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How Environmental Parameters Controls Metabolic Pathways to Hydrogen

Karin Willquist, Ahmad Zeidan, **Ed van Niel**

1 Introduction

Anthropogenic CO_2 emissions to the atmosphere have generally been recognized as the major contribution to global warming and the associated climate changes. Therefore, several measures have been made to decrease the CO_2 emissions; e.g., the European Union strives at substituting 20% of the liquid fuel with biofuels by 2020. In recent years, much effort has been devoted to rendering biofuel production economically competitive with regard to fossil fuels. In this quest, the choice of the raw material is of central concern. First-generation biofuels are produced from sucrose and starch-rich material, which are also used for human consumption - a fact that drives up market prices. Based on this, more focus should be directed to second-generation biofuels produced from lignocellulosics materials as well as domestic and industrial wastes, which would significantly reduce the cost of the fuel, leading to it becoming competitive. Moreover, lignocellulosics constitute the most naturally abundant raw materials [1].

Biohydrogen is a typical example of an environmentally friendly, second-generation biofuel. It has a high molar energy content and it can be produced from both lignocellulosic and waste materials [2-5]. When sugar-based materials are used to produce H_2, the biohydrogen process is called dark fermentation. For a cost-effective dark fermentation process, high H_2 yields have to be obtained at relatively elevated partial H_2 pressures (P_{H2}) to eliminate the need for continuous stripping of the produced H_2 from the bioreactor and accordingly reduce the central cost of subsequent gas upgrading [6]. The current status for mesophilic co-cultures is an H_2 yield of ≤ 2 mol/mol hexose, and thus a substrate-conversion efficiency of merely 17%. In addition, these yields are usually obtained at low P_{H2} [7]. These low yields and the requirement for a low P_{H2} are major obstacles that need to be overcome before biohydrogen production as we know it today can be industrially feasible [7]. As for yields, a theoretical maximum of 4 mol H_2 per mol hexose can be obtained by using clostridia, thermophilic bacteria or Archaea.

The genus *Caldicellulosiruptor* consists of extreme thermophilic bacteria which have high potential for industrial biological hydrogen production due to their ability to: i) produce high yields of hydrogen (>3.6 moles of hydrogen per mole of C6 sugar), ii) metabolise a wide spectrum of carbohydrates including both pentose and hexose sugars, and iii) break down hemicelluloses and many other complex polysaccharides. The genome of the most studied member of this genus, *C. saccharolyticus*, has been recently sequenced enabling an increased insight into its metabolic network [8].

Interestingly, *C. saccharolyticus* can produce high H_2 yields [10] and are at the same time insensitive to relatively high P_{H2} (Willquist, K. Zeidan A., Pawar S. and. van Niel E.W.J, manuscript in preparation). However, its tolerance to different environmental parameters

needs to be further explored. This study is focusing on the physiology of *C. saccharolyticus* and the effect of enviromics on its growth and H_2 yields. It aims at unraveling several of the physiological characteristics which, in many perspectives, make *C. saccharolyticus* a superior H_2 cell factory.

2 Hydrogenomics

A major advantage of using *C. saccharolyticus* as a model H_2 cell factory is that its genome has been sequenced and protocols for genome-wide analysis of its transcriptome have been developed, thus facilitating the understanding of its physiology [8]. It has a relatively small genome with 2,970 kbp and 2,760 protein-coding open reading frames (ORF). From the genome-curation project, several new insights were obtained such as the fact that *C. saccharolyticus* possesses an unusually high number of transposons and transposable derivatives, as opposed to other organisms [8]. Since transposons increase the number of gene variations in the genome, a large amount can enhance the adaptive ability of this organism. Such an adaptation has obvious benefits process-wise, since it allows *C. saccharolyticus* to grow under a variety of environmental conditions. However, this may also have a negative impact on the stability of the process.

The bioinformatics study also shows that *C. saccharolyticus* possesses several polysaccharide-degrading enzymes, an unusually high number of ATP binding cassette (ABC) transporters [8] for the uptake of monomers and dimers and only one phosphotransferase (PTS) system, which is believed to be used for fructose transport [8, 9]. In addition, it possesses a complete gene setup for the Embden-Meyerhof-Parnas (EMP) pathway and the non-oxidative part of the pentose phosphate pathway (PPP; [8, 10]) to convert these sugars to building blocks and energy. No essential genes for the Entner Doudoroff pathway (EDP) have been found [8, 10], and this observation has been supported by ^{13}C-NMR analysis, demonstrating the EMP as the sole pathway for glucose oxidation in *C. saccharolyticus* [10]. Overall, the genome analysis revealed that *C. saccharolyticus* is well equipped for utilizing starch and lignocellulytic materials [8]. These indications are well in line with the growth analysis results which confirm the wide carbon utilization spectrum and its ability to grow and produce H_2 from complex lignocellulosic material (e.g. Miscanthus hydrolysate; [2]) and paper waste [11].

