Comparative study of biological hydrogen production by pure strains and consortia of facultative and strict anaerobic bacteria

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Abstract

In this paper, a simple and rapid method was developed in order to assess in comparative tests the production of binary biogas mixtures containing CO_2 and another gaseous compound such as hydrogen or methane. This method was validated and experimented for the characterisation of the biochemical hydrogen potential of different pure strains and mixed cultures of hydrogen-producing bacteria (HPB) growing on glucose.

The experimental results compared the hydrogen production yield of 19 different pure strains and sludges : facultative and strict anaerobic HPB strains along with anaerobic digester sludges thermally pre-treated or not. Significant yields variations were recorded even between different strains of the same species by *i.e.* about 20% for three *Clostridium butyricum* strains. The pure *Clostridium butyricum* and *pasteurianum* strains achieved the highest yields i.e. up to 1,36 mol H_2/mol glucose compared to the yields achieved by the sludges and the tested *Escherichia* and *Citrobacter* strains.

1. Introduction

For some decades, anaerobic digestion producing fuel gas has been largely investigated and many industrial processes are currently available for methane generation, an interesting renewable energy provider from various carbon sources (Cervantes et al., 2006; De Lemos Chernicharo, 2007; Lens et al., 2004; Verstraete et al., 2009). Since a large number of microorganisms have different and successive contribution in the methanogenic fermentation the process has been divided in four steps: hydrolysis, acidogenesis, acetogenesis, and eventually methanogenesis (Ding & Wang, 2008; Levin et al., 2007). Compared to the other microflora involved in methane production, the methanogenic Archaea group usually contains only a few strains and are restrained to very specific growth conditions. As a consequence, more scientific literature is available about operating parameters (e.g. pH or substrate loading rate) affecting the performances of methanogenic microorganisms rather than about the diversity and specific metabolism of the microflora involved in the first three anaerobic digestion stages. Recently, a great interest has been shown for H_2, an intermediate biogas produced during acido- and aceto-genesis. Biologically produced hydrogen is a promising energy vector with some advantages and complementarities compared to methane : mainly no carbon dioxide emissions from hydrogen combustion and its potential use in fuel cells for higher energetic efficiency (30-45%, low heating value) than in internal combustion engines (25-30%) without co-generation (Sammes et al., 2005). Nevertheless, though the H_2 production rate is about ten times higher than the theoretical CH_4 production rate, the hydrogen produced in anaerobic digesters is rapidly consumed by methanogenic bacteria. In a two-step process, *i.e.* when acido- and aceto-genesis are carried out in a different vessel than methanogenesis, it is feasible to generate separated biogas flows containing either H_2 or CH_4 depending on specific operating parameters such as the pH (Antonopoulou et al., 2010; Ding & Wang, 2008).

According to the highest yields reported by these authors, the mean volumetric H_2 and CH_4 potential would be in a stoichiometric ratio of 2:2 to 2:3.

Many investigations have been carried out in order to improve the H_2 production from organic matter. The results achieved and reviewed by many authors (Bartacek et al., 2007; Das & Veziroglu, 2008; Wang & Wan, 2009a; Wang & Wan, 2009b; Wang & Wan, 2009c) are useful for performance improvements of the whole anaerobic digestion process, whether performed in one or two stages. However, to our knowledge, no study has been conducted to compare a large number of pure strains and mixed cultures of HPB in the same experimental conditions.

Some decades ago, Owen et al. (1979) developed a bioassay method to monitor the biochemical methane potential (BMP) of different organic materials. Many studies have used this method (Hansen et al., 2004; Gunaseelan, 2004; Raposo et al., 2008; Rodriguez et al., 2005) and adapted it to characterize other microorganisms such as sulphate-reducing bacteria (Hiligsmann et al., 1998). The basic approach of the BMP test is to incubate a small amount of the organic material with an anaerobic inoculum and specific minerals and nutrients. Methane generation in the culture vessels, usually serum bottles, is monitored by simultaneous measurement of gas volume and gas composition using syringes or water replacement equipment and gas chromatography respectively (GC-TCD or GC-FID). Other systems have been developed for monitoring gas evolution in multiple closed vessels (Angelidaki et al., 1998). These systems use specific electronic devices and are usually expensive or need high expertise.

Recently, the BMP test has been adapted to investigate the metabolism of carbohydrates fermentation and hydrogen production by different H_2-producing strains and sludges (Lin et al., 2007; Ntaikou 2008; Panagiotopoulos et al., 2009; Pattra et al., 2008). However, the culture medium and preparation are difficult to carry out and

frequent sampling for biogas volume and composition analysis, by fastidious gas chromatography method, are not suitable, particularly when it is necessary to maintain pure cultures.

In our works, an easy-to-operate method was developed in order to assess binary biogas mixtures –H_2 and CO_2 or CH_4 and CO_2- production and composition by different strains and consortia from different substrates. This paper describes especially the experimental and biogas monitoring procedures for fermentative H_2-CO_2 production from glucose. The experimental culture procedure is an adaptation of the BHP assay developed by Lin et al. (2007), also based on the BMP assay described by Owen et al. (1979). By comparison to the relatively complex media used by these authors, our method uses a simple medium containing glucose as the carbohydrate source, peptone and yeast extract as the organic nitrogen sources, cysteine as the reducing agent, a phosphate buffer and magnesium sulphate salt. Moreover, a lower inoculum *i.e.* 2.5 % v/v was used in order to assess the adaptation and growth abilities of the strains and sludges. The biogas monitoring method is based on water replacement method. It was adapted *i.e.* using sterile syringes and CO_2-absorbing solution instead of water to assess both biogas production and composition at different incubation time avoiding culture contamination by other strains. Therefore, this biogas monitoring method does not require an expensive gas chromatographer. However it was validated in this paper by comparing the hydrogen production yields obtained in different cultures by both methods. .

The purpose of the experimental researches reported here is to comprehensively compare the hydrogen production yield and rate of different facultative and strict anaerobic HPB strains and anaerobically digested sludges. The relationship between the metabolites and H_2 production has also been investigated taking into account the

different equations (Eq. 1 to 6) of the metabolic pathways reported below (Tanisho et al., 1998). It should be noted that formic acid would be produced in the cell from by-products of the glucose metabolism. The strains were chosen according to the literature as high H_2 producers from the *Enterobacteriacea* group (*Escherichia coli* and *Citrobacter freundii*) and the *Clostridium* genus (11 strains). The sludges were collected from both a completely stirred digester (CSAD) and an UASB anaerobic digester cultured with or without thermal pre-treatment to enrich the acidogenic microflora.

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)
Lactic acid production	$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$	(3)
Succinic acid production	$C_6H_{12}O_6 + 2CO_2 + H_2 \rightarrow 2(CH_2COOH)_2 + 2H_2O$	(4)
Formic acid production	$CO_2 + H_2 \rightarrow CHOOH$	(5)
Ethanol production	$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$	(6)

2. Materials and methods

2.1. Inocula and treatment conditions

Different facultative and strict anaerobic strains and two anaerobically digested sludges were used as inocula for comparative tests in serum bottles. The pure strains *Clostridium butyricum* DSM2478 (named *C. butyricum* 1 in this paper), *C. butyricum* DSM2477 (named *C. butyricum* 2 in this paper), *C. pasteurianum* DSM525, *C. aminovalericum* DSM1283, *C. aurantibutyricum DSM793, C. puniceum* DSM2619, *C. saccharolyticum* DSM2544, *C. polysaccharolyticum* DSM1801, *C. acetobutylicum* DSM792, *C. thermosaccharolyticum* DSM571 (also known as *Thermoanaerobacterium thermosaccharolyticum* (O-Thong et al., 2008)) and *C. thermosulfurigenes* DSM2229 *were* obtained from the DSMZ collection (Germany). The strain *Escherichia coli* ATCC10536 was obtained from the ATCC collection. The lyophilized strains were first

