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Simultaneous hydrogen production and consumption in Anaerobic mixed culture fermentation

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Abstract
The aim of the present study is to investigate the relevance of homoacetogenic H_2 consumption on the bio-hydrogen yield and products distribution in mixed culture fermentation. A hybrid anaerobic reactor was operated for 93 days with variable pH and organic loads between 8-16 g glucose/L·d for this purpose. High initial H_2 yield decreased gradually to an equivalent of 0.02-0.4 mol H_2/mol glucose consumed. The distribution of the dissolved organic products was influenced strongly by reactor pH, while the overall H_2 yield was not. Low H_2 yield is attributed mainly to homoacetogenesis at pH > 4.6 and to reduced products formation at pH < 4.6. Simultaneous hydrogen production and consumption occurred and at least 22 % of the produced molecular hydrogen, mainly from butyrate fermentation, was used for the reduction of CO_2 to acetate.

Keywords: Fermentation; Homoacetogenesis; Hydrogen production; Hydrogen consumption; Products distribution.

1. Introduction

The use of mixed cultures in biotechnology offer several advantages compared to the use of pure cultures, such as: higher product yields and growth rates, stable culture (by stable associations between microorganisms), better substrate utilization and diverse metabolic capabilities [1]. Mixed cultures are widely applied for the treatment of wastewater and organic solid waste. The energy output as methane from anaerobic processes is advantageous compared to the energy requirement of aerobic treatment. By inhibiting methanogenic bacteria a variety of organic acids (formate, acetate, lactate, propionate, butyrate, valerate) and solvents (ethanol, butanol, acetone) can also be produced through anaerobic fermentation, products that are useful as industrial substrates for various processes, including microbial electrohydrogenesis. Identification of criteria to control product formation is required to better exploit the biotechnological potential in mixed culture fermentation [2].

Mixed cultures have also been applied for direct hydrogen production by anaerobic fermentation. Several advantages compared with other methods to produce hydrogen are described and has been studied for different conditions and reactor designs [3]. Hydrogen yield, however, often do not exceed the equivalent of two mol H_2/mol glucose, even though theoretically glucose can provide 2 or 4 mol hydrogen per mol glucose consumed for facultative anaerobes and strictly anaerobes, respectively [4]. Several reviews [5, 6] have mentioned that due to "inefficient metabolic pathways" the viability of macro-scale production of hydrogen is limited. Dinamarca and Bakke [7] attributed low hydrogen yields to the combined effects of

reduced products formation, molecular hydrogen consumption by homoacetogenic bacteria, and to the hydrogen equivalents consumption for the reduction of pyruvate and biomass formation.

Figure 1 illustrates the catabolic pathways involved in glucose fermentation in mixed cultures. Glucose oxidizes to pyruvate by the reduction of NAD^+ to NADH. For the process to continue, in absence of other electron acceptors like oxygen, nitrate or sulfate, NAD^+ needs to be regenerated. Strictly anaerobes (Figure 1A) convert pyruvate to acetyl-CoA by reducing ferrodoxin (Fd), with the action of pyruvate ferrodoxin oxidoreductase, which is then oxidized by a hydrogenase that in turn regenerates oxidized ferrodoxin (Fd_{ox}) and molecular hydrogen (H_2).

