glucose (28) | | | | |
| | | | | | | | | w/o | 13 ± 0.6 | 1.24 ± 0.06 | 8.71 ± 0.35 | 0.36 ± 0.02 | **[31]** |
| | | | | | | | | N$_2$ | 14 ± 0.7 | 2.06 ± 0.09 | 10.04 ± 0.5 | 0.34 ± 0.02 | |
| | | | | | | | | N$_2$ plus pH control | 18 ± 0.9 | 1.98 ± 0.1 | 12.62 ± 0.53 | 0.25 ± 0.01 | **[115]** |
| Gas sparging | *T. neapolitana* | 77 | SB | 150 | 0.12/0.04 | | | | |
| | | | | | | | | w/o | - | 1.82 ± 0.09 | 64.28 ± 2.83 | 33.48 ± 1.47 | **[110]** |
| | | | | | | | | N$_2$ | - | 3.24 ± 0.14 | 81.42 ± 3.49 | 36.77 ± 2.04 | |
| | | | | | | | | N$_2$ | - | 1.14 ± 0.07 | 40.30 ± 3.5 | 37.68 ± 1.7 | |
| | | | | | | | | N$_2$ | - | 2.20 ± 0.13 | 71.94 ± 3.66 | 50.62 ± 2.38 | |
| | | | 80 | SB | 0.12/0.03 | | | | |
| | | | | | | | | Glucose (28) | 25.7 ± 0.1 | 2.5 ± 0.06 | 27.3 ± 0.8 | 8.6 ± 0.2 | **[70]** |
| | | | | | | | | CO$_2$ | 28.3 ± 1.0 | 2.9 ± 0.21 | 22.1 ± 0.9 | 11.3 ± 0.1 | 3.0 ± 0.3 |
### Table 2. Cont.

| Parameter | Organism                | T (°C) | Culture Type | Mixing Speed (rpm) | Reactor/Working Volume (L) | Substrate Loaded (mmol/L) | Operational Parameter | Substrate Consumed (mmol/L) | Products |
|-----------|-------------------------|--------|--------------|-------------------|---------------------------|---------------------------|-----------------------|---------------------------|----------|
|           |                         |        |              |                   |                           |                           |                       | H₂ yield ^a | AA (mmol/L) | LA (mmol/L) | ALA (mmol/L) | But (mmol/L) | Ref. |
| Gas sparging | T. neapolitana     | 80     | SB no        | 0.12/0.03        | Glucose (28)              | N₂                       | 21.7 ± 0.6             | 2.5 ± 0.03       | 30.2 ± 0.4      | 2.2 ± 0.02 | 1.9 ± 0.3  |             |     |
|           | T. maritima           | 80     | SB no        | 0.12/0.03        | Glucose (28)              | N₂                       | 23.2 ± 1.0             | 1.9 ± 0.06       | 25.5 ± 0.5      | 5.3 ± 0.8  | 2.4 ± 0.06 |             |     |
|           | T. naphthophila       | 80     | SB no        | 0.12/0.04        | Glucose (28)              | N₂                       | 19.9 ± 0.6             | 2.0 ± 0.1        | 18.3 ± 0.3      | 1.6 ± 0.2  | 2.3 ± 0.3  |             |     |
|           | T. petrophila          | 80     | SB no        | 0.12/0.05        | Glucose (28)              | N₂                       | 13.3 ± 1.10            | 2.20 ± 0.20      | 15.70 ± 0.10   | 1.40 ± 0.06 | 0.80 ± 0.10 | 1.80 ± 0.05 |     |
|           | T. caldifontis         | 70     | SB no        | 0.12/0.05        | Glucose (28)              | N₂                       | 10.90 ± 1.10           | 2.60 ± 0.10      | 16.70 ± 3.60   | 2.20 ± 0.50 | 3.20 ± 0.90 |             |     |
|           | T. profunda            | 60     | SB no        | 0.12/0.05        | Glucose (28)              | N₂                       | 15.20 ± 0.90           | 1.80 ± 0.03      | 16.50 ± 1.50   | 2.30 ± 0.40 | 6.60 ± 0.70 |             |     |
|           | Pseudot. hypogea       | 70     | SB no        | 0.12/0.05        | Glucose (28)              | N₂                       | 8.80 ± 1.10            | 1.10 ± 0.30      | 6.40 ± 0.10     | 0.10 ± 0.00 | 2.90 ± 0.10 |             |     |
|           | Pseudot. elfii         | 70     | SB no        | 0.12/0.05        | Glucose (28)              | N₂                       | 4.30 ± 0.10            | 0.50 ± 0.10      | 3.10 ± 0.20     | 0.10 ± 0.00 | 3.40 ± 0.30 |             |     |
|           | Pseudot. lettingae     | 70     | SB no        | 0.12/0.05        | Glucose (28)              | N₂                       | 7.00 ± 0.90            | 2.00 ± 0.20      | 8.30 ± 0.06     | 0.20 ± 0.03 | 4.20 ± 0.30 |             |     |
|           |                         |        |              |                   |                           | CO₂                      | 6.70 ± 0.70            | 2.10 ± 0.10      | 7.80 ± 0.30     | 0.10 ± 0.01 | 10.0 ± 0.30 |             |     |
|           | Gas sparging | Pseudot. subterranea | 70 | SB no | 0.12/0.05 | Glucose (28) | N₂ | 23.10 ± 2.10 | 1.80 ± 0.20 | 30.60 ± 6.90 | 16.20 ± 4.60 | 9.50 ± 10.40 |             |     |
|           |                         |        |              |                   |                           | CO₂                      | 27.00 ± 1.40           | 1.40 ± 0.10      | 31.90 ± 7.90   | 10.70 ± 4.00 | 20.0 ± 8.00 |             |     |
|           | Pseudot. thermarum     | 80     | SB no        | 0.12/0.05        | Glucose (28)              | N₂                       | Complete                | 1.8 ± 0.02       | 30.00 ± 2.20   | 6.50 ± 0.20 | 1.10 ± 0.07 |             |     |
|           |                         |        |              |                   |                           | CO₂                      | Complete                | 1.50 ± 0.10      | 24.80 ± 0.70   | 5.60 ± 0.60 | 2.20 ± 0.20 |             |     |
| Bioass (g CDW/L)| T. neapolitana | 80 | Flask | 300 0.25/0.2 | Glucose (28) | N₂ | 0.46 3.2 ± 0.04 | 2.39 34.3 ± 0.6 | 10.9 ± 0.4 | 0.91 2.9 ± 0.06 | 2.44 32.9 ± 0.8 | 12.2 ± 0.8 | 1.33 3.4 ± 0.01 | 2.58 32.3 ± 0.2 | 11.5 ± 0.5 | 1.74 3.0 ± 0.04 | 2.37 31.4 ± 1.1 | 14.7 ± 0.7 | [88] |
## Table 2. Cont.