3 Effect of Dissolved H_2 Concentration on Hydrogen Yield and Productivity

For practical reasons the P_{H2} is generally used as a measure for H_2 tolerance. The currently quoted critical P_{H2}, defined as the partial H_2 pressure at which lactate formation is initiated, in *C. saccharolyticus* is 10-20 kPa [12]. This critical P_{H2} was determined in batch cultivations on sucrose with no gas sparging in a closed bioreactor. However, in similar experiments, using the same conditions but with xylose as the substrate, the P_{H2} in the headspace reached 60 kPa before the metabolism is partly shifted to lactate (Willquist K., Zeidan A., Pawar S. and van Niel E.W.J., manuscript in preparation). In addition, novel results have shown that during batch growth of *C. saccharolyticus* on glucose (5 g/L), lactate formation was triggered in the deceleration phase (the transition phase between logarithmic and stationary growth phases), which was independent of the P_{H2} (Willquist, Zeidan, Pawar and van Niel, manuscript in

preparation), i.e. during sparging with N_2, lactate formation was triggered at a P_{H2} of 6.3 kPa, whereas without sparging exponential growing cells initiated lactate formation at a P_{H2} of 30 kPa. Moreover, growth and H_2 production were still observed in continuous cultures at a low dilution rate ($0.05h^{-1}$) at a P_{H2} of 67 kPa. However, at this high P_{H2}, the metabolism had shifted predominantly (57%) to lactate, thereby decreasing the H_2 productivity and thus the dissolved H_2 concentration substantially (Ljunggren, Willquist, Zacchi and van Niel, submitted to Biotech. Biofuels).

In a comparative study on another *Caldicellulosiruptor* species, namely *C. owensensis,* we have also found a clear sugar-dependent H_2 tolerance pattern. In this organism, however, a dramatic increase in lactate production was observed when it was grown under elevated P_{H2} on xylose but not on glucose. As compared to acetate, the formation of one mole of lactate is accompanied by (i) the consumption of an extra mole of NADH, (ii) production of one mole less of both ATP and reduced ferredoxin and, most importantly, (iii) production of no H_2. Accordingly, the induction of lactate formation is most probably a regulatory response to changes in the energy and/or redox state of the cells at different growth phases and on different carbon sources.

Due to the lack of a clear pattern in the critical P_{H2} values found for each fermentation condition, one may wonder whether P_{H2}, a parameter for H_2 in the gas phase, is an appropriate measure of the critical H_2 concentration for growth and lactate formation. Since the cells are in the liquid phase, they are confronted with the dissolved H_2, which therefore should be a better choice as a parameter. In a recent work (Ljunggren, Willquist, Zacchi and van Niel, submitted to Biotech. Biofuels) we have demonstrated that the dissolved H_2 concentration is indeed a better parameter, being a function of H_2 productivity and the mass transfer rate, rather than the P_{H2}. The mass transfer rate is, in turn, a function of the gas sparging rate and the stirring rate. In addition, cultivations performed in closed systems without sparging have lower mass transfer rates. Thus, in that case the dissolved H_2 concentration and the P_{H2} are even further from equilibrium (Henry constant) than in a sparged and open system. This means that the critical P_{H2} determined in a closed system gives a more underestimated value than when determined in an open system. This corresponds well with the data acquired in our studies.

As a consequence of H_2 saturation in the liquid, the cells will decrease their H_2 productivity to avoid exceeding the critical dissolved H_2 concentration at which growth is inhibited, for instance by shifting the metabolism to lactate to re-oxidize NADH. Consequently, the NADH/NAD ratios remained low in continuous cultures even in the absence of sparging (Willquist K., Zeidan A., Pawar S. and van Niel E.W.J., manuscript in preparation).

4 Effect of CO_2 as a Sparging Gas on Growth and Hydrogen Production

As described above H_2 yields and productivities are influenced by the dissolved H_2 concentration. As a consequence, several studies have been carried out to remove H_2 from the liquid phase with an appropriate sparging gas [13]. Sparging increases the mass transfer of H_2 from the liquid to the gas phase (Ljunggren M., Willquist K., Zacchi G. and van Niel E.W.J., submitted to Biotech. Biofuels), which generally results in higher H_2 yields [14]. Although N_2 is commonly used as a sparging gas for lab-scale H_2 production, it is not a cost-

effective at an industrial scale since it is inert, thus difficult to remove from the effluent gas stream [6]. Instead, CO_2 might be an appropriate candidate since it can be more easily separated from H_2 and since it is already a product of the same fermentation process [6].

