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Effect of the Nitrogen Source on the Hydrogen Production Metabolism and hydrogenases of *Clostridium butyricum* CWBI1009

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Abstract

Investigations were carried out to determine the effect of various concentrations of organic and ammonium nitrogen sources on fermentative hydrogen production by the strain *Clostridium butyricum* CWBI1009. The results indicated that the H_2-producing metabolism of the strain is favoured within the range (0.56-0.062 g_N/L) of peptone and $(NH_4)_2SO_4$. Optimal overall performance (*i.e.* 1.43 ±0.08 mol H_2/mol glucose and 1.08 ±0.03 mL_{H2}/h, respectively) was achieved with 0.062 g_N/L of casein peptone. The study of the amino acid uptake and the gene expression pattern for four [FeFe]-hydrogenases and the nitrogenase showed that nitrogen was in excess in all the experiments

with a nitrogen concentration above 0.062 g_N/L and, at that optimal concentration, the expression of the *HydB2* gene would be responsible for the much higher H_2 yield.

Keywords : biohydrogen; *Clostridium*; nitrogen; hydrogenase; amino acid

1. **Introduction**

Hydrogen is regarded as an attractive energy vector for the future due to its clean and direct combustion characteristics. Moreover, a hydrogen-based economy could reduce our dependence on fossil fuels. However, hydrogen is mainly found as part of molecular compounds and currently its production is largely dependent on the use of fossil fuels such as natural gas. This results in the release of huge amounts of non renewable CO_2 and consequently to global warming and environmental degradation [1, 2]. Alternatively, hydrogen produced by microorganisms from renewable biomass is promising since the whole process can be considered as carbon neutral [3].

Among the different microbiological routes to hydrogen, dark fermentation using facultative and/or strict anaerobic bacteria has received major attention. Most of the extensive research on this topic has focussed on the advantages of hydrogen production combined with waste treatment [4-7] since the major

limiting factor in the scaling up of dark fermentation is the high cost of the main feedstock and some other nutrients to promote growth and metabolism.

Pure sugars are frequently used as the carbon source and casein peptone or yeast extract as the organic nitrogen source. For industrial-scale applications they should be replaced by readily available and cheap carbohydrate-rich residues and ammonia-based substitutes in order to decrease process costs [2, 6, 8].

Nitrogen-containing nutrients (*e.g.* ammonia, vitamins, and proteins) have a critical impact on fermentation performance since nitrogen is required for cell replication, maintenance, metabolism, and the production of carbohydrate-hydrolysing enzymes [9, 10]. Moreover, the literature clearly indicates that acidogens such as *Clostridium sp.* and *Enterobacter sp.* can utilise not only organic nitrogen (*e.g.* proteins, amino acids), but also ammonia [9, 11, 12]. Therefore, the use of suitable pretreated residues containing ammonia (e.g. animal manure) should also be considered in biohydrogen production, since it is widely available and its disposal represents a significant cost if serious risks of air and water pollution are to be avoided [13]. As a consequence, there is a need to deeply investigate the nitrogen requirements for H_2-producing strains and their impact on bacterial metabolism and major enzymes.

The *Clostridium butyricum* CWBI1009 strain was selected for the study due to its high hydrogen production activity while fermenting different carbon sources (glucose, disaccharides or starch) [14-16]. Moreover, the strain achieved very

high performance (up to 3.4 mol H_2/mol glucose) in immobilised cultures [17]. Hydrogen is produced via pyruvate-ferredoxin oxidoreductase (PFOR) and, at a lower extent, via NADH-ferredoxin oxidoreductase (NFOR) [18, 19]. Both enzymatic complexes transfer electrons to [FeFe]-hydrogenases which generate hydrogen by a reversible reduction of protons accumulated during fermentation. This mechanism controls the electron flow within the cell and therefore determines the direction of by-product synthesis [20].

However, little is known about the physiological and biochemical properties of clostridial [FeFe]-hydrogenases and their role in the hydrogen metabolism [19, 20] since most of the investigations focussed on the commonly known CpI (HydA) from *Clostridium pasteurianum* [21]. *Clostridium butyricum* CWBI1009 possesses four different [FeFe]-hydrogenases, three of which (*hydA2*, *hydB2*, *hydB3*) are monomeric intracellular enzymes and one (*hydA8*) is associated with the heterotrimeric hydrogenase cluster [19, 20].

The objectives of this study were to further optimize the nitrogen source for biohydrogen production by the strain *Clostridium butyricum* CWBI1009. This was carried out by monitoring the H_2 yields (mole of H_2 produced per mole of glucose consumed), production rates (mole of H_2 produced per hour) and the formation of soluble by-products in 270 mL batch cultures at different ammonium and peptone concentrations. In addition, particularly relevant conditions were selected for amino acid fingerprinting (HPLC-UV) and

expression pattern analysis (RT-qPCR) of the four [FeFe]-hydrogenase genes using specifically designed primers [22, 23]. The same was done for the nitrogenase gene since its role in hydrogen production by *Clostridium butyricum* CWBI1009 appears to depend on the environmental conditions [19].

2. **Materials and methods**

2.1. Bacterial strain and culture media

The strain cultured in this work, *Clostridium butyricum* CWBI1009 (accession no. GU395290), was previously isolated from an anaerobic sludge [14]. The strain was grown in variously formulated buffered synthetic media previously used for growth and hydrogen production with *Clostridium* and *Enterobacteriaceae* [11, 14, 16]. The MDT culture medium (without the added variable mineral or organic nitrogen source) contained, per litre of deionised water, glucose monohydrate (5g), yeast extract (0.5g), Na_2HPO_4 (5.1g), KH_2PO_4 (1.2g), $MgSO_4.7H_2O$ (0.5g), cysteine hydrochloride monohydrate (0.5g). L-cysteine hydrochloride was used to ensure strictly anaerobic conditions in the media and its effect was confirmed by measured redox potential ranging from -200 to -250 mV. To determine the impact on hydrogen production of an organic nitrogen source (casein peptone) compared to an ammonia-based substitute ($(NH_4)_2SO_4$), each of the two sources was used in a separate experiment at 10 concentrations ranging from 0.025 to 2.3 g of total nitrogen/L added to MDT

105 medium (g_N/L). They supplement the basal nitrogen content of 0.055 and 0.034
106 g_N/L related to the yeast extract and cysteine initial concentration in MDT
107 medium, respectively. The total amount of nitrogen in the medium compounds
108 was calculated according to the supplier's product description and confirmed by
109 measuring the total nitrogen and ammonia content using HACH kits for total
110 nitrogen and ammonia and a HACH DR/2010 spectrometer. All the chemicals
111 used were of analytical or extra pure quality and were supplied by Merck, VWR
112 and Sigma. Casein peptone and yeast extract were supplied by Organotechnie
113 (La Courneuve, France).

114 **2.2. Experimental set-up**

115 BHP (Biochemical Hydrogen Potential) tests were carried out to assess the
116 influence of both nitrogen sources in 270 mL serum bottles (200 mL working
117 volume). The sterile carbon source (glucose monohydrate in solution in
118 deionised water) was added separately (to a final concentration of 5 g/L) to
119 prevent Maillard reactions between the carbohydrates and the amino acids. The
120 L-cysteine hydrochloride monohydrate was also added separately to obtain a
121 final concentration of 0.5 g/L.

122 After the preparation of the MDT medium and adjustment of the pH to 7.3 the
123 bottles were sterilised and cooled down before inoculation with 3 mL
124 suspension from a fresh (incubated for 48 h) preculture tube. The bottles were
125 then capped with a butyl stopper as described by Lin *et al.* [24] and flushed with

nitrogen to remove the remaining oxygen in the gas phase before incubation at 30 °C. Each BHP test was conducted in triplicate at least and all the experimental conditions were monitored until biogas production had completely ceased (*i.e.* 96 h with casein peptone and 144 h with ammonium sulfate).

