Effect of pH on glucose and starch fermentation in batch and sequenced-batch mode with a recently isolated strain of hydrogen-producing *Clostridium butyricum CWBI1009*

Julien Masset[a]*, Serge Hiligsmann[a], Christopher Hamilton[a], Laurent Beckers[a], Fabrice Franck[b], Philippe Thonart[a]

[a] *Walloon Centre of Industrial Biology, B40 – P70, University of Liege, 4000 Liege, Belgium*
[b] *Laboratory of Vegetal Biochemistry, B22, University of Liege, 4000 Liege, Belgium*

*Corresponding author:

Tel.: +32 43663999

fax: +3243662862

E-mail address: j.masset@ulg.ac.be

Abstract

This paper reports investigations carried out to determine the optimum culture conditions for the production of hydrogen with a recently isolated strain *Clostridium butyricum CWBI1009*. The production rates and yields were investigated at 30 °C in a 2.3 l bioreactor operated in batch and sequenced-batch mode using glucose and starch as substrates. In order to study the precise effect of a stable pH on hydrogen production, and the metabolite pathway involved, cultures were conducted with pH controlled at different levels ranging from 4.7 to 7.3 (maximum range of 0.15 pH unit around the pH level). For glucose the maximum yield (1.7 mol H_2 mol^{-1} glucose) was measured when the pH was maintained at 5.2. The acetate and butyrate yields were 0.35 mol acetate mol^{-1} glucose and 0.6 mol butyrate mol^{-1} glucose. For starch a maximum yield of 2.0 mol H_2 mol^{-1} hexose, and a maximum production rate of 15 mol H_2 mol^{-1} hexose h^{-1} were obtained at pH 5.6 when the acetate and butyrate yields were 0.47 mol acetate mol^{-1} hexose and 0.67 mol butyrate mol^{-1} hexose.

Keywords: Clostridium butyricum; starch; hydrogen production; pH; batch; sequenced-batch

1. Introduction

Dependence on fossil fuels as our primary energy source is a significant cause of global warming, environmental degradation, and health problems [1, 2]. Hydrogen (H_2) is a promising energy vector for the future since CO_2 is not released during its combustion [3-5]. At present hydrogen is produced by chemical methods, such as steam reforming or partial oxidation of fossil fuels, which involve the release of large quantities of greenhouse gases into the atmosphere. Biological hydrogen production by "dark-fermentation" of organic waste or effluent is a promising means of producing renewable energy from waste products [6]. The main means of producing hydrogen via dark-fermentation involve either facultative anaerobic enterobacteriaceae or strict anaerobic bacteria of the geni *Clostridium* and *Ruminococcus*. Enterobacteriaceae use formate, an intermediate of glucose metabolism, to produce hydrogen through catalytic action of the formate hydrogen lyase complex with a theoretical yield of two mol of hydrogen per mol of glucose consumed. The alternative metabolic pathway involving strict anaerobic bacteria has a maximum yield of four mol of hydrogen per mol of glucose [4]. Clostridia can extract energy from carbohydrates using various different metabolic pathways which are promoted or inhibited by the prevailing culture conditions. Each pathway is characterized by a specific metabolite such as acetate, butyrate, ethanol, lactate or formate. The acetate and butyrate pathways are the only ones which involve the release of molecular hydrogen, *i.e.* 4 mol hydrogen per mol glucose with acetate production and 2 mol hydrogen per mol glucose with butyrate production. Thus the butyrate/acetate ratio can be used as a reliable indicator of the efficiency of fermentative hydrogen production and of the metabolic pathways used during glucose fermentation [7].

Although the *Clostridium* genus is promising for fermentative hydrogen production, few investigations have used pure strains to make a detailed study of the optimal conditions for hydrogen production [8-11].

Many authors have reported that initial pH has a marked effect on hydrogen production from carbohydrate substrates. However, since most of these studies investigated mixed cultures of microorganisms or pure cultures without pH control, little is known about the precise impact of a stable pH on the metabolic pathways and hydrogen production rates and yields of specific strains. Various studies have indicated that the optimal pH for the degradation of simple substrates is between 4.5 and 7 [8, 10, 12-18]. In the investigations reported here a pure *Clostridium butyricum* strain was cultured in a 2.3 l bioreactor at different controlled pH levels. The hydrogen production rate and yield and the proportions of the soluble products resulting from the fermentation of glucose and starch were studied. In addition the bioreactor was operated in batch and sequenced-batch mode in order to study the effect of a large inoculum and inhibition by metabolites such as volatile fatty acids (VFA) or alcohols.

2. Materials and Methods

2.1. Media and reactor setup

To ensure a viable culture for a long time, 1 ml of culture was transferred weekly in 25 ml hermetically stoppered tubes completely filled with sterile MDT medium and incubated at 30 °C. The MDT medium contained, per litre of deionised water : glucose monohydrate (5 g), casein pepton (5 g), yeast extract (0.5 g) Na_2HPO_4 (5.1 g), KH_2PO_4 (1.2 g), $MgSO_4.7H_2O$ (0.5 g), cysteine hydrochloride (0.5 g).

The PCA (Plate Count Agar) medium for the aerobic purity check contained, per litre of deionised water : glucose monohydrate (1 g), casein pepton (5 g), yeast extract (2.5 g), agar (15 g).

Pre-cultures for bioreactor experiments were obtained by transferring 5 ml of inoculum into 250 ml serum bottles with 200 ml of sterile MDT medium, degassed with nitrogen, and incubated for 24 hours at 30 °C.

Batch and sequenced-batch fermentations were carried out in a laboratory-scale bioreactor (Biolafitte manufacture) composed of a 2.3 l glass vessel with double envelope and a stainless-steel lid equipped with septum, shaft with blades, 0.20 µm (Midisart, Sartorius) gas filters and tubing for sampling, gas inlet, gas outlet and medium removal or addition. The bioreactor containing 1.5 l of water and the ingredients for the MDT medium (except cysteine and glucose) was autoclaved at 120 °C for 20 minutes, and then cooled under nitrogen gas, prior to injection of 200 ml of the inoculum. Autoclaved cysteine and glucose solutions were added sterilely during inoculation. Needles placed through the septum were used to control the pH level (METTLER TOLEDO combined probe) by automatic addition of sterile 1.5 N sodium hydroxide (maximum range of 0.15 pH unit around the pH level). During fermentation the bioreactor was maintained at 30 ° C and stirred at 60 rpm.

