Optimisation of culture conditions for biological hydrogen production by *Citrobacter freundii* CWBI952 in batch, sequenced-batch and semicontinuous operating mode

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Abstract
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Investigations were carried out to determine the effect of the pH, the nitrogen source, iron and the dilution rate (h^{-1}) on fermentative hydrogen production from glucose by the newly isolated strain *Citrobacter freundii* CWBI952. The hydrogen production rate (HPR), hydrogen yield, biomass and soluble metabolites were monitored at 30 °C in 100 mL serum bottles and in a 2.3 L bioreactor operated in batch, sequenced-batch and semicontinuous mode. The results indicate that hydrogen production activity, formate biosynthesis and glucose intake rates are very sensitive to the culture pH, and that additional formate bioconversion and production of hydrogen with lower biomass yields can be obtained at pH 5.9. In a further series of cultures casein peptone was replaced by $(NH_4)_2SO_4$, a low cost alternative nitrogen source. The ammonia-based substitute was found to be suitable for H_2 production when a concentration of 0.045 g/L $FeSO_4$ was provided. Optimal overall performances (ca. an HPR of 33.2 mL H_2/L.h and a yield of 0.83 $mol_{H2}/mol_{glucose}$) were obtained in the semicontinuous culture applying the previously optimized parameters for pH, nitrogen, and iron with a dilution rate of 0.012 h^{-1} and degassing of biogas by N_2 at a 28 mL/min flow rate.

1. Introduction

In recent years policy makers have started looking for alternatives to fossil fuels, not only to counter the threat of global warming, but also to reduce the risk of overdependence on imported oil and gas supplies. The major alternative, nuclear energy, has an inherent problem of waste management. Hydropower is a mature technology, but is subject to site restrictions. Solar and wind power are well developed energy technologies, but are highly susceptible to climatic conditions. By contrast with fossil fuels hydrogen, whether burned directly or used in fuel cells, is intrinsically a clean energy vector with near zero carbon emissions. However the main current method of producing hydrogen, steam reforming of methane, involves the release of large quantities of greenhouse gases. So although hydrogen already accounts for around 2 % of world consumption of energy, its more widespread adoption as a fuel is still limited by several challenges [1-3]. Consequently there has been increasing interest in recent years in the biological production of hydrogen using microorganisms: mainly algae and bacteria in which the generation of molecular hydrogen is an essential part of the energy metabolism, since it provides a way of eliminating excess electrons [4]. The two main exemplars of such biochemical pathways are photosynthetic microorganisms such as *Chlamydomonas reinhardtii* (oxygenic) or *Rhodobacter sphaeroides* (anoxygenic) and fermentative bacteria such as *Enterobacteriacea* (facultative anaerobe species) or *Clostridium* (obligate anaerobe species) [5-8]. The approach with the greatest commercial potential is fermentative hydrogen generation (dark fermentation) coupled with proton exchange membrane fuel cells (PEMFC). This type of system is very promising since it allows the production of hydrogen from a wide variety of renewable resources such as carbohydrate waste from the agricultural and agro-food industries or processed urban waste and sewage [9, 10]. Hydrogen generation from such renewable biomass would reduce our dependence on fossil fuels and decrease carbon dioxide emissions [11, 12]. Furthermore dark fermentation process units are feasible at mesophilic temperatures and at pressures requiring very little energy input.

The highest hydrogen production rates (HPR) have been obtained with *Enterobacteriacea,* which use formate - an intermediate in the glucose metabolism - to promote the formation of molecular hydrogen via the catalytic action of formate hydrogen lyase (FHL) [13]. The FHL complex consists of

a formate dehydrogenase (FDH-H), a [Fe-Fe]-hydrogenase (HYD-3) and electron transfer mediators such as 2[Fe_4S_4]-ferredoxin and NADH. The degradation of 1 mole of formate by FDH-H produces 2 H^+ moles which are subsequently reduced by the action of HYD-3, providing 1 mol H_2 ($HCOOH \rightarrow H_2 + CO_2$). Reoxidation of the NADH by NADH-ferredoxin oxidoreductase followed by the interaction of high potential ferredoxin with HYD-3 produces another mol of H_2 ($NADH + H^+ \rightarrow NAD^+ + H_2$), resulting in a final theoretical conversion yield of 2 $mol_{H2}/mol_{glucose}$ [14, 15]. In practice the experimental yield ranges from 0.37 to 1.9 $mol_{H2}/mol_{glucose}$ depending on key factors such as pH and temperature [16-19], nitrogen source [20, 21], iron concentration [22, 23] and, in semicontinuous and continuous cultures, the dilution rate of the medium in the bioreactor [24, 25]. Higher yields can be obtained with pure *Clostidium* strains since their theoretical conversion yield is 4 $mol_{H2}/mol_{glucose}$. However their HPRs are lower than those of *Enterobacteriacea* and the required culture conditions are more difficult to maintain. Furthermore, *Enterobacteriacea* can provide anaerobic conditions without the need for expensive reducing agents [26, 27].

The aim of the study described in this paper was to characterize the fermentative hydrogen production of pure *Citrobacter freundii* CWBI952 cultures and determine the optimum conditions for sustainable cost effective production. Initially the effect of pH on hydrogen yields, biomass and metabolite concentrations was investigated in order to find the optimum pH for H_2 production. Subsequent investigations examined outcomes when the casein peptone nitrogen source was replaced with a cheaper ammonia-based source. Finally the effect of the dilution rate was studied in a semicontinuous bioreactor using the ammonia-based nitrogen source and running the reactor vessel with all the previously optimized parameters.

2. Materials and methods

2.1. Isolation of the strain and identification test

The strain was isolated from a sample of cow manure cultured in a medium for isolating sulfate reducing bacteria (Postgate's medium E [28, 29]) and it rapidly disrupted the agar. One mL of inoculum was successively diluted in 9 mL of sterile peptoned water (consisting of: 2 g/L Tween 80, 5 g/L NaCl and 1 g/L casein peptone). One mL of each dilution was then added to 25 mL sterile

tubes and mixed with 24 mL of Postgate's medium E maintained in fluid state at 43°C. After incubation at 30 °C the tube was broken at a convenient point; a white colony was withdrawn with a platinum loop and transferred successively on to agar plates prepared with PCA medium (containing 1 g/L glucose monohydrate, 5 g/L casein peptone, 2.5 g/L yeast extract and 15 g/L agar). Isolated colonies developed after 1 day of incubation and one of them was then transferred to 250 mL serum bottles for BHP tests (as described in Material and Methods, see section 2.3.). Based on the fact that the volume and hydrogen content of the biogas produced by the different samples were similar (i.e. 80 ± 2 mL and 51 ± 4 %), the cultures were considered to contain pure strains. Identification was carried out by 16S rRNA gene amplification and sequencing. Bacterial cell lysates were used to amplify the 16S rRNA gene with universal bacterial primers 16S27F and 16S1492R in a 50 µL reaction volume under the following conditions: initial denaturation at 94 °C during 5 min, followed by 36 cycles with denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 2 min. The PCR products of the correct size obtained in this way were purified with the GeneJET™ PCR Purification Kit (Fermentas). Sequences were determined by GIGA (Genomic Facility, Liège, Belgium) using the 27F and 1492R primers.

