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Direct and Indirect Effects of Increased CO₂ Partial Pressure on the Bioenergetics of Syntrophic Propionate and Butyrate Conversion

Pamela Ceron-Chafla,* Robbert Kleerebezem, Korneel Rabaey, Jules B. van Lier, and Ralph E. F. Lindeboom

ABSTRACT: Simultaneous digestion and in situ biogas upgrading in high-pressure bioreactors will result in elevated CO₂ partial pressure (pCO₂). With the concomitant increase in dissolved CO₂, microbial conversion processes may be affected beyond the impact of increased acidity. Elevated pCO₂ was reported to affect the kinetics and thermodynamics of biochemical conversions because CO₂ is an intermediate and end-product of the digestion process and modifies the carbonate equilibrium. Our results showed that increasing pCO₂ from 0.3 to 8 bar in lab-scale batch reactors decreased the maximum substrate utilization rate (ṙmax) for both syntrophic propionate and butyrate oxidation. These kinetic limitations are linked to an increased overall Gibbs free energy change (ΔG°Overall) and a potential biochemical energy redistribution among syntrophic partners, which showed interdependence with hydrogen partial pressure (pH₂). The bioenergetics analysis identified a moderate, direct impact of elevated pCO₂ on propionate oxidation and a pH-mediated effect on butyrate oxidation. These constraints, combined with physiological limitations on growth exerted by increased acidity and inhibition due to higher concentrations of undissociated volatile fatty acids, help to explain the observed phenomena. Overall, this investigation sheds light on the role of elevated pCO₂ in delicate biochemical syntrophic conversions by connecting kinetic, bioenergetic, and physiological effects.

INTRODUCTION

High-pressure anaerobic digestion (HPAD) has been proposed as a technology for in situ biogas upgrading,5 able to achieve a CH₄ content >90%, after which the produced CH₄ is in principle suitable for further direct use in, for example, (decentralized) gas grid injection or advanced industrial processes. HPAD takes advantage of the large difference in solubility between CH₄ and CO₂, which is most pronounced at high pressures in a digester equipped with a pressure valve for biogas release. However, by letting the pressure rise, the CH₄ content increases in the headspace, whereas CO₂ and other ionizable gases such as H₂S dissolve in the liquid. Thus far, the effects of increased dissolved CO₂ on the overall performance of the high-pressure system have hardly been studied beyond accumulating acidity.5 As far as the authors are aware, limited attention has been paid to its possible impact on metabolic conversion routes and degradation rates.

CO₂ has multiple roles in biological systems such as electron acceptor, carbon donor, intermediate, and end-product of biochemical reactions, and contributes to the aquatic buffer system via the carbonate equilibrium.5 These multiple roles complicate studies searching for a mechanistic description of the response to increased CO₂ partial pressure (pCO₂) in natural and engineered environments, except for the bacteriostatic effects of high pCO₂ applied for sterilization purposes at 40–300 bar and 20–50 °C. The bacteriostatic action leads to cytoplasm acidification, cell rupture, and inactivation of key enzymes and transport proteins.8–10 The impact of “moderate” pCO₂ from 0.1 up to 10 bar is less comprehensively described and is mainly attributed to a decreased intracellular pH.9 However, pH reduction by itself does not explain the reduced microbial activity of denitrifying bacteria observed by Wan et al.10 because of dissolved CO₂ concentrations up to 30,000 ppm. These authors proposed that elevated pCO₂ caused direct inhibition of the carbon metabolism, electron transport chain, enzymatic activity, and substrate consumption at the expense of increased buffer concentration to prevent a pH drop.3,10,11
Research on the impact of moderate pCO₂ on methano-
genesis is limited to observations relevant to oil reservoirs. Operational conditions of 50 bar pressure, 10% pCO₂, and temperature of 55 °C resulted in a shift from syntrophic acetate oxidation (SAO) to acetlastic methanogenesis (AcM). 12 The effects of CO₂ supplementation at atmospheric pressure in anaerobic digesters (ADs) are better documented in literature; when accompanied by stoichiometric H₂ provision, it enhances CH₄ production because of promoted hydrogenotrophic methanogenesis (HyM). 13 Also, exogenous CO₂ can be indirectly converted to CH₄ via homoacetogenesis coupled to AcM. This mechanism has been proposed to explain the increased CH₄ production after CO₂ direct injection in (a) pilot-scale AD treating food waste 5,14 and (b) two-phase AD-treating sewage. The accompanying electron donor was not highlighted; nonetheless, this role could be performed by additional H₂ coming from reduced acidogenesis 15 or after the release of other hydrolyzed material from cell lysis. 

