Investigation of the links between mass transfer conditions, dissolved hydrogen concentration and biohydrogen production by the pure strain *Clostridium butyricum* CWBI1009

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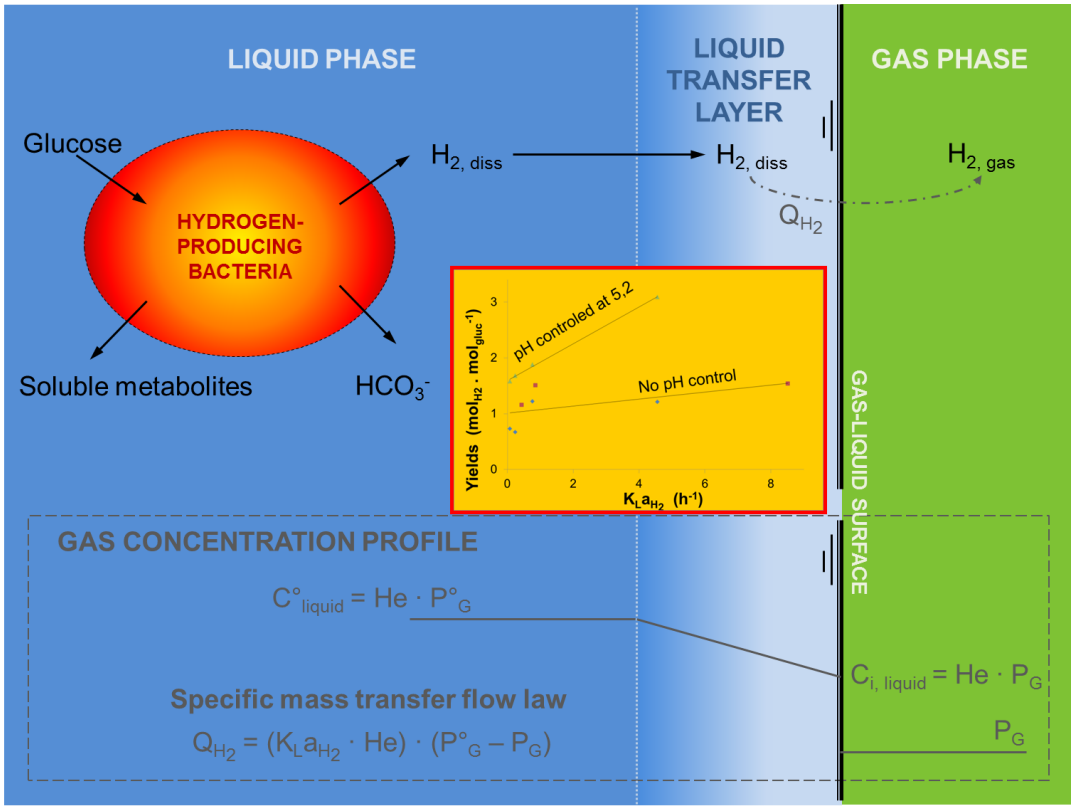
Graphical abstract



Abstract

Fermentative hydrogen production has often been described as inhibited by its own gas production. In this work, hydrogen production by *Clostridium butyricum* was investigated in batch Biochemical Hydrogen Potential (BHP) tests and in a 2.5 L Anaerobic Sequenced Batch Reactor (AnSBR) under different operating conditions regarding liquid-to-gas mass transfer. Through the addition of both stirring up to 400 RPM and nitrogen sparging, the yields were enhanced from 1.6 to 3.1 $mol_{H2} \cdot mol_{glucose}^{-1}$ and the maximum hydrogen production rates from 140 to 278 $mL \cdot h^{-1}$. These original results were achieved with a pure *Clostridium* strain. They showed that hydrogen production was improved by a higher liquid-to-gas hydrogen transfer resulting in a lower dissolved hydrogen concentration in the culture medium and therefore in a lower bacterial inhibition. In addition, biohydrogen partitioning between the gas and the liquid phase did not conform to Henry's Law due to critical supersaturation phenomena up to seven-fold higher than the equilibrium conditions. Therefore dissolved hydrogen concentration should be systematically measured instead of the headspace hydrogen partial pressure. A model was proposed to correlate H_2 production yield and rate by the pure *C. butyricum* strain CWBI1009 with mass transfer coefficient K_La.

Keywords: biohydrogen, *Clostridium butyricum*, dissolved hydrogen concentration, mass transfer coefficient, hydrogen partial pressure, supersaturation

1. Introduction

A future hydrogen economy is widely considered as a sustainable solution to the environmental, economic and societal issues resulting from massive use of fossil fuels and associated greenhouse gas emissions. However, 95% of current world hydrogen production is achieved using CO_2-releasing fossil fuels. Therefore, renewable H_2 production processes have been investigated in many studies [1–3]. Fermentative biohydrogen production, also called

dark fermentation, is one of the promising alternatives that can use the renewable organic fraction present in wastewater or agricultural residues. The general equations, depending on the microorganisms and their specific metabolism, are:

Acetic acid production: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (1)

Butyric acid production: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$ (2)

Formic acid production and dismutation: $C_6H_{12}O_6 \rightarrow \ldots + HCOOH \rightarrow CO_2 + H_2$ (3)

Equation (3), related to Enterobacteriaceae metabolism, is not complete since the group releases a mix of acids containing formic acid that is further enzymatically dismuted in hydrogen and carbon dioxide [4]. The dark fermentation process is advantageous since it leads to the production of renewable energy at relatively low operating costs (e.g. low heating requirements, low substrate cost), requires no light and may be easily integrated with anaerobic digesting processes or in wastewater treatment plants [4, 5].

Biohydrogen production technology suffers from limited development due to several factors that strongly influence the process (e.g. pH, temperature, inoculum size and type, operating conditions and design of the reactor). Taking this into account, previous studies have mainly focussed on the simultaneous improvement of hydrogen production rates (HPR) and conversion yield, currently reaching about 60% of the maximum stoichiometric yield of 4 $mol_{H2} \cdot mol_{glucose}^{-1}$ [3, 4, 6]. Hydrogen partial pressure has often been cited as having a major impact on process performance but has rarely been investigated [7–11]. Hydrogen partial pressure indirectly plays a critical role in the biochemical equilibria involved in the conversion of the substrate to hydrogen and consequently in determining the metabolic pathway.

Indeed, during anaerobic digestion, bacteria consume organic compounds to produce volatile fatty acids (VFA), alcohols, CO_2 and H_2. When the dissolved hydrogen reaches a critical

concentration (corresponding to a partial pressure of 60 Pa), the bacterial metabolism shifts, due to thermodynamic limitation [12, 13], from the production of maximum yields of H_2 with co-production of acetate to other metabolic pathways (e.g. lactate, ethanol) with lower H_2 yield and co-production of butyrate. This mechanism allows the bacteria to continue their activity and growth by maintaining the pool of $NADH/NAD^+$ electron carriers and producing the ATP energy molecule. However, since these biochemical pathways are unfavourable for achieving maximum hydrogen production [4, 14, 15], several authors have proposed various techniques to decrease the concentration of metabolic gases soluble in the liquid phase [6].

Gas sparging has proved to be an efficient method to maintain maximum hydrogen production even though it leads to biogas dilution and higher cost for hydrogen recovery. Improvement of biogas volumetric production of up to 120% has been achieved, depending on the nature of the flushing gas, the flow rate and the reactor configuration [16, 17]. Non-sparging techniques using mechanical stirring [18–20], hydrogen-separating membranes [21] or headspace modification (under vacuum, high pressure or gas adsorption [8, 9, 11, 22–25]), have also led to significant improvements in hydrogen yield (approaching the theoretical maximum of 4 $mol_{H2} \cdot mol_{glucose}^{-1}$). To date, the specific mechanisms enhancing the H_2 yields in pure or mixed culture have not been found but several hypotheses have been proposed [6]. Improvement of hydrogen yield could be linked to (1) negative feedback on the regulation of the NFOR enzyme, (2) lower substrate availability for hydrogen-consuming microorganisms in mixed cultures and/or (3) decreased inhibition of CO_2 [6].