2.3. Monitoring and analytical methods

Biogas analysis

The biogas produced in the bottle experiments was collected by means of sterile syringes as described by Hamilton *et al.* [11]. Water supplemented with 9 N KOH was used in replacement equipment designed by Hiligsmann *et al.* [16] in order to sterilely monitor biogas composition (H_2 and CO_2), except for the last non-sterile gas sample which was analysed by gas chromatography. The proportion of hydrogen gas was determined using a gas chromatograph (GC: Hewlett Packard 5890 Series II, UK). The chromatograph was fitted with a thermal conductivity detector (TCD) and a 30 m x 0.32 mm GAS PRO GSC capillary column (Altech) in series with a 20 m x 0.25 mm CarboPLOT P7 column (Chrompak). The temperatures of the injection, the TCD chambers and the oven were maintained at 90°, 110° and 55 °C respectively. Nitrogen was used as the carrier gas in the column at a flow rate of 20 mL/min.

Glucose and soluble metabolites analysis

Culture samples were collected regularly to operate measurements and to harvest culture components. The samples were centrifuged at 12000 g for 30

147 seconds and the supernatants were filtered through a 0.2 µm cellulose acetate
148 membrane (Sartorius Minisart). The analyses were performed using an Agilent
149 1100 series HPLC and an Agilent ChemStation was used for instrument control
150 and data acquisition (Agilent Technologies, CA, USA). For the quantification of
151 glucose, ethanol, lactate, acetate, formate and butyrate, the HPLC was equipped
152 with a Supelcogel C610-H column preceded by a Supelguard H precolumn
153 (oven temperature 40 °C) and a differential refraction index detector (RID,
154 detection cell maintained at 35 °C). An isocratic mobile phase with 0.1% H_3PO_4
155 (in MilliQ water) was applied at a flow rate of 0.5 mL/min for a duration of 35
156 min at a maximum pressure of 60 bars.
157 *Amino acids analysis*
158 For the separation of amino acids by reverse phase chromatography, the HPLC
159 was fitted with a Zorbax Eclipse XDB-C_{18} column (150 × 4.6 mm, 5 µm,
160 Agilent) preceded by a Phenomenex C_{18} security guard column (4.0 × 3.0 mm,
161 Phenomenex). The mobile HPLC grade phases A (40 mmol/l Na_2HPO_4 at pH
162 7.8) and B (45% acetonitrile, 45% methanol, 10% water) were filtered through a
163 0.22-micron Millipore Durapore PVDF membrane filter. Automatic pre-column
164 derivatisation with 9-Fluorenylmethyl chloroformate (OPA-3MPA) and o-
165 phthalaldehyde 3-mercaptopropionic acid (FMOC) was performed at room
166 temperature, in accordance with the injector programs listed in Table 1, using 1
167 µL of sample. After the derivatisation 0.5 µL of the mixture was injected for

each chromatographic separation. Primary amino acids were derivatised online with OPA-3MPA and identified with a photodiode-array UV detector at 338 nm. Hydroxyproline and proline were derivatised by adding FMOC to OPA-3MPA and detected at 262 nm. The chromatographic separation was obtained using the elution gradient indicated in Table 2 with a column temperature of 40 °C. The column was initially conditioned as follows: mobile phase B was pumped for 25 min at a flow rate of 2 mL/min, followed by 15 min of 10% methanol at 1.0 mL/min and 20 min of mobile phase A (pH 7.8) at 2 mL/min. The flow rate was constant at 2.0 mL/min throughout the analysis. The run time, injection to injection, was of 27 min. In order to maintain column integrity, a mixture of 90% isopropanol and 10% methanol was run through it after every 15 injections. The linearity and accuracy of the method were assessed using three different concentrations (1, 5 and 10 mg/mL) of amino acid standard mixtures injected in triplicate. All common 20 amino acid standards were obtained from Sigma Chemical. FMOC, OPA-3MPA and borate buffer were obtained from Agilent.

2.4. RT-qPCR

RNA was extracted using a RiboPure^{TM}-Bacteria (Ambion) and cDNA was prepared as described in the Reverse Transcription System manual (Promega); as previously described [19]. Specific primers and probes for the 4 [FeFe]-hydrogenases of *Clostridium butyricum* CWBI1009, *i.e. hydA2*, *hydA8*, *hydB2* and *hydB3* and *16SrRNA* were designed in this study. Primers targeting the

reference genes, *i.e. recA*, *gyrA* were as described before [23] and are given in Table 3. The quantitative PCR amplifications were carried out with a Mini Opticon (BioRad) apparatus.

The DNA template used for a standard curve was prepared as previously described [23]. For the gene expression analysis 1 µL of cDNA was used. The total volume of the PCR mix was 25 µL. Each reaction consisted of 1 x PCR mix (ABsolute^{TM} Blue QPCR SYBR$^{®}$ Green Fluorescein Mix or ABsolute^{TM}Blue QPCR mix, Thermo Scientific) with each primer or hydrolysis probe (HPLC cleaned, Biomers, Germany) at a final concentration of 150 nM. Each sample was analysed in triplicate. A 'no template' control was included in each run. The specificities of the primers were verified at the end of each qPCR reaction by performing a melting curve analysis (SybrGreen based quantification). The standard curve preparation and the cycling conditions for SybrGreen chemistry were as previously described [23]. For the probe-based chemistry the initial denaturation of 15 min was followed by 40 cycles of denaturation at 95 °C for 15 s and a primer annealing/amplification step at 60 °C for 30 s. The reaction efficiency was calculated as factor specific using the equation $E=10^{-1/slope}$ [25].

For gene expression analysis the relative expression levels were calculated with a Relative Expression Software Tool 2009, REST© [26]. To estimate the up- and down-regulation of the genes analysed, the Cqs obtained were compared

with those of the reference genes and with an external standard control gene. The stability of the 3 reference genes was determined using the BestKeeperExcel-based tool [27].

3. **Results and discussion**

Very few information is currently available about the impact of different types and concentrations of nitrogen sources on hydrogen production by pure *Clostridium butyricum* and on the associated by-products [9, 28]. Therefore, the experiments reported here were carried out to study the effect of 10 different concentrations of casein peptone (11.2% total nitrogen, average molecular weight 681 Da) and 10 equivalent concentrations of $(NH_4)_2SO_4$. In order to enable easy comparisons, the nitrogen content in the different media was reported in terms of total nitrogen.

Aiming to rapidly survey a large range of potentially interesting conditions, BHP tests were carried out in 270 mL serum bottles without pH regulation. No more biogas production could be measured after 96 h of incubation with casein peptone and 144 h with $(NH_4)_2SO_4$. The hydrogen production evolved according to a sigmoidal curve as reported in former experiments with the same *Clostridium* strain in BHP tests with 5 g/L casein peptone [14, 16]. The H_2 yields (mole of H_2 produced per mole of glucose consumed), H_2 production rates (mole of H_2 produced per hour) and the release of soluble by-products were

monitored for the 20 different conditions. In order to better understand the large differences that were observed in H_2 production activity, three conditions (0.062 and 0.056 g_N/L casein peptone and 0.56 g_N/L $(NH_4)_2SO_4$) were selected for a study of (i) the amino acid distribution (HPLC-UV) and of (ii) the gene expression pattern (RT-qPCR) for the four [FeFe]-hydrogenases and for the nitrogenase, all of which had previously been identified in the genome of *Clostridium butyricum* [20] using specifically designed primers [19, 23].