2.2. Composition of the fermentation media

The growth of the strain was carried out in different modified synthetic media adapted from Ueno [30] and widely used for anaerobic bacterial growth and biological production of hydrogen by *Enterobacteriaceae* and *Clostridium.* The composition of the media was changed depending on the parameters being studied. The standard synthetic medium A was rich in organic nitrogen. This medium was used to determine the optimum pH and the fermentation profile for the strain and as a control test condition in the serum bottle experiments. It contained: 5 g/L glucose monohydrate, 5 g/L casein peptone, 0.5 g/L yeast extract, 2 g/L KH_2PO_4 and 0.5 g/L $MgSO_4.7H_2O$. Medium B was used in serum bottles to study the effect of using an ammonia-based nitrogen source on hydrogen production (with an equivalent N content and replacing $MgSO_4$ with $MgCl_2$ to avoid higher SO_4 concentration compared to the former medium). It consisted of: 5 g/L glucose monohydrate, 6 g/L $(NH_4)_2SO_4$, 0.5 g/L yeast extract, 2 g/L KH_2PO_4 and 0.4 g/L $MgCl_2.6H_2O$. Medium C, which was

used to investigate the effect of iron when using an ammonia-based nitrogen source, had the same composition as medium B except for the addition of 0.125 g/L $FeSO_4$. The first batch sequence of the sequenced-batch cultures in the 2.3 L bioreactor was started with medium C. After complete depletion of the glucose, 20 % of the culture medium was racked and an identical volume of medium C (supplemented with 25 g/L glucose monohydrate) was added in order to perform a second culture sequence with the same initial substance concentrations. This procedure had already been used successfully for 8 successive sequences with medium A yielding similar performances in every sequence (results not shown). A third culture sequence was carried out in a similar way by racking 68 % of the culture medium and adding an identical volume of medium B, supplemented with glucose monohydrate (7.35 g/L). The semicontinuous experiment (see section 3.5) was preceded by a batch phase during which the substances were at similar concentrations to our estimates for the beginning of the third sequence of the sequenced-batch experiment, taking into account NH_4^+ and iron incorporation by the biomass and the dilution of the substances during the previous sequences. This medium (medium D) contained : 5 g/L glucose monohydrate, 3 g/L $(NH_4)_2SO_4$, 0.5 g/L yeast extract, 2 g/L KH_2PO_4, 0.4 g/L $MgCl_2.6H_2O$ and 0.03 g/L $FeSO_4$. The medium added semicontinuously to the bioreactor (medium E) contained : 20 g/L glucose monohydrate, 2.4 g/L $(NH_4)_2SO_4$, 0.4 g/L yeast extract, 1.6 g/L KH_2PO_4, 0.32 g/L $MgCl_2.6H_2O$ and 0.03 g/L $FeSO_4$. Compared to medium D, the glucose monohydrate was tested at 20 g/L to approach the carbon load of agro-industry wastewaters and the other compounds at a 80 % proportion (except for iron sulfate maintained at 0.03 g/L). Hydrochloric acid and sodium hydroxide were used to adjust the pH to 8.5 in all media before autoclaving. After sterilization the pH was ca. 7.1. All the chemicals used were of analytical or extra pure quality and were supplied by Merck, UCB and Sigma. Casein peptone and yeast extract were supplied by Organotechnie (La Courneuve, France).

2.3 Experimental procedures and culture conditions

Pure colonies of *C. freundii* CWBI952 were maintained at 4 °C on PCA solid medium. For fresh inoculum conservation 1 mL of culture was transferred weekly to 25 mL of sterile peptoned medium

A and stored at 30°C. To allow a quick characterization of the isolates and of the influence of iron on hydrogen production of the strain when using an ammonia-based substitute, BHP (Biochemical Hydrogen Potential) tests were carried out respectively in 250 or 100 mL sterile glass serum bottles filled with 200 or 50 mL of medium A, B or C. The sterile carbon source (glucose monohydrate in solution in deionized water) was added separately to obtain a final concentration of 5 g/L. After the medium had been prepared with the pH adjusted to 8.5 and sterilized the glucose solution was added and the medium was then inoculated with a single colony collected with a platinum loop from a previously spread PCA medium plate. The bottles were capped with a butyl stopper as described by Lin et al. [31] however the use of a reducing agent and flushing with nitrogen gas was not necessary since the bacteria consume any oxygen present before entering in anaerobiosis. The bottles were then incubated at 30 °C and monitored after 22 hours of culture.

The batch, sequenced-batch and semicontinuous cultures were run in the same 2.3 L laboratory scale bioreactor (Biolafitte) consisting of a glass vessel with a double envelope and a stainless steel lid equipped with septa, a shaft with 2 Rushton turbines (4 blades, height 10 mm, diameter 45 mm), 0.2 µm gas filters, and tubing for sampling, gas inlet, gas outlet and medium removal or addition. The glass vessel was fitted with a lateral glass overflow tube to remove any liquid in excess of ~ 2 L (working volume) due to overfilling during the semicontinuous operations. Needles were inserted through a septum to control the pH (Mettler Toledo 465 35 90 K9/250 combined probe) by automatic addition of sterile 2.5 N potassium hydroxide. The temperature was maintained at 30 °C and stirring was constant at 110 RPM. The bioreactor containing 1.8 L of deionized water and the ingredients for the different media except glucose was autoclaved at 120 °C for 20 minutes and then cooled under nitrogen gas. The pre-cultures were obtained by transferring 25 mL of inoculum into a 1 L bottle containing 250 mL of sterile medium A, C or D before incubation for 24 hours at 30°C and inoculation of the bioreactor with 10 % of its liquid capacity (200 mL). The batch cultures were monitored until conversion of the carbon substrate was complete (*i.e.* one day) to determine the optimum pH and the fermentation profile for the strain. The first batch phase and the following 2 sequences of the sequenced-batch experiment were also monitored for one day in order to monitor the effect over time of iron when using $(NH_4)_2SO_4$ as a nitrogen source. The two dilutions of the