Increased CO₂ also induces changes in microbiome activity, diversity, community structure, and microbial interactions. The last one is of vital importance in ADs, which rely on syntrophy to overcome thermodynamic limitations for the conversion of intermediate compounds, namely propionate and butyrate. 17,18 The accumulation of these intermediates correlates with reactor disturbance because of the increased organic loading rate, pH changes, and unpaired acidogenesis and methanogenesis. 19 Since these conversions operate close to thermodynamic equilibrium, subtle variations in substrate/product concentrations and environmental conditions can modify the actual Gibbs free energy change (ΔG°) of a specific pathway. 20 The effects of elevated CO₂ on syntrophic interactions have been studied in subsurface environments destined for geological carbon storage. 21,22 Bioenergetic simulations have shown different outcomes on the ΔG° of the intermediate reactions: the energetic feasibility of substrate oxidation and acetlastic methanogenic conversions decreased, whereas the contrary occurred for HyM. 22,23 As a consequence of the apparent thermodynamic control exerted by pCO₂, specific bacterial metabolisms might be promoted or inhibited. 24

In our present work, we studied the impact of elevated pCO₂ on the kinetics and bioenergetics of the syntrophic conversion of propionate and butyrate. It is hypothesized that an increase in the overall available Gibbs free energy for substrate conversion, because of increased pCO₂, could provoke an imbalance in the energy share among syntrophic partners that might translate into kinetic limitations. A scenario analysis is proposed to understand the individual and combined effects of pCO₂ and pH on the bioenergetics of syntrophic conversions. Furthermore, the relationship between bioenergetic and kinetic data is evaluated through a correlation analysis aiming to provide insight into the system response to changing available energy.

### MATERIALS AND METHODS

**Experimental Setup and Reactor Operation.** Five initial operational pCO₂ that is, 0.3, 1, 3, 5, and 8 bar, were selected for the experimental treatments based on pH equilibrium calculations performed with the hydrogeochemical software PHEREEQC (version 3, USGS). The application of an elevated buffer concentration of 100 mM as HCO₃⁻ in the system allowed to maintain circumneutral pH, despite the elevated pCO₂. Batch experiments at 0.3 and 1 bar were carried out at atmospheric pressure in 250 mL Schott bottles sealed with rubber stoppers. In parallel, the elevated pressure experiments were performed in 200 mL stainless-steel pressure-resistant reactors (Nantong Vasia, China). The experiments were conducted at a liquid: gas ratio of 1.5:1 and inoculum/substrate ratio of 2:1 g COD g VSS⁻¹. The liquid medium consisted of macronutrient and micronutrient stock solutions (6 and 0.6 mL L⁻¹, respectively) prepared according to Lindeboom et al. 1 and 1 g of COD L⁻¹ of the substrates propionate or butyrate.

The headspace of bottles and reactors was replaced with N₂ gas (>99%) to ensure anaerobic conditions after filling. Then, the bottles were flushed with the corresponding gas mixture: 70:30% N₂/CO₂ for 0.3 bar pCO₂ or >99% CO₂ for 1 bar pCO₂. Elevated pressure reactors were subjected to three consecutive pressurization-release cycles to ensure complete N₂ replacement by CO₂ (>99%) at the intended pressure. Temperature and agitation speed were controlled using an incubator shaker (Innova 44, Eppendorf, USA) set to 35 ± 1 °C and 110 ± 10 rpm. Pressure was online-monitored using digital sensors (B + B Thermo-Techniek, Germany) and a microcontroller (Arduino Uno, Italy). The experiments had a fixed duration of 14 days.