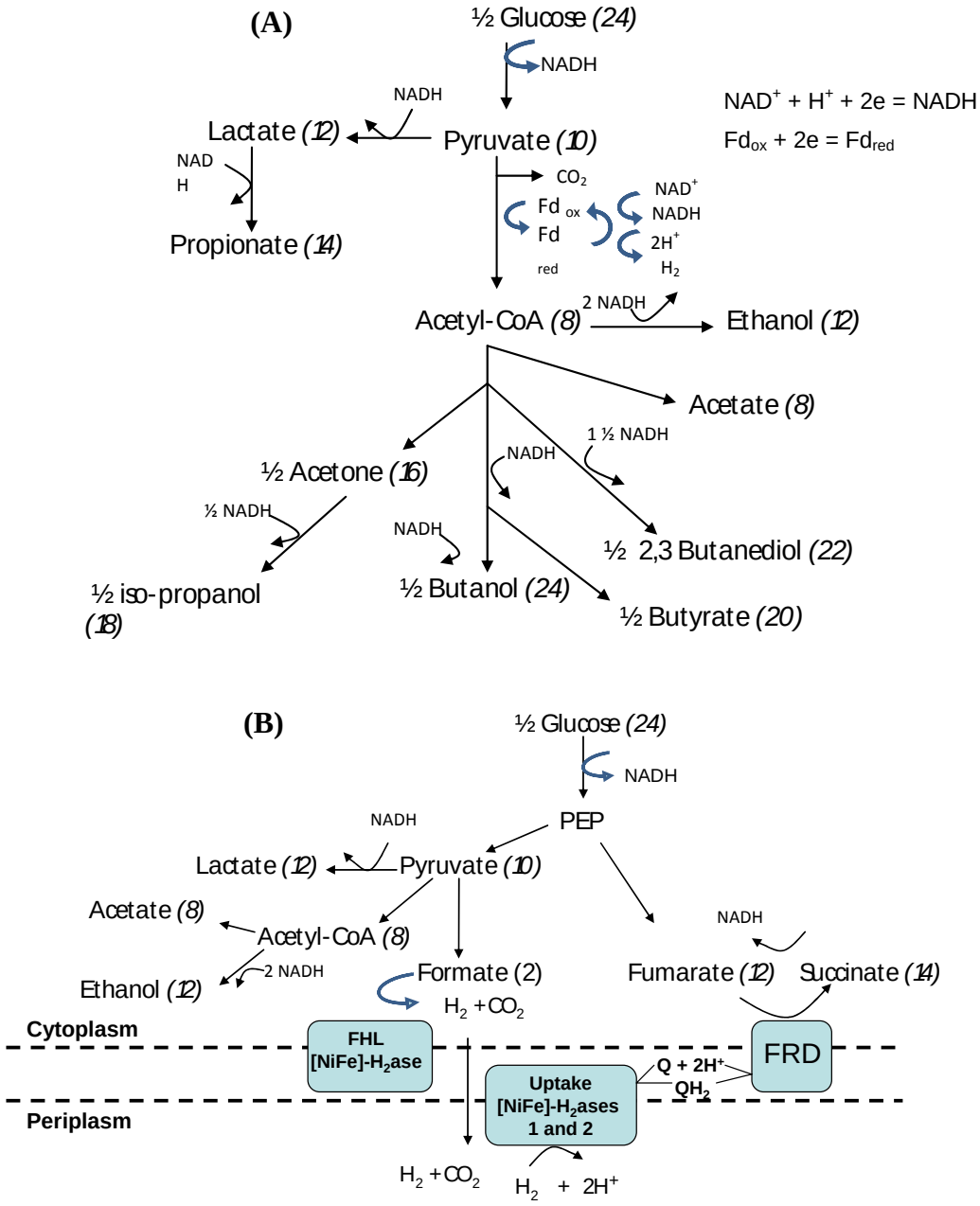

Figure 1. Expected metabolic pathways in: (A) Anaerobic fermentation by strictly anaerobes. Adapted from Evans and Furlong [8] and Lee et al. [9]. (B) Facultative anaerobic fermentation. Adapted from Mathews and Wang, [10]; Murarka, [11]. Numbers in parenthesis indicate degree of reduction per mol of compound. Abbreviations: FHL = formate hydrogen lyase; FRD = fumarate reductase, PEP = phosphoenol-pyruvate; Q = quinine pool; QH2 = quinol

The maximum yield of hydrogen, 4 mol H_2/mol glucose, is achieved when acetate is the sole final organic product. A yield of 2 mol H_2/mol glucose is produced with butyrate as end-product. The formation of lactate, propionate and alcohols serve as electron sinks, for the regeneration of NAD^+, consequently lowering H_2 yields. In facultative anaerobes (Figure 1B) pyruvate is broken down to formate and Acetyl-CoA by pyruvate formate-lyase. Acetyl-CoA is further transformed to acetate and ethanol, or lactate, depending on the environmental conditions [12]. Formate cleavage is the dominant mechanism for hydrogen generation in facultative anaerobes [13]. The fate of pyruvate is controlled by the catalytic capabilities available and the redox balance of the cell.

The experimental study presented here investigate the relevance of homoacetogenic bacteria for the overall H_2 yield and the general effects on product distribution under various continuous flow operation conditions, in mixed culture fermentation. All relevant biochemical pathways are considered. The effects of culture history and pH on the product distribution are also evaluated. The general intention of this research is to contribute in the identification of criteria to control product formation, to better exploit the biotechnological potential in mixed culture fermentation.

2. Materials and methods

2.1 Sludge inoculum

The inoculum came from an anaerobic digester, AD, treating primary sludge at the domestic wastewater treatment plant, Porsgrunn, Norway. The mixed AD operates semi-continuously (fed 4 times per day) at 40 ºC and 12 days hydraulic retention time, HRT. The sludge retrieved from the AD effluent was first sieved at 500 µm and then heat treated at 104 ºC for 12 hours to kill non-spore forming organisms, such as methanogens. The inoculum was kept at the experimental temperature (35 °C) for two days after the heat treatment procedure, prior to the start-up of the experiment.

