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Dark fermentation: isolation and characterization of hydrogen-producing strains from sludges

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Summary. To improve bacterial hydrogen production, ten hydrogen-producing strains belonging to *Clostridium* spp. were isolated from various sludges under low vacuum. Hydrogenogenesis by dark fermentation in batch cultures of these strains was optimal at about 35 ºC and an initial pH of 6.5, which for all strains gradually dropped to *ca.* pH 4 during the fermentation. *Clostridium roseum* H5 and *C. diolis* RT2 had the highest hydrogen yields per total substrate (120 ml H_2/g initial COD). Substrate consumption alone by *C. beijerinckii* UAM and *C. diolis* RT2 reached 573 and 475 ml H_2/g consumed COD, respectively. Butyric acid fermentation was predominant, with butyrate and acetate as the major by-products and propionate, ethanol, and lactate as secondary metabolites. The acetate:butyrate ratios and fermentation pathways varied depending on the strains and environmental conditions. Hydrogenogenesis was studied in greater detail in *C. saccharobutylicum* H1. In butyric acid fermentation by this representative strain, acetoacetate was detected as an intermediate metabolite. Hydrogenogenesis was also analyzed in an enrichment culture, which behaved similarly to the axenic cultures. [**Int Microbiol** 2013; 16(1):53-62]

Keywords: *Clostridium* spp. · hydrogen production · dark fermentation · pH and temperature optimization · fermentation pathways · kinetic glucose degradation

Introduction

Environmental pollution resulting from the use of fossil fuels together with the world's increasing energy demand have stimulated the search for renewable and environmentally friendly sources of energy. Hydrogen (H_2) is a promising alternative energy carrier: it is renewable, with a high energy yield. It is also clean, producing only water when it burns, unlike other renewable energy sources such as methane [33]. Furthermore, its use in hydrogen-fuel cells makes it a promising alternative to hydrocarbons to power land vehicles. Hydrogen is currently obtained by thermal cracking (reformation) of natural gas and the electrolysis of water, both of which are energy-consuming processes. In contrast to these physico-chemical methods, biological processes using renewable sources for H_2 production, i.e., photo-fermentation or dark fermentation, are economical and environmentally friendly [6]. Photo-fermentation requires sunlight and is performed by algae, cyanobacteria, and anaerobic photosynthetic bacteria. However, despite its high yields of H_2, photo-fermentation has several drawbacks, such as the need for light energy and thus the diffi-

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culty of designing efficient photo-reactors [8]. Dark fermentation involves the conversion of organic substrates to H_2 by anaerobic microorganisms, including different species of *Clostridium*, syntrophobacteria, and enteric bacteria such as *Enterobacter*. The advantages of dark fermentation over other processes include better process economy because of lower energy requirements, process simplicity, higher rates of hydrogen production, and the use of inexpensive wastes as raw materials.

Clostridium species are the most significant microorganisms in anaerobic H_2 dark fermentation [12,32,36]. These bacteria ferment sugars or proteins to yield organic acids (mainly acetate, propionate, butyrate, and lactate), alcohols (ethanol), carbon dioxide, and molecular H_2 (also referred to as biohydrogen, specifiying its biological origin).

The production of biohydrogen by dark fermentation (hydrogenogenesis) is limited by the fact that the oxidation of reduced pyridine nucleotides and ferredoxin associated with H_2 formation is inhibited by high partial pressures of H_2 (P_{H2}) [2,13]. Moreover, at high P_{H2}, the metabolism of *Clostridium* switches from acid formation (acidogenesis) to solvent formation (solventogenesis) such that H_2 is no longer released [35]. Consequently, for efficient hydrogenogenesis, the prevention of H_2 accumulation (i.e., high P_{H2}) in the headspace and liquid medium of the dark fermentation system is of utmost importance. Most studies, disregarding this fact, have focused on systems in which the H_2 produced is allowed to accumulate. However, in an increasing number of studies continuous H_2 removal was shown to significantly increase hydrogen yields. For example, experiments in which the fermenters are flushed with dinitrogen gas have increased H_2 yields by 1.5- to 1.7-fold [17,27]. A recent study reported an impressive 11-fold increase in H_2 yield during dark fermentation, achieved by the continuous removal of H_2 from the medium using a fuel-type electrochemical cell [2]. Apart from maintaining low P_{H2}, other factors must be optimized for efficient dark fermentation, particularly the inoculum [15,22], the temperature [20,34], the pH [14,18,34], and the substrate, all of which are critical in defining end-product formation and thus H_2 production.

In this study, we developed an innovative approach—applying a low vacuum in the headspace of the fermentation system, thus maintaining low P_{H2} and preventing product inhibition—for the isolation of H_2-producing strains from various sources. As part of our efforts to optimize the system, ten hydrogen-producing *Clostridium* strains were isolated. Data on the effect of pH and temperature on H_2 production by the isolated strains and an enrichment culture are presented and discussed. Additionally, we analyzed intermediary products of H_2-producing fermentations and conclude this work with a discussion of the respective metabolic pathways and reaction kinetics. Our results provide valuable information for the optimization of dark fermentation and the achievement of efficient hydrogen production.