| Parameter | Organism | T (°C) | Culture Type | Mixing Speed (rpm) | Reactor/Working Volume (L) | Substrate Loaded (mmol/L) | Operational Parameter | Substrate Consumed (mmol/L) | H<sub>2</sub> yield<sup>a</sup> | AA (mmol/L) | LA (mmol/L) | ALA (mmol/L) | But (mmol/L) | Ref. |
|-----------|----------|--------|--------------|-------------------|---------------------------|--------------------------|------------------------|--------------------------|------------------|-------------|-------------|-------------|-------------|------|
| pH        | T. neapolitana subsp. capnolactica | 80    | SB          | no                | 0.12/0.03                | Glucose (28)             | w/o pH control         | 18.54 ± 0.15            | 1.78 ± 0.29       | 22.76 ± 0.40 | 11.35 ± 0.62 |             | [67] |
|           |          |        |              |                   |                           | 0.01M MOPS               | 26.42 ± 0.05            | 3.27 ± 0.18            | 26.65 ± 0.87       | 14.23 ± 0.22 |             |             |             |      |
|           |          |        |              |                   |                           | 0.01M TRIS               | 25.55 ± 0.06            | 3.10 ± 0.10            | 26.77 ± 0.29       | 12.08 ± 0.89 |             |             |             |      |
|           |          |        |              |                   |                           | 0.01M HEPES              | 25.99 ± 0.03            | 2.85 ± 0.40            | 25.56 ± 0.49       | 13.58 ± 0.88 |             |             |             |      |
|           |          |        |              |                   |                           | 0.01M HCO<sub>3</sub>−   | 25.62 ± 0.10            | 2.20 ± 0.30            | 22.82 ± 0.84       | 14.63 ± 3.23 |             |             |             |      |
|           |          |        |              |                   |                           | 0.01M phosphate          | 26.17 ± 0.26            | 2.78 ± 0.40            | 24.70 ± 0.59       | 14.92 ± 0.25 |             |             |             |      |
| pH        | T. neapolitana | 75    | CSABR       | 300               | 3.0/1.0                   | Glucose (28)             | w/o pH control         | 21.98 ± 1.11            | 2.05 ± 0.1       | 30.81 ± 1.5   | 3.33 ± 0.22  |             | [113] |
|           |          |        |              |                   |                           | plus pH control          | 27.47 ± 1.39            | 3.2 ± 0.16            | 38.3 ± 2.0        | 1.77 ± 0.11  |             |             |             |      |
|           |          |        |              |                   |                           | Xylose (33.3)            | w/o pH control         | 29.77 ± 1.46            | 1.84 ± 0.09       | 34.47 ± 1.66  | 3.77 ± 0.22  |             |             | [113] |
|           |          |        |              |                   |                           | plus pH control          | 31.83 ± 1.6             | 2.22 ± 0.11            | 41.8 ± 2.0        | 1.66 ± 0.11  |             |             |             |      |
| pH        | T. neapolitana | 75    | CSABR       | 300               | 3.0/1.0                   | Sucrose (14.6)           | w/o pH control         | 13.78 ± 0.7             | 3.52 ± 0.18       | 33.13 ± 1.65  | 3.11 ± 0.11  |             |             | [113] |
|           |          |        |              |                   |                           | plus pH control          | 14.69 ± 0.06            | 4.95 ± 0.25            | 35.47 ± 1.83      | 2.11 ± 0.11  |             |             |             |      |
|           |          |        |              |                   |                           | w/o pH control          | 29.44                   | 1.85 ± 0.09            | 34.97 ± 1.66      | 3.88 ± 0.22  |             |             |             |      |
|           |          |        |              |                   |                           | pH = 6.5                | 32.57                   | 2.71 ± 0.14            | 49.62 ± 2.50      | 3.44 ± 0.11  |             |             |             |      |
|           |          |        |              |                   |                           | pH = 7.0                | 32.9                    | 2.84 ± 0.14            | 50.29 ± 2.50      | 4.00 ± 0.22  |             |             |             |      |
|           |          |        |              |                   |                           | pH = 7.5                | 31.77                   | 2.23 ± 0.11            | 41.96 ± 2.16      | 1.89 ± 0.11  |             |             |             |      |
| pH        | T. neapolitana | 75    | SB          | no                | 0.04/0.12                | Glycerol (108.6)         | w/o HEPES               | 16.96 ± 0.8             | 1.23 ± 0.06       | 9.14 ± 0.45   |             |             | [116] |
|           |          |        |              |                   |                           | 0.05 M HEPES             | 28.26 ± 1.4             | 2.73 ± 0.14            | 22.35 ± 1.05      |             |             |             |             |      |
|           |          |        |              |                   |                           | w/o NaHCO<sub>3</sub>     | 25.9 ± 1.3              | 2.8                   | 44.5 ± 5.4           | 12.5 ± 2.69  |             |             |             | [31] |
|           |          |        |              |                   |                           | NaHCO<sub>3</sub> 14 mM | 25.4 ± 2.1              | 1.7                   | 30.5 ± 4.9          | 18.0 ± 0.6   |             |             |             |      |
|           |          |        |              |                   |                           | NaHCO<sub>3</sub> 20 mM | 23.2 ± 1.9              | 1.0                   | 44.4 ± 8.2          | 9.2 ± 2.7    |             |             |             |      |
|           |          |        |              |                   |                           | NaHCO<sub>3</sub> 40 mM | 6.2 ± 0.8               | 2.7                   | 18.0 ± 4.3          | 0.7 ± 1.5   |             |             |             |      |
| pH        | T. neapolitana | 80    | B           | 250               | 3.8/1.0                   | Glucose (28)             | w/o IA                  | -                     | 438 ± 22        | 7.49 ± 0.33  | 3.55 ± 0.22  |             |             | [122] |
|           |          |        |              |                   |                           | 1.5 g/L IA               | -                     | 619 ± 30         | 11.49 ± 0.5  | 1.66 ± 0.0  |             |             |      |