136 cultured on the DSMZ recommended medium and then transferred in 25 ml
137 hermetically stoppered tubes filled with sterile MDT medium (Hamilton et al., 2010).
138 The strains *C. butyricum* CWBI1009 and *Citrobacter freundii* CWBI952 were isolated
139 and identified by the authors (Hamilton et al., 2010; Masset et al., 2010). The
140 facultative anaerobic strains were maintained at 4 °C on PCA solid medium and the
141 Clostridia strains in liquid MDT medium containing a lower glucose concentration (2
142 g/l) in order to promote spore formation after the growth phase. For fresh inoculum
143 conservation 1 mL of culture was transferred twice a week to 25 mL of sterile MDT
144 medium and incubated at 30°C (55°C for thermophilic strains).
145 The CSAD sludge was collected from a 20 liters stirred anaerobic digester used in
146 CWBI for BMP assays of different agro-food organic wastes. This lab-scale digester
147 was inoculated two years ago with a sludge collected from a full-scale anaerobic
148 digester treating the activated sludge from a municipal wastewater treatment plant. The
149 UASB sludge was collected from an UASB anaerobic digester treating organic
150 wastewater from sugar-beet and bioethanol industries. In order to enrich the hydrogen-
151 producing bacteria, both sludges were heat treated at 80 °C for 10 and 30 min.
152 Untreated sludges were also used as control.
153

154 **2.2. Experimental procedures and culture conditions**

155 The BHP test developed in this study is a modified version of the biochemical
156 hydrogen potential (BHP) test developed by Lin et al. (Lin et al., 2007). The tests were
157 carried out in 250 ml serum bottles filled with 200 ml of MDT culture medium. The
158 MDT medium contained, per liter of deionized water: glucose monohydrate (5 g),
159 casein peptone (5 g), yeast extract (0.5 g), Na_2HPO_4 (5.1 g), KH_2PO_4 (1.2 g),
160 $MgSO_4.7H_2O$ (0.5 g), L-cysteine hydrochloride (0.5 g). The bottle containing the
161 medium (except cysteine and glucose) with the pH adjusted to 8.5 with NaOH 5N was

autoclaved at 120 °C for 20 minutes. The glucose monohydrate was sterilized separately in aqueous solution in order to prevent Maillard reactions between carbohydrates and amino acids. The aqueous cysteine solution was sterilized in hermetically stoppered tubes to prevent its oxidation by ambient air. After the medium had been prepared, sterilized and cooled down, the glucose and cysteine solutions were added sterilely and the medium was inoculated with 5ml of inoculum. The bottle was capped tightly with a sterile rubber septum, flushed with sterile nitrogen and then incubated at a temperature of 30°C (55°C for thermophilic strains as described by Wang et al. (2009)). Each BHP test was conducted at least in triplicated experiments. Purity check of *Clostridium* and *Enterobacteriacea* cultures were realized by spreading a 100 µL sample or diluted sample, respectively, on a PCA Petri dish before incubation at 30°C for 24 to 48 h.

2.3. Monitoring and analytical methods

Water supplemented with KOH 9 N was used in a 100 ml-replacement equipment (Figure 1 A) to monitor the biogas production and composition of the BHP tests. After sterilizing the rubber (using flamed ethanol) of the culture vessel, the biogas samples were collected (Figure 1 B) with a graduated syringe (20 or 50 ml Terumo medical PEHD syringe) and a sterile needle before its transfer into the KOH solution through the immerged hole (H; Figure 1 C). Carbon dioxide absorption enabled the measurement of the other gases volume. A rubber (R) is used to maintain the thin layer neopren tube (T, resistant to strong basic conditions) used to fill the graduated equipment with KOH by gas suction (Figure 1 D) and gas removal (Figure 1 E) via another syringe (S) and tubes closed by Hoffman clamps (C1 and C2). The absorption

potential of the KOH solution was regularly controlled with gas mixtures containing 0, 20, 35, 80 and 100 % CO_2. Nitrogen, hydrogen and carbon dioxide content can be determined for each gas sample according to the equation below (Eq. 7 to 12) taking into account the initial nitrogen gas volume inside serum bottles gas phase and the further dilutions with the biologically produced gases.

The composition of biogas was validated using a gas chromatographer fitted with a thermal conductivity detector as described elsewhere (Hamilton et al., 2010).

Culture samples were centrifuged at 13000 g for 10 min and the supernatants were filtered through a 0.2 µm cellulose acetate membrane (Sartorius Minisart). The glucose, ethanol, formate, acetate, propionate, butyrate, lactate and succinate were analyzed using a HPLC equipped with a differential refraction index detector as described formerly (Masset et al., 2010). The data on the concentrations of glucose and metabolites present in the culture medium were used to calculate the mass balance (MB) of glucose conversion into the major soluble metabolites using the method reported elsewhere ((Hamilton et al., 2010).

The total volumetric hydrogen production $V_{HT} = \sum_{i=1}^{n} V_{H,i}$ (7)

where $V_{H,i}$ is the volumetric hydrogen production measured at the i^{th} sampling

therefore $V_{H,i} = (V_{S,i} + V_T)\ \%_{H,i} - V_T \,.\, \%_{H,i-1}$ (8)

where $V_{S,i}$ is the total biogas volume sampled at the i^{th} sampling

V_T is the total headspace volume in the culture vessel

$\%_{H,i}$ is the hydrogen content in the gas phase measured at the i^{th} sampling

$\%_{H,i-1}$ is the hydrogen content in the gas phase measured at the $(i-1)^{th}$ sampling

Since $\%_{H,i} = 1 - \%_{N,i} - \%_{C,i}$ (9)

where $\%_{N,i}$ and $\%_{C,i}$ are respectively the nitrogen and carbon dioxide content in the gas phase measured at the i^{th} sampling,

214 and $\%_{N,i} = \frac{V_{N,i}}{V_T} = \left(\frac{V_T}{V_T + V_{S,i}} \right) \%_{N,i-1}$ (10)

215 and $\%_{C,i} = \frac{V_{C,i}}{V_T} = \left(\frac{V_{S,i} - V_{R,i}}{V_{S,i}} \right)$ (11)

216 where $V_{R,i}$ is the biogas volume recovered after injection of $V_{S,i}$ in the KOH

217 replacement equipment at the i^{th} sampling

218 then Eq. 8 becomes $V_{H,i} = (V_{S,i} + V_T) \cdot (1 - \%_{N,i} - \%_{C,i}) - V_T \cdot (1 - \%_{N,i-1} - \%_{C,i-1})$

219 or $V_{H,i} = (V_{S,i} + V_T) \cdot \left[1 - \%_{N,i-1} \left(\frac{V_T}{V_T + V_{S,i}} \right) - \left(1 - \frac{V_{R,i}}{V_{S,i}} \right) \right]$

220 $- V_T \cdot \left[1 - \%_{N,i-1} - \left(1 - \frac{V_{R,i-1}}{V_{S,i-1}} \right) \right]$ (12)

221 For i = 1 : $\%_{N,i} = \%_{N,i-1} = 100\ \%$; $V_{R,i} = V_{S,i}$; $V_{R,i-1} = V_{S,i-1}$

222

223

224 **3. Results and discussion**

225 **3.1. Validation of the biogas monitoring method**

226 Seven cultures of HPB strains and sludges were carried out (in four replicates) in order
227 to validate the biogas monitoring method described in section 2.3. Indeed, the biogas
228 production and composition in the culture vessels were monitored using the new
229 method and the H_2 content in biogas was also measured by gas chromatography after
230 96 h of incubation *i.e.* when no more glucose was consumed and the pH had decreased
231 below 5.
232 Basically, the culture procedure described in section 2.2 was experimented on the pure
233 *Clostridium* strain (*Clostridium butyricum* CWBI1009) and two anaerobically digested
234 sludges without a pretreatment or pretreated at 80°C for 10 or 30 minutes. After 48, 72
235 and 96 h of incubation the biogas produced in the serum bottles was sterilely collected
236 and transferred to the replacement equipment filled with potassium hydroxide for