Materials and methods

Sources of microorganisms. The following sources were used as inocula for the isolation of hydrogen-producing bacteria: (i) granular sludge from a full-scale upflow anaerobic sludge bed (UASB) reactor treating wastewater from a brewery (Mahou SA, Alovera, Spain); (ii) sludge from an anaerobic digester treating municipal solid wastes (Madrid, Spain); (iii) activated sludge from an aerobic domestic wastewater treatment plant (Universidad Autónoma de Madrid [UAM], Cantoblanco, Madrid, Spain); (iv) sediments from an acidic river (Río Tinto, Huelva, Spain).

Culture media. The bacteria were cultured in synthetic (MR) medium containing 280 mg NH_4Cl/l, 328 mg K_2HPO_4/l, 100 mg $MgSO_4$/l, 500 mg $NaHCO_3$/l, and 2 g sucrose/l, 1 g meat extract/l, 500 mg yeast extract/l as carbon source, and 1 ml of micronutrient solution [31]. The initial pH was adjusted to 7.5. Solid media were prepared by adding 15 g bacteriological agar/l to MR medium.

Preparation of enrichment cultures and isolation of axenic cultures. Prior to isolating the strains, we prepared enrichment cultures under low vacuum in 120-ml glass serum bottles containing 60 ml of medium. Anoxic conditions were produced by flushing the headspace with N_2:CO_2 (80:20) and adding L-cysteine (40 mg/l). The medium was inoculated with 1g of homogenized sludge, and then incubated under partial vacuum at 30 °C for 30 days. The nutrients were renewed weekly. Cultures producing significant amounts of H_2 were inoculated onto MR agar plates and incubated at 30 °C for 72 h. Single colonies were selected and grown in MR in order to establish pure cultures of the H_2-producing strains (see Phylogenetic analysis, next). An enrichment culture (EC), obtained from granular sludge after 5 months of incubation under the conditions described above and with periodically renewed medium was monitored along with the axenic cultures.

Phylogenetic analysis. Single colonies from the MR agar plates were inoculated into 25-ml serum bottles containing 5 ml of MR and incubated at 37 °C overnight. Cells from bottles with high H_2 yields were collected for sequencing. One ml of liquid culture was centrifuged (10,000 rpm, 15 min) and the pellet was washed with 1 ml of phosphate buffered saline, centrifuged (10,000 rpm, 15 min), and re-suspended in 100 µl of distilled water. The suspension was heated at 94 °C for 10 min and centrifuged (15,000 rpm, 5 min). Five µl of the resulting supernatant was PCR-amplified using the primers 27f and 1492r [21]. The amplicons were assembled and the consensus sequence corrected manually for errors using DNA Baser 3.0. The sequences were compared to those in the 2011 GenBank database of NCBI [http://www.ncbi.nlm.nih.gov/] using the Basic Local Alignment Search Tool (BLAST) algorithm and the 2011 version of the tool Classifier of the Ribosomal Database Project [http://rdp.cme.msu.edu/].

Optimization of pH and temperature. Tests for the optimization of pH and temperature were conducted in 120-ml serum bottles containing 20 ml of MR. Each bottle was inoculated to an initial optical density (OD_{660}) of 0.001 with one of the axenic cultures grown to exponential phase. The conditions were those described in "Culture media" except for the variable tested (pH or temperature). The tested pH values were 5, 5.5, 6.5, and 7.5. The temperature optimization experiment was carried out at 25, 30, 35, and 40 °C. All tests were done in triplicate. Hydrogen production, OD, and pH were analyzed after 14, 24, and 48 h.

Metabolic and kinetic studies. Studies of the metabolic pathways and growth kinetics of the strains and enrichment culture were carried out using glucose (4 g/l) as the sole carbon source, added to the mineral medium used in MR medium. Batch tests were followed over 2 days until glucose depletion. The main organic metabolites as well as pH, H_2, CO_2, and total suspended solids (TSS) were monitored periodically. All tests were done in triplicate.