Most studies investigating the effect of hydrogen on its own production have considered the indirect influence of H_2 partial pressure by headspace measurements. Partial pressure (P_G) is related to the dissolved saturated concentration (C_S) by Henry's Law (equation 4). However this law is only applicable at equilibrium, i.e. when the transfer to gaseous phase of volatile molecules produced by microorganisms in liquid medium is efficient enough to avoid gas

accumulation in any phase. By contrast, when for instance the mixing state of the culture is unfavourable for gas transfer from the liquid to the gas phase, some mass transfer limitations appear and the hydrogen may accumulate in the liquid medium to reach a supersaturated concentration [26, 27].

$$C_S = He \cdot P_G \quad (4)$$

Therefore, if not efficient enough, the kinetic related to gas transfer would be the rate-limiting step of the biohydrogen production process. As a consequence, the evaluation of HPR would

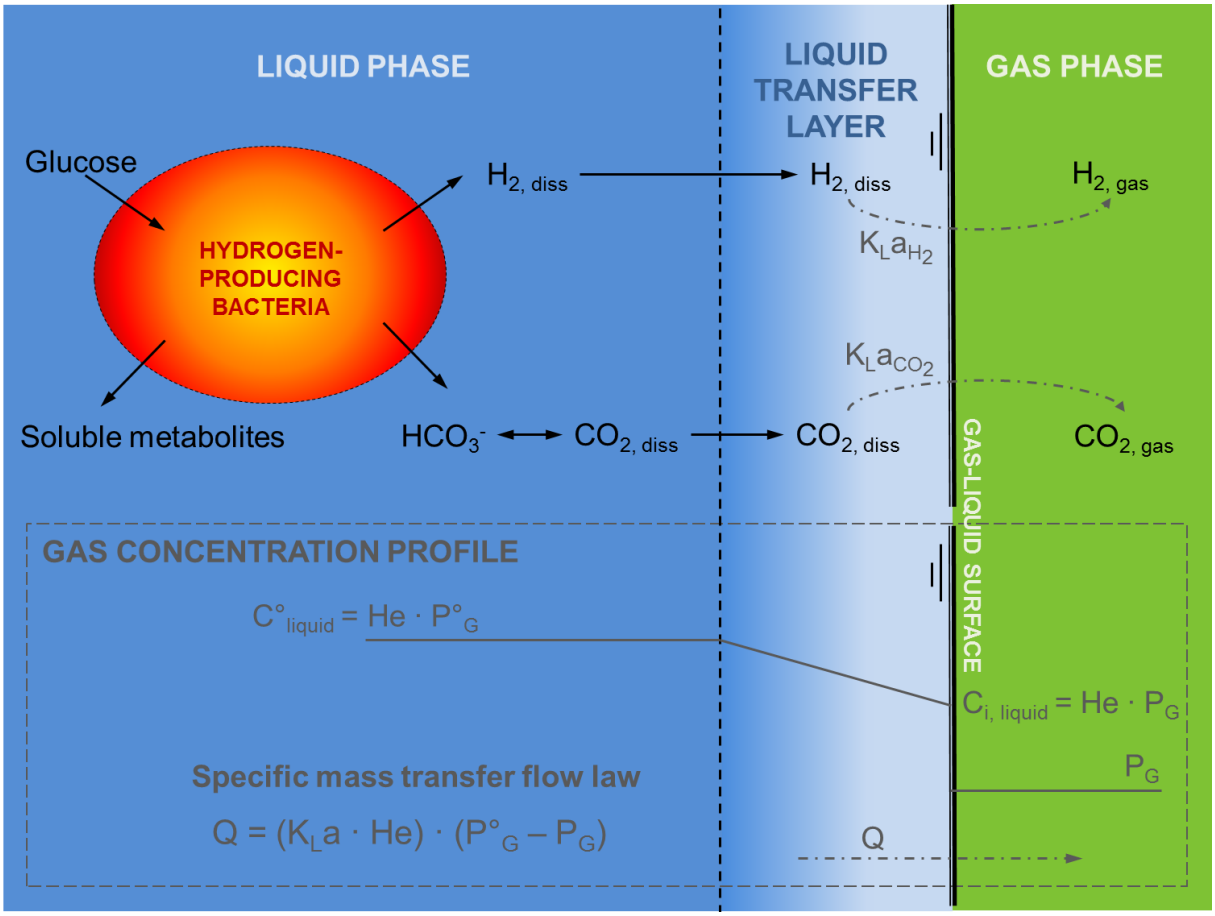


Figure 1: Scheme illustrating the different steps involved in the transfer of hydrogen from the production sites in bacteria to the gas phase of the bioreactor. The liquid-to-gas transfer rate is limited by the liquid film surrounding the gas bubbles. Liquid film displays a laminar flow resulting in gradient development of H_2 concentration. In the bulk liquid the H_2 concentration differs from the concentration calculated at the interface according to the Henry equilibrium. The rate of H_2 transfer through the liquid film can be expressed by considering the K_La parameter. Adapted from Treybal [31] and Kraemer and Bagley [8].

correspond to the measurement of the rate-limiting step instead of the real HPR of the bacteria [27, 28]. Only a few authors have taken into account mass transfer and hydrogen accumulation in the media simultaneously to assess their influence on hydrogen production performance [20, 26, 29, 30].

Regarding the global mass balance (equation 5), the gaseous mass flow Q transferred through a specific gas–liquid interface expressed by unit of time and by unit of liquid volume depends firstly on the global mass transfer coefficient K_La (in h^{-1}, which is directly linked to the mixing state of the media and the gas–liquid interfacial area) and secondly on the potential of transfer $P°_G - P_G$ (linked to the gas partial pressure P_G and the liquid concentration of gas dissolved at the equilibrium $C°_L = He \cdot P°_G$). Henry's constant (He, in $mol \cdot L^{-1} \cdot atm^{-1}$) depends on the nature of the gas considered and the liquid medium [31].

$$Q = K_La \cdot He \cdot (P°_G - P_G) \quad (5)$$

The most important mass transfer limitation lies within the liquid phase, more specifically in the narrow liquid layer at the interface characterised by a gradient of concentration (Figure 1). The K_La coefficient, describing the mass transfer resistance in the equation (5), allows the comparison between different bioreactors or hydrodynamic conditions. Its measurement in anaerobic media was developed by Pauss et al. [27] in a continuous bioreactor operating in steady-state conditions. However, our experiments were run in batch or sequenced batch reactors in unsteady conditions. Therefore, a method based on reverse dynamic gassing-out (in contrast with the classical dynamic gassing-out method performed for aerated bioreactors) was applied as reported by Kraemer and Bagley [29].

While other authors such as the teams of Bagley [29] or Gaddy [32, 33] investigated fermentative hydrogen-producing bioreactors with other strains or mixed cultures, to our knowledge, no study with pure *Clostridium* strain have used the K_La parameter to link

hydrogen production performance (rate and yields) and hydrogen supersaturation in the liquid media with the mixing state of the culture. In this study, the three parameters were simultaneously investigated with pure *Clostridium butyricum* CWBI1009. Therefore it is possible to directly correlate bacterial performance (HPR and H_2 production yield) with the bioreactor operating conditions without potential interference from competitive or synergistic microorganisms as with mixed cultures. Moreover, the expected correlation should be useful for comparing different bioreactor designs with the same strain to broaden understanding of bioproduction of hydrogen.

In the first experiments Biochemical Hydrogen Potential (BHP) tests were run in four different mass transfer conditions. Further experiments were carried out in a pH-controlled Anaerobic Sequencing Batch Reactor (AnSBR) operated under different mixing and degassing conditions. The volume of hydrogen produced and the metabolites released in the media were monitored and the corresponding dynamics were modeled on the basis of the Gompertz equation. The measured gaseous partial pressure was compared with theoretical hydrogen concentration in the medium at equilibrium. The hydrogen mass transfer coefficient was also estimated for each bioreactor.

2. Material and methods

2.1. Cultures and bioreactors set-up

The strain cultured in this paper for the production of hydrogen was *Clostridium butyricum* CWBI1009 (*C. butyricum*), previously isolated and identified in the laboratory. *C. butyricum* was conserved and grown with glucose monohydrate at 5 $g \cdot L^{-1}$ as substrate, in a rich MDT medium [34]. PCA medium in Petri dishes was used for purity check of liquid samples at the end of each culture [34]. All the procedures and media cited above were fully described by

Masset *et al.* [34]. The production of hydrogen in different mixing or degassing conditions was investigated in batch BHP tests and in 2.5 L AnSBR.

The BHP experiments were carried out for 96 hours in 270 mL serum bottles with 200 mL of liquid medium according to the method reported by Hiligsmann *et al.* [35]. Four conditions were investigated in independent triplicate experiments: vertical non-stirred (VNS) and horizontal non stirred (HNS) bottles, developing respectively gas-liquid interfacial area of 28 cm^2 and 63.5 cm^2 respectively; vertical stirred bottles (VS) with orbital shaking at 120 rpm, and horizontal stirred bottles (HS), filled with reticulated polyurethane cubes (1.5 cm x 1,5 cm size; specific surface area +/- 1800m^2/m^3, Type Filtren TM30, Recticel, Belgium) as a biomass carrier and rolled on their horizontal axis at 20 rpm. In order to measure the pH and collect the liquid and gas samples for analysis, twelve bottles were prepared for each condition and three of them were opened every day after the measurement of the biogas volume (the experiments were carried out in triplicates in order to estimate standard errors).

