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Effect of Elevated Hydrogen Partial Pressure on Mixed Culture Homoacetogenesis

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ABSTRACT

This study aimed to systematically investigate the effect of elevated hydrogen partial pressure on mixed culture homoacetogenesis in the range of 1–25 bar. Seven batch experiments were performed at different initial headspace pressures, i.e., 1, 3, 5, 10, 15, 20, and 25 bar. The 15 bar batch showed the highest gas uptake rate (6.22 mol $h^{-1}L^{-1}$) and volatile fatty acids synthesis (3.55 g L^{-1}) by a final microbial consortium that was found to be largely reduced in complexity compared to the original inoculum culture and dominated by members of the *Pseudomonadaceae* and *Clostridiaceae*. Product distribution shifted from acetate to C_3-C_5 acids at a pressure above 15 bar. 15 bar was found to be the optimum elevated pressure for the used mixed culture fermentation medium and biodiversity used in this study, and pressure above 15 bar inhibited the microbial consortia and resulted in lowered gas uptake rate and product synthesis.

1. Introduction

Syngas is a key product of biomass pyrolysis and gasification processes, contains carbon monoxide (CO), hydrogen (H_2) and carbon dioxide (CO_2) (Grimalt-Alemany et al., 2018). It can be converted to biofuels through bacteria-mediated acidogenesis, solventogenesis, and methanogenesis or thermochemical processes like Fischer-Tropsch (Daniell et al., 2012). Acetate is the most common metabolic intermediate, further converted to biogas in an anaerobic digestor (Anukam et al., 2019; Geppert et al., 2016). Biogas production from syngas is a sustainable approach in the field of clean biofuel production (Torri et al., 2020). Utilizing syngas to produce biofuels brings sustainable value addition (Daniell et al., 2012) and reduces the alternative cost of carbon capture and storage.

Homoacetogens are a group of acetogenic bacteria that have the capability to ferment syngas into acids and alcohols (Mohammadi et al., 2011). Fischer et al. (1932) first reported that the homoacetogens with capabilities to use CO_2 and H_2 as carbon and energy sources, respectively, to produce acetate (Drake, 2012), Equation (1). However, Wood and Ljungdahl presented the detail reduction pathway in the late 1980 s. They identified the acetyl-CoA as an essential intermediate (Diekert & Wohlfarth, 1994). Therefore, referred to as Wood Ljungdahl Pathway and the acetyl-CoA pathways. Moreover, the theory behind the WLP is briefly presented in section 1.1 to ensure the good flow of understanding.

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \ \Delta G^0 = -74.3 \text{ kJ/mol} \quad (1)$$

The formation of metabolic intermediates is essentially influenced by the concentration of chemical compounds in the liquid phase, primarily on mass transfer at the gas–liquid interface (Cuff et al., 2020; Mulat et al., 2017; Phillips et al., 2017; Yasin et al., 2019). The solubility of CO and H_2 are 60 and 1056 times less than CO_2 (Phillips et al., 2017), respectively. Temperature and partial pressure of the gaseous species are the main parameters that influence the gas solubility in a liquid medium (Pereira et al., 2013; Phillips et al., 2017). Temperature and pressure impact syngas fermentation have been studied for many years (Conrad & Wetter, 1990; Kundiyana et al., 2011; Shen et al., 2020; Stoll et al., 2019; Van Hecke et al., 2019). However, H_2 in the syngas mixture needs more attention because of its lower solubility. The H_2 utilization rate by bacteria depends on H_2 partial pressure, fermentation product concentrations such as Volatile fatty acids (VFAs) available in the medium, and the mass transfer rates (Dinamarca et al., 2011).

According to Henry's law, a rise in partial pressure of H_2 in the headspace increases the gas solubility, referred in Equation (2).

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Where C_{H_2} is the H_2 concentration (mol L^{-1}) in the liquid medium, y_{H2} is the H_2 mole fraction (at gas phase), P_T is the total headspace pressure (atm) and H_{H2} (atm mol^{-1} L) is Henry's law constant of the H_2 gas (Kantzow & Weuster-Botz, 2016; Phillips et al., 2017).

$$C_{H_2} = y_{H2}\frac{P_T}{H_{H2}} \quad (2)$$

However, microorganisms also could be sensitive to partial pressure (Abubackar et al., 2011) and the dissolved gas tension. Their pressure tolerability could decide the upper limit of partial pressure that can be applied, depending on the type of microorganisms (Van Hecke et al., 2019).

Even though it is substantiated that rising partial pressure increases the gas–liquid mass transfer, to the best of our knowledge, there is not enough research in this field conducted over 10 bar H_2 headspace pressure on mixed culture homoacetogenesis process (Stoll et al., 2018; Van Hecke et al., 2019). This is because of the higher cost of operation and process safety concerns; however, it has become economically viable in recent years due to the technological advancement. Therefore, this study is a first attempt to evaluate the impact of highly elevated H_2 partial pressure up to 25 bar on a sludge-based mixed culture homoacetogenic medium.

1.1. The Wood-Ljungdahl pathway

The Wood-Ljungdahl (WLP) pathway has been extensively studied over several decades because it is an attractive and sustainable way of fixing CO_2 to mitigate global warming and produce valuable chemicals such as acetate and ethanol (Fernández-Naveira et al., 2017; Hu et al., 2011; Saady, 2013; Stoll et al., 2018). The carbon in the CO_2 molecule is at the highest possible oxidation state (+4). H_2 gas acts as the reducing equivalent/electron donor and donates electrons to this CO_2 fixation process, which results in acetate as the primary product. Since many pieces of literature explain the WLP in detail, a brief flow diagram is present in Fig. 1 with relevant sequenced chemical reactions (Bertsch & Müller, 2015; Hu et al., 2011; Liew et al., 2016; Saady, 2013; Schuchmann & Müller, 2014; Wilkins & Atiyeh, 2011). In brief, acetate production via the WLP pathway consists of two branches of reactions, i.e., methyl branch and carbonyl branch. The methyl branch consists of several reductive steps to reduce CO_2 to the methyl group ($-CH_3$), while the carbonyl branch is a shorter reduction branch where CO_2 is reduced into CO/carbonyl group (C═O) by two units of reducing equivalent. Methyl, carbonyl groups and coenzyme merge to form acetyl-CoA, which is further converted into acetate. The H_2 gas oxidation produces reducing equivalents, facilitated by electron bifurcating hydrogenase enzyme (Bertsch & Müller, 2015).

2. Material and methods

In this section, culture enrichment, experimental and analytical methodology, and microbiome analysis procedures are explained in detail.