Analytical methods. Hydrogen and CO_2 were quantified by gas chromatography using a Bruker 450-GC coupled with a thermal conductivity detector (TCD) and a Varian CP2056 0.6 m ×1/8″ Ultimetal Cromosorb GHP 100–120 mesh column in bypass. The temperature of the injection, TCD chambers, and oven was maintained at 150, 200, and 50 °C, respectively. Nitrogen was used as the carrier gas in the column at a flow rate of 25 ml/min. Volatile fatty acids (VFAs) were quantified by high-performance liquid chromatography coupled with a refraction index detector (HPLC-RID) (Varian Prostar 350 RID, Agilent) using a sulfonated polystyrene resin in the protonated form (67H type) as the stationary phase (Varian Metacarb 67H 300 mm) and sulfuric acid (0.25 mM in milliQ water) as the mobile phase at a flow rate of 0.8 ml/min. The column temperature was set at 65 °C. Non-common metabolites were identified by HPLC coupled to a diode array detector (Varian DAD 330) and a triple quadrupole ion trap mass spectrometry detector (HPLC-DAD-MS) with positive and negative electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) (Varian 1200L). The same 67H column described above served as the stationary phase and 0.5 M formic acid as the mobile phase at a flow rate of 0.6 ml/min. The column temperature was set at 65 ºC. All the other analyses were done according to standard methods [3].

Results and Discussion

Isolation of hydrogen-producing strains. Ten H_2-producing strains were isolated from the four sources listed in Materials and methods: three (H1, H5, H17) from the UASB reactor, four (R4, R6, R12, R14) from the anaerobic digester, one from the activated sludge of a domestic wastewater treatment plant (UAM), and two from sedi-

Table 1. Hydrogen-producing strains isolated from different sources

Strain isolated	Query length	Species of *Clostridium* with higher homology	Source*	Similarity (%)
H1	1170	*C. saccharobutylicum* *C. acetobutylicum* *C. saccharoperbutylacetonicum*	1	99
H5	1293	*C. roseum* *C. beijerinckii*	1	100
H17	1180	*C. butyricum*	1	100
R4	1093	*C. butyricum*	2	99
R6	1095	*C. butyricum*	2	96
R12	1328	*Clostridium* sp.	2	96
R14	1335	*Clostridium* sp.	2	96
RT1	1342	*C. beijerinckii*	4	100
RT2	1337	*C. diolis* *C. beijerinckii* *C. acetobutylicum*	4	100
UAM	1371	*C. beijerinckii* *C. diolis* *C. acetobutylicum*	3	100

*1: Anaerobic granular sludge from a UASB reactor treating brewery wastewater.
2: Sludge from an anaerobic digester of municipal solid wastes.
3: Activated sludge from an aerobic domestic waste treatment plant.
4: Anaerobic sediments from a river.

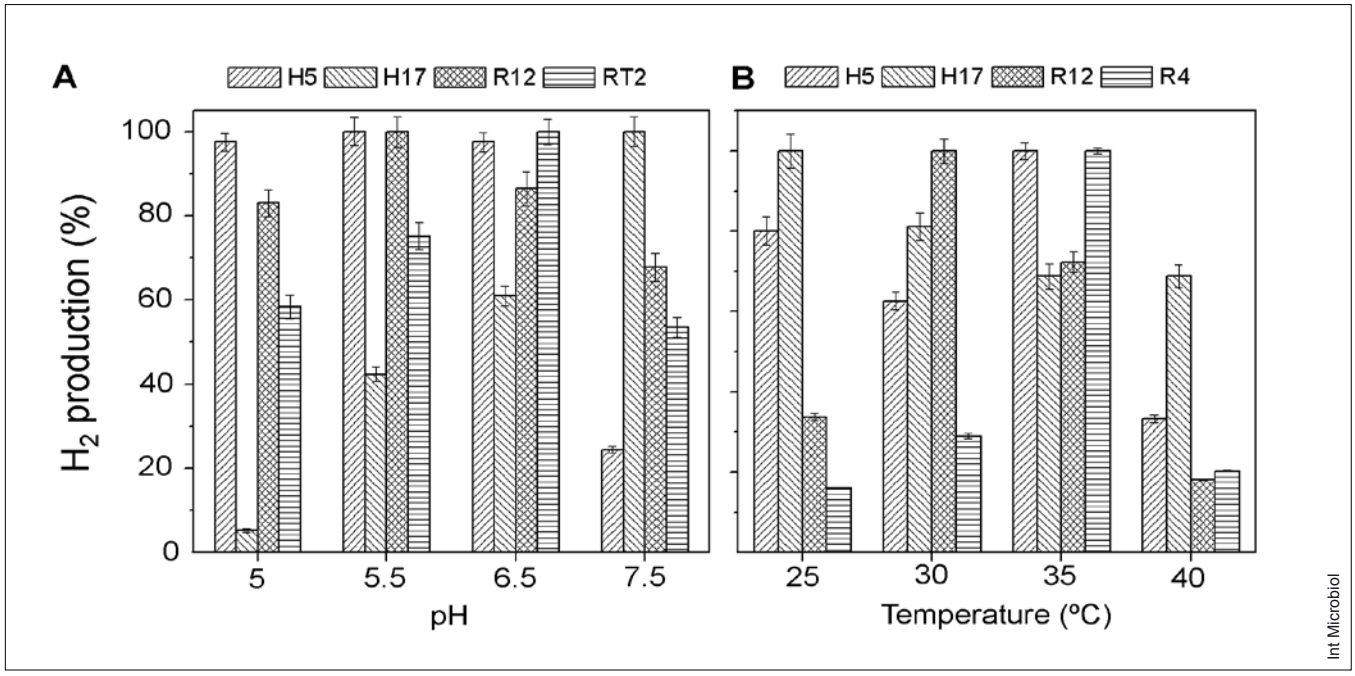

Fig. 1. Effect of pH (**A**) and temperature (**B**) on hydrogen production by representative strains. The results are presented as percentages relative to maximum hydrogen production by each strain.

