

Review

## Hydrogen Production by the Thermophilic Bacterium *Thermotoga neapolitana*

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**Abstract:** As the only fuel that is not chemically bound to carbon, hydrogen has gained interest as an energy carrier to face the current environmental issues of greenhouse gas emissions and to substitute the depleting non-renewable reserves. In the last years, there has been a significant increase in the number of publications about the bacterium *Thermotoga neapolitana* that is responsible for production yields of H<sub>2</sub> that are among the highest achievements reported in the literature. Here we present an extensive overview of the most recent studies on this hyperthermophilic bacterium together with a critical discussion of the potential of fermentative production by this bacterium. The review article is organized into sections focused on biochemical, microbiological and technical issues, including the effect of substrate, reactor type, gas sparging, temperature, pH, hydraulic retention time and organic loading parameters on rate and yield of gas production.

**Keywords:** thermophilic bacteria; fermentation; hydrogen; lactic acid; carbon dioxide; biomass; renewable energy; process kinetics; energy carrier; green-house gas

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## 1. Introduction

Anaerobic digestion of organic material is regarded as a potential method for hydrogen (H<sub>2</sub>) production from biomass [1,2]. Besides simple carbohydrates (e.g., glucose) or polymers such as starch and cellulose, the process utilizes a wide range of organic compounds as substrate, including organic wastes and agro-industrial matrices [3–9]. Considering that such residues are abundant, cheap, renewable and biodegradable, H<sub>2</sub> production by fermentation of this material is potentially competitive over conventional process [5] and technically more feasible than other biological methods, including photofermentation and photobiolysis. Furthermore production of H<sub>2</sub> from organic substrates is viewed as an environmentally friendly process because of its potential to yield clean energy while reducing waste and greenhouse gas emissions. Although a more detailed life cycle assessment of feedstock materials is required to fully understand the environmental impact of the whole process, the possible implications on climate change have prompted growing attention to the fermentative production of H<sub>2</sub> in recent years [10].

Chemotrophic H<sub>2</sub> production can be operated at mesophilic (25–40 °C), thermophilic (40–65 °C) or hyperthermophilic (>80 °C) temperatures [11–13], but the process in heated cultures benefits from thermodynamically favorable reactions [14,15]. Although metabolic activity sharply drops outside the optimum temperature range, increase of temperature accelerates reaction rates and offers a number of technical advantages including reduction of viscosity, improvement of mixing efficiency, reduced risk of contamination and no need for reactor cooling [16]. In addition, the high operating temperature enhances hydrolysis rate of complex substrates and, generally speaking, thermophiles can more effectively utilize complex sugars, e.g., cellulose, than mesophiles [17,18]. Furthermore, hyperthermophilic conditions suffer less from inhibition due to H<sub>2</sub> partial pressure and, in the case of microbial consortia, are less sensitive to H<sub>2</sub> consumers like methanogens [19,20].

In the last years, pure cultures of the hyperthermophilic eubacterium *Thermotoga neapolitana* has shown promising results for fermentative H<sub>2</sub> production from several organic substrates [21]. In a recent paper [22], we have also shown that *T. neapolitana* can yield significant amounts of lactic acid without affecting H<sub>2</sub> synthesis, thus offering novel applications for the fermentative process. Here we critically review the most recent data on H<sub>2</sub> production by *T. neapolitana* and discuss the challenges and future prospects of H<sub>2</sub> production using this bacterium.

## 2. Taxonomy of *Thermotoga neapolitana*

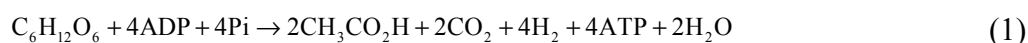
Originally isolated from shallow submarine hot spring nears Lucrino in the Bay of Naples in 1986 [23,24], *T. neapolitana* is a gram-negative bacterium that grows between 55 and 90 °C with an optimal growth temperature of 80 °C [23,24]. The species belongs to the order *Thermotogales* (Phylum *Thermotogae*, class *Thermotogae*) that have, until the recent report of *Mesotoga prima* [25], been exclusively comprised of thermophilic or hyperthermophilic organisms. The order includes an

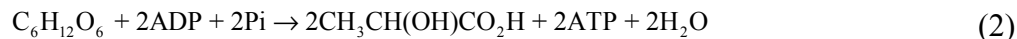
assembly of rod-shaped, non-sporulating bacteria that are characterized by an unconventional outer envelope called the “toga”, which forms a large periplasmic space at the poles of each rod [26–28]. Although it has been shown that these regions could be involved in the formation of multicellular rods [29], the physiological role of the large periplasm remains unknown. *Thermotogales* also synthesizes many polysaccharide hydrolases, some exposed on the cell surface, that allow utilization of diverse sources of carbon [30–39].

The phylogenetic position of *Thermotogae* is still unresolved, even if many studies agree to place members of this phylum among the deepest branches of bacteria, and, thus, as prime candidates for evolutionary studies [21,40]. Based upon different phylogenetic approaches, the class *Thermotogae* is divided into three orders (*Thermotogales*, *Kosmotogales* and *Petrotogales*) containing four families (*Thermotogaceae*, *Fervidobacteriaceae*, *Kosmotogaceae* and *Petrotogaceae*) and 10 genera (*Thermotoga*, *Thermosipho*, *Fervidobacterium*, *Geotoga*, *Petrotoga*, *Marinitoga*, *Thermococcoides*, *Kosmotoga*, *Oceanotoga*, and *Defluviitoga*). The genus *Thermotoga* currently includes eleven species, i.e., *T. maritima*, *T. neapolitana*, *T. thermarum*, *T. elfii*, *T. subterranea*, *T. hypogea*, *T. petrophila*, *T. naphthophila*, *T. lettingae*, *T. caldifontis*, and *T. profunda*, that thrive in marine hydrothermal vents, oil reservoir sites and volcanic springs [21]. Recently, Bandhari and Gupta [41] proposed to split the current genus *Thermotoga* into two evolutionary distinct groups. According to this last classification, the original genus *Thermotoga* retains only the species *T. maritima*, *T. neapolitana*, *T. petrophila*, *T. naphthophila*, *Thermotoga* sp. EMP, *Thermotoga* sp. A7A and *Thermotoga* sp. RQ2 while the other *Thermotoga* species (*T. lettingae*, *T. thermarum*, *T. elfii*, *T. subterranea* and *T. hypogea*) belong to the new genus *Pseudothermotoga* [41].

