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Effect of Different Partial Pressures on H_2 Production with *Parageobacillus thermoglucosidasius* DSM 6285

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Abstract: The ability of *Parageobacillus thermoglucosidasius* to produce H_2 from CO via the water–gas shift (WGS) reaction makes it a compelling microorganism for biofuels research. Optimizing this process requires evaluating parameters such as pressure. This study aimed to understand how H_2 production is affected by increasing CO, N_2, and H_2 partial pressures to 1.0, 2.0, and 3.0 bar. Increasing CO partial pressure can improve the solubility of the gas in the liquid phase. However, raising CO partial pressure to 3.0 bar had an inhibitory effect, delaying and reducing H_2 production. By contrast, increasing N_2 and H_2 partial pressures to 3.0 bar had positive effects, reaching a H_2 production of 9.2 mmol and 130 mmol, respectively. Analysis of the electron balance at the end of the fermentation process showed that the selectivity toward H_2 production reached 95%, with the remainder of electrons deriving from CO and glucose directed at organic acid production, mainly acetate, followed by formate.

Keywords: *Parageobacillus thermoglucosidasius*; hydrogen; water–gas shift reaction; CO-dehydrogenase; partial pressure

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1. Introduction

Hydrogen (H_2) is a versatile energy carrier with the highest energy content per unit weight (142 kJ/g). Furthermore, it can be transported and is safer to handle than natural gas, and its zero-carbon nature makes it a promising energy alternative to dwindling fossil fuels [1–5]. Current industrial H_2 production practices are hampered by high costs and are often non-carbon neutral, and hence there has been increasing research on the development of biological means for H_2 production, with focus on improving the yield and the emergent renewable technologies [6,7]. Biological H_2 production strategies of interest include direct and indirect bio-photolysis, microbial electrolysis cells, photo and dark fermentations, and combined systems such as photo-electrochemical electrolysis [3,4,8,9]. Recently, the biological water–gas shift (WGS) reaction has been added to this group of processes [3,5,8].

This area of biohydrogen production research centers on the use of hydrogenogenic carboxydotrophic mesophilic or thermophilic microorganisms capable of performing the WGS reaction, where carbon monoxide (CO) reacts with water under anaerobic conditions to produce hydrogen and CO_2 [10,11]. A waste gas, forming part of synthesis gas from various industrial process, CO expands the substrate range for carbon-neutral biological H_2 production [5,8]. In particular, the Gram-positive thermophile *Parageobacillus thermoglucosidasius* presents a robust microorganism for WGS-driven hydrogen production [12], and it is a versatile microorganism with industrial significance able to degrade starch, hemicellulose, cellulose, and lignocellulose [13]. Unlike other hydrogenogenic carboxydotrophs, which are strict anaerobes, this bacterium is a facultative anaerobe, and has even been observed

to undertake the WGS reaction under low oxygen levels [14,15]. Various parametric optimizations have been investigated to improve the hydrogen yield from CO oxidation by *P. thermoglucosidasius*, including factors such as temperature, media composition, pH, inoculum size, and age of the inoculum [15]. One parameter that has received limited attention is the effect of pressure on *P. thermoglucosidasius* hydrogenogenesis and how it affects H_2 production, as well as the microorganism metabolism.

When considering gas fermentations, Henry's law applies, where a proportional relationship exists between the concentration of the gases in the liquid phase (where the microorganism resides) and the total headspace pressure [16]. This suggests that more CO would be available for the microorganism to use as substrate and concomitantly increase the H_2 yield. However, this represents a challenge, as the toxicity of CO to microbial cells also tends to increase [17]. *P. thermoglucosidasius* has been shown to cope with toxicity, as CO is present during initial aerobic growth [18]. Alternatively, a lower hydrogen partial pressure in the headspace facilitates the mass transfer of hydrogen from the liquid to the gas phase [16].

Understanding the effects of different partial pressures of gases on the WGS process could therefore be used to increase H_2 yield and thereby optimize the biohydrogen process. In this study, the effect of increasing CO, H_2, and N_2 partial pressures (pCO, pH_2, and pN_2) on WGS-driven H_2 production by *P. thermoglucosidasius* was evaluated.