**Inoculum Selection.** Preliminary experiments of propionate anaerobic conversion under 1 bar pCO₂ were conducted in triplicates using three mesophilic inocula collected from (A) sludge digester-treating excess sewage sludge, (B) UASB reactor-treating sugar beet wastewater, and (C) anaerobic membrane bioreactor-treating food industry wastewater. The three inocula were characterized in terms of physicochemical parameters (Supporting Information, Table S1), and inoculum C was selected for the experiments here described (Supporting Information, Figure S1).

**Analyses.** Experiments were carried out in triplicate incubation; however, because of the small working volume of the reactors (200 mL), a sampling strategy for liquid and gas samples was designed that enabled us to account for replicate variability, minimizing disturbance of the batch incubations (Supporting Information, Table S2). Headspace composition and volatile fatty acids (VFAs) were analyzed using gas chromatography (7890A GC system, Agilent Technologies, US). In the first one, gas samples (5 mL) taken twice per week at atmospheric pressure were measured via a thermal conductivity detector and directed through an HP-PLOT Molsieve GC column (30 m length × 0.53 mm inner diameter × 25 μm film thickness). Helium was used as the carrier gas at a constant flow of 10 mL min⁻¹. The oven and detector were operated at 45 and 200 °C, respectively. In the second one, VFAs were determined according to Ghisami et al. 25 Total and soluble COD, total suspended solids, volatile suspended solids (VSS), and pH were measured at the beginning and end of the experiment according to Standard Methods. 26

**Estimation of Kinetic Parameters.** The modified Gompertz equation 27

\[ y = A \times e^{-\lambda t + \lambda t \times e^{\lambda (t - t_0)}} \]  

where \( y \) represents the substrate concentration (mg L⁻¹), \( \lambda \) is the lag phase (day), \( \lambda_{\text{max}} \) is the maximum substrate utilization rate (mg L⁻¹ day⁻¹), \( A \) is the maximum substrate concentration (mg L⁻¹), and \( t \) is the time (days), was used to fit the data from the atmospheric and pressure experiments.
The kinetic parameters were estimated using nonlinear minimization methods from the package nls tools in R (v3.6.1).

**Bioenergetic Calculations.** \( \Delta G^R_{R} \), the actual Gibbs free energy change for the reactions, was calculated according to

\[
\Delta G^R_{R} = \Delta G^R_{R}^0 + RT \sum_{i=1}^{n} Y_{Si}^R \ln(a_{Si})
\]

where \( \Delta G^R_{R}^0 \) is the Gibbs free energy at pH 7 and 308.15 K, \( R \) is the gas constant (8.31 J K\(^{-1}\) mol\(^{-1}\)), \( T \) is the temperature in kelvin, \( Y_{Si}^R \) is the stoichiometric coefficient of compound \( i \), and \( a_{Si} \) is the molar concentration of compound \( i \). \( \Delta G^R_{R} \) was corrected for temperature using the Gibbs–Helmholtz equation. \( \Delta G^R_{R} \) were taken from Heijnen and Kleerebezem.

**Estimation of Potential Biochemical Energy Distribution in Syntrophic Oxidation of Propionate and Butyrate.** The stoichiometry of the overall syntrophic reaction and the intermediate catabolic reactions is presented in Table 1. From the acetotrophic reactions, only AcM was included in the analysis because SAO was considered unlikely to occur under our experimental conditions and initial community composition (Supporting Information, Figure S2). The stoichiometric coefficients of AcM and HyM for each substrate were calculated using the modified Gompertz equation, the significance levels of which are presented in Table 2.
correspond to the balance of the formed species during the oxidation.17 At the initial adjusted circumneutral pH, the dissolved inorganic carbon corresponds to H$_2$CO$_3$ and HCO$_3^-$. H$_2$CO$_3$ can be expressed in terms of pCO$_2$ using Henry’s law with its proportionality constant ($k_h$) corrected by temperature. The equations, as presented in Table 1, are deliberately written in terms of the H$^+$ concentrations and pCO$_2$ to illustrate the effect of these variables on the thermodynamic calculations.