2.2. Isolation and identification test

The strain was isolated from a culture in serum bottles with MDT medium in which glucose had been replaced by non-sterile rice starch for laundry (Remy, Belgium). The additional inoculum provided by this substrate led to high H_2 production. The culture at the completion of gas production was treated at 80 °C for 10 min. One ml of inoculum was successively diluted in 25 ml stoppered glass tubes containing 24 ml of sterile TSC (Tryptose Sulfite

Cycloserine) agar medium (Merck) and maintained in molten state at 43 °C. This medium was used in order to grow isolated colonies in anaerobic conditions. The tubes were closed hermetically and then incubated at 30 °C. After one week the tubes were broken at convenient points in order to sample black colonies (due to precipitation of iron sulfide). Single colonies were selected and placed in a 5 ml tube containing the liquid TSC culture medium for 3 days and then transferred to 250 ml serum bottles for BHP (Biochemical Hydrogen Potential) tests [7]. Since the hydrogen production by the different samples was similar, *i.e.* 132 ± 11 ml, the cultures were considered to contain pure strains. Strain identification was carried out by examination under a transmitted light microscope (Olympus, CH-2) and by 16S rDNA/ITS (Internal Transcribed Spacer) gene sequencing. The genomic DNA was extracted from the cells using the Promega extraction kit (Madison, USA) and the 16S rDNA and ITS gene were amplified by PCR. Bacterial cell lysates were used to amplify the 16S rDNA Eurogentec (Seraing, Belgium) primers $16SA_1$ and $16SA_2$ (Table 1) under the following temperature profile: initial denaturation at 95 °C during 5 min, followed by 25 cycles with denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec and elongation at 72 °C for 2 min. For ITS universal bacterial primers R16 and R23 from Eurogentec (Seraing, Belgium) (Table 1) were used under the following temperature profile: initial denaturation at 95 °C during 5 min, followed by 30 cycles with denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 2 min. Amplification of 16S rDNA was performed in a volume of 50 µl containing: 5 µl of bacterial genomic DNA solution obtained as above, 5 µl of 10 x PCR reaction buffer (50 mM KC1, 10 mM Tris-HCl (pH 9 at 25 °C), 0.5 % (w/v) triton® X-100), 1 µl of each dNTP (Deoxynucleotide triphosphates) 10 µM, 5µl of $MgC1_2$ 25 mM, 1 µl of each primer 10 µM and 0,2 µl of Taq polymerase (Promega). The PCR products obtained in this way were purified with the Microcon® YM-100 (Millipore) Kit. Sequences were

127 determined by ABI Prism BigDye Terminator v3.1 Cycle Sequencing kit (Applied
128 Biosystems).
129
130
131 The ARDRA (Amplified Ribosomal DNA Restriction Analysis) test was carried out by
132 restriction digestion of the amplified 16S rDNA and the ITS rDNA fragments for 1 h at 37 °C
133 in 20 µl reaction mixture containing 15 µl of the PCR product solution, 2 µl of incubation
134 buffer and 15 U of one of the following restriction enzymes, EcoRI, CfoI, MboI, AccII
135 (Eurogentec). Restriction digests were subsequently analysed by 2 % (w/v) agarose gel
136 electrophoresis. The gel was stained in a solution containing ethidium bromide and
137 photographed in UV light.
138
139 **2.3. Analytical methods**
140 Cell concentration was determined by microscopic observations on a Bürker counting
141 chamber (mean of 10 counts) after dilution and cell settlement in a 0.4 % (final concentration)
142 fresh formaldehyde solution.
143 Absence of aerobic contaminants during fermentation was verified by spreading 0.1 ml of
144 culture on PCA medium plates and incubating for 48h at 30 °C. The absence of bacterial
145 growth confirmed the absence of aerobic contaminants.
146 Collected liquid samples were centrifuged at 13000 g for 10 min and the supernatants
147 obtained were filtered through a 0.2 µm cellulose acetate membrane (Minisart Sartorius) and
148 analysed by HPLC for glucose, ethanol, lactate, acetate, formate and butyrate. The HPLC
149 analysis was carried out using an Agilent 1110 series (HP Chemstation software) with a
150 Supelcogel C-610H column preceded by a Supelguard H pre-column (oven temperature 40
151 °C), 0.1 % H_3PO_4 (in milliQ water) as the isocratic mobile phase at a flow rate of 0.5 ml min^{-1}

and a differential refraction index detector (RID, heated at 35 °C). The process lasted for 35 min at a maximum pressure of 60 bars. The data for the glucose and metabolite concentrations were used to calculate the mass balance (MB) of the glucose conversion using the equation:

$$MB = \frac{\sum N_i \cdot \Delta C_i}{N_G \cdot \Delta C_G} \qquad (1)$$

where N_i is the number of carbon atoms in a molecule of metabolite i; ΔC_i is the concentration of metabolite i effectively produced (*i.e.* the difference between the final and initial concentrations for a given culture sequence); N_G is the number of carbon atoms in the glucose molecule (*i.e.* 6) and ΔC_G is the concentration of the glucose consumed during the culture sequence (Eq.(1)).

The proportion of hydrogen gas was determined using a gas chromatograph (GC) (Hewlett-Packard 5890 Series II) 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^{-1}. Water supplemented with 9 N KOH was used in replacement equipment to monitor the biogas production of the batch and sequenced-batch bioreactors.

3. Results and discussion

3.1. Isolation and identification of the hydrogen-producing strain

The strain was isolated following the isolation procedure described in Material and Methods. The bacteria were mobile and rod shaped and approximately 0.5-2 x 2.5-8 µm in size. Long filaments were occasionally present. Endospores, when observed, were central or

subterminalis as described in Bergey's Manual of Systematic Bacteriology[19]. The bacteria grew anaerobically in presence of organic carbon source and produced hydrogen in presence of carbohydrates. To further characterize the strain, sequencing of the 16S rDNA and ITS was carried out (accession number in Genbank GU395290). The sequences obtained were compared with others available using Gene Runner® and the strain was identified as *Clostridium butyricum*. ARDRA genotyping of the 16S rDNA was also performed and confirmed the identification. The strain is kept at -80 °C in the laboratory culture collection.

3.2. Determination of optimal pH for H_2 production from a glucose substrate in batch reactor mode

The effect of pH on fermentative H_2 production by the pure *Clostridium butyricum* strain was investigated in a 2.3 l batch bioreactor equipped with pH regulation (as described in Materials and Methods). Tests were conducted at eight different pH levels ranging from 4.7 to 7.3 while keeping other operating conditions constant (stirring, temperature, pressure, initial culture medium). The test at pH 5.2 ± 0.15 provided the most promising results: figure 1 illustrates the related profiles for the intake of the glucose substrate and the production of hydrogen, VFA and ethanol. The results show that the various VFAs and H_2 are all primary metabolites of the glucose intake. Similar profiles of the first order were recorded at 4.85±0.1 and 5.4±0.1.

The hydrogen production rates, plotted against time, for the eight different pH tests are indicated in figure 2. A lag phase of about 3 to 5 hours was observed at all pH levels. Subsequently the production rate increased exponentially reaching a maximum level which was maintained, for all the tests except those at pH 6.3 and 6.7, until substrate depletion.

In figure 3 maximum H_2 production yields are indicated for different pH conditions with the related final VFA and ethanol concentrations. The maximum hydrogen production yield and production rate, i.e. 1.69 mol H_2 mol^{-1} glucose or 211 ml H_2 g^{-1} glucose and 9.59 mmol H_2 h^{-1} or 126 ml H_2 h^{-1} l^{-1} respectively, were obtained at a pH of 5.2. These optimum pH and yields are in accordance with other studies with pure or mixed cultures [13, 15, 20-22]. Since at this pH level butyrate and acetate concentrations also peaked, overall performance for the process was at a maximum.