2. Materials and Methods

2.1. Microorganism and Media

P. thermoglucosidasius DSM 6285 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and was conserved in glycerol (80%) stocks at −80 °C. The cultivation of *P. thermoglucosidasius* DSM 6285 was performed in modified Luria Bertani (mLB) medium containing, yeast extract (5 g/L), NaCl (5 g/L), tryptone (10 g/L), 1.25 mL/L of NaOH (10 g/L), and 1 mL/L of each of the stock solutions of nitrilotriacetic acid (1.05 M), $MgSO_4 \cdot 7H_2O$ (0.59 M), $CaCl_2 \cdot 2H_2O$ (0.91 M), and $FeSO_4 \cdot 7H_2O$ (0.04 M), previously filter-sterilized. *P. thermoglucosidasius* was cultured at 60 °C as described below in the inoculum preparation. For the subsequent fermentations, a modified ammonium sulfate medium (mASM) was used, with the following content: citric acid (8.7 mM), $MgSO_4$ (20.2 mM), K_2SO_4 (10 mM), NaH_2PO_4 (22.6 mM), $CaCl_2$ (0.8 mM), $(NH_4)_2SO_4$ (25 mM), and 1 mL each of the filter-sterilized trace elements: H_2SO_4 (6 mM), $CuSO_4$ (0.1 mM), $CoSO_4$ (0.2 mM), $ZnSO_4$ (0.5 mM), $FeSO_4$ (2.29 mM), $NiSO_4$ (0.3 mM), $MnSO_4$ (0.9 mM), and H_3BO_3 (0.1 mM). The pH was adjusted to 6.8 with 4 M NaOH. The ASM medium was supplemented per liter with 0.02 mM biotin (Carl Roth, Karlsruhe, Germany), 20 mL 50×-MEM amino acids solution, 10 mL 100×-MEM non-essential amino acids solution, and 10 mL 100×-MEM vitamin solution (Thermo Scientific, Schwerte, Germany); all supplementary solutions were previously filter-sterilized; and added after the main components of the media were autoclaved. Finally, glucose was added (1 g/L final concentration).

2.1.1. Inoculum Preparation and Bottle Fermentation

Precultures were prepared by adding 300 µL of glycerol stock to 200 mL of mLB medium in 500 mL shake flasks, which were then incubated at 60 °C and 120 rpm under aerobic conditions in an Infors Thermotron (Infors AG, Bottmingen, Switzerland). After 14 h, a calculated volume of 30 mL of the inoculum was added to 1.5 L stainless steel bottles (DURAN® GL 45) containing 600 mL mASM. Initially, the bottles were prepared with an atmosphere of 50% CO and 50% air at standard pressure (1 bar); afterwards, the pressure in the bottles was increased by adding up either CO, H_2, or N_2 up to a given partial pressure for each gas (Table 1). The bottles were then incubated at 60 °C and 120 rpm in the Infors Thermotron (Infors AG, Bottmingen, Switzerland). The fermentations were evaluated over a duration of 168 h.

Table 1. Gas composition for the different experiments.

Experiment	Initial Partial Pressure (bar)					Total Pressure (bar)
	CO	**N_2**	**H_2**	**O_2**	**CO_2**	
No overpressure	0.523	0.482	0	0.133	0.001	1.139
CO (1 bar)	0.976	0.803	0	0.226	0.001	2.006
CO (2 bar)	1.524	0.870	0	0.238	0.002	2.634
CO (3 bar)	2.102	1.201	0	0.329	0.002	3.634
N_2 (1 bar)	0.508	1.390	0	0.129	0.001	2.028
N_2 (2 bar)	0.496	2.429	0	0.123	0.001	3.049
N_2 (3 bar)	0.436	3.073	0	0.138	0.002	3.650
H_2 (1 bar)	0.541	0.487	0.999	0.136	0.001	2.164
H_2 (2 bar)	0.573	0.362	1.908	0.139	0.001	2.983
H_2 (3 bar)	0.675	0.213	2.777	0.111	0.002	3.778

2.1.2. Analytical Methods

The gas composition was evaluated at different time points in each fermentation by drawing 3 mL samples of gas from the headspace and injecting each sample into a 3000 Micro GC gas analyzer (Inficon, Bad Ragaz, Switzerland) connected to 10 m Molsieve and 10 m PoraPLOT Q columns. Gas compositions were calculated according to the ideal gas law as previously described [15].