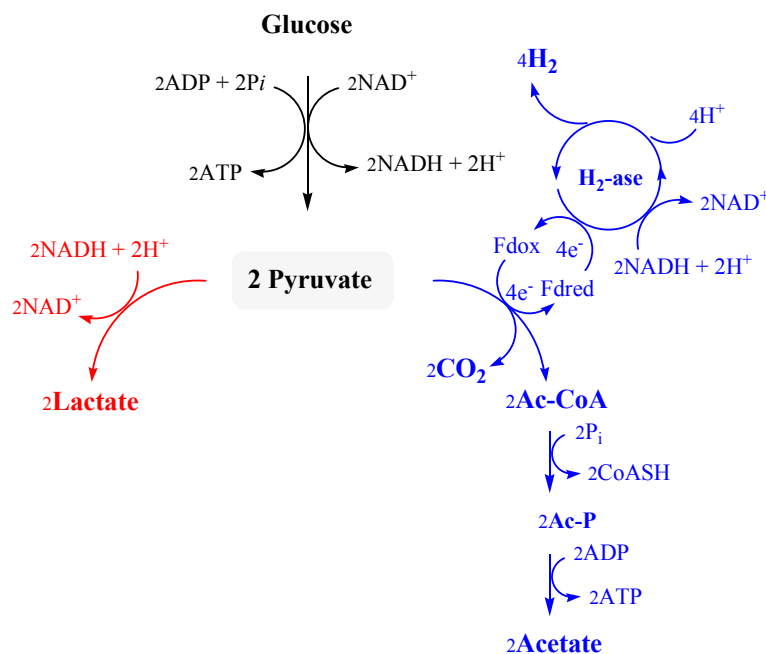
### 3. Dark Fermentation Pathway in *Thermotoga neapolitana*

Chemotropic production of H<sub>2</sub> is a respiration process using H<sup>+</sup> as electron acceptor. The biochemical synthesis by bacteria of the genus *Thermotoga* entails catabolism of carbohydrates even if different members of the genus have the ability to use a large variety of substrates that, for example in *T. elfii*, include also sulfur compounds [42]. As in the related species *T. maritima* [43,44], *T. neapolitana* harvests energy mainly by glycolysis via the Embden-Meyerhoff pathway (EMP) [45]. EMP is the most common route for oxidation of glucose (and other hexoses) and supplies energy (ATP), reducing equivalents (NADH) and pyruvate, that undergoes terminal oxidation (acetate) or is used for biosynthesis (e.g., acetyl-CoA). According to the classical model of fermentation, generally referred to as Dark Fermentation (DF), 4 mol of H<sub>2</sub> can be theoretically produced per mole of consumed glucose [46]. This molar ratio between H<sub>2</sub> and glucose is usually referred to as the Thauer limit and represents the highest yield that can be achieved by a sugar-based fermentation by thermophilic bacteria. As fermentative H<sub>2</sub> production is a mean to dispose of electrons, there is a direct relationship between the biogas yield and the type of the organic products that are concurrently released during the process. Yield is optimized only when all glucose is converted to acetate because NADH and electrons are fully consumed to produce the energy carrier (Equation (1)). On the other hand, in a redox neutral process, no H<sub>2</sub> is produced when lactic acid is the organic product released in the medium (Equation (2)).





As shown in Figure 1, acetate production is driven by formation of additional ATP but, when  $\text{H}_2$  accumulates and consumption of NADH stops, pyruvate is diverted away for the synthesis of other organic substrates, mostly lactate that is produced by lactate dehydrogenase (LDH) with the concomitant oxidation of NADH. Lactate levels reported during fermentation by *Thermotoga* species vary from trace amounts up to levels rivaling those of acetate [44,47–49]. Low levels of alanine and ethanol have been also reported in *T. neapolitana* [21,45,50].



**Figure 1.** Streamlined biochemical pathway for fermentative  $\text{H}_2$  production, adapted from Reference [22]. Water is omitted for simplicity.

The mechanism behind the high  $\text{H}_2$  yields achieved by *T. neapolitana* is likely related to the unique characteristic of the heterotrimeric [FeFe]-hydrogenase that is present in the bacterium. Hydrogenases ( $\text{H}_2\text{ase}$ ) constitute a family of enzymes that efficiently reduce protons to  $\text{H}_2$  in many anaerobic microorganisms. Sequence analysis on the three proteins that form the  $\text{H}_2\text{ase}$  of *T. maritima*, which has more than 90% homology with that of *T. neapolitana*, suggests that the  $\beta$  subunit is a flavoprotein that accepts electrons from NADH, and the  $\gamma$  subunit transfers electrons from the  $\beta$  subunit to the catalytic  $\alpha$  subunit. The catalytic site (the so-called H cluster) shows the most complex Fe-S structure characterized to date and requires the specific action of three highly conserved proteins to be assembled [51]. Despite the detailed knowledge of the active site, how the endergonic reaction of  $\text{H}_2$  production is accomplished under physiological conditions is not clear. In fact, the reduction of  $\text{H}_2\text{ase}$  by NADH is an energetically unfavorable reaction and the reaction is typically influenced by environmental conditions such as pH, cell growth rate and  $\text{H}_2$  partial pressure. In many thermophilic bacteria and several *Clostridium* species, the transfer of electrons to proton ion by [FeFe]- $\text{H}_2\text{ase}$  requires the presence of NADH-Ferredoxin oxidoreductase (NFOR). In this reaction, it is suggested that the oxidized ferredoxin (Fd) is reduced by NADH, which is formed during carbon metabolism. Then, the electrons in Fd are transferred to protons by [FeFe]- $\text{H}_2\text{ase}$  to form molecular  $\text{H}_2$  (Figure 1) [52].

Recently Schut and Adams proposed a novel model for H<sub>2</sub> production for *Thermotoga* species based on the synergistic effect of NADH and reduced Fd [44]. According to this study, H<sub>2</sub>ase of *T. maritima* concurrently oxidizes reduced Fd and NADH in a 1:1 ratio in order to reduce the H<sup>+</sup> ions. Ferredoxin is cyclically produced by pyruvate Fd oxidoreductase (POR) during oxidation of pyruvate to acetyl coenzyme A (Figure 1). This mechanism that couples an exoergonic reduction with an endoergonic reduction has been called “bifurcating” [53] and it is proposed to correspond to a novel type of energy conservation. Thus, energy from the oxidation of Fd drives the unfavorable oxidation of NADH in *T. maritima* [44] and the hyperthermophilic bacterium has the ability to achieve H<sub>2</sub> yields close to the Thauer limit. According to this mechanism, H<sub>2</sub> production by H<sub>2</sub>ase of *T. neapolitana* is influenced by factors that affect either NADH or reduced Fd. Furthermore, the composite mechanism of this H<sub>2</sub>ase is consistent with the complexity of the trimeric structure, which is much greater than that of the typical Fd-dependent, single subunit [Fe-Fe]-H<sub>2</sub>ase found in *Clostridium* spp. [44].

