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# Bio-Hydrogen Production from Cheese Whey by Dark Fermentation

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This paper focuses on the production of bio-hydrogen by dark fermentation of de-proteinized (by membrane ultra-filtration) cheese whey, an abundant waste stream of the food industry. This waste stream contains mainly lactose, which was also used as reference substrate in bio-hydrogen production tests. Those tests were conducted in batch by using a thermally treated anaerobic sludge as inoculum of fermentative bacteria. Tests were performed under different buffering conditions and initial substrate concentrations. The maximum hydrogen production yield (2.8-3.6 mol $H_2$ mol lactose$^{-1}$) was obtained when the initial substrate was between 9 and 15 g COD $L^{-1}$, and by adding an appropriate amount of an organic buffer. This yield is slightly lower than the theoretical one of 4 mol $H_2$ mol lactose$^{-1}$ which is expected when both acetic and butyric acids are produced. The biogas contained 47 % of $H_2$ and 53 % of $CO_2$. The residual soluble end products were mainly acetic and butyric acid at a molar ratio of approximately 1:1. Significant formic acid concentrations were also observed, whereas no solventogenesis products were detected.

## 1. Introduction

Hydrogen ($H_2$) is the most promising in the succession of fuel evolution, with the highest energy content per unit weight (142 kJ $g^{-1}$) and is now universally accepted as an environmentally safe, renewable energy resource, alternative to fossil fuels that doesn't contribute to the greenhouse effect. Bio-hydrogen has gained attention as a sustainable and renewable alternative to the conventional methods for $H_2$ production. Among the known biochemical routes to produce $H_2$, dark fermentation (DF) of various kinds of biomass is a promising one (Hallenbeck and Benemann, 2002). Attractive substrates include waste streams containing carbohydrates such as lignocellulosic (Toscano et al., 2013) or starchy material (Markowski et al., 2012), sucrose containing substrates such as sugar beet (Karaoglanoglou et al., 2012) and molasses (Noebauer and Schnitzhofer, 2011), and other di/monosaccharide sugars such as lactose and glucose (Davila-Vazquez et al., 2008). Lactose is an interesting substrate because it is present in by-products from the dairy industry, such as cheese whey, that represents around 85-90 % of the total volume of processed milk and it is a potential substrate for fermentative processes (Prazeres et al., 2012). Cheese whey management has been focused on the development of technical strategies to valorise instead of treating this by-product because of the presence of valuable compounds such as proteins and lactose. Each liter of cheese whey contains about 50 g of lactose and 10 g of proteins with a high nutritional and functional value (Prazeres et al., 2012). Physicochemical treatments, membrane separation processes in particular, are utilized to obtain proteins and lactose concentrates from cheese whey (Prazeres et al., 2012). A strategy to valorise this by-product is to use microfiltration or ultrafiltration to separate proteins as a first step, whereas the effluent, containing mainly lactose, is a excellent substrate for a second step of dark fermentation.
The aim of this work was to study hydrogen production from lactose and ultra-filtered cheese whey (UF-CW) by DF, in batch experiments. Different initial substrate and buffer concentrations were evaluated in terms of their effect of the $H_2$ molar yield (mol $H_2$ mol$^{-1}$ lactose) and in the composition of the end products of the DF process.