2.2 Reactors design and start up

The experiment was run in an up flow hybrid anaerobic reactor as shown in Figure 2. The reactor was built from an acrylic glass tube with an effective volume of 780 mL. One third of the reactor volume was filled with expanded clay aggregates (4-10 mm "Filtralite") as fixed biofilm carriers to increase reactor biomass retention. The bottom section of the reactor was mixed with a magnetic stirrer. A glass dome in the upper part of the reactor was used as a gas trap to avoid gas leakage. Outlet liquid and gas were separated outside the reactor. The reactor was kept at 35 °C in an incubator.

The reactor was completely filled with heat-treated conditioned sludge at the start up. Argon was then flushed through the reactor to ensure anaerobic conditions, and about 150 mL of synthetic feed (described below) was pumped in for an initial batch operation. Continuous flow operation at 30 hours HRT started after the observation of a strong gas production. Effluent liquid and gas flow was recorded daily as liquid and gas samples were taken. The organic load was changed during the experiment as shown in Table 1.

2.3 Synthetic media

The synthetic media was based on D-glucose (10-20 g/L) prepared from the following stock solutions in g/L: A) NH_4Cl = 100; NaCl = 10; $MgCl_2 \cdot 6H_2O$ = 10; $CaCl_2 \cdot 2H_2O$ = 5. B) K_2HPO_4 = 300. C) $MnSO_4 \cdot H_2O$ = 0.04; $FeSO_4 \cdot 7H_2O$ = 2.7; $CuSO_4 \cdot 5H_2O$ = 0.055, $NiCl_2 \cdot 6H_2O$ = 0.1; $ZnSO_4 \cdot 7H_2O$ = 0.088; $CoCl_2 \cdot 6H_2O$ = 0.05; H_3BO_3 = 0.05. D) A 10 times concentrated vitamin solution described by Wolin et al. [14]. Glucose and 10, 2, 2, 1 mL/L of solutions A, B, C and D, respectively, were dissolved in distilled water. The mixed feed solution was autoclaved at 121 ºC for 90 minutes. After autoclaving, a sterilized $NaHCO_3$ buffer solution was added to achieve a final concentration of 3.5 g/L of buffer in the feed. Additional K_2HPO_4 was also included, at variable concentrations, to the feed bottle from day 60, to gradually increase the reactor pH. 6 g/L of K_2HPO_4 was used to achieve a pH of 5.3 in the reactor outlet.

Table 1. Organic loads applied as synthetic feed glucose concentrations

Time from start-up (days)	Glucose ($kg/m^3 \cdot d$)
0-51	8
52-64	12
65-80	16
81-93	12

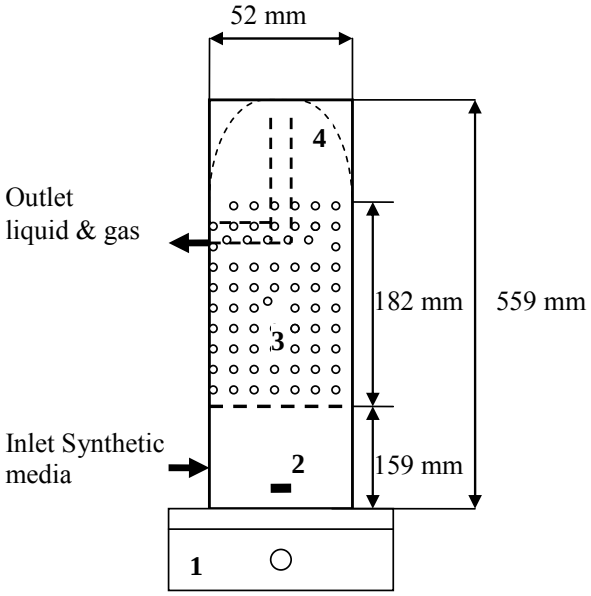

Figure 2. Upflow hybrid anaerobic reactor. 1- Magnetic stirrer, 2- Magnetic bar, 3- Expanded clay biofilm medium, 4- Glass dome gas trap

2.4 Analytical methods

Filtered outlet samples (0.45 μm) were analyzed for volatile fatty acids (VFAs), ethanol, chemical oxygen demand (COD) and glucose concentration. Gas composition (% v/v) and volume (mL) were also measured. VFAs (lactate, formate, acetate, propionate, butyrate, valerate) were analyzed with a Dionex ICS-3000 reagent-free ion chromatograph (IC) system, equipped with an IonPac AS16 4 mm column. Ethanol was analyzed by gas chromatography (Hewlett Packard 6890) with a flame ionization detector and a capillary column (FFAP 30 m, inner diameter 0.250 mm, film 0.25 μm). The oven was programmed to go from 80 ºC, hold for one minute, to 180 ºC at a rate of 30 ºC/min, and then to 230 ºC at a rate of 100 ºC/min. The carrier gas used was helium at 24 mL/min. The injector and detector temperatures were set to 200 and 250 ºC, respectively. COD was analyzed according to US standard 5220D [15]. Glucose was analyzed using the phenol-sulphuric acid method described by Dubois et al. [16].