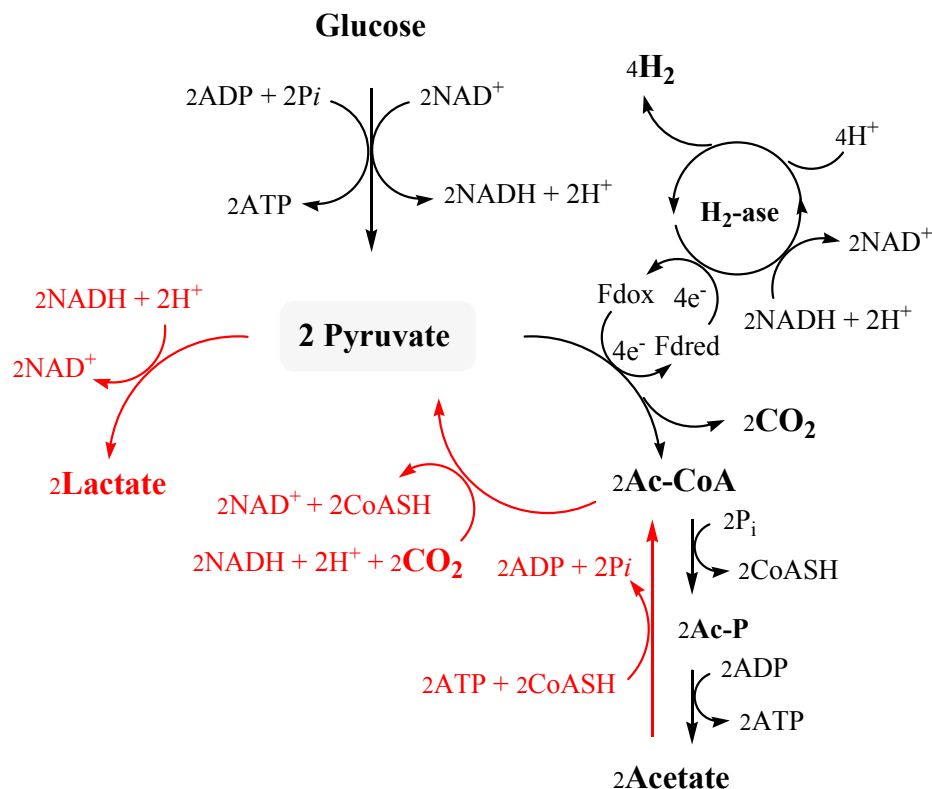
#### 4. Production of Lactic Acid and H<sub>2</sub> by Capnophilic Lactic Fermentation

*T. neapolitana* and the other taxonomically-related species, such as *T. maritima*, *T. petrophila*, *T. naphthophila*, *T. caldifontis*, *T. profunda*, *Pseudothermotoga thermarum*, *P. elfii*, *P. subterranea*, *P. hypogea* and *P. lettingae* have been targeted for biological production of H<sub>2</sub> because of yields approaching the theoretical maximum value (Thauer limit) of 4 mol H<sub>2</sub>/mol glucose [46]. According to Figure 1, this result can be achieved only if all of the reducing equivalents from glucose oxidation are used to reduce protons to H<sub>2</sub>. Nevertheless, as discussed above, in practice these reducing equivalents are also employed for biosynthetic purposes or formation of other fermentation products. Thus, the high H<sub>2</sub> yields and low production of biomass that have been reported for *T. neapolitana* suggest that pyruvate is only partially used in other metabolic transformations under standard operating conditions [54].

Inflow of gases is the most commonly reported method for removing oxygen and H<sub>2</sub> from bacterial cultures in closed reactors [55,56]. Use of CO<sub>2</sub> as gas sparging significantly increases the rate of both glucose consumption and hydrogen production even if there was no improvement of the overall productivity and molar yield that remained substantially unchanged in comparison with N<sub>2</sub> [22]. Paradoxically, CO<sub>2</sub> stimulated also synthesis of lactic acid. Feeding experiments with labeled precursors clearly proved that at least part of exogenous CO<sub>2</sub> is biologically coupled with acetyl-CoA to give lactic acid when the cultures are stripped by CO<sub>2</sub> gas or enriched in sodium bicarbonate. The process recycles glycolysis-derived acetyl-CoA or employs exogenous acetate with ATP consumption. In this latter case the overall outcome is a conversion of equimolar concentration of acetate and carbon dioxide into lactic acid according to reaction Equation (3).



The fermentative CO<sub>2</sub>-dependent synthesis of lactic acid and hydrogen was named capnophilic lactic fermentation (CLF) and, as suggested in Figure 2, it put forward the possibility to fully convert sugar to lactic acid (or other reduced derivatives of pyruvate) without affecting hydrogen synthesis by means of an additional consumption of reducing equivalents deriving from other cellular processes [57].



**Figure 2.** Proposed model of capnophilic lactic fermentation, adapted from [57]. Water is omitted for simplicity.

To date, CLF has been described only in *T. neapolitana* but the pathway is likely to occur in other species of the order *Thermotogales*. The key enzyme of the process is a Pyruvate Synthase (also named Pyruvate Oxido-Reductase) that utilizes reduced ferredoxin as source of electrons [57]. In *Thermotogales* reductive carboxylation of Ac-CoA likely requires the pool of Fd that is also involved in hydrogen production. Role of Fd as efficient reductant in pyruvate synthesis has been demonstrated *in vitro* with *Clostridium thermoaceticum* [58] and suggested *in vivo* for methanogenic archaea, such as *Methanosarcina barkeri* [59]. It is noteworthy that the sequence of pyruvate oxido-reductase of this last organism has a good relation to those of *T. neapolitana* and *T. maritima* [57]. CLF is an example of biological sequestration of carbon dioxide by coupling with an exogenous substrate (acetate, glucose, etc.) and release of the end-product (lactic acid) outside of the cell. Since *T. neapolitana* does not convert  $\text{CO}_2$  to the reduced organic compounds required for cell metabolism, the above mechanism is not related to the autotrophic fixation known in other anaerobes. In fact, unlike known autotrophic [60,61] and heterotrophic [62,63] pathways for carbon dioxide assimilation, the capnophilic metabolism of *T. neapolitana* implies complete excretion of  $\text{CO}_2$  after fixation in lactic acid and no synthesis of reduced organic compounds required for cell metabolism.