237 carbon dioxide absorption as described in section 2.3. Also, taking into account the
238 initial nitrogen gas volume inside the gas phase and its further dilutions by the
239 produced biogas, it was possible for each gas sampling to assess the gas composition
240 and therefore the volumetric biogas and H_2 production. The cumulative biogas and H_2
241 production are presented on Figure 2 A and B respectively. Each dot on the figure
242 represents the average value over the 4 measurements. Standard deviations are not
243 depicted on the graph for better reading; however the average standard deviation is
244 about 25±2 ml and 15±1 ml respectively. The profiles are discussed in section 3.2.
245
246 Figure 3 shows the H_2 production yields calculated after 96 h (A) as the molar ratio
247 between the cumulative H_2 production (determined by the new biogas monitoring
248 method) and the glucose consumed and (B) the cumulative biogas production
249 (expressed in mole of gaseous mixture per mole of glucose consumed) multiplied by
250 the H_2 content measured by gas chromatography. It is to notice that the results obtained
251 by both measurement methods are similar. Indeed, the overall mean difference between
252 the results of Figure 3 A and B calculated over the four replicates of the 7 different
253 samples is about 2.5 % and the standard deviation is about 5.7 %. Moreover, in similar
254 conditions, regarding the medium and environmental parameters, Chen et al. (2005)
255 achieved similar H_2 yields with *C. Butyricum* compared with the results shown in
256 Figure 3 with the *C. butyricum* pure strain. Therefore, it can be assumed that the
257 method developed in this paper has been validated and would be useful for other binary
258 biogas potential assays e.g. CH_4/CO_2 biogas mixture produced by methanogenic
259 bacteria. It has been tested successfully for anaerobic digestion of different substrates
260 such as glucose, cellulose, straw and other (hemi-) cellulosic materials (results not
261 shown). Compared to other biogas monitoring methods the one described here has
262 many advantages. It is less expensive than the system developed by Angelidaki et al.

263 (1998) for monitoring gas evolution in multiple closed vessels. It is also simpler than
264 the experimental and mathematical method developed by Hansen et al. (2004) for
265 determination of methane potential of solid organic wastes. Furthermore, our method
266 gives direct information on biogas production and does not require a gas
267 chromatographer neither a GC-TCD (Gunaseelan, 2004; Lin et al., 2007) nor a GC-FID
268 (Hansen et al., 2004), even if it is a relatively usual equipment in laboratories. This
269 simple method is then suitable for small companies and low-income countries to assess
270 the biogas potential from organic by-product or waste.
271
272 **3.2. Comparative H_2 production by mixed cultures and a pure *Clostridium* strain**
273 The results reported in Figure 2 and 3 also allow comparing performances of the pure
274 *Clostridium* strain and the different sludges. A low inoculum size was used in order to
275 assess the adaptation and growth faculties of the strain and sludges. This explains the
276 longer lag phase compared to those mentioned in other strudies (Baghchehsaraee et al.,
277 2008; Lin et al., 2007) with inoculum about ten times higher than the inoculum volume
278 used in our experiments. Similar observation was deduced from Chen's 5 L-cultures
279 inoculated with 1.2 % of preculture (Chen et al., 2005). In our experiments, the purity
280 check on PCA medium of the *Clostridium* culture asserted that the culture was
281 maintained pure. The biogas and H_2 curves have relatively similar trends showing that
282 biogas composition is relatively constant along incubation time. The *Clostridium* pure
283 strain has the highest H_2 production rate (HPR), *i.e.* 1.2 ml/h, during the initial 48 hours
284 and the culture from UASB heat-treated for 10 min has the overall highest HPR, i.e. 2
285 ml/h recorded between 48 and 72 hours of culture. No significant difference was
286 observed between the heat-treated cultures from stirred anaerobic digester (CSAD) nor
287 between the untreated and heat-treated UASB microflora . By contrast, the untreated
288 culture from the stirred digester led to relatively low H_2 production. As foreseen both

289 autoclaved sludges and pure culture (121°C for 20 min) did not show any growth
290 (results not shown).
291 By comparison to the results shown on Figure 2, a lower difference is observed
292 between the H_2 yields recorded for the heat-treated UASB sludges compared to the
293 related H_2 cumulative curves. Therefore, higher HPR were related to a higher glucose
294 intake rate. H_2 yields are then more relevant to compare different cultures. The yields
295 achieved *i.e.* about 1 mol H_2 per mole glucose consumed are lower than those
296 mentioned by other authors (Alalayah et al., 2008; Baghchehsaraee et al., 2010; Lin et
297 al., 2007; Pattra et al., 2008). This trend is related to the environmental parameters in
298 the culture medium that are largely affected by either the initial pH and buffer
299 concentration or by the H_2 partial pressure (Das & Veziroglu, 2008). The latter is
300 related to the initial gas composition inside the gas phase and to the total pressure that
301 is affected by the initial gas to liquid phase ratio. The pH and pressure parameters are
302 subject to relatively large variations during the culture *i.e.* in our experiments about 3
303 pH unit and 2 bars. Therefore, our culture conditions are more restrictive compared to
304 the culture conditions usually used in other small vessels experiments with higher
305 volumetric gas to liquid ratio (Alalayah et al., 2008; Baghchehsaraee et al., 2010; Lin et
306 al., 2007; Pattra et al., 2008). However the yields reported in this paper are comparable
307 to those reported for H_2-producing bioreactors (Bartacek et al., 2007; Chen et al., 2005;
308 Fang & Liu, 2002; Masset et al., 2010).
309 As already shown by Baghchehsaraee et al.(2008), the untreated anaerobic sludges
310 achieved lower H_2 yields compared to the treated sludges. No significant difference
311 was recorded for the sludge treated at 80°C for 10 or 30 minutes as for the treatment at
312 65° C or 80°C conducted by Baghchehsaraee. Moreover, the pure culture and heat-
313 treated cultures from both digesters achieved similar yields.
314

As shown in Table 1, the mass balance (MB) of glucose conversion into soluble metabolites is similar to each inoculum and limited to 77 ± 5%. This indicates that about 25% of the glucose is converted into biomass, 25 % into butyrate, 15% into formate and lactate and 8% into acetate and into carbon dioxide . No propionate was detected at the end of the culture. These results are in accordance with those reported by other authors (Lin et al., 2007; Masset et al., 2010; Skonieczny & Yargeau, 2009; Wang et al., 2005). Ethanol was only produced by the untreated UASB sludge probably due to the presence of *Enterobacteriaceae* (Hamilton et al., 2010). In the glucose metabolic pathways ethanol, formate and lactate productions indicate a lack of potential hydrogen production that should be overcome for industrial prospects with optimized culture conditions. From this point of view, the culture conditions seems more adapted for the pure *Clostridium* strain. Indeed, the glucose conversion MB showed a lower concentration of these metabolites and higher H_2 production yields were obtained.

3.3. Comparative H_2 production by pure facultative and strict anaerobic strains

In order to characterise hydrogen production of different pure strains, the BHP test method was carried out on two facultative anaerobic bacteria, *i.e. Citrobacter freundii* and *Escherichia coli*, and eleven other strict anaerobic HPB strains of the genus *Clostridium*. They were chosen amongst the mesophilic and thermophilic highest H_2 producers from a large range of carbohydrates including starch. Three different strains of *C. butyricum* have also been compared. The results are shown in Figure 4 presenting the H_2 production yields and H_2 biogas content after 96 h of culture (in triplicate except for *C. puniceum* that was quite difficult to culture : only one from the three cultures grew). This strain seems less adapted to the experimented culture conditions. The *Clostridium* strains yielded the highest amount of H_2 and total biogas, *i.e.*

approximately 20% and 30% more than the amount produced by the untreated sludge (Figure 2) and the other pure strains.