To evaluate the growth of *P. thermoglucosidasius* and metabolites production during the fermentation, 1 mL liquid samples were collected through the bottle stoppers. The absorbance (OD_{600}) of each sample was measured using the Ultrospec 1100 pro spectrophotometer (Amersham Biosciences, Uppsala, Sweden). The remaining liquid samples were centrifuged at 13,000 rpm for 10 min and the supernatants were transferred into 1.5 mL HPLC vials to evaluate the presence of glucose, acetate, ethanol, formate, lactate, succinate, propionate, butyrate, and valerate. HPLC was performed to quantify the metabolites present in each sample with an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a wavelength detector and refractive index detector with a 50 × 7.8 mm long pre-column (model Rezex ROA-Organic Acid H^+ 8% guard column) and a 300 × 7.8 mm, 8 µm long separation column (model Rezex ROA-Organic Acid H^+ 8% column). A sample volume of 10 µL was injected with a 0.6 mL min^{-1} flow rate for 40 min per sample. A 5 mM H_2SO_4 solution was used as the mobile phase, and a column temperature of 55 °C was selected. Data acquisition and analysis were performed with the software Chemstation (C.01.07 27, Agilent Technologies, Santa Clara, CA, USA). The electron selectivity for the different partial pressures experiments was calculated as explained in the Supplementary Material.

3. Results

3.1. Effect of Increased CO Partial Pressure on H_2 Production

The pCO was increased in the fermentations to evaluate its enhancing or inhibitory effect on hydrogenogenesis. H_2 production commenced after 24 h in the fermentation with no overpressure (pCO: 0.523 bar) and reached a maximum of 16.2 mmol, H_2 corresponding to a pCO of 0.574 bar, after 72 h (Figure 1A). Increase of pCO invariably resulted in a delay in the start of H_2 production. Increasing pCO to 1 bar resulted in the commencement of hydrogenogenesis after 48 h. However, due to higher availability of CO in the headspace, the production of H_2 increased to 63 mmol, corresponding to a pH_2 of 1.39, by 96 h post-inoculation (Figure 1A).

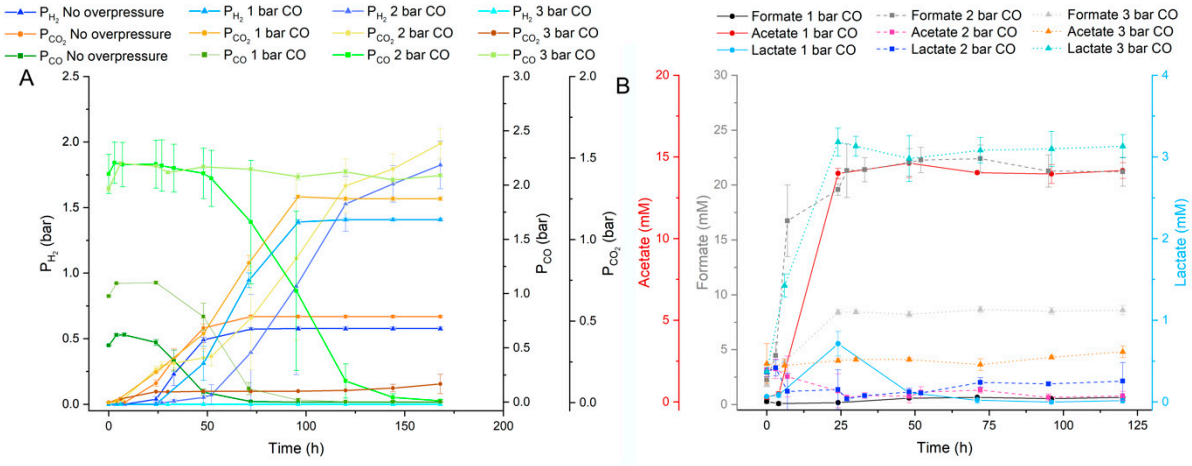

Figure 1. (**A**) Gas partial pressures during fermentations with different pCO. The blue lines denote H_2 production, while the green and orange lines represent CO and CO_2. (**B**) Formate, acetate, and lactate production in the different pCO. Formate, acetate, and lactate are indicated in grey, red, and blue, respectively.

A further increase of pCO to 2 bar delayed the start of H_2 production to 52 h post-inoculation, with H_2 reaching a concentration of 26.6 mmol after 168 h, with a pH_2 of 1.42 bar. At the highest pCO (3 bar), no evident CO consumption or hydrogen production was observed (Figure 1A). The hydrogen production rate reached a maximum of 0.21 mmol d^{-1} at 96 h with 1 bar pCO, while at the same time, it reached 0.03 mmol d^{-1} with 2 bar pCO (Supplementary Figure S1). The volumetric H_2 production rate was 7.44 and 1.08 L H_2 L^{-1} d^{-1} for 1 bar and 2 bar pCO, respectively. On the other hand, oxygen was depleted in all experiments within the first 24 h of fermentation, except for the 3 bar pCO, and it remained constant from 24 to 120 h (Supplementary Figure S2).