141 medium, using a peristaltic pump after racking off an identical volume, were carried out to prevent
142 any by-product inhibition from interfering with the parameters under study. In the semicontinuous
143 culture the bioreactor was first run in batch mode for one day before dilution of the medium. The
144 effect of the dilution rate and the influence of ammonia assimilation on microbial growth for cost-
145 effective H_2 production were then investigated during 9 days in semicontinuous mode. A time slot
146 sequencer was connected to a peristaltic pump (Verder Autoclude EV) for additions of sterile
147 substrate and to an electric valve for removal of excess medium from overfilling. When investigating
148 the effect of the first dilution rate (0.009 h-1) additions and removals were made every 120 min
149 based on the glucose consumption rate (0.45 g/L.h) during the batch phase. Removal took 30
150 seconds and each addition was set to take 15 min.
151
152 ***2.4. Monitoring and analytical methods***
153 Cell concentration was determined by consecutive dilutions in peptoned water. A 100 µL sample of
154 the three final dilutions were spread on PCA Petri dishes before incubation at 30°C for 18 to 24 h.
155 This method was also used to confirm the absence of microorganisms other than *Citrobacter*
156 *freundii* CWBI952. Culture samples were collected regularly to make measurements and harvest
157 culture components. These samples were centrifuged at 13000 g for 10 min and the supernatants
158 were filtered through a 0.2 µm cellulose acetate membrane (Sartorius Minisart). The HPLC analyses
159 for glucose, ethanol, lactate, acetate, formate and succinate were performed using an Agilent 1110
160 series HPLC equipped with a Supelcogel C 610H column preceded by a Supelguard H precolumn
161 (oven temperature 40 °C) and a differential refraction index detector (RID, detection cell maintained
162 at 35 °C). An isocratic mobile phase consisting of 0.1 % H_3PO_4 (in MilliQ water) was used at a flow
163 rate of 0.5 mL/min. The method lasted for 35 min at a maximum pressure of 60 bars. The data on
164 the concentrations of glucose and metabolites present in the culture medium were used to calculate
165 the mass balance (MB) of glucose conversion into the major soluble metabolites using the equation:
166 $MB = \sum N_I \, . \, \Delta C_I / N_G . \Delta C_G$; where N_I is the number of carbon atoms in a molecule of metabolite I; ΔC_I
167 is the concentration of metabolite produced during the culture sequence I; N_G is the number of
168 carbon atoms in the glucose molecule (6) and ΔC_G is the concentration of glucose consumed during

the culture sequence. The proportion of hydrogen gas was determined using a gas chromatograph (GC) (Hewlett Packard 5890 Series II, UK) 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, 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. Water supplemented with KOH 9 N was used in replacement equipment to monitor the biogas production of the batch and sequenced-batch cultures. In the semicontinuous culture and in the preceding batch culture, the GC was fitted with a 1.2 m x 5 mm stainless steel column packed with Porapak Q Supelco (80/100 mesh). The biogas was constantly extracted by degassing with nitrogen (28.6 mL/min) gas and automatically analyzed on GC for H_2 composition every 30 min via a 250 µL injection loop (Valco, Canada). HACH (USA) kits 26069 45 and method 1003 were used for the determination of the ammonia concentration in the harvested culture samples. The readings were carried out with a HACH DR/2010 photospectrometer.

3. Results

3.1. Isolation and identification of the hydrogen producing strain

The isolated bacteria were observed to be gram negative, mobile and approximately 1 x 3-4 µm in size. Colonies were generally 2-4 mm in diameter, smooth, slightly convex, opaque with a shiny surface and an entire edge as described in Bergey's Manual of Systematic Bacteriology [32]. The bacteria grew aerobically in presence of organic carbon and produced hydrogen through formate bioconversion under anaerobic conditions. To further characterize the strain, the sequenced 16S rRNA gene (accession number in Genbank EU373418.1) was aligned to the 20 most similar sequences obtained using the SeqMatch option of the Ribosomal Database Project (http://rdp.cme.msu.edu/). Compared to the type strain *Citrobacter freundii* DSM 30039, our strain has ambiguities at 7 positions out of an alignment of 1440 positions. In one region at positions 455, 460, 472 and 477 (*E. coli* positions), our strain has C/T, A/T, T/G and G/A whereas the type strain has C, T, G and G. These positions in helix 18 are base paired. In a second region at positions 1134, 1137, and 1140, our strain has A/G, A/C and C/T whereas the type strain has G, C, and C.

These ambiguities are in helix 43 and the first one is base paired with the last one. The last ambiguity is also present in the sequences of the CDC62164 and YRL11 strains. These ambiguities probably represent microheterogeneities between operon copies. If we exclude these ambiguous positions from the alignment, the sequence of our strain is identical to that of the type's 16S rRNA; we therefore designated it as *Citrobacter freundii* CWBI952.

3.2. Effect of pH on glucose metabolism and H_2 production

Since pH variations within a narrow range is known to lead to large fluctuations in metabolic activity [33, 34], optimization of pH conditions was determined as a first essential step in promoting hydrogenesis by the fermentative bacteria. The effect of pH on fermentative H_2 production by the pure *C. freundii* CWBI952 strain was investigated in a 2.3 L batch bioreactor equipped with pH regulation. The tests were conducted at eight different pH levels ranging from 3.6 to 8.2 (± 0.1) which were maintained and monitored for one day while keeping other operating conditions constant (stirring at 110 RPM, temperature at 30 °C, atmospheric pressure and initial medium A). Our results are in line with the work of Yokoi et al. with *E. aerogenes* [35] and Oh et al. with *Citrobacter* sp. Y19 [36] which indicated that the optimum pH for H_2 production is situated within a weakly acidic range (*i.e.* pH 5 to 6). We found that H_2 production by *C. freundii* CWBI 952 underperformed at pH 3.6 and at 8.2, reaching an optimum at pH 5.9 and yielding 0.63 $mol_{H2}/mol_{glucose}$ (**Fig 1.A**). This optimum pH is very different from that observed for cell growth, namely 7.2. These results are also consistent with the data of Tanisho et al. with *E. aerogenes* since they observed a Δ_{pH} of 1.2 between these two optima (ca. 5.8 and 7.0) with yields ranging from 0.52 to 1.58 mol_{H2}/mol_{hexose} at pH 5.8 depending on their culture conditions [37, 38]. The results presented in **Fig. 1.B** highlight the impact of pH on the glucose intake rate and its conversion to formate (according to the MB calculation; see section 2.4). At low and at high pH values, namely pH 4.5 and 8.2, the glucose intake rate was very low and the growth of the strain was limited. As expected the higher glucose intake rate, ca. 0.7 $g_{glucose}/h$, was obtained at the optimum pH for growth. At pH 5.9 the glucose intake rate and biomass decreased by about 30 % and 11 % of the glucose was converted to formate. Formate accumulation in the glucose metabolic pathways of