2.1. Homoacetogenic culture enrichment

The anaerobe seed sludge was collected from a biogas digester at Knarrdalstrand wastewater treatment plant, Porsgrunn, Norway. The sludge went through several pretreatment steps to obtain the desired fermentation quality. First, a 600 μm sieve was used to eliminate coarse impurities such as plastic and woody debris. It was then incubated for seven days at 35 °C for further thickening and depleted the remaining degradable organic matters. The thickened sludge was heat-treated at 105 °C for 48 h to obliterate

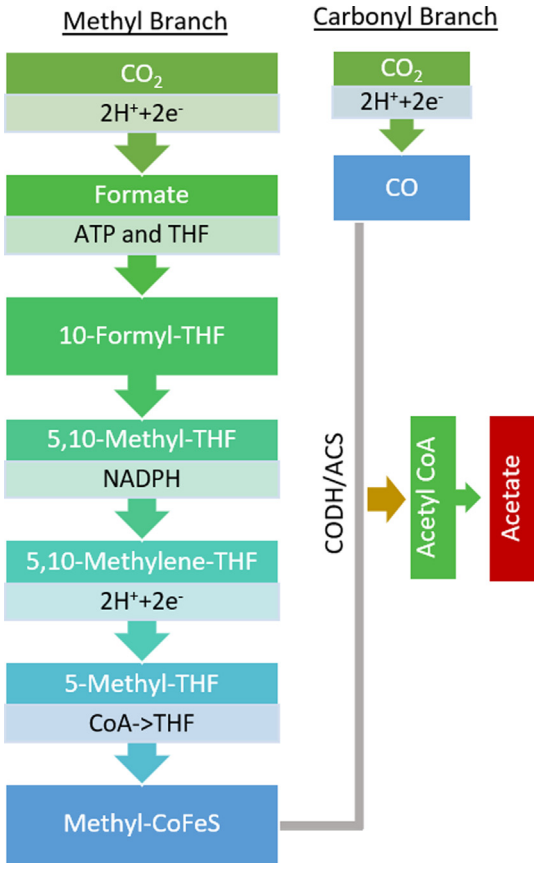

Fig. 1. *The WLP for acetate synthesis;* (). *adapted from* Drake, 2012

methanogens (Sivalingam et al., 2021). While the methanogens are obliterated, homoacetogens remain in the sludge in the form of spores. Following the heat treatment process, the sludge was left to cool down to room temperature and was used as an inoculum. The treated inoculum density in terms of volatile and total solids ratio (VS/TS) was 0.41. A salt solution (10 mL/L inoculum), a vitamin solution (1 mL/ L inoculum), and a mineral solution (1 mL/ L inoculum) were added to the inoculum to make a nutrient base for microbial growth. The nutrient base medium was prepared according to a similar study performed earlier (Dinamarca & Bakke, 2009; Sivalingam & Dinamarca, 2021). The content of the nutrient base is listed in table 1.

2.2. Fermentation reactor and experimental methodology

A stainless steel 640 mL pressure vessel (BR-500, Berghof, Eningen, Germany) was used as the fermentation reactor. The reactor

Table 1
Content of nutrient base media used to support the growth of homoacetogenic culture.

Vitamin solution (g/L)	Mineral solution (g/L)	Salt solution (g/L)
Biotin: 0.02	$MnSO_4{\cdot}H_2O$: 0.04	NH_4Cl: 100
Folic acid: 0.02	$FeSO_4{\cdot}7H_2O$: 2.7	NaCl: 10
Pyridoxine hydrochloride: 0.1	$CuSO_4{\cdot}5H_2O$: 0.055	$MgCl_2{\cdot}6H_2O$: 10
Riboflavin: 0.05	$NiCl_2{\cdot}6H_2O$: 0.1	$CaCl_2{\cdot}2H_2O$: 5
Thiamine: 0.05	$ZnSO_4{\cdot}7H_2O$: 0.088	
Nicotinic acid: 0.05	$CoCl_2{\cdot}6H_2O$: 0.05	
Pantothenic acid: 0.05	H_3BO_3: 0.05	
Vitamin B12: 0.001		
p-aminobenzoic acid: 0.05		
Thioctic acid: 0.05		

comprises a digital manometer (LEO-3, Keller, Winterthur, Switzerland) connected to the computer via RS485 interface to log the pressure every 10 min. The Control Center Series-30 from the Keller software package was used as the automatic pressure logging platform. A mechanical stirrer (BG 65X50, Dunkermotoren, Bonndorf, Germany) continuously mixed the fermentation medium at 200 rpm.

The experimental plan is to run seven batch experiments, where only the initial H_2 partial pressure was changed from 1 to 25 bar. First the pressure vessel was filled with 300 mL treated inoculum with added nutrient base and sodium bicarbonate, which leaves 340 mL headspace. Nitrogen gas was purged for 5 min to push out the air from the inoculum and the headspace; subsequently, the residual nitrogen was flushed out with pure H_2 gas for 2 min to ensure the anaerobic environment. Then the reactor was pressurized to desired values according to the experimental plan, thus 1, 3, 5, 10, 15, 20, and 25 bar manometric pressure, respectively in independent batch experiments, using the H_2 gas (H_2 gas Laboratory 5.5 = $\geq$ 99.9995 %, Linde Gas AS, Oslo, Norway).

All experiments were performed at ambient temperature, 25 °C. Since the study aims to evaluate the impact of the H_2 partial pressure, 3.4 g/L sodium bicarbonate ($NaHCO_3$) was added to the inoculum as the dissolved inorganic carbon source (Gardner et al., 2013; Sivalingam & Dinamarca, 2021). Though the inoculum contains intrinsic inorganic carbon originated from bacterial biomass decay, $NaHCO_3$ was added to corroborate appropriate culturing conditions. The initial pH of the inoculum was 8.5, which was neither adjusted nor controlled throughout the experiments. Such higher pH ensures that added bicarbonate will remain in the liquid medium at equilibrium with carbonate ion without escaping to the headspace as carbon dioxide gas (Tchobanoglous et al., 2014).

During each experiment, H_2 in the headspace diffuses into the bulk-liquid. As the inoculum consumes it, the headspace pressure decreases over time. The experiments were completed when H_2 consumption rate becomes significantly low, indicating depletion of the carbon source, HCO_3^-. The 1 bar batch was re-pressurized two times to reach no further change in pressure, while the other batches reached this state with one time pressurizing. Because the research aim is to perform repeated batch process only replenishing the initial H_2 partial pressure between batch experiments.

Various analyses were performed on the liquid medium are described in section 2.3. Experimental conditions were identical for all seven batch cultivations, except the initial H_2 headspace pressure.

2.3. Analytical methodology

Volatile fatty acids (VFAs), pH, total solids (TS), ammonium and volatile solids (VS) were analyzed at the beginning and the end of each batch experiments. The pH was measured by a Beckman 390 pH-meter (Beckman Instruments, Indiana, USA). The fermentation products/VFAs were quantified by gas chromatography (PerkinElmer, Clarus 500, Massachusetts, USA) equipped with a capillary column (length 25 m × 0.25 mm diameter × film 0.2 µm) and Flame Ionization Detector (FID) having H_2 as the carrier gas (45 mL/min). The injector and detector temperatures were 270 °C and 250 °C, respectively. The initial oven temperature was set at 80 °C and kept constant for 0.7 min, then let it rise by 25 °C/min until it reached 200 °C. Subsequently, a 20 °C/min ramp-up rate was assigned to achieve 240 °C. A Spectroquant® Pharo 300 UV/VIS photometer (Merck KGaA, Darmstadt, Germany) was used to quantify the ammonium concentration according to the standard method (2500 A) of the America Public Health Association (APHA, 1995). Volatile solids were determined according to US standard 2540 E (APHA, 1995).