Changes in the metabolite profiles were also observed with increased pCO. With a pCO of 1 bar, acetate production was the highest, while for 2 and 3 bar pCO, formate and lactate, respectively, were prominently produced (Figure 1B). More specifically, with 1 bar pCO, acetate production commenced after 24 h, and increased to a maximum of 14.17 mM after 120 h. Formate production stayed below 0.66 mM, while lactate peaked (0.71 mM) after 24 h and subsequently decreased to 0.02 mM (Figure 1B). With 2 bar pCO, 22.4 mM of formate was observed after 72 h and subsequently remained constant, while lactate (0.25 mM) and acetate (0.73 mM) production reached their maximum after 120 h and 72 h, respectively (Figure 1B).

Under 3 bar pCO, formate (8.6 mM) and acetate (2.5 mM) reached their relatively low maxima after 72 h and 24 h, respectively, while fermentation with this partial pressure resulted in the highest amount of lactate being produced, reaching 3.2 mM after 24 h and remaining stable throughout the remainder of the fermentation (Figure 1B). For all the fermentations, the glucose in the media was depleted by 24 h post-inoculation, except for the highest pCO (3 bar), where glucose content decreased to 4 mM and remained constant until the end of the fermentation. This correlates with the lack of consumption of CO under this partial pressure, underlying the inhibition in the fermentation (Supplementary Figure S3). Additionally, it further correlates with the slight O_2 concentration observed with 3 bar pCO, as both oxygen and glucose consumption should normally occur in parallel.

3.2. Effect of Increased N_2 Partial Pressure on H_2 Production

To understand the effects of overall gas pressure on hydrogenogenesis, pN_2 was increased by adding up to 3 bar of pressure. Under no overpressure, H_2 production was observed after 24 h, with 1.0 mmol (pH_2: 0.03 bar), increasing up to 16.4 mmol by 96 h (pH_2: 0.5 bar). With 1 bar pN_2, H_2 production was first observed after 24 h, with 7.9 mmol (pH_2: 0.2 bar) and 22.1 mmol (pH_2: 0.5 bar) of H_2 produced after 29 h and 72 h of fermentation,

respectively (Figure 2A). Increasing the pressure in the system to 2 bar pN_2 produced 6.8 mmol (pH_2: 0.2 bar) and 17.6 mmol (pH_2: 0.5 bar) of H_2 at the same time points. At the highest pN_2 (3 bar), H_2 production commenced earlier, 5.7 mmol of H_2 (pH_2: 0.3 bar) already observed after 24 h of fermentation, reaching a peak of 9.2 mmol H_2 (pH_2: 0.6 bar) after 48 h and afterward remaining stable. The maximum hydrogen production rate was observed at 48 h with 0.13, 0.11, and 0.07 mmol d^{-1}, corresponding to 1, 2, and 3 bar pN_2 (Supplementary Figure S1). Similarly, the volumetric H_2 production rate was 1.58, 0.94, and 0.40 L H_2 L^{-1} d^{-1} for the increasing pN_2 of 1, 2, and 3 bar. Increased pN_2 also affected the growth of the microorganism; the specific growth rate was reduced from 0.145 to, 0.116, 0.090, and 0.075 h^{-1}, with 1, 2, and 3 bar pN_2, respectively.

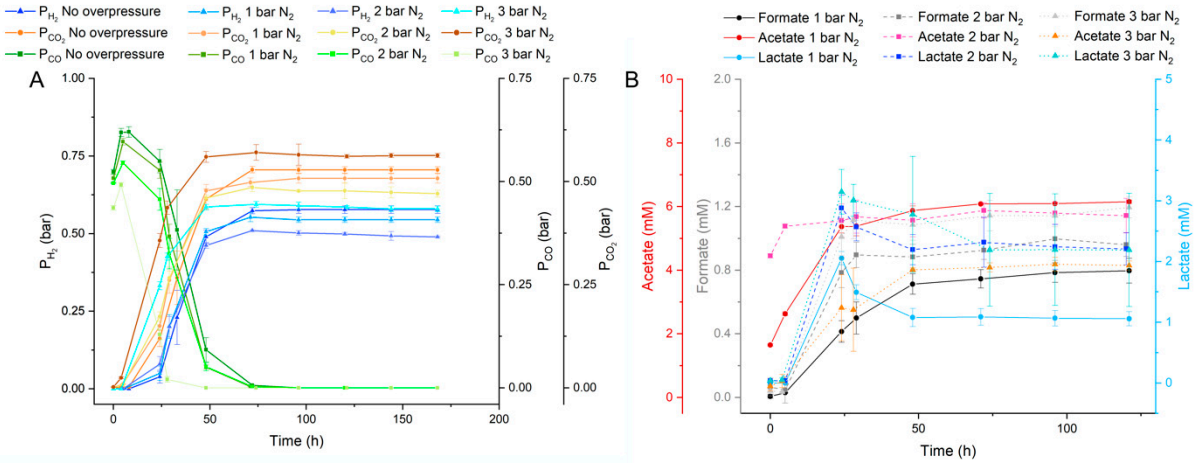

Figure 2. (**A**) Gas partial pressures during fermentations with different pN_2. The blue lines denote H_2 production, while the green and orange lines represent CO and CO_2. (**B**) Formate, acetate, and lactate production in the different pCO. Formate, acetate, and lactate are indicated in grey, red and blue, respectively.