Ethanol was released at lower amounts than the other metabolites. At pH levels below 6 ethanol production tended to occur during the stationary growth phase as already noted by other authors [23, 24]. Therefore the negligible amounts of ethanol production in these experiments indicate that the Clostridium population consisted mainly of vegetative cells.

For all the pH levels investigated the final concentrations of lactate, acetate and butyrate were in relatively narrow ranges, *i.e.* 8±1.5; 10.5±1.8 and 15±2 mM respectively. However formate production increased from 6 mM to 31 mM with pH rising from 4.7 to 7.3. Although Wang *et al* [25] obtained similar hydrogen and total VFA yields with a *C. butyricum* strain in a 1.5 l bioreactor with pH maintained at 6.5, the main metabolite they analysed was lactate (60 mM), followed by acetate (22 mM) and then butyrate (15 mM). Similar results for ethanol and VFA concentration ranges at different pH levels were reported by Chen [21] with acetate as the main metabolite followed by butyrate and ethanol.

As shown in Table 2, the mass balance of glucose conversion into soluble metabolites is limited to 70% ± 3% for the cultures carried out at pH levels below 6. This indicates that about 30% of the glucose is converted into CO_2 and biomass. Assuming a mean 35% CO_2 content in the biogas [26], the CO_2 contribution in the mass balance would be about 13%.

Therefore the biomass yield would account for 17% of the glucose consumed. Similar results were obtained at pH levels higher than 6, in which the mass balance reached about 80% and the CO_2 contribution was estimated to be 5 %. These results are in accordance with the 12% calculated from Wang's data [25]. In addition biomass stabilisation was also apparent during our experiments from the counts on a Bürker chamber : at the end of the cultures the biomass population ranged, independently of pH, from 1.2 $x10^8$ to 2.5 x 10^8 cells per ml.

In the glucose metabolic pathways ethanol, formate and lactate productions indicate a lack of potential hydrogen production. Therefore the potential for additional H_2 production can be stoichiometrically estimated from the quantity of metabolite that was not converted in the bioreactor [27], e.g. for lactate, 850 mL at pH 5.2 and 1200 mL at pH 6.7. Investigations into ways of realizing this potential would advance the prospects for industrialization.

3.3. Investigation of H_2 production from glucose in sequenced-batch reactor mode

Industrial application of this biological process requires the use of a continuous or sequenced-batch mode. Therefore a sequenced-batch reactor (SBR) mode was investigated in order to optimize the process by testing different culture conditions while using the same methodology as described above for the batch tests. The SBR mode was studied at pH 5.2 ± 0.15 after running the culture in batch mode under the same conditions. A former series of tests involved a simple addition of 5g of glucose at the end of the batch culture. Since no further hydrogen was produced under these conditions, even at pH 4.85 and 5.3, the results are not reported here. Additional hydrogen production only took place when the pH level was increased to 7. A second series of experiments involved 4 sequences of removal-addition of 20 % (450 ml) of the culture medium and addition of glucose up to the standard initial concentration, *i.e.* 5 g l^{-1} glucose monohydrate. These 4 sequences followed the batch culture

and each sequence was started after complete depletion of the glucose added previously. Finally a third series of 3 sequences was carried out with removal-addition of 40 % of the culture medium and, as before, addition of glucose monohydrate to 5g l^{-1}.

Mean values were calculated for the H_2 production rates and yields during the active period of each series, *i.e.* while hydrogen production was effective. The results of the 8 sequences, including the batch culture (referred to as sequence 1), are shown in figures 4. Sequence 1 achieved similar results to those mentioned in figure 1. The duration of the active period when H_2 production was effective increased progressively from sequence 1 through sequence 5 going from 26 hours to 36, 48, 71, and finally 74 hours. So although the mean H_2 production yield did not change markedly, the production rates decreased sharply. By contrast the conditions tested in sequences 6 to 8 were favourable for H_2 production since the active period stabilized at about 46 h. Also H_2 production rates increased again reaching levels similar to those measured during sequence 1 and yields increased by about 50 %. The maximum yield, *i.e.* 2.3 mol H_2 mol^{-1} glucose, was twice as high as the yield per mole hexose reported by Chen et al. [21] in SBR with a sucrose substrate.

The major cause for the decrease of production rate would be VFA inhibition. In fact as indicated in figure 4, the total VFA concentration measured at the end of sequence 5 was twice as high as the value measured after the batch culture (sequence 1). The increase can be mainly attributed to acetate and butyrate release that approaches a total of 90mM. The results are consistent with the conclusions of Wang [25] who investigated the inhibitory effect of metabolites on fermentative H_2 production in mixed cultures. They showed that performance tends to decrease with increasing ethanol and VFA concentrations. Moreover acetate had the

highest inhibitory effect since H_2 production rate and yields dropped by 50% when the initial concentration was increased from 50 to 100 mM and from 100 to 200 mM, respectively.

To better understand how each sequence contributed to the production of VFAs and ethanol, the mass balance of glucose conversion into metabolites has been plotted in Table 3. The results show a similar 70% level for all sequences meaning that biomass formation is stabilized. In addition the metabolite distribution analyzed for sequences 1 to 5 confirms the literature trends since both increasing of acetate and butyrate initial concentration induced change in metabolite distribution with decrease of acetate proportion and increase of butyrate proportion [25]. The trends are less obvious in sequences 6 to 8 where acetate and butyrate production correlate with the related metabolite concentrations in culture medium. It should be noted that the higher H_2 production performance observed in sequences 7 and 8 are related to higher butyrate and acetate yields and a lower butyrate/acetate ratio. These results are in close agreement with metabolic studies reporting higher H_2 yields with the acetate pathway.

3.4. Effect of pH on H_2 production from starch in sequenced-batch reactor mode

Many industrial activities involve the discharge of large volumes of effluent loaded with starch. Yokoi has shown the feasibility of producing H_2 from starch using *Clostridium* strains in association with an *Enterobacter* strain [28, 29]. More recently Chen studied H_2 production from starch using pure strains of *C. butyricum* and *C. pasteurianum* and mixed cultures [9]. They achieved higher H_2 yields with pure *C. but.* strains. However pH was not controlled (initial pH was 7.5) and H_2 production rates were low compared to those of mixed cultures. In our investigations *C. but.* was cultured in a 2.3 l SBR with soluble starch as the sole carbohydrate source (starch concentration calculated as half the COD of the glucose added in MDT medium in order to avoid starch settlement during sterilization). Then using the

previous methodology a batch culture with glucose was followed by four sequences carried out successively at pH 5.2, 5.4, 5.6 and 5.8. The hydrogen production yields, presented in figure 5, are similar to those indicated for sequences 7 and 8 in figure 4. The optimal pH level for hydrogen production from starch by *Clostridium butyricum CWBI1009*, as well as the optimal pH for H_2 production from glucose, are comparable to those found in the literature and vary depending on the substrate [9-11]. By comparison with the yields recorded for the glucose substrate, the H_2 yields for the starch substrate seem less affected by pH than those for the glucose substrate. By contrast the H_2 production rate from starch decreased sharply at pH levels below 5.6. Moreover, at this optimal pH the hydrogen production rate was about 5 times lower than that recorded for glucose substrate. These results highlight the impact of pH on starch hydrolysis and are consistent with the work of Chen showing that the activity of amylases strongly decreases when the pH is close to 5 [30]. Therefore it seems reasonable to suggest that starch hydrolysis could be the rate-limiting step for H_2 production and that pH 5.6 is optimum for the overall enzymatic and metabolic kinetics. This is in close agreement with earlier works [18, 31, 32].

