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

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Keywords: Clostridium, hydrogen production, biogas potential, pure strain, sludge

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 CO2 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 H2/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 H₂, 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 H₂ production rate is about ten times higher than the theoretical CH₄ 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 H₂ or CH₄ 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 H₂ and CH₄ potential would be in a stoichiometric ratio of 2:2 to 2:3.

Many investigations have been carried out in order to improve the H₂ 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 H₂-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 –H₂ and CO₂ or CH₄ and CO₂- production and composition by different
strains and consortia from different substrates. This paper describes especially the
experimental and biogas monitoring procedures for fermentative H₂-CO₂ 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 CO₂-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 H₂ 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 H₂ 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  \[ \text{C}_6\text{H}_12\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2 \] (1)

Butyric acid production  \[ \text{C}_6\text{H}_12\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2 \] (2)

Lactic acid production  \[ \text{C}_6\text{H}_12\text{O}_6 \rightarrow 2\text{CH}_3\text{CHOHCOOH} \] (3)

Succinic acid production  \[ \text{C}_6\text{H}_12\text{O}_6 + 2\text{CO}_2 + \text{H}_2 \rightarrow 2(\text{CH}_2\text{COOH})_2 + 2\text{H}_2\text{O} \] (4)

Formic acid production  \[ \text{CO}_2 + \text{H}_2 \rightarrow \text{CH}_2\text{O} \] (5)

Ethanol production  \[ \text{C}_6\text{H}_12\text{O}_6 \rightarrow 2\text{CH}_3\text{CH}_2\text{OH} + 2\text{CO}_2 \] (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 lyophilized before being used in the experiments.
cultured on the DSMZ recommended medium and then transferred in 25 ml hermetically stoppered tubes filled with sterile MDT medium (Hamilton et al., 2010). The strains *C. butyricum* CWBI1009 and *Citrobacter freundii* CWBI952 were isolated and identified by the authors (Hamilton et al., 2010; Masset et al., 2010). The facultative anaerobic strains were maintained at 4 °C on PCA solid medium and the Clostridia strains in liquid MDT medium containing a lower glucose concentration (2 g/l) in order to promote spore formation after the growth phase. For fresh inoculum conservation 1 mL of culture was transferred twice a week to 25 mL of sterile MDT medium and incubated at 30°C (55°C for thermophilic strains).

The CSAD sludge was collected from a 20 liters stirred anaerobic digester used in CWBI for BMP assays of different agro-food organic wastes. This lab-scale digester was inoculated two years ago with a sludge collected from a full-scale anaerobic digester treating the activated sludge from a municipal wastewater treatment plant. The UASB sludge was collected from an UASB anaerobic digester treating organic wastewater from sugar-beet and bioethanol industries. In order to enrich the hydrogen-producing bacteria, both sludges were heat treated at 80 °C for 10 and 30 min. Untreated sludges were also used as control.

2.2. **Experimental procedures and culture conditions**

The BHP test developed in this study is a modified version of the biochemical hydrogen potential (BHP) test developed by Lin et al. (Lin et al., 2007). The tests were carried out in 250 ml serum bottles filled with 200 ml of MDT culture medium. The MDT medium contained, per liter of deionized water: glucose monohydrate (5 g), casein peptone (5 g), yeast extract (0.5 g), Na₂HPO₄ (5.1 g), KH₂PO₄ (1.2 g), MgSO₄.7H₂O (0.5 g), L-cysteine hydrochloride (0.5 g). The bottle containing the 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 steriley 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 % CO2. 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 $V_{H,i} = (V_{S,i} + V_T) \%_{H,i} - V_T \cdot \%_{H,i-1}$ (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,
\[ \text{and } \%_{N,i} = \frac{V_{N,i}}{V_T} = \left( \frac{V_T}{V_T + V_{S,i}} \right) \%_{N,i-1} \]  
\[ \text{and } \%_{C,i} = \frac{V_{C,i}}{V_T} = \left( \frac{V_{S,i} - V_{R,i}}{V_{S,i}} \right) \]  

where \( V_{R,i} \) is the biogas volume recovered after injection of \( V_{S,i} \) in the KOH replacement equipment at the \( i^{th} \) sampling.

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

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

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

3. Results and discussion

3.1. Validation of the biogas monitoring method

Seven cultures of HPB strains and sludges were carried out (in four replicates) in order to validate the biogas monitoring method described in section 2.3. Indeed, the biogas production and composition in the culture vessels were monitored using the new method and the H\(_2\) content in biogas was also measured by gas chromatography after 96 h of incubation \( i.e. \) when no more glucose was consumed and the pH had decreased below 5.

