

# Electron bifurcation mechanism and homoacetogenesis explain products yields in mixed culture anaerobic fermentations

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## ABSTRACT

Anaerobic fermentation of organic wastes using microbial mixed cultures is a promising avenue to treat residues and obtain added-value products. However, the process has some important limitations that prevented so far any industrial application. One of the main issues is that we are not able to predict reliably the product spectrum (i.e. the stoichiometry of the process) because the complex microbial community behaviour is not completely understood. To address this issue, in this work we propose a new metabolic network of glucose fermentation by microbial mixed cultures that incorporates electron bifurcation and homoacetogenesis. Our methodology uses NADH balances to analyse published experimental data and evaluate the new stoichiometry proposed. Our results prove for the first time the inclusion of electron bifurcation in the metabolic network as a better description of the experimental results. Homoacetogenesis has been used to explain the discrepancies between observed and theoretically predicted yields of gaseous H<sub>2</sub> and CO<sub>2</sub> and it appears as the best solution among other options studied. Overall, this work supports the consideration of electron bifurcation as an important biochemical mechanism in microbial mixed cultures fermentations and underlines the importance of considering homoacetogenesis when analysing anaerobic fermentations.

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## 1. Introduction

The recent advances in environmental technologies brought a change of paradigm regarding the organic waste materials. They are now seen as potential renewable sources for a plethora of bioprocesses (Agler et al., 2011; Angenent et al., 2004; Kleerebezem et al., 2015) and the generation of added-value products, from otherwise waste materials, is now a promising avenue to reduce the dependency on fossil fuels, to decrease the amount of untreated pollutants released to the environment and to work towards a more sustainable and circular economy. Among these bioprocess, anaerobic systems are one of the most attractive options. The

absence of O<sub>2</sub> as strong electron acceptor limits the biomass growth and allows the generation of high energy density products that could be used as biofuels or chemical building blocks (Agler et al., 2011). So far, one of the successful examples is the anaerobic digestion, which has become a well-established technology for biogas production. However, the use of biogas as biofuel is limited because its low volumetric energy density and the low price of the natural gas. Consequently, other avenues should be considered for organic wastes valorisation.

Most of the novel initiatives for obtaining higher added-value products from organic wastes involve the use of mixed culture fermentations (MCFs) (Kleerebezem et al., 2015). These are anaerobic processes in which methanogenesis is inhibited and substrates are converted into a mixture of short carboxylates, also known as volatile fatty acids (VFAs; e.g. acetate, propionate or butyrate), alcohols (mainly ethanol), H<sub>2</sub> and CO<sub>2</sub>. VFAs are already valuable products themselves but they also can act as substrates or building blocks for several other bioprocesses producing biofuels, bioplastics (polyhydroxyalkanoate family) or biosolvents. Alternatively, VFAs

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can be used for electricity generation in microbial fuel cells or for H<sub>2</sub> production (Agler et al., 2011; Angenent et al., 2004; Kleerebezem et al., 2015; Kleerebezem and van Loosdrecht, 2007). Moreover, H<sub>2</sub> is a valuable by-product of MCFs because it has potential as biofuel and energy vector (Bundhoo and Mohee, 2016; Hallenbeck and Ghosh, 2009). Conversion of organic wastes into a short carboxylate pool and CO<sub>2</sub> and H<sub>2</sub> is envisioned as one of the bio-refinery platforms: the carboxylate platform (Agler et al., 2011).

The use of mixed culture systems has several advantages: these are systems that do not need sterilization, can be operated continuously and can treat complex substrates owing to their broad functional diversity (Kleerebezem and van Loosdrecht, 2007). However, these processes involve fundamental challenges because mixed cultures are complex and uncharacterized. So far, we are not able to fully understand the mechanisms that control open communities of microorganisms, which makes the design of such processes a difficult task.

In MCFs the absence of an external acceptor of electrons limits the energy available and therefore the fermentative catabolic reactions run close to the thermodynamic equilibrium. This restricts the growth of the microbial population (Carballa et al., 2015; Rodríguez et al., 2009). But it also opens the possibility of directing the process towards those products of interest by modifying some operational conditions. Changes in the operational conditions push the microbial community to adapt to the selective pressures of the system and therefore, the outcome of the process could be controlled (González-Cabaleiro et al., 2015; Kleerebezem and van Loosdrecht, 2007; Rodríguez et al., 2006; Temudo et al., 2007).

Experimentation in MCFs shows that there is a tight relationship between the operational conditions and the products obtained (Fang and Liu, 2002; Horiuchi et al., 2002; Karadag and Puhakka, 2010; Temudo et al., 2007; Yu and Fang, 2003; Zhang et al., 2015; Zoetemeyer et al., 1982). How this relationship works is not clear enough and for this reason, we have not succeeded in directing product formation in MCFs. Studies reported in literature are usually conceived from an experimental point of view and do not analyse the mechanistic causes of the shifts in the product distribution. Only a handful of studies looked to understand the underlying reasons of these product spectrum changes and some included them in mathematical models (González-Cabaleiro et al., 2015; Groeger et al., 2017; Kleerebezem et al., 2008; Mosey, 1983; Rodríguez et al., 2006; Zhang et al., 2013). However, even though the predictions from the successive models have improved, there are differences when compared with the experimental observations. For example, at low pH butyrate is predicted to be the main product in González-Cabaleiro et al. (2015) or Kleerebezem et al. (2008) but experimentally both butyrate and acetate are obtained (Fang and Liu, 2002; Temudo et al., 2007; Zoetemeyer et al., 1982). These discrepancies might be due to the use of an incomplete metabolic network that does not describe accurately the stoichiometry of the process.