The AnSBR experiments were run in a laboratory-scale tank reactor (Biolafite manufacture; gas-liquid interfacial area of 115 cm^2) of 2.5 L total volume fully equipped with temperature, pH and agitation control. The culture was set up in two sequences with 2.3 L culture medium [36]. A first batch sequence at uncontrolled-pH decreasing from 7.3 down to 5.2 was achieved for cell enrichment. It was followed by a second sequence with pH control at 5.2 after removal/addition of fresh culture medium (40% of the initial 2.3 L volume as described elsewhere; [34]). Bioreactors preparation, start-up and sequential operation along with samples collection were already detailed elsewhere [34]. The bioreactors were operated at three different stirring speeds (0, 100 and 400 rpm) by three Rushton propellers (with four blades) placed at an equal distance on the agitation axis. Furthermore, a fourth condition was tested with 400 rpm stirring and nitrogen sparging in the liquid at a flow rate of 1.65 $L \cdot h^{-1}$

(based on the optimum 12 mL/min .L reported by Kraemer and Bagley [29]), through a porous stainless steel sparger placed at the bottom of the tank.

2.2. Hydrogen production measurement

The biogas produced in the BHP tests was collected daily in each bottle by sterile syringe and needle through the butyl septum. Injection of the collected biogas in a 9N KOH measurement system for CO_2 sequestration allowed the determination of hydrogen content and volumetric hydrogen production by gas balance as already described by Hiligsmann *et al.* [35].

The 2.5L AnSBR was connected to a flow meter for continuous biogas monitoring (MGC10 and Rigamo program V2.2, Ritter, D). A second digital flow meter (TG05/5, Ritter) was placed in series for the results validation. Headspace gas characterisation was performed by on-column injections of 50 µL gas samples on CarboPLOT P7 column (Varian Chrompack, NL) in a gas chromatography system (HP8950 SeriesII) equipped with a TCD detector, using either nitrogen or helium as carrier gas (respectively for hydrogen and for nitrogen/methane/carbon dioxide detection). A detailed procedure can be found elsewhere [37].

Experimental volumetric data were fitted on a modified Gompertz equation by using the "Solver" function of the Excel software (Microsoft Office 2007) [18, 38]. The three parameters of the fit curve were the hydrogen production potential (P, in mL or L), the maximum HPR (R_m, in $mL_{H2} \cdot h^{-1}$) and the lag phase (λ, in h).

2.3. Analytical methods

There is no standard method for the accurate measurement of dissolved hydrogen. Commercial dissolved gas sensors used in the food and beverage industry are usually expensive and specific to their application. The method used here for dissolved hydrogen

measurement was based on the static headspace gas chromatography presented by Kraemer and Bagley [26]. A 2 mL liquid sample was collected by a 2.5 mL Gastight syringe (Hamilton, CH) and injected in sealed vials of 8.68 mL already containing 4 mL of 3 M H_2SO_4. After 48 hours, the equilibrium (according to Henry's Law) between the liquid and gas phases was assumed to be attained. Gas composition was analysed by on-column gas chromatography as already described (section 2.2). Calibration was achieved with 5% ± 0.1% H_2 in N_2 gas mixture (Air Liquide, BE) as the external standard. The dissolved hydrogen concentration in liquid phase (in $mmol \cdot L^{-1}$) was calculated according to the method described by Kolb and Ettre [39], *i.e.* by multiplying by 56.1 the measured hydrogen partial pressure in the vial headspace (coefficient taking into account the total vial volume, the total liquid volume and the sample volume). The measurement of dissolved hydrogen concentration in BHP tests was carried out in triplicate before releasing the biogas (*i.e.* under overpressure).

The soluble metabolites were measured on liquid samples centrifuged at 13000 rpm for 3 min. The supernatants were filtered through a 0.2 µm cellulose acetate filter (Minisart, Sartorius). The glucose, lactate, succinate, formate, acetate, propionate, ethanol and butyrate were analysed using a HPLC (1100, Agilent) equipped with a differential refraction index detector as described formerly [34]. The concentrations measured in the culture medium were used to evaluate the carbon mass balance (MB) of glucose conversion in the soluble metabolites using the method of calculation reported by the same authors.

2.4. K_La measurements

The volumetric mass transfer coefficient (K_La in h^{-1}) is the most suitable parameter to describe the hydrogen gas transfer efficiency (or resistance) within the liquid phase depending on the mixing state of the media [40, 41]. The K_La in the different experiments was measured by dynamic-gasing out method as already reported [29, 42]. The measurements were made in

the same operating conditions (reactor size, liquid volume, stirring speed, N_2 sparging rate, temperature) but with the reactor being filled with water, and either with oxygen or hydrogen gas (water was used instead of the culture medium, with similar density and viscosity, in order to enable further and easy comparisons with results achieved in other environmental conditions). Oxygen measurement allowed simpler procedure by contrast with the hydrogen measurements. The liquid was first degassed with N_2, then saturated by 99.99% O_2 or H_2 bubbling in the liquid under intensive mixing conditions during 15 to 30 min. When the saturation was reached, the decrease of dissolved gas concentration was recorded by a dissolved oxygen probe (polarographic probe, WTW, D) for O_2 (from 100% down to 21% *i.e.* the oxygen concentration in air) and by headspace technique described in section 2.3. for H_2 (from 100% down to 0%). In conditions without gas sparging the dissolved gas concentration decreased by natural exchange through liquid-gas exchange surface. The oxygen probe data were recorded every 30 sec whereas liquid samples for hydrogen concentration were collected every 2 to 60 min depending on the K_La result estimated by O_2 experiment. Indeed, the K_La of poorly soluble gases is known to be proportional to the square root of their diffusivity (D) as stated in the equation (6), and validated in Table 4, allowing an estimation of the K_La_{H2} based on the K_La_{O2} [27]. The data of decreasing concentration of dissolved gas were fitted to the exponential curve for desorption (equation (7)) as described by Shizas and Bagley [43] and resulted in a K_La value.

$$K_La(O_2) \cdot K_La(H_2)^{-1} = [D(O_2) \cdot D(H_2)^{-1})]^{1/2} \quad (6)$$

$$C = C_0 \cdot \exp(-K_La \cdot t) \quad (7)$$

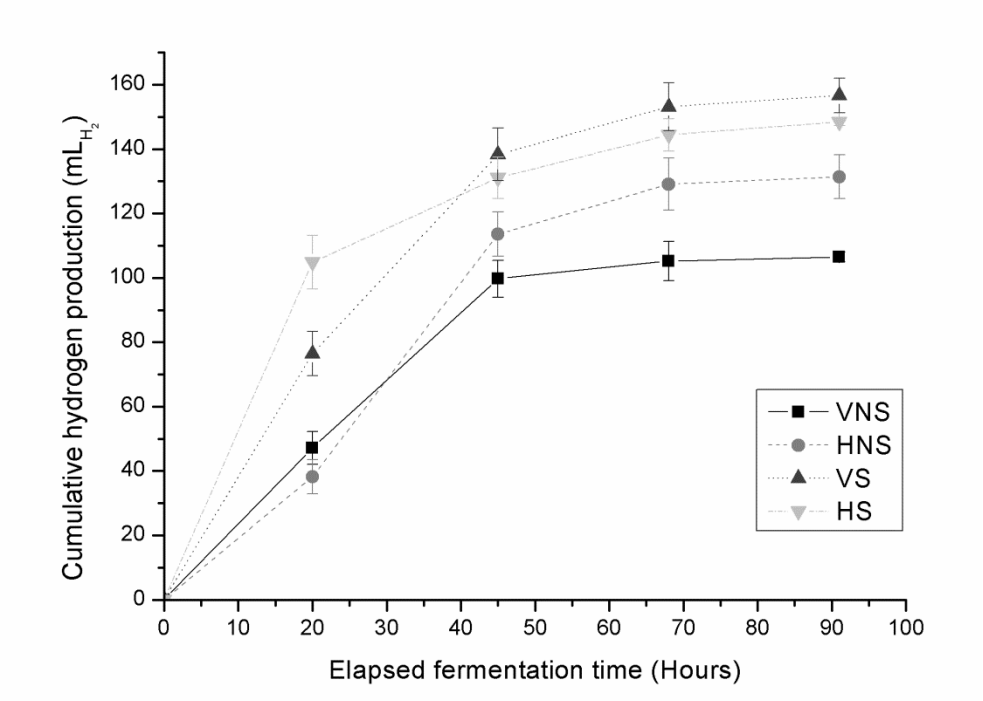


Figure 2: Cumulative hydrogen production by *C. butyricum* in 200 mL BHP tests carried out in different mass transfer conditions *i.e.* Vertical (V) and Horizontal (H), Non-Stirred (NS) and Stirred (S) conditions. The standard deviation bars are calculated on three independent experiments.

3. Results and discussion

3.1. Biohydrogen production and dissolved H_2 concentration in BHP tests

The production of hydrogen by pure *C. butyricum* cultures was investigated in different stirring and degassing conditions. The first series of experiments was carried out in 200 mL BHP tests. The volume of biogas produced was measured daily and hydrogen production was calculated after CO_2 absorption in KOH displacement equipment. Four different mass transfer conditions were investigated: the flask oriented in vertical or horizontal positions (denoted V or H); and in stirred or not-stirred conditions (denoted S or NS). The hydrogen production curves (Figure 2) show classic sigmoidal profiles with short lag phases. Both stirred cultures (VS and HS) produced hydrogen faster than the non-stirred cultures. Indeed, production after 20 h of incubation in stirred cultures was already as high as the hydrogen volume reached

after 96 h of VNS culture. After 96 h, the production of hydrogen in VS and HS (156.6 mL and 148.4 mL, respectively) was 40% greater than the volume achieved in both NS conditions. The HNS experimental conditions achieved a 23% greater final H_2 volume than the VNS (131.4 mL and 106.5 mL for HNS and VNS, respectively). The NS cultures also had lower HPR than the stirred cultures.