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## 2. Materials and methods

Food purity grade lactose and UF-CW (Table 1) from Grana Padano cheese production were used in this study. A total of 25 batch tests were performed under mesophilic conditions (35±0.5 °C) at food to biomass (F/M) values of 0.3-10 g COD g $VS^{-1}$. The hydrogen production was measured by using a commercial laboratory instrument (AMTPS, Bioprocess Control, Sweden). AMPTS (Automatic Methane Potential Test System) is a multi-channel analyzer for studying biogas production from bio-samples, comprising temperature controlled bottle reactors with overhead stirrers, connected to scrubbing solution units (NaOH, 3 M) where $CO_2$ is fixed, whereas hydrogen passes to a sensor chamber, that measure the volumetric production by the principle of liquid displacement. This measuring unit has a resolution of approximately 10 mL. A data acquisition system allows flow-rate data to be recorded continuously.
Each bottle was filled with substrate (lactose or cheese whey), inoculum (2.3 g VS $L^{-1}$), mineral medium (OECD, 2006) (50 mL) and different amounts of an organic buffer (2- (N-morpholino) ethanesulfonic acid monohydrate, MES) (0.045 M, 0.090 M, and 0.135 M). Then deionized water was added to the final working volume (540 mL). The suspension pH was adjusted to approx. 6.2 using HCl 10 M or NaOH 2 M. The headspace was purged with nitrogen gas, then samples were equilibrated at 35 °C and experiments started (duration: 20 – 260 h).
The inoculum was obtained from two anaerobic sludge samples (A, B) collected from a WWTP (Wastewater Treatment Plant) digester located near Milano (IT). To inhibit hydrogen-using microorganisms, such as homoacetogens and methanogens, the sludge was thermically pre-treated (90 °C, 30 min) in order to eliminate all microorganisms except for spore-forming fermenting bacteria (Angenent et al., 2002).
To assess the effectiveness of the pre-treatment, blank bottles were prepared by mixing the pre-treated inoculum and the mineral medium (blank type 1) and pre-treated inoculum, mineral medium and sodium acetate (blank type 2) to evaluate the endogenous hydrogen production (blank type 1) and the possible residual methanogenic activity (blank type 2). Both blank samples showed a negligible biogas production.
Total and Volatile Solids (TS, VS), COD were analysed according to APHA (2005). Biogas composition in the headspace of reactor bottles ($H_2$, $CO_2$ and $CH_4$) was measured at the end of the test by gas chromatography (MICROGC 3000, Agilent Technologies, Germany) using a Molsieve column (Molsieve 10m/PPU 3m Gc, temperature 90 °C and pressure 15 psi) and Argon as gas-carrier. The concentration of fermentation soluble end products was also measured by High Performance Liquid Chromatography, performed using a Polyspher OA KC (300-7.8 mm) Column (Merk®) equipped with a protective precolumn and a UV 210 nm detector. The eluting solution was 0.005 M $H_2SO_4$, the flow rate was 0.4 mL $min^{-1}$, and the column temperature was maintained at 30 °C.

*Table 1: Characteristics of UF-CW*

| Parameter | Unit | Value | Parameter | Unit | Value |
|---|---|---|---|---|---|
| COD | g $L^{-1}$ | 157 | Salinity | g $L^{-1}$ | 13.9 |
| Total organic carbon | g $L^{-1}$ | 42.0 | pH | | 6.2 |
| Volatile solids | g $L^{-1}$ | 135 | Alkalinity | mg $CaCO_3$ $L^{-1}$ | 1,500 |
| $BOD_5$ | g $L^{-1}$ | 114 | Conductivity | µS $cm^{-1}$ | 10,000 |
| $BOD_{30}$ | g $L^{-1}$ | 144 | Phosphate | mg P $L^{-1}$ | 820 |
| BMP | NL $CH_4$ g $COD^{-1}$ | 0.3 | Total phosphorous | mg P $L^{-1}$ | 1,040 |
| Grease | g $L^{-1}$ | 0.59 | Ammonia | mg N $L^{-1}$ | 27.0 |
| Protein | g $L^{-1}$ | 3.44 | Total Kjehldahl Nitrogen | mg N $L^{-1}$ | 735 |
| Lactose | g $L^{-1}$ | 113 | Total nitrogen | mg N $L^{-1}$ | 759 |

## 3. Results and discussion

### 3.1 Effects of substrate type (lactose; cheese whey) on hydrogen production

Table 2 and Figure 1 show the effect of substrates type (lactose; UF-CW) on hydrogen production by dark fermentation. MES concentration was equal to 0.045 M for all tests. From a kinetic point of view, UF-CW and lactose behave differently. Tests performed with UF-CW showed a max volumetric $H_2$ production rate approximately twice faster than that of lactose tests (Table 2). Differently, as for stoichiometry, the $H_2$ molar yield (mol $H_2$ produced form 1 mol of lactose) is comparable, with values of 2.6 mol $H_2$ mol $lactose^{-1}$ at an initial COD of 6 g COD $L^{-1}$ and 2.9-3 mol $H_2$ mol $lactose^{-1}$ at 9 and 12 g COD $L^{-1}$, for both the

substrates. These results are probably related to the presence of some substances that enhanced the microbial conversion rate of lactose to hydrogen in UF-CW, such us metals or macro/micro-nutrients important for the synthesis of enzymes required in the DF process, that contain complex metallo-clusters as active sites, or of proteins and enzymes involved in these metallo-clusters formation (Hallenbeck and Benemann, 2002).