Although all strains were chosen for their high H_2 production, wide hydrogen yield variations were recorded depending on the species, even for strains of the same species. Indeed, H_2 yields of the three *C. butyricum* species varied in a 20% range including the results achieved with *C. butyricum* CWBI1009 in the former experiments (Figure 3). However, the biogas composition was quite similar (69 ± 8 % of H_2) compared to the average H_2 content of 60 ± 10 % calculated over the whole group of *Clostridium* strains tested. Lin et al. (2007) observed similar variations between different *Clostridium* strains e.g. a ratio of 1,27 between the H_2 yields of *C. Butyricum* and *C. Acetobutylicum* compared to a mean ratio of about 2,1 estimated in our results. Our resulting yields for *Clostridia* are however lower, probably due to more restrictive environmental parameters as already mentioned in section 3.2. The low H_2 production and H_2 content in the biogas recorded for *C. aminovalericum* should be related to the pH that did not decrease below 6 for this strain (pH 4,8 ± 0,4 for the other *Clostridium* strains at the end of culture) although most of the glucose was consumed. Moreover, as shown in Table 2, the glucose amount converted by this strain to ethanol was relatively high : high ethanol yield (28,79 %) related to a low glucose conversion yield to metabolites (69,26 %).

The thermophilic *Clostridium* strains (cultured at 55°C) achieved interesting yields but lower than the highest mesophilic H_2 producers. The yields (H_2 and soluble metabolites shown on Table 2) recorded for *C. thermosaccharolyticum* are similar to those (0.96 mole H_2 produced per mole glucose) achieved at 60°C by O-Thong et al. (2008) with another strain of the same species. It is to mention that relatively few investigations have been carried out on glucose with *Clostridium* thermophilic pure strains and most

365 of the studies with sludges achieved higher H_2 yields with thermophilic than mesophilic
366 culture conditions (Bartacek et al., 2007; Shin et al., 2004; Zhang et al., 2003).
367 Compared to the hydrogen yields, the soluble metabolites yields (Table 2) also vary
368 significantly between the *Enterobacteriacea* family and the *Clostridium* genus, even
369 between the different strains of the *Clostridium* genus. The average sum of the
370 converted glucose into metabolites is similar to that mentioned for the experiments
371 reported in section 3.2 (77 %); the standard deviation (20 %) is however larger. That
372 implies that about 25% of the glucose is used for biosynthesis but it varies between 11
373 and 35 %, even for the three different strains of the *C. butyricum*. The butyrate yields
374 and the acetate + butyrate yields are the highest for the *C.butyricum* and *C.*
375 *pasteurianum* strains which are also the highest H_2 producers. This trend is consistent
376 to the results of other authors (Lin et al., 2007; Wang et al., 2008). By comparison to
377 the results of Table 2 for the strains *C. butyricum* and *C. acetobutylicum*, the
378 percentages calculated from the results of Lin et al. (2007) were similar for acetate and
379 about 25 % and 3-fold higher for butyrate respectively. By contrast, Lin et al. reported a
380 quite low lactate production and a 3-fold higher CO_2 production. These differences
381 should be related to the different composition of the medium used by Lin et al, and in
382 our experiments. However, it should be noticed that *C. pasteurianum* converted about
383 twice more glucose to CO_2 (17 ± 1 %) than the other strains (7,5 ± 3,5 %). Therefore,
384 this H_2 producer seems less interesting for an industrial process, particularly a two-
385 stage anaerobic digestion process producing hydrogen and methane since a high CO_2
386 production in the first hydrogen-producing stage would be considered as a partial lack
387 of methane potential in the second stage.
388 The highest yields in lactate were reported with *C. acetobutylicum*. Both *C.*
389 *Saccharolyticum* and *thermosaccharolyticum* converted about 30 % of the carbon from
390 the glucose to ethanol. *Enterobacteriacea* and *C. aminovalericum* strains also produced

391 large concentration of ethanol. These strains except the latest also produced high
392 amounts of lactate.
393
394 **4. Conclusions**
395 The method developed in this paper for the evaluation of the biogas production and its
396 composition has many advantages. It is relatively cheap and easy to operate and gives
397 direct information without the need of a gas chromatographer. Moreover it is suitable
398 for other binary biogas potential assays e.g. CH_4/CO_2 biogas production from different
399 substrates.
400 The results of the comparative tests show that the hydrogen yields vary significantly
401 between the 19 different pure strains and sludges, even between different strains of the
402 same species *i.e.* about 20% variation. Therefore, this paper asserts that strain selection
403 is an important way in order to optimize anaerobic digestion processes with H_2
404 production.
405
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Figures caption

Fig. 1. Experimental setup and operating procedure used to monitor the biogas composition of the BHP tests : KOH replacement equipment (A); gas sampling operation (B); gas transfer for carbon dioxide absorption in KOH (C); equipment filling with KOH (D); gas removal from syringe (E) (the arrows L1 and L3 indicate the displacement of the syringe piston and L2 the displacement of the KOH level in the replacement equipment).

Fig. 2. Investigation of biogas (A) and H_2 (B) production from glucose by the pure *C. butyricum* CWBI1009 and two sludges from stirred (CSAD) and UASB anaerobic digesters without a pretreatment or pretreated at 80°C for 10 or 30 minutes.

Fig. 3. Investigation of H_2 production yields from glucose by the pure *C. butyricum* CWBI1009 and two sludges from stirred (CSAD) and UASB anaerobic digesters without a pretreatment or pretreated at 80°C for 10 or 30 minutes. Hydrogen yields are calculated as (A) the molar ratio between the cumulative H_2 production (determined by the new biogas monitoring method) and the glucose consumed and (B) the cumulative biogas production (expressed in mole of gaseous mixture per mole of glucose consumed) multiplied by the H_2 content measured by gas chromatography (errors bars meaning standard deviation on replicates).

Table 1. Metabolite analysis during H_2 fermentative production from glucose by the pure *C. butyricum* CWBI1009 and two sludges from stirred (CSAD) and UASB anaerobic digesters without a pretreatment or pretreated at 80°C for 10 or 30 minutes.

Fig. 4. Investigation on H_2 production yields (A) from glucose by different facultative and strict anaerobic pure strains and H_2 content in biogas (B) (errors bars meaning standard deviation on replicates).

Table 2. Metabolite analysis during H_2 fermentative production from glucose by different facultative and strict anaerobic pure strains.