## 5. Substrate Metabolism by *Thermotoga neapolitana*

As discussed above, extreme thermophiles are capable of producing  $\text{H}_2$  yields close to the theoretical Thauer limit of  $4.0 \text{ mol H}_2/\text{mol}$  of glucose. In addition, the theoretical maximum yield for xylose, sucrose and glycerol are  $3.33 \text{ mol H}_2/\text{mol}$  xylose,  $8.0 \text{ mol H}_2/\text{mol}$  sucrose and  $3.0 \text{ mol H}_2/\text{mol}$

glycerol under dark fermentation. Glucose is the substrate that gives the highest production of H<sub>2</sub> with *T. neapolitana*. In batch experiments with this sugar, independent studies have reported H<sub>2</sub> yield higher than 3.5 mol/mol and production rate ranging from 23 to 50 mL/L/h at pH of 7.5 and temperature of 80 °C [45,64]. As already mentioned above, the bacterium can also efficiently use a wide range of other substrates ranging from simple to complex sugars including ribose, xylose, fructose, sucrose, maltose, lactose, galactose, starch, and glycogen (Table 1) [21,24,50,65–71].

Waste glycerol from bio-diesel manufacturing is currently considered an attractive and abundant feedstock for fermentation process. Batch tests conducted by Maru *et al.* [69] have demonstrated that 2.65 mol of H<sub>2</sub> can be produced per mole of glycerol by using the bacterium *T. neapolitana* at a glycerol concentration of 2.5 g/L. Ngo and Sim [72] also reported that the bacterium transforms pure glycerol and crude waste glycerol with approximately similar H<sub>2</sub> production (447 ± 22 and 437 ± 21 mL/L, respectively). According to these authors, these yields are better than those reported with mesophilic bacteria [73,74] and addition of itaconic acid to buffer the culture medium further increased this productivity with both substrates. It is notable that a prediction model built on comparative analysis of the genomes of *T. maritima* and *T. neapolitana* put forward that this latter species should not be able to metabolize a number of sugars, including cellotetraose. However, experimental assessment proved that the bacterium grows on this substrate despite that the model predicted an incomplete cellotetraose transport complex. Proteomic analysis of glucose and cellotetraose revealed two possible new gene clusters that may be associated with transport of these sugars [75].

Cappelletti *et al.* [65] showed that *T. neapolitana*, *T. maritima*, *T. petrophila* and *T. naphthophila* produce about 2.95 mol of H<sub>2</sub> per mol of glucose equivalent from molasses and 2.5 mol of H<sub>2</sub> per mol of glucose equivalent from cheese whey, whereas 2.7–2.8 mol of H<sub>2</sub> per mol of glucose equivalent were produced on carrot pulp hydrolysates containing glucose, fructose and sucrose as main sugars [50]. Enzymatic hydrolysis of the polysaccharide fraction prior to fermentation increased the H<sub>2</sub> yield of almost 10% to 2.3 g/kg of hydrolyzed carrot pulp. Lignocellulosic substrates (e.g., crop residues) were tested for H<sub>2</sub> production with some standard pretreatment to wash out lignin [70,76,77]. Thermo-chemical pretreatment (*i.e.*, heat, ammonia soaking and dilute H<sub>2</sub>SO<sub>4</sub> soaking) were found to be effective pretreatment techniques to remove lignin and enhance availability of simple sugars for H<sub>2</sub> production. According to Ngo *et al.* [76] 2.8 mol of H<sub>2</sub> per mol of xylose equivalent are produced by *T. neapolitana* in a pH-controlled continuously stirred anaerobic bioreactor sparged with N<sub>2</sub> gas. Similar results have also been reported with rice straw pretreated with ammonia soaking and diluted sulfuric acid [70]. Algal biomass (*Chlamydomonas reinhardtii*) pretreated by heat-HCl or Termamyl<sup>®</sup> enzymatic hydrolysis has been also used as substrate of *T. neapolitana* to give 2.5 mol of H<sub>2</sub> per mol of glucose equivalent [71]. Without pretreatment, a slightly lower yield (2.2 mol/mol of glucose equivalent) was produced by fermentation of laminarans derived from the marine diatom *Thalassiosira weissflogii* [66].

**Table 1.** H<sub>2</sub> production from various substrates by hyperthermophilic eubacterium *T. neapolitana*. B = batch; FB = fed-batch; AA = Acetic acid; LA = Lactic acid; EtOH = Ethanol.

Carbon Source	Substrate Load (g/L)	Culture Type	T(°C)/Start pH	Mixing Speed (rpm)	Reactor Volume (mL)	Working Volume (mL)	H <sub>2</sub> Yield	Byproducts	Ref.
Glucose	5	B	80/7.5	250	3800	1000	2.8 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[22]
Glucose	5	B	80/7.1	250	2400	600	3.5 ± 0.1 mol H <sub>2</sub> /mol glucose <sup>a</sup>	AA, LA, CO <sub>2</sub>	[45]
Glucose	10	B	72/7.0	350	2000	1000	3.5 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[50]
Glucose	20	B	72/7.0	350	2000	1000	3.4 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[50]
Glucose/Fructose 7:3	10	B	72/7.0	350	2000	1000	3.3 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[50]
Glucose/Fructose 7:3	20	B	72/7.0	350	2000	1000	3.0 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[50]
Fructose	10	B	72/7.0	350	2000	1000	3.4 mol H <sub>2</sub> /mol fructose	AA, LA, CO <sub>2</sub>	[50]
Fructose	20	B	72/7.0	350	2000	1000	3.2 mol H <sub>2</sub> /mol fructose	AA, LA, CO <sub>2</sub>	[50]
Carrot pulp hydrolysate	10	B	72/7	350	2000	1000	2.7 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub> , EtOH	[50]
Carrot pulp hydrolysate	20	B	72/7	350	2000	1000	2.4 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub> , EtOH	[50]
Glycerol	5	B	75/7.5	-	120	40	2.7 ± 0.1 mol H <sub>2</sub> /mol glycerol	AA, LA, CO <sub>2</sub>	[64]
Molasses	20	B	77/8.5	100	116	40	2.6 ± 0.1 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[65]
Cheese whey	12.5	B	77/8.5	100	116	40	2.4 ± 0.1 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[65]
Diatom <sup>b</sup> water soluble sugars	2	B	80/7.5–8	250	3800	500	1.9 ± 0.1 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[66]
Glucose	5	B	80/8.0	200	120	60	3.8 ± 0.4 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[67]
Arabinose	5	B	80/8.0	200	120	60	3.8 ± 0.5 mol H <sub>2</sub> /mol arabinose	AA, LA, CO <sub>2</sub>	[67]
Xylose	5	B	80/8.0	200	120	60	3.4 ± 0.3 mol H <sub>2</sub> /mol xylose	AA, LA, CO <sub>2</sub>	[67]
Potato steam peels	10	B	75/6.9	350	2000	1000	3.8 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[68]
Glycerol	2.5	B	80/7.3	200	120/240	25/50	2.6 mol H <sub>2</sub> /mol glycerol	AA, LA, CO <sub>2</sub>	[69]
Rice straw	10	B	75/7.5	150	120	40	2.7 mmol H <sub>2</sub> /g straw	-	[70]
Algal <sup>c</sup> starch	5	B	75/7–7.4	150	120	40	2.5 ± 0.3 mol H <sub>2</sub> /mol glucose	-	[71]
Glycerol	1–10	B	75/7.5	-	120	40	620 ± 30 mL H <sub>2</sub> /L glycerol	AA, LA, CO <sub>2</sub>	[72]
Xylose	5	B	75/7.5	300	3000	1000	2.8 ± 0.1 mol H <sub>2</sub> /mol xylose	AA, LA, CO <sub>2</sub>	[76]