$\Delta G^\circ_b$ for the reactions presented above can be affected by pCO$_2$, pH, or by a combined interaction. The nature of the effect will depend on the role of the parameter in the catabolic reaction, meaning it acts as a reagent, product, or is not directly involved. As well, the magnitude of the effect might be amplified because of an initially less negative $\Delta G^\circ_b$. A scenario analysis was performed to understand the impact of changing pCO$_2$ and pH on the $\Delta G^\circ_b$ of the overall and intermediate catabolic reactions. The resulting calculations, subsequently, were used to estimate the change in the potential biochemical energy share. A summary of input parameters in each scenario (A, B, and C) is presented in Table S3, Supporting Information. The calculations were performed using a pH$_3$ value of 1 × 10$^{-5}$ bar, typical for ADs14 and at which syntrophic reactions become thermodynamically feasible.17

**Statistical Analysis.** Spearman’s rank-order correlation coefficient ($r_s$) was calculated via the function rcorr() of the package “Hmisc” in R (v3.6.1),28 ordered using hierarchical clustering and plotted using the package “corrplot.”32

### RESULTS AND DISCUSSION

**Effect of Elevated pCO$_2$ on the Anaerobic Substrate Conversion and Metabolite Production Rate.** Subplots A and C, as presented in Figure 1, show the decrease in substrate conversion rates in the experimental treatments at increased pCO$_2$ ranging from 0.3 to 8 bar during the 14 days. The reduction in $r_{\text{max}}$ was further quantified using the process parameters extracted from the data-fitting to the modified Gompertz equation, as presented in Table 2. Data from the 8 bar pCO$_2$ experiment are not included because it was not possible to determine the kinetic parameters accurately. Increasing pCO$_2$ from 0.3 to 5 bar led to a 93% reduction in $r_{\text{max}}$ for propionate, whereas for butyrate, the $r_{\text{max}}$ dropped by 57%. The calculated specific $r_{\text{max}}$ for propionate at 0.3 bar pCO$_2$ is already in the low range of the values proposed in the literature: 150—292 mg propionate g VSS$^{-1}$ day$^{-1}$. In the case of butyrate, the specific $r_{\text{max}}$ at 0.3 bar pCO$_2$ was 1 order of magnitude lower than the inferior boundary of the theoretical range: 3.9—10.9 g butyrate g VSS$^{-1}$ day$^{-1}$.33 For both cases, elevated pCO$_2$ resulted in a concomitantly increase in the lag phase ($\lambda$), which is likely associated with inadequate levels of adaptation to operational conditions. A considerable effect on the production and consumption of acetate was not evident in
the propionate experiment; however, for butyrate, a decrease in acetate production occurred (Figure 1B,D). Lower methane production was observed in the propionate experiment only at 8 bar $p_{\text{CO}_2}$ while it appeared already at 5 bar $p_{\text{CO}_2}$ for butyrate (Figure 2A,B).

Hansson and Molin first reported the adverse effects of $p_{\text{CO}_2}$ on the propionate and butyrate anaerobic conversion rate.34 These authors observed a decrease of 70% in the $r_{\text{smax}}$ in propionate degradation when increasing $p_{\text{CO}_2}$ from 0.2 to 1 bar. The effect for butyrate was not significant, as opposed to our current work in which we identified an 18% reduction in $r_{\text{smax}}$ at a comparable $p_{\text{CO}_2}$ increase. In a previously reported experiment, using suspended pressure-cultivated inoculum that originated from anaerobic granular sludge degrading propionate,5 it was shown that 5 bar $p_{\text{CO}_2}$ caused a 93% reduction in the $r_{\text{smax}}$. This value agrees with the calculations presented here (Table 2).

Figure 3. Change in the overall available Gibbs free energy ($\Delta G_{\text{Overall}}$) during mesophilic syntrophic (A) propionate oxidation and (B) butyrate oxidation at 0.3, 1, 3, 5, and 8 bar initial $p_{\text{CO}_2}$ calculated with measured concentrations of reactants and products during the experimental period. Aqueous concentrations were used (in mol L$^{-1}$), the partial pressure of gases (in bar), $T = 35 ^\circ \text{C}$, and a theoretical value of $p_{\text{H}_2} = 1 \times 10^{-5}$ bar.