2.4. Microbiome analysis by 16s rRNA gene metabarcoding

Total metagenomic DNA was extracted from the pellet of various amounts of the samples using the Quick DNA-Fecal/ Soil Microbe DNA Miniprep Kit (Zymo Research) according to the manufacturer's protocol. Sequencing amplicon libraries were generated by PCR following the "16S Metagenomic Sequencing Library Preparation, Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System" protocol (Illumina part number 15044223 rev. B). Internal parts of the 16S ribosomal RNA (rRNA) gene, covering variable regions V3 and V4, were PCR-amplified with the KAPA HiFi HotStart ReadyMix (KAPA Biosystems) and the primers 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG-3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACTACHVGGGTATCTAATCC-3′ and purified with the Agencourt AMPure XP kit (Beckman Coulter). The Nextera XT Index Kit was used to add sequencing adapters and multiplexing indices by PCR, and the products were purified by Agencourt AMPure XP followed by quantification on a Qubit v2 using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). Pooled DNA libraries were sequenced on an Illumina MiSeq sequencer using the MiSeq Reagent Kit v3 in the 2x·300 bp paired-end mode. After sequencing, raw sequencing reads were demultiplexed, filtered, combined, and taxonomically classified by the Metagenomics Workflow within MiSeq Reporter v. 2.5.1 (Illumina), generating abundance tables and biodiversity indices like Phylogenetic diversity and Shannon index, which were further processed in Microsoft Excel.

3. Results and discussion

3.1. H_2 gas consumption and product formation

Fig. 2 presents 7 time series plots for different start pressures, i.e., 1, 3, 5, 10, 15, 20, and 25 bar. Only the 1 bar batch's pressure reached close to zero during incubation and was then repeatedly re-pressurized to 1 bar until the gas consumption stopped. For 16.7 h, pressure remained approximately unchanged for the 1, 3, and 5 bar experiments, while higher pressure batches showed an immediate reduction in headspace pressure. The impact of elevated pressure could be the reason for such instant pressure reduction; according to equation (2), the increase pressure-gradient, consequently, increase the H_2 molar transfer rate that results in the headspace pressure reduction. However, equation (2) does not explain the impact of the constant pressure observed at the beginning of every batch, almost the first 10 h. It could be the lag phase of the microbial community. The one bar experiment took around 13 days to reach the saturated gas consumption level, while other batches took only 4 – 5 days. In order to evaluate the amount of total consumed H_2, cumulative consumed H_2 graphs are presented in Fig. 3.

The one bar batch cultivation showed a cumulative H_2 consumption of 39 mmol after 300 h. The other batch cultivations all reached maximum cumulative H_2 consumption much earlier by 120 h. Up to 15 bar, the total amount of dissolved H_2 gas consumed increased with the partial pressure applied, thus 21.1 mmol in 3 bar, 32.18 mmol in 5 bar, 36.56 mmol in 10 bar and 47.24 mmol in 15 bar batches. The consumed H_2 in the 15 bar batch was approximately five times higher than the 1 bar batch cultivation. The 20 and 25 bar batches consumed respectively 43.27 and 47.75 mmol H_2, which do not comply with the observed trend in pressure versus consumed H_2 from 1 bar to 15 bar batches. This result indicates that the partial pressure improves gas solubility and uptake rate until 15 bar, while further increase apparently affects the uptake rate negatively.

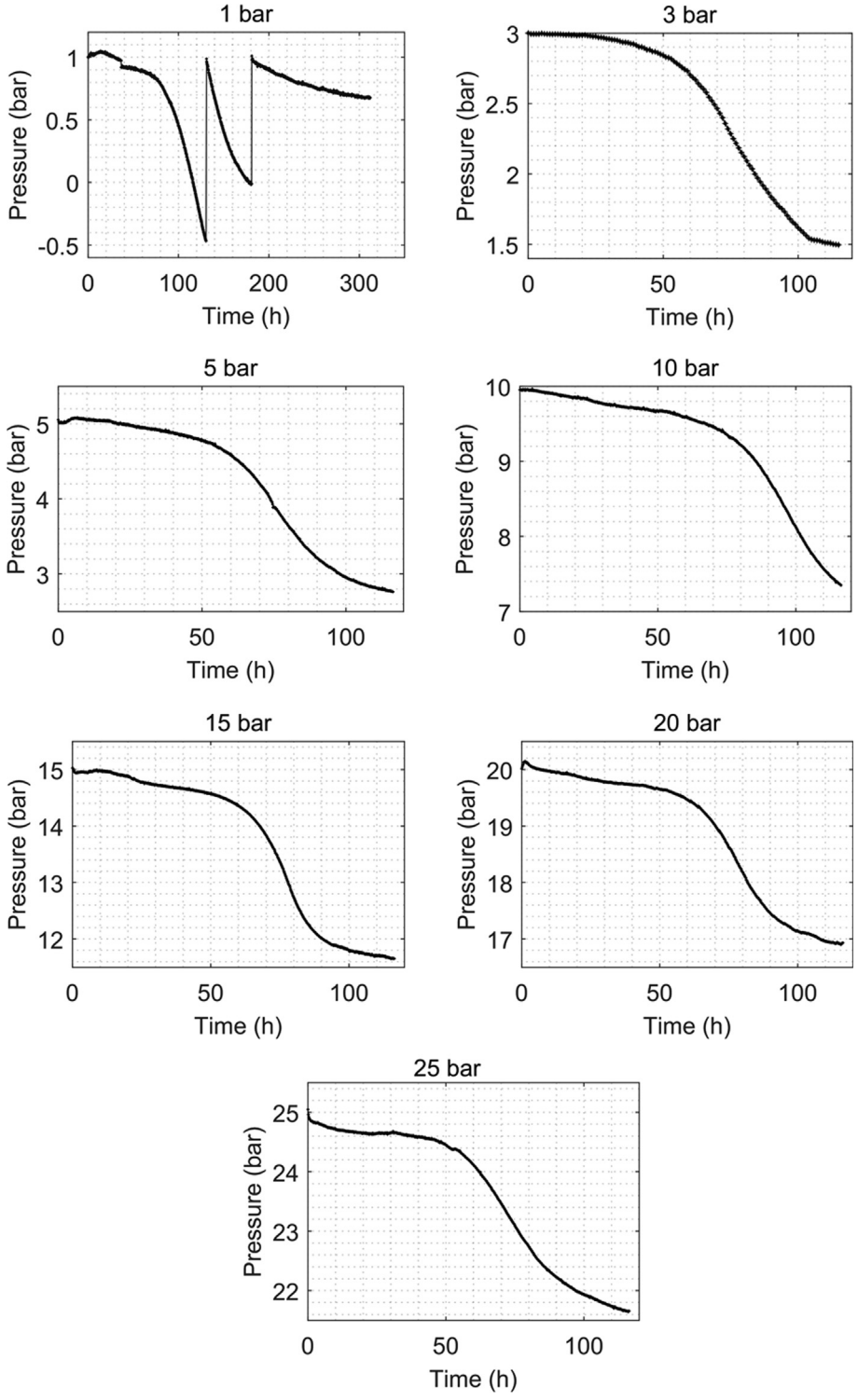

Fig. 2. Headspace pressure time series.