Biogas flow was measured with a "milli-gas counter" (Ritter). Gas composition (H_2, CO_2 and CH_4) was quantified by gas chromatography (Hewlett Packard 5890A) equipped with a thermal conductivity detector and two columns connected in parallel: Column 1, CP-Molsieve 5A (10 m x 0.32 mm) and Column 2, CP-PoraBOND Q (50 m x 0.53 mm). The gas carrier was argon at 7 bar pressure. The oven temperature was kept constant at 40 ºC.

3. Results and discussion

3.1 General reactor performance

The overall hydrogen yield, $Y_{H2/G}$, during the first 20 days varied close to 2 mol H_2/mol glucose consumed (Figure 3A). Low hydrogen yield, equivalent to 0.02 to 0.4 mol H_2/mol glucose, was observed after 30 days. During constant feed load operation the yield range narrows to 0.02-0.2 mol H_2/mol glucose, which is equivalent to a 200-20 fold decrease compared to the maximum theoretical hydrogen yield. Effluent glucose concentration was low, with > 98 % consumption, except in transition periods (unintentionally caused by the change to fresh feed) and at the maximum load tested (between days 65 and 80; organic load = 17 kg COD/m^3·d). Biogas composition was approximately 50 % H_2 and 50 % CO_2 during the first 20 days of operation. Gas composition then gradually changed to an average of 24 % H_2 and 76 % CO_2 during the last 50 days (Figure 3B). In the microbial metabolism, as described by Figure 1, the CO_2 and H_2 production is highly coupled. The amounts of H_2 and CO_2 during the first 20 days comply with this, while the observed uncoupled gas production later shows that H_2 or electron-equivalents are also being consumed.

Hydrogen yields lower than theoretically predicted from the oxidized acids concentrations, has been previously reported in UASB reactors by Gavala and collaborators [17] and, Yu and Mu [18]. These

authors observed high acetate and butyrate concentration, low reduced products concentration, and low hydrogen yield, indicating homoacetogenic activity. The coexistence of hydrogen producer and consumers in a continuous flow mixed (non-methanogenic) culture was first suggested by Hussy et al. [19]. They observed a decrease in hydrogen yield if acetate or propionate concentration increased, suggesting hydrogen consumption by homoacetogens and propionate producers. Also Siriwongrungson et al. [20] found H_2 and CO_2 consumption subsequent to butyrate degradation in continuous culture at 55 °C. Even though molecular hydrogen consumption has been recognized as a possible hindrance for sustainable hydrogen production [21-24], simultaneous H_2 production and consumption has not been rigorously confirmed in continuous flow mixed culture reactors.

Implications of hydrogen consumption (molecular hydrogen or equivalents) on the overall hydrogen yield are previously discussed by Dinamarca and Bakke [7]. The formation of reduced products, biomass growth and homoacetogenesis are hinders for sustainable mixed culture hydrogen production. Microbial electrohydrogenesis can become a method to overcome these hiders. If so, the fermentation investigated here, with low hydrogen yields and mainly organic products, may serve as an appropriate pretreatment of complex organic wastes. Such anaerobic fermentation may be useful for the production of organics for a variety of other applications also. The relative effects of each of the bioprocesses involved on the product formation and its correlation with operational parameters, such as pH, HRT, temperature and buffer systems, are not well understood. pH and HRT effects are investigated further here to increase the understanding of product distribution in mixed cultures.