Figure 4. Effect of changing selected operational parameters on the $\Delta G^*_R$ in the proposed scenarios for the syntrophic conversions. Scenario A—partial pressure of $\text{CO}_2$ ($p_{\text{CO}_2}$) in propionate and butyrate conversion (A and D, respectively). Scenario B—$\text{pH}$ in propionate and butyrate conversion (B and E, respectively). Scenario C—concomitant effect of $\text{pH}$ and $p_{\text{CO}_2}$ on propionate and butyrate conversion (C and F, respectively). Lines represent the $\Delta G^*_R$ for the intermediate biochemical reactions: dotted-purple ($\Delta G_{\text{HyM}}$), dashed-orange ($\Delta G_{\text{Pr-Ox}}$ or $\Delta G_{\text{Bu-Ox}}$), short-dash-dotted green ($\Delta G_{\text{AcM}}$), and solid black (overall reaction—$\Delta G_{\text{Overall}}$). The experimental conditions ($p_{\text{H}_2}$, $p_{\text{CO}_2}$, and $\text{pH}$) that remained fixed during the calculation are included for reference in the upper part of the subplots. Values are presented as log $p_{\text{CO}_2}$ for data linearization purposes. Concentrations of liquid reactants (mol L$^{-1}$) and gases (bar) correspond to the initial experimental conditions at $T = 35 ^\circ \text{C}$ presented in the heading of Table S3, Supporting Information.

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The reactions of syntrophic conversions has not been thoroughly elucidated in literature. We tried to gain further insight into the individual and combined effects of elevated pCO₂ and pH on the bioenergetics using scenario analysis. By such analysis, possible bioenergetic limitations caused by an increase in the ΔG₂overall value might be identified.

Figure 5 visualizes the change in the ΔG₂overall value when the parameters pCO₂ and pH are independently and concomitantly modified in syntrophic propionate and butyrate conversion. Lines represent the change in Gibbs free energy at increasing pCO₂ or decreasing pH for the intermediate biochemical reactions: substrate oxidation (ΔGpr - ΔGbu - ΔGbu-Ox), short-dash-dotted green (AcM = ΔGAcM), and solid black (overall reaction = ΔG₂overall). The experimental conditions (pH, pCO₂, and pH₂) that remained fixed during the calculation are included for reference in the upper part of the subplots. Values are presented as log pCO₂ and log pH₂ for data linearization. Concentrations of liquid reactants (mol L⁻¹) and gases (bar) correspond to the initial experimental conditions at T = 35 °C presented in the heading of Table S3, Supporting Information.

conversion calculated using the actual concentrations of reactants during the atmospheric and pressure experiments and at pH₂ = 1 × 10⁻⁵ bar. Results showed a less steep increasing trend over time for ΔG₂overall from 1 bar pCO₂ onward, indicating that the two syntrophic reactions became less energetically feasible because of decreased substrate consumption or product accumulation. At day 0, the ΔG₂overall at 0.3 bar pCO₂ for propionate oxidation was −85.0 J mol⁻¹ for butyrate oxidation. At 8 bar pCO₂, the ΔG₂overall for propionate increased to −78.0 compared to −137.9 J mol⁻¹ for butyrate. The calculated dissimilarity in the ΔG₂overall of the reactions (≈40%) might have weakened the driving force to carry out propionate conversion at increased values of pCO₂ at atmospheric and pressurized conditions. This observation relates well with what Kleerebezem and Stams¹⁸ proposed in their metabolic network analysis of syntrophic butyrate conversion, where they highlighted the possibility of a lowered specific reaction rate as a function of increased Gibbs free energy change of the catabolic reaction.

ΔG₂overall responds to direct and indirect changes in biochemical reactions.¹⁸ A deliberate change in the concentration of one or more biochemical species is considered a direct intervention. A change in the concentration of the species induced by the modification of another operational parameter is an indirect intervention. The predominance of a direct or indirect effect of increased pCO₂ on the ΔG₂overall and intermediate biochemical reactions of syntrophic conversions has not been thoroughly elucidated in literature.
conditions, there is a marginal increase in $\Delta G_{\text{Overall}}$ for the conversion of both substrates (C and F).