The metabolites released during each sequence at different pH levels are shown in figure 5 and the carbon balance in Table 4. When determining the VFA concentrations at the end of starch fermentation, maximum acetate (6mM) and butyrate (8.3mM) concentrations were observed at pH 5.4. It was also at this pH level that lactate (0mM), formate (4.9mM) and ethanol (1.4mM) were at their lowest concentration. Likewise for H_2 production from glucose substrate (sequences 7 and 8 in table 4), butyrate and acetate are the major metabolites and about 70 % of the starch is converted into VFAs or ethanol . Whereas lactate and formate were not produced with the glucose substrate at optimal pH, 10 % of the starch was converted

into formate whatever the pH level. The butyrate/acetate ratio, *i.e.* about 1.3, mentioned by Chen et al. [9] is similar to those calculated from the data in figure 5, but it was lower than those obtained during the glucose fermentation (1,8). In addition they also analyzed propionic and valeric acids that were not detected in our cultures.

4. Conclusions

In this study the strain *Clostridium butyricym CWBI1009* was characterized as a new hydrogen-producing strain and some culture conditions (pH, substrates and operation mode) were optimized to maximize production of hydrogen. The first step was to determine the optimal pH for cultures using glucose or starch as the substrate. The results confirm that low pH variations caused large variations in the activity of hydrogenases and metabolic pathways [14, 33]. For glucose the maximum yield (1.7 mol H_2 mol^{-1} glucose) was measured at pH 5.2. For starch a maximum yield of 2.0 mol H_2 mol^{-1} hexose and a maximum production rate of 15 mol H_2 mol^{-1} hexose h^{-1}, were obtained at pH 5.6 These results are relevant to the prospects for using effluents from agro-food industries as an organic substrate for fermentative H_2 production. However since such effluents often contain various different substrates, determining and maintaining an optimum intermediate pH level will be crucial to achieve efficient fermentation of the substrate mixture. For instance a sharp decrease (80%) in the hydrogen production rate for starch occurred when the pH decreased from 5.6 to 5.2, while the yield only declined by 14%.

Carrying out the investigations with a pure strain at various different controlled pH levels while tracking all the potential metabolites (*i.e.* formate, acetate, ethanol, lactate and butyrate), provided a better understanding of the metabolic pathways involved and the factors

affecting them. The results highlight the important role of parameters such as pH, the nature of the carbohydrate substrate, the growth phase, VFA concentrations and inoculum. Indeed for instance, significant differences in metabolite distribution have been evidenced by comparing our results and those of Wang [25] obtained with another *Clostridium butyricum* strain cultured under similar conditions. In our experiments H_2-producing glucose fermentation at optimum pH 5,2 released acetate and butyrate at respectively 11.2 and 17.1 mM meaning that about 12 % and 40 % of the initial glucose carbon content were converted in these compounds, respectively. Bearing this in mind tests in a sequenced-batch bioreactor were designed to promote the metabolic pathways which release butyrate and acetate, since these pathways are the only ones producing H_2 from carbohydrates.As a result, a 50 % increase in H_2 yield was obtained with removal-addition of 40 % of the culture medium and 15 % more glucose was converted in acetate and butyrate. In conclusion an analysis of the mass balance of the various metabolites converted from the carbohydrate substrate should be considered in any future studies.

5. Acknowledgements

J. Masset et S.Hiligsmann 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). F. Franck is senior research associate of the FRS-FNRS. This work was also supported by an ARC project (Action de Recherches Concertées ARC-07/12-04) and the Walloon Region.

6. References

[1] Sivaramakrishna D, Sreekanth D, Himabindu V,Anjaneyulu Y, Biological hydrogen production from probiotic wastewater as substrate by selectively enriched anaerobic mixed microflora, Renew. Energ. (2009) 937-40
[2] Levin DB, Pitt L,Love M, Biohydrogen production: prospects and limitations to practical application, Int. J. Hydrogen. Energ. 29 (2004) 173-185
[3] Lee M-J, Song J-H,Hwang S-J, Effects of acid pre-treatment on bio-hydrogen production and microbial communities during dark fermentation, Bioeressour. Technol. 100 (2009) 1491-1493
[4] Kraemer JT,Bagley DM, Improving the yield from fermentative hydrogen production, Biotechnol. Lett. 29 (2007) 685-695
[5] Jo JH, Lee DS,Park JM, The effects of pH on carbon material and energy balances in hydrogen-producing Clostridium tyrobutyricum JM1, Bioeressour. Technol. 99 (2008) 8485-8491
[6] Gomez X, Cuetos MJ, Prieto JI,Moran A, Bio-hydrogen production from waste fermentation: Mixing and static conditions, Renew. Energ. 34 (2009) 970-975
[7] Lin P-Y, Whang L-M, Wu Y-R, Wei J, Hsiao C-J, Li S-L,Chang JS, Biological hydrogen production of the genus Clostridium: metabolic study and mathematical model simulation, Int. J. Hydrogen. Energ. (2007) 1728-35
[8] Skonieczny MT,Yargeau V, Biohydrogen production from wastewater by Clostridium beijerinckii: Effect of pH and substrate concentration, Int. J. Hydrogen. Energ. 34 (2009) 3288-3294
[9] Chen SD, Lee KS, Lo YC, Chen WM, Wu JF, Lin CY,Chang JS, Batch and continuous biohydrogen production from starch hydrolysate by Clostridium species, Int. J. Hydrog. Energy. 33 (2008) 1803-1812
[10] Pan C-M, Fan Y-T, Zhao P,Hou H-W, Fermentative hydrogen production by the newly isolated Clostridium beijerinckii Fanp3, Int. J. Hydrogen. Energ. 33 (2008) 5383-5391
[11] Liu Y, Yu P, Song X,Qu YB, Hydrogen production from cellulose by co-culture of Clostridium thermocellum JN4 and Thermoanaerobacterium thermosaccharolyticum GD17, Int. J. Hydrogen. Energ. 33 (2008) 2927-2933
[12] Zhao QB,Yu HQ, Fermentative H-2 production in an upflow anaerobic sludge blanket reactor at various pH values, Bioeressour. Technol. 99 (2008) 1353-1358
[13] Chen WM, Tseng ZJ, Lee KS,Chang JS, Fermentative hydrogen production with Clostridium butyricum CGS5 isolated from anaerobic sewage sludge, Int. J. Hydrogen. Energ. 30 (2005) 1063-1070
[14] Ying Z,Yang ST, Effect of pH on metabolic pathway shift in fermentation of xylose by Clostridium tyrobutyricum, J. Biotechnol. 110 (2004) 143-157
[15] Fang HHP,Liu H, Effect of pH on hydrogen production from glucose by a mixed culture, Bioeressour. Technol. 82 (2002) 87-93
[16] Kataoka N, Miya A,Kiriyama K, Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria, Water. Sci. Tech. 36 (1997) 41-47
[17] Chen CC,Lin CY, Using sucrose as a substrate in an anaerobic hydrogen-producing reactor, Adv. Environ. Res. 7 (2003) 695-699
[18] Van Ginkel S, Sung SW,Lay JJ, Biohydrogen production as a function of pH and substrate concentration, Environ. Sci. Tech. 35 (2001) 4726-4730
[19] Bergey's, Bergey's manual of systematic bacteriology, London, 1984
[20] Wang JL,Wan W, Experimental design methods for fermentative hydrogen production: A review, Int. J. Hydrogen. Energ. 34 (2009) 235-244
[21] Chen WH, Sung S,Chen SY, Biological hydrogen production in an anaerobic sequencing batch reactor: pH and cyclic duration effects, Int. J. Hydrogen. Energ. 34 (2009) 227-234