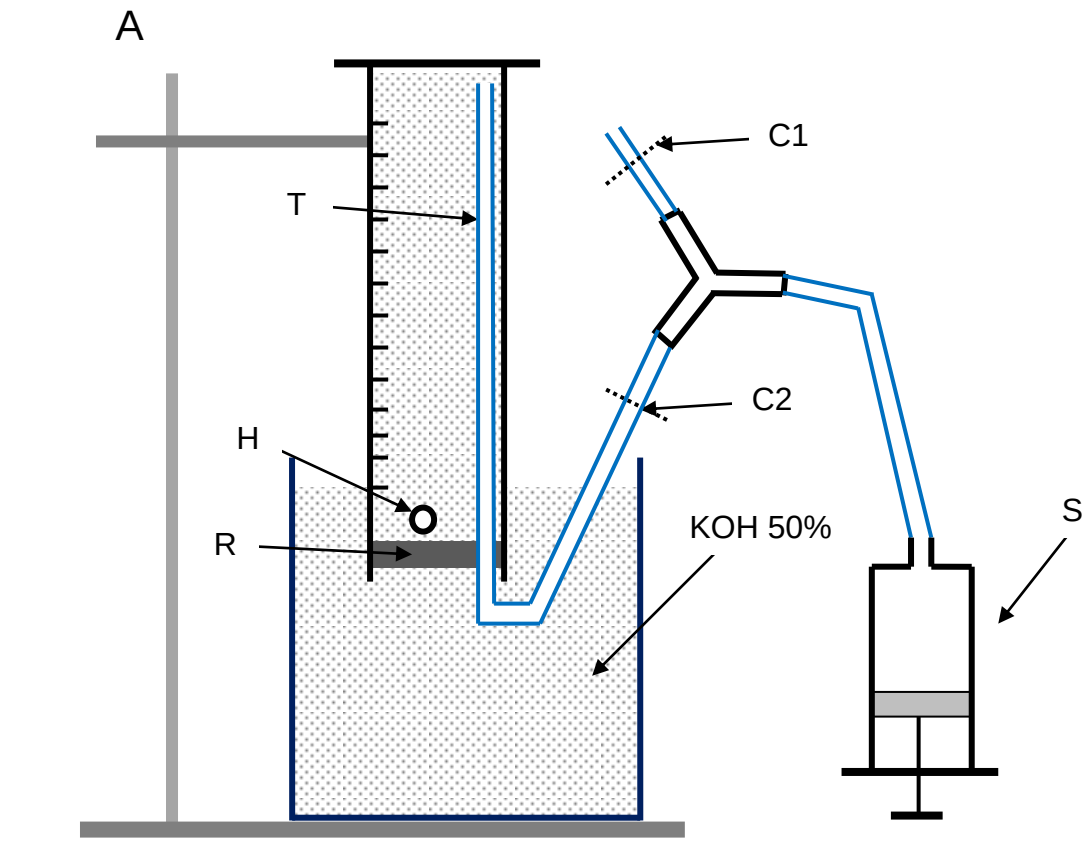
Table

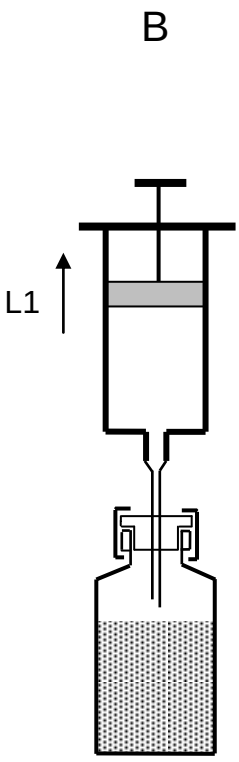
	Carbon converted from glucose (%)						
	Lactate	Formate	Acetate	Ethanol	Butyrate	CO_2	Sum
C. butyricum CWBI	8.77	12.07	8.15	0.00	32.05	7.33	68.38
CSAD	16.50	10.85	11.33	0.00	36.85	4.08	79.61
CSAD 10' 80°C	13.57	15.01	8.82	0.00	33.79	6.66	77.85
CSAD 30' 80°C	12.66	14.09	7.76	0.00	35.34	5.60	75.44
UASB	19.12	13.67	9.97	2.01	24.85	8.89	78.51
UASB 10' 80°C	24.97	7.19	1.34	0.00	39.70	10.75	83.95
UASB 30' 80°C	15.18	13.36	6.85	0.00	34.61	8.33	78.32

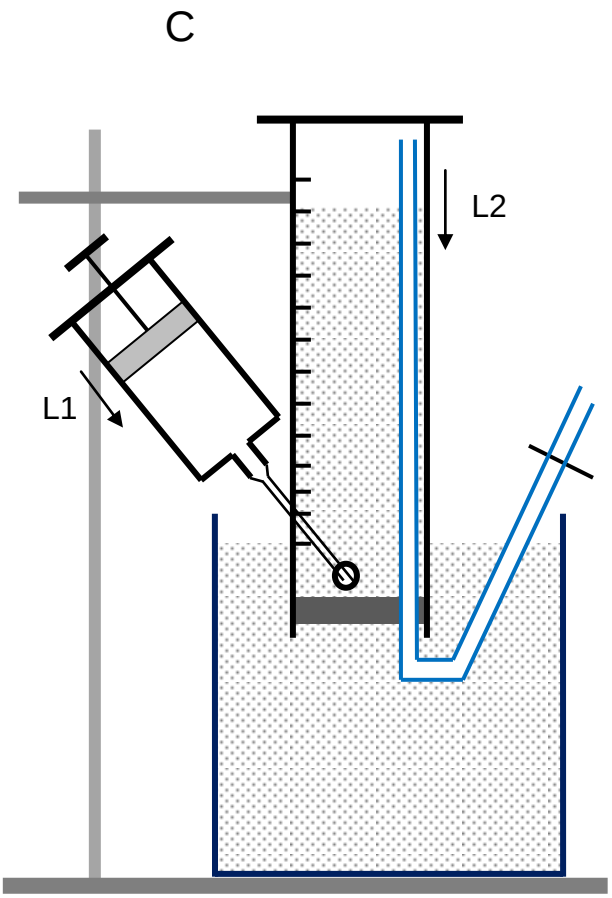
Table

	Carbon converted from glucose (%)						
	Lactate	Formate	Acetate	Ethanol	Butyrate	CO_2	Succinate
C. butyricum 1	18.97	7.21	10.45	1.08	29.15	7.25	0.00
C. butyricum 2	11.52	9.05	12.73	13.68	33.94	8.48	0.00
C. butyricum CWBI	12.32	5.72	8.90	13.29	17.81	7.38	0.00
C. pasteurianum	23.43	2.46	10.09	4.39	22.31	16.89	0.00
C. saccharolyticum	23.66	6.22	12.78	32.11	7.49	6.50	0.00
C. acetobutylicum	39.45	0.69	4.96	7.78	13.61	7.09	0.00
C. aminovalericum	17.71	2.38	8.44	28.79	6.91	5.04	0.00
C. aurantibutyricum	18.72	0.45	5.96	0.73	11.02	10.01	0.00
C. puniceum	25.46	4.18	7.70	5.44	20.38	2.22	0.00
C. thermosaccharolyticum	27.35	1.14	10.43	30.33	9.94	9.37	0.00
C. thermosulfurigenes	26.12	1.18	9.59	16.11	7.09	7.11	0.00
Citrobacter freundii	29.20	0.00	24.40	22.40	0.00	2.30	10.50
Escherichia coli	30.00	0.00	18.50	23.00	0.00	10.00	0.00

Figure







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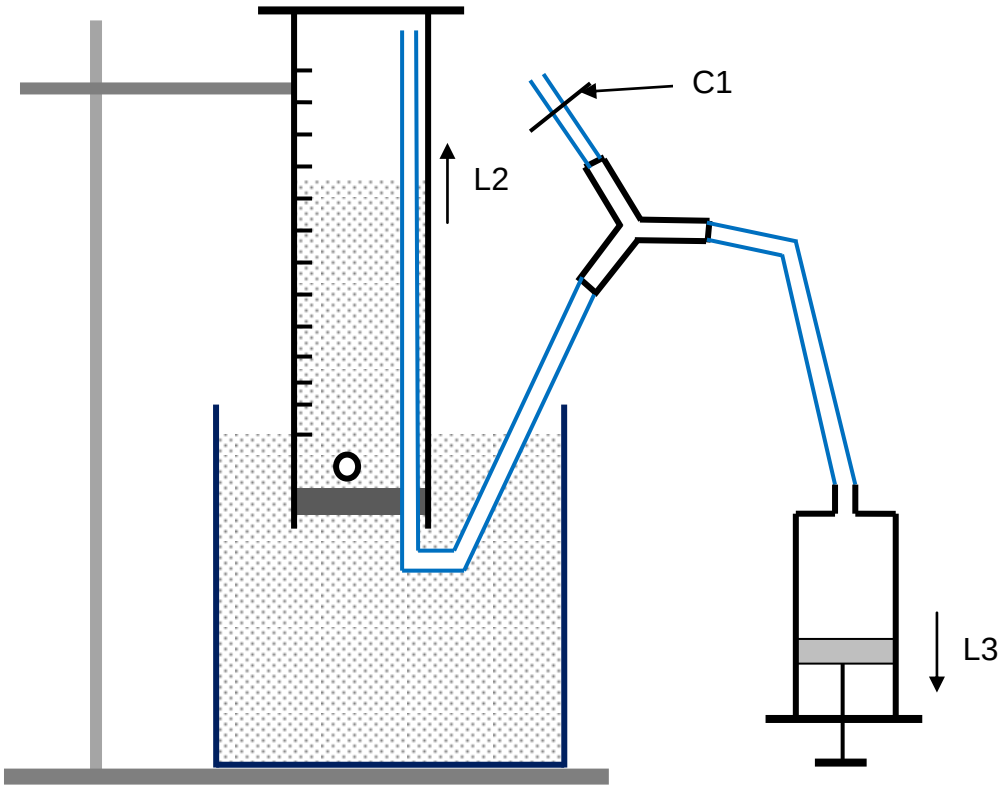