3.1. H_2 production activity

Casein peptone as nitrogen source

The control sample (indicated by a black arrow on Fig. 1) contained the casein peptone concentration used in previous laboratory studies, *i.e.* 5 g/L containing 0.56 g_N/L total nitrogen [11, 14]. With this sample, a total of 181.5 ±7.79 mL_{biogas} were produced after 96 h of culture and, due to release of acid metabolites, the pH of the medium dropped from 7.6 to 4.7. Based on an average biogas H_2 content of 68.6 ±1.4% (Fig. 1.A) it was calculated that 107.6 ±6.2 mL_{H2} (Fig. 1.B) and 73.9 ±6.2 mL_{CO2} were produced, yielding 1.10 ±0.05 mol H_2/mol glucose (Fig. 1.C) at a maximum production rate of 0.98 ±0.04 mL_{H2}/h (Fig. 1.D).

This H_2 yield is similar to that reported elsewhere in similar conditions [16] and was only about half of that recorded with the same strain and medium in 2.3L batch bioreactors, but at a fixed optimum pH of 5.2 [14]. The large difference

252 can be explained by the absence of pH regulation (ΔpH up to 3 units) and an
253 increase in pressure in the gas phase (up to 0.5 bar) during growth in BHP tests.
254 The determination of the optimal concentration of casein peptone for hydrogen
255 production was carried out by testing 10 concentrations ranging from 0.025 to
256 2.3 g_N/L. With the sample containing twice the nitrogen content of the control
257 sample, the cumulative H_2 production, H_2 yields and production rates decreased
258 by 14%, 20%, and 21%, respectively. The trend was confirmed with a further
259 increase of concentration up to 2.3 g_N/L (Fig. 1).
260 Since casein peptone concentrations higher than 0.56 g_N/L were not beneficial to
261 hydrogen production, the nitrogen content was decreased aiming to determine a
262 lower concentration limit. A decrease from 0.56 to 0.25 g_N/L had a significant
263 positive impact on the H_2 yields and production rates (Fig. 1.C and 1.D).
264 Surprisingly this trend continued until the nitrogen content was reduced to 0.062
265 g_N/L. This 8-fold decrease resulted in a 30% increase in the H_2 yield compared
266 to the control sample (Fig. 1.C, from 1.10 ±0.05 for the control sample to 1.43
267 ±0.08 mol H_2/mol glucose).
268 However below 0.062 g_N/L a very strong decrease occurred for both the H_2 yield
269 and the H_2 production rate: 27% and 38% respectively (Fig. 1.C and 1.D). This
270 shift may have been due to a lack of sufficient available nitrogen for cellular
271 anabolism and basal metabolism [9]. This assumption was supported by the
272 detection of increasing residual glucose at lower concentrations of casein

peptone (Table 4, column 2). Therefore, as suggested in the literature [9, 28, 29], the uptake of the substrate seems to be highly dependent on the nitrogen content of the medium.

Based on these results the lower limit for optimized hydrogen production by *Clostridium butyricum* CWBI1009 was determined to be ca. 0.062 g_N/L of organic nitrogen. This finding is highly relevant for cost reduction in biotech applications, comparing to control or other conditions with similar peptone-based nitrogen source. Other opportunities to reduce cost could be investigated through less expensive mineral nitrogen source or cheap residual organic source providing the most essential amino acids for hydrogen production metabolism. The experiments reported below deal with culture medium containing ammonium or amino acids and further analyses to assess their specific consumption by the *Clostridium* strain.

Bacto-tryptone and bacto-casamino acids as nitrogen source

To determine the effect of the complexity of the organic nitrogen source, tests were also carried out with easily assimilated pure nitrogen sources, namely bacto-tryptone (small peptides resulting from casein pancreatic digest) and bacto-casamino acids (mostly free amino acids), at 6 different concentrations (ranging from 0.56 to 0.056 g_N/L, see Supplementary Figure). The performances were compared to that obtained for equivalent conditions with casein peptone.

Under all the tested conditions the biogas H_2 content was on average 10% higher and a similar increase in H_2 yields was also recorded. In addition, the H_2 production rates were much higher than those achieved for the equivalent nitrogen concentration of casein peptone. However, since the H_2 yields were very similar to those for the control sample with casein peptone (*i.e.* around 1 mol H_2/mol glucose) and considering the extremely high price of these pure nitrogen sources, they were not investigated further. Interestingly the decrease in H_2 production activity was observed at exactly the same low concentration as with the casein peptone source. These results enable to consider an optimal organic nitrogen content of ca. 0.062 g_N/L.

Ammonium sulfate as nitrogen source

The prospect of using an ammonia-based substitute is of particular interest for biotech applications since the cost of the nitrogen source could radically be about 10-fold lower comparing to casein peptone. Therefore, we studied the effect of 10 different $(NH_4)_2SO_4$ concentrations (Fig. 1) used as a replacement nitrogen source. Compared to the control sample the H_2 production rates decreased by about 35% (Fig. 1.D). This observation was in line with the results of Liu *et al.* [30] which showed a correlation between a prolonged lag phase and increasing $(NH_4)_2SO_4$ concentrations. However, the H_2 yields obtained with the $(NH_4)_2SO_4$ nitrogen source (*i.e.* a maximum of 1.11 ±0.04 mol H_2/mol glucose

315 at 0.25 g_N/L) were comparable to those for the control sample (Fig. 1.C). These
316 trends for H_2 production yields and rates are consistent with the global benefit
317 reported by Whang *et al.* [12] in continuous bioreactor fed by glucose and either
318 peptone or ammonium nitrogen source. Analysis of the H_2 production activity
319 showed that the inhibition pattern described above for low and high organic
320 nitrogen contents was also observed with $(NH_4)_2SO_4$ (Fig. 1).
321 It has been shown that ammonia at high concentrations can inhibit fermentative
322 hydrogen production, probably by changing the intracellular pH of the
323 bacterium, increasing maintenance energy requirements or inhibiting specific
324 enzymes such as the hydrogenase [9, 29-31].
325 In line with Liu *et al.* [30] we found that at high concentrations (*e.g.* 2.3 g_N/L)
326 ammonia strongly inhibited fermentative H_2 production. By contrast Salerno *et*
327 *al.* [29] found that the hydrogen yield was not affected by ammonia and
328 averaged 1.07 ±0.04 mol H_2/mol glucose at 10 g_N/L ammonia. However they
329 used a heat-treated sludge as inoculum that might involve microorganisms
330 decreasing the inhibition effect caused by ammonia.
331 On the contrary, in the present study a pure isolate of *Clostridium butyricum* was
332 used and the H_2 yield decreased when the ammonia concentration was outside a
333 given range (*i.e.* from 0.56 to 0.062 g_N/L). This further demonstrated in
334 accordance with Bisaillon *et al.* [31], Liu *et al.* [30] and Wang *et al.* [32] that
335 decreasing the ammonia concentration could improve the H_2 yield. However, it

should be noted that widely differing optimal ammonia concentrations have been reported for dark fermentation in the literature i.e. from 0.01 g_N/L reported by Bisaillon *et al.* [31] up to 10 g_N/L by Salerno *et al.* [29]. This can be explained by the very large differences between the studies regarding the seed sludge and substrate used.

Impact of carbon-to-nitrogen ratio and glucose uptake

In our results, an optimal 0.062 g_N/L of organic nitrogen was evidenced which lead to about 50% higher H_2 yield than with the same nitrogen content of ammonium sulfate and 30% higher H_2 yield than at the optimal ammonium concentration i.e. at a 4-fold higher nitrogen concentration leading to a H_2 production rate about 25% lower. Regarding the related carbon-to-nitrogen ratio (C/N) that enables easier comparisons of results achieved in different experimental conditions, the 29.3 value corresponding to the optimum 0.062 g_N/L is similar to that calculated from Cheong and Hansen [33] at optimal 0.8 g_N/L with ammonium nitrogen source. However, they did not record a significant difference in biohydrogen production compared with experiments with 2 g_N/L (i.e. C/N = 12). This trend is consistent with the results reported in our experiments with ammonium content varying from 0.062 to 0.56 g_N/L (i.e. C/N in the range 3 - 30) and those of Wang *et al.* [32] with C/N in the range 8 – 80. Lin and Lay [34], also using a simple carbon source (glucose) in batch

experiments, recorded an optimum C/N ratio of 47 within the range 40 -130. By contrast to our study, their H_2 production yields and rates decreased severely (>50%) for a slightly lower C/N of 40. Moreover, only H_2 yield decreased with C/N ratio increasing upto 98. Indeed, they report about a 2-fold decrease that is far less than the differences recorded in our results with C/N upto 72 for both H_2 production yields and rates (e.g. a 5-fold decrease for H_2 production rate by comparison to that at the optimum level).