[22] Kim JK, Nhat L, Chun YN,Kim SW, Hydrogen production conditions from food waste by dark fermentation with Clostridium beijerinckii KCTC 1785, Biotechnol. Bioproc. Eng. 13 (2008) 499-504
[23] Kapdan IK,Kargi F, Bio-hydrogen production from waste materials, Enzym. Microb. Tech. 38 (2006) 569-582
[24] Hawkes FR, Dinsdale R, Hawkes DL,Hussy I, Sustainable fermentative hydrogen production: challenges for process optimisation, Int. J. Hydrogen. Energ. 27 (2002) 1339-1347
[25] Wang XY, Jin B,Mulcahy D, Impact of carbon and nitrogen sources on hydrogen production by a newly isolated Clostridium butyricum W5, Int. J. Hydrogen. Energ. 33 (2008) 4998-5005
[26] Chong ML, Rahim RA, Shirai Y,Hassan MA, Biohydrogen production by Clostridium butyricum EB6 from palm oil mill effluent, Int. J. Hydrogen. Energ. 34 (2009) 764-771
[27] Matsumoto M,Nishimura Y, Hydrogen production by fermentation using acetic acid and lactic acid, J. Biosci. Bioeng. 103 (2007) 236-241
[28] Yokoi H, Maki R, Hirose J,Hayashi S, Microbial production of hydrogen from starch-manufacturing wastes, Biomass & Bioenergy. 22 (2002) PII S0961-9534(02)00014-4
[29] Yokoi H, Tokushige T, Hirose J, Hayashi S,Takasaki Y, H-2 production from starch by a mixed culture of Clostridium butyricum and Enterobacter aerogenes, Biotechnol. Lett. 20 (1998) 143-147
[30] Chen SD, Shen DS, Chen WM, Lo YC, Huang TI, Lin CY,Chang JS, Dark hydrogen fermentation from hydrolyzed starch treated with recombinant amylase originating from Caldimonas taiwanensis On1, Biotechnol. Lett. 23 (2007) 1312-1320
[31] Lay JJ, Modeling and optimization of anaerobic digested sludge converting starch to hydrogen, Bioeressour. Technol. 68 (2000) 269-278
[32] Khanal SK, Chen WH, Li L,Sung SW, Biological hydrogen production: effects of pH and intermediate products, Int. J. Hydrogen. Energ. 29 (2004) 1123-1131
[33] Dabrock B, Bahl H,Gottschalk G, Parameters affecting solvent production by clostridium-pasteurianum, Appl. Environ. Microbiol. 58 (1992) 1233-1239

Figure 1. Hydrogen production (ml H_2), and glucose, ethanol and VFA concentration (mM) plotted against time (hours) in 2.3 l batch cultures with *C. butyricum CWBI1009* at pH 5.2. —◆—Glucose —■—Lactate —□—Formate —●—Acetate —○—Ethanol —✳—Butyrate —★—Hydrogen volume

Figure 2. Hydrogen production rate per litre of culture medium (ml H_2 h^{-1} l^{-1}) plotted against time (hours) in 2.3 l batch cultures at different pH levels. —■—pH 7.3 —□—pH 6.7 —●—pH 6.3 —○—pH 5,85 — ▲ —pH 5,4 — △ —pH 5,2 —◆—pH 4,85 — ◇ —pH 4,7

Figure 3: Metabolite analysis of *Clostridium butyricum CWBI1009* glucose fermentation at different pH levels in a 2.3 l batch bioreactor. Hydrogen production yield (mol H_2 mol^{-1} glucose), hydrogen production rate (mmol H_2 mol^{-1} gluc h^{-1}) and final VFA concentrations (mM) —■—Lactate —□—Formate —●—Acetate —○—Ethanol —✳—Butyrate —★—Hydrogen yield —☆—Hydrogen production rate.

Figure 4 : Metabolite analysis of *Clostridium butyricum CWBI1009* glucose fermentation in a 2.3 l sequenced-batch bioreactor. Hydrogen production yield (mol H_2 mol^{-1} glucose), hydrogen production rate (mmol H_2 mol^{-1} gluc h^{-1}) and final VFA concentrations (mM) —■—Lactate —□—Formate —●—Acetate —○—Ethanol —✳—Butyrate —★—Hydrogen yield —☆—Hydrogen production rate.

Figure 5: Metabolite analysis of *Clostridium butyricum CWBI1009* starch fermentation at different pH levels in a 2.3 l sequenced-batch bioreactor. Hydrogen yield (mol H_2 mol^{-1} hexose), hydrogen production rate (mmol H_2 mol^{-1} hexose h^{-1}) and final VFA concentrations —■—Lactate —□—Formate —●—Acetate —○—Ethanol —✳—Butyrate —★—Hydrogen yield —☆—Hydrogen production rate.

Table 1: Sequence primer used to amplify 16S rDNA and ITS

Table 2: Metabolite analysis of *Clostridium butyricum CWBI1009* glucose fermentation at different pH levels in a 2.3 l batch bioreactor

Table 3: Metabolite analysis of *Clostridium butyricum CWBI1009* glucose fermentation in a 2.3 l sequenced-batch bioreactor.

Table 4: Metabolite analysis of *Clostridium butyricum CWBI1009* starch fermentation at different pH levels in a 2.3 l sequenced-batch bioreactor.