Lactate and formate production increased in line with the increase in pN_2. Whereas formate accumulated to 0.79 mM at the end of fermentation with 1 bar pN_2, it reached 0.96 mM and 1.19 mM with 2 and 3 bar pN_2, respectively (Figure 2B). Maximum lactate production was observed after 24 h for all evaluated pN_2 levels, with 2.05 mM, 2.88 mM, and 3.14 mM produced with 1, 2, and 3 bar of pressure, respectively. Afterwards, lactate production decreased by 1 mM in all the pN_2 levels and remained stable. By contrast, acetate production was highest (6.15 mM after 120 h) with 1 bar pN_2 and the lowest (4.14 mM after 120 h) with 3 bar pN_2.

3.3. Effect of H_2 Partial Pressure on H_2 Production

Increasing pH_2 up to 3 bar allowed us to evaluate product inhibition. With 1 bar pH_2, an increase in H_2 from an initial amount of 37.8 mmol to 46.2 mmol (an increase of 8 mmol) was observed after 24 h, while increases of 11.9 mmol and 15.1 mmol of H_2 were observed with 2 bar (initial 69.1 mmol H_2) and 3 bar (initial 97.9 mmol H_2) pH_2, respectively (Figure 3A). The highest amount of H_2, 130 mmol, was seen after 120 h with 3 bar pH_2. Production of hydrogen commenced after 4 h of fermentation, with 0.39, 0.70, and 0.97 mmol d^{-1} for 1, 2, and 3 bar pH_2, respectively (Supplementary Figure S1). A similar trend was observed with the volumetric H_2 production rate, with 1.03, 4.05, and 7.64 and L H_2 L^{-1} d^{-1} for the increasing pH_2 of 1, 2, and 3 bar. The specific growth rate was negatively affected by increased pH_2, reducing from 0.145 h^{-1} with no overpressure to 0.062 h^{-1} when increasing the pH_2 to 3 bar.

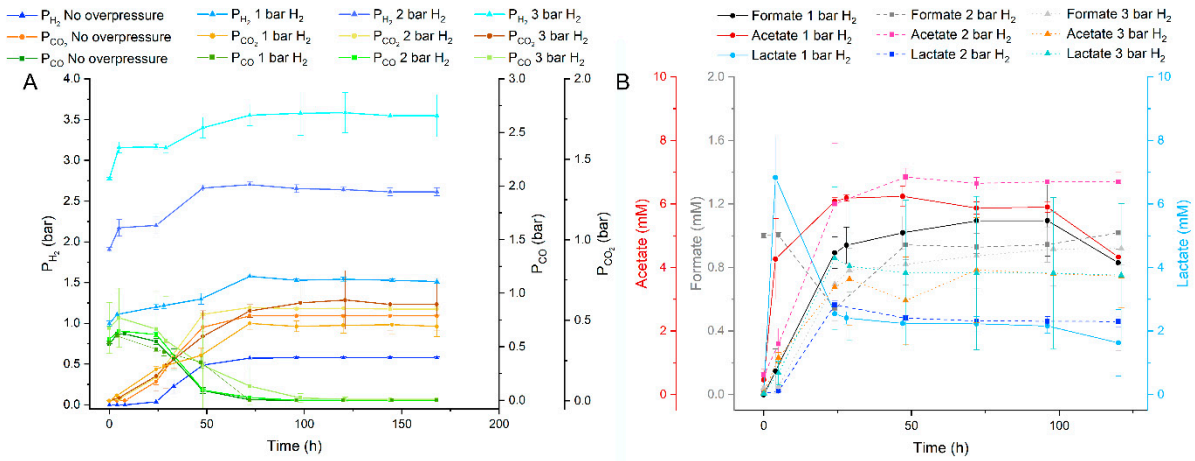

Figure 3. (**A**) Gas partial pressures during fermentations with different pH_2. The blue lines denote H_2 production, while the green and orange represent CO and CO_2. (**B**) Formate, acetate, and lactate production in the different pCO. Formate, acetate and lactate are indicated in grey, red, and blue, respectively.