*Table 2: Effects of substrates type on volumetric $H_2$ production rate and on molar yield*

| Substrate | Lactose | UF-CW | Lactose | UF-CW | Lactose | UF-CW |
|---|---|---|---|---|---|---|
| Initial COD (g COD $L^{-1}$) | 6 | 6 | 9 | 9 | 12 | 12 |
| $H_2$ molar yield (mol $H_2$ mol lactose$^{-1}$) | 2.6 | 2.6 | 3.0 | 2.9 | 2.9 | 3 |
| Max volumetric $H_2$ production rate (NmL $H_2$ $h^{-1}$ g $VS^{-1}$) | 25.9 | 43.8 | 21.0 | 43.4 | 32.6 | 57.3 |

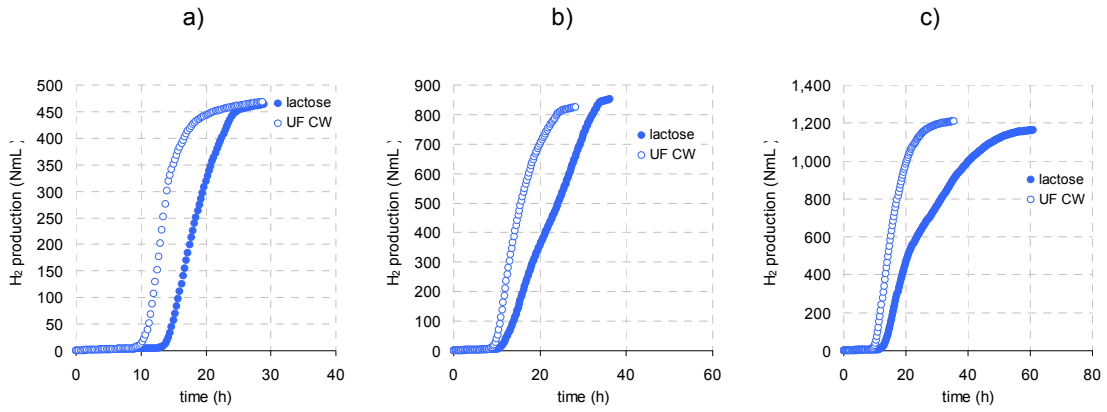


*Figure 1: Cumulative hydrogen production: results related to experiments with initial COD of 6 g COD $L^{-1}$ (a), 9 g COD $L^{-1}$ (b) and 12 g COD $L^{-1}$ (c)*

**3.2 Effects of initial substrate and buffer concentrations on hydrogen production**

Results shown in Figure 2 reveal the influence of initial substrate (COD) and buffer (MES) concentration on the $H_2$ molar yield. Since no significant differences were found between bio-hydrogen production yield from lactose and UF-CW all data are presented in the chart, without differentiating among the substrate. The initial COD concentration played a relevant role in hydrogen production. Below 9 g COD $L^{-1}$ the $H_2$ molar yield was suboptimal, ranging from 0.25 to 2.5 mol $H_2$ mol lactose$^{-1}$. Between 9 and 15 g COD $L^{-1}$, values of 2.8-3.6 mol $H_2$ mol lactose$^{-1}$ were observed, with a composition of the biogas produced of 47 % of $H_2$ and 53 % of $CO_2$.

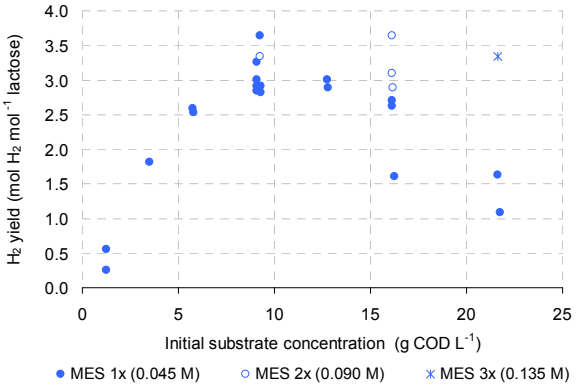


*Figure 2: $H_2$ molar yield as a function of initial substrate (either UF-CW or lactose) concentration and buffer (MES) concentration*

At higher COD concentrations (>15 g COD $L^{-1}$), the acidification effect due to Volatile Fatty Acids (VFAs) formation by hydrogen producing bacteria was more relevant (see section 3.4). As a result, the $H_2$ molar yield shows a clear decreasing trend at the lower MES concentration (0.045 M). By comparison, at higher MES initial concentration, the $H_2$ yield increased by 39 % (MES, 0.090 M) and 146 % (MES, 0.135 M), at initial COD concentrations of 16 and 22 g COD $L^{-1}$, respectively.
Figure 3 shows the dependence of the $H_2$ molar yield from the MES/lactose molar ratio, this parameter indicating the grade of buffering capacity available in each test. These results suggest an optimal MES/lactose molar ratio approximately between 2 and 2.5 mol MES mol lactose$^{-1}$. Under these buffering conditions, the pH at the end of the batch test remained between 5 and 5.5.