Table 5 (column 2) shows the effect of the $(NH_4)_2SO_4$ on the substrate uptake efficiency. In line with Wang *et al.* [32] the glucose uptake rate was inhibited when ammonia was present as the sole nitrogen source. Furthermore, as with casein peptone, we observed a strong increase of glucose accumulation when the $(NH_4)_2SO_4$ content decreased from 0.062 g_N/L to 0.056 g_N/L. This was associated with a 37% reduction and an 11% reduction in the H_2 yield and production rate, respectively.

3.2. Soluble metabolite production

Tables 4 and 5 summarise the effect of casein peptone and $(NH_4)_2SO_4$ on the synthesis of soluble metabolites. The analysis was carried out by HPLC-RID and showed that the main metabolites produced by *Clostridium butyricum* CWBI1009 in the control sample were formate (14.39 ±1.96 mM), butyrate (12.91 ±2.27 mM) and acetate (9.45 ±1.30 mM). Compared to cultures in

bioreactors [14], more reduced products such as lactate (3.51 ±1.41 mM) and ethanol (1.47 ±0.31 mM) were detected.

As suggested by Liu *et al.* [35], Masset *et al.*[14] and Lin *et al.* [24] this could be due to both the absence of pH regulation and the increasing hydrogen supersaturation in the liquid phase that are inherent characteristic of BHP batch tests. When using 0.062 g_N/L casein peptone (the concentration at which the maximum H_2 yield of 1.43 ±0.08 mol H_2/mol glucose was obtained), the increased H_2 yield compared to the control sample could not be explained as the result of actetate- and/or butyrate-type fermentation, since both acetate and butyrate final concentrations were significantly lower than in control sample [14, 24]. By contrast the 50% decrease in the level of formate would suggest that other metabolic pathways contributed to the H_2 yield improvement [20]. For the test condition with a casein peptone concentration of 0.056 g_N/L, a sharp decrease in H_2 production activity was observed. This is in line with the findings of Lin *et al.* [24], since an increase in lactate and ethanol production and a decrease in butyrate–type fermentation were also observed.

By contrast, the presence of 0.56 g_N/L $(NH_4)_2SO_4$ favours butyrate production. This is consistent with the results reportedby Wang *et al.* [28] and Salerno *et al.* [29]. The increase in butyrate implies that there was a gradual shift from acetate- to butyrate-type fermentation [24]. Additionally formate production remained high at all $(NH_4)_2SO_4$ concentrations and did not correlate with the amount of

399 residual glucose detected, suggesting that the increased formate production
400 could be associated with a decrease in the activity of formate-tetrahydrofolate
401 ligase which catalyses the initial uptake of C_1 units (one carbon units, [19]).
402 Compared to other studies with the *Clostridium butyricum* W5 isolate [28, 36],
403 *Clostridium butyricum* CWBI1009 did not produce large amounts of lactate
404 under any of the conditions tested. Furthermore in other studies [14] we showed
405 that our isolate further converted lactate to H_2 (2 mol of lactate + 1 mol of
406 acetate leading to butyrate formation + an additional 1 mol of H_2) and concluded
407 that *Clostridium butyricum* CWBI1009 has better potential than the W5 isolate
408 for future biotech applications. Interestingly this trend is confirmed when using
409 an ammonia-based substitute since our isolate produced similarly low amounts
410 of lactate whether growing with a casein peptone or an ammonia-based nitrogen
411 source.
412 Out of the 20 test conditions described in the previous section, four were
413 selected for further analysis in order to better understand the differences
414 observed in H_2 production: (i) the control sample with casein peptone (0.56
415 g_N/L), (ii) the sample providing the highest H_2 yield (0.062 g_N/L casein
416 peptone), (iii) the sample associated with the sharpest drop in the H_2 production
417 rate (0.056 g_N/L casein peptone) and finally (iv) an ammonia-based sample with
418 a nitrogen content equivalent to that of the control sample (0.56 g_N/L
419 $(NH_4)_2SO_4$). The study consisted of a comparison between the control sample

and each of the last three samples. This was done by quantifying the amino acids present in the culture supernatants (HPLC-UV) and by analysing the gene expression patterns (RT-qPCR) for the four [FeFe]-hydrogenases and the nitrogenase of *C. butyricum* CWBI1009.

3.3. Amino acid distribution

The quantitative analysis (HPLC-UV) of 20 proteogenic amino acids in the culture supernatants was carried out initially and after 96 h of fermentation. The comparison of the control sample (0.56 g_N/L of casein peptone) with the sample associated with the highest H_2 yield (0.062 g_N/L casein peptone) confirm that in the control sample only a small fraction of the available nitrogen was assimilated and, as a consequence, that the higher level of available nitrogen did not favour high H_2 yields. This was also confirmed by another analysis which showed that only 15% of the available organic nitrogen was incorporated into the biomass (results not shown). At 0.062 g_N/L much more efficient amino acid assimilation (+5.73 mM amino acids uptake compared to the control sample, Fig. 2.A) was observed with the strongest incorporation for MET, ARG and GLY.

Since the 0.056 g_N/L casein peptone sample and the control sample have very similar distribution patterns in Fig. 2.A and Fig. 2.B, it can be assumed that the sharp 50% decrease in production rates recorded with the lower casein peptone concentration could not be explained by a significant difference in the

441 assimilation of a specific amino acid. Moreover the average 35% decrease in H_2
442 production rates observed with the $(NH_4)_2SO_4$ sample would be related to the
443 high ATP cost of *de novo* glutamate biosynthesis to fix ammonia in the
444 GS/GOGAT cycle [29].
445 Interestingly the amino-acid profile for the ammonia-based sample (Fig. 2.C)
446 clearly indicated that *C. butyricum* CWBI1009 is capable of biosynthetising
447 (mainly ARG, MET, ALA) and fermenting (mainly CYS, ASN, LEU)
448 proteogenic amino acids. This characteristic has been demonstrated for other
449 *Clostridium* strains (*e.g. C. pasteurianum*), but experimental evidence was
450 previously lacking for *C. butyricum*. It should be noted that the high apparent
451 incorporation of CYS indicated by the results in Fig. 2 could also be related to
452 the addition of pure L-CYS at 0.5 g/L to provide the required anaerobic
453 conditions before inoculation.
454 **3.4. [FeFe]-hydrogenase and nitrogenase expression patterns**
455 It is now recognised that molecular microbial ecology tools can greatly facilitate
456 scientific investigation of the processes involved in environmental
457 biotechnology. Therefore Real-Time Quantitative PCR was used for the four
458 [FeFe]-hydrogenases and the nitrogenase (nifH subunit) identified in the
459 genome of *C. butyricum* to provide a deeper insight into the role played by these
460 two enzyme groups [19].

To correlate the expression patterns with an increase or a decrease in H_2 production activity we compared the control sample (0.56 g_N/L casein peptone) with the other three above-mentioned conditions (namely, 0.062 g_N/L and 0.056 g_N/L casein peptone and 0.56 g_N/L $(NH_4)_2SO_4$).