In this work, we propose modifications to the most commonly used metabolic networks for glucose in MCFs, with the aim of improving the prediction of experimental results reported in literature. The novel biochemical mechanism of electron bifurcation (EB), fully described elsewhere, was included (Buckel and Thauer, 2013; Herrmann et al., 2008; Li et al., 2008; Peters et al., 2016). We also propose that homoacetogenesis (HA) (Dinamarca et al., 2011) is the most reasonable hypothesis for correctly explaining the gaseous species yields.

## 2. Materials and methods

A metabolic network defines the global stoichiometry of the process as a compilation of the pathways that a single microorganism or a microbial population can catalyse. These pathways are

described with all the intermediate metabolic steps, including the energetic coupling sites and the coenzymes involved (e.g. coenzyme A or NADH). To check if including EB in a metabolic network for glucose fermentation helps improving its prediction capacity, we first constructed a reference metabolic network.

### 2.1. Building the reference metabolic network

The metabolic pathways of the major products observed when glucose is fermented by open microbial communities are included in the reference network (Fig. 1). The products considered in this reference metabolic network are: acetate, ethanol, propionate, lactate and butyrate (Angenent et al., 2004; Fang and Liu, 2002; González-Cabaleiro et al., 2015; Hoelzle et al., 2014; Horiuchi et al., 2002; Lengeler et al., 1999; Mohd-Zaki et al., 2016; Rodríguez et al., 2006; Temudo et al., 2008, 2007; Zhang et al., 2013; Zoetemeyer et al., 1982). All these products derive from a common glycolytic process (glucose conversion to pyruvate) in which the degradation of glucose starts with the *Embden-Meyerhof-Parnas* pathway (EMP) producing two moles of pyruvate per mole of glucose. Two other degradation pathways of glucose can be found in prokaryotes microorganisms: *Entner-Doudoroff* (ED) and *Pentose-Phosphate pathway* (PPP). ED pathway is reported to be related with aerobic environments and to be used to mainly metabolise sugar acids (Peekhaus and Conway, 1998). PPP pathway is mainly used for anabolic purposes (i.e. to obtain the needed metabolites in biomass generation) (Kruger and Von Schaewen, 2003). But the fact that EMP pathway has an ATP yield twice as big as the ED pathway strongly suggests selecting EMP as the glycolysis route in our metabolic network. These reasons, and the fact that in literature EMP for glycolysis is ubiquitous (Buckel and Thauer, 2013; González-Cabaleiro et al., 2015; Hoelzle et al., 2014; Kleerebezem et al., 2008; Mosey, 1983; Rodríguez et al., 2006; Temudo et al., 2009, 2008, 2007; Zhang et al., 2013; Zoetemeyer et al., 1982), lead us to only consider the EMP pathway in the metabolic network.

Pyruvate is a node in the network: the different branches arise from it. The three-carbon compounds, propionate and lactate, are directly yielded from pyruvate. Acetate, ethanol and butyrate (compounds with an even number of carbons) need a first decarboxylation of pyruvate to yield acetyl-CoA, in which an equimolar mixture of H<sub>2</sub> and CO<sub>2</sub> or formate is produced. The production of H<sub>2</sub> and CO<sub>2</sub> versus formate is in a thermodynamic equilibrium ruled only by the pH (González-Cabaleiro et al., 2015; Hoelzle et al., 2014; Rodríguez et al., 2006; Temudo et al., 2007). Direct formate conversion to H<sub>2</sub> and CO<sub>2</sub> is very close to the thermodynamic equilibrium ( $\Delta G^m = -0.3$  kJ/mol formate) and it is catalysed by formate dehydrogenases. For simplicity, and because both options are equivalent in mass and redox terms, we assume that only H<sub>2</sub> and CO<sub>2</sub> are yielded at this step (Batstone et al., 2002).

#### 2.1.1. Electron carriers in the metabolic network

Reduction and oxidation reactions happening in the metabolic network involve several electron carriers (EC). In the proposed network we consider two of them: ferredoxin (Fd<sub>red</sub>/Fd<sub>ox</sub>) and NADH (NADH/NAD<sup>+</sup>). Fd<sub>red</sub>/Fd<sub>ox</sub> is characterized by its low redox potential ( $E^0 \approx -400$  mV and  $E' \approx -500$  mV (Buckel and Thauer, 2013)) and because is the only EC capable of reducing protons to H<sub>2</sub>. It is considered that all the Fd<sub>red</sub> produced will eventually be used to produce H<sub>2</sub> by cytoplasmatic ferredoxin:proton reductases (Ech). Based on this assumption and on the experimental H<sub>2</sub> yields of MCFs, it is widely accepted that Fd<sub>ox</sub> is only reduced in the pyruvate decarboxylation to acetyl-CoA resulting in a maximal theoretical yield of 2 mole of H<sub>2</sub> per mole of glucose consumed (Kleerebezem et al., 2008; Temudo et al., 2007). The couple NADH/NAD<sup>+</sup> has a higher redox potential than Fd<sub>red</sub>/Fd<sub>ox</sub> ( $E^0 = -320$  mV (White et al.,