ments from the Río Tinto (RT1, RT2). All of the isolates belonged to the genus *Clostridium* (Table 1). The 16S rRNA gene sequences have been deposited in the GenBank database under accession number JX575125–JX575134. The four sludge samples studied proved to be appropriate sources of hydrogen-producing bacteria. Indeed, diverse clostridia and syntrophobacteria have repeatedly been isolated from granular and other kinds of sludge, accounting for their frequent use as inocula in studies focusing on hydrogen production [10,12,22]. The sludge and enrichment cultures from which the strains were isolated were submitted to low vacuum, which allowed continuous removal of the gaseous products from the headspace, thus keeping the P_{H2} low. This innovative method boosts the growth of H_2-producing microorganisms by avoiding the product inhibition caused by high P_{H2}.

Although hydrogen-producing bacteria are diverse [16,18], the medium used in these experiments, rich in sugars and proteins, may have selected *Clostridium* spp., which are typical H_2 producers associated with the fermentation of carbohydrates [4,12,37] and proteins [30] to acetate, butyrate, carbon dioxide, and organic solvents [29].

Effect of pH and temperature. Figure 1A shows the production of H_2 at different initial pH settings. In most cases, pH 6.5 was optimal for H_2 production. Strains H1 (not shown in the figure) and H5 (no significant differences between pH 5.5 and 6.5), R12 (optimal pH of 5.5), and H17 (pH 7.5) were the exceptions to this general trend. In several reports, pH 5.5–6.5 was reported to be the optimal range for H_2 production [7,19,26]. Indeed, in at least two studies evaluating the effect of pH on fermentative biohydrogen production by isolated *Clostridium* species H_2 production rates and yields were shown to peak at an initial pH 6 [11,24]. However, other studies have not found that the initial pH profoundly affects H_2 production [9], which concurs with our results for *Clostridium* R12 and for the enrichment culture. In our system, the initial optimal pH for *C. butyricum* H17 (7.5) was close to that reported for *C. butyricum* ATCC 19398, *C. acetobutyricum* M12, and *C. tyrobutyricum* FYa102, all of which produced maximum amounts of hydrogen at pH 7.2 [25].

The effect of temperature on hydrogen production is shown in Fig. 1B. With the exception of strains H17 (optimum production at 25 °C) and R12 (30 °C), the highest H_2 production was recorded at 35 °C. Note that the effect of temperature in the range studied was limited for some strains (H17), yet dramatic for others (R4, R12). The highest temperature tested (40 ºC) had a negative effect on the growth of all ten strains, and especially on the growth of isolates from the municipal solid waste digester and the enrichment culture. Differences in the behavior of hydro-