<sup>a</sup> Yield calculated as mmol/L/substrate mmol/L.
| Parameter | Organism          | T (°C) | Culture Type | Mixing Speed (rpm) | Reactor/Working Volume (L) | Substrate Loaded (mmol/L) | Operational Parameter | Substrate Consumed (mmol/L) | H₂ yield | AA (mmol/L) | LA (mmol/L) | ALA (mmol/L) | But (mmol/L) | Ref. |
|-----------|-------------------|--------|--------------|-------------------|---------------------------|--------------------------|------------------------|--------------------------|-----------|-------------|-------------|--------------|-------------|------|
| Temp. (°C)| T. neapolitana     | 60     | SB           | 75                | 0.26/0.05                 | Glucose (14)             | 60                     | 2.2 *                     | 2.04 ± 0.05 | 2.0         | n. d        |              |              | [65] |
|          |                   | 65     |              |                   |                           |                          | 60                     | 5.0 *                     | 3.09 ± 0.3  | 7.0         | 0.05        |              |              |      |
|          |                   | 70     |              |                   |                           |                          | 70                     | 8.5 *                     | 3.18 ± 0.02 | 11.5        | 0.45        |              |              |      |
|          |                   | 77     |              |                   |                           |                          | 77                     | 11.0 ± 0.5 *              | 3.85 ± 0.28 | 16.5        | 0.85 ± 0.1  |              |              |      |
|          |                   | 85     |              |                   |                           |                          | 85                     | 11.0 ± 0.5 *              | 3.75 ± 0.49 | 18.0 ± 1.0  | 1.25 ± 0.05 |              |              |      |
| Oxygen   | T. maritima        | 80     | B            | 150               | 2.30/1.53                 | Glucose (20)             | w/o O₂                  | 17.41                    | 38.09 b     | 18.05       | 4.36        | 1.60 ± 0.2   |              | [129]|
|          |                   |        |              |                   |                           |                          | with O₂                 | 19.30                    | 31.75 b     | 18.27       | 5.45        | 1.30 ± 0.2   |              |      |
3. Nitrogen Containing-Compounds

Nitrogen sources (N-sources) are essential for bacterial life for the synthesis of cellular components like nucleic acids, proteins, and enzymes [133,134]. Yeast extract (YE), tryptone, and ammonium chloride (NH₄Cl) have been identified as highly efficient and versatile organic N-sources in laboratory practices. It is widely demonstrated that most of the Thermotogae members can use yeast extract and tryptone to grow and metabolize carbohydrates [1,10,77,108,135,136].

Numerous efforts were made to replace YE by combining casamino acids and amino acids, but Pseudot. elfii failed to grow on these alternative substrates. The biogas yields of cultures grown with other N-sources were about 4–14% of those with YE (Table 3) [108].

Experiments with different concentrations of YE and tryptone were performed to identify their optimal and minimal concentrations in growth media [64,108,122,137,138]. YE and tryptone are sufficient to ensure growth and hydrogen production without additional carbon sources in Pseudot. elfii (Table 3) [108]. van Niel et al. [108] used media with various concentrations of YE and tryptone to ferment glucose by Pseudot. elfii [108]. They discovered that increasing the contents of both YE and tryptone from 2 g/L to 5 g/L improved H₂ production (14.8 vs. 28.8 mmol/L) but higher contents did not further improve hydrogen and acetic acid production; high levels of both YE and tryptone only increased acetic acid production in medium lacking other C-sources [108].