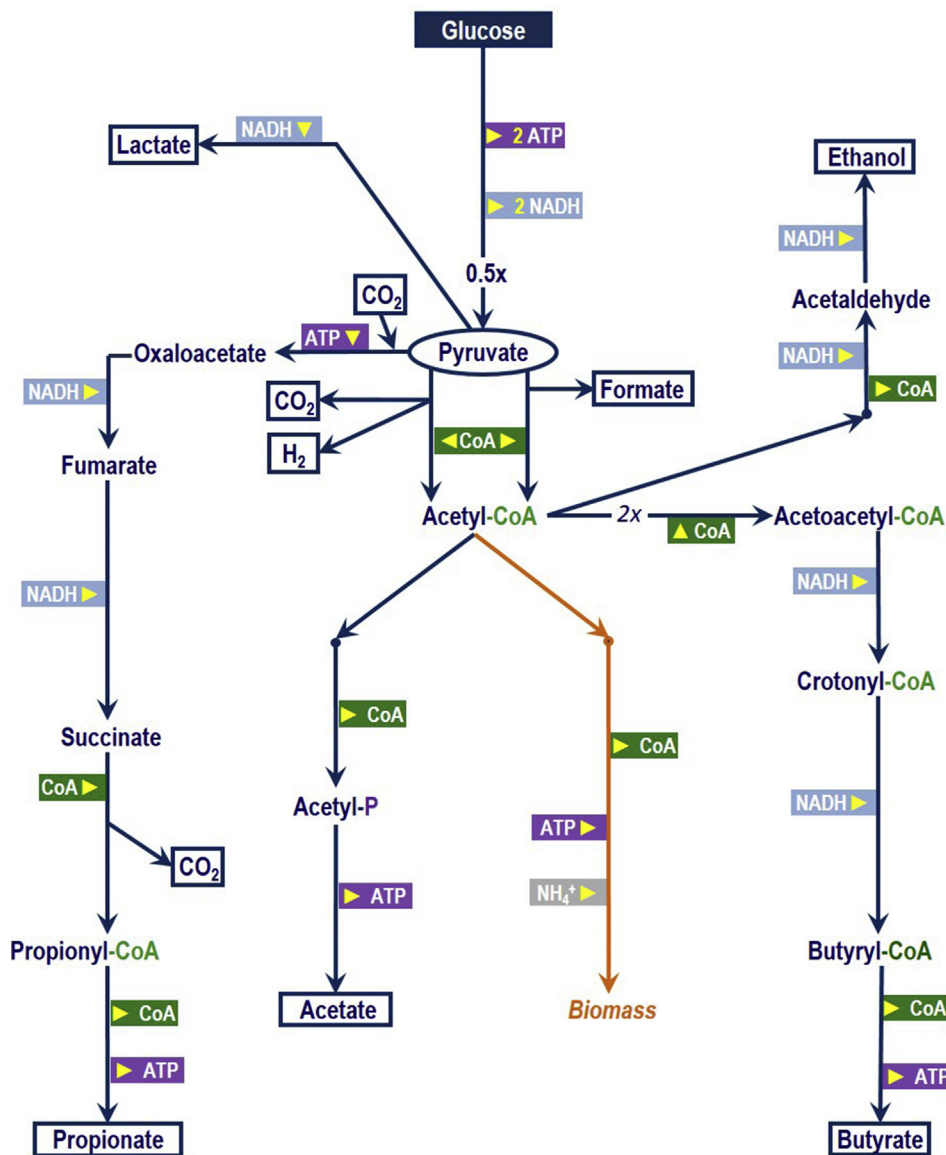
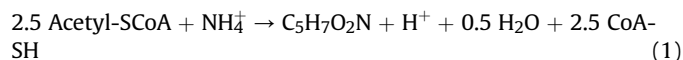


Fig. 1. Reference metabolic network of glucose fermentation with mixed cultures used in this study.

2012),  $E' = -280$  mV (Buckel and Thauer, 2013)) and it is involved in most of the redox reactions occurring in the metabolic network (González-Cabaleiro et al., 2015; Kleerebezem et al., 2008).

### 2.1.2. Anabolism

The metabolic network is closed by including the consumption and production of chemicals in the anabolic process. Following McCarty (2007), we considered anabolism as a process that uses acetyl-CoA as substrate to produce biomass, which has a lumped chemical formula of  $C_5H_7O_2N$  (Eq. (1)). This biomass formula has the same oxidation state as acetyl-CoA ( $\gamma = 4$ ), therefore no further redox reactions are needed. However, producing acetyl-CoA from glucose implies that the anabolism carries an extra production of  $H_2$ ,  $CO_2$  and  $NADH$  (Fig. 1) and this must be considered in the global stoichiometry.



### 2.2. Methodology

At steady state ECs must maintain the balance between oxidised and reduced forms to keep the redox neutrality of the system. Therefore, when an EC is reduced or oxidised, it needs to be regenerated. In fermentations all  $Fd_{red}$  is assumed to be oxidised producing  $H_2$  (section 2.1.1).  $NADH$ , on the contrary, cannot produce  $H_2$  directly as the redox potential of the  $NADH/NAD^+$  couple is higher than that of the proton reduction even in the most favourable fermentative conditions (González-Cabaleiro et al., 2015; Kleerebezem et al., 2008). Therefore, the  $NADH$  produced during glycolysis must be consumed in other places of the metabolic network. In consequence, a complete and accurate metabolic network results in a neutral  $NADH$  balance of the data analysed.