225 *Enterobacteriacea* indicates a gain of potential hydrogen production. Therefore the potential for
226 additional H_2 can be stoichiometrically estimated from the quantity of formate that is not converted in
227 the bioreactor [39]. For example at pH 5.9 and 7.2 the glucose mass balance for the accumulated
228 formate reached 11 and 4.1 % which could potentially lead, after dismutation into CO_2 and H_2, to an
229 increase in yield of 0.27 $mol_{H2}/mol_{glucose}$ and 0.10 $mol_{H2}/mol_{glucose}$ respectively.
230
231 ***3.3 Fermentation profile* at the optimum pH for H_2 production**
232 **Fig. 2.A** characterizes the cell growth and H_2 production capacity of *C. freundii* CWBI952 in a batch
233 culture with the peptoned medium A monitored for one day at pH 5.9. The growth curve depicts a
234 short lag phase of 2 hours followed by exponential growth at a rate of 0.53 h^{-1}, reaching 1.45 10^{+09}
235 CFU/mL after 8 hours. H_2 production was observed very soon after inoculation and was continuous,
236 totaling 825 mL H_2 over 24 hours. The maximum hydrogen production rate (HPR), ca. 26.4 mL_{H2}/L.h,
237 and yield, ca. 0.63 $mol_{H2}/mol_{glucose}$, were recorded during the exponential growth phase. Glucose
238 utilization and the fermentation profile are illustrated in **Fig. 2.B**. The monitored soluble compounds
239 include glucose, volatile fatty acids (VFAs) and ethanol. The chromatographic analysis showed that
240 the primary metabolites on completion of fermentation were ethanol (24.4 mM), lactate (15.7 mM)
241 and acetate (15.0 mM) followed by formate (4.8 mM) and succinate (4.2 mM). Since only formate
242 bioconversion leads to hydrogen gas generation, any production of other metabolites involves a
243 diversion away from potential hydrogen production [14]. Although Oh et al. [36] reported similar
244 patterns with *Citrobacter* sp. Y19 in 165 mL serum bottles with no pH control; they did not detect
245 lactate as a primary metabolite of pyruvate degradation. In a more recent study [13], they found that
246 ethanol, lactate and succinate were the main by-products of glucose. This is generally consistent
247 with the results in **Fig. 2.B**, but our data did not indicate transformation of succinate into propionate
248 in any of our cultures. During the stationary phase H_2 production continued due to further
249 bioconversion of the residual formate. About 445 mL H_2 was produced during this phase, but at 50
250 % of the maximum HPR. Given that metabolization of the formate started after 8 hours, resulting in
251 the bioconversion of 8.7 mM after 15.5 hours of growth, the formate bioconversion rate was
252 calculated to be 2.7 times slower than its accumulation rate. And since that at pH 4.5 and 8.2

253 formate accounted for only 0.9 % and 0.2 % of glucose transformation (**Fig 1.B**) the rate limiting
254 factor probably resides in the pathway converting formate (via the FHL complex) into H_2 and CO_2
255 [14, 40]. The mass balance of glucose conversion into the major metabolites is summarized in **table**
256 **1** (column 4) and reaches a total of 92.7 %. The CO_2 content in the biogas measured by GC was
257 49 % therefore the CO_2 contribution in the total mass balance can be estimated to be 9.6 % of
258 92.7 % with biomass accounting for the remaining 7.3 % of the glucose consumed. The same
259 experiment was carried out in a 15 L batch bioreactor: the carbon recovery and the glucose intake
260 rate observed, 91.3 % and 0.5 $g_{glucose}$/h respectively, were not significantly different (results not
261 shown).
262
263 ***3.4 Effect of iron concentration on hydrogen production with an ammonia-based nitrogen***
264 ***source***
265 The common use of casein peptone as a nitrogen source is not economically viable in an industrial
266 prospect. Ammonia-based substitutes have already been applied successfully in mixed cultures [20]
267 or *Clostridium* cultures [41] whereas it has been less studied with pure *Enterobacteriacea*. *C.*
268 *freundii* CWBI952's ability to produce H_2 when growing with $(NH_4)_2SO_4$ was first investigated in 100
269 mL bottles with no pH regulation (BHP tests, see section 2.3). The first experimental data, collected
270 after 22 hours of growth, indicated that when using peptone as the nitrogen source (medium A),
271 biomass and hydrogen yield reached 1.4 10^{+09} CFU/mL and 0.22 $mol_{H2}/mol_{glucose}$. In terms of mass
272 balance lactate was the main soluble by-product followed by acetate, ethanol, and succinate
273 representing in all 88.8 % of the initial glucose (**table 1**, column 1). The yield obtained with the
274 serum bottles was only about one third of the yield obtained in the 2.3 L bioreactor using the same
275 medium (ca. 0.63 $mol_{H2}/mol_{glucose}$). This can be explained by the absence of pH regulation (ΔpH up
276 to 3 units) and increasing pressure in the gas phase (up to 0.5 bars) during growth of the strain.
277 When replacing casein peptone by an equivalent mineral nitrogen containing source, *i.e.* 6 g/L
278 $(NH_4)_2SO_4$ and no iron added (medium B), similar cell concentrations were obtained (1.1 10^{+09}
279 CFU/mL), but no biogas was produced. Furthermore high formate accumulation (8.7 ± 1.5 mM) and
280 residual glucose (9.4 ± 1 mM from an initial 25 mM concentration) were detected. By contrast when