However, the changes in pressure gradients (gas uptake rate) are not clearly seen in Fig. 3; therefore, the gas uptake rates are presented separately in Fig. 4. Increasing gas solubility by elevating partial pressure is always possible if it is considered as the physical process only. However, the increasing dissolved H_2 tension may negatively impact the microbial gas uptake rate.

The characteristics and state of the microbial inoculum could represent a key limiting factor, as the assemblage may be sensitive to factors such as pressure, pH, temperature, and other physio-chemical parameters. However, in this systematic experimental approach, only the H_2 partial pressure was the parameter changed throughout all the batches indicates the pressure as the critical parameter for H_2 gas consumption. This is in line with previous studies that earlier showed that acetogens are very diverse and primarily respond to inoculum (Van Hecke et al., 2019). Increasing pressure on microorganisms produce changes cell structures and

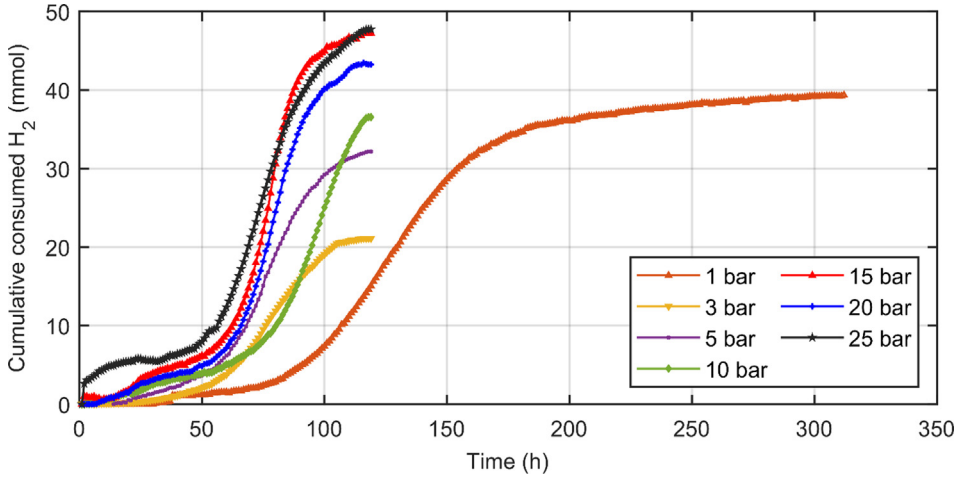

Fig. 3. *Cumulative consumed* H_2 *gas profiles.*

cell functions, sometimes cause for inhibition or cell death (Mota et al., 2013); however not many detail studies have been available particularly about how acetogens behave in different pressures.

The one bar batch reached the maximum gas uptake rate (1.78 mol $h^{-1}L^{-1}$) after 125 h, while the other batches reached their maxima after 70 – 80 h, except for the 10 bar batch that took around 100 h. The 15 bar experiment achieved the highest uptake rate (6.22 mol $h^{-1}L^{-1}$) among all seven batches. Overall, from 1 bar to 15 bar batches, we can see a clear upward trend in maximum uptake rate, consistent with pressure increment. However, the 20 and 25 bar batches showed contradictory behavior. At 20 bar batch, the maximum uptake rate dropped slightly to 5.05 mol h^{-1}-L^{-1} and decreased nearly half of the 15 bar test's uptake rate at 25 bar test (3.79 mol $h^{-1}L^{-1}$). This remarkable reduction in the gas uptake rate explains that pressure above 15 bar inhibits the gas uptake rate, which could be due to microorganisms are inhibited by high dissolved gas tension. As shown in Fig. 2, the pressure time series for 20 and 25 bar batches indicate that the final pressure of the batches at the end of experiments was above 15 bar, i.e., 17 and 21.5 bar, respectively. This is a clear evidence that our inoculum is sensitive to pressures higher than 15 bar.

3.2. Fermentation product synthesis

The acetic acid and total VFAs concentrations with relevant pressures for all seven batches are presented in Fig. 5. The total VFAs concentration grew along with pressure from 1 bar to 15 bar, followed a gradual downward trajectory at 20 and 25 bar tests. The total VFAs consisted of 90 % acetic acid. From 1 to 15 bar, the balance 10 % was contributed by propionic acid, except 3 bar batch, for which only acetic acid could be detected as the fermentation product. Among the seven batches, the highest concentration of the acetic acid (3 g/L) was observed in the 15 bar batch, for which also the highest concentration of total VFAs (3.55 g/L) was detected. This fermentation product analysis confirms that 15 bar is the optimum pressure for this particular mixed culture fermentation, yielding both the highest gas uptake rate and the highest VFA product yield, primarily limited by the added inorganic carbon (bicarbonate salt) in the fermentation medium. The depletion of bicarbonate was ensured stoichiometrically (Equation (1)), which shows that the consumed H_2 and produced acetic acid are more than the available bicarbonate stoichiometric ratio.

In addition to acetic and propionic acid synthesis, the 20 and 25 bar batches showed in addition small amounts of isobutyric and isovaleric acid production. However, all these medium-chain VFAs contributed less than 5 % of the total VFAs production. Ethanol production was observed only for the 25 bar batch and in very small amounts (less than 1 %). All these VFAs concentration are presented in FigureS1 under supplementary section. Although the concentrations of medium-chain VFAs are significantly lower than the short-chain VFAs, this result indicates that elevated headspace pressure can change microorganisms' metabolism, resulting in a different fermentation product spectrum. A similar study performed by Oswald et al. (2018), noticed that increasing partial pressure of H_2 and CO_2 on *Clostridium ljungdahlii* shifted the primary fermentation product acetate to formate (Oswald et al., 2018). However, in this study, acetate shift from formate was not observed; this could be due to different fermentation mediums. Oswald et al., used the pure *Clostridium ljungdahlii* culture, while mixed culture is used in this study which is dominated by *Pseudomonadaceae* and *Clostridiaceae.*

3.3. Physiochemical analysis

pH was measured at the beginning and the end of each experiment, tabulated in Table 2. Overall, an increment in pH from 8.5 to 9.1 – 9.6 was observed. Even though all seven batches produced significant amounts of VFAs, none of them showed a pH reduction. The possible reasons for such a rise in pH are discussed in general.