Table 1. Cont.

Carbon Source	Substrate Load (g/L)	Culture Type	T(°C)/Start pH	Mixing Speed (rpm)	Reactor Volume (mL)	Working Volume (mL)	H <sub>2</sub> Yield	Byproducts	Ref.
Glucose/Xylose 7:3	10–28	B	80/6.8	350	2000	1000	2.5–3.3 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[77]
Cellulose	10–28	B	80/6.8	350	2000	1000	2.0–3.2 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[77]
Xylose	5	B	75/7.0	300	3000	1000	1.8 ± 0.1 mol H <sub>2</sub> /mol xylose	AA, LA, CO <sub>2</sub>	[78]
Glucose	7.5	B	77/8.5	100	119	40	1.3 ± 0.1 mmol H <sub>2</sub> /g glucose	AA, LA, CO <sub>2</sub>	[79]
Molasses	20	B	77/8.5	100	119	40	1.8 ± 0.1 mol H <sub>2</sub> /g glucose	AA, LA, CO <sub>2</sub>	[79]
Cheese whey	12.5	B	77/8.5	100	119	40	1.04 ± 0.05 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[79]
Glucose	5	FB	75/7.5	300	3000	1000	3.2 ± 0.2 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[80]
Xylose	5	FB	75/7.5	300	3000	1000	2.2 ± 0.1 mol H <sub>2</sub> /mol xylose	AA, LA, CO <sub>2</sub>	[80]
Sucrose	5	FB	75/7.5	300	3000	1000	4.9 ± 0.2 mol H <sub>2</sub> /mol sucrose	AA, LA, CO <sub>2</sub>	[80]
Glucose	2.5	B	77/7.5	75	160	50	3.8 ± 0.3 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[81]
Glucose	5	B	70/8.5	75	160	50	24% H <sub>2</sub> (v/v) headspace <sup>d</sup>	CO <sub>2</sub>	[82]
Glucose	7	B	77/7.5	150	120	40	3.2 ± 0.1 mol H <sub>2</sub> /mol glucose	AA, LA, CO <sub>2</sub>	[83]
Xylose	4	B	77/7.5	150	120	40	2.2 ± 0.1 mol H <sub>2</sub> /mol xylose	AA, LA, CO <sub>2</sub>	[83]
Glucose	5	B	70/8.5	-	160	50	25%–30% H <sub>2</sub> (v/v) headspace <sup>d</sup>	AA, LA, CO <sub>2</sub>	[84]
Cellulose <sup>e</sup>	5	B	75–80/7.5	150	120	50	0.25 ± 0.01 mol H <sub>2</sub> /mol glucose	AA, CO <sub>2</sub>	[85]
Cellulose derivative	5	B	75–80/7.5	150	120	50	0.77 ± 0.04 mol H <sub>2</sub> /g glucose	AA, CO <sub>2</sub>	[85]
Cellulose	5	B	80/7.5	150	120	40	2.2 mol H <sub>2</sub> /mol glucose	AA, CO <sub>2</sub>	[85]
Starch	5	B	75–80/7.5	150	120	50	1.4 ± 0.1 mL H <sub>2</sub> /g glucose	AA, CO <sub>2</sub>	[85]

<sup>a</sup> excluding the estimated contribution from protein; <sup>b</sup> *Thalassiosira weissflogii*; <sup>c</sup> *Chlamydomonas reinhardtii*; <sup>d</sup> yield not reported; <sup>e</sup> *Miscanthus giganteus*.

There are conflicting reports on the effect of protein lysates on growth and H<sub>2</sub> production by *T. neapolitana*. Maru *et al.* [69] noticed that reduced level of yeast extract (YE) negatively affects H<sub>2</sub> production but increasing concentration from 2 to 4 g/L did not induce significant change in gas evolution. On the contrary, increasing YE concentration from 1 to 4 g/L improved biomass and H<sub>2</sub> production in cultures of *T. neapolitana* on glycerol as reported by other independent studies [72,73]. No effect is reported through increasing the concentration of protein lysates above 5 g/L. Cappelletti *et al.* [65] reported that partial production of H<sub>2</sub> can be due to metabolism of tryptone soy broth (TSB) whereas the contribution of YE is null. On the other hand, transformation of peptone, tryptone and YE yields 10%–15% to the total H<sub>2</sub> production according to d'Ippolito *et al.* [45] and Eriksen *et al.* [67].

## 6. Systems Integration

According to Levin *et al.* [86], H<sub>2</sub> production by dark fermentation is considered the most practically applicable process for production of the energy carrier. However, as shown in Figure 1, only 2 mol of carbon from the substrate (*i.e.*, glucose) are fully oxidized to CO<sub>2</sub> and only 4 mol H<sub>2</sub> are formed. Thus, according to the dark fermentation model, a fermentative H<sub>2</sub> production can only convert, even in an optimal condition, less than 33% of the energy from the substrate (*e.g.*, glucose). On the other hand, the transformation efficiency can be significantly improved (theoretically up to 12 mol H<sub>2</sub> per mol of glucose) if a second biological process allows for the complete oxidation of the residual products released by the thermophilic process. In particular, photo-heterotrophic fermentation of organic acids produced by *T. neapolitana* is hypothetically entitled to produce a further 8 mol of H<sub>2</sub>, thus reaching the maximum possible yield of 12 mol of H<sub>2</sub>.