To verify the proposed metabolic network, we compared it with the experimental data by calculating the  $NADH$  balance (Eq. (2)). With the stoichiometry given by the network, we calculate the amount of corresponding  $NADH$  formed and consumed according

to the experimental products yields (moles of product formed per mole of substrate consumed in the system). If the NADH balance is neutral, this means that the metabolic network represents accurately the stoichiometry of the process. This can be used to compare different metabolic networks or to check whether a modification in a network improves its accuracy.

$$\sum_{i=N} v_{\text{NADH},i} \cdot y_i = 0 \quad (2)$$

where  $v_{\text{NADH},i}$  is the NADH stoichiometric coefficient associated with the product  $i$  and  $y_i$  is the yield of the product  $i$  (moles of product  $i$  per mole of glucose consumed).  $N$  is the total number of products. The result of the summation at steady state must be zero.

### 2.3. Experimental data used

The experimental work of [Temudo et al. \(2007\)](#) (hereafter termed Temudo experiments) was selected as it is the most comprehensive data set available on glucose fermentation using mixed cultures. It consists in a series of experiments using glucose as substrate (4 g/L) at pH values from 4 to 8.5. The hydraulic retention time (average time that a compound remains inside the reactor, HRT) is 8 h for pH from 5.5 to 8.5 and 20 h for pH from 4 to 5.5 (pH 5.5 was tested twice at different HRT). The volume of the reactor is 2 L with 1 L of heading space and it was operated in continuous mode at 30 °C. To keep an anaerobic environment, N<sub>2</sub> was flushed in the liquid phase at a 200 ml/min flow rate. They measured the yields of volatile fatty acids (acetate, propionate, lactate, and butyrate), ethanol, formate, CO<sub>2</sub>, H<sub>2</sub>, biomass and other minor products, closing the electron and carbon balances within a 10% of confidence (the values of the experimental yields are presented in [Supplementary Material Section A](#)). At neutral and high pH the proportion of inorganic carbon in the form of bicarbonate ion (HCO<sub>3</sub><sup>-</sup>) should be considered and it was estimated assuming liquid-gas equilibrium and acid-base equilibrium. The experimental set up was designed to ensure full substrate consumption and that steady state is truly reached. To use these data in our work, formate production was considered equivalent to the sum of H<sub>2</sub> and CO<sub>2</sub> production.

## 3. Results and discussion

### 3.1. Incorporation of the electron bifurcation into the metabolic network

EB is an enzymatic mechanism by which an endergonic electron transfer reaction is coupled with a sufficiently exergonic one. In this way, the energy surplus of the exergonic reaction is used to drive the endergonic one ([Peters et al., 2016](#)). EB was first hypothesised ([Herrmann et al., 2008](#)) in the crotonyl-CoA reduction with NADH in the butyrate synthesis pathway as a possible way for additional energy conservation. The reduction of crotonyl-CoA to butyryl-CoA ( $E' = -37$  mV) when combined with the oxidation of NADH ( $E' = -280$  mV), creates a highly exergonic step that could be used to drive endergonic reactions. The mechanism was then detected ([Li et al., 2008](#)) linking the highly exergonic and irreversible NADH-mediated reduction of crotonyl-CoA with the endergonic Fd<sub>ox</sub> reduction by NADH. Overall, when one mole of crotonyl-CoA is reduced to butyryl-CoA, one mole of Fd<sub>ox</sub> is reduced and two moles of NADH are oxidised ([Fig. 2](#)).

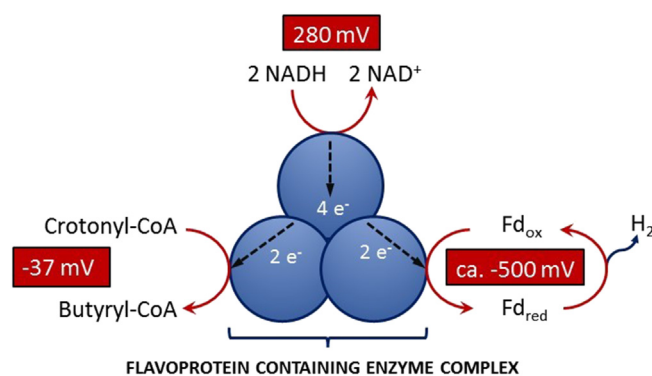
The set of reactions is catalysed by a cytoplasmic enzyme complex (butyryl-Coa dehydrogenase/Etf complex) containing flavoproteins ([Buckel and Thauer, 2013](#)). The Fd<sub>red</sub> yielded is re-oxidised producing H<sub>2</sub> (section 2.1.1), increasing thus the global

H<sub>2</sub> yield of the system to a theoretical maximum of 2.7 moles of H<sub>2</sub> per mole of glucose (35% more than without EB). Therefore, EB could explain why higher ratios than 2 moles of H<sub>2</sub> per mole of glucose are found in some works ([Davila-Vazquez et al., 2008](#); [Hallenbeck and Ghosh, 2009](#); [Jungermann et al., 1973](#); [Kapdan and Kargi, 2006](#); [Petitdemange et al., 1976](#); [Ren et al., 2016](#)). So far, EB was only observed using purified enzymes from *C. kluyveri* ([Buckel and Thauer, 2013](#); [Li et al., 2008](#)). However, there is no fundamental impediment to its occurrence in living *C. kluyveri* or *C. pasteurianum*, as proposed by [Buckel and Thauer \(2013\)](#), or in MCFs. For these reasons, in the new metabolic network we propose to include EB in the butyrate pathway ([Fig. 3](#)).