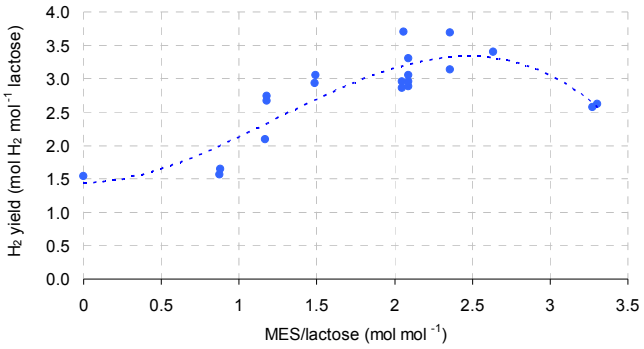


*Figure 3: Effect of the MES to lactose molar ratio (MES/lactose) on $H_2$ production*

The max $H_2$ molar yield observed in our study was between 3.3 and 3.6 mol $H_2$ mol lactose$^{-1}$ at COD ≥ 9 g $L^{-1}$ and at MES/lactose ratio of 2-2.5 mol mol$^{-1}$. By comparison, due to thermodynamic constraints, the maximum stoichiometric yield of 8 mol of $H_2$ can be expected from 1 mol of lactose, when only acetic acid is the end product, whereas a maximum of 4 mol $H_2$ mol$^{-1}$lactose can be achieved when more reduced metabolites, such as ethanol and butyric acid, are also produced (Davila-Vazquez et al., 2008), as observed in this study (see section 3.4). Experimental $H_2$ molar yield data using lactose or cheese whey as the substrate of dark fermentation has been previously reported in literature: values of 2.7-3.6 and 2.1-3, and 2.7-4.1 and 1.8-5.1 were reported by Davila-Vazquez et al. (2008) and Prazeres et al. (2012) for batch and continuous reactors, respectively.

**3.3 Effects of inoculum on hydrogen production**

Figure 4 compares data obtained by using two sludge inocula taken from the same digestor in different period, pretreated under similar conditions (all tests were performed with MES buffer at 0.045 M). These data indicate that higher yields were obtained by using sludge sample B (+57 % on average with respect to sludge sample A), in spite of the fact that they were both collected from the same digester. This finding suggests that the inoculum, besides initial COD and buffer concentration, is a key factor for increasing and optimizing hydrogen production by DF.

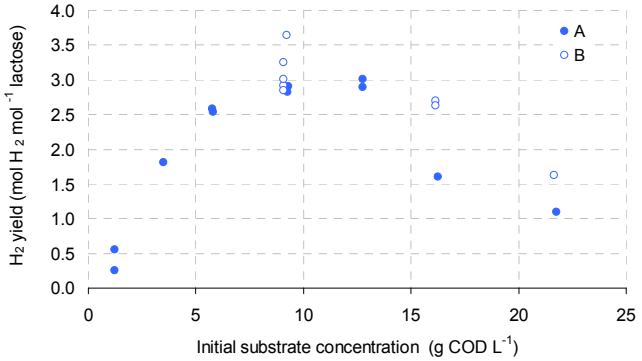


*Figure 4: $H_2$ molar yield as a function of initial substrate (COD) and the seeded sludge sample (all tests were performed with MES buffer at 0.045 M)*