With the 0.062 g_N/L casein peptone sample the highest H_2 yield (1.43 ±0.08 mol H_2/mol glucose) occurred with up-regulation of the *hydB2* and *hydB3* hydrogenase genes (Fig. 3.A). However the basal expression level of *hydB2* (measured as the number of cDNA copies per 1000 cDNA copies of 16S rRNA, Fig. 4) was higher than that of *hydB3*, which suggests that the increased H_2 yield can be attributed to the activity of the HydB2 enzyme. This is confirmed by the similar basal expression level of the *hydB2* hydrogenase gene (Fig. 4) recorded in the control sample and in the experiment with 0.056 g_N/L that led to a sharp decrease in H_2 production activity (Fig. 1).

This assumption about the key role of the HydB2 enzyme was also supported by another study comparing the expression patterns of the *Clostridium butyricum* CWBI1009 hydrogenase genes at the optimal pH value for growth (pH 7.3) and for H_2 production (pH 5.2) [19]. In that study we also suggested that the increased H_2 production at low pH could be attributed to the HydB2 enzyme.

Regarding *hydA2* gene that has the closest identity match with *Clostridium pasteurianum* CP1, the results of the present study show similar expression levels under the control condition and both the 0.056 g_N/L casein peptone and

0.56 g_N/L $(NH_4)_2SO_4$ test conditions. By contrast, under the 0.062 g_N/L casein peptone condition, *hydA2* was slightly down-regulated (Fig. 3.A) whereas its basal expression level was higher than that of the other three hydrogenase genes (Fig. 4).

The differences observed in the relative expression levels in the three test samples and the control sample (Fig. 3) show that the nitrogenase gene was very sharply down-regulated with 0.062 g_N/L peptone and 0.56 g_N/L $(NH_4)_2SO_4$, and at a lower extent with 0.056 g_N/L casein peptone. This suggests that with 0.062 g_N/L casein peptone H_2 production was essentially hydrogenase mediated. The quite high basal expression level recorded for the nitrogenase in the control sample (Fig. 4), while the H_2 yield was lower than with 0.062 g_N/L casein peptone, suggests that nitrogenase-mediated H_2 production by *Clostridium butyricum* CWBI1009 may be less efficient than hydrogenase-mediated H_2 production.

4. **Conclusion**

This research aimed to investigate fermentative hydrogen production by *Clostridium butyricum* CWBI1009 with different nitrogen sources. The results show increasing glucose accumulation when casein peptone or $(NH_4)_2SO_4$ concentration decreased below 0.062 g_N/L. This concentration of peptone leads to the optimal overall performance *i.e.* an H_2 yield and production rate of 1.43

503 mol H_2/mol glucose and 1.08 mL_{H2}/h, respectively. The amino acid distribution
504 and the gene expression pattern for hydrogenases and nitrogenase confirm that,
505 at a higher nitrogen concentration, there was an excess of nitrogen. These results
506 are promising for industrial implementation of the bioprocess. They enable,
507 based on the carbon and nitrogen content of the main substrates, to futher
508 determine the optimized conditions for operating dark fermentation in (semi-
509)continuous mode leading to minimum residues and maximum H_2 production
510 yields and rates. They also confirm the key role of *HydB2* in the optimisation of
511 hydrogen production by clostridia. Further investigations of this specific enzyme
512 in larger scale bioreactors and especially in biofilm bioreactors providing better
513 H_2 gas transfer should be encouraged.
514
515 **Acknowledgements**
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520 Recherche dans l'Industrie et l'Agriculture, Fonds de la Recherche
521 Scientifique).
522
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Table captions

Table 1 : Injection and derivatisation program for HPLC-UV analysis of amino acids.

Table 2 : Mobile phase composition and gradient conditions for HPLC-UV analysis of amino acids.

Table 3 : Specific primers and probes used in this study. [1] AT – PCR annealing temperature. [2] HP – hydrolysis probe.

Table 4 : Glucose conversion and distribution of soluble metabolites produced by *Clostridium butyricum* CWBI1009 after 96 h of culture in 270 mL serum vessels with casein peptone as nitrogen source. SD, Standard Deviation on triplicates; N.D., No data available.

Table 5 : Glucose conversion and distribution of soluble metabolites produced by *Clostridium butyricum* CWBI1009 after 144 h of culture in 270 mL serum vessels with $(NH_4)_2SO_4$ as nitrogen source. SD, Standard Deviation on triplicates.

Figure captions

Figure 1 : *Clostridium butyricum* CWBI1009 grown with 5 g/L glucose in 270 mL batch bottles with 10 different concentrations of casein peptone and $(NH_4)_2SO_4$ expressed in terms of total nitrogen content. [A] Biogas H_2 content, [B] Cumulative H_2 production, [C] H_2 yield, [D] H_2 production rate. At least three biological replicates were performed for each condition. The control sample is indicated by a black arrow.

Figure 2 : Amino acid distribution, compared to the control sample (0.56 g_N/L casein peptone), in the culture supernatant of *Clostridium butyricum* CWBI1009 grown with 5 g/L glucose in 270 mL batch cultures. [A] 0.062 g_N/L casein peptone, [B] 0.056 g_N/L casein peptone and [C] 0.56 g_N/L $(NH_4)_2SO_4$. The

691 samples were derivatised online and analysed by HPLC-UV at 262 nm for
692 hydroxyproline and proline and at 338 nm for the other amino acids.
693
694 Figure 3 : Differential expression of [FeFe]-hydrogenase genes (*hydA2, hydA8,*
695 *hydB2* and *hydB3*) and the nitrogenase gene (*nifH)* of *Clostridium butyricum*
696 CWBI1009 and comparison of the gene expression patterns. [A] 0.062 g_N/L and
697 [B] 0.056 g_N/L casein peptone and [C] 0.56 g_N/L $(NH_4)_2SO_4$ compared to 0.56
698 g_N/L casein peptone (control sample). The standard bar graph visualisation was
699 replaced by a statistical whisker-box plot to provide additional information
700 about the skew of the distributions that would not be available by simply
701 plotting the sample mean. *Statistically not different from the control sample,
702 $p>0.05$.
703
704 Figure 4 : Basal expression of [FeFe]-hydrogenase genes and the nitrogenase
705 gene of *Clostridium butyricum* CWBI1009 with casein peptone and $(NH_4)_2SO_4$.
706 The basal expression level is shown as the number of cDNA copies per 1000
707 cDNA copies of 16S rRNA.
708
709 Supplementary Figure 1 : *Clostridium butyricum* CWBI1009 grown with 5 g/L
710 glucose in 270 mL batch bottles with different concentrations of bacto-tryptone
711 and bacto-casamino acids, expressed in terms of the total nitrogen. [A] Biogas

712 H_2 content, [B] Cumulative H_2 production, [C] H_2 yield, [D] H_2 production rate.

713 At least three biological replicates were performed for each condition.

714

Table 1

Line	Function	Amount	Reagent
1	borate buffer		
2	draw	0.5 μL	Sample
3	mix	3.0 μL in air, 400 μL/min, 2 times	
4	wait	0.50 min	
5	draw	0.0 μL	Water-needle wash
6	draw	1.0 μL	OPA-3MPA
7	mix	3.5 μL in air, 400 μL/min, 6 times	
8	draw	0.0 μL	Water-needle wash
9	draw	0.5 μL	FMOC
10	mix	4.0 μL in air, 400 μL/min, 6 times	
11	draw	32.0 μL	water
12	mix	18.0 μL in air, 400 μL/min, 2 times	
13	inject	(0.5 μL)	

Table 2

Time (min)	**Mobile phase A** (40 mmol/l Na_2HPO_4, pH 7.8)	**Mobile phase B** (45% acetonitrile, 45% methanol, 10% water)
0.00	100.0	0.0
1.90	100.0	0.0
8.00	79.7	21.3
19.30	44.8	55.2
19.80	0.0	100.0
23.50	0.0	100.0
24.40	100.0	0.0
25.20	100.0	0.0