Purple nonsulphur (PNS) bacteria are a non-taxonomic group of microorganisms that are attractive for the biological production of H<sub>2</sub> from biomass (reviewed in [87]). A few studies have also demonstrated that these microorganisms can be successfully integrated into a two-step process to produce H<sub>2</sub> in combination with dark fermentation. The first report of a two-stage process with *T. neapolitana* by Uyar and coworkers [88] showed that *Rhodobacter capsulatus* effectively produces hydrogen when the concentration of acetate is lower than 60 mM in the spent medium of the thermophilic bacterium. Interestingly these authors also noticed that addition of iron II in the range of 0–29 μM to the culture medium (*i.e.*, to the spent medium of thermophilic bacterium) of *R. capsulatus* increased the hydrogen production in a significant manner (1.37 L H<sub>2</sub>/L culture in effluent media supplemented with iron and vitamins; 0.30 L H<sub>2</sub>/L culture in effluent media supplemented only with vitamins). More recently, we have repeated the experiment with *T. neapolitana* and *Rhodospseudomonas palustris* by replacing the traditional conditions of DF (dark fermentation) with those of CLF (capnophilic lactic fermentation) [89]. To achieve photo-fermentation by a mutant strain of *R. palustris* [90], *T. neapolitana* was grown under reduced level of NaCl and nitrogen-containing compounds. According to Uyar *et al.* [88], the spent broths of the thermophilic bacterium were only supplemented with Fe-citrate and phosphate buffer. The combined microbial system gave 9.4 mol of hydrogen per mole of glucose consumed during the anaerobic process, which is the best production yield so far reported for conventional two-stage batch cultivations [89]. The results also proved that

CLF can be used for inducing a metabolic switch in *T. neapolitana* that brings actual improvements of hydrogen yields in combination with photofermentation.

The advantages of using biomass for H<sub>2</sub> production range from the mitigation of CO<sub>2</sub> and other pollutant emissions, to reduction of environmental and economical costs for disposing wastes. Limitations in use of biomass are mainly due to the seasonal availability of agro wastes, costs of their collection and incomplete use of the organic matter. In this view, microalgal biomass is an attractive alternative since algal cultivation can theoretically run continuously with no restriction due to seasonal cycle and can yield large amounts of biomass of constant composition. Furthermore, fermentation of algal feedstock can be associated with production of biofuels or by-products of high value. *T. neapolitana* directly produces H<sub>2</sub> by fermentation of the biomass of the green alga *Chlamydomonas reinhardtii* with molar yields (1.8–2.2 mol/mol glucose equivalent) depending on pretreatment methods [71]. *T. neapolitana* possesses genes encoding both for a 1,3-β-glucosidase BglB (laminaribiase) and a 1,3-β-glucanase LamA (laminarinase) that are able to completely degrade chrysolaminarin, the storage polysaccharides of diatoms, to glucose with a synergic action [91,92]. Accordingly, the bacterium fermented the water-soluble fraction of the marine diatom *Thalassiosira weissflogii* without any pretreatment [66]. Production (434 mL/L in 24 h; 18.1 mL/L/h) and yield (2.2 mol H<sub>2</sub>/mol glu. eq) of H<sub>2</sub> on diatom extracts containing 2 g/L of sugar equivalent were just slightly lower than those achieved by fermentation of glucose (809 mL/L in 24 h; 33.7 mL/L/h; 3.0 mol H<sub>2</sub>/mol glu) and pure chrysolaminarin (643 mL/L in 24 h; 26.8 mL/L/h; 3.2 mol H<sub>2</sub>/mol glu. eq).

## 7. Bioreactor Configuration

Several bioreactor configurations such as continuously stirred tank reactors (CSTRs), fluidized bed reactors (FBRs), packed bed reactors (PBRs), up-flow anaerobic sludge blanket (UASB) reactors, anaerobic sequencing batch reactors (AnSBRs), high rate/hybrid reactors, and membrane biological reactors (MBRs) have promising prospects for dark fermentation processes [14]. Table 2 reports the CSTR used for fed-batch and continuous reactors studied for H<sub>2</sub> production by suspended and immobilized cells of *T. neapolitana*. CSTRs operate continuously and the bulk inside the reactor is mixed uniformly. However the mixing rate depends on the reactor geometry and power input [93]. CSTRs favor mass transfer among biomass, substrates and gases, and are effective in temperature and pH bulk control. However, CSTR can experience biomass washout, when the loading and the dilution rate increases.

The biomass washout is less probable in attached biomass reactors (e.g., FBRs, PBRs, UASB) where inert material is used to support and contain the bacteria, thus providing a high concentration of cells and, consequently high solids retention time, high organic load, high mass transfer efficiency and high tolerance for shock loads [78,94,95]. Several inert materials have been used successfully as support for *T. neapolitana* growth, *i.e.*, coir, bagasse, loofah sponge, expanded clay, diatomaceous clay, activated carbon, polysaccharide gels (e.g., alginate, k-carrageenan, agar, chitosan), synthesized materials (e.g., polyvinyl alcohol (PVA), silicone, polyacrylamide, urethane foam and polymethyl methacrylate) [26,96–99], ceramic porous carriers (*i.e.*, biomax) [65,79], and porous glass beads [78]. The batch fermentation tests conducted by Ngo and Bui have shown that the H<sub>2</sub> production rate and H<sub>2</sub> yield of the immobilized cells reached the highest values of 5.64 ± 0.19 mmol H<sub>2</sub>/L/h and

1.84 ± 0.1 mol H<sub>2</sub>/mol xylose, respectively, which were 1.7- and 1.3-fold higher than those with free cells [78]. Synthetic hydrogels based on methacrylate derivatives with buffer capacity also effectively supported cell growth and hydrogen production [96,100]. In particular, the use of hydrogel with positive charge and amine groups doubled hydrogen production rate compared with suspension cultures. Both sugar metabolism and hydrogen synthesis were affected positively by neutralization of the acidic side-products of the fermentation, *i.e.*, acetate and lactate. The presence of positively charged groups on the inert support proved to be critical to promote the colonization of the polymeric material by a great number of *T. neapolitana* cells laying in a biofilm-like arrangement.