When there was a low level of YE (2 g/L) but no tryptone, productions of H₂ and acetic acid remained low, suggesting that tryptone served as an energy source like YE (Table 3) [108]. Although the amino acid compositions of the two N-sources are fairly similar, tryptone contains abundant peptides, a preferred form of amino acids by many bacteria [138]. In another study [122], T. neapolitana biomass increased along with the increase of YE concentrations in the range of 1.0–4.0 g/L but not with higher YE concentrations (5.0–6.0 g/L) [122]. The H₂ production plateaued at 420 mL/L in T. neapolitana growing on glycerol with 1.0–4.0 g/L YE [122]. Experiments in T. maritima and T. neapolitana revealed that with over 2 g/L YE, there was a clear increase of acetic acid production, and hydrogen counted up to 30-33% of the total gas in the headspace, even though a mild reduction in glucose consumption occurred (Table 3) [64,138].

Nevertheless, low concentrations (2–4 g/L) of YE are still able to support productivity and bacterial growth [64,108,122,138]. d’Ippolito et al. [30] reported that 2 g/L of both tryptone and YE contributed to 10–15% of the total fermentation products in T. neapolitana [30]. Balk et al. [75] demonstrated that Pseudot. lettingae was able to degrade methanol in around 30 days in the presence of 0.5 g/L YE, whereas the substrate degradation did not occur when YE was omitted [75]. In contrast, the fermentation of T. neapolitana with glucose occurred in a medium without YE, even though the total glucose consumption without YE was attained in 30 h rather than 12 h. H₂ and acetate amounts were half in the medium without YE, (Table 3) [135].

The impact of an inorganic N-source on Thermotogae fermentation, such as NH₄Cl, has not been extensively studied, but the presence of NH₄Cl has often been associated with either exopolysaccharide (EPS) formation in T. maritima or alanine production in T. neapolitana [62,129,136,139]. It is not clear how NH₄Cl stimulates EPS production, but it might involve processing the surplus of reducing equivalents. For example, some organisms produce EPS as a mechanism to transport reducing equivalents out of the cell [140].

Han and Xu [61] demonstrated that a surplus of NH₄Cl could partially substitute YE and tryptone in an optimized medium for auxotrophic Thermotoga sp. RQ7 strain [61].

4. Sodium Chloride and Phosphate

All members of the phylum Thermotogae showed great adaptability to a wide range of salinity levels (Table 1), although the optimal concentrations of NaCl vary among the members. Geotoga, Oceanotoga, and Petrotoga species can survive in environments comprised of 10% NaCl, while P. mexicana can live in up to 20% NaCl (Table 1) [10,12,95].
In contrast, species of the genus *Fervidobacterium* can tolerate salt concentrations up to 1% [5,79–81,83]. Among the species of the genus *Mesotoga*, *Ms. infera* exhibited the lowest tolerance of NaCl (Table 1).

NaCl at 20 g/L was reported to be optimal for *T. neapolitana* growing on either glucose or glycerol when hydrogen production is concerned [64,105,106,108,110,116]. Recently, the effect of different NaCl concentrations (0–35 g/L) on the CLF process was explored in *T. neapolitana subs. capnolactica* using glucose as the carbon source [67]. H2 synthesis and biomass growth were reduced by 15% and 25%, respectively, when NaCl was increased to 35 g/L (Table 3). Similarly, acetic acid production decreased from 26.1 ± 4.7 mM with 10 g/L NaCl to 23.2 ± 0.8 mM with 35 g/L NaCl. In contrast, high NaCl levels had a positive impact on lactic acid production, which increased 7.5-fold (2.8 ± 0.3 mM at 0 g/L NaCl vs. 21.6 ± 6.2 mM at 35 g/L NaCl), without affecting the overall H2 yields (Table 3) [67]. Pradhan and coworkers [67] suggested a possible involvement of NaCl in a sodium ion gradient that potentially fuels ATP synthesis and transport processes [67]. This creates a bioenergetic balance and supplies necessary reducing equivalents to convert acetic acid into lactic acid under CLF conditions (Figure 1) [67,118,119]. Similarly, another study [141] on H2-producing *Vibrionaceae* showed that increasing NaCl levels from 9 to 75 g/L enhanced lactic acid synthesis [141].

Regarding phosphate species, they have a strong buffering ability to mitigate pH fluctuation caused by the accumulation of volatile fatty acids [142]. Phosphate deficiency induced an increase in lactic acid production and a small decrease in H2 formation, suggesting a slight shift of the *T. maritima* metabolism towards lactic acid production. Besides its role as a macro-element, phosphate can also interact with calcium, favoring H2 production [141,143]. Saidi and co-workers [52] showed that *T. maritima* struggled to produce H2 at the same rate when there was an oversupply of calcium but an undersupply of phosphate in the medium [52]. For unknown reasons, phosphate exceeding 50 mM has been suggested to inhibit *Pseudot. elfii* growth [108].
Table 3. Effect of organic nitrogen source and NaCl on *Thermotoga* fermentation. AA: Acetic acid; LA: Lactic acid; ALA: Alanine; YE: Yeast extract; Tryp: Tryptone; CA: Casamino acids; V: Vitamins solution [108]; aa: Amino acids (cysteine, alanine, asparagine, proline, glutamine, serine, and tryptophan, added at 0.2 g/L each). Experiments were performed in different bioreactor configurations: B = Batch; SB = Serum bottles. \( \text{H}_2 \) column: \( a \) % \( \text{H}_2 \) = calculated setting hydrogen production yield on medium with yeast extract to 100%; \( b \) mmol \( \text{H}_2 \)/L medium; \( c \) mL \( \text{H}_2 \)/L culture; \( d \) mol \( \text{H}_2 \)/mol glucose. * Values extrapolated from the graphical representation of data.