With 1 bar pH_2, acetate accumulated to 6.24 mM after 48 h, remained stable until 96 h, and subsequently decreased to 4.33 mM by 120 h of fermentation. A similar trend was observed with 2 bar pH_2, where acetate accumulated to 6.85 mM by 48 h and remained relatively stable until 120 h, with a production of 6.70 mM at this time point. A further increase of pressure to 3 bar pH_2 led to a decrease in acetate, with the highest acetate amount (3.92 mM) after 72 h.

Figure 3B shows formate production with 1 bar pH_2 increased to 0.89 mM after 24 h and stabilized at a highest concentration of 1.09 mM after 72 h. By contrast, with 2 bar pH_2, there was consumption of the initial amount (1.0 mM) of formate, decreasing to 0.54 mM after 24 h. However, it subsequently recovered to a concentration of 1.01 mM after 120 h. Formate trends with 3 bar pH_2 were similar to what was observed with 1 bar, as formate increased to 0.69 mM after 24 h, with the highest accumulation (0.92 mM) after 120 h of fermentation. Production of lactate only increased to 6.8 mM after 4 h of the fermentation with 1 bar pH_2 but then decreased to 1.62 mM after 120 h of fermentation. Lower and later peaks of lactate production were observed with 2 and 3 bar pH_2.

3.4. Selectivity of the WGS Process with Different CO, N_2, and H_2 Partial Pressures

The electron balances of the fermentation reactions with different pCO, pN_2, and pH_2 were evaluated. In the experiment with no overpressure, more than 98% of electrons from CO were directed into H_2. Similarly, for the experiments with increased pN_2 and pH_2, the fermentation process was highly selective towards H_2 production, where more than 95% of electrons derived from CO were converted into H_2, regardless of the level of partial pressure of the selected gas (Figure 4A,B). This is followed by acetate as the main metabolite, with less than 5% of electrons from CO and glucose being routed into organic acids. The same trend was observed with 1 and 2 bar pCO. However, when applying 3 bar pCO (Figure 4C), as H_2 production was inhibited, the electrons were primarily derived from glucose and acetate and routed into organic acids.