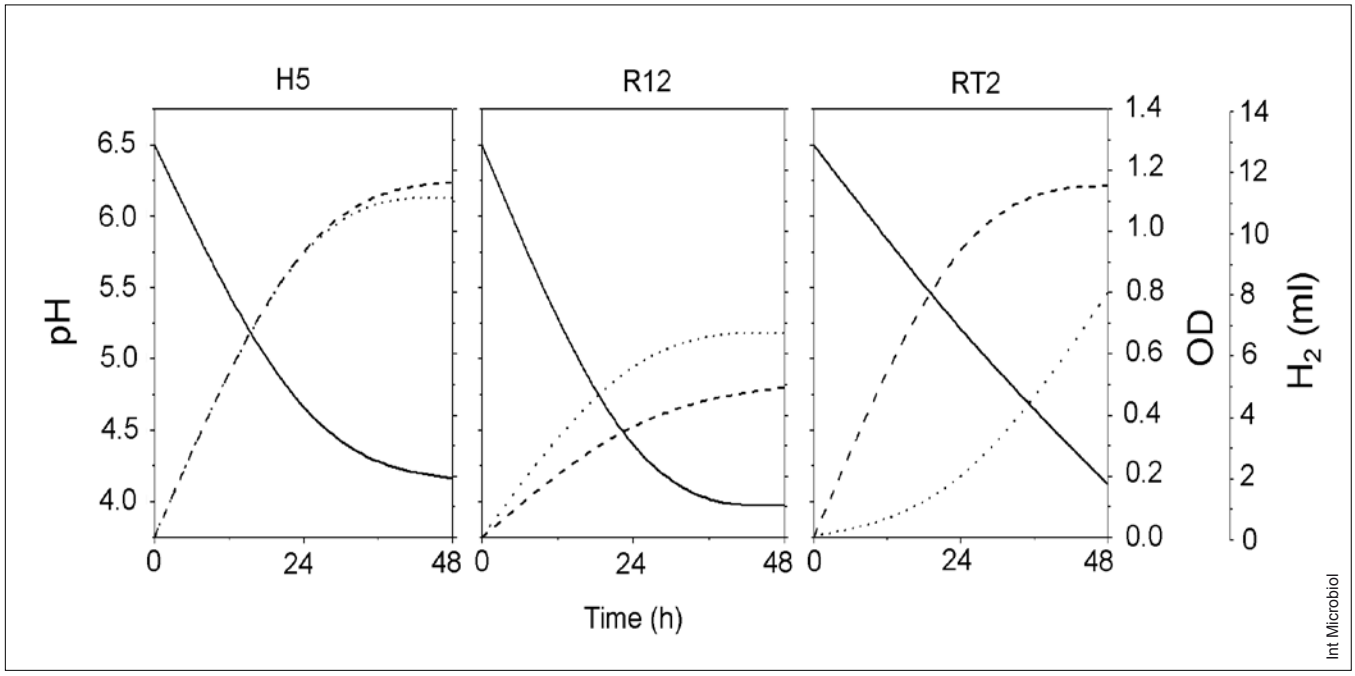

Fig. 2. Growth curve (dot), hydrogen production (dash), and pH trend (solid) for strains H1, H5, and R12 during batch fermentation at 30 °C and pH 6.5 during the first 48 h.

gen-producing strains due to temperature had been described previously [1,10,23].

General trends and hydrogen yields during batch fermentation. Figure 2 shows the time-course of hydrogen production by three of the isolated strains growing in MR as well as the pH and OD of the respective cultures. In most cases (i.e. H5, R12) H_2 production mirrored the growth curve (determined by OD) of each strain, and was accompanied by a decrease in the pH of the culture due to the accumulation of acids. In some case (i.e., for strain RT2), the apparent growth rate is low over the first 24 h, while the H_2 production rate is high. From this moment on, probably due to the low pH, the cell number increases but stop producing H_2. A summary of the results obtained in the fermentation studies is provided in Table 2.

The hydrogenogenesis potential ranged from 120 ml/[g of initial chemical oxygen demand (CODi)] for *C. roseum* H5 and *C. diolis* RT2 to nearly 70 ml/g CODi for *C. butyricum* H17 and R4. H_2 production levels of 140 ml/g starch by *C. pasteurianum* [26], 150 ml/g sucrose by *Clostridium* sp. [34], 134 ml/g molasses by a mixed culture [26], and almost 180 ml H_2 per g maltose [39] and g lactose [11,39] have been reported, indicating that yields are lower when complex wastes are used as substrates. In other studies, much lower amounts of H_2 were achieved: 2.7 ml H_2/g COD of food waste by *C. beijerinckii* KCTC [19] and 9.1 ml H_2/g of raw sludge by raw sludge seed inocula [5]. Some authors consider only the COD consumed when calculating H_2 yields. Tang et al. [34], for example, reported a yield of 319 ml H_2/g COD of cattle waste consumed. The yields obtained in our study were mostly higher than 300 ml H_2/g COD consumed, or even above 500 ml H_2/g COD consumed for *C. diolis* RT2, *C. beijerinckii* UAM, and the enrichment culture (Table 2). It is not easy, however, to compare hydrogen production in different experiments because dark fermentation is influenced by many factors, including the type of inoculum, the pH, the temperature, and the nature of the substrate. The high hydrogen production rates obtained in our experiments can perhaps be ascribed to the use of the complex MR medium, which might have promoted the growth of both carbohydrate and protein fermenters.

Fermentation products. The decrease in pH observed during the batch experiments may have resulted from the accumulation of VFAs during acid fermentation by the strains. The major by-products accumulated were the VFAs butyrate and acetate (Table 3). When grown on MR medium, strains H1, H5, RT1, RT2, and UAM mainly

Table 2. pH, OD, COD, and hydrogen produced at the end of experiments for the isolates and the enrichment culture