| Parameter | Organism   | T (°C) | Culture Type | Mixing Speed (rpm) | Reactor/Working Volume (L) | Substrate Loaded (mmol/L) | Operational Parameter | Substrate Consumed (mmol/L) | Products |
|-----------|------------|--------|--------------|--------------------|--------------------------|--------------------------|----------------------|--------------------------|----------|
|           |            |        |              |                    |                          |                          |                      |                          | H2       |
|           |            |        |              |                    |                          |                          |                      |                          | AA       |
|           |            |        |              |                    |                          |                          |                      |                          | LA       |
|           |            |        |              |                    |                          |                          |                      |                          | ALA      |

| Parameter | Organism   | T (°C) | Culture Type | Mixing Speed (rpm) | Reactor/Working Volume (L) | Substrate Loaded (mmol/L) | Operational Parameter | Substrate Consumed (mmol/L) | Products |
|-----------|------------|--------|--------------|--------------------|--------------------------|--------------------------|----------------------|--------------------------|----------|
|           |            |        |              |                    |                          |                          |                      |                          | H2       |
|           |            |        |              |                    |                          |                          |                      |                          | AA       |
|           |            |        |              |                    |                          |                          |                      |                          | LA       |
|           |            |        |              |                    |                          |                          |                      |                          | ALA      |

| Parameter | Organism   | T (°C) | Culture Type | Mixing Speed (rpm) | Reactor/Working Volume (L) | Substrate Loaded (mmol/L) | Operational Parameter | Substrate Consumed (mmol/L) | Products |
|-----------|------------|--------|--------------|--------------------|--------------------------|--------------------------|----------------------|--------------------------|----------|
|           |            |        |              |                    |                          |                          |                      |                          | H2       |
|           |            |        |              |                    |                          |                          |                      |                          | AA       |
|           |            |        |              |                    |                          |                          |                      |                          | LA       |
|           |            |        |              |                    |                          |                          |                      |                          | ALA      |

| Parameter | Organism   | T (°C) | Culture Type | Mixing Speed (rpm) | Reactor/Working Volume (L) | Substrate Loaded (mmol/L) | Operational Parameter | Substrate Consumed (mmol/L) | Products |
|-----------|------------|--------|--------------|--------------------|--------------------------|--------------------------|----------------------|--------------------------|----------|
|           |            |        |              |                    |                          |                          |                      |                          | H2       |
|           |            |        |              |                    |                          |                          |                      |                          | AA       |
|           |            |        |              |                    |                          |                          |                      |                          | LA       |
|           |            |        |              |                    |                          |                          |                      |                          | ALA      |

References: [108], [64], [136]
### Table 3. Cont.

| Parameter          | Organism                        | T (°C) | Culture Type | Mixing Speed (rpm) | Reactor/Working Volume (L) | Substrate Loaded (mmol/L) | Operational Parameter | Substrate Consumed (mmol/L) | Products | Ref.  |
|-------------------|--------------------------------|--------|--------------|--------------------|----------------------------|---------------------------|------------------------|---------------------------|----------|-------|
| NaCl (g/L)        | *T. neapolitana* subsp. *capnolactica* | 80     | SB           | no                 | 0.12/0.03                  | Glucose (28)              | w/o                    | 25.62 ± 0.07                | H2       | [67]  |
|                   |                                |        |              |                    |                            |                           | NaCl (5)               | 26.00 ± 0.14                | AA       |       |
|                   |                                |        |              |                    |                            |                           | NaCl (10)              | 26.12 ± 0.16                | LA       |       |
|                   |                                |        |              |                    |                            |                           | NaCl (20)              | 25.96 ± 0.11                | ALA      |       |
|                   |                                |        |              |                    |                            |                           | NaCl (30)              | 25.68 ± 0.25                |          |       |
5. Sulfur-Containing Compounds