According to inorganic carbon species equilibrium and pH dependence (Dodds & Dodds, 2002), pH above 8.5 ensures that there is no CO_2 exchange between the headspace and the fermentation medium in our reactors, but the bicarbonate and carbonate species will be in equilibrium (Eq. (3)).

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \quad (3)$$

During the fermentation, process bicarbonate is consumed by the homoacetogens at the expense of H_2 (Equation (4)) (Angelidaki et al., 2011) gas which causes a subsequent leftward shift in equation (3). Such bicarbonate consumption causes a reduction in protons, resulting in an increase in pH.

$$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O \quad (4)$$

Sodium ions (Na^+) are freely available in the fermentation medium due to $NaHCO_3$ added at the beginning of each experiment. The produced acetic acid (pKa = 4.7) will be in the carboxylate ion form (de-protonated) due to the high pH of the fermentation medium (>8.5). Half of the acid will be de-protonated at the pH of pKa, and more will be de-protonated while the pH increases above 4.7 (Trček et al., 2015). Therefore, the de-protonated acetic

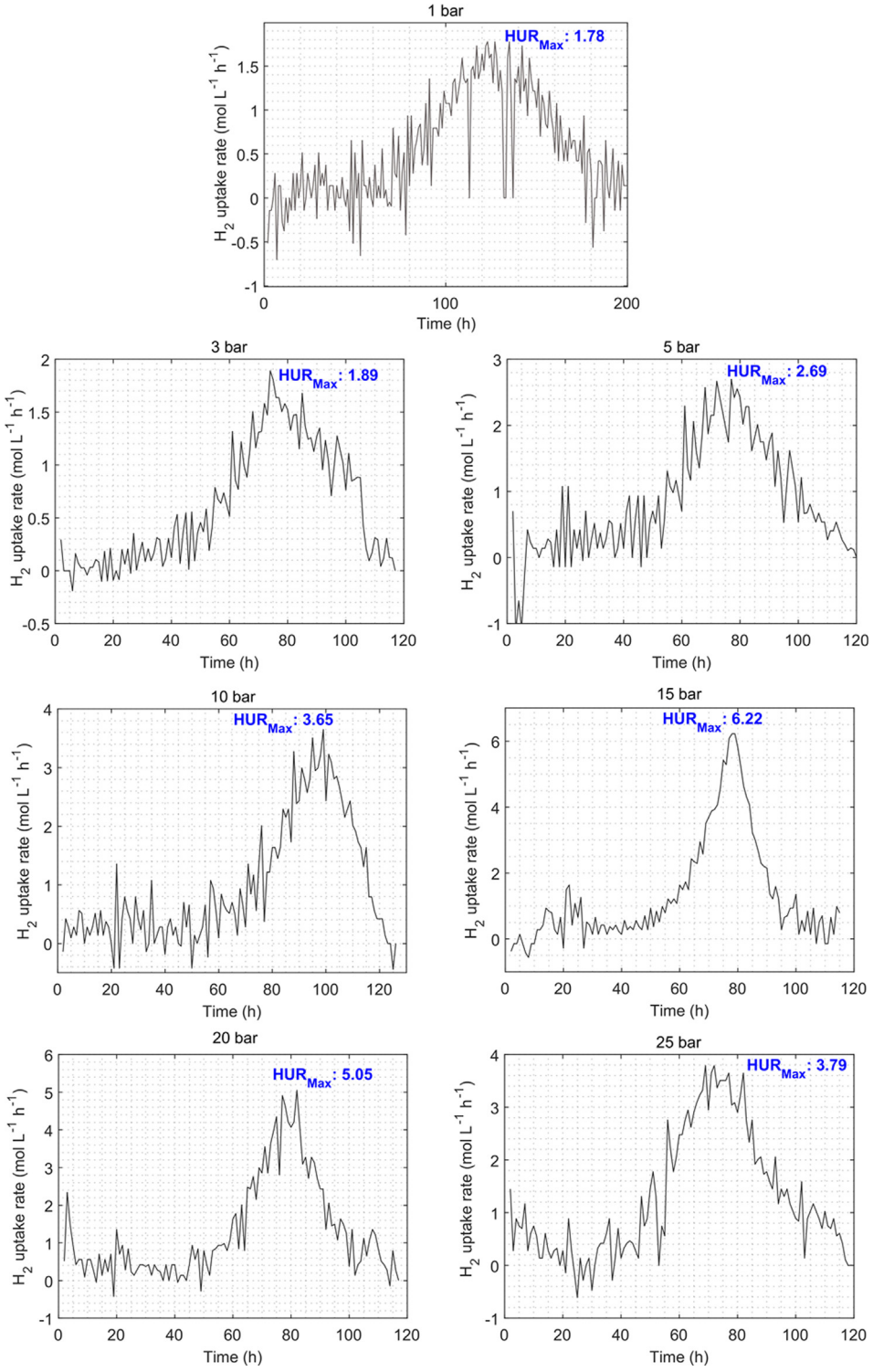

Fig. 4. *H_2 gas uptake rate time series. (Maximum H_2 gas uptake rate (HUR_{Max})* is denoted in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acid (CH_3COOH^-) and the free sodium ion could form sodium acetate (CH_3COONa). Sodium acetate is a conjugated base, which could trap protons (H^+) from the water and leaves hydroxyl ion (OH^-). The increment in the OH^- concentration could be one possible cause of the pH rise.

Ammonia (NH_3) is usually produced during the anaerobic digestion process due to the breakdown of proteins molecules (Yenigün & Demirel, 2013), which consequently increases the pH by capturing protons from water molecules and leaves hydroxyl ions in the liquid medium. A slight increment in ammonium concentration was observed in our experiments. The ammonium concentration of the inoculum at the start of the experiments was 573 ± 20 mg/L and increased slightly up to 609 ± 22 mg/L towards the end of experiments (Table 2). This shows that protein breakdown and utilization of the amino acids as carbon source could be another possible reason for the observed pH increment.

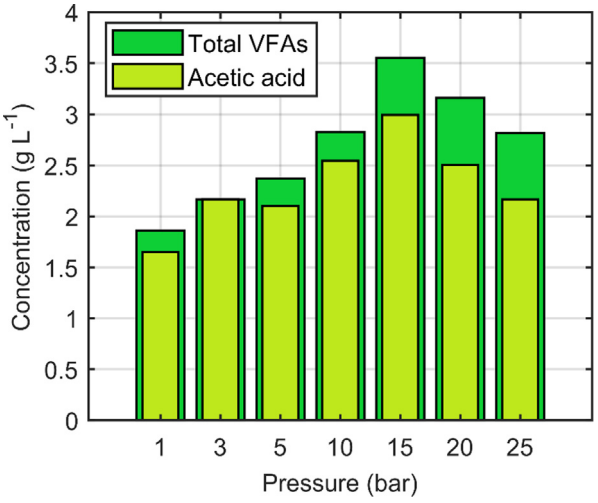

Fig. 5. Acetic acid and total VFAs concentration variations in all seven batches.

However, more control experiments are needed to figure out the individual contributions of above discussed processes.