However, CO_2 is an inadequate choice of sparging gas for H_2 production by *C. saccharolyticus* as it negatively influences its growth rates and H_2 productivity [13]. Higher P_{CO2} values increase the concentration of dissolved CO_2, which hydrolyzes to bicarbonate and protons. This requires the addition of larger amounts of a caustic agent to maintain the fermentation pH, which in total contributes to an increased osmotic pressure and a higher environmental burden [13].

5 Effect of Osmotic Pressure on Growth and Metabolic Shift

As discussed above, *C. saccharolyticus* has several advantages as a H_2 cell factory such as its elevated H_2 yields, its relatively large tolerance to high P_{H2}, and the ability to grow on complex sugars. However, it also presents some major disadvantages which should be considered before it can be used industrially. Primarily, its sensitivity to osmotic pressures should be addressed. Growth of *C. saccharolyticus* is inhibited by osmolarities above 0.218 ± 0.005 osm/kg H_2O [13]. The critical molarity when growth is completely ceased is estimated to be 400-425 mM [12]. To avoid growth inhibition, sparging with CO_2 should be avoided and a maximum glucose concentration of 5 g/L should be used. The latter would obviously have a negative effect on the productivity. In addition, osmolaritiesabove 0.218 ± 0.005 osm/kg H_2O were found to induce cell lysis in *C. saccharolyticus* [13]. Increased protein and DNA concentrations in the culture supernatant were strong indications of lysis, but it remains to be investigated what can be the cause.

Interestingly, an increased osmolarity also triggers lactate formation in *C. saccharolyticus* (Ljunggren M., Willquist K., Zacchi G. and van Niel E.W.J., submitted to Biotech. Biofuels). The effect of osmotic pressure could be that elevated osmolarities inhibit growth in *C. saccharolyticus* [13]. This leads to a metabolic shift to lactate due to an induction of lactate dehydrogenase (LDH) activity, which will be explained below.

6 Regulation of Metabolic Shift to Lactate

During non-limited growth conditions, acetate and hydrogen are the soleproducts in *C. saccharolyticus* cultures, hence the superior H_2 yields. However, as described above, when the growth is inhibited and the cells enter a deceleration phase, the metabolism is partly directed to lactate [15]. To describe this observation a kinetic model of LDH activity was developed [15] and the influence of vital metabolites such as ATP, PPi, NADH and NAD were determined ([15]; Bielen A., Willquist K., Engman J., van der Oost J, van Niel E. W. J. and Kengen S.W.M., accepted for publication in FEMS Microb. Lett.).

Kinetic analysis of LDH activity revealed that the enzyme is regulated through i) competitive inhibition by pyrophosphate (PPi, ki=1.7mM) and NAD (ki=0.43mM) and ii) allosteric activation by FBP (300%), ATP (160%) and ADP (140%). In addition, metabolite analysis showed that the ATP/PPi decreased by an order of magnitude during the deceleration phase Bielen A., Willquist K., Engman J., van der Oost J, van Niel E. W. J and Kengen S.W.M., accepted for publication in FEMS Microb. Lett.) resulting in an induction of LDH activity [15].

Moreover, the decrease in growth resulted in a sudden increase in NADH/NAD ratio and subsequently decrease in the same as a result of an increased lactate flux [15]. The activation of LDH by ATP indicates that *C. saccharolyticus* uses LDH as a means to adjusts its flux of ATP and NADH production.

Other tools used in this study include metabolic flux analysis and pathway- as well as genome-wide mathematical modelling.

7 H_2 Production by Designed Co-cultures of Caldicellulosiruptor Species

Although using an undefined microbial consortium is a common practice during fermentative H_2 production, little attention has been given to using a mixture of known organisms in a defined co-culture. The usefulness of applying *C. saccharolyticus* in biological H_2 production was brought into a wider context through testing the performance of designed co-cultures of this bacterium with other members of the genus, including *C. owensensis* and *C. kristjanssonii*. The fermentations were carried out on a mixture of glucose and xylose, the major sugars in lignocellulosic hydrolysates. The H_2 yield obtained with *C. saccharolyticus*–*C. kristjanssonii* co-culture was approaching the maximum theoretical stoichiometry (3.8±0.2 mol H_2/ mol hexose equivalent), and was significantly higher than that of the pure culture of either organism. This suggests the existence of synergy between both organisms on hydrogen production. In addition, a number of other potential advantages should be expected by using a co-culture instead of a pure organism, including an increased probability of biofilm formation as well as extending the spectrum of substrate utilization. However, these experiments were carried out in batch mode and the stability of the co-culture in a continuous system remained questionable. Accordingly, we have developed a quantitative real-time PCR protocol for monitoring each species in the co-culture and it is currently being used to assess the possibility of their co-existence in a continuous system under carbon and non-carbon limitation. This is particularly important since in practical application chemostat conditions are more likely to be used for H_2 production.

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