All members of the phylum *Thermotogae* reduced sulfur-containing compounds such as elemental sulfur (*S*<sub>0</sub>), thiosulfate (Thio), and polysulfide to hydrogen sulfide (*H*<sub>2</sub>S), which is produced at the expense of *H*<sub>2</sub> (Table 1) [1,4,29,76,144,145]. Sufficient supply of sulfur-containing compounds seems to be critically important; due to a large requirement for Fe-S clusters by the hydrogenase (containing 20 atoms of Fe and 18 atoms of S), PFOR, and other enzymes (Figure 1) [123,146]. In the literature, the effect of sulfur sources has been widely explored. The reduction of *S*-sources is considered an electron-sink reaction to deplete the surplus of electron power [3,98,107,147]. It is well known that the growth of most anaerobic bacteria of the phylum *Thermotogae* is stimulated by *S*-sources, but not dependent on them [1,29,52,53,75,107,125,126,144]. Generally speaking, the substrate consumption rate is benefited from a sulfur supply in the medium, except for the methanol fermentation in *Pseudot.* lettingae, which is reduced by *S*-containing compounds (19.7 mmol/L w/o *S*-source, 18.7 mmol/L with Thio and 10.6 mmol/L with *S*<sup>0</sup>) (Table 4). Members of the *Mesotoga* genus are able to oxidize sugars, although with low efficiency, only when *S*<sup>0</sup> is used as the terminal electron acceptor [26,27,66,148,149]. This process gives acetic acid, CO<sub>2</sub>, and sulfide (2 mol of acetate and 4 mol of sulfide per mol of glucose), with no or trace amounts of *H*<sub>2</sub> (Table 4) [27]. After 250 days of *Ms.* prima cultivation, 9.21 ± 0.13 mmol/L of acetate was measured in the presence of *S*<sub>0</sub> rather than 1.67 ± 0.21 mM obtained in its absence (Table 4) [27]. Fadhlaoui and collaborators [27] argued that the metabolic differences between *Thermotoga* spp. and *Ms.* prima strains are related to the absence of a bifurcating [FeFe]-hydrogenase and the accumulation of NADH in *Ms.* prima, leading to growth inhibition in the absence of an external electron acceptor [27]. However, *Ms.* prima and *Ms.* infera strains grew more efficiently in a syntrophic association with a hydrogenotrophic microbial partner that serves as a biological electron acceptor compared to growing *Mesotoga* in a pure culture with sulfur as electron acceptor [26,27]. Boileau et al. [107] investigated the different responses of fermentation performance to different *S*-sources (Table 4) [107]. Among these compounds (Table 4), thiosulfate, cysteine, and *Na*<sub>2</sub>*S* were the most efficient ones to optimize *T.* maritima glucose fermentation (Table 4) [107]. Biogas production and glucose utilization increased in the order of no *S*-source < DMSO < *S*<sup>0</sup> < Thio < Methionine (Met) < *Na*<sub>2</sub>*S* < Cysteine (Cys) (Table 4) [107]. Moreover, *Na*<sub>2</sub>*S* and Cys increased acetic acid production 3-fold and *H*<sub>2</sub> production 2-fold (Table 4). Thiosulfate seemed to promote lactic acid formation (0.8 ± 0.1 mM w/o *S*-source and 6.3 ± 0.6 mM with Thio) without affecting other products [107]. Surprisingly, lactic acid was dependent on thiosulfate concentration (0.3 mol/mol glucose w/o Thio and 0.6 mol/mol glucose with 0.24 mmol Thio), even though the proportion between lactic and acetic acid yields remained constant (Table 4). DMSO had no significant impact on *T.* maritima fermentation parameters (Table 4) [107].

In the presence of thiosulfate, the growth and glutamate production of *Fervidobacterium* is stimulated; however, *S*<sup>0</sup> does not seem to help overcoming the *H*<sub>2</sub>-feedback inhibition (Table 4) [32,80,88,144]. *P.* olearia, *P.* sibirica, and *Ts.* africanus produced small amounts of ethanol (0.17 mM for both *Petrotoga* species and 0.79 mM for *Ts.* africanus) only in the absence of *S*-sources (Table 4) [93,145]. *Pseudot.* lettingae produced L-alanine, at the expense of acetate, only when thiosulfate or *S*<sup>0</sup> was present in the medium using methanol as the substrate (Table 4) [75]. Meanwhile, the presence of thiosulfate or *S*<sup>0</sup> resulted in increased production of acetic acid and decreased production of alanine in *Pseudot.* hypogea, *Ts.* melaniensis, *Ts.* geolei, *P.* olearia, and *P.* sibirica cultures, using glucose or xylose as the carbon source (Table 4) [77,87,90,93]. When *S*<sup>0</sup> is available, no hydrogen could be detected in *Mn.* hydrogenitolerans growing on glucose [101].

*Thermotoga* members have been widely employed to degrade different organic wastes, and their degradation significantly benefited from the presence of a reducing agent [51–54,113,116,138]. It is noteworthy to mention that high concentrations of thiosulfinate, a volatile organo-sulfur compound found in organic wastes, has an inhibitory effect on *T.* maritima growth [54]. Similarly, Tao et al. [150] demonstrated that thiosulfinate inhibited the *H*<sub>2</sub> production by mesophilic seed sludge when co-fermenting food wastes [150].
### Table 4: Effect of sulfur compounds on *Thermotogae* fermentation.

| Organism     | Carbon Source (mM) | Sulfur Source (mM) | Substrate Consumed (mmol/L) | Products mmol/L Culture | Ref. |
|--------------|--------------------|-------------------|-----------------------------|-------------------------|------|
| *T. maritima* | Glucose (25)       | w/o               | 7.1 ± 0.4                   | 21.3 ± 2.1              |      |
|              |                    | DMSO **           | 9.2 ± 0.5                   | 28.7 ± 2.9              |      |
|              |                    | S0 **             | 16.6 ± 0.8                  | 46.1 ± 4.6              |      |
|              |                    | Met **            | 18.3 ± 0.9                  | 53.3 ± 5.3              |      |
|              |                    | Thio **           | 17.5 ± 0.9                  | 47.3 ± 4.7              |      |
|              |                    | Cys **            | 20.4 ± 1.0                  | 58.5 ± 5.8              | [107]|
|              |                    | Na2S **           | 20.4 ± 1.0                  | 54.9 ± 5.5              |      |
|              | Glucose (60)       | w/o Thio          | 17.7 ± 1.9                  | 25.0 ± 2.2              |      |
|              |                    | Thio (0.01)       | 20.0 ± 1.1                  | 31.0 ± 2.3              |      |
|              |                    | Thio (0.03)       | 28.0 ± 1.5                  | 57.9 ± 4.8              |      |
|              |                    | Thio (0.06)       | 38.5 ± 2.0                  | 73.3 ± 5.9              |      |
|              |                    | Thio (0.12)       | 45.7 ± 2.5                  | 99.7 ± 8.3              |      |
|              |                    | Thio (0.18)       | 45.4 ± 2.2                  | 86.9 ± 8.2              |      |
|              |                    | Thio (0.24)       | 43.8 ± 2.2                  | 88.6 ± 8.9              |      |
|              | Glucose (20)       | w/o               | 13.70                       | 36.09                   | n.d. |
|              |                    | Thio (20)         | 13.55                       | 4.02                    | 0.80 | 14.45|
|              |                    | Thio (20)         | 13.90                       | 16.07                   | 16.12|
|              |                    | Thio (20)         | 13.90                       | 16.07                   | 16.12|
|              | Glucose (20)       | w/o               | 14.00                       | 31.67                   | 18.27|
|              |                    | Thio (20)         | 13.90                       | 16.07                   | 16.12|
| *T. neapolitana* | Glucose (20)       | w/o               | 19.70                       | n. d.                   |      | [145]|
|               |                    | Thio (20)         | 19.70                       | n. d.                   | 13.70|      | [75] |
|               |                    | S0 (2%)           | 10.6                        | n. d.                   | 11.2 |      |
| *Pseudot. lettingae* | Methanol (20) | w/o               | 8.60                        | 29.03                   | 4.49 | 1.71 | 15.08|
| *Pseudot. hypogea* | Glucose (20)    | w/o               | 8.60                        | 29.03                   | 4.49 | 1.71 | 15.08|