281 0.125 g/L $FeSO_4$ were added to the culture medium (medium C), biomass reached 1.1 10^{+09}
282 CFU/mL and a yield of 0.23 $mol_{H2}/mol_{glucose}$ was obtained. In this case the metabolites accounted for
283 74.9 % of glucose utilization and the formate was entirely converted. Lee [23], using anaerobic
284 mixed microflora, reported that a ferrous iron concentration of 20 mg/L could have an inhibitory
285 effect on hydrogenase activity. Nevertheless our data indicated that no suppression of the hydrogen
286 production activity had occurred in our experiments with 6 times this level of ferrous iron. However,
287 Yang [42] has suggested that the accessible iron in the culture medium was often overestimated
288 due to precipitation phenomena. Our observations confirm this statement: after sterilization ferrous
289 sediments were formed as a consequence of the precipitation of compounds such as $Fe(OH)_2$,
290 $FeCO_3$. When the VFA concentration gradually increased (as pH decreased), the precipitate was
291 dissolved and ferrous iron gradually became available to the strain, maintaining bacterial growth
292 with no inhibitory influence on H_2 production.
293 The effect of iron on ammonia-based cultures was further investigated in a 2.3 L sequenced-batch
294 reactor with pH regulation since H_2 production can only be increased if the iron is present within the
295 appropriate range of concentration [22]. In order to avoid ferrous iron flocculation pH was
296 maintained at an optimum of 5.9 during the experiment. The bioreactor was run with 3 sequences: a
297 first batch sequence was followed by 2 successive sequences with removal-addition of 20 % of the
298 culture medium and then 68 % with no addition of ferrous compounds. All the sequences were
299 started after complete depletion of the glucose (*i.e.* one day). During the first batch phase, with 6 g/L
300 $(NH_4)_2SO_4$ and 0.125 g/L $FeSO_4$ (medium C), no hydrogen was produced but biomass reached 6.7
301 10^{+08} CFU/mL (**table 1**, column 5). In accordance with Lee's results [23], H_2 production might have
302 been suppressed by a decrease in hydrogenase activity due to iron inhibition of cellular enzymes.
303 Wang [43] also reported that hydrogen production activity decreased gradually with increasing
304 ferrous iron concentration. In the serum bottles, when using the same medium, 0.23 $mol_{H2}/mol_{glucose}$
305 were obtained due to the lower levels of iron as ferrous sediments formed. By contrast during the
306 former batch culture pH was controlled at 5.9 leading to an increase in iron levels and the
307 associated inhibitory effect on H_2 production. The second sequence did not restore hydrogen
308 production activity, yielding only 0.01 $mol_{H2}/mol_{glucose}$. With the third sequence the resulting final

309 $FeSO_4$ concentration of 0.045 g/L. Under these conditions a yield of 0.58 $mol_{H2}/mol_{glucose}$ was
310 obtained, which is comparable to the yield obtained with peptone medium A, ca. 0.63
311 $mol_{H2}/mol_{glucose}$. The carbon mass balance calculation indicated that 79.9 % of the consumed
312 glucose was converted into the detected metabolites and, compared to the batch culture with
313 peptone medium illustrated in **Fig 2.** , the carbon recovery from glucose decreased by 12.8 %. This
314 metabolic deviation may be explained by a decrease in the glucose degradation efficiency occurring
315 when ammonia is present as the main nitrogen source. Several mechanisms for ammonia inhibition
316 have already been proposed, such as a change in the intracellular pH, increase of maintenance
317 energy requirement, and inhibition of specific enzymatic reactions [20, 44].
318
319 ***3.5 Effect of the dilution rate on H_2 production***
320 In this experiment the production of hydrogen by *C. freundii* CWBI952 was studied in a
321 semicontinuous culture in order to investigate the influence of the dilution rate on growth, $(NH_4)_2SO_4$
322 assimilation and hydrogen production activity (**Fig. 3.**). In line with the findings of Kraemer et al. [45]
323 and Mizuno et al. [46] a flow of nitrogen gas (28.6 mL of N_2/min) was used to expel the biogas to the
324 gas chromatographer and to further sparge the culture medium decreasing inhibition effect of
325 hydrogen partial pressure. A first batch phase was performed with medium D and led to higher
326 yields and hydrogen production rates (HPR) compared to previous experiments in the bioreactor
327 confirming the positive effect of degassing the medium. After one day of batch culture, the reactor
328 was operated in semicontinuous mode at 4 different dilution rates (0.009, 0.012, 0.018 and 0.024 h^{-1})
329 for 9 days with a substrate feed of 20 $g_{glucose}$/L (medium E) to approach the typical biochemical
330 organic demand (BOD) of biodegradable organic matter from agro-industry wastewaters. At this
331 concentration glucose accumulation was not detected except at a dilution rate of 0.024 h^{-1}. Given
332 that the culture was semicontinuous the resulting curve of HPR versus time has a saw-toothed
333 appearance due to the periodic dilution of the carbon substrate (**Fig. 3.A**). In addition a reduction in
334 the HPR amplitude can be observed when the dilution rate increases. This could be related to the
335 more regular sequential addition of glucose. As shown in **table 2**, the maximum H_2 yield of 0.95
336 $mol_{H2}/mol_{glucose}$ was obtained with a dilution rate of 0.009 h^{-1} before growth restriction took place

probably as a consequence of by-product inhibition. The hydrogen yield then decreased to 0.74 $mol_{H2}/mol_{glucose}$ when the dilution rate was increased to 0.018 h^{-1}. The opposite trend was observed for HPR since, in accordance with the findings of Zhang et al. [47], our data indicated that when the dilution rate was doubled (from 0.009 to 0.018 h^{-1}), the HPR nearly doubled too. At 0.009 h^{-1}, the assimilation of $(NH_4)_2SO_4$ was lower (ca. 64 mg_N/L) than at 0.018 h^{-1} (ca. 99 mg_N/L), confirming that microbial activity peaked when approaching the washout point [12]. However dilution rates of 0.018 h^{-1} or higher resulted in lower overall performance due to growth inhibition by the accumulated metabolites. During the former 24 h batch culture the total mass balance increased by 8.6 % compared to the first sequenced-batch culture using 6 g/L $(NH_4)_2SO_4$ as the nitrogen source (detailed in **table 1**, column 5). This indicated that a decrease by half in $(NH_4)_2SO_4$ allowed more effective uptake and degradation of the glucose. It should be noted that total mass balance remained very stable at 86.0 ± 2.1 % at all dilution rates. This result indicates that the differences in performance reside principally in the dissimilarities in the fermentation profiles. **Fig. 3.C** illustrates the significant accumulation of soluble metabolites. In fact, except for the dilution rate of 0.024 h^{-1}, under which metabolites were gradually removed from the medium, ethanol, acetate and succinate accumulated independently of the dilution rate. By contrast lactate and formate concentrations varied in line with the dilution rate and the H_2 production activity. As shown in **table 2**, yields gradually decreased as glucose conversion to lactate and formate increased. HPRs, however, were highly dependent on formate bioconversion into H_2 and CO_2, but independent of lactate accumulation. For example during the first day at a dilution rate of 0.009 h^{-1} lactate and formate started to accumulate at a higher rate and after 24 h the formate had been totally converted leading to an increase of 6.0 % in H_2 yield and a 120 % increase in HPR (from 15.6 to 36.6 $mL_{H2}/L.h$). In addition lactate production increased by 11.9 % when switching from a batch to a semicontinuous culture at a dilution rate of 0.024 h^{-1}. This suggests a stronger activation of lactate dehydrogenase (LDH) in the glucose catabolic pathway with high coenzyme ratios inducing lactate production and a concomitant loss of potential hydrogen production [48, 49]. Bearing this in mind the optimum trade off point for improved H_2 yield and higher HPR was found to be a dilution rate of 0.012 h^{-1}.