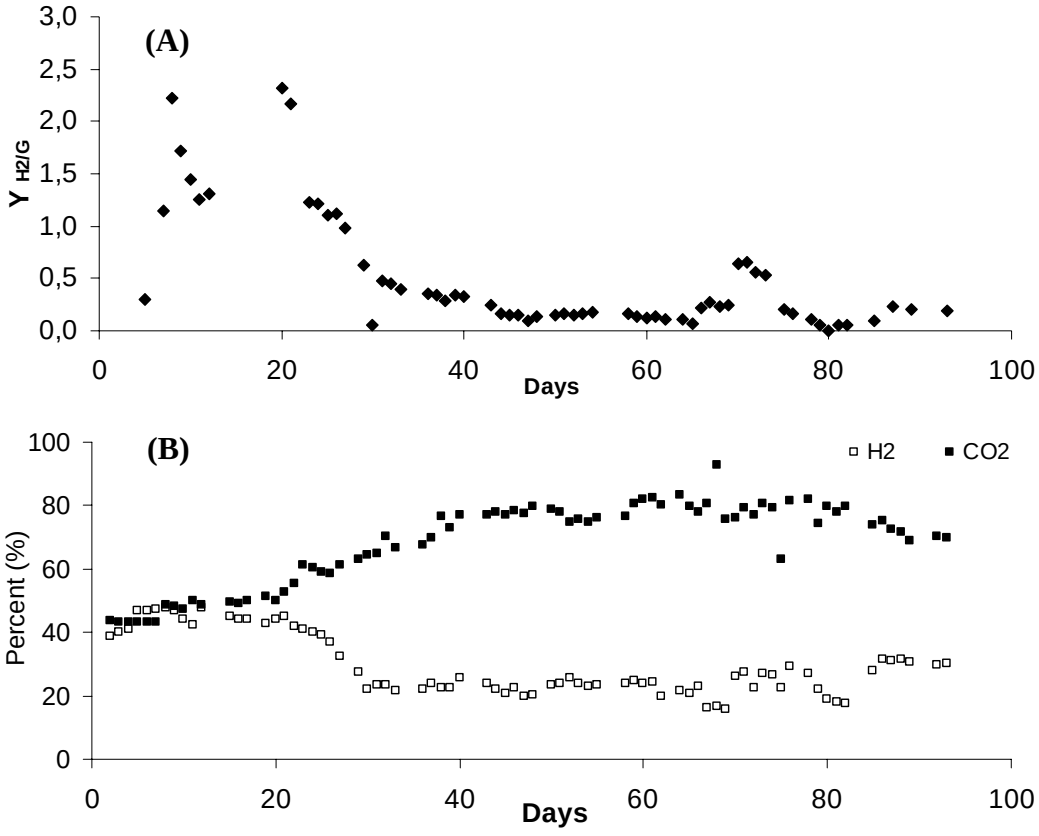

Figure 3. Exhaust gas. (A) hydrogen yield (mol H_2/mol glucose consumed) and; (B) Gas composition (% v/v)

3.2 Homoacetogenic activity

Headspace hydrogen consumption between 4 to 62 mmol H_2/L sludge·d has been previously obtained in batch experiments in our laboratory [25]. The simultaneous hydrogen production and homoacetogenic activity is further investigated by three different approaches based on the recorded observations (η= 57) during 93 days of operation. In each observation glucose consumption and product concentrations (formate, acetate, lactate, propionate, butyrate, valerate, ethanol, H_2 and CO_2) were measured.

3.2.1 Carbon balance

A carbon balance (as mmol C/L), Eq. 1, was calculated for each set of observations.

$$\mathbf{C}_{VFAs}+\mathbf{C}_{Ethanol}+\mathbf{C}_{eq,CO2}+\mathbf{C}_{Biomass}+\mathbf{C}_{unknown}-\mathbf{C}_{Glucose}=0 \quad (1)$$

where:

$\mathbf{C}_{VFAs}$: Equivalent carbon for the measured concentration of volatile fatty acids; $\mathbf{C}_{Ethanol}$: Equivalent carbon for the measured concentration of ethanol; $\mathbf{C}_{eq,CO2}$: Corresponding stoichiometrical equivalent carbon (from heterotrophic metabolism) produced as CO_2 for each measured product; $\mathbf{C}_{Biomass}$: Calculated by assuming that 8 % of the carbon glucose consumed goes to biomass; $\mathbf{C}_{unknown}$: Represents not measure products (as $COD_{unknown} = COD_{dissolved} - COD_{VFAs,e} + COD_{Ethanol,e}$); $\mathbf{C}_{Glucose}$: Measured consumed glucose as carbon equivalent.

Fixation of CO_2, using H_2 as electron acceptor, implies that the total carbon balance, as shown in Eq. 1, will result in values higher than zero. Assuming heterotrophic formation of all the measured products will cause an overestimation of the $\mathbf{C}_{CO2,e}$ equivalent carbon because of autotrophic addition of acetate to the bulk liquid. Additionally by assuming $\mathbf{C}_{unknown}$ equal to zero, to be conservative, we obtain that about 30 % (η= 17) of the observations are higher than zero, implying autotrophic fixation of CO_2.