Basically, the culture procedure described in section 2.2 was experimented on the pure \( \text{Clostridium} \) strain (\( \text{Clostridium butyricum} \) CWBI1009) and two anaerobically digested sludges without a pretreatment or pretreated at 80\(^{\circ}\)C for 10 or 30 minutes. After 48, 72 and 96 h of incubation the biogas produced in the serum bottles was steriley collected and transferred to the replacement equipment filled with potassium hydroxide for...
carbon dioxide absorption as described in section 2.3. Also, taking into account the initial nitrogen gas volume inside the gas phase and its further dilutions by the produced biogas, it was possible for each gas sampling to assess the gas composition and therefore the volumetric biogas and H$_2$ production. The cumulative biogas and H$_2$ production are presented on Figure 2 A and B respectively. Each dot on the figure represents the average value over the 4 measurements. Standard deviations are not depicted on the graph for better reading; however the average standard deviation is about 25±2 ml and 15±1 ml respectively. The profiles are discussed in section 3.2.

Figure 3 shows the H$_2$ production yields calculated after 96 h (A) as 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. It is to notice that the results obtained by both measurement methods are similar. Indeed, the overall mean difference between the results of Figure 3 A and B calculated over the four replicates of the 7 different samples is about 2.5 % and the standard deviation is about 5.7 %. Moreover, in similar conditions, regarding the medium and environmental parameters, Chen et al. (2005) achieved similar H$_2$ yields with *C. Butyricum* compared with the results shown in Figure 3 with the *C. butyricum* pure strain. Therefore, it can be assumed that the method developed in this paper has been validated and would be useful for other binary biogas potential assays e.g. CH$_4$/CO$_2$ biogas mixture produced by methanogenic bacteria. It has been tested successfully for anaerobic digestion of different substrates such as glucose, cellulose, straw and other (hemi-)cellulosic materials (results not shown). Compared to other biogas monitoring methods the one described here has many advantages. It is less expensive than the system developed by Angelidaki et al.
(1998) for monitoring gas evolution in multiple closed vessels. It is also simpler than the experimental and mathematical method developed by Hansen et al. (2004) for determination of methane potential of solid organic wastes. Furthermore, our method gives direct information on biogas production and does not require a gas chromatographer neither a GC-TCD (Gunaseelan, 2004; Lin et al., 2007) nor a GC-FID (Hansen et al., 2004), even if it is a relatively usual equipment in laboratories. This simple method is then suitable for small companies and low-income countries to assess the biogas potential from organic by-product or waste.

3.2. Comparative H₂ production by mixed cultures and a pure Clostridium strain

The results reported in Figure 2 and 3 also allow comparing performances of the pure Clostridium strain and the different sludges. A low inoculum size was used in order to assess the adaptation and growth faculties of the strain and sludges. This explains the longer lag phase compared to those mentioned in other studies (Baghchehsaraee et al., 2008; Lin et al., 2007) with inoculum about ten times higher than the inoculum volume used in our experiments. Similar observation was deduced from Chen's 5 L-cultures inoculated with 1.2 % of preculture (Chen et al., 2005). In our experiments, the purity check on PCA medium of the Clostridium culture asserted that the culture was maintained pure. The biogas and H₂ curves have relatively similar trends showing that biogas composition is relatively constant along incubation time. The Clostridium pure strain has the highest H₂ production rate (HPR), i.e. 1.2 ml/h, during the initial 48 hours and the culture from UASB heat-treated for 10 min has the overall highest HPR, i.e. 2 ml/h recorded between 48 and 72 hours of culture. No significant difference was observed between the heat-treated cultures from stirred anaerobic digester (CSAD) nor between the untreated and heat-treated UASB microflora. By contrast, the untreated culture from the stirred digester led to relatively low H₂ production. As foreseen both...
autoclaved sludges and pure culture (121°C for 20 min) did not show any growth (results not shown).

By comparison to the results shown on Figure 2, a lower difference is observed between the H₂ yields recorded for the heat-treated UASB sludges compared to the related H₂ cumulative curves. Therefore, higher HPR were related to a higher glucose intake rate. H₂ yields are then more relevant to compare different cultures. The yields achieved i.e. about 1 mol H₂ per mole glucose consumed are lower than those mentioned by other authors (Alalayah et al., 2008; Baghchehsaraee et al., 2010; Lin et al., 2007; Pattra et al., 2008). This trend is related to the environmental parameters in the culture medium that are largely affected by either the initial pH and buffer concentration or by the H₂ partial pressure (Das & Veziroglu, 2008). The latter is related to the initial gas composition inside the gas phase and to the total pressure that is affected by the initial gas to liquid phase ratio. The pH and pressure parameters are subject to relatively large variations during the culture i.e. in our experiments about 3 pH unit and 2 bars. Therefore, our culture conditions are more restrictive compared to the culture conditions usually used in other small vessels experiments with higher volumetric gas to liquid ratio (Alalayah et al., 2008; Baghchehsaraee et al., 2010; Lin et al., 2007; Pattra et al., 2008). However the yields reported in this paper are comparable to those reported for H₂-producing bioreactors (Bartacek et al., 2007; Chen et al., 2005; Fang & Liu, 2002; Masset et al., 2010).