The inclusion of EB implies the consumption of one extra mole of NADH. This helps cells to decrease the reducing potential generated by the initial glycolysis. At the same time, it modifies the global stoichiometry of the process, as now the butyrate yielded from glucose requires the production of more oxidised products to close the electron balance of the fermentation. Without considering EB, the two moles of NADH formed in glycolysis were consumed in the butyrate pathway ([Fig. 1](#)). However, with EB three NADH are consumed ([Fig. 3](#)), which means that a higher share of the substrate can be oxidised to “energy rich” intermediates with concomitant substrate level phosphorylation (SLP). In essence, EB is an indirect way of conserving energy as reducing protons to H<sub>2</sub> saves substrate as electron acceptor and therefore microbes have the opportunity to harvest more energy from it ([Buckel, 1998](#); [Buckel and Thauer, 2013](#)). [Table 1](#) shows that ATP yields increases by 11% when considering EB in the butyrate formation pathway.

To determine whether these modifications give a better fit with the experimental data, we applied the methodology described in section 2.2. The NADH balances were calculated as per Eq. (2) using the measured products and biomass yields of Temudo experiments and the NADH stoichiometric coefficients given by the metabolic networks for each product. Stoichiometric coefficients for the production and consumption of NADH of both networks and an example of the calculation of the NADH balance are included in the [Supplementary Material](#) (Section B and Section C, respectively).

The new metabolic network shows improvements in the NADH balance ([Fig. 4](#)) primarily at low pH (pH ≤ 5.5) which correlates with high butyrate production. This likely indicates that without considering EB, the butyrate pathway is not described adequately. Butyrate is produced also at pH 7.75 and again the error without considering EB is sensibly higher than when EB is included in the metabolic network. This suggests that the inclusion of EB explains better the experimental results reported in Temudo experiments. Following the same methodology, we analysed other experimental data sets on MCF of glucose available in the literature ([de Kok et al.,](#)



**Fig. 2.** Electron Bifurcation mechanism in NADH mediated Crotonyl-CoA reduction in butyrate production pathway ([Buckel and Thauer, 2013](#)).

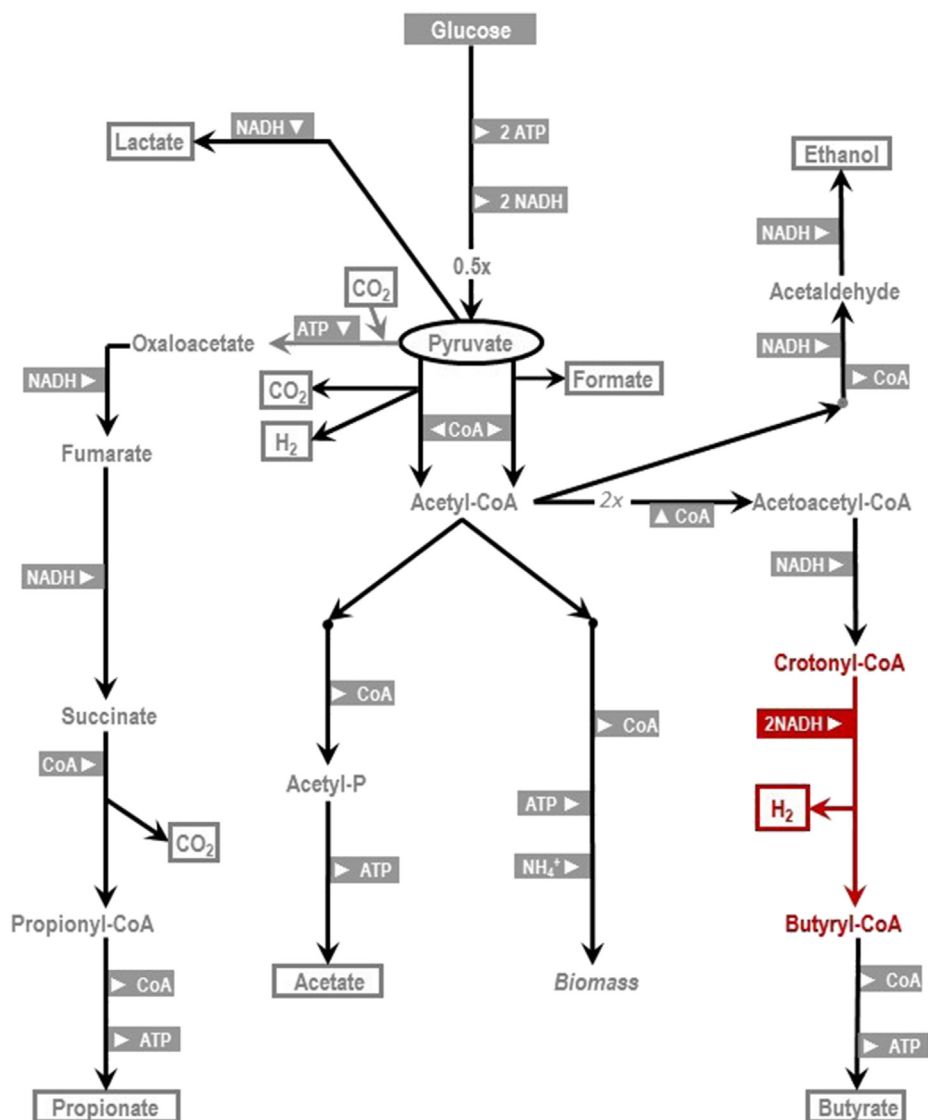


Fig. 3. Metabolic network including EB in the butyrate pathway.