3.2.2 Correlation between oxidized, reduced products and H_2 production

A positive correlation between formation of oxidized products (acetate, butyrate) and hydrogen production, and a negative correlation between the reduced products (lactate, propionate, ethanol) formation, and H_2 production is expected in hydrogen producing reactors. Electrons from pyruvate oxidation will end up either as H_2, reduced products or biomass, as described previously. Observations contrary to the expected correlations are evidences of autotrophic H_2 consumption. Correlations given as R^2 values for sets of data during periods of increasing or decreasing products concentrations and for all the observations were calculated. The data distributions for all observations are present Table 2, while data from two specified periods are presented in Figure 4, as an example. No correlations (R^2) higher than 0.5 were found for the complete data set (Table 2, n = 57). The analysis for the first 19 days of operation is in accordance with the expected correlations, when just oxidized products were present (Figure 4). Acetate and butyrate show a clear negative (R_1^2 = -0.974) and positive (R_1^2 = +0.905) correlation, respectively, with H_2. This implies that homoacetogenic hydrogen consumption is the main reason for lower H_2 yields (than the theoretical expected from heterotrophic metabolism). The correlation is lost when the analysis is expanded to 30 days (n=19; R_2^2 = -0.0222 and R_2^2 = +0.0287) (Figure 4). This includes the period when hydrogen yield drops. Decreasing hydrogen production did correlate with increasing propionate concentration (R^2 = -0.752) between days 26-33.

Table 2. Correlation between H_2 production against reduced and oxidized products (in mmol electrons/L, n=57)

	ΣOxidized	ΣReduced	Acetate	Butyrate	Lactate
H_2	+0.457	-0.368	+0.132	+0.497	-0.329

The observed correlations show that the process fulfills some of the criteria for a hydrogen-producing reactor in the early stages (Figure 4; butyric acid correlated with H_2), but not completely, and the required correlations are ~zero in the final stages. The correlations also show that butyrate is the main source of the produced hydrogen and acetate is the main sink. Acetate is clearly influenced by the activity of homoacetogens even in the early stages, with a strong negative correlation to H_2, as hydrogen is consumed to produce acetate. Lactate shows no significant relation with decreasing hydrogen production. Reduction in the H_2 concentration when the carbon flow is bypassing the CoA-pathway, shifting from butyric and acetic acid to lactate production is not observable because most of the H_2 was already consumed by homoacetogens. The observed correlation between decreasing hydrogen production with increasing propionate concentration (R^2 = -0.752) between days 26-33 implies that propionate production influence hydrogen yield, and on average 6.6 % of the total electron distribution went to propionate at this stage.