Concerning the intermediate reactions at 20 bar pCO$_2$ in scenario A, $\Delta G_{\text{Pr-Ox}}$ increased by 44%, and $\Delta G_{\text{Bu-Ox}}$ remained constant because CO$_2$ is not a reaction product. Regarding the methanogenic reactions, $\Delta G_{\text{AcM}}$ increased by 30%, whereas $\Delta G_{\text{HyM}}$ decreased by 40% for both substrates (A and D). The pH decrease to 5.5 in scenario B did not strongly affect the reactions where H$^+$ ions are not produced, that is, $\Delta G_{\text{Py-Ox}}$ and $\Delta G_{\text{HyM}}$. Contrastingly, $\Delta G_{\text{Bu-Ox}}$ increased by 32% and $\Delta G_{\text{AcM}}$ decreased by 27% and 28% for the propionate- and butyrate-fed assays, respectively, suggesting enhanced energetic feasibility of this reaction (B and E). In scenario C, $\Delta G_{\text{Pr-Ox}}$ and $\Delta G_{\text{Bu-Ox}}$ changed analogously to scenario A. $\Delta G_{\text{AcM}}$ remained the same in the entire pCO$_2$ range, which could be attributed to the simultaneous variation of pCO$_2$ annulling the pH effects on the bioenergetics. The behavior of $\Delta G_{\text{HyM}}$ resembled scenario A because of the absent effect of H$^+$ production (C and F).

Scenario A highlighted the adverse effects of increased pCO$_2$ on the bioenergetics of syntrophic reactions. In this regard, Jin and Kirk$^{22}$ postulated that increasing pCO$_2$ from 0 to 30 bar in simulated non-buffered and buffered aquifer systems made SAO and AcM less energetically feasible, whereas the contrary was calculated for HyM. Moreover, they proposed additional effects of elevated pCO$_2$ on biochemical reactions because of induced changes in aqueous speciation, ionic strength, and in the reduction potential of redox couples such as H$^+$/H$_2$. Kato et al.$^{21}$ found that increasing pCO$_2$ from 0 to 1 bar strongly suppressed syntrophic activity in a model bacterial consortium for SAO, including the bacterium Thermacetogenium phaeum and the archaea Methanothermobacter thermautotrophicus and Thermacetogenium phaeum. They established a 91% reduction in the $r_{\text{max}}$ of acetate, coincidently occurring when $\Delta G_{\text{AcM}}$ became higher than $-20$ kJ mol$^{-1}$, which is considered the smallest quantum to sustain life.$^{17}$ In our experiments, $r_{\text{max}}$ values decreased when pCO$_2$ increased from 0.3 to 8 bar, and the most significant drop also occurred when, theoretically, $\Delta G_{\text{Bu-Ox}}$ was higher than $-20$ kJ mol$^{-1}$ (Supporting Information, Table S4).

Scenario B showed that decreasing pH modifies the bioenergetics of syntrophic propionate and butyrate conversion in a different direction than elevated pCO$_2$. Interestingly, pH can directly change the $\Delta G_{\text{R}}$ when reactions produce or consume protons and indirectly as a result of modified chemical speciation.$^{35,36}$ From the bioenergetics point of view, proton (H$^+$)-consuming reactions, namely syntrophic oxidation and AcM (Table 1), could be promoted when decreasing pH inside a physiologically reasonable range. The more negative $\Delta G_{\text{Overall}}$ value in this scenario indicates a potential increase in the driving force to carry out the syntrophic reaction. Nonetheless, this might be compromised by physiological limitations and enhanced toxicity effects$^{37}$ observed at decreased pH levels, particularly in the case of methanogenic populations.$^{38}$ In consequence, bioenergetics does not suffice to elucidate the detrimental effects observed on the syntrophic conversions if pH is considered as the main explanatory variable.