**Table 1**  
Stoichiometry and ATP yield for butyrate pathway with and without EB.

Butyrate production		ATP yield (mol/mol glucose)
Without EB	Glucose → Butyrate + 2 H <sub>2</sub> + 2 CO <sub>2</sub> + H <sup>+</sup>	3
With EB	Glucose → 0.66 Butyrate + 0.66 Acetate + 2.67 H <sub>2</sub> + 2 CO <sub>2</sub>	3.33

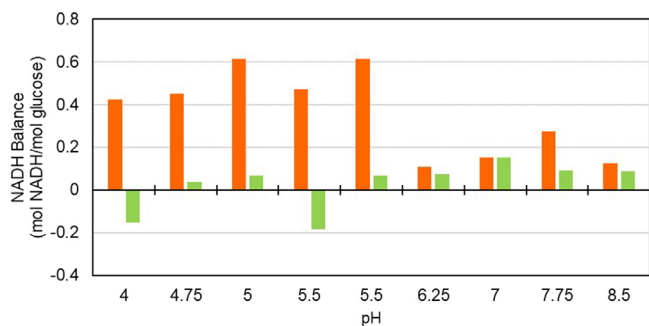
2013; Fang and Liu, 2002; Horiuchi et al., 2002; Mohd-Zaki et al., 2016; Temudo et al., 2009, 2008; Zoetemeyer et al., 1982). These data sets are not as comprehensive as Temudo experiments: in some cases, information on the minor products yields is not available or the gas production information is incomplete. As a result, the application of the methodology only provides qualitatively information. Nevertheless, the results confirm the conclusions presented here regarding Temudo experiments (Supplementary Material Section D).

### 3.2. Imbalanced gaseous species

Inclusion of EB alters the NADH balance of the network but also implies a higher overall H<sub>2</sub> yield (section 3.1), and therefore a yield

ratio of H<sub>2</sub> to CO<sub>2</sub> higher than one. Some authors argue that the H<sub>2</sub> to CO<sub>2</sub> ratio should be equal to 1 because the only step in the metabolic network where H<sub>2</sub> and CO<sub>2</sub> are produced is in the pyruvate decarboxylation (González-Cabaleiro et al., 2015; Kleerebezem et al., 2008; Temudo et al., 2007). This seems to be confirmed by Temudo experiments, and apparently contradicts our EB hypothesis.

We calculated the theoretical yields of H<sub>2</sub> and CO<sub>2</sub> and compared them with the yields reported in Temudo experiments. The theoretical yield values are those calculated considering the product spectrum of the experiment and following the stoichiometry presented in the metabolic network including EB (Fig. 3). In Fig. 5, H<sub>2</sub> theoretical yields (yellow bars) are higher than the experimental ones (grey bars). This can suggest that the difference



**Fig. 4.** Absolute error for the NADH balance with the different metabolic networks considered for the experimental data presented by [Temudo et al. \(2007\)](#). ■ results of the metabolic network without electron bifurcation and ■ results of the metabolic network considering electron bifurcation.

is because EB is not occurring after all. At the same time, CO<sub>2</sub> theoretical yields (yellow bars) are higher than the measured yields (grey bars) and this cannot be explained by an incorrect assumption of EB. To explain this imbalance, we proposed two hypotheses: ferredoxin regeneration without H<sub>2</sub> formation and homoacetogenesis.

### 3.2.1. Ferredoxin regeneration without H<sub>2</sub> formation

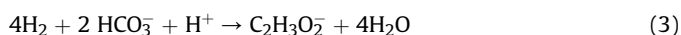
We calculated the yield of H<sub>2</sub> by assuming that all the Fd<sub>red</sub> produced (from the pyruvate decarboxylation step and from the EB mechanism) is re-oxidised by cytoplasmic hydrogenases (Ech) and produces H<sub>2</sub>. This is the most reported form of regenerating Fd<sub>ox</sub> ([Buckel and Thauer, 2013](#); [González-Cabaleiro et al., 2015](#); [Hoelzle et al., 2014](#); [Kleerebezem et al., 2008](#); [Temudo et al., 2007](#)) but Fd<sub>ox</sub> could be regenerated in other ways. The two electrons of Fd<sub>red</sub> can be used in anabolic reactions, presumably in highly endergonic reactions in which NAD(P)H is not a sufficiently strong electron donor ([Buchanan and Arnon, 1970](#)). For example, it was reported that some *Clostridia* species lack the enzymes needed to produce the anabolic-related NADPH during glycolysis and their only NADPH source is through ferredoxin-NADPH oxidoreductase ([Jungermann et al., 1973](#)). Alternatively, Fd<sub>red</sub> can participate as electron donor in other catabolic reactions either directly or transferring first its electrons to NAD<sup>+</sup> via the enzymatic complex ferredoxin-NAD oxidoreductase (RnfA-G enzyme complex), which is usually associated with energy conservation through the creation of an electrochemical Na<sup>+</sup>/H<sup>+</sup> gradient ([Biesterveld et al., 1994](#); [Buckel and Thauer, 2013](#); [Fonknechten et al., 2010](#); [Herrmann et al.,](#)