4. Conclusions

This paper has shown that the recently isolated *Citrobacter freundii* CWBI952 can be used to maintain anaerobic conditions and produce hydrogen via dark fermentation of glucose in batch, sequenced-batch and in semicontinuous cultures. The growth and generation of hydrogen by the pure strain was accompanied by the production of ethanol, lactate, acetate, formate and succinate but no propionic acid. Investigations into the effect of ambient factors such as the pH, the nitrogen source, the iron concentration in batch and sequenced-batch tests and the dilution rate in a semicontinuous culture highlighted important trends which allowed the following conclusions to be drawn. pH must be carefully regulated given its major impact on the hydrogen production activity of the strain. The optimal pH value (ca. 5.9) was shown to be a favorable condition for formate bioconversion and for the production of H_2 with lower biomass yield. While an organic nitrogen source such as casein peptone was found to be suitable for bacterial growth and hydrogen production, $(NH_4)_2SO_4$ proved to be an excellent low-cost substitute. Within a certain concentration range $FeSO_4$ (ca. 0.045 and 0.03 g/L respectively tested in sequenced-batch and semicontinuous cultures) enhanced the HPR and hydrogen yield when growing the strain on $(NH_4)_2SO_4$. In semicontinuous culture an increase of the dilution rate from 0.009 to 0.018 h^{-1} led to a doubling of the HPR from 24.6 to 40.2 mL_{H2}/L.h, but also to a 20 % decrease in yields from 0.95 to 0.69 $mol_{H2}/mol_{glucose}$. Optimal overall performances (an HPR of 33.2 mL_{H2}/L.h and a yield of 0.83 $mol_{H2}/mol_{glucose}$) were obtained when combining the previously optimized parameters for the pH (5.9), nitrogen (2.4 g/L $(NH_4)_2SO_4$), and iron (0.03 g/L $FeSO_4$) with a dilution rate of 0.012 h^{-1} and degassing of the biogas by N_2 at a 28 mL/min flow rate. These findings should contribute to a better understanding of the processes involved in the optimization of hydrogen production by enteric bacteria and facilitate efforts to obtain enhanced performance and reduced costs by identifying key parameters such as pH conditions, the nitrogen substrate, the iron concentration and the dilution rate. Furthermore the study shows that the selection of the strain and the control of its optimized culture conditions have a real impact on bioreactor performance. Nevertheless since the overall yields obtained in the present study are lower than the theoretical maximum and lower than some others reported in the literature further research is needed in this area. Among the options that

393 could be explored are the introduction of suspended or immobilized cells in continuous cultures, and
394 hybrid approaches using a pure *Clostridium* strain in association with *Enterobacteriacea* to initiate
395 H_2 production.
396
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405
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Caption Table 1

Table 1. Metabolite synthesis and performance of H_2 production with *Citrobacter freundii* CWBI 952 growing in 100 mL, 2.3 L batch and sequenced-batch cultures.

Table 1

	100 mL serum bottles			2.3 L bioreactor			
				Batch	Batch	Sequence 2	Sequence 3
Nitrogen source and iron sulfate concentration in the medium	Peptone without $FeSO_4$	$(NH_4)_2SO_4$ without $FeSO_4$	$(NH_4)_2SO_4$ with 0.125 g/L $FeSO_4$	Peptone without $FeSO_4$	$(NH_4)_2SO_4$ with 0.125 g/L $FeSO_4$	$(NH_4)_2SO_4$ with 0.125 g/L $FeSO_4$[a]	$(NH_4)_2SO_4$ with 0.045 g/L $FeSO_4$[a]
Mass balance (%)							
Ethanol	22.4	14.7	18.1	23.7	18.8	15.2	18
Lactate	29.2	26.1	23.8	28.3	23.5	29.2	26.8
Acetate	24.4	26.4	21.22	16.5	14.7	12.2	13.1
Succinate	10.5	3.8	8.7	9.7	9.4	8.4	9.6
Formate	0	11.8	0	4.9	3.9	4.5	3.4
CO_2	2.3	0	3.1	9.6	0	0.2	8.9
Total	88.8	82.9	74.9	92.7	70.3	69.7	79.9
Biomass (CFU/mL)	$1.4\ 10^{+09}$	$1.1\ 10^{+09}$	$1.1\ 10^{+09}$	$1.2\ 10^{+09}$	$6.7\ 10^{+08}$	$6.1\ 10^{+08}$	$7.1\ 10^{+08}$
Yield ($mol_{H2}/mol_{glucose}$)	0.22	0	0.23	0.63	0	0.01	0.58
Hydrogen production rate ($ml_{H2}/L.h$)	27.5	0	29	26.4	0	0.54	31.32

a. estimated due to removal/addition of culture medium

Caption Table 2

Table 2. Metabolite synthesis and performance of H_2 production with *Citrobacter freundii* CWBI 952 growing on $(NH_4)_2SO_4$ in a 2.3 L semicontinuous culture at different dilution rates.