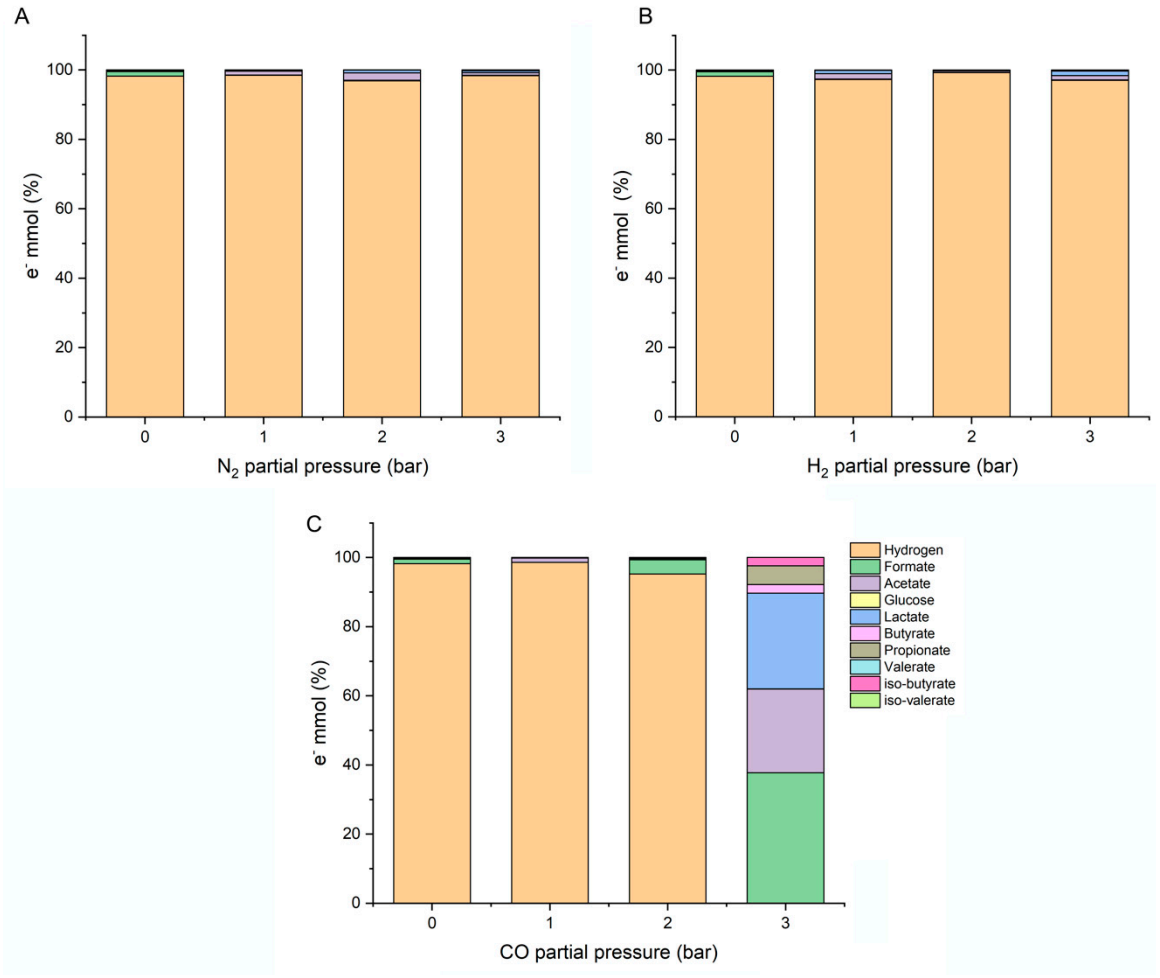

Figure 4. Electron selectivity for the different (**A**) N_2, (**B**) H_2, and (**C**) CO partial pressures.

4. Discussion

4.1. Effect of Increasing Partial Pressure of Gases on H_2 Production

The presence of H_2 could lead to product inhibition, as described for the thermophilic bacterium *Caldicellulosiruptor saccharolyticus*, where H_2 production ceased after reaching a pH_2 of 0.56 bar [19]. Similarly, an increase in pH_2 had a detrimental effect on H_2 yield and decreased sugar utilization efficiency by *Clostridium butyricum* TM-9A, where the total H_2 production decreased 2.6-fold when the pH_2 increased from 0.1 to 0.2 bar [17].

The increase of pH_2 up to 3 bar in our study had no inhibitory effect on H_2 production, and following the WGS reaction, all the CO present in the system was converted into H_2 with a selectivity higher than 95%. This agrees with a study where pH_2 up to 1.25 bar had no effect on *P. thermoglucosidasius* H_2 production [20]. In contrast, the increase of pH_2 in a study evaluating different gas release strategies (uncontrolled, intermittent, and constant) demonstrated that increasing pH_2 was inhibitory to dark fermentative H_2 production by a mixed anaerobic microbial consortium and that the highest H_2 production was obtained with the constant gas release, denoting the lowest pH_2 [21].

An increase in pH_2 primarily resulted in a reduction of acetate and formate, while lactate production was higher at 3 bar pH_2. Changes in the metabolite profile in response to high pH_2 have also been observed in *T. thermosaccharolyticum* W16 [22]. In anaerobic bacteria, the end product formation can be greatly affected by the pH_2, with a metabolic shift towards reduced metabolites such as ethanol and lactate [23]. This was also documented in a study with *Clostridium ljungdahlii*, where the increase of pH_2 to 1.52 bar shifted the product ratio to ethanol; however, an increase of pH_2 up to 3 bars caused a decrease in the gas consumption rates [24]. In *Chlamydomonas reinhardtii*, a high pH_2 caused inhibition of

H_2 production, in addition to changes in metabolism towards reduced products; therefore, a low pH_2 improved H_2 yields exponentially in the latter case [25].

The effect of total pressure on *P. thermoglucosidasius* hydrogenogenesis was evaluated by increasing the pN_2 up to 3 bar. A similar experiment with selected *Clostridium* spp. in an anaerobic biodisc reactor with a pressure increase of 0.18 above atmosphere pressure (0.89 bar) decreased H_2 yield by 19.5% [26]. In another experiment with *Clostridium acetobutylicum*, hydrogen yield declined by 40% in comparison with a strategy releasing constant gas [27]. In our case, while no difference in CO consumption was observed in our study at pN_2 up to 3 bar, slight decreases in H_2 production were noted rather than the expected increase in yield. However, given the negligible effects, total pressures above 3 bar should be evaluated for effect on H_2 yield.