A residual amount of glucose in the media was detected at the end of the culture (Table 1) since the BHP experiments were run at uncontrolled pH, decreasing from pH 7.6 to pH 4.8 due to acid metabolite production. As a consequence, glucose consumption stopped due to low pH or VFA accumulation inhibiting bacterial growth. However, the bacteria could limit

Table 1: Evolution of glucose and metabolites concentrations in mM [A] and carbon mass balance in % of glucose converted [B] after 96 hours of incubation of *C. butyricum* in 200 mL BHP tests carried out in different mass transfer conditions *i.e.* Vertical (V) and Horizontal (H), Non-Stirred (NS) and Stirred (S) conditions (standard deviation calculated on triplicates).

A	Glucose	Lactate	Formate	Acetate	Ethanol	Butyrate
VNS	2,82 ± 0,26	2,5 ± 0,19	21,16 ± 0,38	6,3 ± 0,19	4,06 ± 0,78	11,14 ± 0,52
HNS	0,81 ± 0,49	2,08 ± 0,1	19,13 ± 1,25	6,45 ± 0,5	1,24 ± 0,36	17,28 ± 2,29
VS	0,61 ± 0,14	0,76 ± 0,57	19,14 ± 2,58	8,33 ± 0,38	0 ± 0	18,98 ± 0,88
HS	1,63 ± 0,03	0,94 ± 0,18	21,63 ± 0,84	7,98 ± 0,37	0 ± 0,59	16,33 ± 0,47

B	Lactate	Formate	Acetate	Ethanol	Butyrate	CO_2	Sum
VNS	6,1 ± 0,39	17,18 ± 0,15	10,24 ± 0,18	6,59 ± 1,21	36,2 ± 1,52	6,61 ± 0,54	82,92 ± 2,22
HNS	4,61 ± 0,29	14,16 ± 1,07	9,54 ± 0,72	1,84 ± 0,58	51,15 ± 7,1	7,55 ± 1,18	88,85 ± 9,96
VS	1,67 ± 1,26	14,04 ± 1,97	12,22 ± 0,63	0 ± 0	55,64 ± 2,27	10,23 ± 1,28	93,79 ± 1,95
HS	4,69 ± 0,44	12,88 ± 0,71	11,78 ± 0,59	0 ± 0	48,22 ± 1,59	11,19 ± 2,1	72,07 ± 0,91

the pH drop by combining two protons with electrons to release H_2. Unfortunately this mechanism is not independent from the metabolic production of acid compounds with one or two moles diatomic H_2 via the acetate and butyrate metabolic pathway. This hypothesis is confirmed here since in both stirred conditions, greater hydrogen production was associated with higher glucose consumption due to a slower decrease in pH.

For better comparison the hydrogen yields were estimated taking into account the residual glucose concentration and therefore are related to the amount of glucose converted. The VNS, HNS, VS and HS cultures yielded 1.16, 1.51, 1.54 and 1.62 $mol_{H2} \cdot mol_{glucose}^{-1}$, respectively. These results are lower than those achieved in bioreactors with pH control ([34] and section 3.2) since pH conditions were not optimal for the whole culture duration.

The concentration of dissolved hydrogen was measured before each biogas measurement in

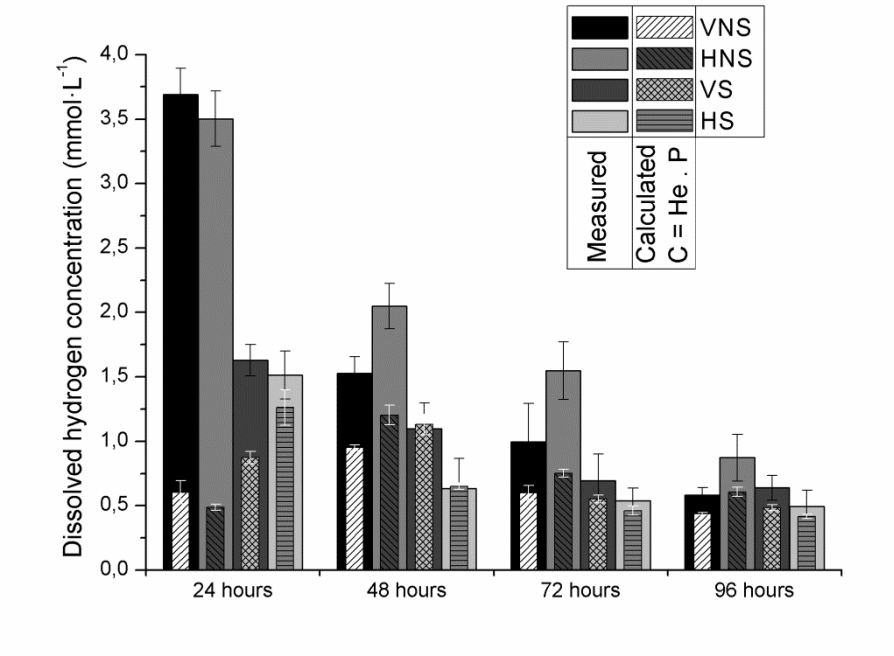


Figure 3: Evolution of the dissolved hydrogen concentration in 200 mL BHP tests carried out in different mass transfer conditions *i.e.* Vertical (V) and Horizontal (H), Non-Stirred (NS) and Stirred (S) conditions. The measured values are compared to the calculated concentration (C) at the gas-liquid equilibrium according to the Henry's Law (with Henry's constant (He) of 7.68 10^{-4} $mol \cdot L^{-1} \cdot atm^{-1}$ [51]) based on partial pressure measurement (P). The standard deviation bars are calculated on triplicates.

the BHP bottles. The measured concentrations were compared to the values estimated at steady-state according to Henry's Law, taking into account the total pressure and the gas composition in the headspace of the bottle (Figure 3). Hydrogen supersaturation was observed, particularly in the VNS and HNS bottles after 20 h of incubation, with measured dissolved hydrogen concentration up to seven times the value at equilibrium. Hydrogen supersaturation was greater in the VNS and HNS bottles, even up to the final measurement at 96 h. By contrast, equilibrium was achieved after 48 h in the VS and HS bottles.

These results emphasise the increased H_2 transfer occurring in stirred conditions that would

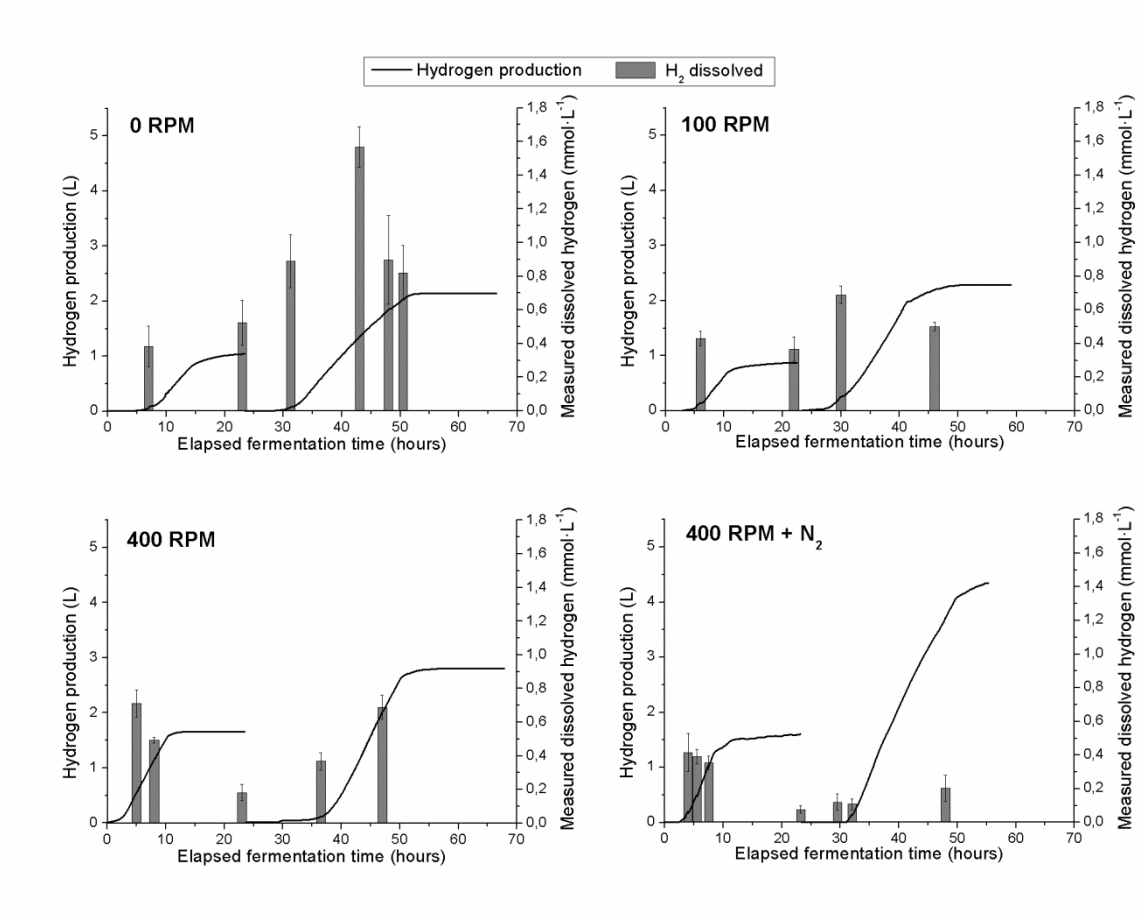


Figure 4: Cumulative hydrogen production and dissolved hydrogen concentration in four different mixing and degassing conditions in 2.5 L AnSBR with *C. butyricum*. Both successive curves for each condition are related to two successive sequences with uncontrolled pH and pH control at 5.2, respectively. Dissolved hydrogen concentration was measured after liquid sampling by gas headspace chromatography. Error bars are calculated on the basis of two separate samples.