Figure 1

Click here to download high resolution image

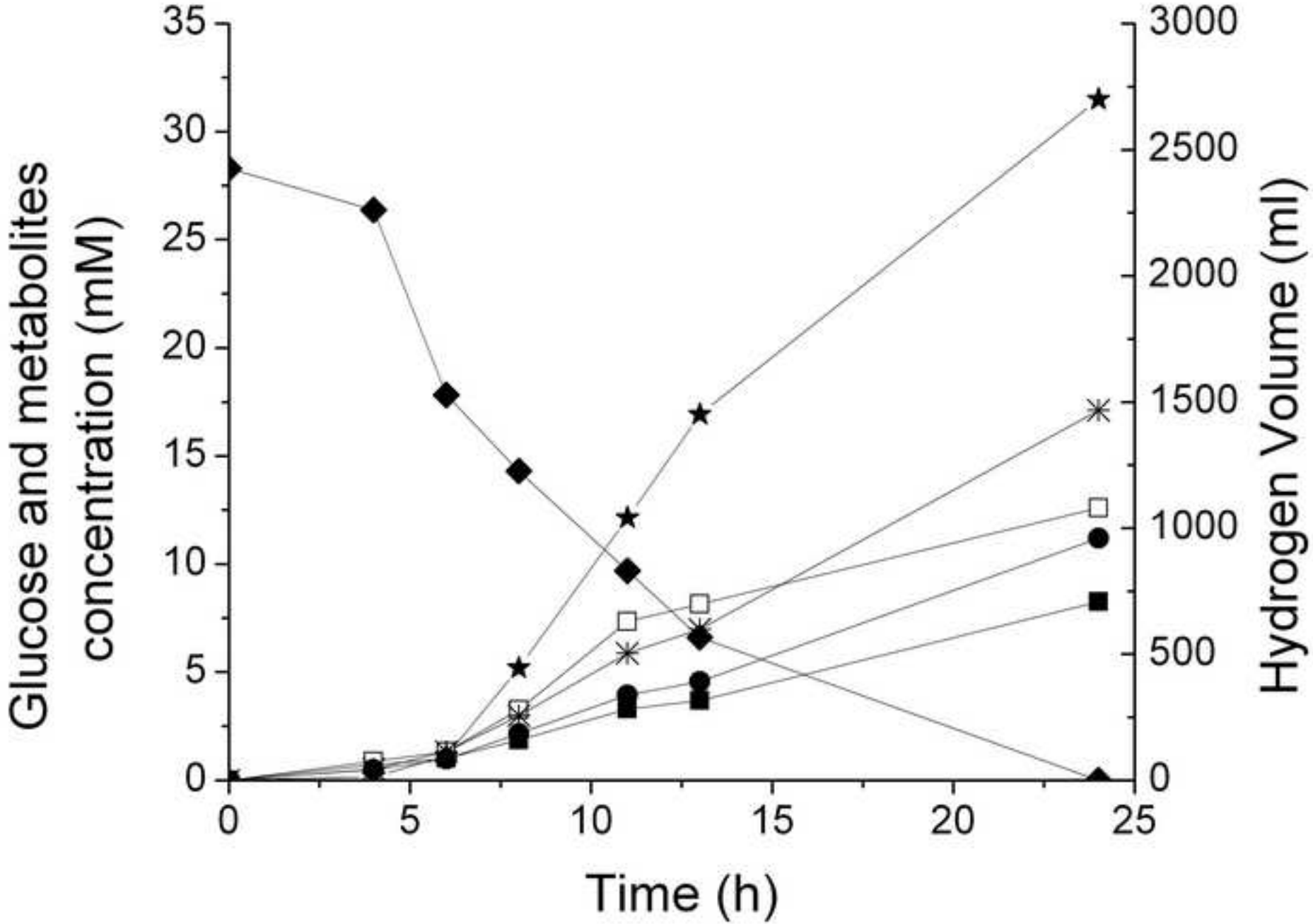

Figure 2

Click here to download high resolution image

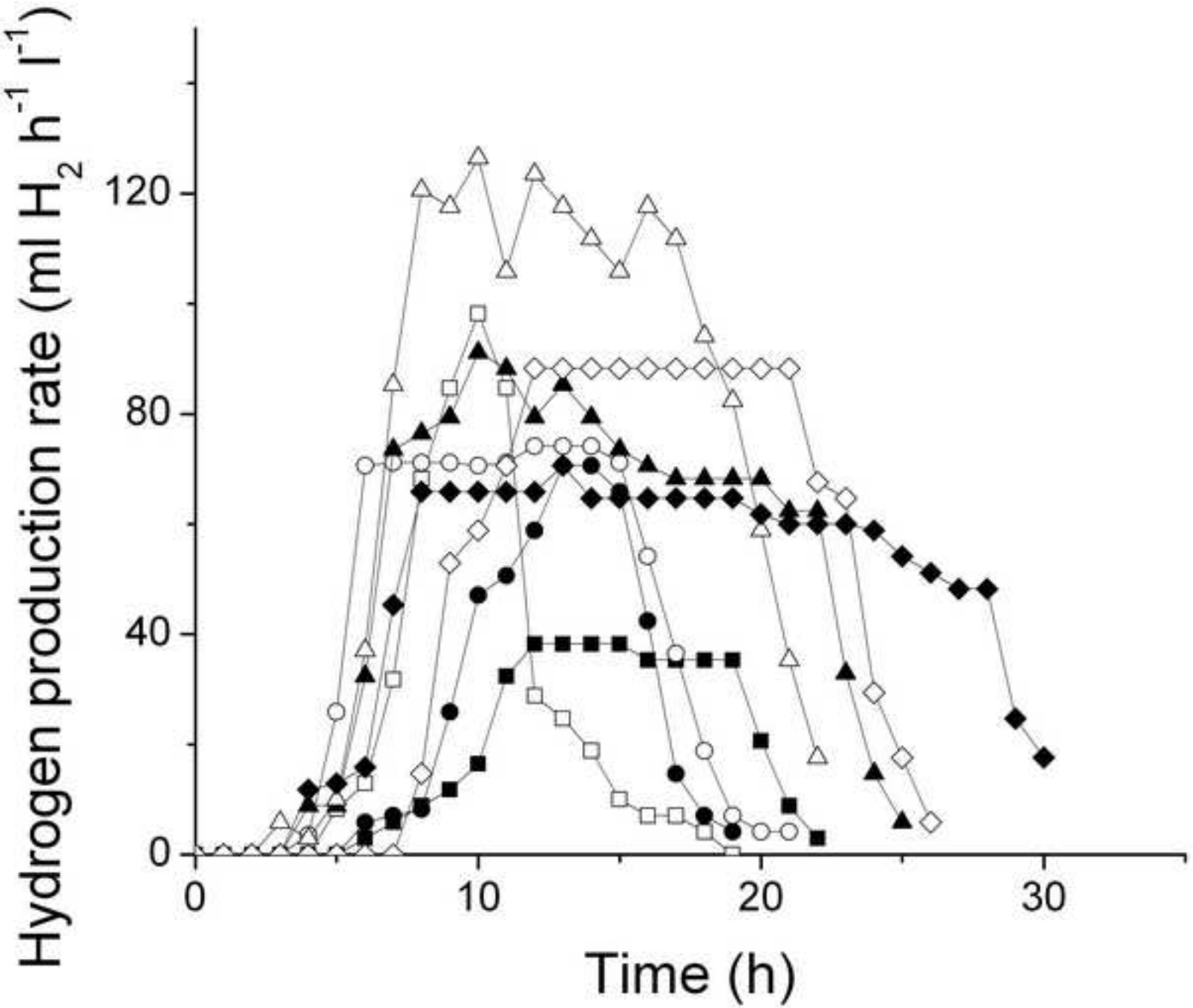

Figure 3

Click here to download high resolution image

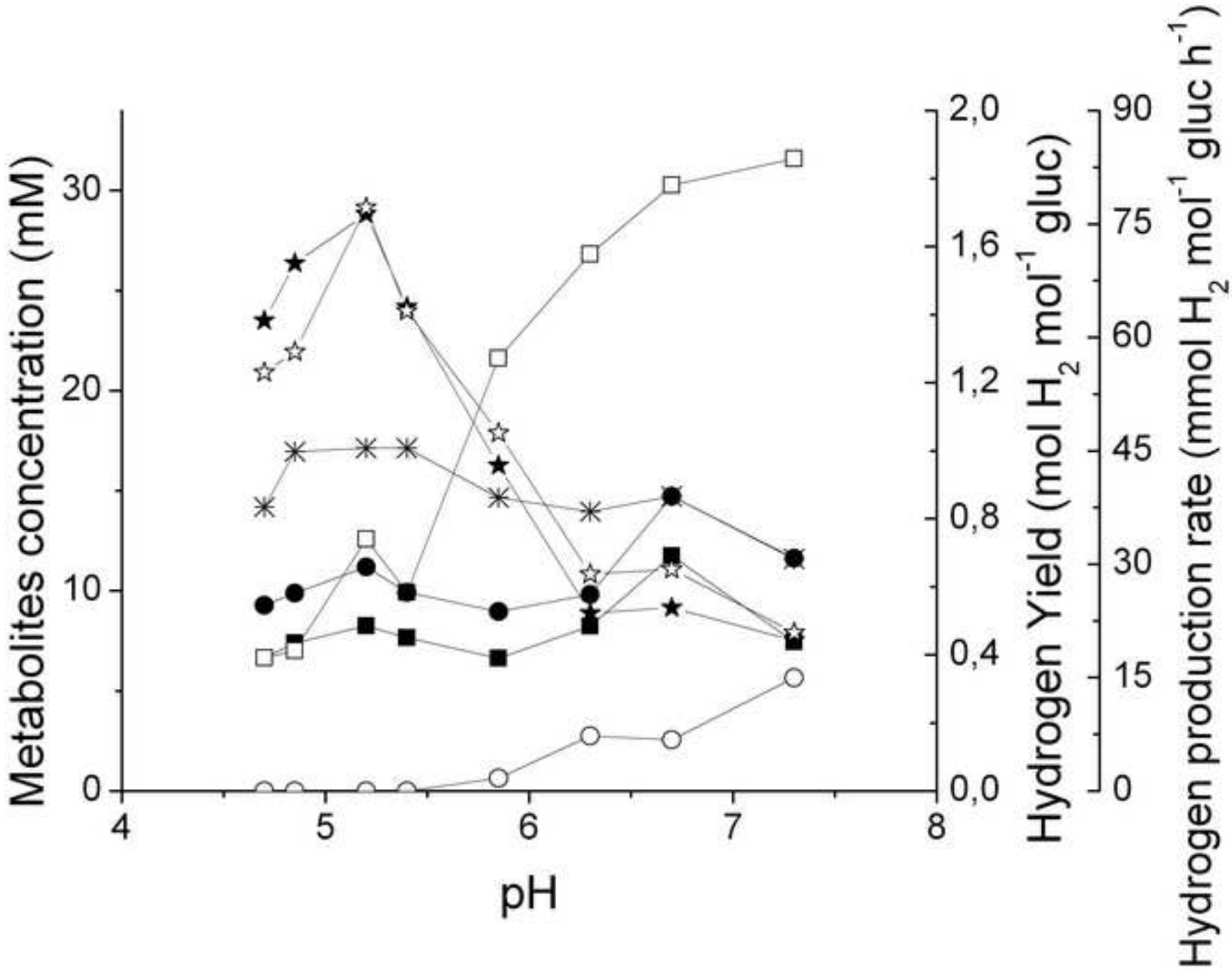

Figure 4

Click here to download high resolution image

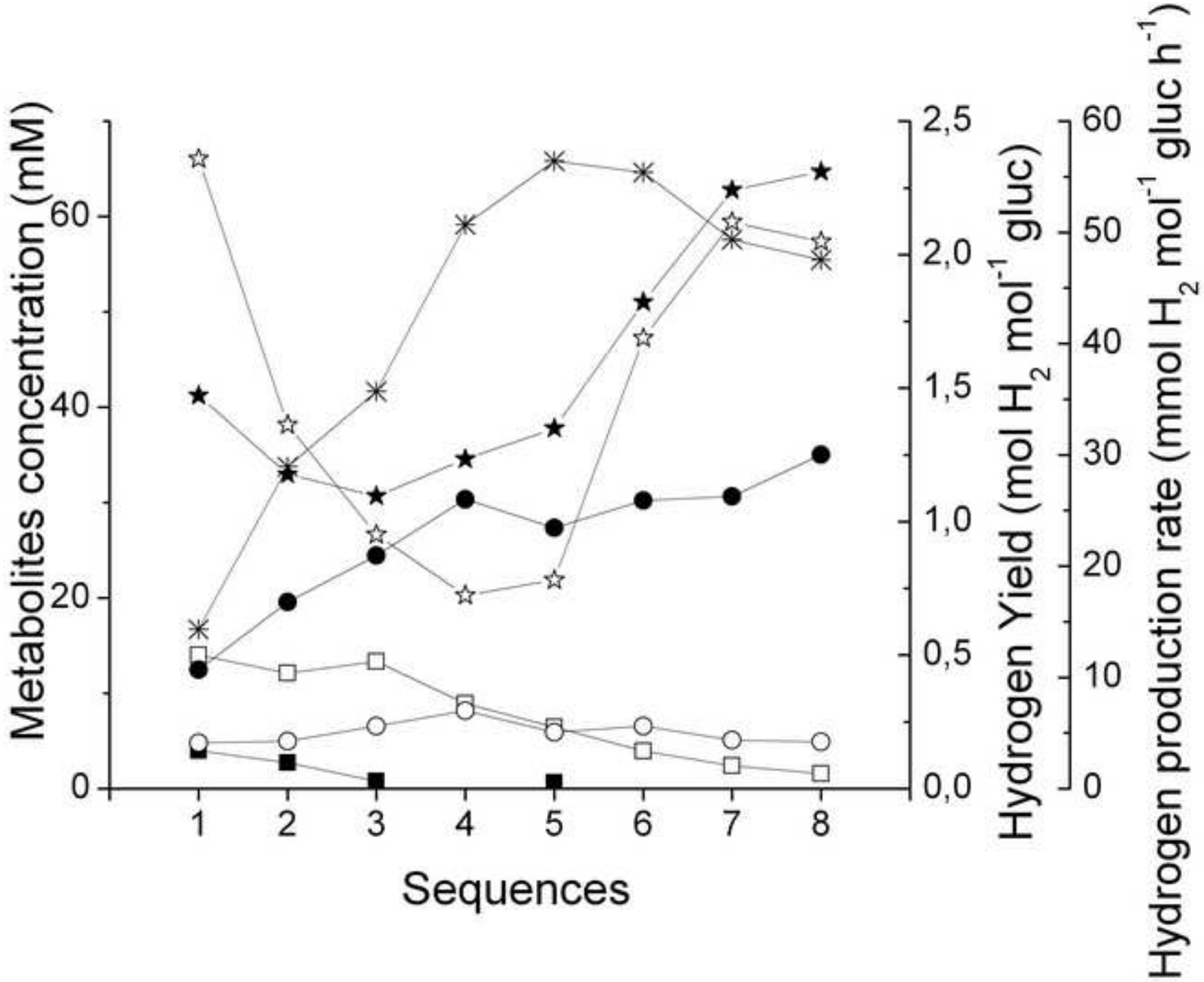

Figure 5

Click here to download high resolution image

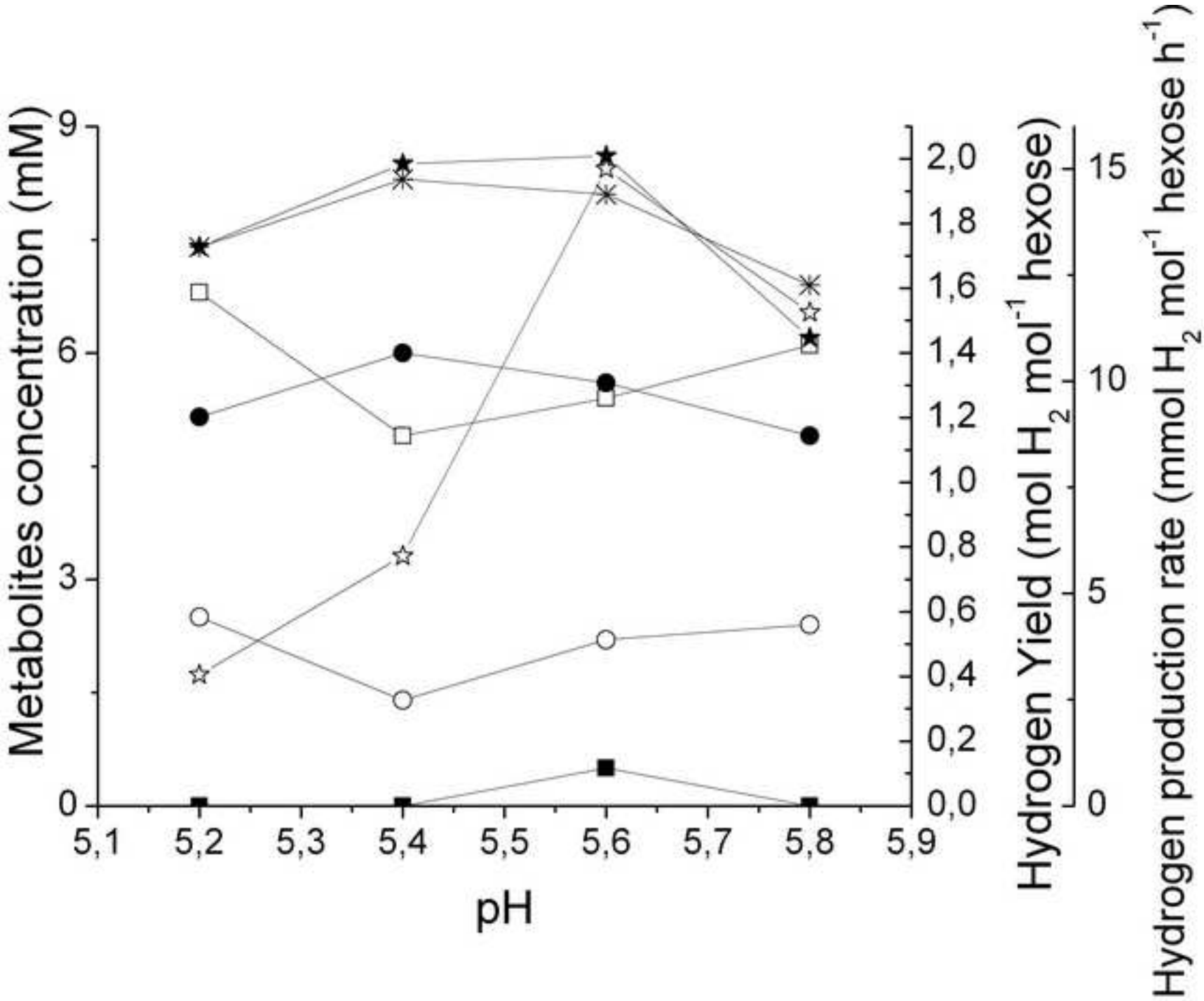

Table 1: Sequence primer used to amplify 16S rDNA and ITS

Primer	Sequence
$16SA_1$	5' –TGGCTCAGATTGAACGCTGGCGGC- 3'
$16SA_2$	5' –TACCTTGTTACGACTTCACCCCA- 3'
R16	5' –KASTGCCAGGGCATCCAC- 3'