AA: Acetic acid; LA: Lactic acid; ALA: Alanine; EtOH: Ethanol; iVal: iValeterate; H2S: Hydrogen sulfide; Glu: Glutamate; DMSO: Dimethyl Sulfoxide; S0: Elemental sulfur; Met: Methionine; Thio: Thiosulfate; Cys: Cysteine; Na2S: Sodium sulfide. * Values extrapolated from the graphical representation of data. ** Concentrations of Sulfur compounds are 0.03 mol equivalent of sulfur. ^ H2 produced millimolar equivalent; b mmol; c µM.
| Organism             | Carbon Source (mM) | Sulfur Source (mM) | Substrate Consumed (mmol/L) | Products mmol/L Culture | Ref.  |
|---------------------|--------------------|--------------------|----------------------------|-------------------------|-------|
|                     |                    |                    | H2  | AA  | LA  | ALA | EtOH | iVal | H2S | Glu  |
| *Pseudot. hypogea*  | Glucose (20)       | w/o                | 7.0 | 9.4 | 5.0 | 1.7 | 1.0  | 0.2  |     | [77] |
|                     |                    | Thio (20)          | 13.0| 0.9 | 19.8| 1.0 | 1.6  | 15.1 |     |      |
| *Pseudot. hypogea*  | Xylose (20)        | w/o                | 12.9| 19.0| 8.9 | 2.4 | 1.0  | 0.2  |     | [77] |
|                     |                    | Thio (20)          | 12.0| 1.8 | 13.7| 1.3 | 1.0  | 7.5  |     |      |
| *Pseudot. elfii*    | Glucose (20)       | w/o                | 3.1 | 8.8 | 4.0 |     |      |      |     |      |
|                     |                    | Thio (20)          | 10.4| 2.0 | 17.9|     |      |      |     |      |
| Ts. geolei          | Glucose (0.28)     | w/o                | 7.0 | 9.3 | 8.5 | 1.2 | 0.5  |      |     | [87] |
|                     |                    | S0 (2%)            | 6.0 | 0.0 | 7.5 | 0.5 |      |      |     |      |
| Ms. Prima Phos Ac3  | Glucose (20)       | w/o                | 1.50±0.20 | <1 | 1.67±0.21 | 1.05±0.25 |      |     | [27] |
|                     |                    | S0                 | 6.57±0.19 | <1 | 9.21±0.13 | 24.40±0.30 |      |     |      |
| Ms. Prima MesG1Ag4.2T| Fructose (20)      | w/o                | 1.00±0.23 | <1 | 0.70±0.41 | 1.18±0.41 |      |     | [92] |
|                     |                    | S0                 | 3.27±0.85 | <1 | 8.48±1.96 | 18.03±5.16 |      |     |      |
| Ts. africamus       | Glucose (28)       | w/o                | 7.20| 16.80| 7.90| <0.2| 0.79 |      | n.d. | [145]|
|                     |                    | Thio (20)          | 7.70| 1.00| 12.40| -   | -   |      |      |      |
| Ts. atlanticus      | Glucose (28)       | w/o                | 5.6 | 12.5| 1.7 |     |      |      | 0.14 | [92] |
|                     |                    | S0 (1%)            | 6.0 | 7.5 | 1.9 |     |      |      | 1.3  |      |
| F. islandicum       | Glucose (20)       | w/o                | 14.20| 21.58| 6.25| 3.98 |      |      | n.d. | [145]|
|                     |                    | Thio (20)          | 16.20| 6.0 | 7.5 | 1.9 |     |      | 1.22 |      |
| F. pennavorans      | Glucose (11)       | w/o                | -   | 0.25| 6.7 | 4.0±0.5 |      |      | 1.3  | [32] |
|                     |                    | Thio (20)          | -   | 0.2 | 6.7 | 4.50 |      |      |      |      |
6. Metal Ions

Typically, hydrothermal ecosystems are enriched with essential micronutrients and trace metals such as soluble and insoluble iron, manganese, cobalt, and molybdenum. Some terrestrial hydrothermal waters are also characterized by chromium and uranium contents of several micrograms per liter [151]. The physiological roles that most of these metals play in microbial metabolism are still largely unknown. It is believed that their functions include energy generation and biosynthesis [151]. In addition, Mn, Fe, Zn, and Co metals are vitally important micro-elements for growth, essential for cellular transport processes, and serve as cofactors for many enzymes [152]. Understanding the physicochemical properties of extreme habitats can help to determine the metal toxicity limits on microbial growth in laboratory settings. Indeed, metal susceptibility tests have been carried out on *T. neapolitana*, *T. maritima*, and *Ts. africanus*, and have identified the following toxicity order: cadmium (1.0–10.0 µM) > zinc (0.01–0.1 mM) > nickel (1.0–5.0 mM) > cobalt (1.0–10.0 mM) [153].