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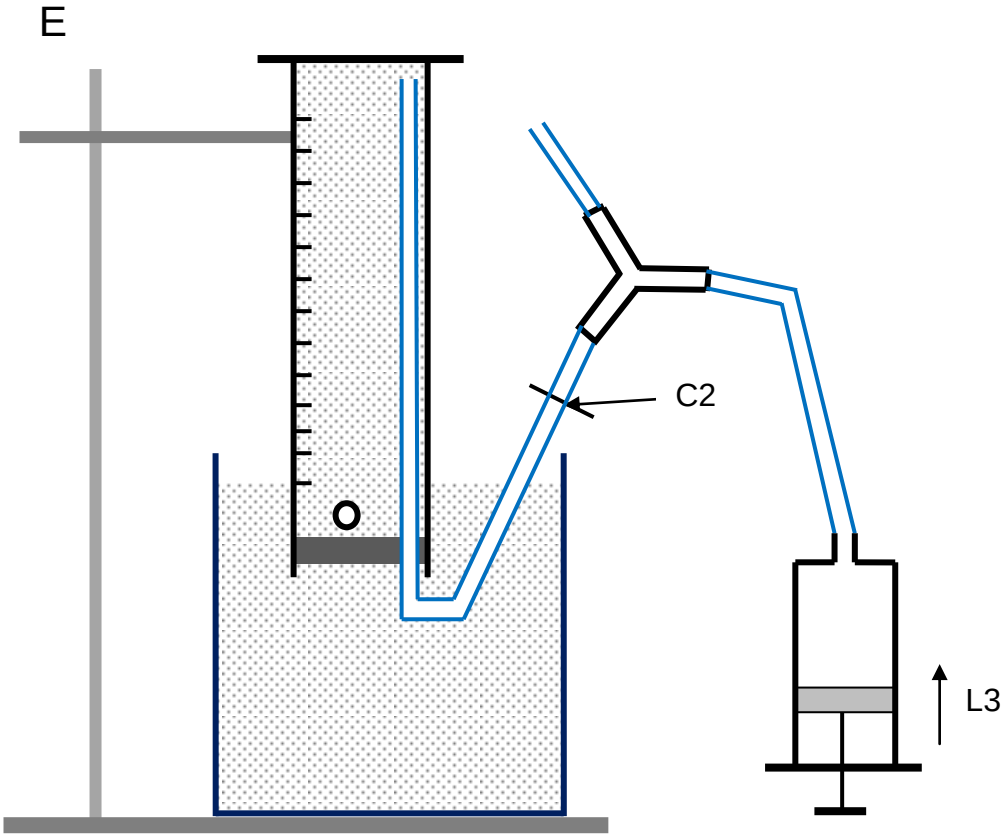
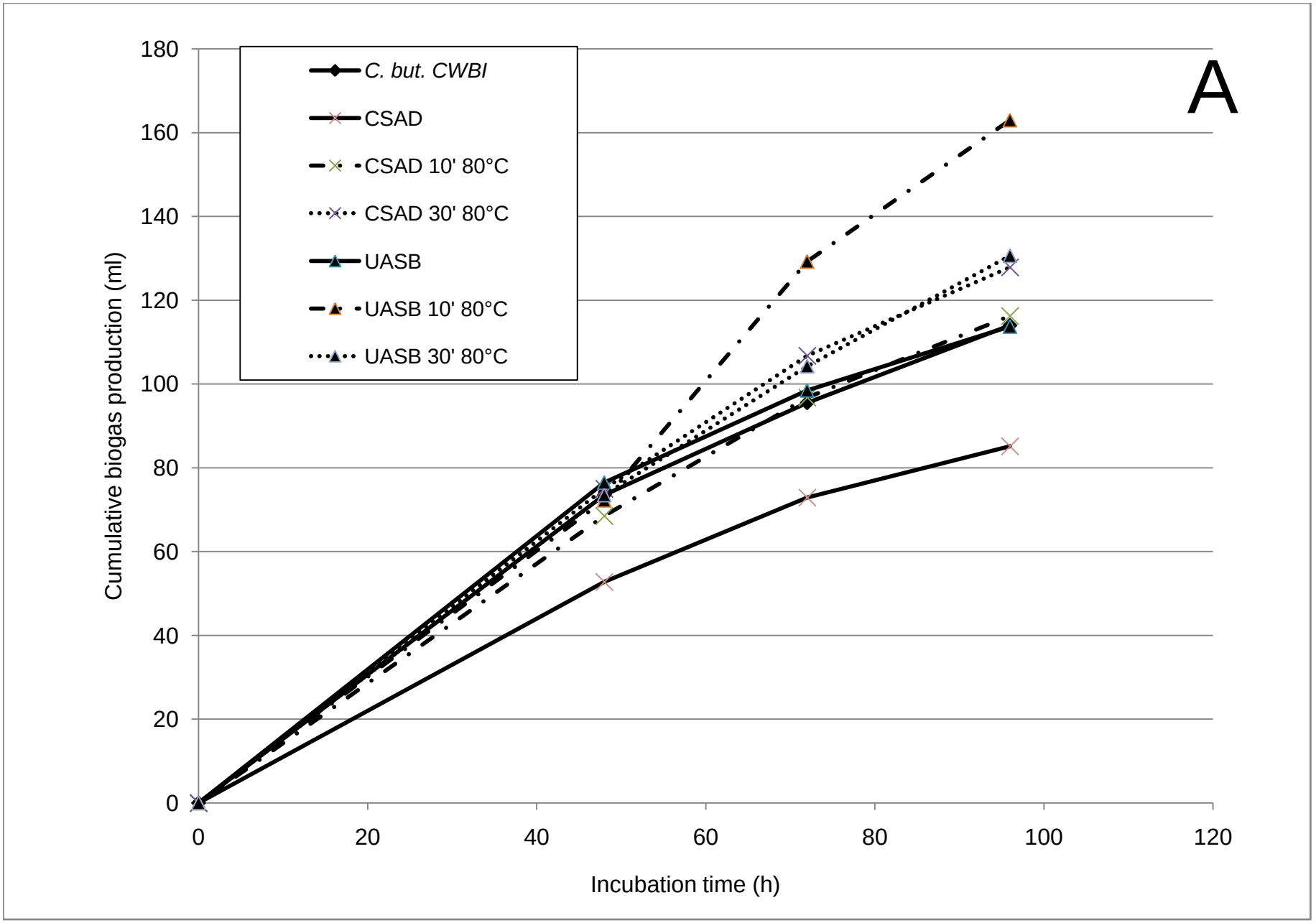
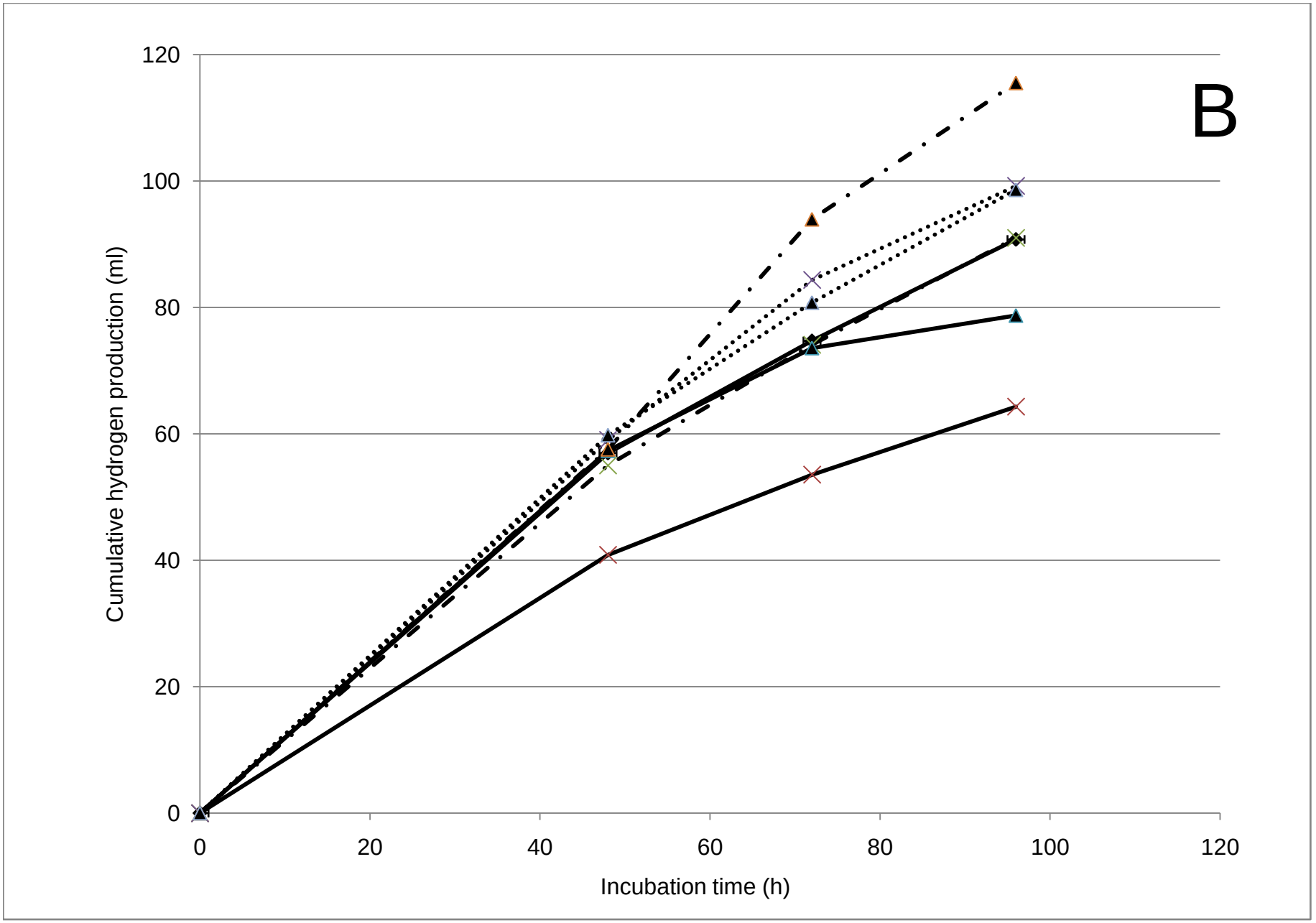