**Elevated pCO$_2$ as a Biochemical Steering Parameter.**

The distribution of available biochemical energy between the syntrophic partners is expected to change because of the direct and indirect effects of increasing pCO$_2$ on $\Delta G_{\text{R}}$ of the overall and intermediate reactions (Supporting Information, Figure S3). In our results, the biochemical energy allocation is proposed under conditions of fixed pH$_2$. Under conditions of changing pH$_2$, pH, and pCO$_2$ (Figure S5, scenarios D, D.1, and D.2), a new thermodynamic equilibrium will be established, which can further modify the biochemical energy distribution among partners in syntrophic propionate and butyrate conversion. Values of pH$_2$ lower than $6 \times 10^{-4}$ bar will have a positive effect on reaction feasibility, whereas higher values will reduce the feasibility “niche.” The impact of increasing pH$_2$ on the available Gibbs free energy has been previously discussed in the literature,$^{39}$ nevertheless, its interaction with increased pCO$_2$ and decreased pH, to the best of our knowledge, has not been thoroughly described. A correlation analysis with hierarchical clustering of bioenergetic and experimental data was performed in order to verify whether the highlighted trends of the scenario analysis were still valid at a varying pH$_2$ (Supporting Information, Figure S4). Two theoretical values were chosen: a typical value for ADs at which syntrophic reactions are thermodynamically feasible (1 $\times 10^{-5}$ bar)$^{41}$ and the lowest detection level of the used gas chromatograph (6 $\times 10^{-8}$ bar). A strong negative correlation was found between pCO$_2$ and $r_{\text{max}}$ ($r = -0.82$, $p < 0.05$) for both propionate and butyrate. Concerning the Gibbs free energy change, a strong negative correlation was encountered only between $\Delta G_{\text{Bu-Ox}}$ and pH ($r = -0.78$, $p < 0.05$). $\Delta G_{\text{AcM}}$ was strongly negatively correlated with $\Delta G_{\text{HyM}}$ ($r = -0.87$, $p < 0.05$), evidencing the role of increasing pCO$_2$ and pH$_2$ in modulating the feasibility of methanogenic reactions.

**Response of Syntrophic Anaerobic Conversion at Elevated pCO$_2$: Possible Physiological Effects.** This study highlighted a possible relation between bioenergetic limitations and the observed kinetic effects occurring because of increased pCO$_2$. However, additional limitations cannot be discarded. For example, in our experiments, the dissolution of CO$_2$ from the headspace could decrease pH levels, irrespective of the applied high buffer concentration (100 mM HCO$_3^-$). Changes in pH disrupt cell homeostasis and impose limitations for growth, maintenance, and metabolic activity. In particular, syntrophic butyrate oxidizers (SBOs) and syntrophic propionate oxidizers (SPOs) demonstrate moderate growth at a pH lower than 6.5$^{40}$ and 6.0$^{41}$ respectively. The increased lag phases and limited conversion under elevated pCO$_2$ could then be explained by the combination of pH effects on, for example, $\Delta G_{\text{Bu-Ox}}$ and physiological limitations affecting SBOs and SPOs at a different extent.

Also, the acidification of the fermentation medium modifies the equilibrium between undissociated and dissociated forms of the VFAs,$^{42}$ further altering cell homeostasis. At the applied pCO$_2$ of 8 bar and resulting equilibrium pH of 5.9, the concentrations of undissociated propionic acid (HPr) were slightly above inhibitory levels, that is, 20 mg L$^{-1}$ HPr$^{43}$ (Supporting Information, Table S5). The concentration of undissociated butyric acid (HBut) remained below 500 mg L$^{-1}$ HBut$^{44}$ proposed in literature as inhibitory for growth in, for example, Clostridium acetobutylicum. Acetic acid concentrations (HAc) remained below indicative inhibitory levels in methanogenesis.$^{37}$ However, the detrimental effects of elevated pCO$_2$ in our experimental treatments were already seen at 1 bar pCO$_2$. Consequently, increased undissociated VFA concentrations do not explain the observed phenomena.

At elevated pCO$_2$, the equilibrium dissolved CO$_2$ concentration in the liquid medium increased from 320 to 8,620 mg L$^{-1}$ (Supporting Information, Table S5). These dissolved CO$_2$ concentrations are in line with values reported by Wan et al.$^{10}$
(3,000–30,000 ppm), which negatively impacted the nitrogen removal efficiency because of increased membrane permeability, thus inhibiting electron transport and protein expression.