The volatile solids (VS) and total solids (TS) ratio (VS/TS) was quantified (Table 2) at the beginning and at the end of each batch to evaluate the biomass growth. The fresh fermentation medium's VS/TS was 0.41 and remained approximately in the same range (±0.03) for the pressure batches from 3 to 15 bar. The one bar batch showed a slightly higher ratio than other because this batch was operated for the longest time (13 days). The batches with 20 and 25 bar showed a remarkable VS/TS ratio reduction, respectively 0.37 and 0.34. This observation adds value to our arguments that microorganisms are inhibited (less biomass synthesis) by elevated pressure above 15 bar. The less biomass synthesis coincides with lower product formation at relevant batches (Fig. 5).

Moreover, the overall stoichiometric equation includes energy and synthesis for acetate synthesis from CO_2 and H_2 is derived (Equation (5)) based on approach presented in (Rittmann and McCarty, 2020), therein stoichiometrically possible ammonium consumption and biomass synthesis were evaluated.

Table 2
Initial and final values of pH, ammonium concentration of the liquid medium, and VS/TS ratio at all seven batches.

Parameters	Raw medium	Pressure (bar)						
		1	3	5	10	15	20	25
pH	8.5 ± 0.5	9.1	9.3	9.2	9.4	9.3	9.7	9.6
VS/TS	0.41 ± 0.004	0.46	0.44	0.43	0.41	0.41	0.37	0.34
NH_4^+ (mg/L)	573 ± 20	600	610	610	601	618	572	654

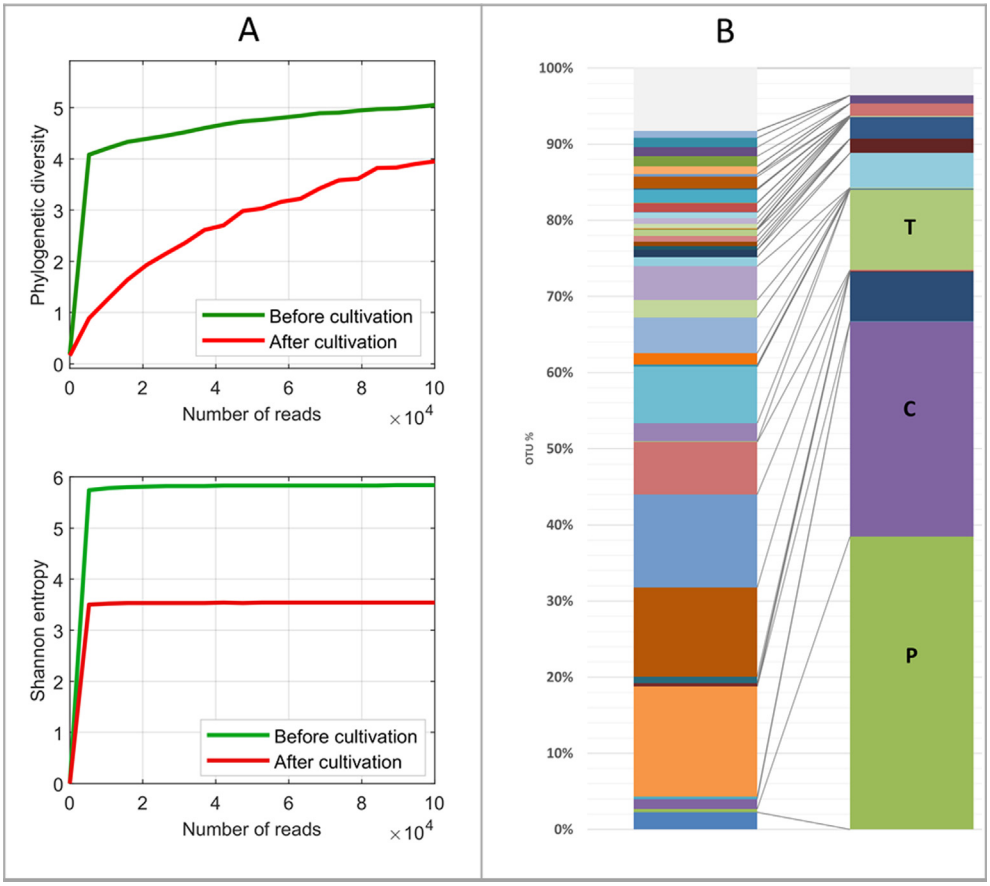

Fig. 6. Changes in the microbial assemblage upon gas fermentation at 15 bar. A: Changes in Phylogenetic diversity and Shannon entropy. B: Changes in community composition at Family level towards a few families known to comprise acetogenic anaerobic taxa that are well-known for the observed metabolic conversions and products. P, Pseudomonadaceae, C, Clostridiaceae, T, Tissierellaceae. For details on Families covered, see Supplementary Figure S2.

$$0.1359CO_2 + 0.5H_2 + 0.114HCO_3^- + 0.0073NH_4^+ \rightarrow 0.1067CH_3COO^- + 0.0073C_5H_7O_2N + 0.3859H_2O \quad (5)$$

Since the ammonium ion is the nitrogen source for the biomass synthesis, the concentration should drop in the fermentation medium, but contradictorily the concentration increased, which elucidates that hydrolysis processes of the remaining organics could be the reason. However, the calculated biomass growth associated with homoacetogenesis is thirty times magnitude lower than the measured VS concentration; therefore, the changes in VS/TS could be a stochastic variation and challenging to correlate with the hydrolysis process. Future studies could investigate the association with hydrolysis, VS and ammonium release in detail.

3.4. Microbiome analysis

In order to assess the changes in microbial community structure and complexity, we performed 16S rRNA gene amplicon sequencing of the original raw sludge sample (before the pretreatment) used as inoculum for all seven batch cultivations at different pressures and the final microbial assemblage of the best performing batch culture at 15 bar. Fig. 6 clearly shows both the reduction of community complexity by means of a reduced phylogenetic diversity and Shannon entropy (Fig. 6A) and the overall change in community composition at family level (Fig. 6B). While the inoculum featured a multitude of families with significant shares in the overall microbial assemblage, the final consortium was with approx. 65 % dominated by *Pseudomonadaceae* and *Clostridiaceae*, while members of the *Tissierellaceae, Porphyromonadaceae*, *Erysipelotrichaceae*, *Peptostreptococcaceae*, *Eubacteriaceae*, and *Ruminococcaceae* held distinguishable shares within the remaining 35 % (Fig. 6B). Species within the two dominating families, *Pseudomonadaceae* and *Clostridiaceae,* were annotated as unknown genera of *Pseudomonadaceae*-2 and or unknown species of *Natronincola-Anaerovirgula*, respectively. Both families and predicted genera/species comprise known, spore-forming anaerobes that play central roles in biogas production and the formation of VFAs (Buettner et al., 2019). In particular, among the Clostridiaceae, many species are capable of fixating carbon dioxide in the presence of H_2 as the energy source using the WLP and performing acetogenesis.