Strains of *Clostridium*	Final pH[a]	Final OD	H_2 (ml)[b]	H_2 (ml/g CODi)[c]	H_2 (ml/g COD removed)
C. saccharobutylicum H1	4.39 ± 0.04	0.75 ± 0.05	7.3 ± 3.0	76.4 ± 31.6	251.7
C. roseum H5	4.16 ± 0.02	1.09 ± 0.02	11.5 ± 0.5	120.3 ± 5.2	396.6
C. butyricum H17	4.35 ± 0.01	0.23 ± 0.09	6.4 ± 0.2	68.0 ± 2.1	300.9
C. butyricum R4	4.28 ± 0.06	0.66 ± 0.03	6.4 ± 0.1	69.0 ± 1.0	336.7
C. butyricum R6	4.00 ± 0.01	0.70 ± 0.04	7.6 ± 0.6	79.5 ± 6.3	263.9
Clostridium sp. R12	3.98 ± 0.05	0.67 ± 0.05	4.2 ± 0.1	51.3 ±1.0	175.0
Clostridium sp. R14	4.02 ± 0.03	0.66 ± 0.05	6.8 ± 0.2	77.4 ± 2.1	345.8
C. beijerinckii RT1	4.23 ± 0.02	0.52 ± 0.04	9.3 ± 0.7	99.4 ± 7.3	341.6
C. diolis RT2	4.12 ± 0.02	0.88 ± 0.05	11.2 ± 0.1	120.3 ± 1.0	475.2
C. beijerinckii UAM	4.17± 0.01	0.88 ± 0.03	7.7 ± 0.1	90.0 ± 1.0	573.3
Enrichment culture	4.46 ± 0.04	1.02 ± 0.01	10.5 ± 1.0	114.1 ± 10.1	500.0

[a]Initial pH: 6.5.
[b]Serum bottles containing 20 ml of medium.
[c]Initial COD varied from 4.6 to 4.78 g/l.

carried out butyrate fermentation. The presence of alcoholic and propionic fermentation can be inferred for strains H1 and H5 cultured on meat extract as a carbon source and glucose, respectively, based on the production of acetate, propionate, and ethanol as final products. Moreover, in strain H1, the alcoholic fermentation pathway was apparently used for glucose fermentation. In strains R12 and R14, lactate was the second most abundant fermentation by-product (data not shown), suggesting heterolactic or mixed-acid fermentation.

The effect of pH and temperature on the fermentation end-products was also studied in enrichment culture. In general, both had limited effects, except at an incubation temperature of 40 °C. At this temperature, H_2 was not released, sucrose was not fully consumed, and metabolism shifted to lactic acid fermentation (data not shown). With respect to pH, the butyrate:acetate ratio increased from 1.8 to 4.6 at an initial pH range of 7.5–5.5, with the amounts of minor products (ethanol and propionate) remaining constant (data not shown).

Metabolic and kinetic studies. Among our isolates, *Clostridium saccharobutylicum* H1 was selected as a representative strain and its metabolism and kinetics were studied in cultures containing 4 g glucose/l. Figure 3 shows the evolution of glucose and its main degradation products over time (Fig. 3A) and the evolution of pH, glucose, and biomass concentration (expressed as volatile suspended solids [VSS]) (Fig. 3B). Glucose evolution is presented in both figures to allow comparisons of glucose consumption and biomass production. Both the CO_2 and the H_2 values (mmol) were corrected for pressure. The results showed that butyrate fermentation was the predominant process involved in glucose fermentation, with 0.1 mmol of butyrate and 0.06 mmol of acetate produced during the fermentation of 0.48 mmol of glucose.

Trace concentrations of lactate were detected during the first hours of the experiment, suggesting the appearance of residual lactic fermentation. However, lactate was consumed during the course of the experiment, consistent with the anaerobic oxidation of lactate to acetate. The accumulation of VFAs led to a drop in pH to around 4 (Fig. 3B). Similar amounts of H_2 and CO_2 (approximately 2 mmol/mmol glucose consumed) were produced, which is in accordance with the maximum H_2/glucose ratio reported thus far [28].

Table 3. Concentrations of fermentation end products (expressed in mM)

Strain	Medium	Acetate	Butyrate	Ethanol	Glucose	Lactate	Propionate	Sucrose
H1	Complex medium MR	7.6	13	2.7	2	–	–	–
H1	With 11.1 mM glucose	4.9	–	18.2	1.6		–	–
H5	Complex medium MR	4.2	18.8	–	1.4	–	0.7	–
H5	With 2g/l meat extract	7.2	1.7	4.6	–	–	3.3	–
RT1	Complex medium MR	11	14	–	1.2	–	1.4	–
UAM	Complex medium MR	5.5	13.4	–	–	1.9	2.2	6