Table 3

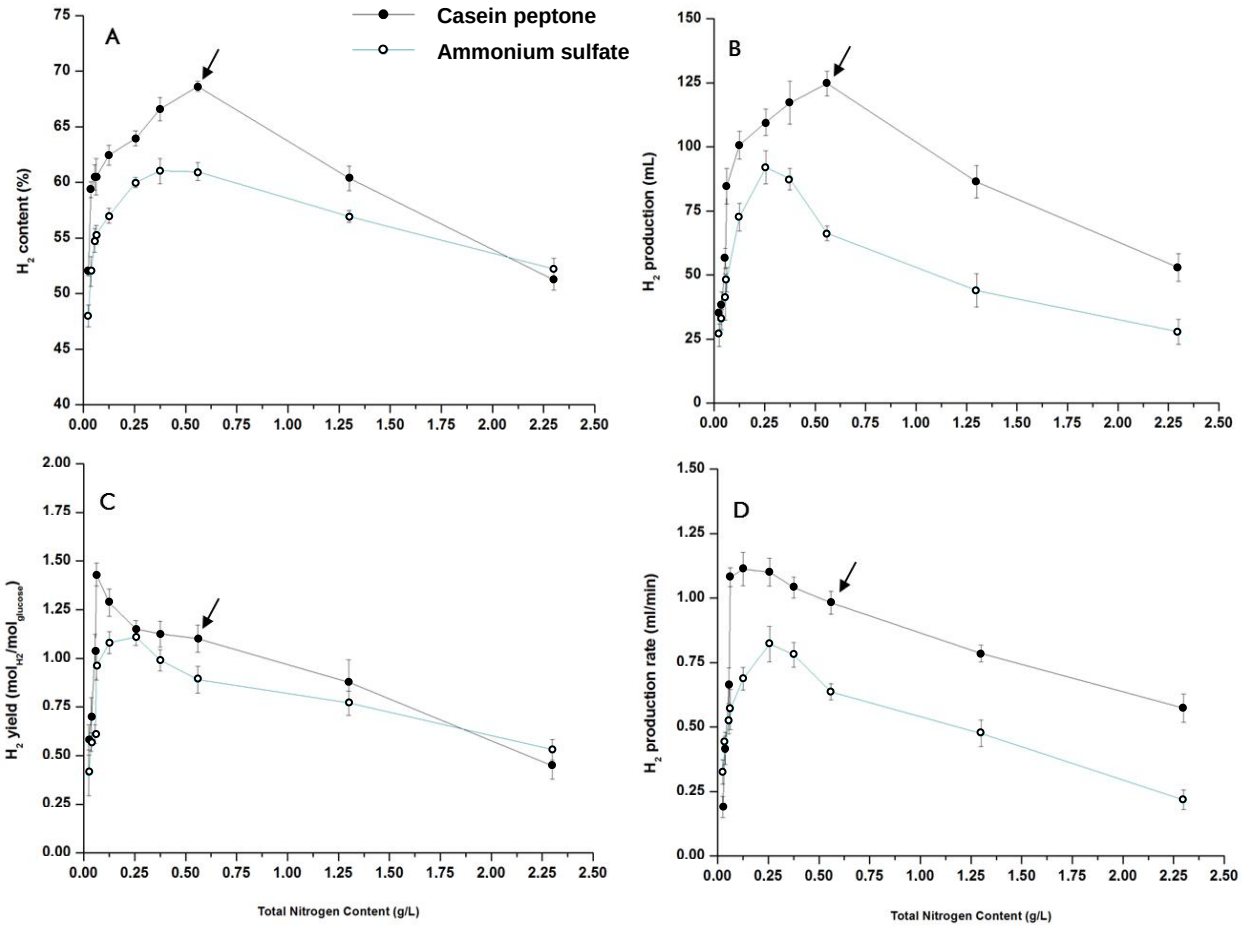
Target gene	Primer name	Sequence 5'→ 3'	Length of product (bp)	AT[1] (°C)
recA	RecA-butF RecA-butR	AAGCATTAGTGCGTTCTGGAG GAATCTCCCATTTCCCCTTC	97	60
gyrA	GyrA-butF GyrA-butR	AGCAATGGGTAGAACTGCATC ATTCTTCGCCATCAACTGCT	95	60
hydA2	ButA2F ButA2R	ATAGTTGCAATGGCTCCTGC TTTCTGCTTGCCTAACCCAT	250	60
hydA8	ButA8F ButA8R	TCTTTGGAGTTACAGGGGGA TTCAGCATTTGCAAGACCAC	188	60
hydB2	ButB2F ButB2R	TGGTGGTGTATCAACTGCTG TTGCATCCCATTCCTTCAAT	168	60
hydB3	ButB3F ButB3R	CAATGGTTGCTACAGGCAGA CAAAAGCATCGAATAACGCA	168	60
16S rRNA	16SButF 16SbutR HP16Sbut[2]	CCTGCCTCATAGAGGGGAAT GAGCCGTTACCTCACCAACT CCGCATAAGATTGTAGTACCG CATGGTACA	143	60