**Table 2.** Continuous and fed-batch operation in CSTRs for *T. neapolitana*.

Substrate	Reactor Volume (L)	Working Volume (L)	Temp. (°C)	Culture Type	Culture Condition	H <sub>2</sub> Yield	References
Glucose/Xylose/ Arabinose	3.0	2.75	80	Suspended cells	Fed-batch	3.8 ± 0.4 mol H <sub>2</sub> /mol glucose; 3.4 ± 0.3 mol H <sub>2</sub> /mol xylose; 3.8 ± 0.5 mol H <sub>2</sub> /mol arabinose	[67]
Glucose/Sucrose/ Xylose	3.0	1.0	75	Suspended cells	Fed-batch	3.2 ± 0.16 mol H <sub>2</sub> /mol glucose; 4.95 ± 0.25 mol H <sub>2</sub> /mol sucrose; 2.2 ± 0.11 mol H <sub>2</sub> /mol xylose	[80]
Xylose	3.0	1.0	75	Immobilized cells	Fed-batch	1.84 ± 0.1 mol H <sub>2</sub> /mol xylose	[78]
Glucose/Cheese whey/Molasses	19.0	15.0	77	Suspended cells	Continuous	1.2 mmol H <sub>2</sub> /L/h for glucose; 0.42 mmol/L/h for cheese whey; 1.3 mmol/L/h for molasses	[79]
Glucose	-	-	80	Immobilized cells	Fed-batch	3.3 mol H <sub>2</sub> /mol glucose	[96]

## 8. Operating Conditions and Kinetics of *Thermotoga neapolitana* Fermentation

### 8.1. Hydraulic Retention Time (HRT)

In the dark fermentative H<sub>2</sub> production, hydraulic retention time (HRT), organic loading rate (OLR) and pH are coupled variables since short HRT and high OLR generally correspond to low pH condition that affects the biomass metabolism. Both high OLR and low HRT represent favorable conditions for H<sub>2</sub> production as such operating conditions inhibit other slow growing bacteria, such as methanogens [101]. A HRT in the range of 0.25–60 h (lower HRT for attached/immobilized biomass and higher HRT for suspended growth biomass) is proved to be suitable for hyperthermophilic dark fermentative H<sub>2</sub> production by *T. neapolitana* in batch, fed-batch and continuous bioreactors using a wide range of substrates such as glucose, sucrose, starch, lignocellulose, organic waste and algal starch [102]. *T. neapolitana* is an exceptionally robust microorganism for H<sub>2</sub> production because of its efficient hydrolytic abilities and adaptability to different culture conditions [77]. Nevertheless, production of H<sub>2</sub> is optimal only in very restricted range of operating conditions. In particular, the bacterium grows in a wide interval of temperatures (*i.e.*, 55–90 °C) but the highest H<sub>2</sub> production occurs between 75 and 80 °C [76].

## 8.2. Working pH

As reported above, culture pH is directly affected by the acidogenic activity. Consequently, pH control by base addition (e.g., NaOH) is critically important to maximize both H<sub>2</sub> production and substrate consumption [45,80,81]. Growth of *T. neapolitana* is inhibited at pH of 4.5 [82], whereas change from 4.0 to 5.5 induces an increase of H<sub>2</sub> content in the headspace from 42% to 64% [103]. Nguyen *et al.* demonstrated that variation of pH in *T. neapolitana* cultures from 5.5 to 7.0 enhances cumulative H<sub>2</sub> production from 125 to 198 mL H<sub>2</sub>/L medium, but further increase to 8.0–9.0 leads to total decline in the biogas evolution [83]. At laboratory scale, the strict control of pH has also suggested the use of compounds with increased buffer capacity such as diacid/monoacid phosphate (HPO<sub>4</sub><sup>2-</sup>/H<sub>2</sub>PO<sub>4</sub><sup>-</sup>), tris (hydroxymethyl) aminomethane (TRIS), 3-(*N*-morpholino) propanesulfonic acid (MOPS), piperazine-*N,N'*-bis(2-ethanesulfonic) acid (PIPES), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) [77,81,82,84]. For large scale application the use of these chemicals is probably economically prohibitive but similar effects could be achieved by use of more convenient products (e.g., CO<sub>2</sub>).

## 8.3. Temperature

The primary fermentation products for *T. neapolitana* across the permissive growth temperature range are H<sub>2</sub>, CO<sub>2</sub>, acetate and small amounts of lactate. Two independent studies on the influence of temperature on H<sub>2</sub> production of *T. neapolitana* and *T. maritima* support a direct correlation with H<sub>2</sub> production and bacterial growth [81,85]. Munro *et al.* reported that rate and amount of glucose consumption and H<sub>2</sub> formation increased by arising the operating temperature from 60 to 77 °C, but there was no significant difference from 77 to 85 °C [81]. Although production of acetate and lactate indicated a difference between 77 and 85 °C, a comparison of the molar yields acetate/glucose and lactate/glucose for the operating temperatures between 65 and 85 °C suggested no significant change in molar yield for the two organic acids.

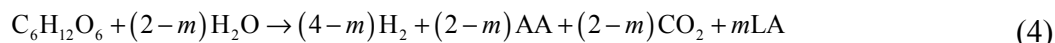
## 8.4. Partial Pressure

The total and partial pressure of gas inside the reactor influences the biomass growth and product formation. According to Schonheit and Schafer [104], H<sub>2</sub> itself inhibits the process in a batch reactor. Van Niel *et al.* reported that H<sub>2</sub> partial pressure less than 20 kPa is required for reactor operating at high temperature (>70 °C) [20]. Partial pressure of H<sub>2</sub> above 20 kPa reverses the metabolic pathway, thereby facilitating the production of more reduced products such as acetone, ethanol, lactate, butanol and alanine [11,50,105]. Experimental data show that use of pure nitrogen as gas sparging and high ratio between headspace volume/culture volume can contain the partial pressure of H<sub>2</sub> below the critical limit in cultures of *T. neapolitana* [45,67,81]. Increase in yield and production of H<sub>2</sub> are reported by N<sub>2</sub> sparging compared to no sparging condition [45,83]. The tolerance of *T. neapolitana* to oxygen is matter of debate. Van Ooteghem *et al.* described significant improvement of H<sub>2</sub> production under microaerobic condition [82,84], whereas Eriksen *et al.*, in line with other studies, reported that *T. neapolitana* can tolerate only low oxygen partial pressure (1% or 1.2 kPa) and

found that 6% O<sub>2</sub> (7.2 kPa) inside the reactor completely inhibits H<sub>2</sub> production and reduces glucose consumption from 12 to 4 μmol/h [106].