Glucose consumption was linked to biomass growth, which indicated the use of a high fraction of glucose by the strains during anabolic processes. Both glucose consumption and biomass growth can be modeled by the following equation system, simplified from the general Monod model:

$$\frac{dG}{dt} = -k_1 \cdot \frac{X}{Y_{x/s}} \quad (1)$$

$$\frac{dX}{dt} = -Y_{x/s} \cdot \frac{dG}{dt} \quad (2)$$

where k_1 is the pseudo-first order kinetics constant (liter per h per mmol glucose), $Y_{X/S}$ is the biomass yield factor (mg VSS/mmol glucose), and X and G are the biomass (mg VSS/l) and glucose concentrations (mM), respectively. Equations (1) and (2) were integrated by the Episode numerical method for Stiff systems, with the initial conditions t = 16, X = 57.8, and G = 31.75, by means of a non-linear least squares minimization of the error, using a simplex algorithm followed by a Powell minimization algorithm (Micromath Scientist 3.0). Fitting glucose and biomass concentrations to this model resulted in the following values for the fitting parameters: $k_1 = 0.00130 \pm 0.00007$ liter per h per mmol glucose, $Y = 13.26 \pm 0.32$ mg VSS produced per mmol glucose consumed, and $R^2 = 0.997$. The fitting curves are depicted in Fig. 3B. The model accurately describes both biomass growth and glucose consump-

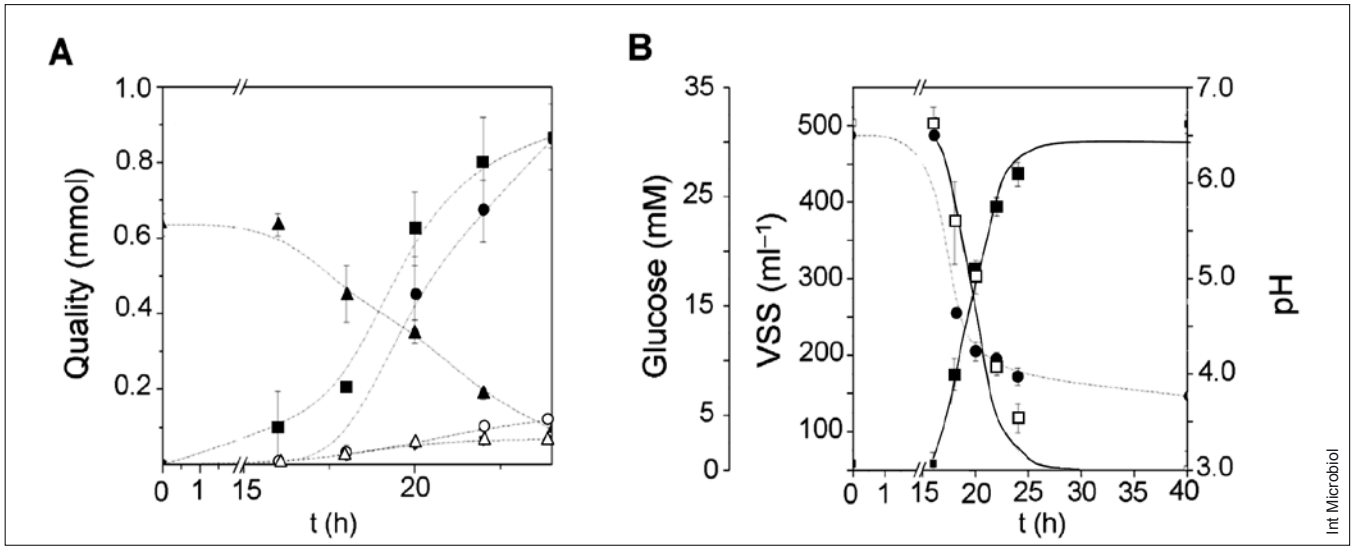

Fig. 3 (A) Time-evolution of glucose (closed triangles), hydrogen (closed squares), CO_2 (closed circles), acetate (open triangles) and butyrate (open circles). (**B**) Time evolution of pH (closed circles), glucose (open squares), and biomass expressed as VSS (closed squares) during the metabolic and kinetic studies. Continuous lines are model fittings.