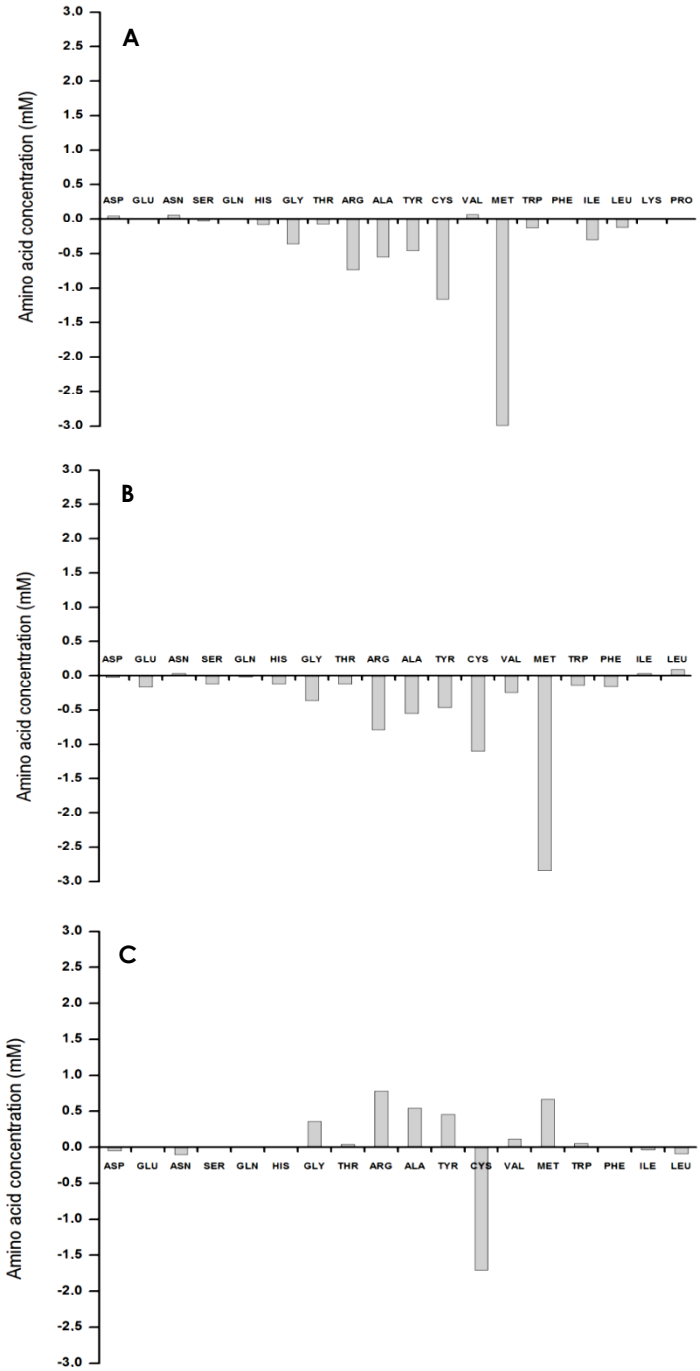
Revised Table 4

Casein peptone concentration (g_N/L)	Glucose mmol ± SD	Lactate mmol ± SD	Formate mmol ± SD	Acetate mmol ± SD	Ethanol mmol ± SD	Butyrate mmol ± SD
2.3	4.93 ± 1.44	6.79 ± 3.91	1.25 ± 0.46	0.87 ± 0.29	5.18 ± 0.97	4.64 ± 1.18
1.3	3.64 ± 0.93	4.95 ± 1.01	1.63 ± 1.01	1.08 ± 0.53	4.81 ± 1.09	4.77 ± 2.19
0.56	0.44 ± 0.09	3.51 ± 1.41	14.39 ± 1.96	9.45 ± 1.30	1.47 ± 0.31	12.91 ± 2.27
0.37	0.11 ± 0.07	3.86 ± 0.82	18.66 ± 1.64	5.61 ± 1.64	0.15 ± 0.08	13.32 ± 1.15
0.25	0.70 ± 0.23	3.91 ± 0.33	15.72 ± 2.52	3.68 ± 1.12	0.88 ± 0.67	9.44 ± 1.19
0.12	8.33 ± 1.79	3.68 ± 0.24	16.32 ± 2.59	3.90 ± 1.17	0.95 ± 0.33	9.46 ± 1.13
0.062	10.58 ± 3.00	2.80 ± 1.50	7.80 ± 2.86	3.11 ± 1.47	3.57 ± 1.14	6.54 ± 1.37
0.056	13.43 ± 1.50	4.10 ± 0.34	6.01 ± 1.70	2.68 ± 0.25	7.59 ± 1.26	2.10 ± 0.56
0.037	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
0.025	15.00 ± 2.04	6.10 ± 1.01	4.46 ± 1.30	1.89 ± 0.21	9.52 ± 1.35	1.29 ± 0.61

Revised Table 5

$(NH_4)_2SO_4$ Concentration (g_N/L)	Glucose mmol ± SD	Lactate mmol ± SD	Formate mmol ± SD	Acetate mmol ± SD	Ethanol mmol ± SD	Butyrate mmol ± SD
2.3	3.47 ± 0.33	3.76 ± 1.07	17.06 ± 2.88	6.02 ± 1.75	3.09 ± 1.75	4.09 ± 1.02
1.3	4.21 ± 1.64	3.10 ± 0.72	17.10 ± 1.72	6.08 ± 1.84	2.22 ± 1.81	3.75 ± 1.70
0.56	3.02 ± 1.55	3.85 ± 1.58	17.01 ± 1.30	6.42 ± 1.36	1.31 ± 0.60	12.73 ± 2.36
0.37	2.67 ± 0.53	3.80 ± 0.87	18.32 ± 2.27	6.05 ± 1.64	1.72 ± 0.76	13.80 ± 1.62
0.25	2.33 ± 0.51	3.55 ± 0.63	17.74 ± 1.17	6.82 ± 2.17	1.13 ± 0.33	12.06 ± 1.98
0.12	2.21 ± 1.11	3.83 ± 0.53	19.08 ± 3.00	6.93 ± 1.95	0.92 ± 0.40	13.09 ± 2.72
0.062	2.01 ± 0.88	3.68 ± 1.21	18.93 ± 1.52	7.34 ± 1.39	1.39 ± 0.54	13.32 ± 1.29
0.056	8.13 ± 1.40	4.07 ± 2.02	13.50 ± 1.92	6.83 ± 1.34	2.12 ± 0.50	2.65 ± 0.99
0.037	11.32 ± 2.56	1.52 ± 1.18	10.52 ± 1.61	5.14 ± 1.68	2.55 ± 0.60	7.13 ± 1.39
0.025	11.92 ± 2.34	2.26 ± 2.48	9.87 ± 2.02	4.63 ± 1.63	3.87 ± 1.41	3.08 ± 0.34


A
B
C
D
Casein peptone
Ammonium sulfate
H2 content (%)
H2 production (mL)
H2 yield (molH2/molglucose)
H2 production rate (ml/min)
Total Nitrogen Content (g/L)
Total Nitrogen Content (g/L)