lead to lower supersaturation in the bacteria culture and two-fold greater H_2 production after 25 h culture than in unstirred conditions. Moreover, supersaturation levels tended to decrease with successive pressure releases at each biogas measurement. Note that no measurement was carried out in the few hours after the sample collection at 20 h when H_2 production and therefore supersaturation might be the greatest in VNS and HNS, preceding a natural decrease of dissolved gas concentration by transfer to the headspace. The higher supersaturation measured in the HNS cultures compared to VNS cultures, irrespective of sampling time after the first 20 h, should be related to the marginal H_2 production that was higher in HNS (e.g.75 mL between 20 h and 45 h samples) than in the VNS (52.6 mL in the same period). This suggests that without stirring the liquid phase transfer is limiting rather than the lack of interfacial area. This is confirmed by the similar H_2 production and supersaturation recorded in VNS and HNS during the first 20 h. Therefore the BHP tests suggest that the production of hydrogen is linked with the dissolved hydrogen concentration in the culture medium rather than with the headspace composition.

3.2. Biohydrogen production and dissolved H_2 concentration in 2.5 L AnSBR

A second series of experiments investigated hydrogen production in a stirred tank reactor operated in sequenced batch mode (AnSBR). Different stirring speeds (0, 100 and 400 RPM) and N_2 gas sparging at 1.65 $L \cdot h^{-1}$ (experiment denoted as 400 RPM + N_2) were tested. For each experimental condition, hydrogen production was monitored during two successive sequences. The first (batch sequence) was run to promote bacterial growth with uncontrolled pH decreasing from 7.3 to 5.2. It was further controlled at pH 5.2, the optimal pH for hydrogen production by the *C. butyricum* strain [34]. The second sequence was run at controlled pH (5.2) in order to promote hydrogen production in optimal conditions. The H_2 production for both sequences was plotted (Figure 4) taking into account continuous biogas flowrate monitoring and regular biogas analysis by gas chromatography. No significant

differences in hydrogen content in the biogas was recorded (around 58% of hydrogen) except for the 400 RPM + N_2 reactor since the biogas was diluted by the addition of N_2 (approximately 2–6% of hydrogen in the headspace). The results confirm the importance of pH control since whatever the stirring conditions the volume of hydrogen was approximately two times greater for the sequences with pH control than for the sequences without pH control. Moreover, the volume of hydrogen produced increased with stirring or sparging conditions.

The hydrogen yields reported in Table 2 have been calculated taking into account the volumetric production of hydrogen at the end of the sequence and the amount of glucose

Table 2: Optical density at 600 nm (OD_{600}), H_2 yields and Gompertz parameters of the hydrogen production by *C.butyricum* in 2.5 L AnSBR with or without pH control. The initial OD_{600} at the inoculation was measured between 0.42 and 0.5. All the R^2 for the Gompertz model were higher than 0.998.

				Gompertz parameter		
		Final OD_{600}	H_2 yields ($mol_{hydrogen} \cdot mol_{glucose}^{-1}$)	Lag time (hour)	Max. HPR ($L \cdot h^{-1)}$	Max. H_2 production (L)
Sequence without pH control	0 RPM	2.14	0.73	7.6	0.13	1.05
	100 RPM	2.1	0.67	5.3	0.142	0.86
	400 RPM	2.6	1.22	2.8	0.253	1.68
	400 RPM + N_2	2.4	1.21	3.7	0.281	1.57
Sequence with pH control at 5.2	0 RPM	2.14	1.58	9.	0.14	2.22
	100 RPM	2.2	1.68	7.6	0.191	2.34
	400 RPM	2.96	1.88	14.9	0.264	2.87
	400 RPM + N_2	4.5	3.09	8.9	0.278	4.2

consumed. Since the culture conditions were similar between the sequences without pH control in AnSBR and the BHP experiments, hydrogen production performance may be compared. The best yields obtained in AnSBR at uncontrolled pH at 400 RPM (1.22 $mol_{H2} \cdot mol_{glucose}^{-1}$) were similar to the lowest yield obtained in BHP tests (VNS conditions; 1.16 $mol_{H2} \cdot mol_{glucose}^{-1}$). Therefore, we conclude that the environmental conditions for H_2 production were less efficient in the stirred AnSBR than in the BHP tests. This will be further discussed in relation with the mass transfer coefficient presented in the section 3.4. However, It should already be noticed that, in the BHP tests, increasing stirring from 0 to 120 RPM resulted in an increase of the yield of approximately 30% from 1.16 $mol_{H2} \cdot mol_{glucose}^{-1}$ (VNS) to 1.54 $mol_{H2} \cdot mol_{glucose}^{-1}$ (VS), whereas in the AnSBR, stirring at 100 RPM did not improve the yields compared to the 0 RPM condition.

By contrast, a significant improvement (yields two-fold greater) was achieved by controlling the pH at 5.2. In the sequences with pH control, the highest yields were achieved in the experiment with 400 RPM + N_2 sparging, reaching 3.1 $mol_{H2} \cdot mol_{glucose}^{-1}$, i.e. approximately 60% higher than the yields recorded without gas sparging in the 400 RPM condition. Such yield improvements have also been described by other authors [3, 6] working with pure cultures and using degassing conditions.

The Gompertz model was fitted to the hydrogen production curves for sequences with or without pH control (Table 2). In the sequences without pH control the lag time was shorter than with pH control since higher initial pH favours bacterial growth instead of biohydrogen production [34]. Maximum HPR increases with stirring speed (in RPM) according to a regression line of 0.295 x RPM + 149 (R^2 = 0.968) in pH-controlled conditions or 0.322 x RPM + 121 (R^2 = 0.976) when pH was not controlled. As in BHP tests, this suggests that HPR, unlike H_2 yields, was not significantly pH-dependent but both were significantly improved when stirring speed increased. By contrast, sparging at 400 RPM did not lead to a

significant increase of HPR. This HPR limitation at 278 $mL \cdot h^{-1}$ (approximately 120 $mL \cdot L_{medium}^{-1} \cdot h^{-1}$) is probably due to other parameters such as substrate availability or cell density since six-fold greater HPR was recorded with the same *Clostridium* strain in bioreactors with biomass immobilisation operated in continuous mode [44]. In addition to the exchange surface area that also affected H_2 yields in BHP tests, these three parameters (exchange surface area, stirring speed and gas stripping) that are involved in H_2 transfer conditions were further investigated in 2.5 L AnSBR.

Different dissolved hydrogen concentrations were measured in the AnSBR depending on culture growth phase and operating conditions (Figure 4). High H_2 concentrations were already reached at the beginning of the exponential growth phase without N_2 stripping irrespective of pH control or stirring conditions. The highest concentrations were achieved in the nearly linear production phase of the sequences with pH control (i.e. after 44 or 47 hours of culture for 0 or 400 RPM respectively). Since the AnSBR was operated at atmospheric pressure with maximal hydrogen concentration of 58% in the gas phase, these values should be compared to the maximum theoretical concentration of 0.45 $mmol \cdot L^{-1}$ of hydrogen dissolved at equilibrium in the liquid phase. Most of the measured values were two to four times greater than the saturation H_2 concentration, except for the experiment at 400 RPM + N_2. Indeed, in this case, a low dissolved H_2 concentration of approximately 0.07 $mmol \cdot L^{-1}$ was measured due to low H_2 partial pressure achieved by constant degassing with N_2. In addition, stirring led to significant improvement in H_2 production since the highest levels of supersaturation and lowest H_2 production yields and rates were measured in non-stirred conditions. In such conditions hydrogen accumulates in the liquid phase and influences bacterial metabolism by inhibiting H_2 production and producing soluble metabolites other than acetate or butyrate.