R23	5' –GGGTGAAGTCGTAACAAG- 3'

Table 2: Metabolite analysis of *Clostridium butyricum CWBI1009* glucose fermentation at different pH levels in a 2.3 l batch bioreactor

	Carbon converted from glucose (%)					
pH	Lactate	Formiate	Acetate	Ethanol	Butyrate	Sum
4,7	14,13	4,21	11,03	0	34,93	66,24
4,85	13,30	4,2	11,83	0	40,62	69,95
5,2	13,53	8,09	11,67	0	40,34	73,63
5,4	13,32	6,78	12,06	2,25	36,89	71,3
5,85	11,08	12,07	10,01	0,72	32,72	66,6
6,3	14,35	15,58	11,40	3,18	32,42	76,93
6,7	20,59	17,68	12,25	2,98	34,41	87,91
7,3	13,52	19,10	14,04	6,83	28,04	81,53

Table 3: Metabolite analysis of *Clostridium butyricum CWBI1009* glucose fermentation in a 2.3 l sequenced-batch bioreactor.

	Carbon converted from glucose (%)					
Sequence	Lactate	Formiate	Acetate	Ethanol	Butyrate	Sum
1	6,74	7,64	14,03	5,35	37,47	71,23
2	0	2,62	12,52	1,47	53,3	69,91
3	0	2,89	14,01	4,2	47,05	68,15
4	0	0	12,25	3,33	58,1	73,68
5	1,64	0	5,05	0	61,6	68,29
6	0	0	15,03	3,28	54,45	72,76
7	0	0,01	17,12	1,62	50,83	69,57
8	0	0,06	19,89	2,17	49,6	71,72

Table 4: Metabolite analysis of *Clostridium butyricum CWBI1009* starch fermentation at different pH levels in a 2.3 l sequenced-batch bioreactor.

	Carbon converted from starch (%)					
pH	Lactate	Formiate	Acetate	Ethanol	Butyrate	Sum
5,2	0	9,7	13,8	6,2	41,6	71,3
5,4	0	7,1	14,9	3,4	46,8	72,2
5,6	2,1	10,2	15,7	5,8	44,8	78,6
5,8	0	8,9	13,8	5,3	39,2	67,2