Attention has also been paid to Fe (III) reduction by thermophilic bacteria, since Fe (III) may work as an external electron acceptor in microbial metabolism [154]. Members of the phylum *Thermotogae* are capable of coupling the reduction of iron with the oxidation of a wide range of organic and inorganic compounds. *T. maritima* reduced Fe (III) into Fe (II) exclusively with molecular hydrogen as an electron donor [154]. Fe (III) reduction has also been reported to stimulate growth and mitigate H₂ inhibition in *Pseudot. lettingae*, *Pseudot. subterranea*, *Pseudot. elfii*, *Ts. affectus*, *Ts. globiformans*, and *Ts. activus* [75,76,88,89,91].

The recently characterized member of the order *Mesoaciditogales*, *A. saccharophila*, changed fermentation end-products when growing with Fe (III), favoring the production of small amounts of acetate, isobutyrate, and isovalerate [14].

Ions and metals are generally supplied in *Thermotogae* growth media through Balch’s oligo-elements solution [155]. The removal of oligo-elements from *T. maritima* cultures resulted in a minor increase in lactic acid production (1.2 vs. 4.3 mmol/L) and a decrease in H₂ productivity (12.4 vs. 8.8 mmol/h/L) [52]. Limitation in iron lowered H₂ production by deviating the fermentation pathway towards the production of more reduced end-products such as lactic acid in mixed cultures [156,157]. Another study [139] highlighted how the supplementation of Fe ions to mixed cultures had pronounced effect on hydrogen activity [139]. Similarly, Fe²⁺ (as well as Co, Ni and Mn) stimulated *Pseudot. hypogea* alcohol dehydrogenase activity (ADH), an iron-containing enzyme involved in alcohol fermentation, by 10–15%, while Zn²⁺ completely inhibited the enzyme activity [158]. On the same base, the inclusion of tungsten in the growth medium of *T. maritima* increased the specific activity of both hydrogenase (by up to 10-fold) and PFOR in cell-free extracts, although the function of tungsten in the metabolism of *T. maritima* is not clear [123,126].

As for magnesium, potassium, and calcium ions, they not only play critical roles in bacterial growth, but also act as enzyme cofactors and ensure the survival of microorganisms in their hot ecosystems, by protecting double-stranded DNA from degradation [159]. The best cell yields were obtained with a low concentration of Mg²⁺ and a high concentration of Ca²⁺ [126]. It would be worthwhile to dig further into the metal ions repercussions on *Thermotogae* metabolism in future research.

7. Conclusions

Steam reforming of methane (CH₄) is currently used to produce hydrogen in the industry, as it is the most economic technology available so far. Producing hydrogen by biological means at an industrial scale remains as a challenge. Within the race to find the best way to generate hydrogen via microbes (e.g., choice of strains, substrates, fermentation conditions), *Thermotogae* seem to have many unique advantages. Optimization of their cultivation conditions is fundamental to improve the overall productivity of the fermentation system and its profitability, which determine the feasibility of replacing the current methods of hydrogen production.

The phylum *Thermotogae* comprises a wide collection of species with astonishing and unique features associated to their original habitats. Extensive research has shown tremen-
ous potentials of using these bacteria in biological production of hydrogen, degradation of wastes, and isolation of thermostable enzymes.

Many factors affect the anaerobic metabolism of Thermotogae species, including operating conditions (shaking, inoculum, gas sparging, and culture/headspace volume ratio), temperature, pH, nitrogen, sulfur-containing compounds, sodium chloride, phosphate, and metal ions. Optimization of these fermentation parameters has been intensively pursued with Thermotoga and Pseudothermotoga species, which are the best hydrogen producers in the phylum. In contrast, little is known regarding other species of the phylum, especially their ability to synthesize desirable biological products.

In general, Thermotogae fermentation is affected by the accumulation of produced biogas and organic acids because they increase hydrogen partial pressure inside of the bioreactor and drastically reduce the pH of the cultivation medium. Consequently, the metabolic process stops before the substrate is completely consumed. Gas sparging, stirring, and adjusting culture/headspace volume ratio can help to overcome the inhibition on growth caused by hydrogen accumulation. Implementing these strategies and adjusting pH during the fermentation process can result in high hydrogen yields and efficient consumption of substrates. A reduction of fermentation time by starting with the right inoculum size could cast favorable great perspectives on the economics of the industrial processes.

This review highlights the importance of nitrogen-containing compounds that need to be supplied to the medium to stimulate bacterial growth. Overall, yeast extract and tryptone are the preferred forms of nitrogen. Sulfur-containing compounds not only play a critical role in bacterial growth but also divert reducing power to selectively produce certain end-products in Thermotogae metabolism.

Until now, the impact of metal ions and salts on the fermentation process has not been well investigated even though it has been demonstrated that they could stimulate many key enzymes involved in various metabolic pathways.

In summary, the extensive data collection of this review offers a great reference for the optimization and development of sustainable bioprocesses based on Thermotogae species and helps to generate insightful perspectives for the exploitation of these anaerobic bacteria in biotechnological processes.

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