As already shown by Baghchehsaraee et al.(2008), the untreated anaerobic sludges achieved lower H₂ yields compared to the treated sludges. No significant difference was recorded for the sludge treated at 80°C for 10 or 30 minutes as for the treatment at 65°C or 80°C conducted by Baghchehsaraee. Moreover, the pure culture and heat-treated cultures from both digesters achieved similar yields.
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 H₂ production, wide hydrogen yield variations were recorded depending on the species, even for strains of the same species. Indeed, H₂ 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 H₂) compared to the average H₂ 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 H₂ 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 H₂ production and H₂ 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 H₂ producers. The yields (H₂ and soluble metabolites shown on Table 2) recorded for *C. thermosaccharolyticum* are similar to those (0.96 mole H₂ 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
of the studies with sludges achieved higher H$_2$ yields with thermophilic than mesophilic
culture conditions (Bartacek et al., 2007; Shin et al., 2004; Zhang et al., 2003).

Compared to the hydrogen yields, the soluble metabolites yields (Table 2) also vary
significantly between the *Enterobacteriacea* family and the *Clostridium* genus, even
between the different strains of the *Clostridium* genus. The average sum of the
converted glucose into metabolites is similar to that mentioned for the experiments
reported in section 3.2 (77 %); the standard deviation (20 %) is however larger. That
implies that about 25% of the glucose is used for biosynthesis but it varies between 11
and 35 %, even for the three different strains of the *C. butyricum*. The butyrate yields
and the acetate + butyrate yields are the highest for the *C. butyricum* and *C.
pasteurianum* strains which are also the highest H$_2$ producers. This trend is consistent
to the results of other authors (Lin et al., 2007; Wang et al., 2008). By comparison to
the results of Table 2 for the strains *C. butyricum* and *C. acetobutylicum*, the
percentages calculated from the results of Lin et al. (2007) were similar for acetate and
about 25 % and 3-fold higher for butyrate respectively. By contrast, Lin et al. reported a
quite low lactate production and a 3-fold higher CO$_2$ production. These differences
should be related to the different composition of the medium used by Lin et al, and in
our experiments. However, it should be noticed that *C. pasteurianum* converted about
twice more glucose to CO$_2$ (17 ± 1 %) than the other strains (7,5 ± 3,5 %). Therefore,
this H$_2$ producer seems less interesting for an industrial process, particularly a two-
stage anaerobic digestion process producing hydrogen and methane since a high CO$_2$
production in the first hydrogen-producing stage would be considered as a partial lack
of methane potential in the second stage.

The highest yields in lactate were reported with *C. acetobutylicum*. Both *C.
Saccharolyticum* and *thermosaccharolyticum* converted about 30 % of the carbon from
the glucose to ethanol. *Enterobacteriacea* and *C. aminovalericum* strains also produced
large concentration of ethanol. These strains except the latest also produced high
amounts of lactate.

4. Conclusions

The method developed in this paper for the evaluation of the biogas production and its
composition has many advantages. It is relatively cheap and easy to operate and gives
direct information without the need of a gas chromatographer. Moreover it is suitable
for other binary biogas potential assays e.g. CH₄/CO₂ biogas production from different
substrates.

The results of the comparative tests show that the hydrogen yields vary significantly
between the 19 different pure strains and sludges, even between different strains of the
same species i.e. about 20% variation. Therefore, this paper asserts that strain selection
is an important way in order to optimize anaerobic digestion processes with H₂
production.

Acknowledgements

S.Hiligsmann and J. Masset contributed equally to the paper. J. Masset is recipient of a
FRIA fellowship (Fonds de la Communauté française de Belgique pour la Formation à
la Recherche dans l’Industrie et l’Agriculture) and L. Beckers is recipient of a FRS-
FNRS fellowship, (Fonds de la Recherche Scientifique). This work was also supported
by an ARC project (Action de Recherches Concertées ARC-07/12-04) and the Walloon
Region.

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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>
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<tr>
<th></th>
<th>Lactate</th>
<th>Formate</th>
<th>Acetate</th>
<th>Ethanol</th>
<th>Butyrate</th>
<th>CO₂</th>
<th>Sum</th>
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<td>Acetate</td>
<td>Ethanol</td>
<td>Butyrate</td>
<td>CO₂</td>
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</tr>
</tbody>
</table>
Figure B

Hydrogen production yield (mol H2/mol glucose)

- C. but. CWBI: 1.05
- CSAD: 0.72
- CSAD 10' 80°C: 1.00
- CSAD 30' 80°C: 1.04
- UASB: 0.71
- UASB 10' 80°C: 1.02
- UASB 30' 80°C: 0.96