Furthermore, Salek et al. showed that there is at least 1 order of magnitude difference in the kinetically controlled rate of physical reactions such as CO2 dissolution and biochemical reactions, such as production of VFAs. This, in turn, may affect the concentration of the various species that are responsible for the reactions used in the thermodynamic calculations, leading to disparities in the calculated and observed bioenergetic effects at specific time points. More accurate pH2 measurements in the low range, for example, <6 × 10⁻⁴ bar, are required to further validate the occurrence of the postulated effects on the feasibility of syntrophic reactions because of concomitant variation of pH2 and pH or pCO2. The possible pCO2 exposure might confer potentials for steering metabolic observed kinetic and bioenergetic aftermath of elevated associated with a lowered pH, and a minor detrimental impact induces pH changes that make the presence of hydrogen and elevated pCO2, particularly role of other electron shuttles, whose appearance is favored by the presence of hydrogen and elevated pCO2, particularly formate, needs to be further addressed.

Elevated pCO2 influences the kinetics and bioenergetics of the syntrophic conversion of propionate and butyrate. Based on this study, we propose that kinetic effects might appear as an evident sign of thermodynamic limitations, which is different for each compound. From detailed bioenergetic calculations, it was concluded that pCO2 increases the ΔG PrOx induces pH changes that make ΔG BuOx more positive, and increases the ΔGOverall of the syntrophic conversion. The more positive ΔGOverall at elevated pCO2 likely induces a redistribution of the available biochemical energy among the syntrophic partners that, if unbalanced, will translate into kinetic constraints. However, the here discussed biochemical energy limitations could not fully explain the strong kinetic effects on the system at increasing pCO2. Presumably, the overall effects resulted from the concomitant impact of reduced thermodynamic feasibility, physiological effects associated with a lowered pH, and a minor detrimental impact of increased concentrations of undissociated VFAs. The observed kinetic and bioenergetic aftermath of elevated pCO2 exposure might confer potentials for steering metabolic pathways, if limitations are overcome. For instance, the use of acclimated inocula and energy-rich substrates such as sugars, proteins, or lipids could minimize the physiological impact of lowered pH and relieve bioenergetic limitations. Under such conditions, the steering potential of elevated pCO2 on biochemical pathways in mixed culture anaerobic conversions could be unraveled.

- ASSOCIATED CONTENT
  - Supporting Information
    The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02022.

Role of inoculum origin on pCO2 response; physicochemical characteristics of initial inocula; microbial community analysis, including Illuma sequencing protocol and absolute abundance results; sampling strategy; input parameters for the bioenergetics scenario analysis of syntrophic propionate and butyrate conversion; calculated values for ΔG PrOx in scenario A; theoretical share of ΔGOverall for each of the proposed scenarios for the syntrophic conversion of propionate and butyrate; correlogram at different pH2 values; and calculation of undissociated acids and carbonate equilibrium species for the anaerobic conversion experiments at 0.3, 1, 3, 5, and 8 bar pCO2 (PDF)

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Notes
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- NOMENCLATURE

\[ \Delta G^\circ_{R} \] Gibbs free energy change for reaction R at standard temperature and pressure (kJ mol⁻¹)

\[ \Delta G^\circ_{R}^{bi} \] Gibbs free energy change for reaction R corrected by biological pH reference value (pH = 7) (kJ mol⁻¹)

\[ \Delta G^\circ_{R}^{a} \] Gibbs free energy change for reaction R corrected by actual operational conditions (kJ mol⁻¹)

\[ \Delta G_{overall} \] Gibbs free energy change for the syntrophic reaction corrected by actual operational conditions (kJ mol⁻¹)

\[ \Delta G_{PrOx} \] Gibbs free energy change for propionate oxidation corrected by actual operational conditions (kJ mol⁻¹)

\[ \Delta G_{BuOx} \] Gibbs free energy change for butyrate oxidation corrected by actual operational conditions (kJ mol⁻¹)

\[ \Delta G_{AcM} \] Gibbs free energy change for acetlastic methanogenesis corrected by actual operational conditions (kJ mol⁻¹)

\[ \Delta G_{HyM} \] Gibbs free energy change for hydrogenotrophic methanogenesis corrected by actual operational conditions (kJ mol⁻¹)
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