Increasing pCO positively affected cell growth and product (ethanol and acetate) formation in *Clostridium carboxidivorans* $P7^T$ [28]. In our study, increasing pCO up to 3 bar resulted in inhibition of hydrogenogenesis. Furthermore, the increase of pCO caused a decrease in the specific growth rate of *P. thermoglucosidasius* by 59.4% at a pressure up to 1.0 bar and by 66.2% when the pCO reached 3.0 bar compared to when no overpressure was applied. Similarly, an increase of pCO from 0 to 0.5 atm was limiting on the specific growth rate of *Citrobacter* sp. Y19, reducing from 0.72 to 0.29 h^{-1} [29]. In *P. thermoglucosidasius*, the production of acetate and lactate increased at a pCO of 2 bar. This may serve as a means for this bacterium to cope with CO toxicity, as there is a shift in the flow of reducing equivalents away from terminal oxidases with increased CO concentration [18]. Furthermore, the conversion of pyruvate to lactate is a well-established mechanism used to restore the balance of NAD^+/NADH during bacterial growth on glucose [30]. A relationship between acetate and pCO was documented in *C. carboxidivorans* $P7^T$, where lower amounts of acetate were produced at higher pCO [28]. An opposite effect was shown in this work, where the increase of pCO caused an overall decrease of acetate from 14.17 mM to 2.5 mM, with 1.0 and 3.0 bar pCO, respectively. It has been observed that CO conversion rate is limited by the gas–liquid mass transfer when evaluating high biomass concentrations; additionally, the dissolved CO concentration affects the specific CO uptake [31].

4.2. Future Perspectives

Increasing the pH_2 up to 3 bar did not have an inhibitory effect on *P. thermoglucosidasius* hydrogenogenesis. Future studies where pH_2 is increased to pressures higher than this could help to understand the limits of product inhibition on the WGS reaction and changes in *P. thermoglucosidasius* metabolism. A study performed with *Clostridium acetobutylicum* showed that when increasing pH_2 up to 14.59 bar, the yield of butanol and ethanol increased by 18 and 13% [32]. In the latter study, the high H_2 concentration caused the hydrogenase system to be inhibited, while the oxidoreductase system received a higher flow of electrons to produce ethanol [32]. The increase of pH_2 up to 25 bar with a mixed culture was evaluated in a series of batch fermentations; when evaluating the partial pressures of 20 and 25 bar, these were inhibited due to the increase in the dissolved gas tension, which could be detrimental for the microorganisms [33]. However, to evaluate higher pressures in the presented study would require a different system, such as a high-pressure resistant reactor.

Influence of CO_2 partial pressure in the present study was not evaluated; however, it has been reported that CO_2 sparging, which increases CO_2 partial pressure, is beneficial for H_2 production when working under gas mixtures. In a study performed with *Thermoanaerobacterium thermosaccharolyticum* W16, it was observed that under high CO_2 partial pressure, the microorganism could produce H_2, while increasing H_2 partial pressure had a negative effect [22]. Future studies should therefore also consider the effect of increased pCO_2 on *P. thermoglucosidasius* hydrogenogenesis.

5. Conclusions

With the exception of 3 bar pCO, *P. thermoglucosidasius* DSM 6285 was capable of producing H_2 under conditions of increased pCO, pN_2, and pH_2. Increasing pCO up to

2 bar, in particular, had a positive effect on hydrogen production, likely due to increased WGS substrate availability. Finetuning of the pCO should be investigated further as a potential strategy for improved hydrogen yield. However, caution should be exercised as inhibition of hydrogenogensis was observed at pCO of 3 bar, suggesting a toxic CO limit for *P. thermoglucosidasius*. The application of higher pH_2 should also be investigated further as it could be crucial to understanding the limit of inhibition of the WGS reaction, and the kinetics of distribution of the organic acids produced under such inhibition.

Regardless of the partial pressure applied, hydrogenogenesis via the WGS is a highly efficient process, with 95% electron recovery from CO and glucose in hydrogen gas, while other metabolites such as acetate, formate, and lactate represent minor electron sinks, suggesting that hydrogenogensis by *P. thermoglucosidasius* via the WGS reaction represents an attractive option for future biohydrogenogenesis approaches.

The potential for scaling up this process in bioreactors utilizing *P. thermoglucosidasius* remains to be fully assessed. While challenges exist, scaling this system up presents an open possibility for improving the biological hydrogen production platform. It is also important to evaluate the influence of different gas mixtures and a range of gas partial pressures to better understand the limitations observed in this study. Such evaluations could provide essential insights into optimizing biohydrogen production. This study serves as a foundation for further research aimed at refining gas phase conditions to enhance hydrogen yields in biological systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10110592/s1, Figure S1: Hydrogen production in the different partial pressures, Figure S2: Oxygen partial pressure for all fermentations, Figure S3: Glucose consumption in all fermentations. Table S1. Conversion factors.

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