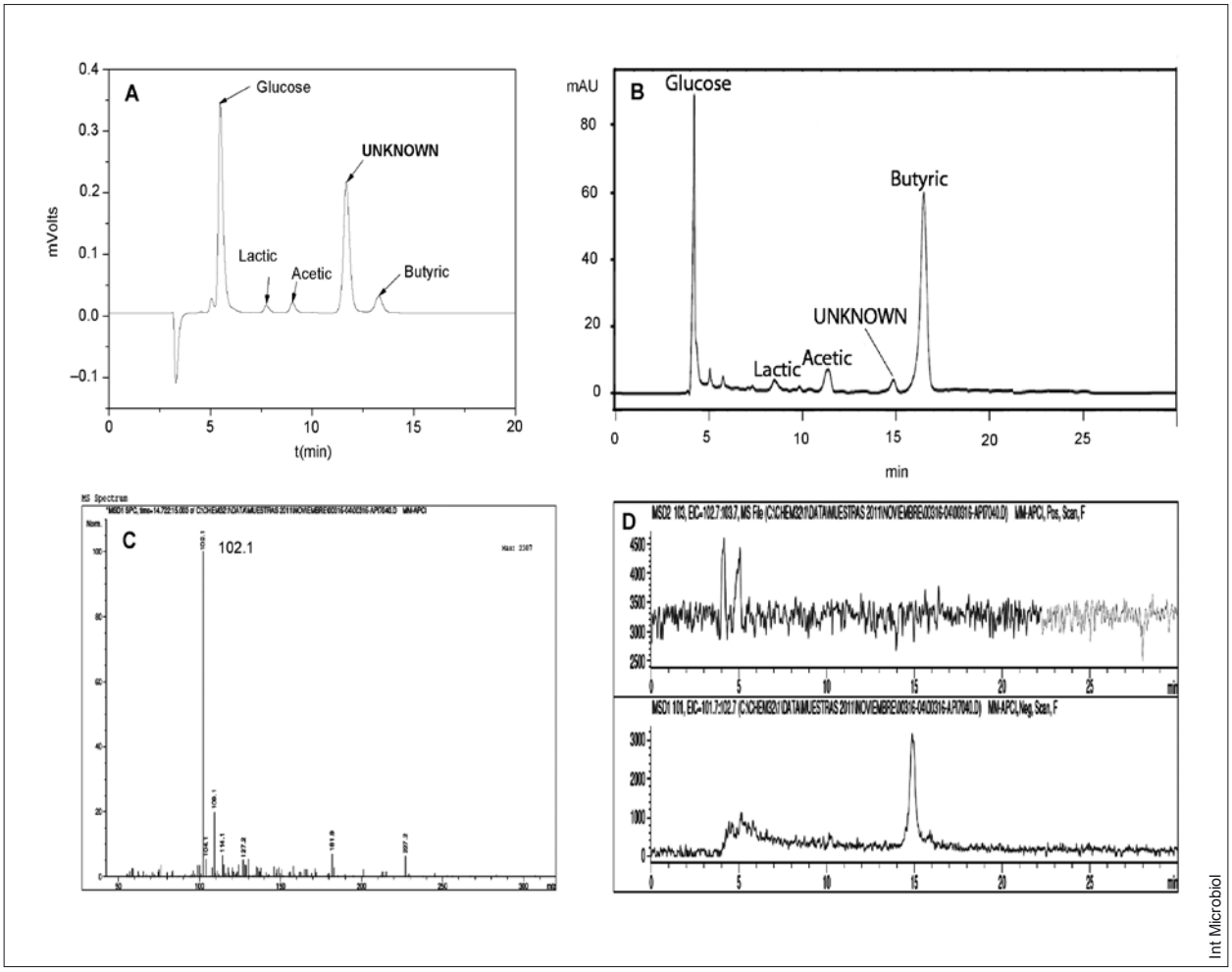

Fig. 4. Representative chromatograms of samples from the metabolic study of strain H1. (**A**) HPLC-RID chromatogram at 18 h. (**B**) HPLC-DAD chromatogram at 24 h. (**C**) HPLC-MS profile of peak at 14.7 min from the HPLC-DAD chromatogram. (**D**) HPLC-MS chromatogram of the 102 g/mol fraction of the HPLC-DAD chromatogram, showing positive (up) and negative (down) ionization of the sample.

tion. Additionally, from these data, a duplication period of 1.87 h could be estimated.

Considering the approximate composition of biomass to be $C_4H_7O_2N$ [38], which corresponds to approximately 50 % molar/molar C and 31 % m/m O, the C and O mass balances were not closed at the end of the experiment. This suggests the presence of other metabolites not considered in the glucose fermentation processes. The HPLC-RID chromatogram at 18 h is presented in Fig. 4. A peak at 11.5 min was detected, indicating that only one other metabolite was produced during glucose fermentation, in addition to those already mentioned. The sample was analyzed by HPLC-DAD-MS at 24 h (Fig. 4B). The same distribution of metabolites was observed, with the detection of an unknown peak at 14.7 min. This peak was analyzed individually by MS, which showed that the predominant molecular mass was 102 g mol^{-1} (Fig. 4C). The ionic profiles indicated that the compound is negatively ionizable, because no cations were detected at the cationic 103 g mol^{-1} analysis whereas a significant peak was detected at the anionic 101 g mol^{-1} analysis (Fig. 4D). Carbon and oxygen mass balances were carried out for all of the samples, and an average C to O ratio of 1.25 ± 0.05 mol C per mol O was calculated for the non-closed mass balance. This result combined with the molecular mass determined in the HPLC-MS analysis (102 g mol^{-1}) suggested that the unknown compound has four C atoms and three O atoms. We therefore propose that the unknown metabolite corresponds to acetoacetic acid ($C_4H_6O_3$), an intermediate in butyric acid fermentation that usually combines with CoA before

butyric acid production. This compound was previously detected in a butyric acid fermentation under pH conditions unfavorable for butyric acid production [13]. Microorganisms adopt the strategy of excreting acetoacetate during butyric acid production when growth conditions again become favorable [13].

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Competing interests. None declared.

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