Table 2

	Dilution rate (h^{-1})					
	Batch	**0.009**[a]	**0.012**	**0.018**	**0.024**	**0.012**
Mass balance (%)						
Ethanol	19.3	21	20.3	19	17.6	19.9
Lactate	23.7	30.9	31.2	33.7	35.6	32.6
Acetate	14.5	14	13.4	13.2	12.1	13.2
Succinate	7.9	7.8	7.1	6.4	6.3	7.1
Formate	0	0	0	2.7	0.9	0.8
CO_2	13.5	14.6	12.8	11.4	10.6	14.1
Total	78.9	88.3	84.8	86.4	83.1	87.7
Biomass (CFU/mL)	$1.2\ 10^{+09}$	$7.8\ 10^{+08}$	$1.4\ 10^{+09}$	$8.2\ 10^{+08}$	$3.4\ 10^{+08}$	$4.5\ 10^{+08}$
Yield ($mol_{H2}/mol_{glucose}$)	0.88	0.95	0.83	0.74	0.69	0.92
Hydrogen production rate ($ml_{H2}/L.h$)	48.2	24.6	33.2	40.2	28.8	25.4

a. at the end of the first day

Figure 1

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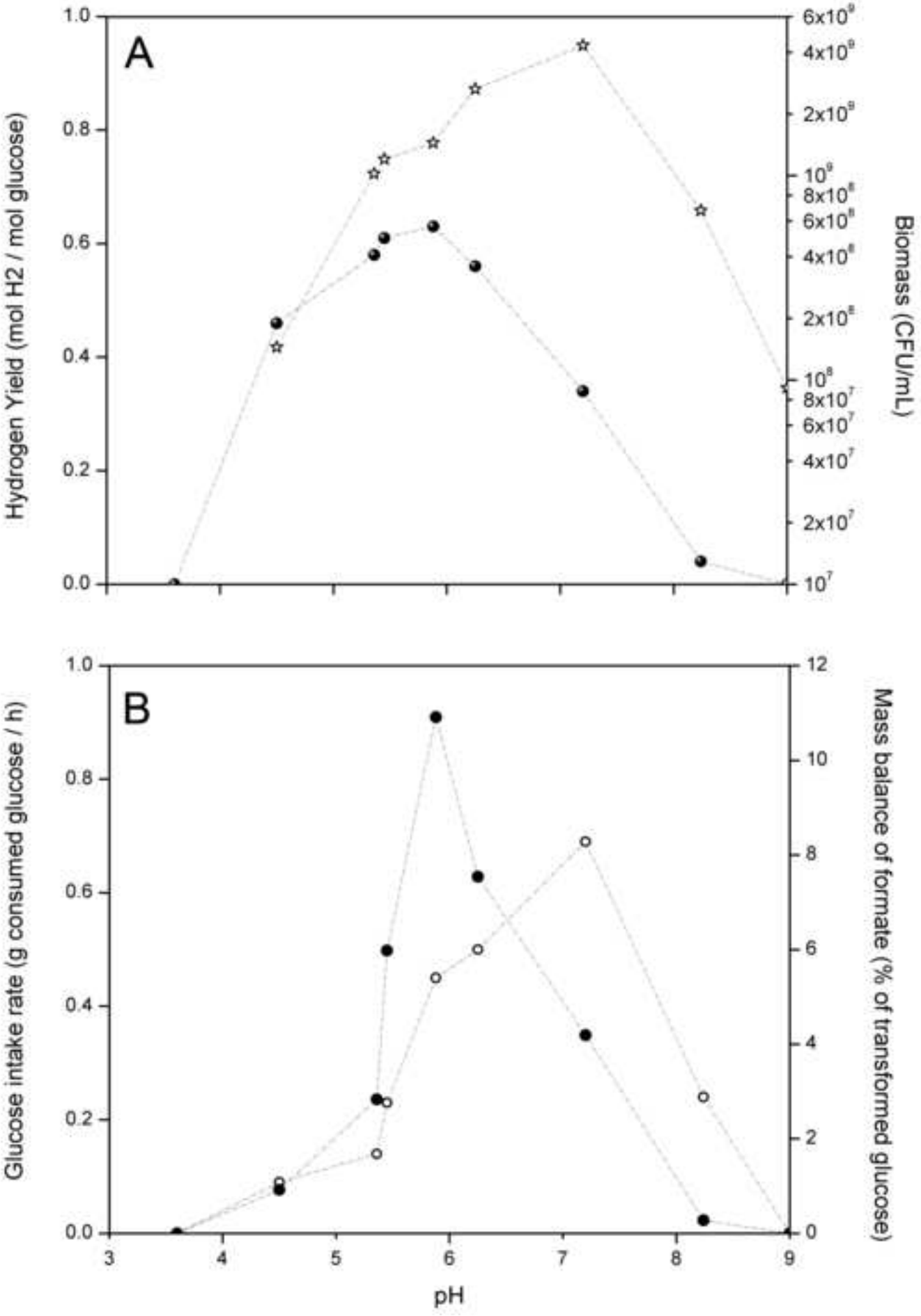

Caption Figure 1

Fig. 1. Investigation of H_2 production by the pure *C. freundii* CWBI952 culture growing on glucose at eight pH values ranging from 3.6 to 8.2 in a 2.3 L batch bioreactor. **(A)** Evolution of H_2 yield (★) and biomass (☆). **(B)** Glucose intake rate (○) and transformation to formate (●).

Figure 2

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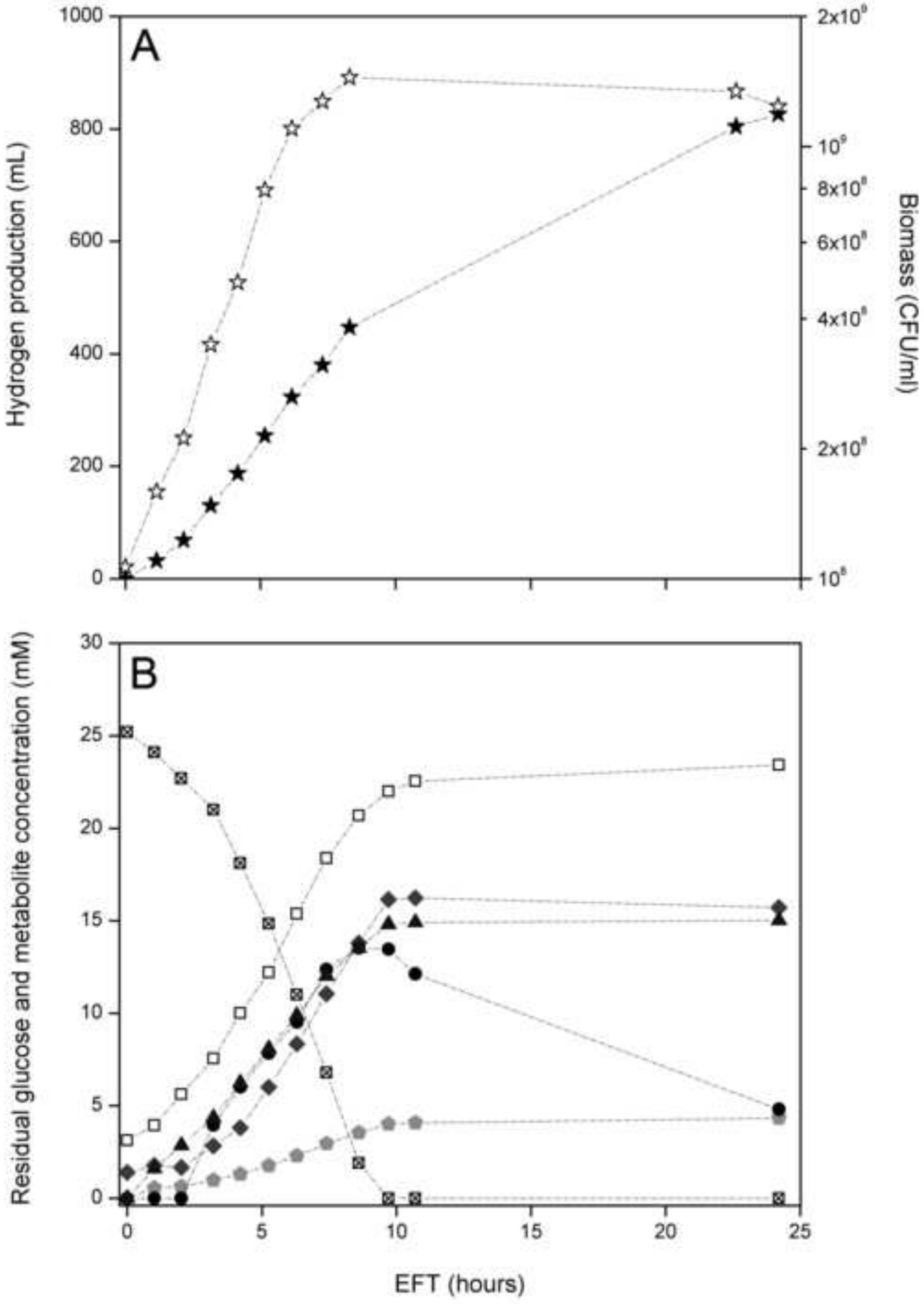