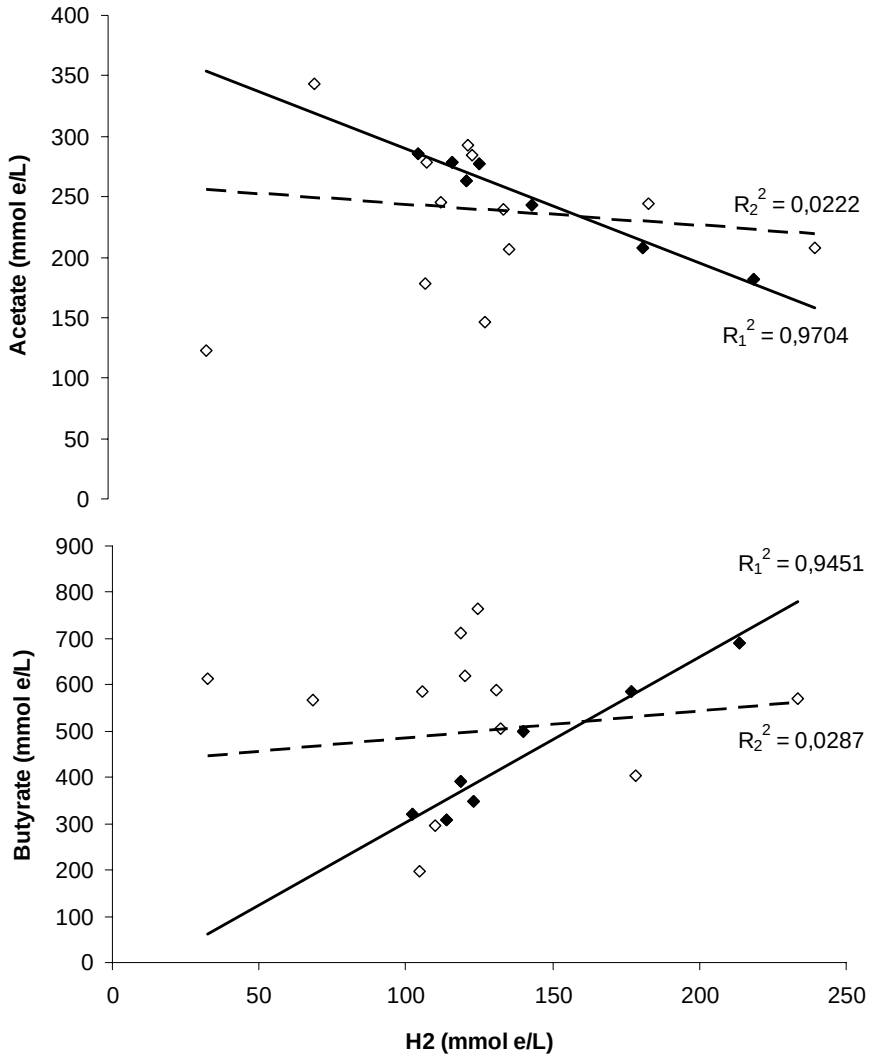

Figure 4. Correlations between acetate and butyrate versus H_2 in mmol electron equivalents for the first 19 days (R_1, n = 9) and the first 30 days (R_2, n = 19) of culture

3.2.3 Graphical observation

A third approach to elucidate the relative importance of hydrogen producers and homoacetogens is by further interpreting the overall products distribution through the whole experiment. In Figure 5 it is show the acids and hydrogen distribution, in terms of mmol electron equivalent by liter (mmol e/L), during the whole study, including perturbations induced by pH adjustments. During the first 20 days the produced hydrogen follows the butyrate formation, but not acetate. Between days 20 and 30 a steep decrease in hydrogen, from 233 to about 5 mmol electron equivalent/L, do not correspond to the profile of oxidized products, neither to an increase in the concentration of reduced products (Figure 5). The total change in the electrons distribution can just be explained partly (< 10 %) by the increase in propionic acid concentration. Furthermore from day 30 the increase observed in the lactic acid concentration did not significantly affect the amount of electrons directed to H_2. Propionate can play a role as an indicator of transitions in such processes, but does not represent a major electron sink in any condition tested.

The overall behavior can be summarized as follows: The hydrogen produced is mainly a product of butyrate fermentation. Butyrate concentration does not, however, always follow hydrogen progression due to varying degree of hydrogen consumption. Acetate is mainly produced autotrophically from H_2 and CO_2 by homoacetogens already from the early stages of the experiment. The shift of carbon flow to lactate fermentation did not produce an observable change in the hydrogen production. H_2 yields lower than the theoretical expected, from the formation of acetic and butyric acids, were observed during the whole study. Low H_2 yield is attributed mainly to homoacetogenesis at pH > 4.6 and to reduced products formation at pH < 4.6.

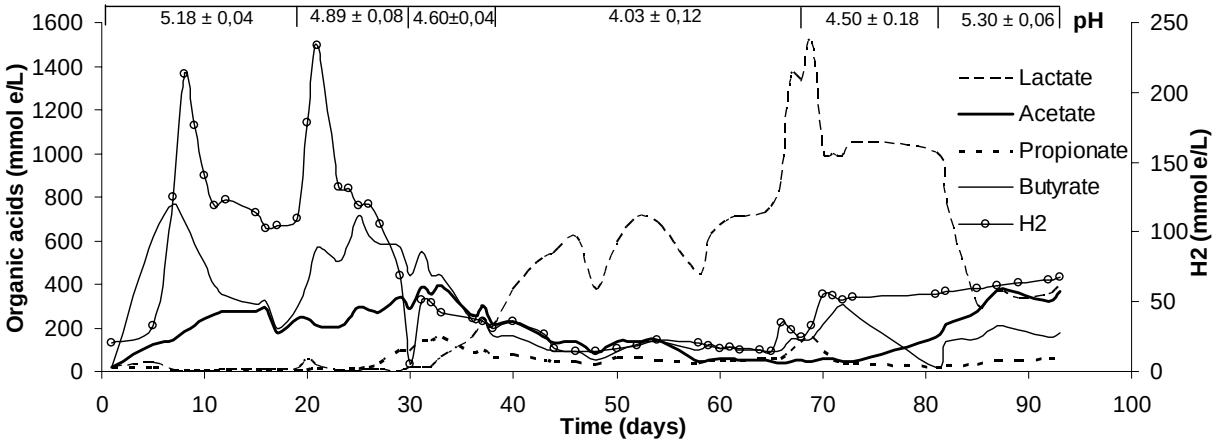

Figure 5. Organic acids distribution in mmol electron equivalents per liter. Formate, valerate, and ethanol are not shown in the figure as they contribute less than 5 %. Hydrogen produced in mmol electrons equivalent and pH values are also given