4. Conclusions

To our knowledge, this study represents the first attempt to systematically assess the effect of highly elevated H_2 headspace pressure on mixed culture homoacetogenesis, demonstrating a remarkable impact on gas uptake rate and VFAs synthesis. The gas uptake rate and the amount of synthesized fermentation products increased with increasing headspace pressure from 1 to 15 bar by 250 %, while higher pressures of 20 and 25 bar had the opposite effect. At 15 bar, an optimum H_2 gas uptake rate of 6.22 mol $h^{-1}L^{-1}$ and the highest concentration of VFAs (3.55 g/L) were determined. Though the consumed amount of H_2 was directly proportional to the elevation in pressure, the reduction in gas uptake rate and product synthesis at pressures higher than 15 bar suggests that the microorganisms were inhibited by elevated pressure (>15 bar). The fermentation medium turned out more alkaline throughout the experiments (pH > 9.3). Higher buffer capacity and higher fermentation medium pH let the bicarbonate to behave as an acid. Therefore, the consumption of bicarbonate increased the pH.

The primary fermentation product was acetate (90 %) in all batches. However, for pressures above 15 bar, the presence of C_3-C_5 acids were enhanced but the acetic acid is still the major product. In addition to C_3-C_5 acids, limited ethanol production was observed at 25 bar. Microbial consortium analysis revealed a significant reduction in the microbial assemblage's complexity obtained through cultivation at 15 bar, with members of the *Pseudomonadaceae* and *Clostridiaceae*, families well-known to include many anaerobic acetogens, representing the majority of OTUs determined by 16S rRNA gene metabarcoding. These observations also provide evidence that elevation in H_2 headspace pressure impacts fermentation metabolic pathways. The results show that 15 bar is the optimum headspace pressure for the used mixed culture fermentation inoculum (Knardalstrand municipal wastewater treatment plant's anaerobic sludge) to accomplish the highest gas uptake rate and enhanced VFAs production. Future research will examine the impact of pH and provide a more detailed analysis of the microbial community's metabolic potential to elucidate these initial findings further.

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CRediT authorship contribution statement

Vasan Sivalingam: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. **Tone Haugen:** . **Alexander Wentzel:** Investigation, Writing – review & editing. **Carlos Dinamarca:** Conceptualization, Methodology, Investigation, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cesx.2021.100118.

References

Abubackar, H.N., Veiga, M.C., Kennes, C., 2011. Biological conversion of carbon monoxide: Rich syngas or waste gases to bioethanol. Biofuels, Bioprod. Biorefin. 5 (1), 93–114. https://doi.org/10.1002/bbb.256.

Angelidaki, I., Karakashev, D., Batstone, D.J., Plugge, C.M., Stams, A.J.M., 2011. Chapter sixteen—Biomethanation and Its Potential. In: Rosenzweig, A.C., Ragsdale, S.W. (Eds.), Methods in Methane Metabolism, Part A, Vol. 494. Academic Press, pp. 327–351. https://doi.org/10.1016/B978-0-12-385112-3.00016-0.

Anukam, A., Mohammadi, A., Naqvi, M., Granström, K., 2019. A Review of the Chemistry of Anaerobic Digestion: Methods of Accelerating and Optimizing Process Efficiency. Processes 7 (8), 504. https://doi.org/10.3390/pr7080504.

APHA (1995). *Standard methods for the examination of water and wastewater*. American Public Health Association, American Water Works Association and Water Environment Federation. Editors: Eaton, A.; Clesceri L., Greenberg.

Bertsch, J., Müller, V., 2015. Bioenergetic constraints for conversion of syngas to biofuels in acetogenic bacteria. Biotechnol. Biofuels 8 (1), 210. https://doi.org/10.1186/s13068-015-0393-x.

Mohammadi, M., Najafpour, G.D., Younesi, H., Lahijani, P., Uzir, M.H., Mohamed, A. R., 2011. Bioconversion of synthesis gas to second generation biofuels: A review. Renew. Sustain. Energy Rev. 15 (9), 4255–4273. https://doi.org/10.1016/j.rser.2011.07.124.

Geppert, F., Liu, D., van Eerten-Jansen, M., Weidner, E., Buisman, C., ter Heijne, A., 2016. Bioelectrochemical Power-to-Gas: State of the Art and Future Perspectives. Trends Biotechnol. 34 (11), 879–894. https://doi.org/10.1016/j.tibtech.2016.08.010.

Buettner, C., von Bergen, M., Jehmlich, N., Noll, M., 2019. Pseudomonas spp. Are key players in agricultural biogas substrate degradation. Sci. Rep. 9 (1), 12871. https://doi.org/10.1038/s41598-019-49313-8.

Conrad, R., Wetter, B., 1990. Influence of temperature on energetics of hydrogen metabolism in homoacetogenic, methanogenic, and other anaerobic bacteria. Arch. Microbiol. 155 (1), 94–98. https://doi.org/10.1007/BF00291281.

Cuff, G., Nelting, K., Trautmann, N., Mohammad-pajooh, E., 2020. Production and upgrading of biogas through controlled hydrogen injection for renewable energy storage. Bioresource Technology Reports 9, 100373. https://doi.org/10.1016/j.biteb.2019.100373.

Daniell, J., Köpke, M., Simpson, S., 2012. Commercial Biomass Syngas Fermentation. *Energies* 5 (12), 5372–5417. https://doi.org/10.3390/en5125372.

Diekert, G., Wohlfarth, G., 1994. Metabolism of homoacetogens. Antonie Van Leeuwenhoek 66 (1-3), 209–221.

Dinamarca, C., Bakke, R., 2009. Apparent hydrogen consumption in acid reactors: Observations and implications. Water Sci. Technol.: J. Int. Assoc. Water Pollut. Res. 59 (7), 1441–1447. https://doi.org/10.2166/wst.2009.135.

Dinamarca, C., Ganan, M., Liu, J., & Bakke, R., 2011. H2 consumption by anaerobic non-methanogenic mixed cultures. Water Sci. Technol. : A J. Int. Assoc. Water Pollut. Res. https://doi.org/10.2166/wst.2011.214.

Dodds, W.K., Dodds, P.W.K., 2002. Freshwater Ecology: Concepts and Environmental Applications. Elsevier Sci. Technol. http://ebookcentralproquest.com/lib/ucsn-ebooks/detail.action?docID=294137.

Drake, H.L., 2012. Acetogenesis. Springer Science & Business Media.

Fernández-Naveira, Á., Veiga, M.C., Kennes, C., 2017. H-B-E (hexanol-butanol-ethanol) fermentation for the production of higher alcohols from syngas/waste gas. J. Chem. Technol. Biotechnol. 92 (4), 712–731. https://doi.org/10.1002/jctb.2017.92.issue-410.1002/jctb.5194.

Gardner, R.D., Lohman, E., Gerlach, R., Cooksey, K.E., Peyton, B.M., 2013. Comparison of CO2 and bicarbonate as inorganic carbon sources for triacylglycerol and starch accumulation in Chlamydomonas reinhardtii. Biotechnol. Bioeng. 110 (1), 87–96. https://doi.org/10.1002/bit.24592.