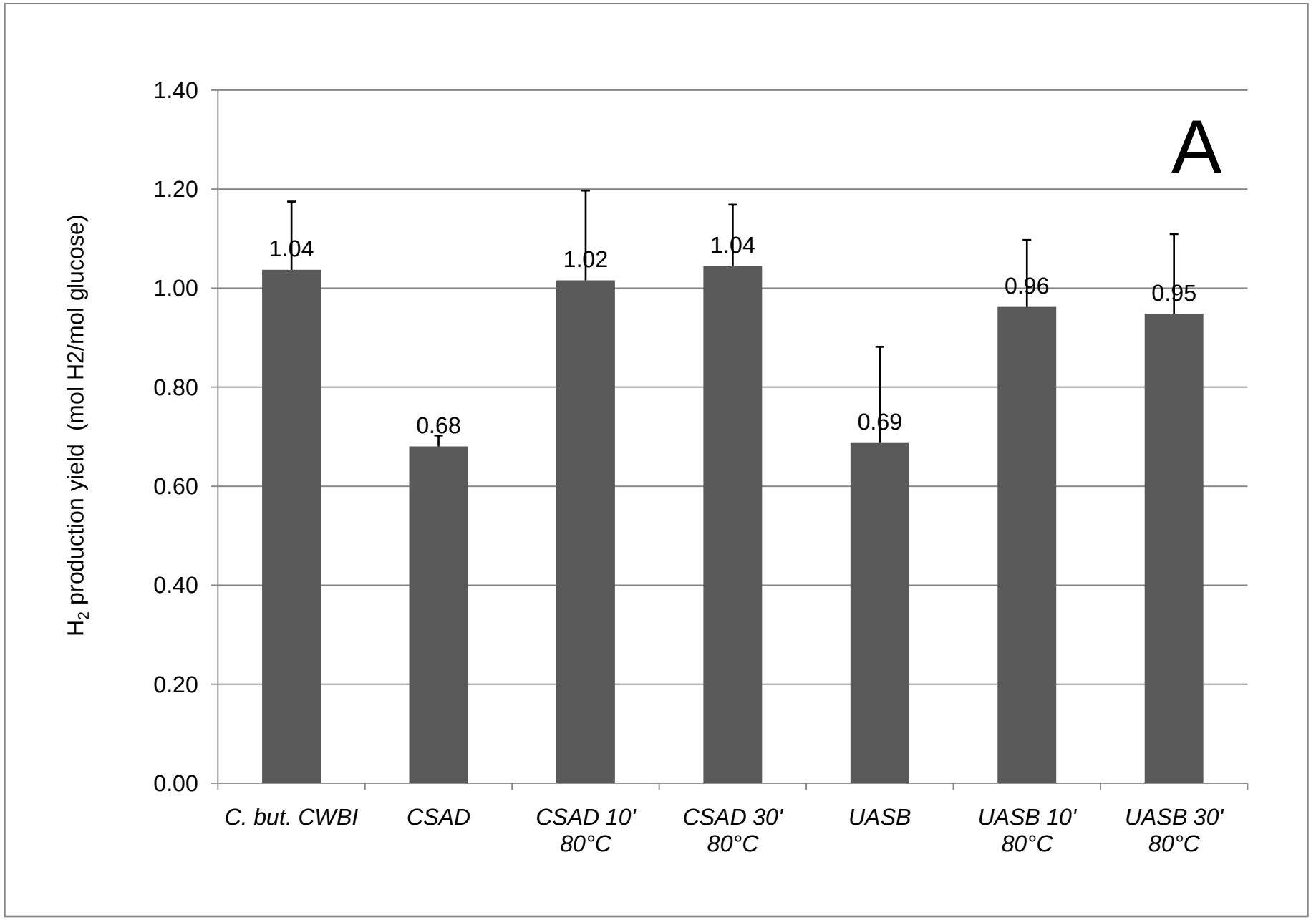
Figure


A
C. but. CWBI
CSAD
CSAD 10' 80°C
CSAD 30' 80°C
UASB
UASB 10' 80°C
UASB 30' 80°C
Cumulative biogas production (ml)
Incubation time (h)
0
20
40
60
80
100
120
140
160
180