Caption Figure 2

Fig. 2. Investigation of H_2 production by the pure *C. freundii* CWBI952 culture growing on glucose at the optimum pH (5.9) in a 2.3 L batch bioreactor. **(A)** Growth curve (☆) and cumulative hydrogen production (★). (**B)** Glucose utilization and fermentation profile (⊠ glucose, □ ethanol, ⬟ succinate, ◆ lactate, ● formate, ▲ acetate).

Figure 3

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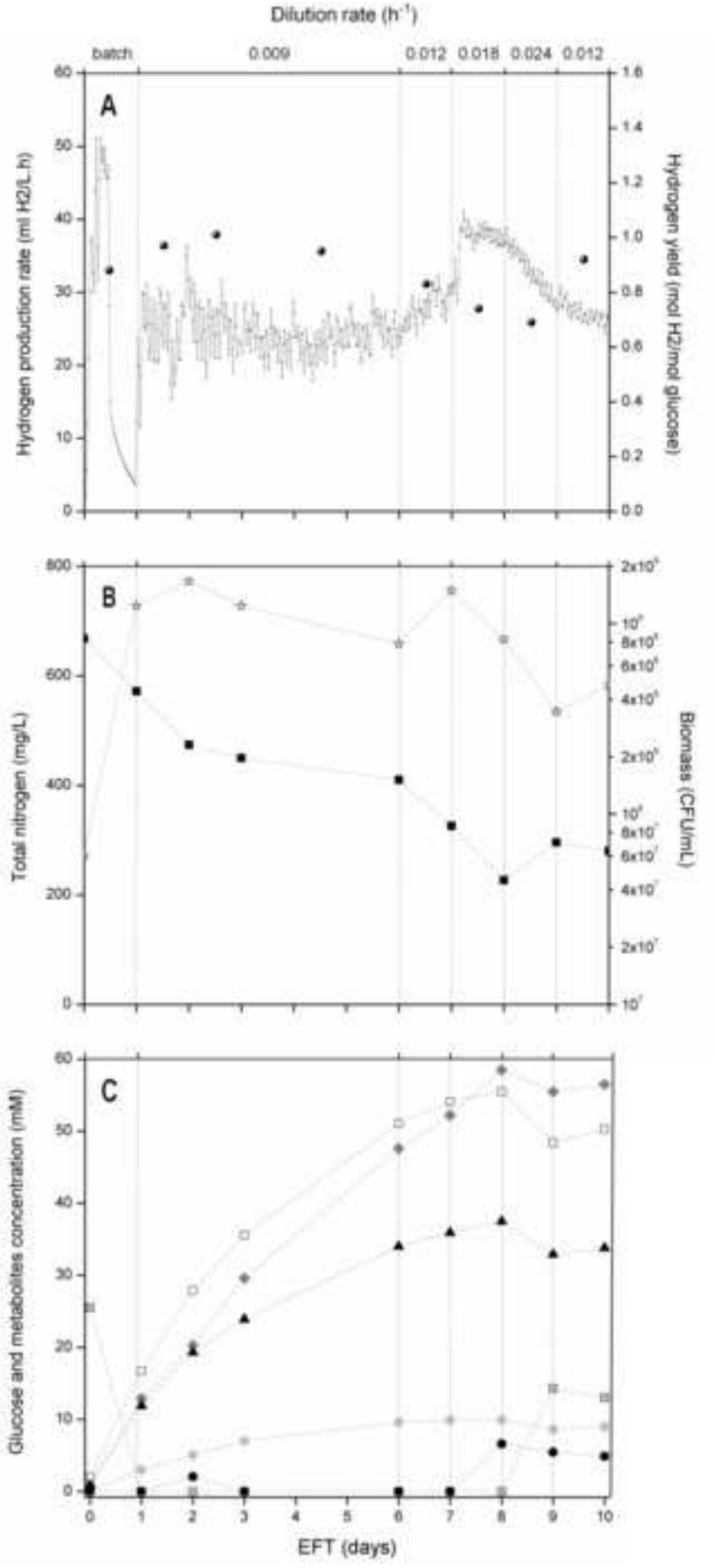

Caption Figure 3

Fig. 3. Effect of various dilution rates (0.009, 0.012, 0.018 and 0.024 h^{-1}) on the H_2 production activity of the pure *C. freundii* CWBI952 growing on glucose in a 2.3 L semicontinuous culture. **(A)** Hydrogen production rate (– • –) monitored during 10 days with GC-TCD and hydrogen yield (◒). **(B)** Total NH_4 (■) and biomass (☆) contained in the effluents. **(C)** Glucose utilization and metabolites concentration (⊠ glucose, □ ethanol, ⬟ succinate, ◆ lactate, ● formate, ▲ acetate).