Grimalt-Alemany, A., Łężyk, M., Lange, L., Skiadas, I.V., Gavala, H.N., 2018. Enrichment of syngas-converting mixed microbial consortia for ethanol production and thermodynamics-based design of enrichment strategies. Biotechnol. Biofuels 11 (1), Scopus. https://doi.org/10.1186/s13068-018-1189-6.

Hu, P., Bowen, S.H., Lewis, R.S., 2011. A thermodynamic analysis of electron production during syngas fermentation. Bioresour. Technol. 102 (17), 8071–8076. https://doi.org/10.1016/j.biortech.2011.05.080.

Kantzow, C., Weuster-Botz, D., 2016. Effects of hydrogen partial pressure on autotrophic growth and product formation of Acetobacterium woodii. Bioprocess Biosyst. Eng. 39 (8), 1325–1330. https://doi.org/10.1007/s00449-016-1600-2.

Kundiyana, D.K., Wilkins, M.R., Maddipati, P., Huhnke, R.L., 2011. Effect of temperature, pH and buffer presence on ethanol production from synthesis gas by "Clostridium ragsdalei". Bioresour. Technol. 102 (10), 5794–5799. https://doi.org/10.1016/j.biortech.2011.02.032.

Liew, F., Martin, M.E., Tappel, R.C., Heijstra, B.D., Mihalcea, C., Köpke, M., 2016. Gas Fermentation—A Flexible Platform for Commercial Scale Production of Low-Carbon-Fuels and Chemicals from Waste and Renewable Feedstocks. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.00694.

Mota, M.J., Lopes, R.P., Delgadillo, I., Saraiva, J.A., 2013. Microorganisms under high pressure—Adaptation, growth and biotechnological potential. Biotechnol. Adv. 31 (8), 1426–1434. https://doi.org/10.1016/j.biotechadv.2013.06.007.

Mulat, D.G., Mosbæk, F., Ward, A.J., Polag, D., Greule, M., Keppler, F., Nielsen, J.L., Feilberg, A., 2017. Exogenous addition of H2 for an in situ biogas upgrading through biological reduction of carbon dioxide into methane. Waste Manage. 68, 146–156. https://doi.org/10.1016/j.wasman.2017.05.054.

Oswald, F., Stoll, I.K., Zwick, M., Herbig, S., Sauer, J., Boukis, N., Neumann, A., 2018. Formic Acid Formation by Clostridium ljungdahlii at Elevated Pressures of Carbon Dioxide and Hydrogen. Front. Bioeng. Biotechnol. 6, 6. https://doi.org/10.3389/fbioe.2018.00006.

Pereira, F. M. R., Alves, M., & Sousa, D. (2013). *Effect of pH and pressure on syngas fermentation by anaerobic mixed cultures*. 1–4.

Phillips, J.R., Huhnke, R.L., Atiyeh, H.K., 2017. Syngas Fermentation: A Microbial Conversion Process of Gaseous Substrates to Various Products. Fermentation 3 (2), 28. https://doi.org/10.3390/fermentation3020028.

Rittmann, B.E., McCarty, P.L., 2020. Environmental Biotechnology: Principles and Applications. Second Edition, McGraw-Hill Education.

Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. Int. J. Hydrogen Energy 38 (30), 13172–13191. https://doi.org/10.1016/j.ijhydene.2013.07.122.

Schuchmann, K., Müller, V., 2014. Autotrophy at the thermodynamic limit of life: A model for energy conservation in acetogenic bacteria. Nat. Rev. Microbiol. 12 (12), 809–821.

Shen, S., Wang, G., Zhang, M., Tang, Y., Gu, Y., Jiang, W., Wang, Y., Zhuang, Y., 2020. Effect of temperature and surfactant on biomass growth and higher-alcohol production during syngas fermentation by Clostridium carboxidivorans P7. Bioresources and Bioprocessing 7 (1), 56. https://doi.org/10.1186/s40643-020-00344-4.

Sivalingam, V., Ahmadi, V., Babafemi, O., Dinamarca, C., 2021. Integrating Syngas Fermentation into a Single-Cell Microbial Electrosynthesis (MES) Reactor. Catalysts 11 (1), 40. https://doi.org/10.3390/catal11010040.

Sivalingam, V., Dinamarca, C., 2021. High Pressure Moving Bed Biofilm Reactor for Syngas Fermentation. Chem. Eng. Trans. 86, 1483–1488. https://doi.org/10.3303/CET2186248.

Stoll, I.K., Boukis, N., Sauer, J., 2019. Syngas Fermentation at Elevated Pressure—Experimental Results. In: European Biomass Conference and Exhibition Proceedings. https://doi.org/10.5071/27thEUBCE2019-3CV.3.4.

Stoll, I., Herbig, S., Zwick, M., Boukis, N., Sauer, J., Neumann, A., Oswald, F., 2018. Fermentation of H_2 and CO_2 with Clostridium ljungdahlii at Elevated Process Pressure – First Experimental Results. Chem. Eng. Trans. 64, 151–156. https://doi.org/10.3303/CET1864026.

Tchobanoglous, G., Burton, F.L., Stensel, H.D., 2014. & Metcalf & Eddy (Boston). Wastewater engineering, Treatment and resource recovery. McGraw-Hill Higher Education.

Torri, C., Pambieri, G., Gualandi, C., Piraccini, M., Rombolà, A.G., Fabbri, D., 2020. Evaluation of the potential performance of hyphenated pyrolysis-anaerobic digestion (Py-AD) process for carbon negative fuels from woody biomass. Renew. Energy 148, 1190–1199. https://doi.org/10.1016/j.renene.2019.10.025.

Trček, J., Mira, N.P., Jarboe, L.R., 2015. Adaptation and tolerance of bacteria against acetic acid. Appl. Microbiol. Biotechnol. 99 (15), 6215–6229. https://doi.org/10.1007/s00253-015-6762-3.

Van Hecke, W., Bockrath, R., De Wever, H., 2019. Effects of moderately elevated pressure on gas fermentation processes. Bioresour. Technol. 293, 122129. https://doi.org/10.1016/j.biortech.2019.122129.

Wilkins, M.R., Atiyeh, H.K., 2011. Microbial production of ethanol from carbon monoxide. Curr. Opin. Biotechnol. 22 (3), 326–330. https://doi.org/10.1016/j.copbio.2011.03.005.

Yasin, M., Cha, M., Chang, I.S., Atiyeh, H.K., Munasinghe, P., Khanal, S.K., 2019. In: Biofuels: Alternative Feedstocks and Conversion Processes for the Production of Liquid and Gaseous Biofuels. Elsevier, pp. 301–327. https://doi.org/10.1016/B978-0-12-816856-1.00013-0.

Yenigün, O., Demirel, B., 2013. Ammonia inhibition in anaerobic digestion: A review. Process Biochem. 48 (5), 901–911. https://doi.org/10.1016/j.procbio.2013.04.012.