3.3 Product distribution

Quantification of the impact of hydrogen consumers on the total hydrogen production and hydrogen yield cannot be determined exactly based on the data obtained in this study and literature data. It can, however, be estimated from the first 30 days, when only a small fraction (~3 % of total measured electrons distribution) of reduced acids was observed. The hydrogen measured was 62 % of the total equivalent of the acetate and butyrate measured, and 78 % of the butyrate equivalent. This implies that homoacetogenesis reduced the hydrogen production by at least 22 % during the first 30 days, causing increased acetate yields.

pH also had a clear effect on the products distribution (Figure 6). Low pH (< 4.5) favored lactate fermentation (days 38-68), while pH between 5.0 - 5.5 favored acetate and butyrate fermentation. Propionate had a maximum concentration at pH of 4.6 but was probably more influenced by process transitions than pH since it dropped gradually lower at this pH (days 31-38). Ethanol was not significantly influenced by pH. On average 5 % of the total equivalents were in the form of ethanol (Figure 6).

Several previous studies on glucose fermentation have demonstrated the strong influence of pH on the product distribution [26-29]. These studies show a decrease in butyrate and hydrogen production and an increase in acetate and ethanol with increasing pH. The authors agree that the results are reproducible, reversible and independent of the inoculum [26-29]. The results from the narrower pH range (4.0 – 5.5) tested here show a similar trend. Higher concentration of acetate at higher pH can be explained as a direct result of the activity of homoacetogens, in accordance with Drake et al. [30], proposing that homoacetogenesis is favored at higher pH. This overlaps the reported optimal pH range (4.0-5.7) for hydrogen production in continuous fermentation [27, 31, 32].

The data in Figure 6 also demonstrate that the history or age of the culture has an effect on the product distribution since it is different in the first and final stages of the experiment, even if the pH is similar (pH = 5.3 and pH = 5.2, respectively). These results can probably be explained by a shift in the microbial community, especially by more lactate fermentative bacteria and by a shift from heterotrophic to autotrophic (hydrogen consuming) metabolism.

4. Conclusion

Simultaneous production and consumption of hydrogen is observed in a continuous flow anaerobic hybrid reactor. Homoacetogens consumed at least 22 % of the produced hydrogen. The low hydrogen yield was due to the combined effects of reduced products formation (especially at pH < 4.6), and molecular hydrogen consumption by homoacetogenic bacteria (especially at pH > 4.6). Fermentation can be optimized, with a minimal loss as hydrogen, for the production of organic acids, e.g. as feed for microbial electrohydrogenesis. The product distribution was strongly influenced by pH while the H_2 yield was not in the range tested.

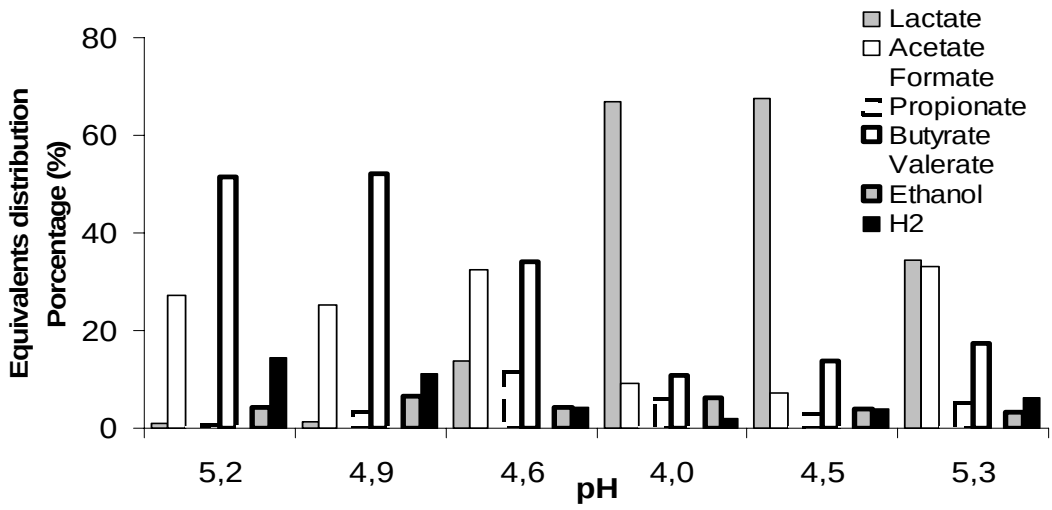

Figure 6. Product distribution as percentage equivalents versus chronological-average effluent pH. Exact pH values and when the observations are made are presented in Figure 5

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