A
B
C
Amino acid concentration (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
ASP GLU ASN SER GLN HIS GLY THR ARG ALA TYR CYS VAL MET TRP PHE ILE LEU LYS PRO
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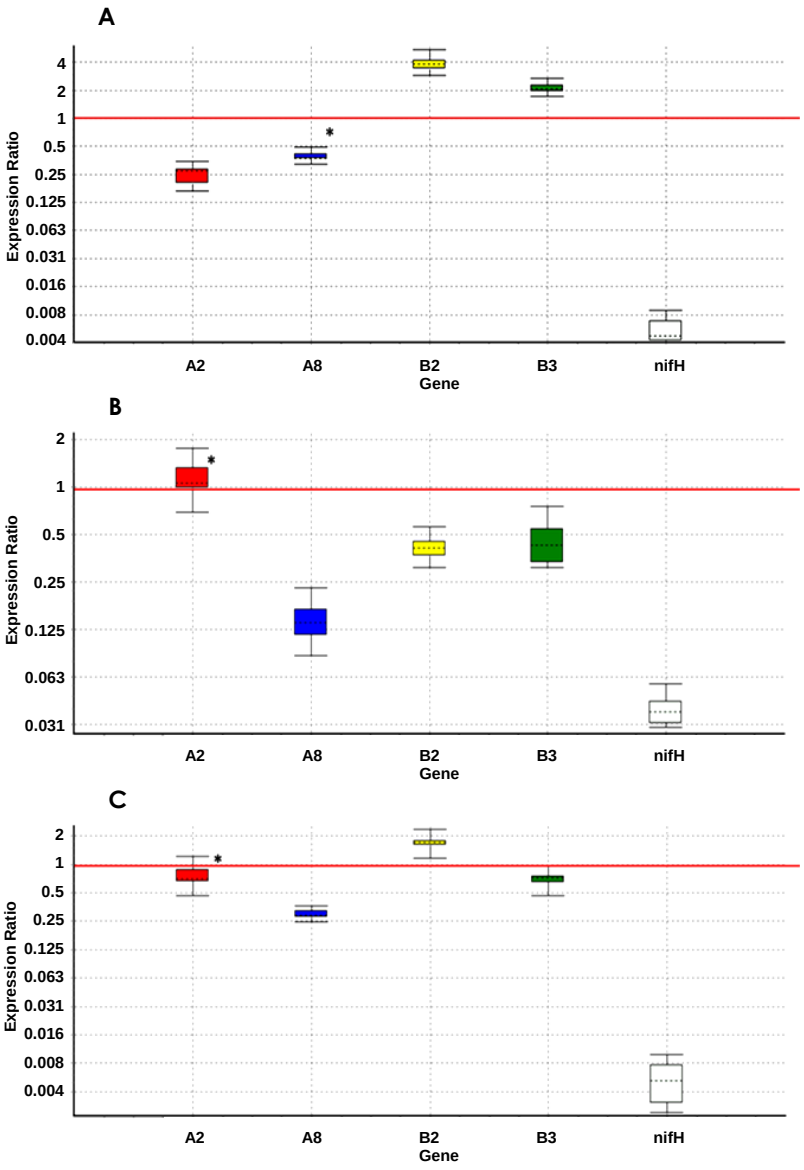
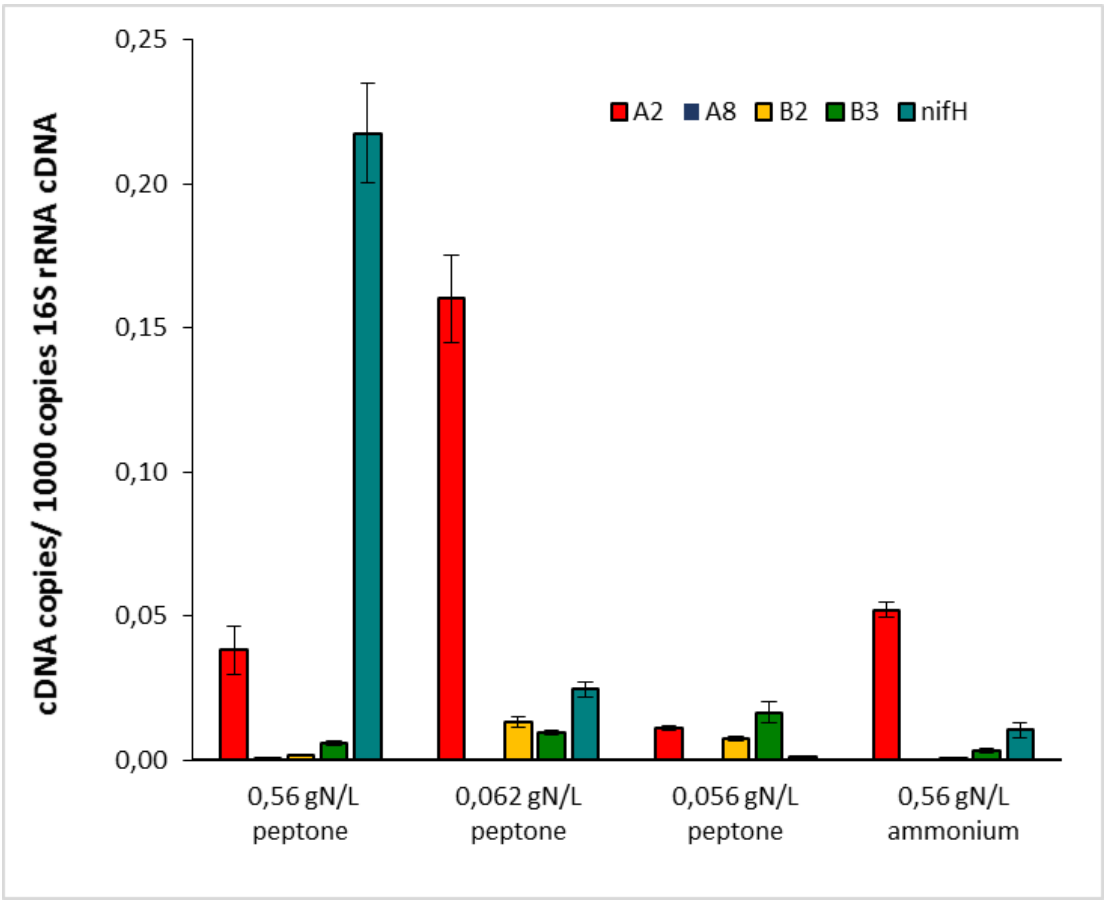
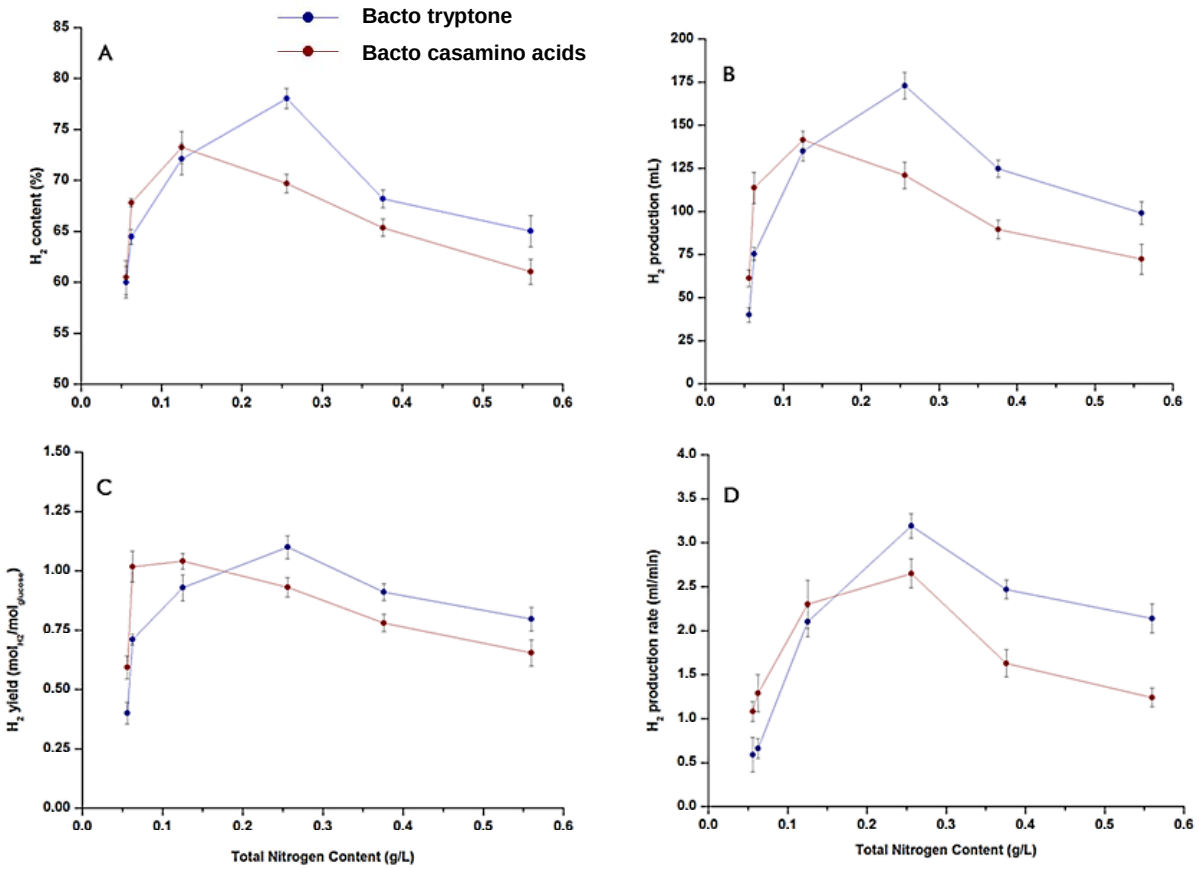

A
Expression Ratio
4
2
1
0.5
0.25
0.125
0.063
0.031
0.016
0.008
0.004
A2
A8
B2
B3
nifH
Gene
B
Expression Ratio
2
1
0.5
0.25
0.125
0.063
0.031
A2
A8
B2
B3
nifH
Gene
C
Expression Ratio
2
1
0.5
0.25
0.125
0.063
0.031
0.016
0.008
0.004
A2
A8
B2
B3
nifH
Gene

Figure 4


cDNA copies/ 1000 copies 16S rRNA cDNA
0,25
0,20
0,15
0,10
0,05
0,00
A2
A8
B2
B3
nifH
0,56 gN/L peptone
0,062 gN/L peptone
0,056 gN/L peptone
0,56 gN/L ammonium


Bacto tryptone
Bacto casamino acids
A
B
C
D
H2 content (%)
H2 production (mL)
H2 yield (molH2/molglucose)
H2 production rate (ml/min)
Total Nitrogen Content (g/L)
Total Nitrogen Content (g/L)