3.3. Metabolite production

The soluble metabolite concentration analysed by HPLC (Table 1A) varied significantly among the different conditions performed in BHP tests. More glucose was consumed in stirred experimental conditions than in non-stirred conditions. In addition, no ethanol was produced in stirred conditions and lactate concentration was about three-fold lower than in the VNS bottles. In contrast, more acetate and butyrate were measured, which are usually related to the production of hydrogen in the *C. butyricum* metabolism. When compared to the other tests the HNS test showed an intermediate metabolite profile.

The mass balance (Table 1B) clearly shows the influence of stirring on metabolite production. Only 36.2% of *C. butyricum* metabolism is targeted towards the major butyric pathway in the VNS bottles, while up to 55.6% of metabolites reflect the butyric pathway in the HS experimental conditions. These results are in accordance with previous studies on hydrogen-producing *Clostridium* strains [8, 34, 45].

The AnSBR experiments confirmed results from the BHP tests (Table 3A). Indeed, increasing stirring speed without stripping resulted in high formate production as well as both a decrease in lactate production and an increase in acetate production. Higher butyrate and H_2 yields were achieved with pH control. In contrast, there was no significant change in butyrate yield with increasing stirring speed, and the greatest butyrate yield was recorded for unstirred AnSBR with pH control, i.e. when the highest H_2 supersaturation and the lowest acetate yield were recorded. In summary, stirring prevented further formate production in sequences with pH control and stripping limited carbon conversion to soluble metabolites (Table 3B).

1.1. K_La measurements

K_La was measured to link the mixing intensity of the different experimental conditions to

Table 3: Evolution of glucose and metabolites concentrations in mM [A] and carbon mass balance in % of glucose converted [B] in four different mixing and degassing conditions in 2.5 L AnSBR with *C. butyricum* in two successive sequences with or without pH control at 5.2 respectively (EFT : effective fermentation time after inoculation; EFT are slightly different since the experiments were not done simultaneously). Negative values are associated with the consumption of the metabolites produced in the first sequence.

A	EFT	Glucose	Lactate	Formate	Acetate	Ethanol	Butyrate
Sequence without pH control							
0 RPM	0	25,68	0,61	3,47	1,11	0,00	1,92
	23	0,00	8,98	20,33	6,87	3,82	15,26
100 RPM	0	24,54	0,59	2,67	1,15	0,00	2,34
	22	1,54	2,45	29,42	12,26	4,00	14,59
400 RPM	0	25,36	0,83	2,77	1,80	0,00	2,62
	23	1,16	3,26	23,62	13,90	2,88	14,78
400 RPM	0	24,97	1,05	4,42	1,47	0,00	2,49
+ N_2	23	1,02	6,16	16,40	11,89	1,58	16,99
Sequence with pH control at 5.2							
0 RPM	23,5	24,30	5,24	10,94	4,26	3,16	8,00
	53,5	0,00	2,83	17,17	8,69	3,44	28,21
100 RPM	22,5	24,37	1,42	17,31	7,02	2,18	8,73
	51	0,00	1,45	18,11	16,04	2,17	24,50
400 RPM	23,5	26,60	2,16	15,26	8,05	2,06	9,34
	72,5	0,00	1,06	14,85	19,80	2,66	26,28
400 RPM	23,5	25,16	3,79	10,28	6,78	1,29	10,88
+ N_2	48	0,00	4,51	9,98	17,11	1,38	24,12

B	Lactate	Formate	Acetate	Ethanol	Butyrate	CO_2	Sum
Sequence without pH control							
0 RPM	16,31	10,94	7,47	4,97	34,64	8,81	83,15
100 RPM	4,04	19,38	16,10	5,80	35,50	8,15	88,97
400 RPM	5,03	14,36	16,68	3,96	33,51	14,76	88,30
400 RPM+N_2	10,67	8,34	14,50	2,20	40,35	14,51	90,57
Sequence with pH control at 5.2							
0 RPM	-4,95	4,28	6,08	0,38	55,45	19,04	80,28
100 RPM	0,07	0,55	12,34	-0,01	43,13	20,23	76,31
400 RPM	-2,08	-0,26	14,73	0,74	42,48	22,75	78,36
400 RPM+N_2	1,42	-0,20	13,69	0,11	35,06	37,26	87,33

hydrogen production performance (yields and HPR), dissolved hydrogen concentrations and the metabolite profiles. K_La is the most significant parameter influencing the mass transfer rate (equation 5) and allows the comparison of different bioreactor configurations. Measurement of this parameter for H_2 gas in BHP tests requires a specific probe that would affect the measurement itself, therefore K_La was determined via O_2 gas experimentation after validating the procedure in a 2.5 L bioreactor. The K_La in the bioreactor was measured in water by dynamic gassing-out with oxygen and hydrogen, denoted $K_La_{(O2)}$ and $K_La_{(H2)}$ respectively. Equation (7) was fitted to the experimental values, giving K_La values. The determination coefficients R^2 obtained for oxygen and hydrogen were greater than 0.998 and 0.987 respectively. The measured K_La for oxygen and hydrogen coefficients were compared and they verified equation (6) at ± 10% of the proportional value of 1.3612 at 30°C (Table 4). This allowed us to estimate $K_La_{(H2)}$ in the bottles from $K_La_{(O2)}$ measurement using the same WTW polarographic probe as in the bioreactor.

The mass transfer coefficient K_La associates the mutually interacting parameters of specific interfacial area and mixing intensity. By increasing these two parameters separately, the H_2 mass transfer and the production of hydrogen were improved. Since the specific interfacial area, denoted by 'a' in the K_La coefficient, is the ratio between the liquid volume and the interfacial area, an increase of the interfacial area of the bioreactor allows an enhancement of the transfer (Table 4). Indeed, as evidenced in the VNS and HNS BHP tests, by doubling the surface of exchange in the bottles, the K_La also increases from 0.32 to 0.63 h^{-1}. By comparison, the AnSBR developed a lower specific surface than the VNS bottles (0.05 $m^2.m^{-3}$ and 0.14 $m^2.m^{-3}$ respectively) resulting in a 5-fold lower K_La in unstirred conditions (0.06 h^{-1}) and 37% lower H_2 yields. The mixing intensity of the culture also strongly influenced the mass transfer coefficient; the maximum stirring speed in the BHP tests and in the AnSBR

Table 4: K_La determination for different conditions in 200 mL BHP tests bottles and in 2.5 L AnSBR containing water by dynamic gasing out method (for O_2) and evolution of dissolved hydrogen concentration; ratio calculated between the K_La determined for O_2 and H_2 (in 2.5 L AnSBR only).

		$K_La\ O_2\ (h^{-1})$	$K_La\ H_2\ (h^{-1})$	K_La_{H2} / K_La_{O2}
BHP bottles	VNS	0.318	n.r.	-
	HNS	0.627	n.r.	-
	VS	6.257	n.r.	-
	HS	n.r	n.r.	-
AnSBR	0 RPM	0.061	0.080	1.328
	100 RPM	0.191	0.246	1.288
	400 RPM	0.524	0.768	1.466
	400 RPM + N_2	3.346	4.556	1.362

n.r. : not recorded data

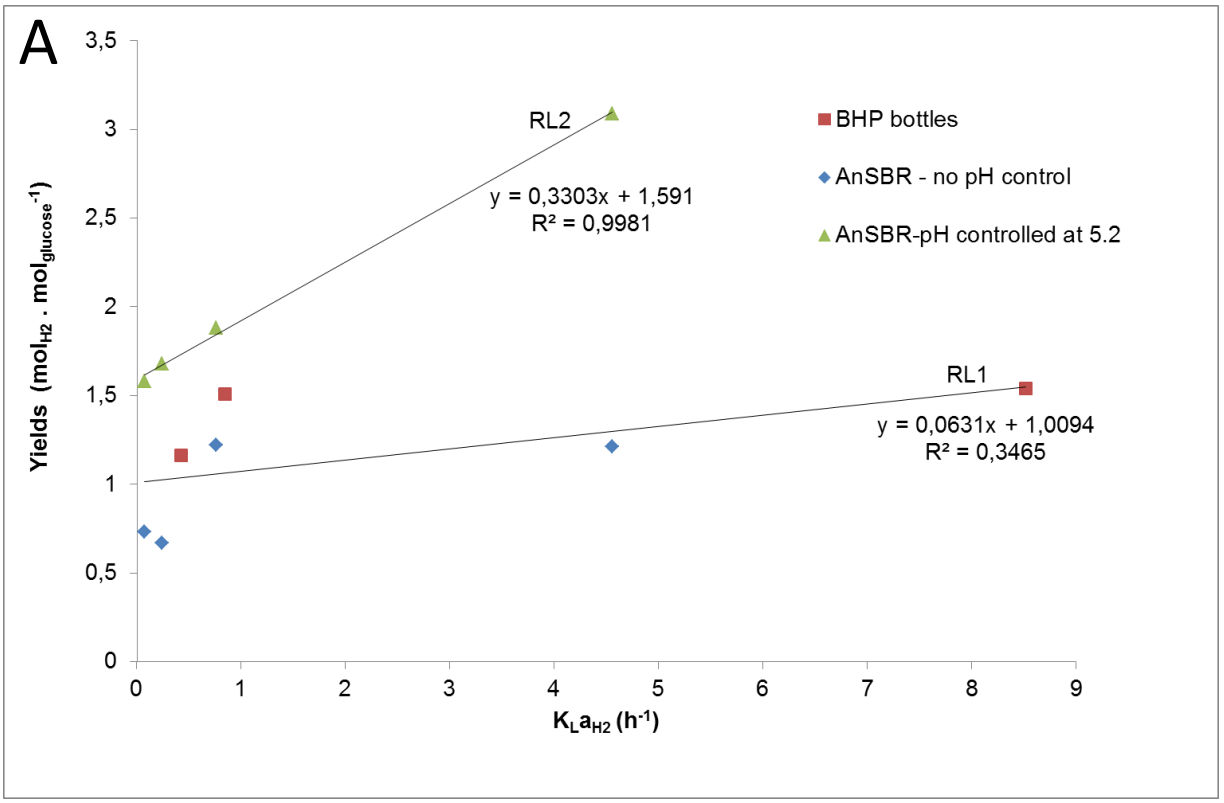
resulted in 20- and 10-fold increases in the K_La, respectively, in comparison to the K_La measured in unstirred conditions. Moreover, the addition of N_2 degassing resulted in another 6-fold increase of K_La (up to 4.56 h^{-1}) and a global 60-fold increase when compared to the unstirred reactor. Therefore, mass transfer needed from 9 min to about 9 h to decrease the concentration of H_2 dissolved in the liquid medium by 50%. Note that the addition of nitrogen influenced the K_La by increasing both the specific interfacial area by dispersing fine bubbles in the media and the mixing state by creating an ascendant flow.