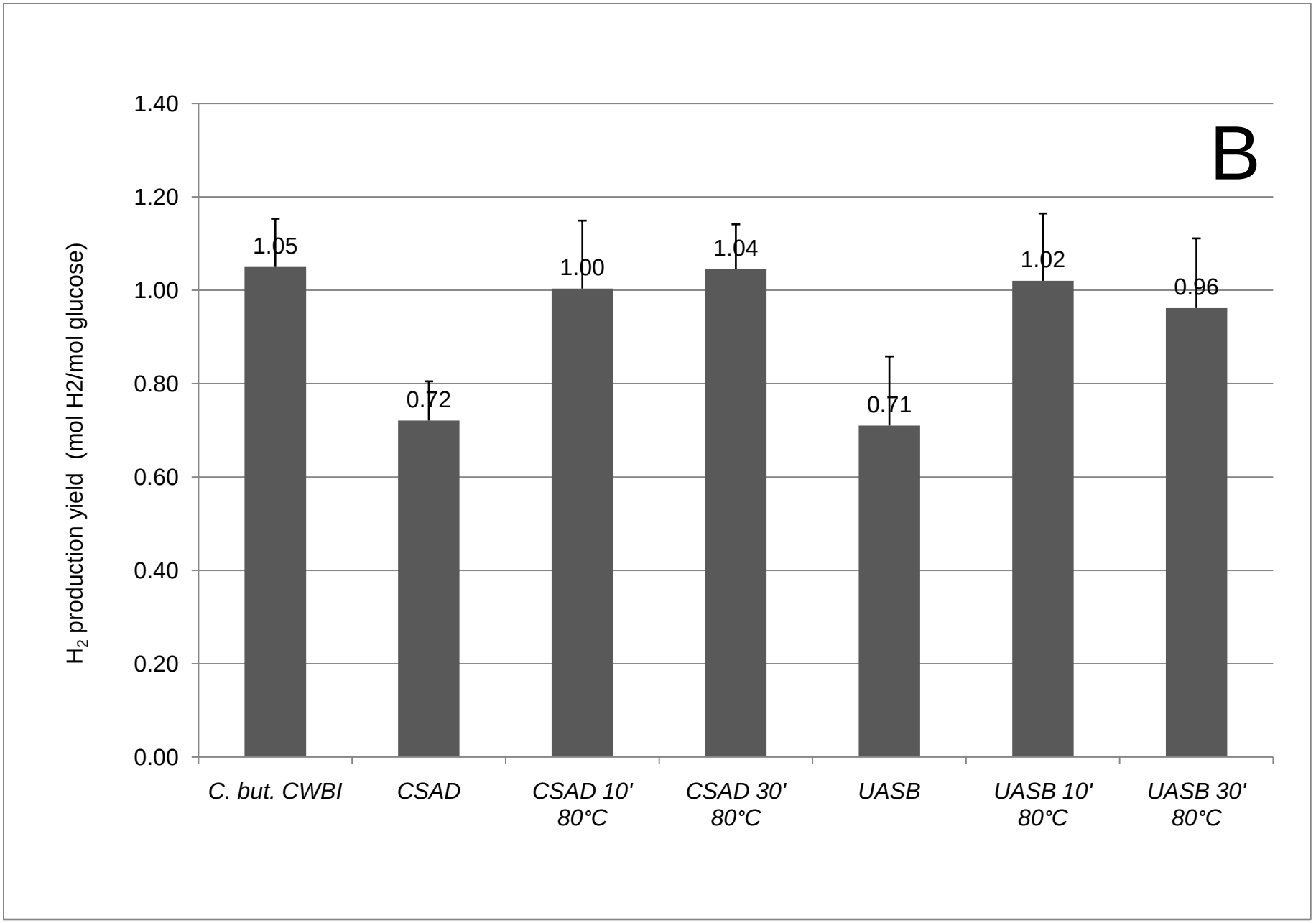
Figure


B
Cumulative hydrogen production (ml)
Incubation time (h)
0
20
40
60
80
100
120

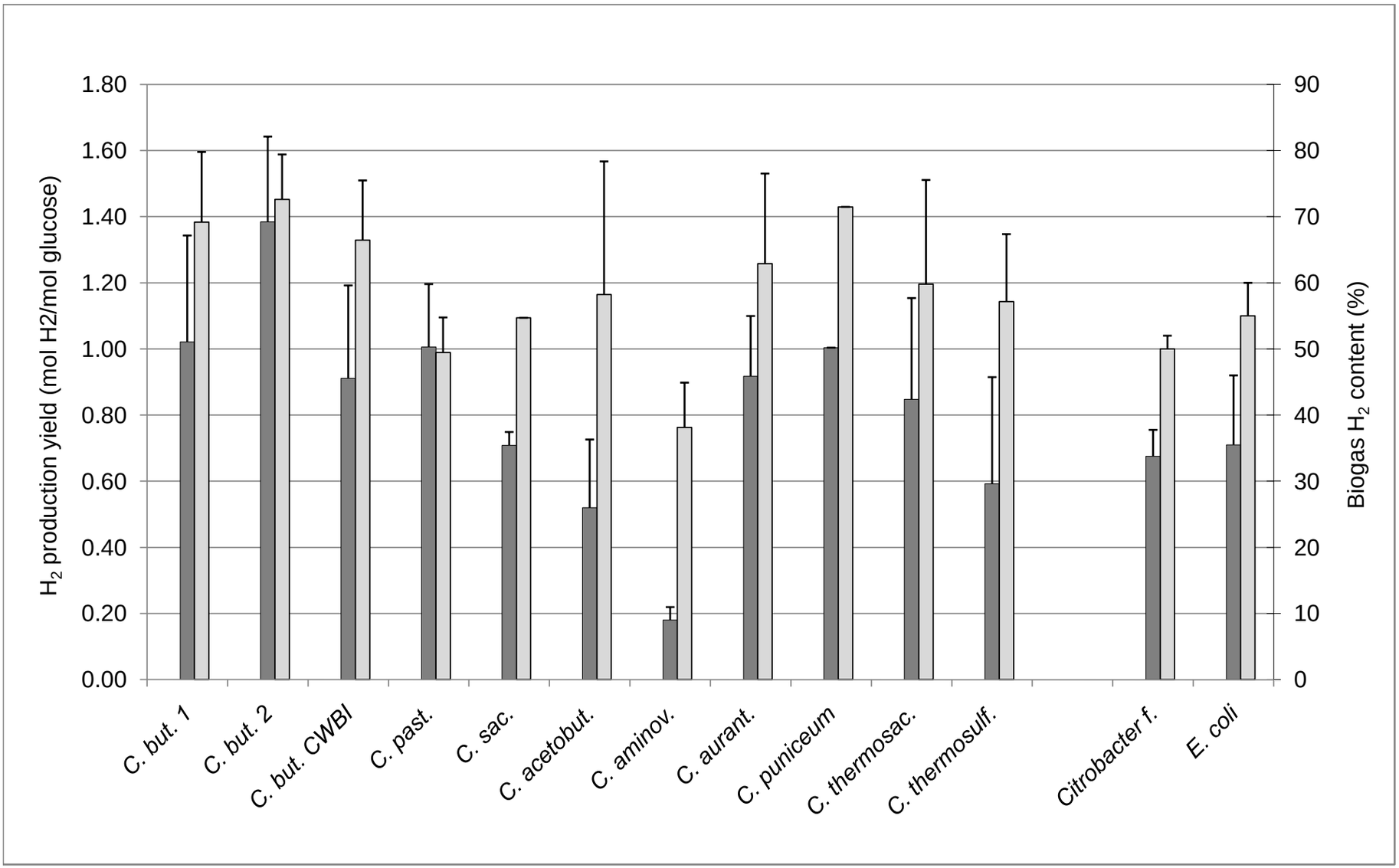
Figure


A
H_2 production yield (mol H2/mol glucose)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.04
0.68
1.02
1.04
0.69
0.96
0.95
C. but. CWBI
CSAD
CSAD 10' 80°C
CSAD 30' 80°C
UASB
UASB 10' 80°C
UASB 30' 80°C

Figure


B
H_2 production yield (mol H2/mol glucose)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.05
0.72
1.00
1.04
0.71
1.02
0.96
C. but. CWBI
CSAD
CSAD 10' 80°C
CSAD 30' 80°C
UASB
UASB 10' 80°C
UASB 30' 80°C

Figure


1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
H2 production yield (mol H2/mol glucose)
90
80
70
60
50
40
30
20
10
0
Biogas H2 content (%)
C. but. 1
C. but. 2
C. but. CWBI
C. past.
C. sac.
C. acetobut.
C. aminov.
C. aurant.
C. puniceum
C. thermosac.
C. thermosulf.
Citrobacter f.
E. coli