[2008](#); [Hugo et al., 1972](#); [Jungermann et al., 1973](#); [Petitdemange et al., 1976](#)). The latter is typically found in microorganisms that cannot produce NADH by others means because they lack the appropriate enzymes or their substrate does not allow for NADH production (when pyruvate is the main substrate, only Fd<sub>red</sub> is yielded in its decarboxylation to acetyl-CoA) ([Petitdemange et al., 1976](#)).

Any of these options decrease the predicted H<sub>2</sub> production and the predicted H<sub>2</sub> to CO<sub>2</sub> ratio but would not explain why the CO<sub>2</sub> predicted yields are also higher than the ones reported experimentally. Moreover, all these options would imply that the NADH stoichiometric coefficient of the butyrate pathway with EB would be the same as without EB (as in the reference metabolic network of [Fig. 1](#)). In consequence, the NADH balances including EB would have the exact values as the ones without including it (orange bars in [Fig. 4](#)). Moreover, the EB mechanism described in the butyrate pathway is reported in literature to regenerate Fd<sub>(ox)</sub> by yielding H<sub>2</sub>, as it is the only way to explain the observed H<sub>2</sub> yields in essays with purified enzymes from *C. kluyveri* ([Li et al., 2008](#)).

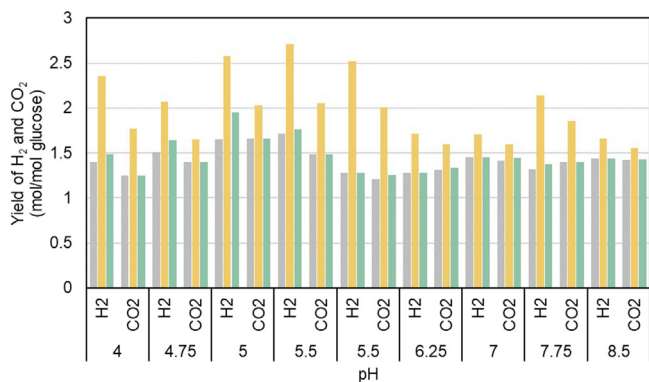
### 3.2.2. Homoacetogenesis

In fermentative conditions HA (Eq. (3)) is reported to play an important role as H<sub>2</sub> consumer. Partially due to the lack of competitors as there are no methanogens or sulphate reducing bacteria ([Bundhoo and Mohee, 2016](#); [Dinamarca and Bakke, 2009](#); [Fang and Liu, 2002](#); [Guo et al., 2010](#); [Hallenbeck and Ghosh, 2009](#); [Karadag and Puhakka, 2010](#); [Saady, 2013](#)). Some authors describe HA as persistent in fermentative conditions ([Saady, 2013](#)) and it is considered the main barrier for H<sub>2</sub> production via anaerobic fermentation ([Dinamarca and Bakke, 2009](#); [Hallenbeck and Ghosh, 2009](#)). Therefore, HA could explain the lower-than-expected H<sub>2</sub> yields found in numerous H<sub>2</sub>-oriented fermentations ([Hallenbeck and Ghosh, 2009](#); [Ren et al., 2016](#)). Energetic calculations show that HA is highly exergonic considering typical H<sub>2</sub> partial pressures observed in acidogenic fermentations (Section E in [Supplementary Material](#)).



Like all autotrophs, the growth rate of known homoacetogens is slow. *Acetobacterium woodii* is reported to have growth rates on H<sub>2</sub>/CO<sub>2</sub> between 0.024 and 0.050 h<sup>-1</sup> ([Peters et al., 2006](#); [Straub et al., 2014](#)), while *Moorella sp.* was reported to have a growth rate of 0.042 h<sup>-1</sup> ([Sakai et al., 2005](#)). Dilution rates in Temudo experiments are 0.042 h<sup>-1</sup> for low pH and 0.125 h<sup>-1</sup> for medium and high pH values, which apparently prevents the presence of homoacetogens. However, homoacetogens can also grow on organic compounds such as glucose, and therefore lower their doubling times down to values of 1.75 h ([Saady, 2013](#)). [Dinamarca and Bakke \(2009\)](#) reported homoacetogens presence in continuous experiments with a HRT of 8 h and [Saady \(2013\)](#) discarded lowering HRT as a successful strategy to avoid HA in fermentations. Therefore, it is feasible to hypothesise the presence of homoacetogens in Temudo experiments.