Nitrogen stripping in the bioreactor also influenced mass transfer by reducing the partial pressure in the headspace (P_G in equation 5) via dilution of the biogas produced. Indeed, a lower gaseous partial pressure P_G would improve the potential of transfer and result in a

higher mass flow rate. Therefore N_2 stripping results in a double effect on the hydrogen mass transfer equation (5) and allowed the high yields achieved in the degassed bioreactor.

The global effect of K_La on hydrogen production performance is shown by plotting K_La values evaluated in the BHP and AnSBR experiments versus the corresponding H_2 yields and HPR (Figure 5). The purpose of Figure 5A is to develop a model between H_2 yields and H_2 mass transfer potential even though the H_2 mass transfer was not directly quantified in 200 mL bottles but estimated from O_2 mass transfer potential after validation of equation (6) with our measurement tools (Table 4). One relation (RL1) describes results achieved without pH control (in bottles and bioreactor) and the other relation (RL2) describes results achieved in the bioreactor with pH controlled at 5.2.

While taking into account the variability of environmental conditions for H_2 production in BHP tests and in AnSBR with or without pH control, these results confirm that H_2 production efficiency can be directly linked to the K_La value of the bioreactor. This means that considerable efforts would be needed in large-scale bioreactors to further increase H_2 yields towards 2 $mol.mol_{glucose}^{-1}$ without pH control and to 3.5 $mol.mol_{glucose}^{-1}$ with pH control. We used different mixing and stripping conditions to develop the regression line RL2 at pH 5.2 (H_2 yield = 0.33 x $K_La_{(H2)}$ + 1.591), showing a similar trend to that obtained by Kraemer and Bagley (H_2 yield = 0.382 x $K_La_{(H2)}$ + 0.987; R^2 = 0.998; [29]) achieved at constant stirring speed but for stripping flow rates varying from 0 to 12 $mL.min^{-1}.L^{-1}$ (taking into account the K_La in basal medium calculated with an average α ratio of 2 [29]). In addition, the H_2 yield achieved with mixed culture [29] in similar environmental conditions to ours was 20–37% lower, confirming the benefit of using pure strains for H_2 production. By contrast, HPR was less affected by an increase of the mass transfer parameter at K_La values greater than 1



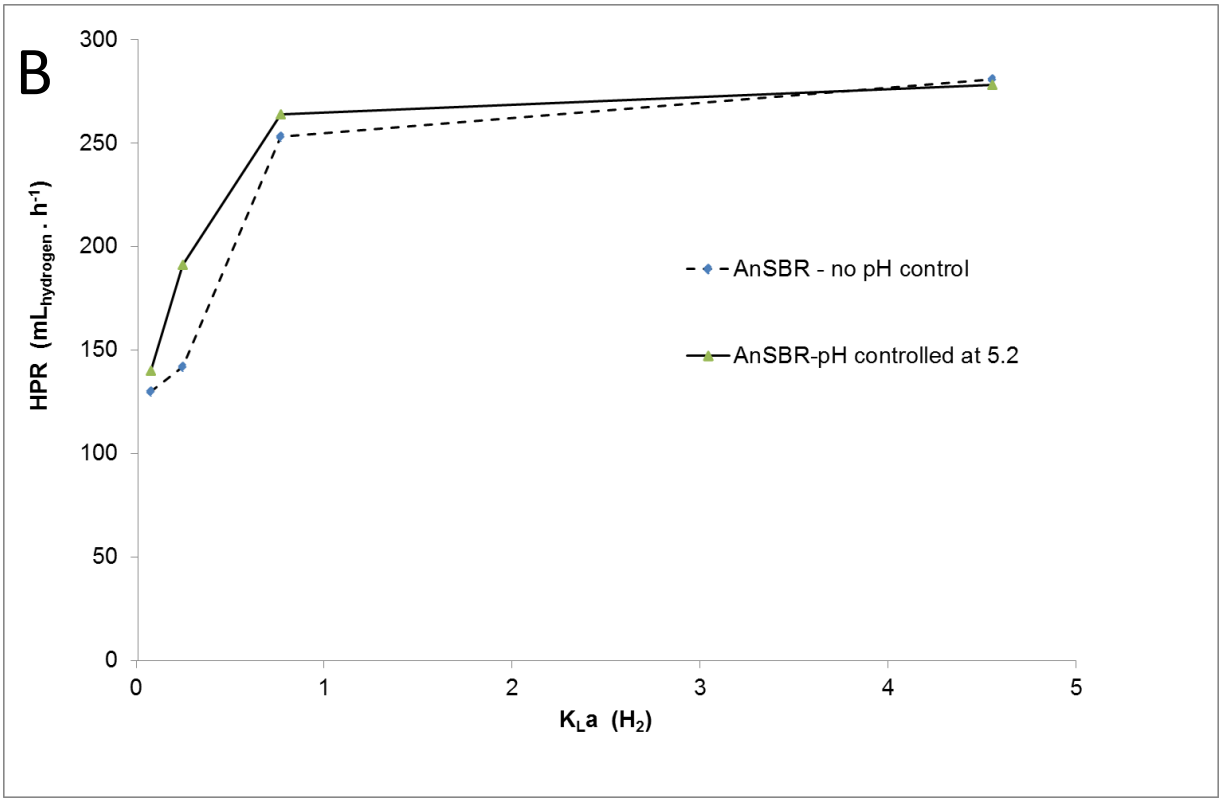


Figure 5: H_2 production yields [A] from glucose converted in BHP tests or in the AnSBR (sequences with or without pH control) and HPR [B] in the AnSBR at different mixing state determined by the K_La parameter. The K_La values for BHP tests were estimated on the basis of the oxygen transfer rate (being proportionally linked to the hydrogen transfer rate by a factor of 1.36). The regression lines are reported either [RL1] for the AnSBR with pH control at 5.2 or [RL2] for the seven experiments in bottles and AnSBR without pH control.

(Figure 5B) probably due to some other limitations. Consequently, since HPR was directly linked to stirring speed but not affected by stripping at 400 RPM, the HPR recorded at 0, 100 and 400 RPM may be fitted by the equations HPR = 0.17 x $K_La_{(H2)}$ + 0.14 (with pH control) or HPR = 0.19 x $K_La_{(H2)}$ + 0.11 (without pH control) at the optimum pH 5.2 ($R^2 > 0.96$). However due to high H_2 supersaturation measured at low K_La values, the apparent HPR equation determined at pH 5.2 would underestimate the 0.17 slope coefficient and overestimate the 0.14 y-intercept coefficient. Nevertheless, further experiments would be needed with regular measurement of the dissolved H_2 concentration to improve this equation by subtracting the physical H_2 mass transfer kinetic. However this contribution would not be higher than 10% of the HPR recorded without stirring since, when estimated from the derivative of the exponential decrease of H_2 concentration versus time (supplement Figure) the H_2 mass transfer from the 2.3 L liquid medium to the headspace increases from 1.2 to 2.5 $mL.h^{-1}$ at a concentration of 0.3 and 0.6 $mmol.L^{-1}$ and up to 6.6 $mL.h^{-1}$ when extrapolating the curve up to the highest H_2 dissolved concentration (i.e. 1.6 $mmol.\ L^{-1}$) recorded during fermentation with pH controlled at 5.2. Moreover, as shown in Figure 4, a global decreasing trend is suggested for the maximum H_2 dissolved concentration with increasing K_La.