### 8.5. Mathematical Modeling and Kinetics

Metabolic transformation of glucose by *T. neapolitana* can be effectively described with the Equation (4).



where  $m$  is a stoichiometric coefficient [22].

A majority of studies have either adapted or modified existing mathematical (or empirical) models to describe the experimental results [107]. Gompertz empirical model (Equation (5)) and International Water Association (IWA) anaerobic digestion model No. 1 (ADM1) are the most popular models to simulate dark fermentation. The Gompertz model is particularly used to estimate the maximum hydrogen production potential and to determine the lag phase for H<sub>2</sub> production [108] but it does not allow the process kinetics to be addressed because of the exclusion of operating conditions (e.g., substrate type and concentration, pH, temperature, and partial pressure of gas mixture) that regulate the fermentation reaction [108].

$$H(t) = P * \exp \left\{ - \exp \left[ \frac{R_m e}{P} (\lambda - t) + 1 \right] \right\} \quad (5)$$

where

$H(t)$  = Cumulative H<sub>2</sub> production (mL/L)

$P$  = H<sub>2</sub> production potential (mL H<sub>2</sub>)

$R_m$  = Maximum H<sub>2</sub> production rate (mL H<sub>2</sub>/h)

$t$  = Incubation/cultivation time (h)

$\lambda$  = Duration of the lag phase (h)

On the other hand, ADM1 is a complete and comprehensive kinetic model based on Monod kinetic Equations (6) [109–112] and has been used often to model DF reactions [79,113,114].

$$\mu = \frac{\mu_{\max} S}{k_s + S} \quad (6)$$

where

$\mu$  = specific growth rate of biomass (h<sup>-1</sup>)

$\mu_{\max}$  = maximum specific growth rate of biomass (h<sup>-1</sup>)

$k_s$  = semi saturation constant (g/L);  $k_s$  equals the substrate concentration at which  $\mu$  equals  $\frac{1}{2} \mu_{\max}$

$S$  = substrate concentration (g/L)

The ADM1-based model and Gompertz empirical model have been extensively used to study H<sub>2</sub> production by fermentative process, but to date there are only two studies with *T. neapolitana*. In pure culture on glucose at 77 °C, Yu and Drapcho [114] reported maximum specific maximum growth rate ( $\mu_{\max}$ ) of 0.94 h<sup>-1</sup> and semi saturation constant ( $k_s$ ) of 0.57 g sugar/L when H<sub>2</sub> and biomass product yields were 0.0286 g H<sub>2</sub>/g glucose and 0.248 g biomass/g glucose, respectively. More recently,

Frasconi *et al.* [79] have studied the kinetic parameters for *T. neapolitana* grown on glucose, molasses and cheese whey by suspended or immobilized cells. The  $\mu_{\max}$  value with immobilized bacteria ( $0.09 \pm 0.05 \text{ h}^{-1}$  for glucose,  $0.19 \pm 0.02 \text{ h}^{-1}$  for molasses and  $0.042 \pm 0.007 \text{ h}^{-1}$  for cheese whey) was found to be significantly higher than with suspended cells ( $0.024 \pm 0.005 \text{ h}^{-1}$  for glucose,  $0.055 \pm 0.005 \text{ h}^{-1}$  for molasses and  $0.033 \pm 0.006 \text{ h}^{-1}$  for cheese whey). On the contrary, the semi saturation constant ( $k_s$ ) was  $0.09 \pm 0.05 \text{ g sugar/L}$  for glucose,  $0.6 \pm 0.2 \text{ g sugar/L}$  for molasses and  $1.2 \pm 0.3 \text{ g sugar/L}$  for cheese whey in the immobilized systems and  $1.1 \pm 0.3 \text{ g sugar/L}$  for glucose,  $0.2 \pm 0.05 \text{ g sugar/L}$  for molasses and  $1.5 \pm 0.5 \text{ g sugar/L}$  for cheese whey with bacterial suspensions [79].

## 9. Conclusions

Among the various technologies, fermentation has many advantages for the biological production of  $\text{H}_2$  and is theoretically feasible for large-scale application particularly from the fermentation of solid wastes [115,116]. Extensive research in the last decades has shown the promising prospect of using pure cultures of the bacterium *T. neapolitana*. Like other hyperthermophilic process, the technology is readily used at laboratory scale with high production rate, low energy demand, easy operation and sustainability. On the contrary little has been done in terms of comparison of cost and effectiveness between *T. neapolitana* and traditional processes that use fossil fuel for production of hydrogen.

*T. neapolitana* has also shown great potential for other applications such as recovery of byproducts with potential economic value in the market *i.e.*, lactic acid. Introduction of capnophilic process for the simultaneous production of  $\text{H}_2$  and lactic acid is very promising and could significantly influence the future of agro-waste management. Clearly, further research is needed to optimize the operating parameters and reactor configurations and more experiments are needed to verify process kinetics and full-scale applicability. Nevertheless, fermentation of organic material by the thermophilic bacterium could be the beachhead of a complete conversion process that generates  $\text{H}_2$  only as a first step.

Agro-food wastes and algal biomass seem to be attractive substrates for fermentation by *T. neapolitana* and thus are considered as feedstock for comprehensive development of biorefineries. Moreover, coupling of *T. neapolitana*-based transformation with other biological processes also seems very promising. In this view, chemotrophic production of hydrogen by hyperthermophilic bacteria has already shown great potential in association with both microalgal cultivations and photofermentation by purple nonsulphur bacteria. Finally, considerable enhancement of the fermentative capacity of *T. neapolitana* can be also expected by metabolic engineering and physiological manipulations of strains, as well as by improvement in reactor configurations [1,117]. In fact, for example the unexpected success of CLF suggests that a huge unexplored gene pool available in nature, with great potential for  $\text{H}_2$  production, is yet to be discovered [118].

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### Author Contributions

Nirakar Pradhan and Laura Dipasquale carried out bibliographic search; Nirakar Pradhan, Laura Dipasquale and Angelo Fontana carried out data analysis; Laura Dipasquale and Angelo Fontana wrote the manuscript; Giuliana d'Ippolito, Antonio Panico, Piet N. L. Lens and Giovanni Esposito corrected the manuscript and improved the discussion.

### Conflicts of Interest

The authors declare no conflict of interest.

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