To check if HA could be responsible for the imbalance in H<sub>2</sub> and CO<sub>2</sub> production, we assumed that HA consumed the difference between the theoretical ([Fig. 5](#) yellow bars) and the measured yield ([Fig. 5](#) grey bars) of either H<sub>2</sub> or CO<sub>2</sub>. Accordingly, the stoichiometry (Eq. (3)) defines the correspondent acetate production and CO<sub>2</sub> or H<sub>2</sub> consumption. An example of calculations is included in section F of the [Supplementary Material](#). Green bars in [Fig. 5](#) show a good fit between the theoretical and measured gaseous yields when HA is considered. The Root-mean-square deviation (RMSD) measures the differences between predicted and observed values ([Table 2](#)). These results suggest unequivocally that HA provides a much better fit to



**Fig. 5.** H<sub>2</sub> and CO<sub>2</sub> yields experimental and predicted by the different stoichiometries. ■ Experimental values ([Temudo et al., 2007](#)) ■ Theoretical yield considering EB but not HA ■ Theoretical yield considering EB and HA.

**Table 2**Values of RMSD for H<sub>2</sub> and CO<sub>2</sub> without considering HA and considering HA.

	Without HA (mol/mol glucose)	With HA (mol/mol glucose)
$\sigma$ (H <sub>2</sub> )	0.789	0.117
$\sigma$ (CO <sub>2</sub> )	0.444	0.020

the experimental data, as values improve by 85% and 95% for H<sub>2</sub> and CO<sub>2</sub>, respectively.

Including HA in the system would also mean that part of the acetate measured did not come through direct glucose degradation but via HA. Consequently, part of the acetate did not produce either the NADH or the CO<sub>2</sub> and the H<sub>2</sub> of the pyruvate decarboxylation, altering the NADH balance. Blue bars in Fig. 6 represent the NADH balance after considering HA. For some of the experiments (especially those at pH 4 and the first at pH 5.5), the consideration of HA implies an increment of the error in the NADH balance. However, these differences might be very well attributed to experimental deviations. For example, carbon recovery in the first experiment at pH 5.5 is around 110%. At the same time, the ratio between butyrate and acetate yields in this experiment is higher than it is in the rest of low-pH experiments (yields are available in section A in the Supplementary Material), which might imply that the measurement of butyrate could be deviated. As butyrate consumes NADH, its consumption could be overestimated and explain the negative deviation at this pH (Stoichiometric coefficients are available in section B in Supplementary Material). At pH 4 the butyrate to acetate ratio also seems to be overestimated and a negative deviation in the NADH balance is again observed. Nevertheless, these deviations can be considered relatively small because for every glucose oxidation to pyruvate two moles of NADH are yielded.

### 3.3. EB in the propionate pathway

Fumarate reduction to succinate in the propionate pathway is a similar step to crotonyl-CoA reduction to butyryl-CoA. They are mechanistically alike (hydrogenation of a double carbon bond) and in both cases their energetics suggest an extra energy conservation ( $E'(\text{crotonyl-CoA}) = -37 \text{ mV}$ ;  $E'(\text{fumarate}) = -5 \text{ mV}$ ). However, the EB mechanism was reported in the crotonyl-CoA reduction of the butyrate pathway but not in the fumarate reduction (Buckel and Thauer, 2013; Li et al., 2008). The energy surplus of this metabolic step in the propionate pathway is reported to be conserved extruding a proton from the cytoplasm and therefore creating an electrochemical proton gradient (Buckel, 2001; Herrmann et al.,

2008). Even though EB was never detected in this step, we assumed it was feasible as there is no theoretical impediment for it. Using the same methodology as above, the NADH balances were calculated assuming EB in both butyrate and propionate pathways and HA (see Supplementary Material, section G). But the results show a worse fit when EB is included in the propionate pathway (the NADH balances increase the error by 23%) and therefore the consideration of EB in the fumarate reduction was discarded.

## 4. Conclusions

The summarised conclusions of this MCFs analysis based on the new detailed stoichiometry proposed are:

1. For first time EB is considered in MCFs yielding butyrate and this consideration returns a better fit between the predicted stoichiometry and the experimental yields measured.
2. EB consideration implies the prediction of the formation of butyrate accompanied by acetate. This was experimentally observed but not theoretically predicted before.
3. The addition of EB revealed an imbalance between the stoichiometry and the observation in the production of the gaseous components, H<sub>2</sub> and CO<sub>2</sub>. This highlights lack of understanding of MCFs stoichiometry.
4. It has been proposed that the imbalance of H<sub>2</sub> and CO<sub>2</sub> could be explained by the occurrence of HA in the MCFs of glucose.

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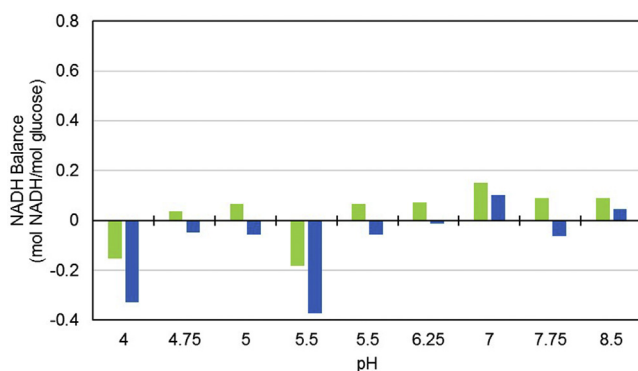
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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.watres.2018.05.013>.

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**Fig. 6.** Absolute error for the NADH/NAD<sup>+</sup> balance with the different stoichiometry considered for the experimental data presented by Temudo et al. (2007). NADH/NAD<sup>+</sup> balances considering: ■ the metabolic network with electron bifurcation ■ the metabolic network with electron bifurcation and assuming homoacetogenesis.

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