2. General discussion

The results achieved in this work clearly show the direct relation between hydrogen yield and hydrogen mass transfer properties in the bioreactor. Many authors have related hydrogen production efficiency to gaseous hydrogen partial pressure or mixing state of the culture. However, these factors impact first on mass transfer which subsequently directly influences the production of hydrogen. While previous studies confirm the results obtained in our study, in this paper we directly quantify the mass transfer parameters and their effect on H_2 production. Indeed, we investigated these factors (the media hydrodynamic related to K_La and the potential of transfer related to the gaseous partial pressure) separately and, to our

knowledge, this paper presents the links between K_La and H_2 production yields and rates for the first time.

Most previous studies used undefined bacterial cultures [27–30] and, as a consequence, the results may be affected by additional parameters such as the presence of hydrogen-consuming communities that are also influenced by H_2 concentration in the culture media. In contrast we used a pure culture of one of the most efficient bacteria strains for biohydrogen production.

Experimental systems that improve mass transfer conditions have significant effects on hydrogen yields and metabolite production. On one hand, the hydrodynamic conditions that have been investigated at different stirring speeds [18, 20, 25, 46] and with different reactor configurations [19, 47] resulted in greater hydrogen production in highly mixed media (turbulent flow) due to higher mass transfer. In our study, the H_2 yield and production rate were 20% and 200% higher, respectively, at 400 RPM compared with unstirred. On the other hand, conditions in the bioreactor headspace leading to a lower hydrogen partial pressure also enhanced hydrogen production performance. This was evidenced by gas flushing with N_2 or H_2 [24, 48, 49], by applying higher [23, 52] or lower total pressure [8, 9, 11, 25, 45, 52]. These techniques allowed achievement of yields closer to the theoretical maximum of 4 $mol_{H2} \cdot mol_{glucose}^{-1}$. Authors also combined both effects by sparging gases directly in the media, therefore affecting simultaneously the hydrodynamic behaviour and hydrogen partial pressure in the bioreactor [16, 17, 29, 50, 51]. Finally, new bioreactors have been proposed with original design or specific carriers that improved liquid distribution and the specific transfer area [44, 47, 52, 53].

Few studies have investigated the relationships between K_La, dissolved hydrogen concentration and hydrogen production performance of pure strains. The improvements reported in these studies were attributed to the thermodynamic equilibrium of the hydrogen

production process, as shown by free-Gibbs energy survey and analysis [10, 15, 46, 54]. They were often directly linked to the hydrogen partial pressure, assuming that equilibrium was achieved in the bioreactors [14, 55]. However, dissolved hydrogen concentration has rarely been measured [26, 27]. We assume that the enhancement of hydrogen mass transfer resulted in a lower dissolved hydrogen concentration and, as a consequence, to a shift in the bacterial metabolism with higher H_2 yields. Our study with a pure strain using both mechanical stirring and gas stripping confirms this hypothesis. Therefore equal attention should be paid to dissolved hydrogen concentration and headspace hydrogen concentration.

Our results also showed that, except at atmospheric pressure and low gaseous hydrogen partial pressure, the liquid medium was supersaturated. In most previous studies, the bioreactors should not be considered at liquid–gas equilibrium since they were less stirred or degassed than in our experiments carried out at 400 RPM with gas stripping. For instance, high supersaturation factors (i.e. the ratio between the measured dissolved concentration and the theoretical concentration at the phase equilibrium) have been reported in H_2-producing reactors (from 3 to 14) and in anaerobic digesters (from 3 to 105) [26]. The maximum supersaturation factors reached in our work were within the same range of values; 7 in the VNS BHP experiment (Figure 3) and 4 in the 0 RPM AnSBR (Figure 4).

Supersaturation should be lowered by enhancing the gaseous mass transfer flow rate to increase H_2 yields and approach the theoretical maximum of 4 $mol_{H2}.mol_{glucose}^{-1}$. While degassing is an effective way to simultaneously increase the K_La and lower the partial pressure, it leads to hydrogen dilution which at high levels is unfavourable, particularly for energetic end-use in industrial processes. Therefore, future research should focus first on the optimal ratio between H_2 dilution level (by degassing techniques) and secondly on bioreactor design and hydrodynamic parameters in order to maximise gaseous mass transfer. The feasibility of scaling-up the process should also be taken into account in research and

development since, for instance, mixing conditions have an influence on hydrodynamic conditions but also on dynamic stress on microorganisms. In our study, the stirring speed of 400 RPM, corresponding to a peripheral speed of 0.94 $m.s^{-1}$ does not seem to stress the bacterial cells. Optimum values should be determined for all parameters (e.g. degassing flowrate, mixing conditions, investment and operational costs for specific bioreactor design) and their resultant effects on bioreactor performance.

3. Conclusions

In this work, we report links between hydrogen production performance, metabolite production profile, dissolved hydrogen concentration and mass transfer conditions for a pure *Clostridium* strain. Both an increase in stirring speed to 400 RPM and stripping with nitrogen improved hydrogen production achieving yields and HPR two-fold higher than without mixing or stripping, up to 3.1 $mol_{H2} \cdot mol_{glucose}^{-1}$ and 278 $mL.h^{-1}$ respectively. In conditions with low mass transfer properties, lower H_2 yields were recorded due to H_2 accumulation in the liquid phase and supersaturation up to seven-fold higher than equilibrium conditions. Therefore a link was determined between the increase of mass transfer coefficient (K_La) reducing dissolved hydrogen concentration in the culture medium and the improvement of hydrogen yields. Consequently, further investigations on H_2 production should measure dissolved H_2 concentration instead of H_2 partial pressure. A direct link between HPR and K_La was only evidenced for K_La up to 0.77 h^{-1}. The link would probably be extended in operating conditions with higher substrate availability or biomass density. Therefore, improved mass transfer efficiency should be studied via optimum mixing and/or degassing conditions in specially adapted bioreactors for approaching theoretical hydrogen production yields and maximising the energy recovery in the hydrogen production process.

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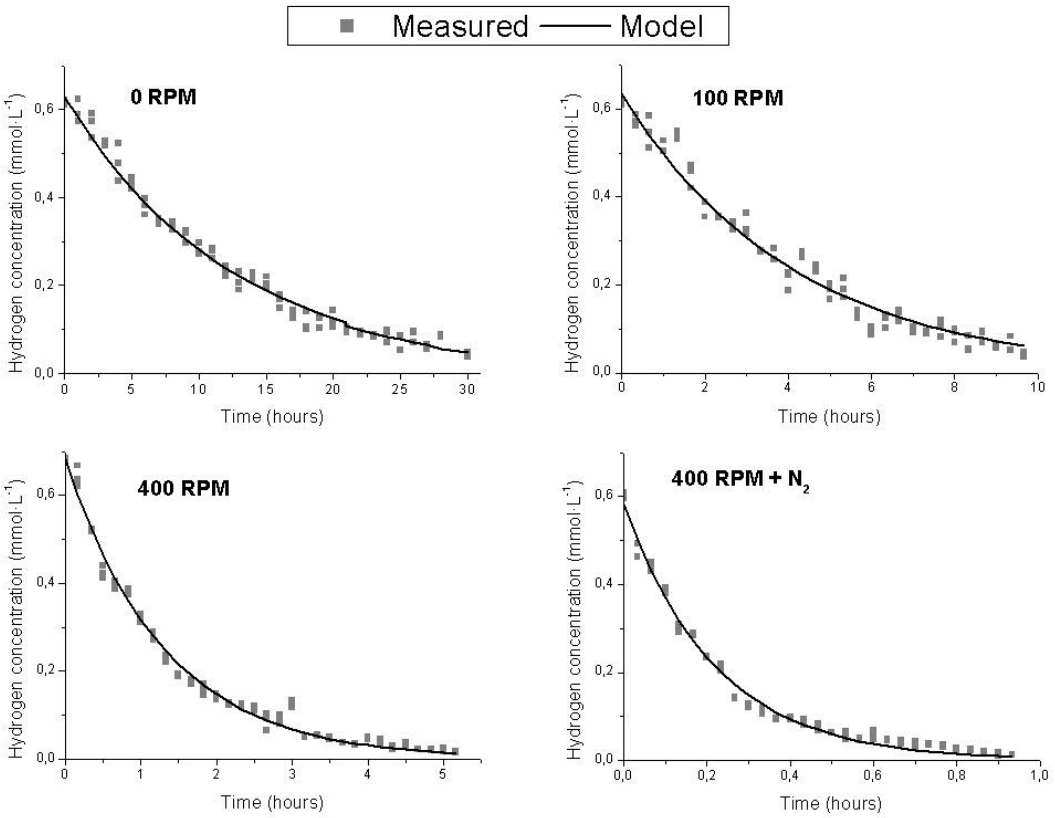
Additional file



Supplementary figure : decreasing exponential hydrogen concentration and fitting curve measured in the 2.5L AnSBR with hydrogen according to the equation (7). Each liquid sample was equilibrated in a headspace vial and injected three times in GC corresponding to the multiple points at the same abscissa. All the R^2 were higher than 0.987.