### 3.4 Evaluation of DF soluble end products

Table 3 and Figure 5 show the concentration of residual substrate (lactose) and soluble end products detected at the end of the test. Residual lactose was relevant only in test 7 where the MES/lactose molar ratio was too low for proper buffering of the acidification associated with the fermentation process. pH rapidly declined to 4.8 resulting in an incomplete hydrolysis of lactose and in a very low $H_2$ molar yield (1.1 mol $H_2$ mol lactose$^{-1}$). With regard to soluble end products, acetic acid and butyric acid were the predominant VFAs (41.1±7.9 % and 41.5±5.2 %, respectively), whereas formic acid accounted for 14.6±7.5 % of the molar apportionment between soluble end products. The presence of butyric acid occurs in a hydrogen-saturated system because at hydrogen partial pressure higher than $10^{-4}$ atm metabolic routes deviate from acetate production to other products (Angenent et al., 2004). Moreover, butyric acid in the undissociated form can be inhibitory to the $H_2$ producing microorganisms (Van Ginkel and Logan, 2005). Taking into account its $pK_a$ value (4.81) this can be relevant at pH below 4.8, but this condition never occurred in these experiments. Also, since acidogenesis shifts to solventogenesis (production of acetone, ethanol, propanol or butanol) below this pH value and with undissociated butyric acid higher than 2-30 mM (Davila-Vazquez et al., 2008), solventogenesis products were not detected.

*Table 3: Test conditions and concentration of residual substrate and soluble end products (all tests were performed with MES buffer at 0.045 M)*

| | Test 1 | Test 2 | Test 3 | Test 4 | Test 5 | Test 6 | Test 7 |
|---|---|---|---|---|---|---|---|
| Substrate | Lactose | Lactose | lactose | UF-CW | UF-CW | UF-CW | lactose |
| Initial COD (g $L^{-1}$) | 5.8 | 9.3 | 12.8 | 5.8 | 9.3 | 12.8 | 21.8 |
| MES/lactose (mol $mol^{-1}$) | 3.2 | 2.0 | 1.5 | 3.2 | 2.0 | 1.5 | 0.9 |
| $H_2$ molar yield (mol $mol^{-1}$) | 2.5 | 2.9 | 2.9 | 2.6 | 2.8 | 3.0 | 1.1 |
| Lactose (mM) | Nd | 1.3 | 0.6 | nd | nd | nd | 16.5 |
| Formic acid (mM) | 9.5 | 12.4 | 8.5 | 0.0 | 11.4 | 11.2 | 22.7 |
| Acetic acid (mM) | 20.2 | 26.2 | 34.3 | 21.5 | 24.4 | 31.5 | 32.1 |
| Butyric acid (mM) | 18.6 | 27.4 | 38.6 | 16.3 | 28.2 | 36.5 | 32.9 |

nd = non detected.

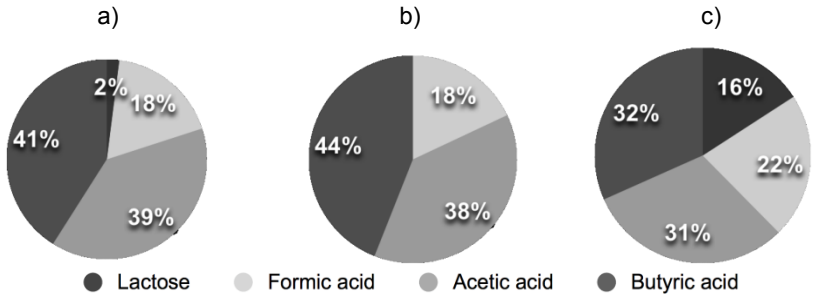


*Figure 5: Molar apportionment between residual substrate and soluble end products: lactose, formic acid, acetic acid and butyric acid: test 2 (a), test 5 (b), test 7 (c)*

## 4. Conclusions

Batch tests of biohydrogen production by dark fermentation of de-proteinized (by membrane ultrafiltration) cheese whey and lactose performed under different buffering conditions and initial substrate concentrations were performed. According to the experimental results, the following conclusions can be drawn:

- $H_2$ production was found to proceed faster with cheese-whey than with lactose, however, similar final $H_2$ molar yields were found for both substrates.

- The initial buffering capacity, obtained by using an organic buffer (MES), was found to strongly affect the $H_2$ yield; an optimal molar ratio of 2 - 2.5 mol MES/mol lactose was found, which allowed to maintain the final pH around 5.5-5; under these conditions yields of 2.5-3.0 mol of $H_2$ per mol of lactose were achieved.
- When using the appropriate buffering capacity, the residual soluble end products were mainly acetic and butyric acid at a molar ratio of approximately 1:1. Significant formic acid concentrations were also observed, whereas no solventogenesis products were detected.
- Inoculum was also found to significantly affect the final $H_2$ molar yield.

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